JOINT APPENDIX 45

US006734171B1

(12) **United States Patent**
Saravolac et al.

(10) **Patent No.: US 6,734,171 B1**
(45) **Date of Patent: *May 11, 2004**

(54) **METHODS FOR ENCAPSULATING NUCLEIC ACIDS IN LIPID BILAYERS**

(75) Inventors: **Edward George Saravolac**, Vancouver (CA); **Yuan-Peng Zhang**, Mountain View, CA (US); **Jeffery J. Wheeler**, Surrey (CA); **Pieter R. Cullis**, Vancouver (CA); **Peter Scherrer**, Vancouver (CA); **Ljiljana D. Kojic**, Vancouver (CA); **Olga Ludkovski**, Port Coquitlam (CA)

(73) Assignee: **Inex Pharmaceuticals Corp.**, Burnaby (CA)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/169,573**

(22) Filed: **Oct. 9, 1998**

Related U.S. Application Data

(60) Provisional application No. 60/063,473, filed on Oct. 10, 1997.

(51) **Int. Cl.**[7] **A61K 48/00**

(52) **U.S. Cl.** **514/44**; 424/450; 435/320.1; 435/455; 435/458

(58) **Field of Search** 424/450; 435/325, 435/320.1, 455, 458; 514/44

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,089,801	A		5/1978	Schneider 252/316
4,460,560	A		7/1984	Tokes et al. 424/1.1
4,501,728	A		2/1985	Geho et al. 424/38
4,544,545	A		10/1985	Ryan et al. 424/1.1
4,617,186	A		10/1986	Schafer et al. 424/78
4,650,909	A		3/1987	Yoakum 568/621
4,728,575	A		3/1988	Gamble et al. 428/402.2
4,737,323	A		4/1988	Martin et al. 264/4.3
4,752,425	A		6/1988	Martin et al. 264/4.6
4,837,028	A	*	6/1989	Allen 424/450
4,861,521	A		8/1989	Suzuki et al. 260/403
4,920,016	A		4/1990	Allen et al. 424/450
4,925,661	A		5/1990	Huang 424/85.91
4,943,624	A		7/1990	Regen 528/301
4,944,948	A		7/1990	Uster et al. 424/450
4,963,367	A		10/1990	Ecanow 424/485
5,008,109	A		4/1991	Tin 424/422
5,013,556	A		5/1991	Woodle et al. 424/450
5,064,655	A		11/1991	Uster et al. 424/450
5,153,000	A		10/1992	Chikawa et al. 424/450
5,185,154	A		2/1993	Lasic et al. 424/450
5,206,027	A		4/1993	Kitaguchi 424/450
5,288,499	A		2/1994	Janoff et al. 424/450
5,356,633	A		10/1994	Woodle et al. 424/450
5,395,619	A		3/1995	Zalipsky et al. 424/450
5,527,528	A		6/1996	Allen et al. 424/178.1
5,552,155	A		9/1996	Bailey et al. 424/450
5,593,622	A		1/1997	Yoshioka et al. 264/4.32
5,631,237	A	*	5/1997	Dzau et al. 514/44
5,705,385	A		1/1998	Bally et al.
5,708,385	A	*	1/1998	Shou et al. 435/320.1
5,753,262	A	*	5/1998	Wyse et al. 424/450
5,820,873	A	*	10/1998	Choi et al. 424/283.1
5,827,703	A	*	10/1998	Debs et al. 435/455
5,885,613	A	*	3/1999	Holland et al. 424/450
5,976,567	A	*	11/1999	Wheeler et al. 424/450
5,981,501	A		11/1999	Wheeler et al.
2002/0192651	A1	*	12/2002	Wheeler 435/6

FOREIGN PATENT DOCUMENTS

CA	1270197	6/1990
CA	2067133	4/1991
CA	2067178	4/1991
CA	1305054	7/1992
EP	0 118 316	3/1983
EP	0 072 111	10/1985
EP	0 220 797	5/1987
EP	0 370 491	5/1990
EP	0 422 543	4/1991
EP	0 482 860	4/1992
EP	526700	2/1993
EP	546951	6/1993
EP	572049	12/1993
EP	0 445 131	4/1994
EP	0 354 855	12/1994
EP	0 496 813	12/1994
EP	0 496 835	5/1995
GB	2185397	7/1987
WO	WO 88/04924	7/1988
WO	WO 90/04384	5/1990
WO	WO 91/05545	5/1991
WO	WO 91/05546	5/1991
WO	WO 93/19738	10/1993
WO	WO 94/07466	4/1994
WO	WO 94/21281	9/1994
WO	WO 94/22429	10/1994
WO	WO 94/26251	11/1994
WO	WO 94/27580	12/1994
WO	WO 95/31183	11/1995
WO	WO 96/34598	11/1996
WO	WO96/37194	11/1996
WO	WO96/40964	12/1996

OTHER PUBLICATIONS

Zelphati et al., Liposomes as a carrier for intracellular delivery of antisense oligonucleotides: a real magic bullet?, 1996, Journal of Controlled Release, vol. 41, pp. 99–119.*

(List continued on next page.)

Primary Examiner—Dave T. Nguyen
(74) *Attorney, Agent, or Firm*—Townsend & Townsend & Crew LLP

(57) **ABSTRACT**

The present invention relates to lipid-based formulations for nucleic acid delivery to cells, methods for the preparation of such formulations and, in particular, to lipid encapsulated plasmids. The compositions are safe and practical for clinical use. In addition, the present invention provides methods for introducing nucleic acids into cells and for inhibiting tumor growth in cells using such lipid-nucleic acid formulations.

19 Claims, 31 Drawing Sheets-

OTHER PUBLICATIONS

Filion et al., Major limitations in the use of cationic liposomes for DNA delivery, 1998, International Journal of Pharmaceutics, vol. 192, pp. 159–170.*
Branch, A good antisense molecule is hard to find, Feb. 1998, TIBS, vol. 23, pp. 45–50.*
Anderson, Human gene therpy, Apr. 30, 1998, Nature, vol. 392, pp. 25–30.*
Verma et al. Gene therapy –promises, problems and propespects, Sep. 18, 1997, Nature, vol. 389, pp. 239–242.*
Abuchowski, et al., "Treatment of L5178Y Tumor–Bearing BDF Mice With a Nonimmunogenic L–Gutaminase–L–Asparginase," *Cancer Treatment Reports,* 63(6):1127–1132 (1979).
Abuchowski, et al., "Immunosuppressive Properties and Circulating Life of Achromobacter Glutaminase–Asparaginase Covalently Attached to Polyethylene Glycol in Man," *Cancer Research Reports*, 65(11–12):1077–1081 (1981).
Berger, Jr., et al., "Preparation of Polyethylene Glycol–tissue Plasminogen Activator Adducts That Retain Functional Activity: Characteristics and Behavior in Three Animal Species," *Blood,* 71(6):1641–1647 (1988).
Blume, et al., "Specific targeting with poly(ethylene glycol)–modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times," 1993, *Biochimica et Biophysica Acta., 1149*:180–184.
Chonn, et al., "Ganglioside G_{M1} and hydrophilic polymers increases liposome circulation times by inhibiting the association of blood proteins," 1992, *J. Liposome Res., 2(3)*:397–410.
Danishefsky, et al., "Total Synthesis of Zincophorin," 1988, *J. Am. Chem. Soc.,* 1988, *110*:4368–4378.
Gregoriadis, et al., "Coupling of ligands to liposomes independently of solute entrapment; observations on the formed vesicles," 1993, *Biochimica et Biophysica Acta., 1147*:185–193.
Huang, et al., "Extravasation and Transcytosis of Liposomes in Kaposi's Sarcom–Like Dermal Lesions Transgenic Mice Bearing the HIV Tat Gene," 1993, *American Journal of Pathology, 143(1)*:10–14.
Hyde, et al., "Correction of the ion transport defect in cycstic fibrosis transgenic mice by gene therapy," 1993, *Nature, 362:*250–255.
Kiso, et al., "A Convenient Synthesis of Sphingosine and Ceramide from D–Xylose or D–Glactose," 1986, *J. Carbohydrate Chemistry, 5(2)*:335–340.
Klibanov, et al., "Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to traget," 1991, *Biochimica et Biophysica Acta., 1062*:142–148.
Liu, et al., "Role of liposome size and RES blockade in controlling biodistribution and tumor uptake of GM_1–containing liposomes," 1992, *Biochimica et Biophysica Acta., 1104*:95–101.
Mori, et al., "Influence of the steric barrier activity of amphipathic poly(ethyleneglycol) and ganglioside GM_1 on the circulation time of liposomes and on the target binding of immunoliposomes in vivo," 1991, *FEBS Letters, 284(2)*:263–266.
Nicolaou, et al., "A Practical and Enantioselective Synthesis of Glycosphingolipids and Related Compounds. Total Synthesis of Globotriaosylceramide (Gb_3)," 1988, *J. Am. Chem. Soc., 110:*7910–7912.
Oikawa, et al., "Selective hydrogenolysis of the benzyl protecting group for hydroxy function with Raney nickel in the presence of the MPM (4–methoxybenzyl) and DMPM (3,4–dimethoxybenzyl) protecting groups," 1984, *Tetrahedron Letters, 25(47)*:5397–5400.
Parr, et al., "Factors influencing the retention and chemical stability of poly(ethylene glycol)–lipid conjugates incorporated into large unilamellar vesicles," Jun. 19–22, 1994, Liposome Research Days Conference—Liposomes: The Next Generation, University of British Columbia.
Parr, et al., "Factors influencing the retention and chemical stability of poly(ethylene glycol)–lipid conjugates incorporated into large unilamellar vesicles," 1994, *Biochimica et Biophysica Acta, 1195*:21–30.
Reed, et al., "Generation of targets for alloreactive CTL using purified H–$2K^k$ in liposomes and polyethylene glycol," 1986, *Molecular Immunology, 23(12)*:1339–1347.
Schmidt, et al., "Lactosylceramides with Unsaturated Fatty Acids—Synthesis and Use in the Generation of Bilayer Membranes," 1987, *Angew. Chem. Int. Ed. Engl., 26(8)*:793–794.
Torchilin, et al., "Targeted accumulation of polyethylene glycol–coated immunoliposomes in infarcted rabbit myocardium," 1992, *The FASEB Journal , 6*:2716–2719.
Wang, et al., "pH–sensitive immunoliposomes mediate target–cell–specific delivery and controlled expression of a foreign gene in mouse," 1987, *Proc. Natl. Acad. Sci. USA, 84*:7851–5855.
Allen, T., *Journal of Liposome Research,* 2(3):289–305 (1992).
Chu et al., *Journal of Liposome Research,* 4(1):361–395 (1994).
Gao, et al., *Journal of Liposome Research,* 3(1):17–30 (1993).
Klibanov, et al., *Journal of Liposome Research,* 2(3):321–334 (1992).
Soehnlein, et al., Seifen, Ole, Fette, Wachse, 115(3):85–86 (1989).

* cited by examiner

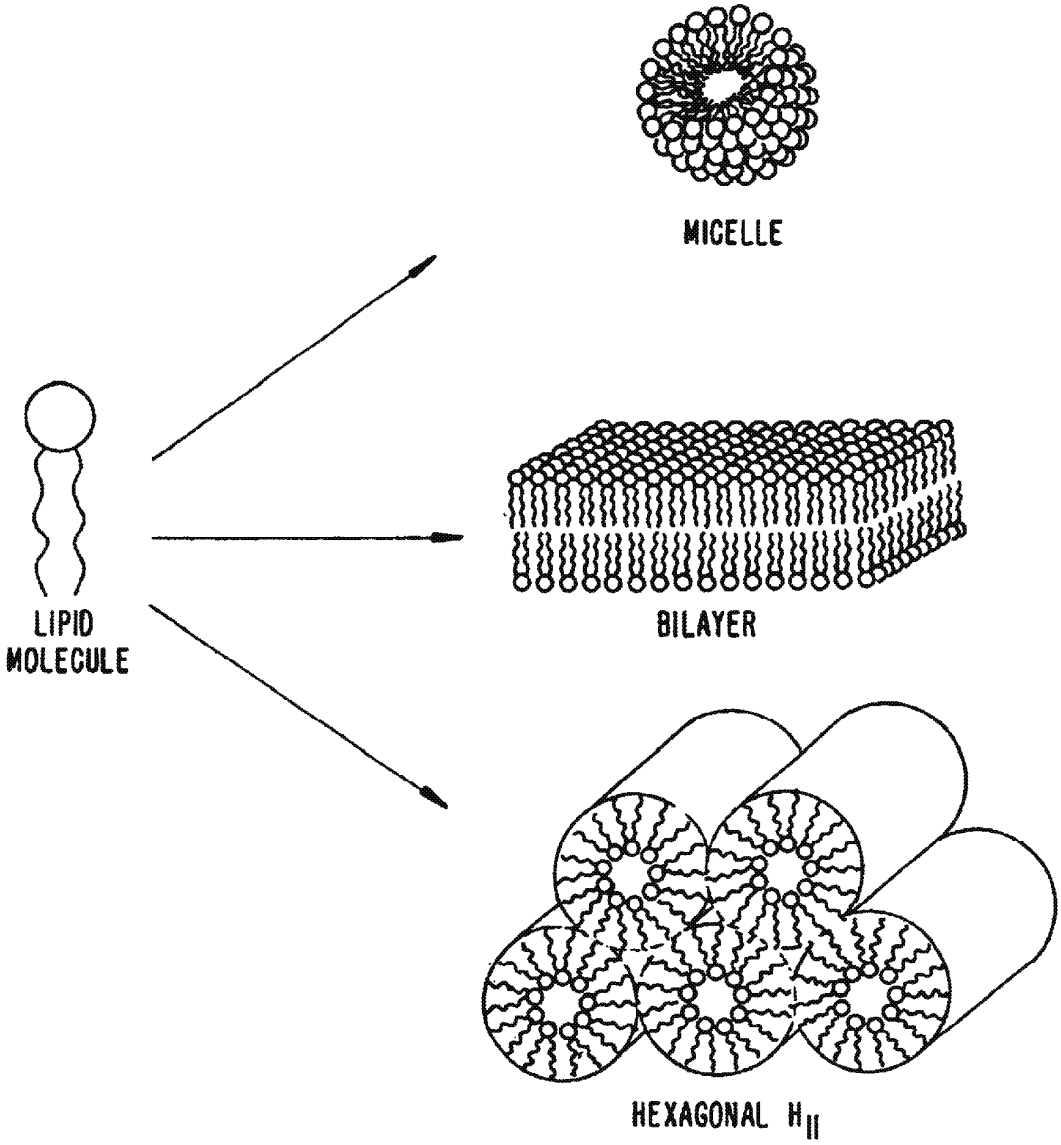


FIG. 1.

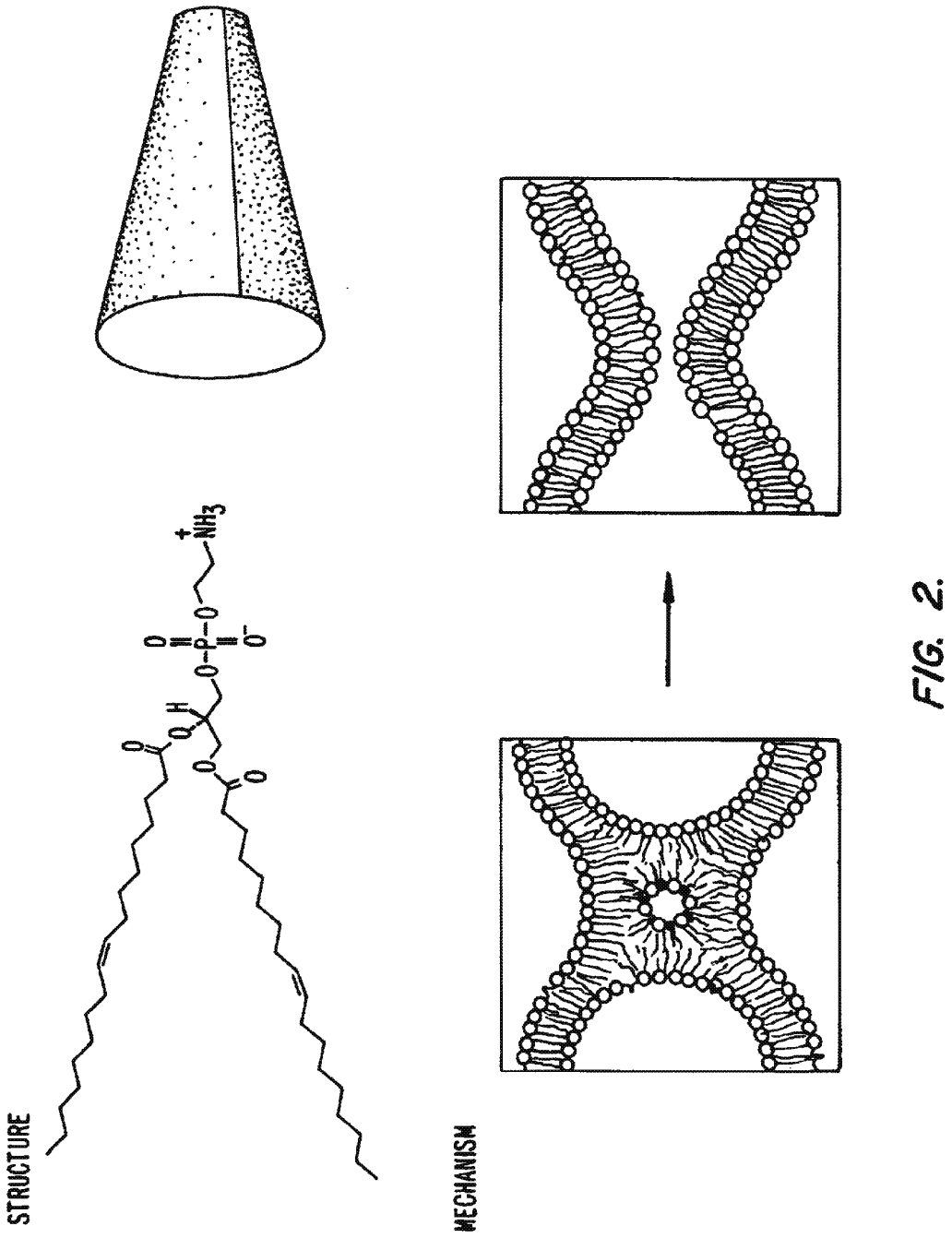


FIG. 2.

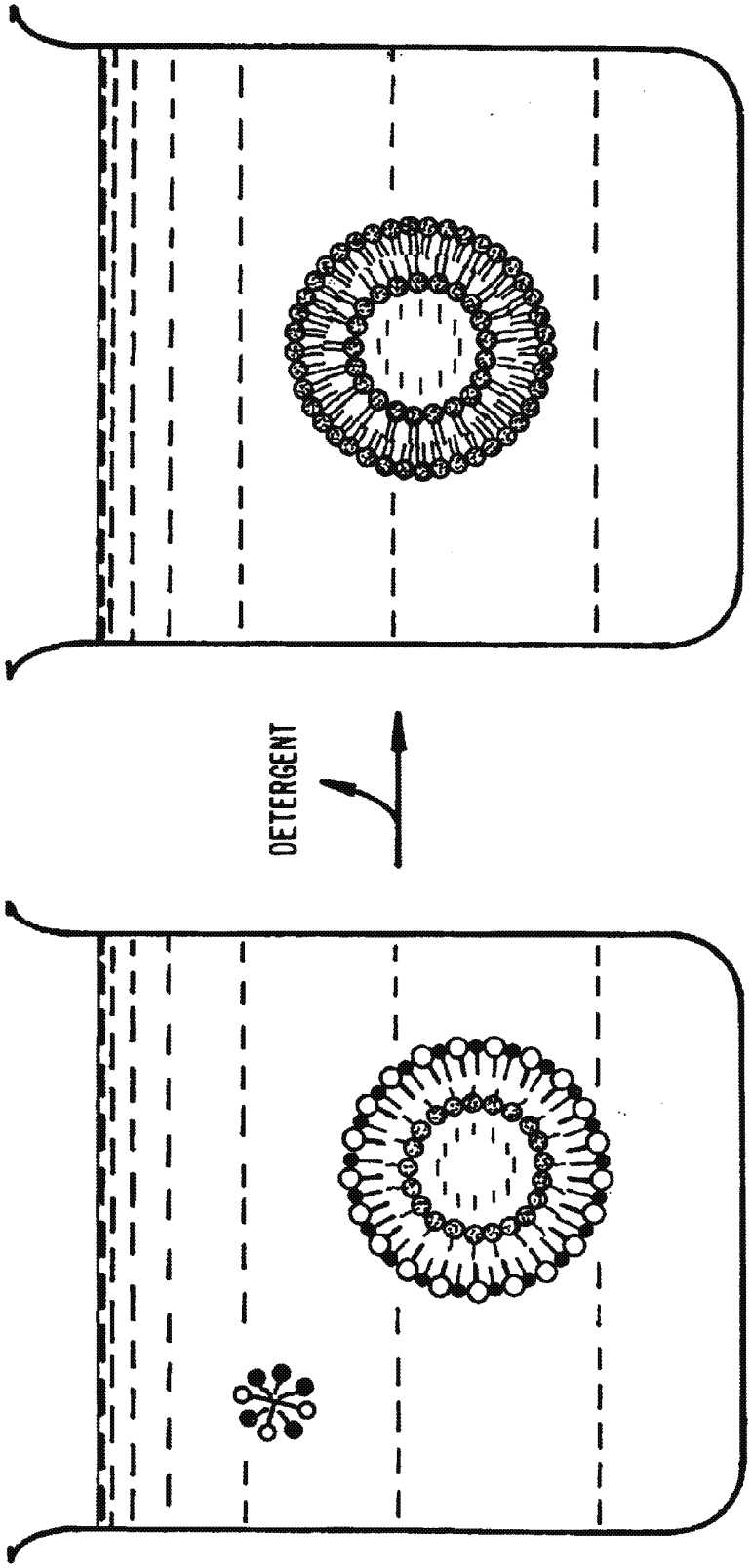
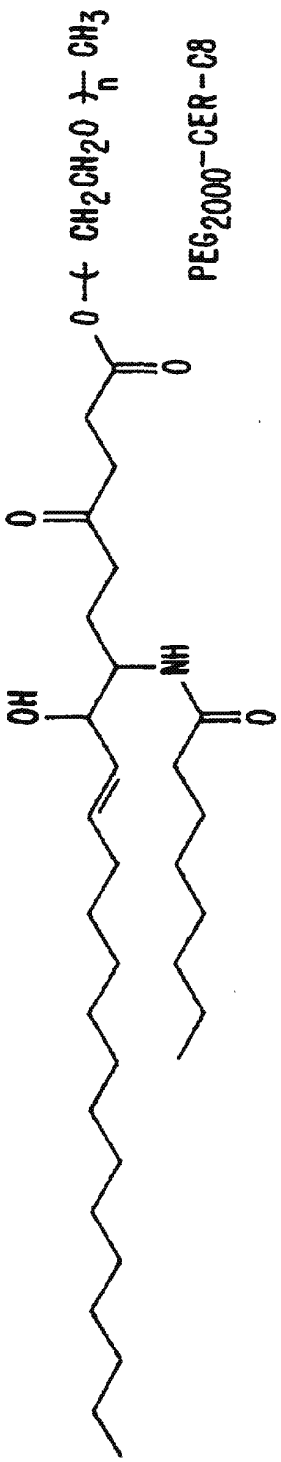


FIG. 3.



CERAMIDE	HALF-TIME FOR DISSOCIATION
PEG_{2000}—CER—C8	< 1 MINUTE
PEG_{2000}—CER—C14	10 MINUTES
PEG_{2000}—CER—C22	22 HOURS

FIG. 4.

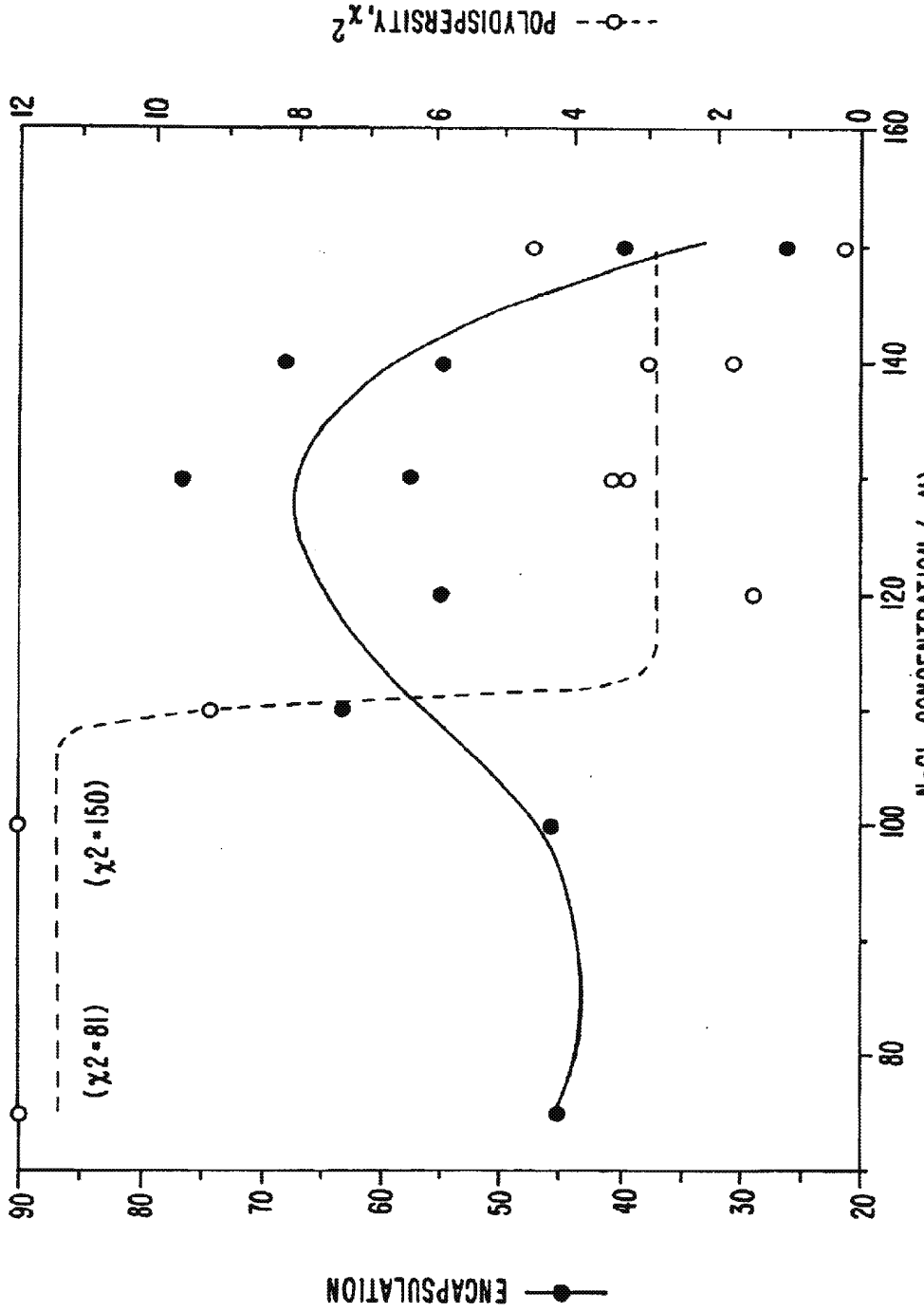

POLYDISPERSITY, χ^2
ENCAPSULATION
NaCl CONCENTRATION (mM)
(χ2=150)
(χ2=81)
8

FIG. 5.

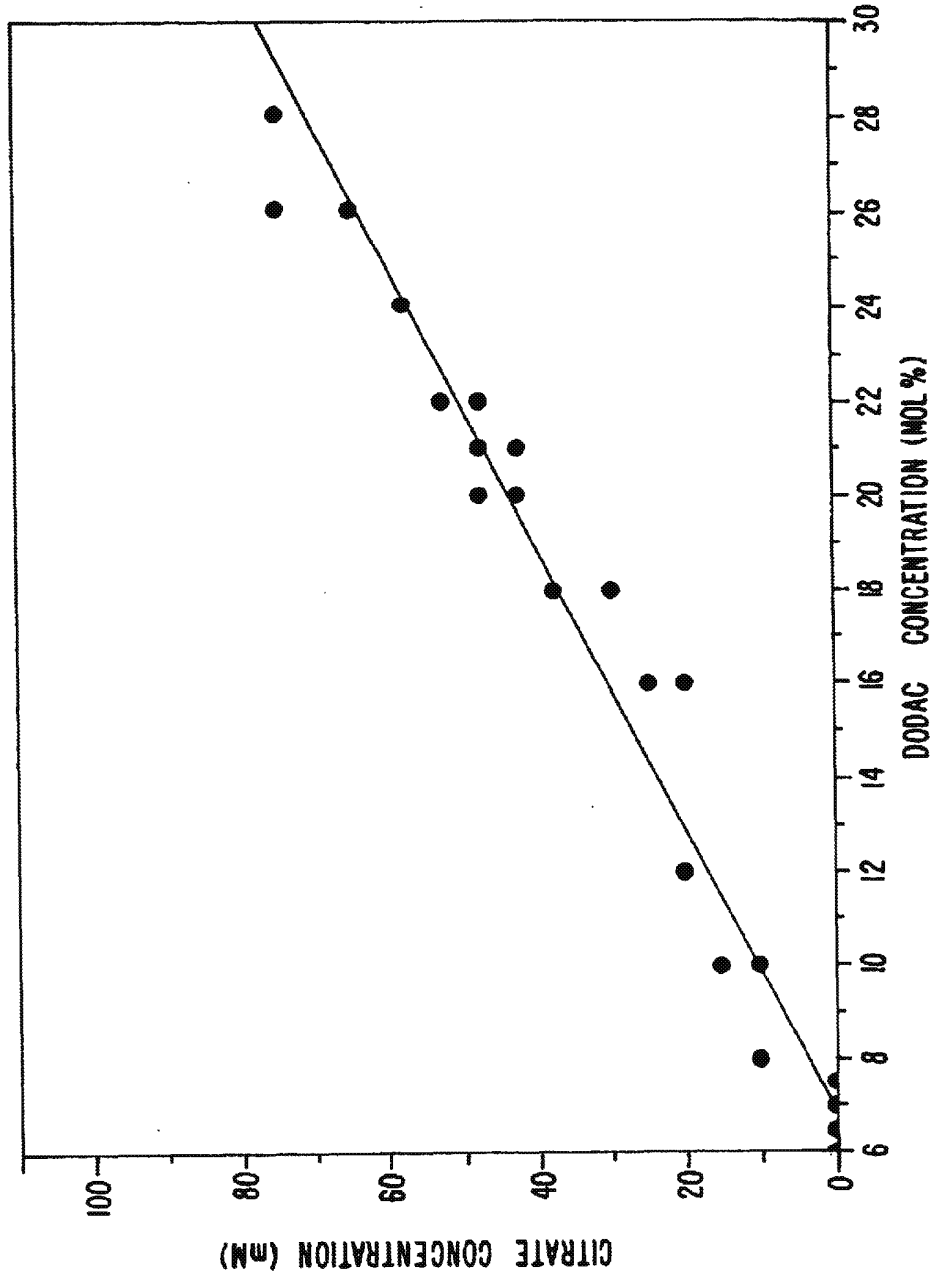

CITRATE CONCENTRATION (mM)
0
20
40
60
80
100
DODAC CONCENTRATION (MOL %)
6
8
10
12
14
16
18
20
22
24
26
28
30

FIG. 6.

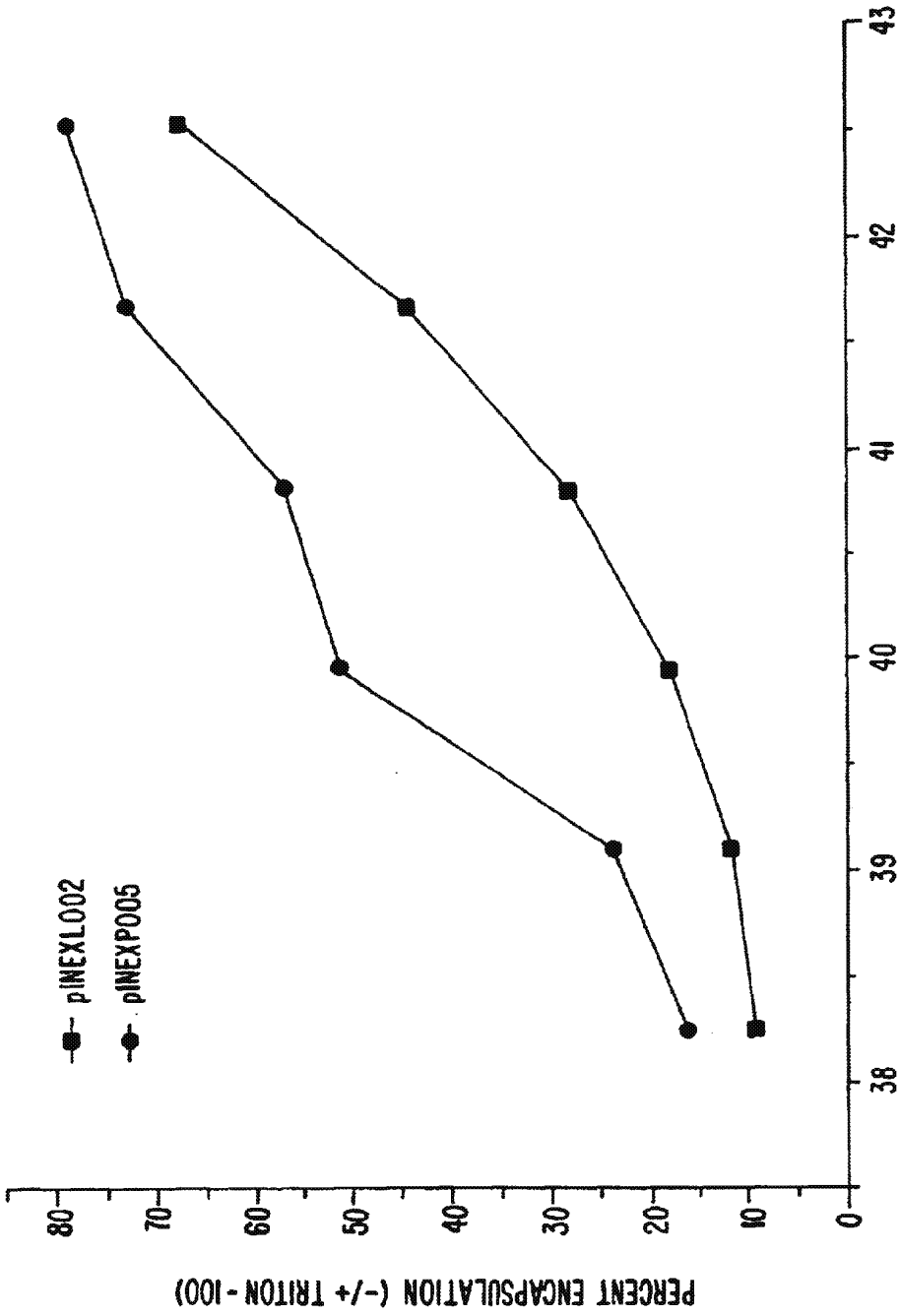

PERCENT ENCAPSULATION (-/+ TRITON - 100)
0
10
20
30
40
50
60
70
80
38
39
40
41
42
43
MOL % DODAC
pINEXL002
pINEXP005

FIG. 7.

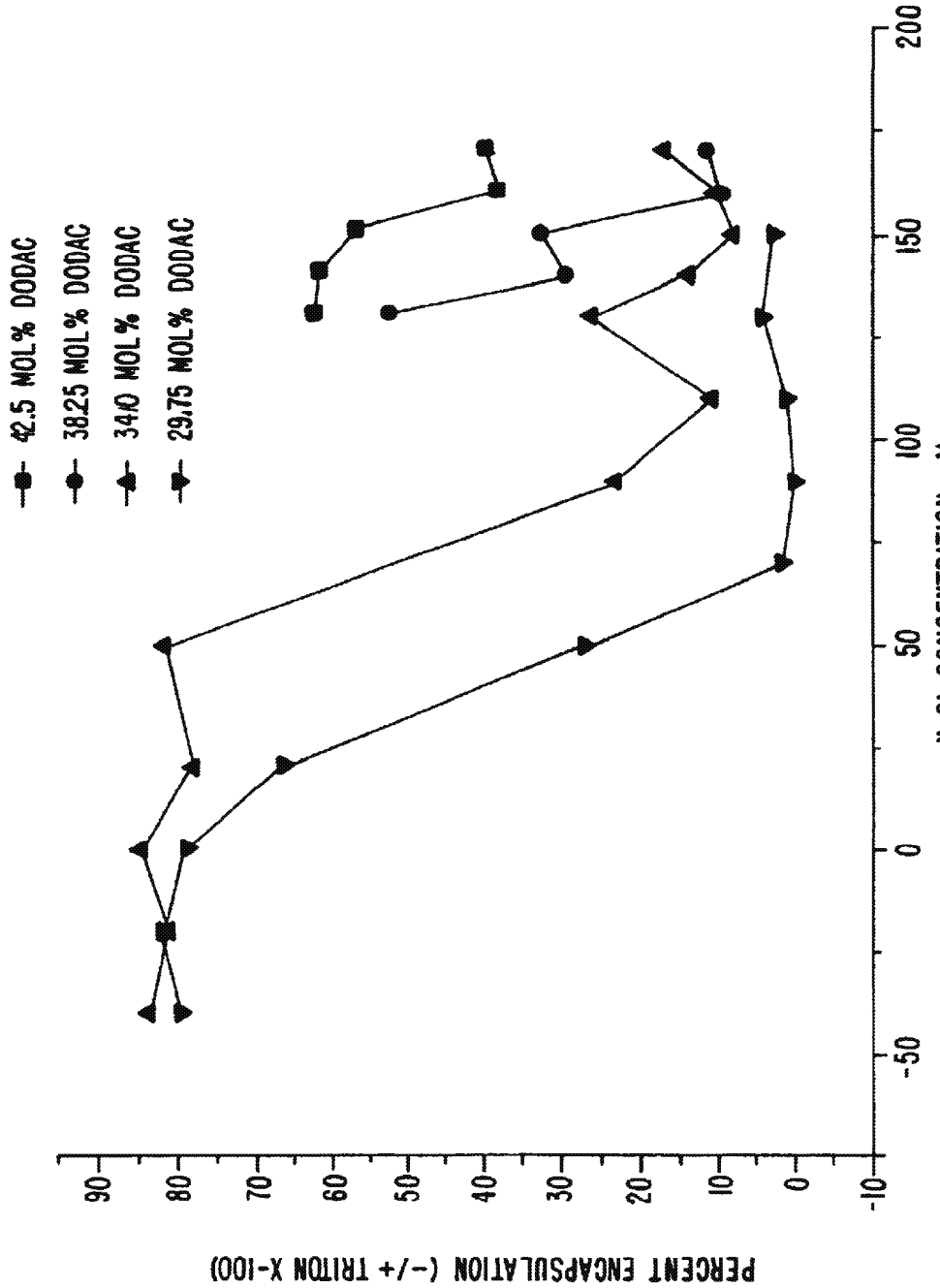

42.5 MOL% DODAC
38.25 MOL% DODAC
34.0 MOL% DODAC
29.75 MOL% DODAC
PERCENT ENCAPSULATION (-/+ TRITON X-100)
90
80
70
60
50
40
30
20
10
0
-10
-50
0
50
100
150
200
NaCl CONCENTRATION mM

FIG. 8.

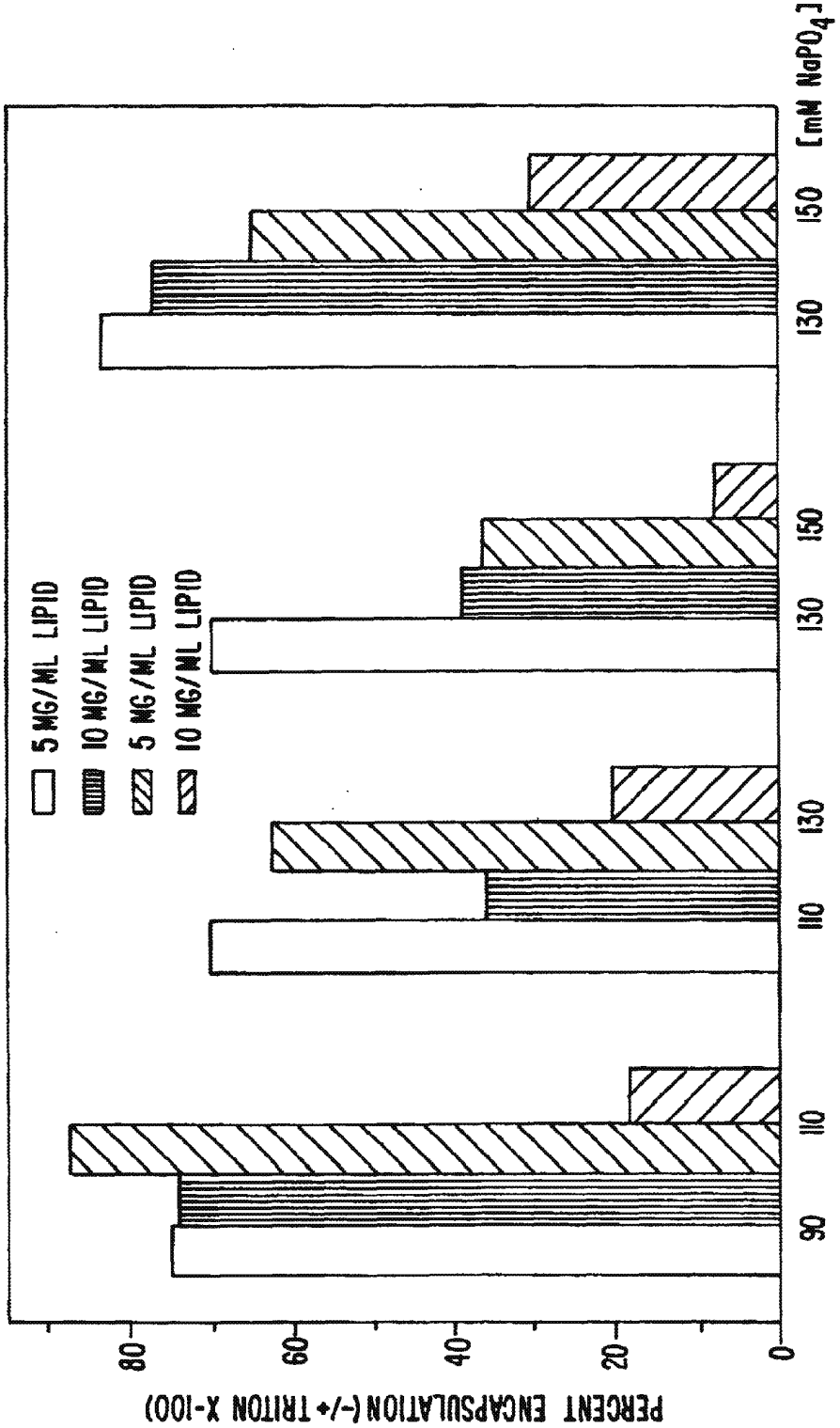

PERCENT ENCAPSULATION (-/+ TRITON X-100)
0
20
40
60
80
5 MG/ML LIPID
10 MG/ML LIPID
5 MG/ML LIPID
10 MG/ML LIPID
20
90
110
24
110
130
27
130
150
30
130
150
[mM NaPO4]
DODAC CONCENTRATION

FIG. 9.

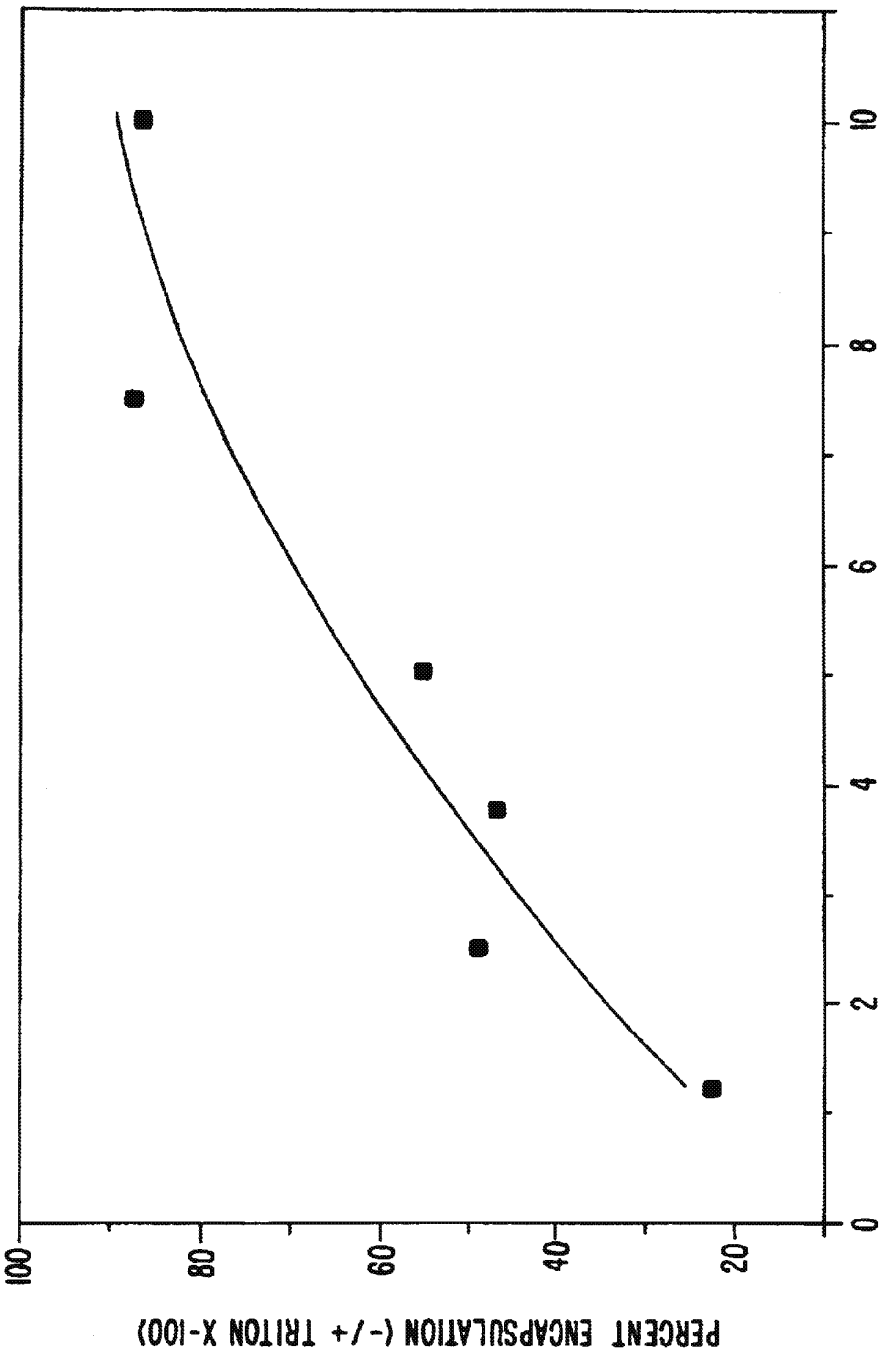

PERCENT ENCAPSULATION (-/+ TRITON X-100)
100
80
60
40
20
0
2
4
6
8
10
TOTAL LIPID CONCENTRATION

FIG. 10.

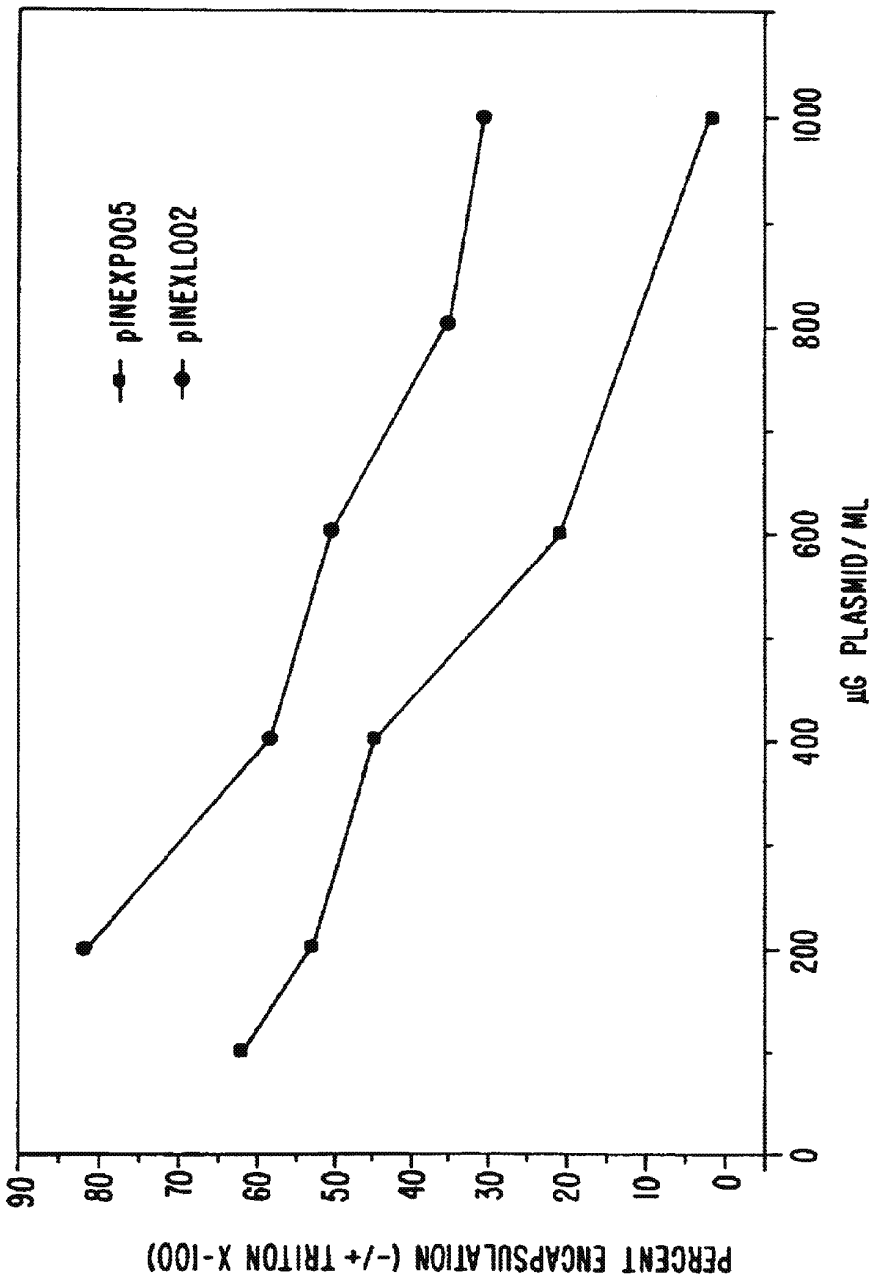

pINEXP005
pINEXL002
PERCENT ENCAPSULATION (-/+ TRITON X-100)
0
10
20
30
40
50
60
70
80
90
µG PLASMID/ML
0
200
400
600
800
1000

FIG 11.

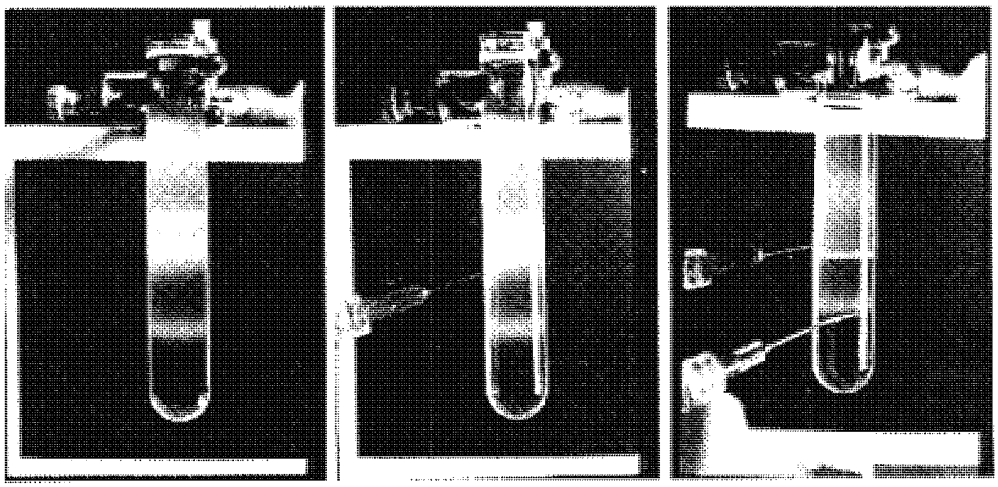


FIG. 12A. FIG. 12B. FIG. 12C.

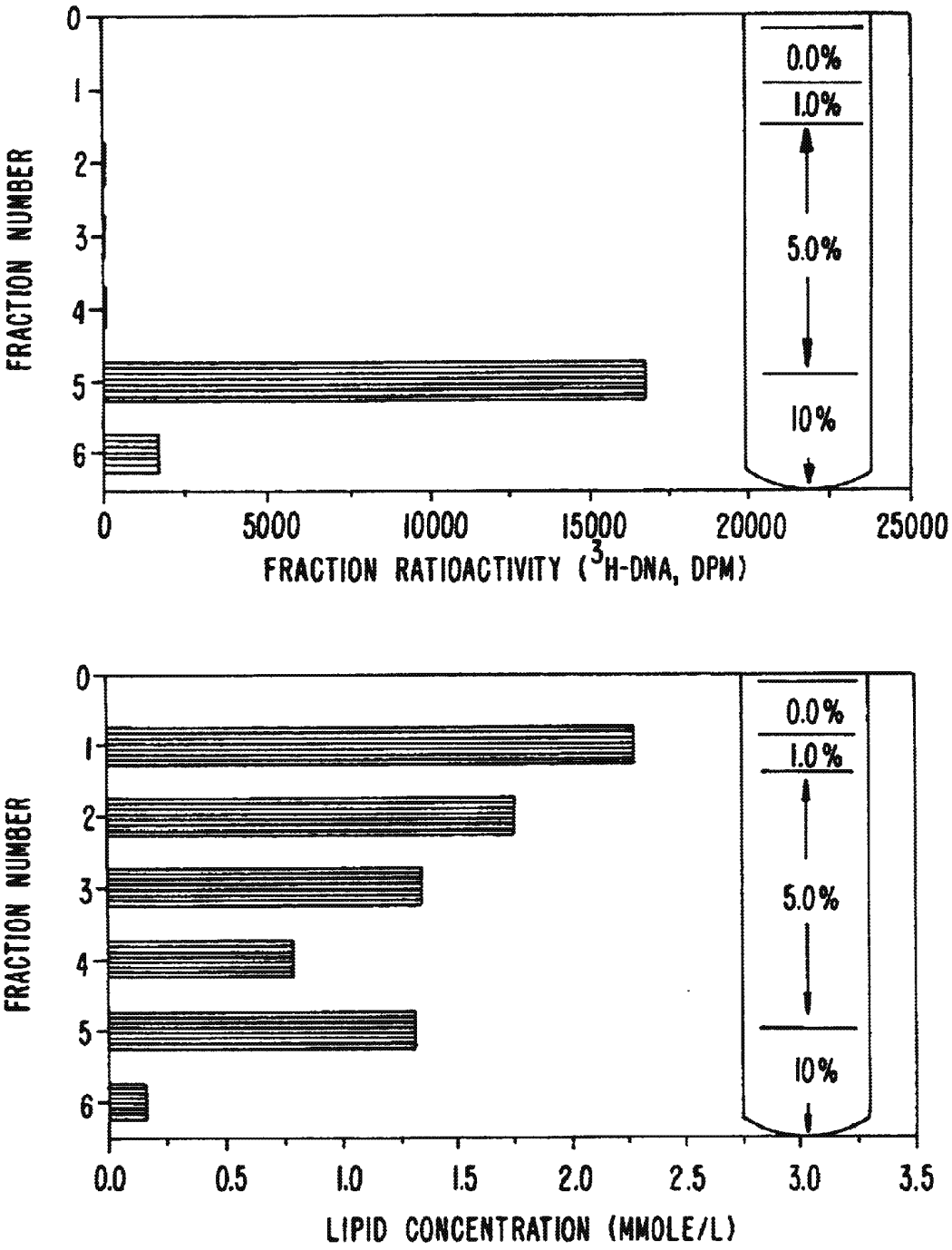

FRACTION NUMBER
0
1
2
3
4
5
6
0.0%
1.0%
5.0%
10%
0
5000
10000
15000
20000
25000
FRACTION RATIOACTIVITY (^{3}H-DNA, DPM)
FRACTION NUMBER
0
1
2
3
4
5
6
0.0%
1.0%
5.0%
10%
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
LIPID CONCENTRATION (MMOLE/L)

FIG. 13.

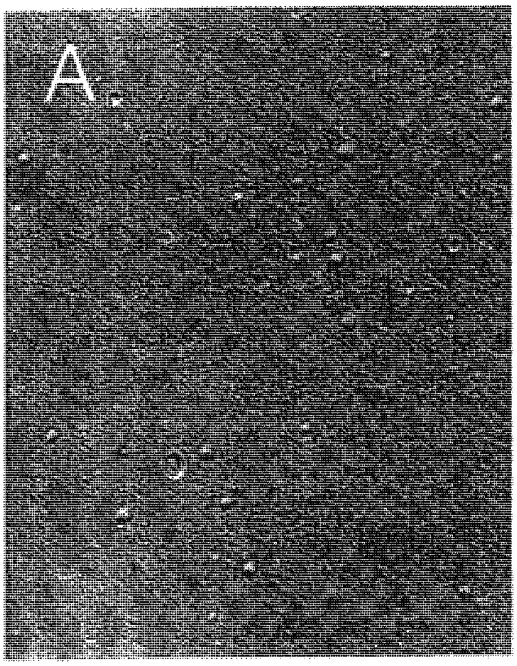


FIG. 14A.

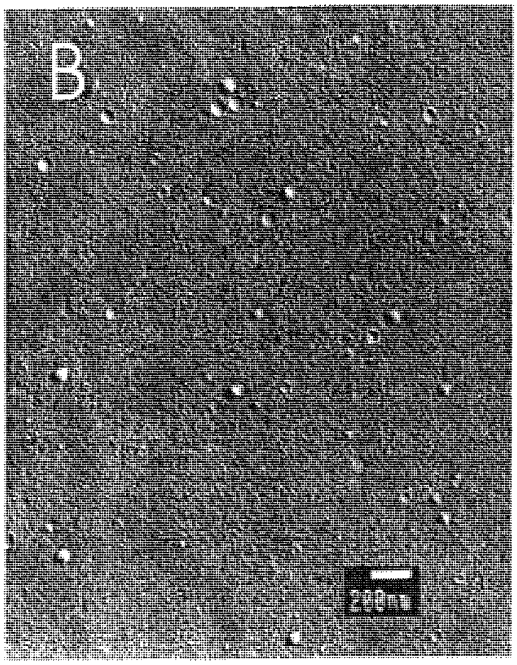


FIG. 14B.

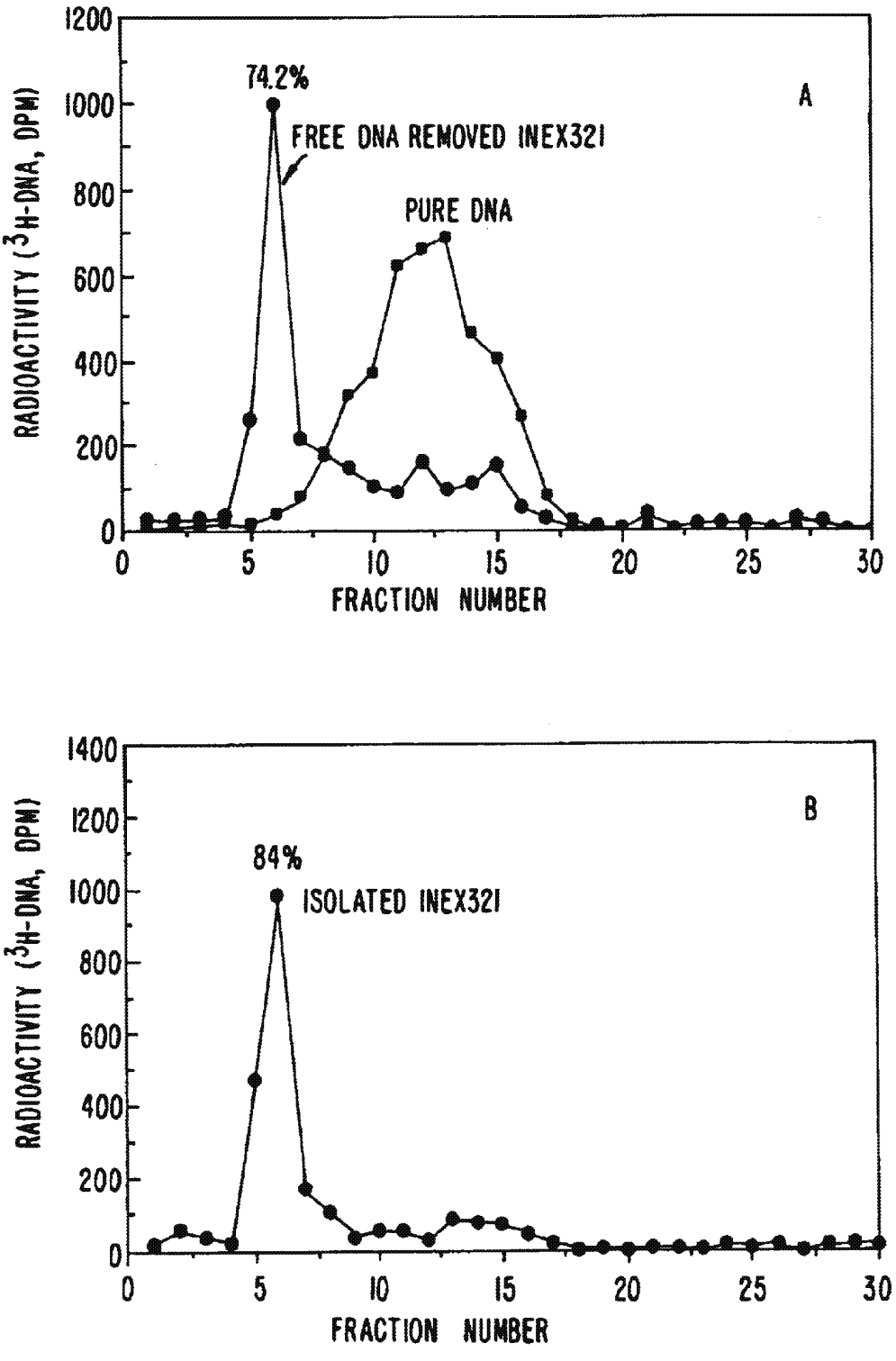

A
74.2%
FREE DNA REMOVED INEX321
PURE DNA
RADIOACTIVITY (^{3}H-DNA, DPM)
FRACTION NUMBER
B
84%
ISOLATED INEX321
RADIOACTIVITY (^{3}H-DNA, DPM)
FRACTION NUMBER

FIG. 15.

LANE	1	2	3	4	5	6	7	8	9	10	11	12
SERUM	–	+	–	–	–	+	–	–	–	+	–	–
DNA'se	–	–	+	+	–	–	+	+	–	–	+	+
CARRIER	+	–	–	+	+	–	–	+	+	–	–	+

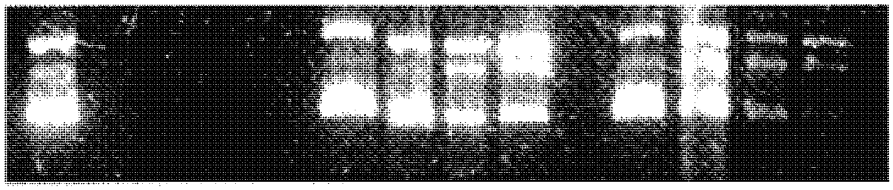


FIG. 16.

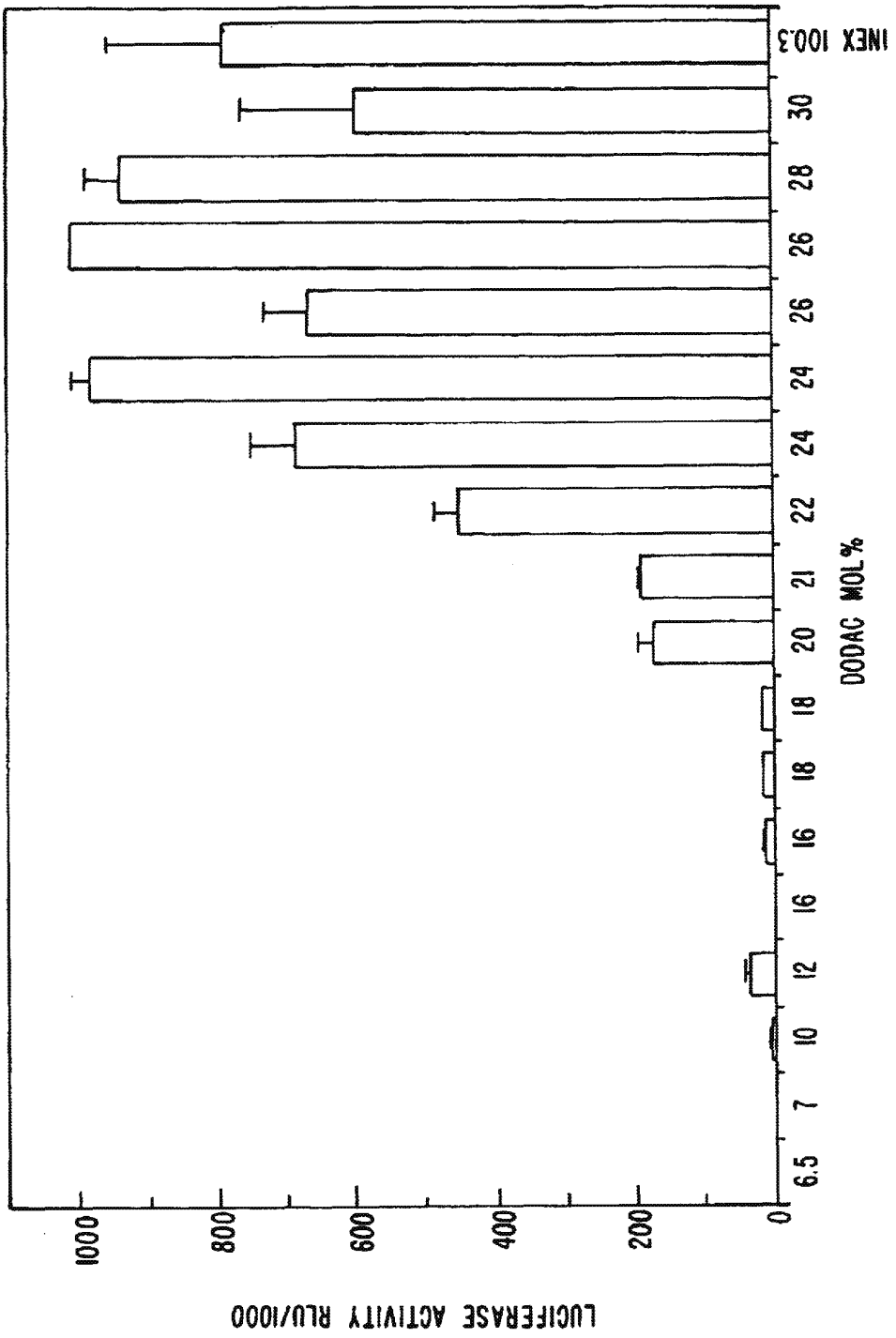

LUCIFERASE ACTIVITY RLU/1000
1000
800
600
400
200
0
DODAC MOL %
6.5
7
10
12
16
16
18
18
20
21
22
24
24
26
26
28
30
INDEX 100.3

FIG. 17.

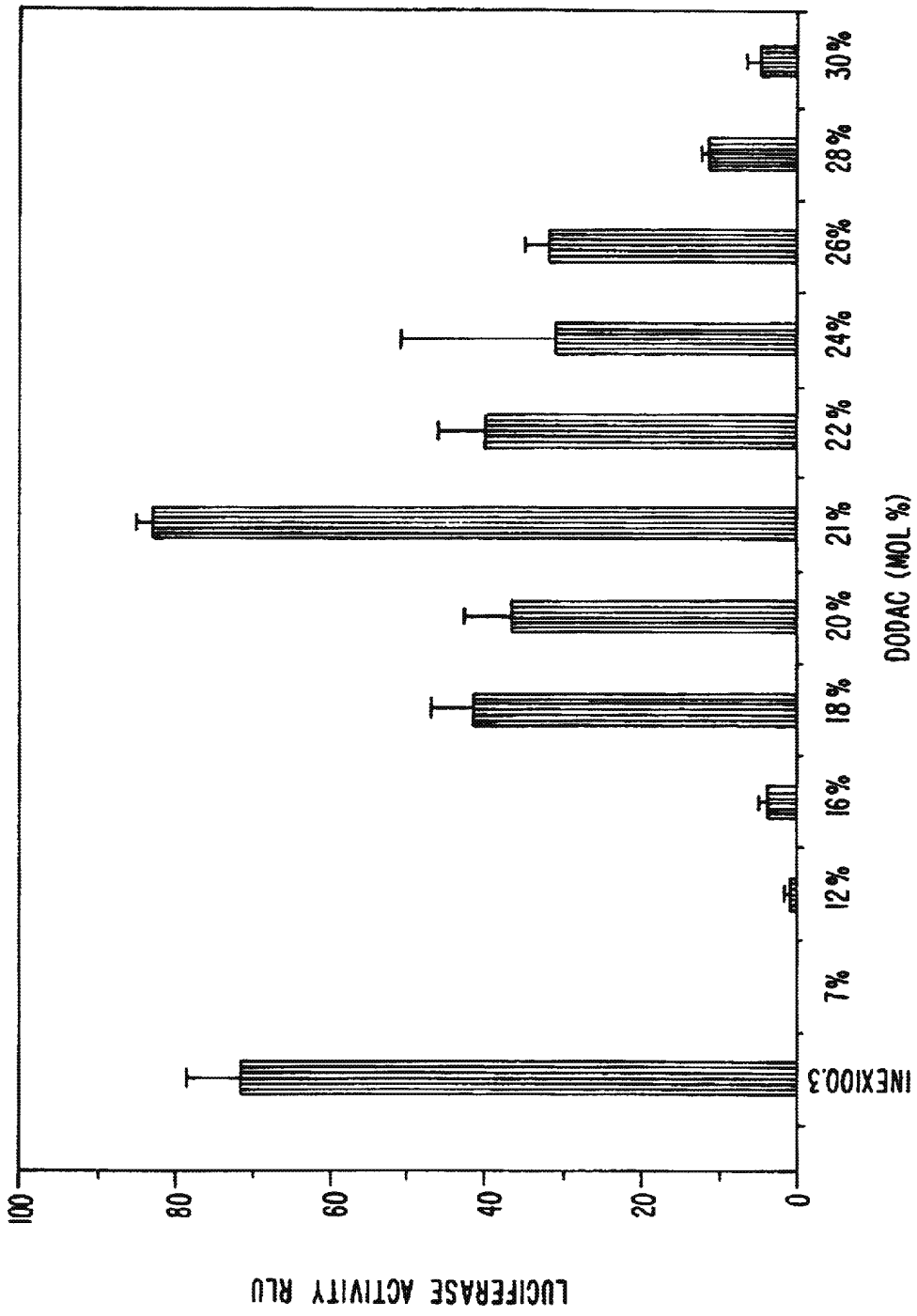

LUCIFERASE ACTIVITY RLU
100
80
60
40
20
0
INE.XIOO.3
7%
12%
16%
18%
20%
21%
22%
24%
26%
28%
30%
DODAC (MOL %)

FIG. 18.

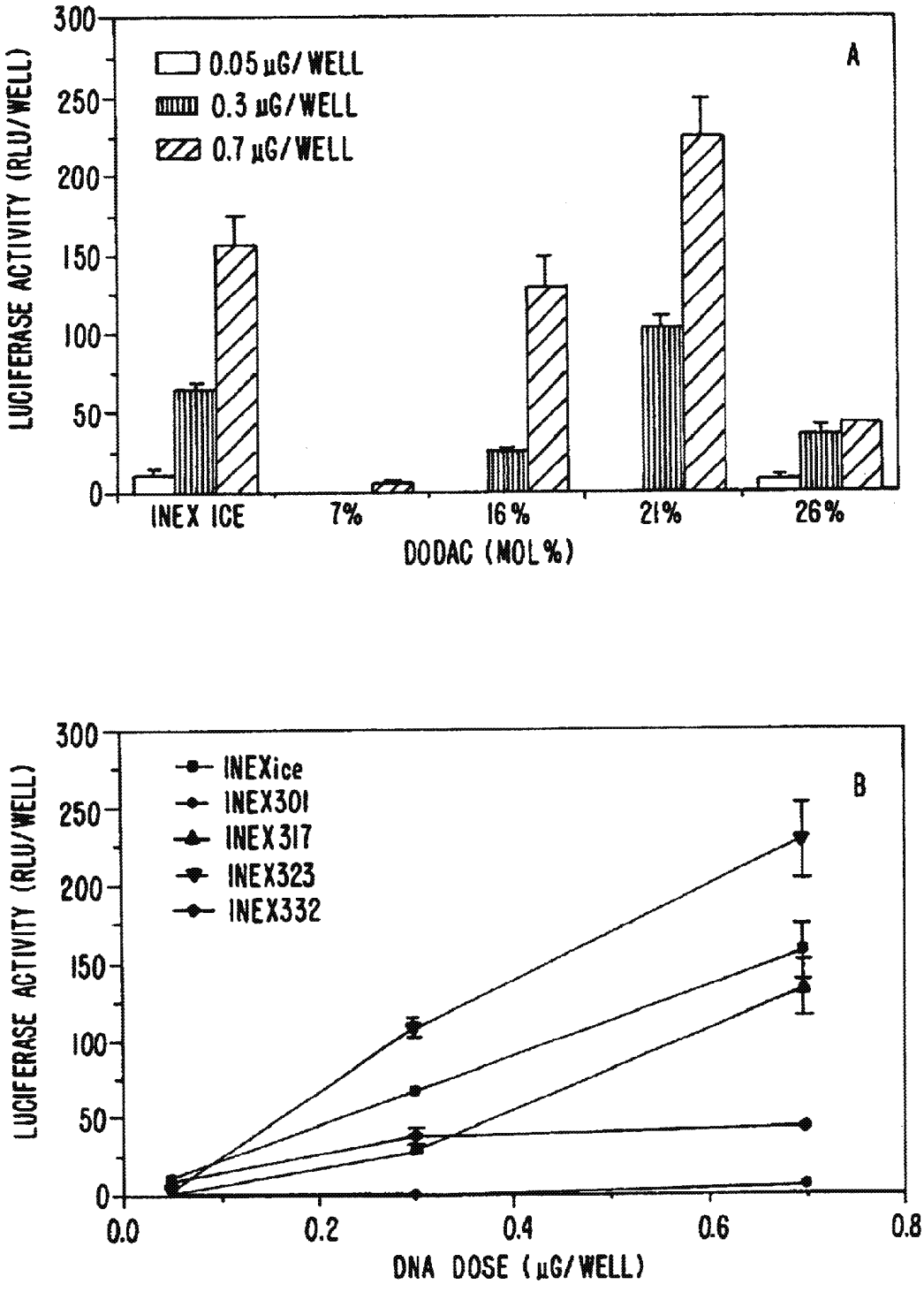

300
250
200
150
100
50
0
LUCIFERASE ACTIVITY (RLU/WELL)
0.05 µG/WELL
0.3 µG/WELL
0.7 µG/WELL
A
INEX ICE
7%
16%
21%
26%
DODAC (MOL%)
300
250
200
150
100
50
0
LUCIFERASE ACTIVITY (RLU/WELL)
INEXice
INEX301
INEX317
INEX323
INEX332
B
0.0
0.2
0.4
0.6
0.8
DNA DOSE (µG/WELL)

FIG. 19.

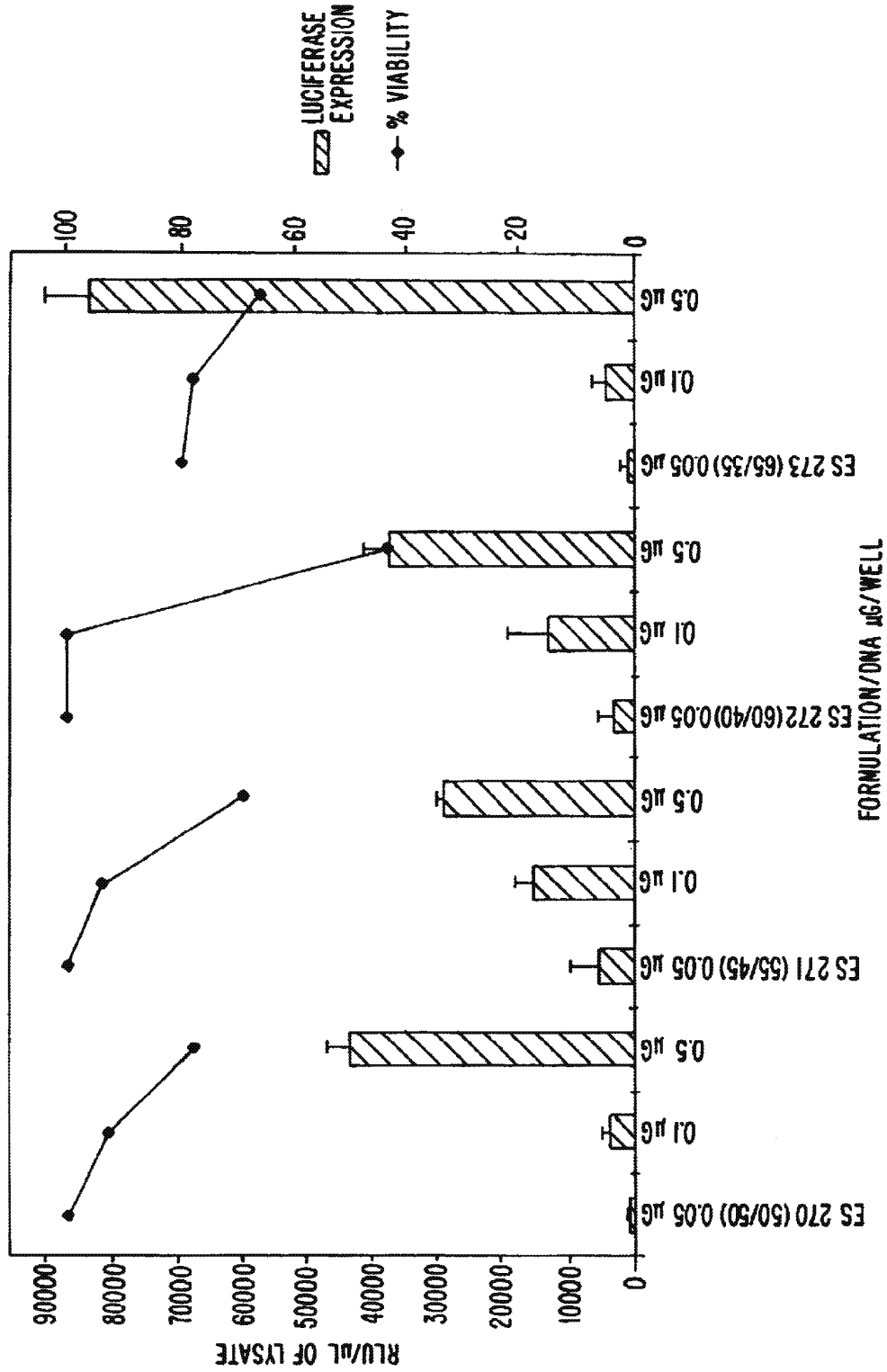

LUCIFERASE EXPRESSION
% VIABILITY
RLU/uL OF LYSATE
FORMULATION/DNA uG/WELL
ES 270 (50/50) 0.05 uG
0.1 uG
0.5 uG
ES 271 (55/45) 0.05 uG
0.1 uG
0.5 uG
ES 272(60/40)0.05 uG
0.1 uG
0.5 uG
ES 273 (65/35) 0.05 uG
0.1 uG
0.5 uG

FIG. 20.

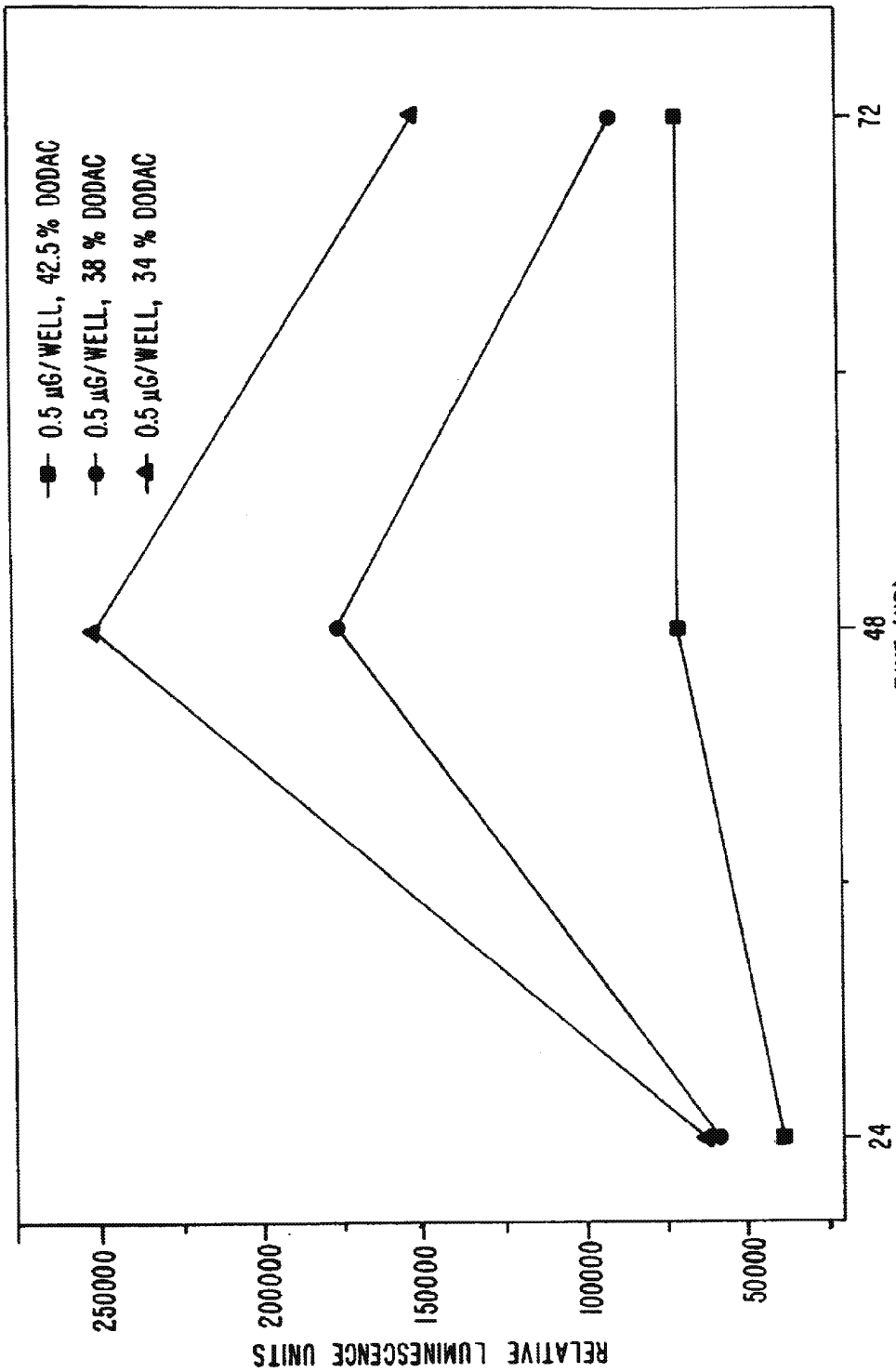
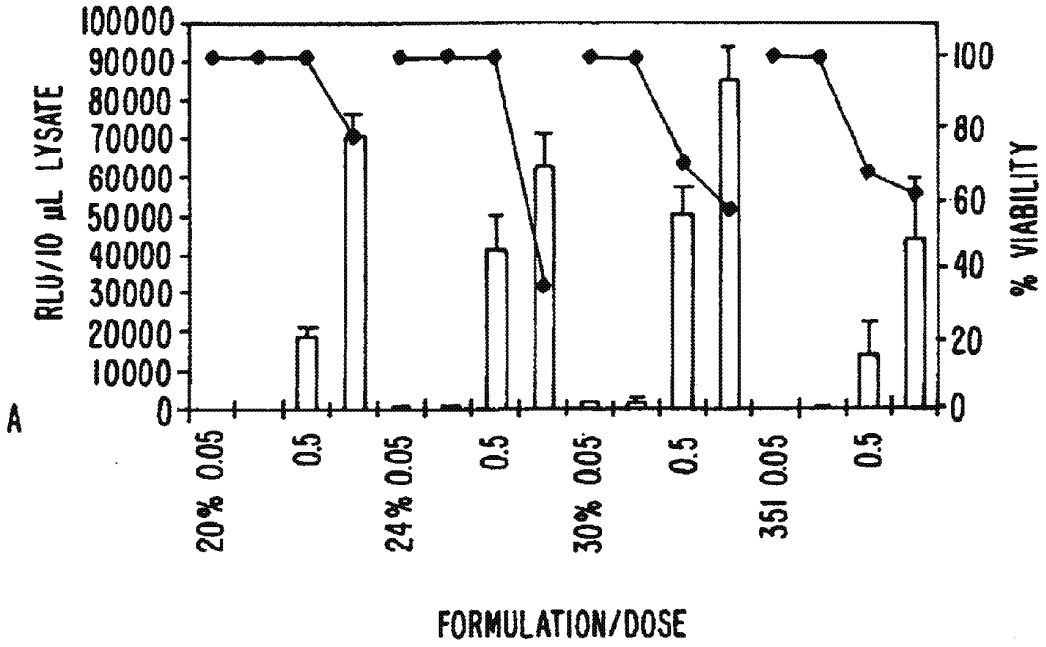

0.5 µG/WELL, 42.5 % DODAC
0.5 µG/WELL, 38 % DODAC
0.5 µG/WELL, 34 % DODAC
RELATIVE LUMINESCENCE UNITS
250000
200000
150000
100000
50000
24
48
72
TIME (HR)

FIG. 2I.


A
RLU/10 µL LYSATE
% VIABILITY
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
100
80
60
40
20
0
20% 0.05
0.5
24% 0.05
0.5
30% 0.05
0.5
351 0.05
0.5
FORMULATION/DOSE

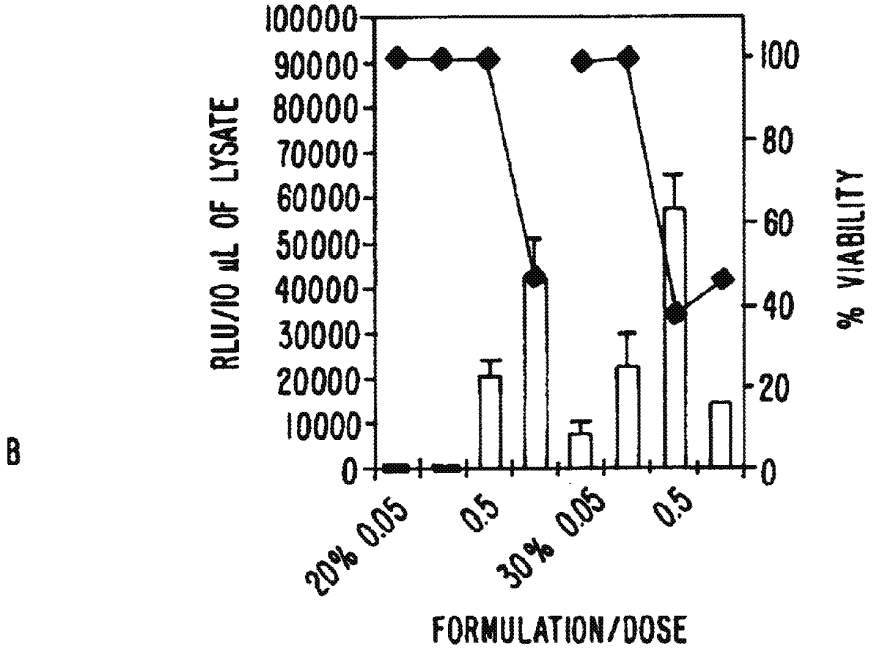

B
RLU/10 µL OF LYSATE
% VIABILITY
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
100
80
60
40
20
0
20% 0.05
0.5
30% 0.05
0.5
FORMULATION/DOSE

FIG. 22.

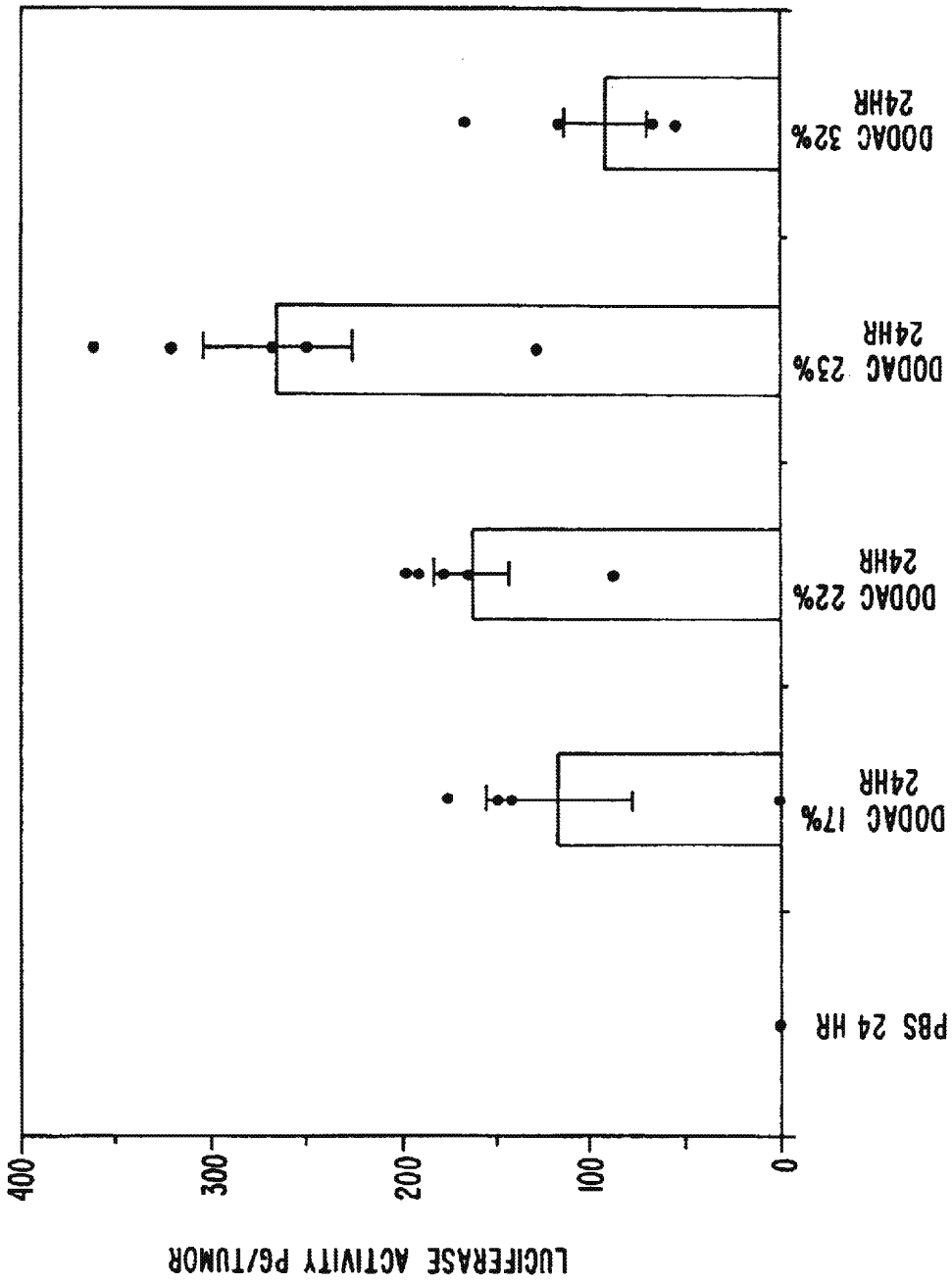

DODAC 32% 24HR
DODAC 23% 24HR
DODAC 22% 24HR
DODAC 17% 24HR
PBS 24 HR
400
300
200
100
0
LUCIFERASE ACTIVITY PG/TUMOR

FIG. 23.

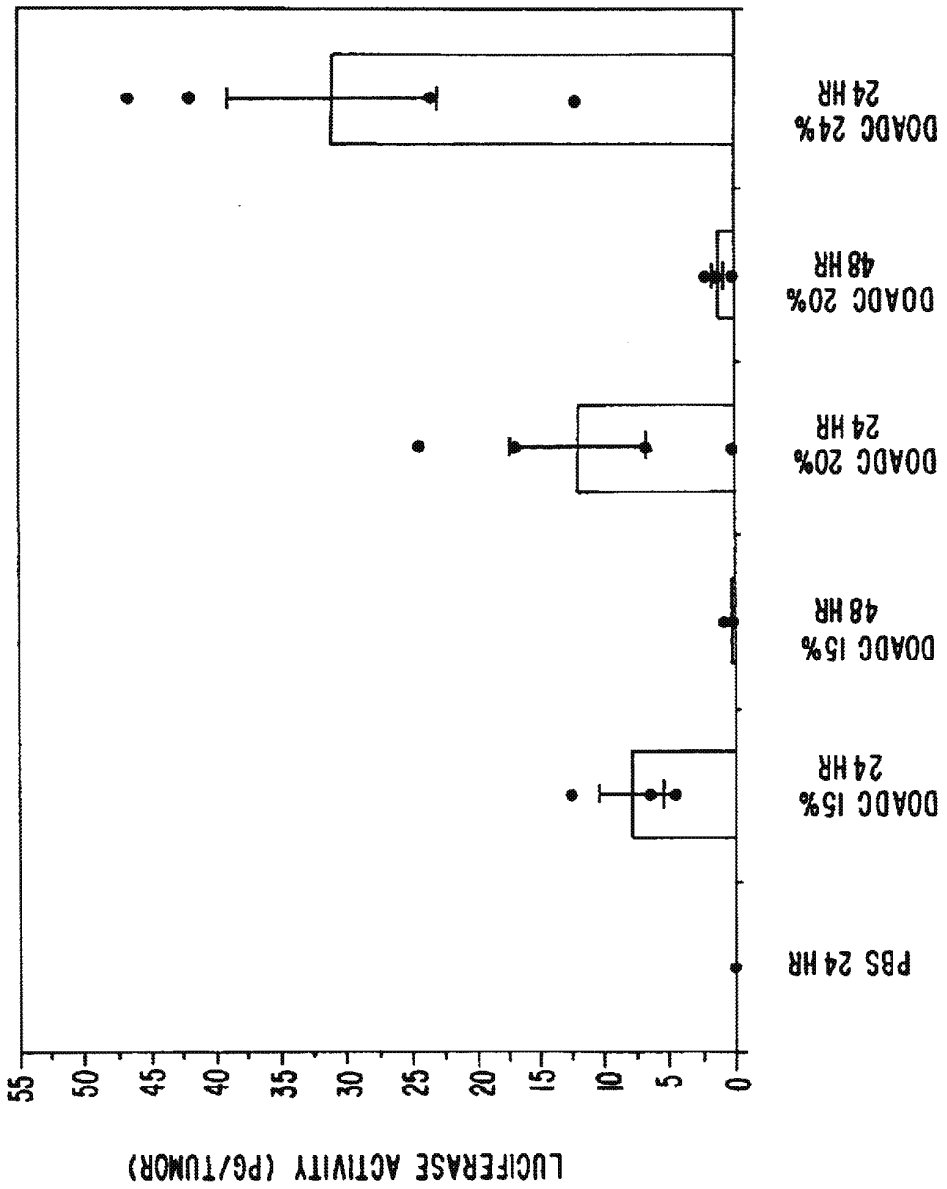

LUCIFERASE ACTIVITY (PG/TUMOR)
55
50
45
40
35
30
25
20
15
10
5
0
PBS 24 HR
DOADC 15%
24 HR
DOADC 15%
48 HR
DOADC 20%
24 HR
DOADC 20%
48 HR
DOADC 24%
24 HR

FIG. 24.

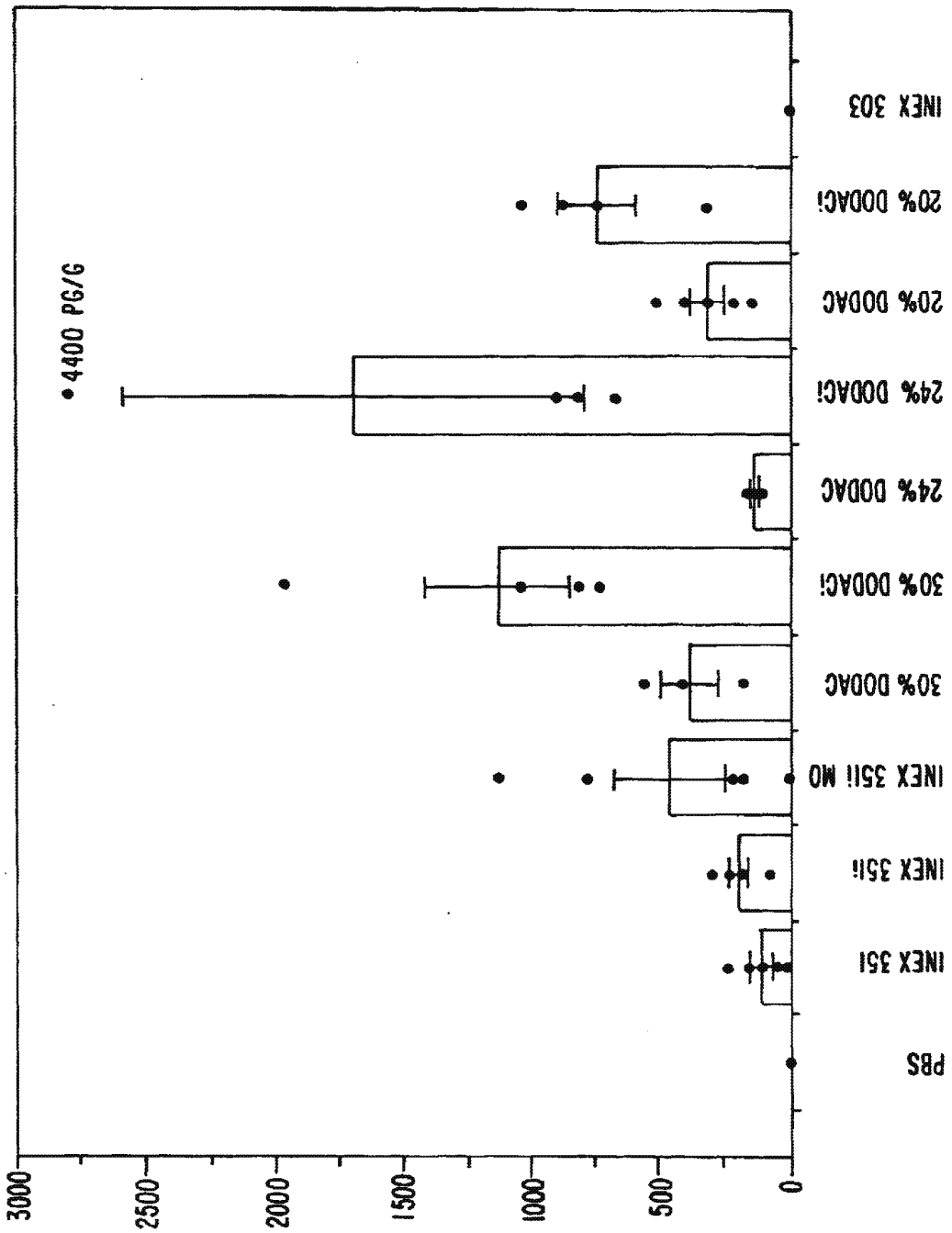

• 4400 PG/G
3000
2500
2000
1500
1000
500
0
INEX 303
20% DODACi
20% DODAC
24% DODACi
24% DODAC
30% DODACi
30% DODAC
INEX 351i MO
INEX 351i
INEX 351
PBS

FIG. 25.

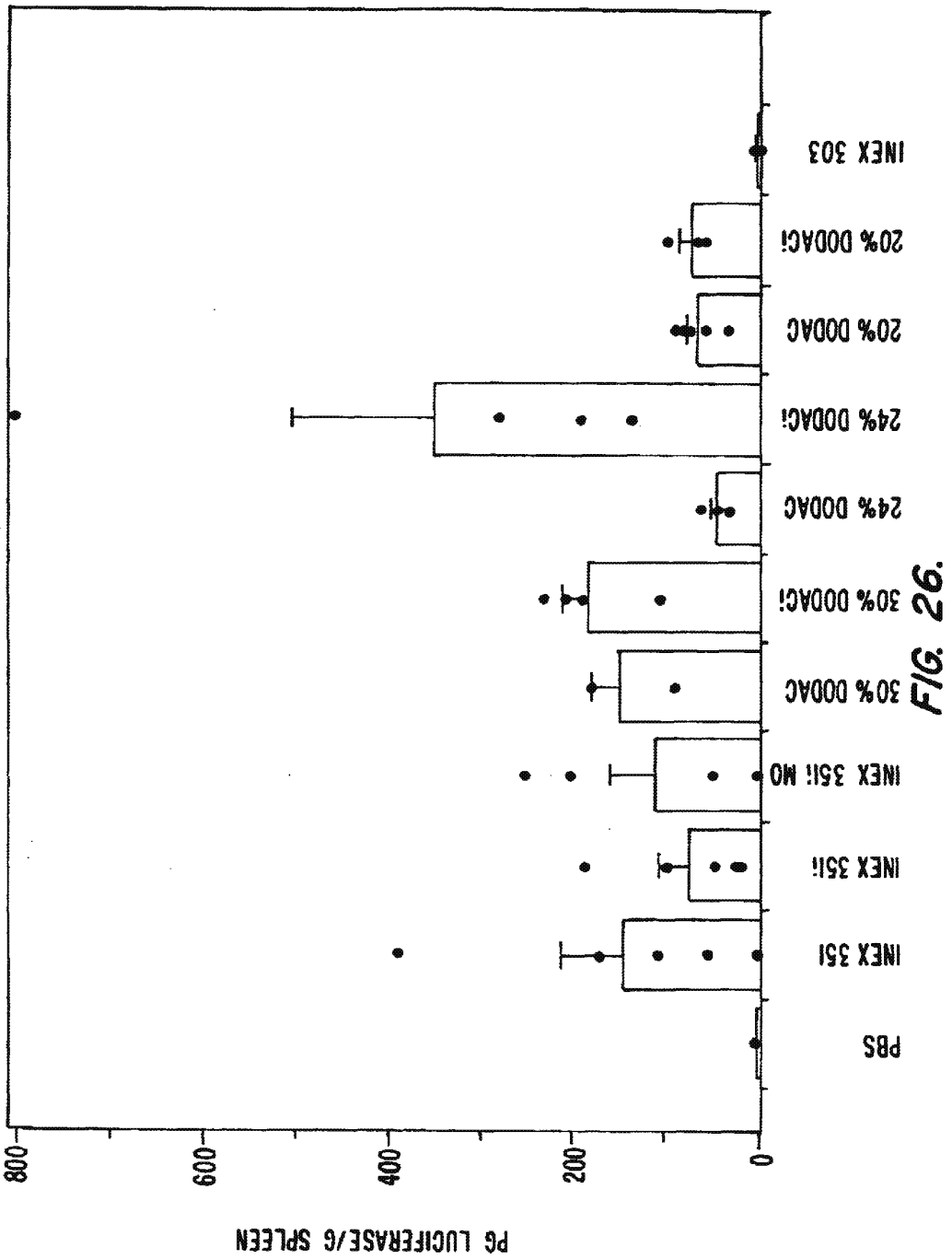

INEX 303
20% DODAC!
20% DODAC
24% DODAC!
24% DODAC
30% DODAC!
30% DODAC
INEX 351! MO
INEX 351!
INEX 351
PBS
800
600
400
200
0
P6 LUCIFERASE/6 SPLEEN

FIG. 26.

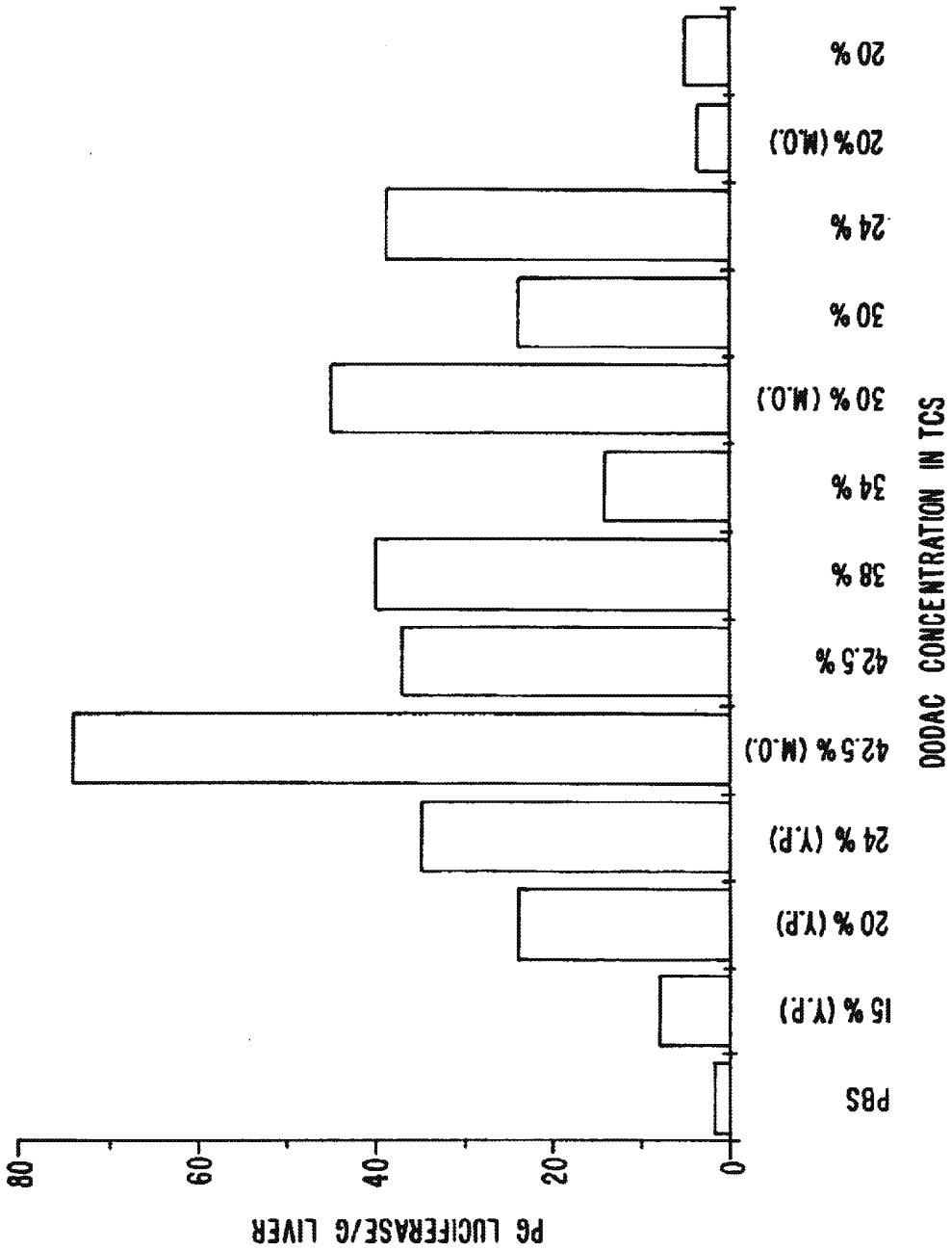

PG LUCIFERASE/G LIVER
0
20
40
60
80
PBS
15 % (Y.P.)
20 % (Y.P.)
24 % (Y.P.)
42.5 % (M.O.)
42.5 %
38 %
34 %
30 % (M.O.)
30 %
24 %
20% (M.O.)
20 %
DODAC CONCENTRATION IN TCS

FIG. 27.

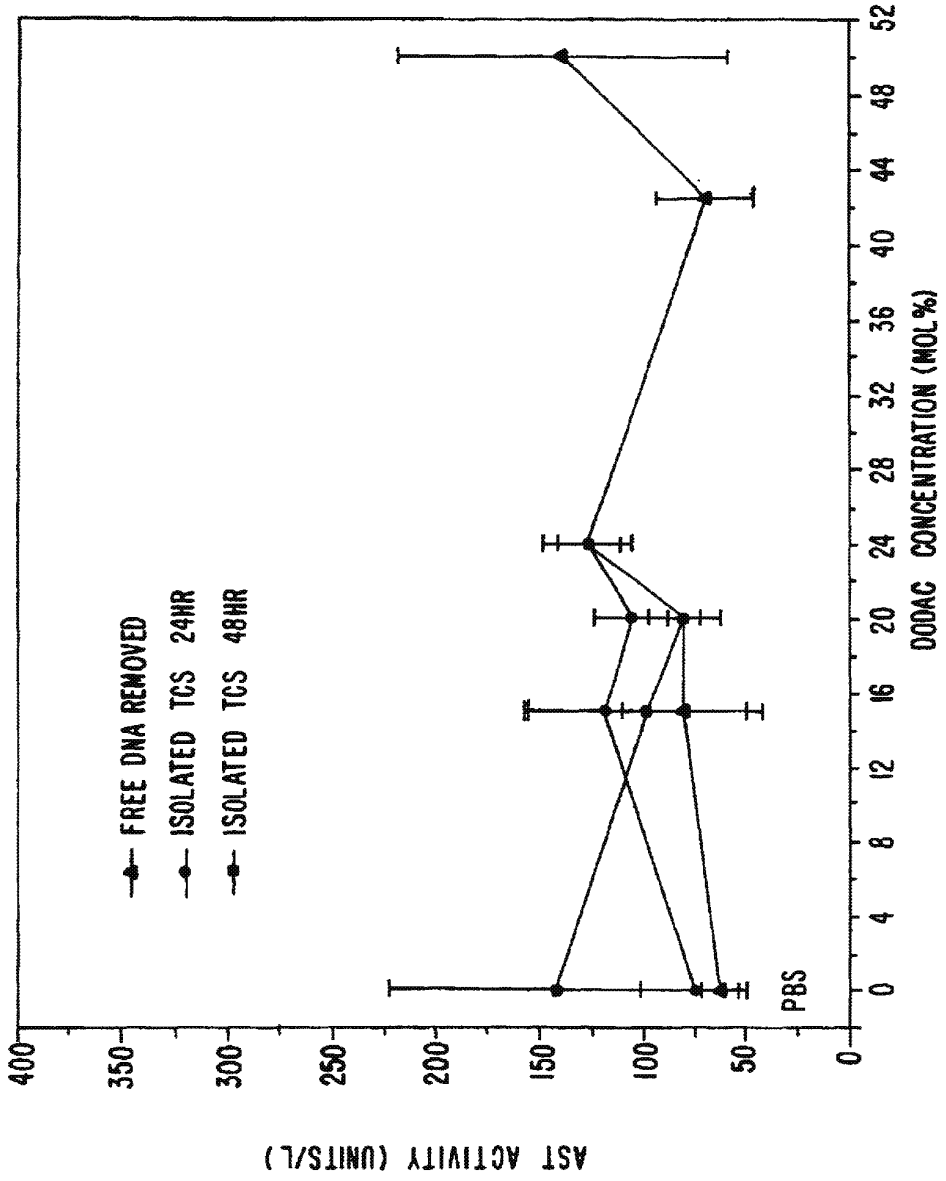

AST ACTIVITY (UNITS/L)
400
350
300
250
200
150
100
50
0
0
4
8
12
16
20
24
28
32
36
40
44
48
52
DODAC CONCENTRATION (MOL %)
PBS
FREE DNA REMOVED
ISOLATED TCS 24HR
ISOLATED TCS 48HR

FIG. 28.

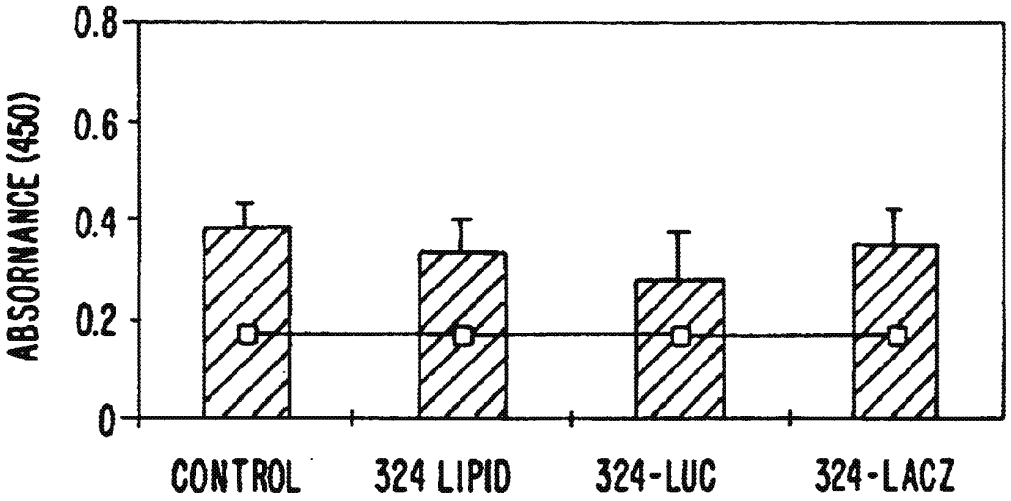

ABSORNANCE (450)
0.8
0.6
0.4
0.2
0
CONTROL
324 LIPID
324-LUC
324-LACZ

FIG. 29.

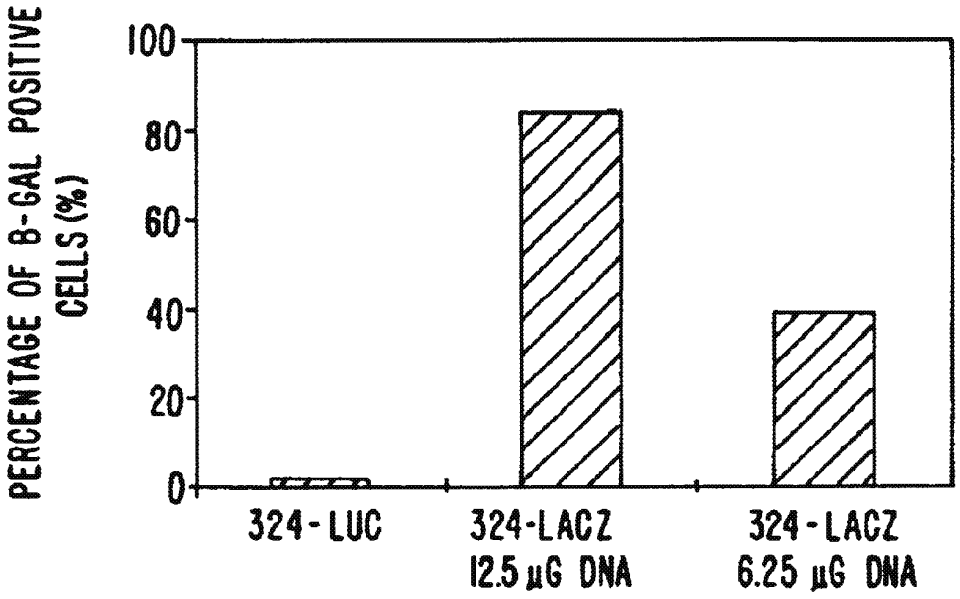

PERCENTAGE OF B-GAL POSITIVE CELLS (%)
100
80
60
40
20
0
324-LUC
324-LACZ 12.5 µG DNA
324-LACZ 6.25 µG DNA

FIG. 30.

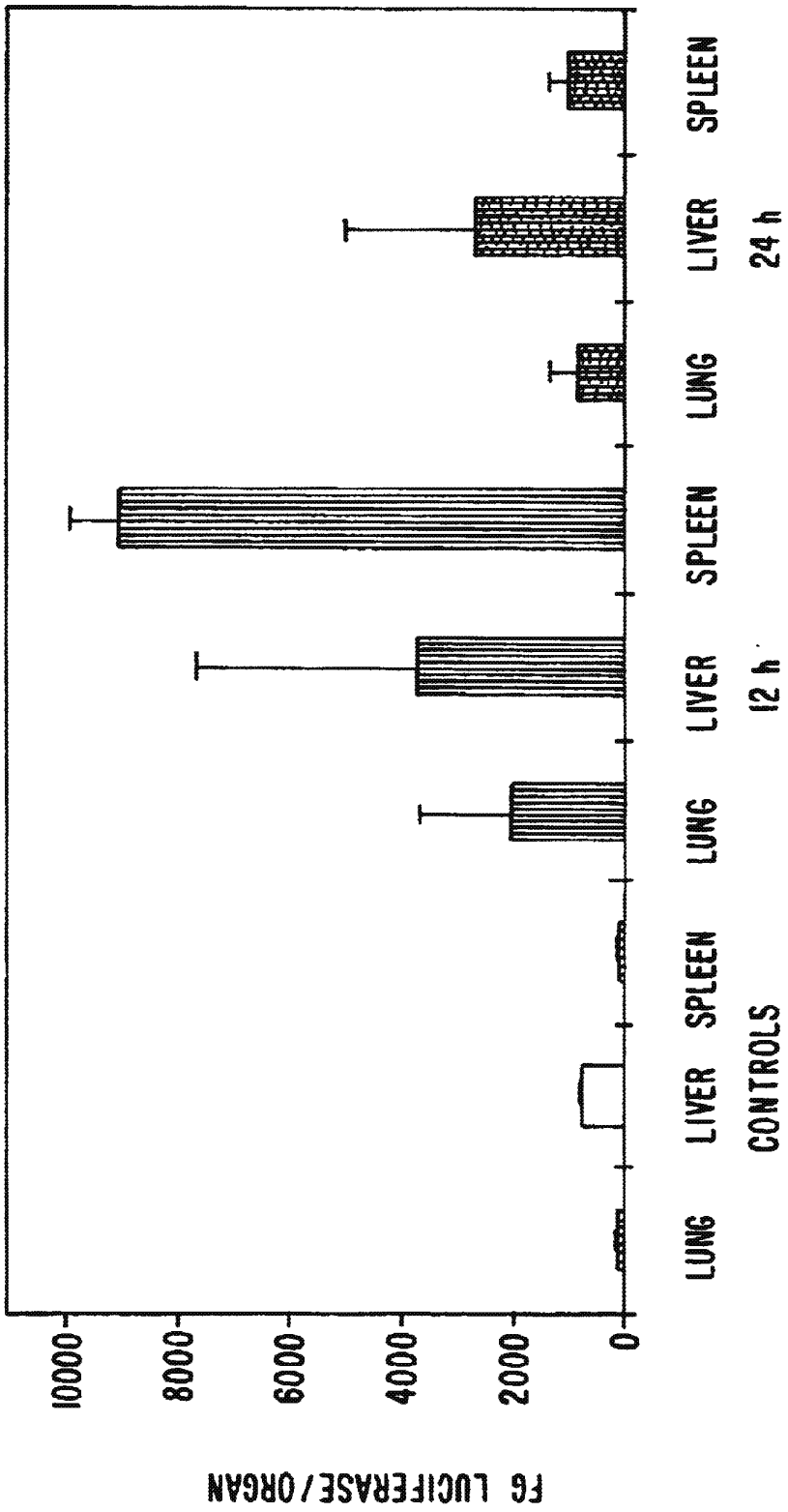

FG LUCIFERASE/ORGAN
0
2000
4000
6000
8000
10000
LUNG
LIVER
SPLEEN
CONTROLS
LUNG
LIVER
SPLEEN
12 h
LUNG
LIVER
SPLEEN
24 h

FIG 31.

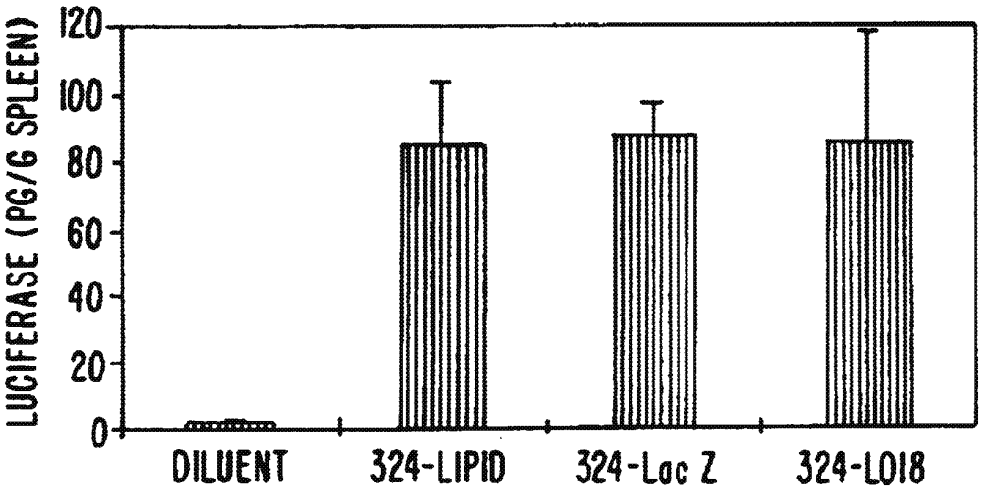

LUCIFERASE (PG/G SPLEEN)
120
100
80
60
40
20
0
DILUENT
324-LIPID
324-Lac Z
324-LO18

FIG. 32.

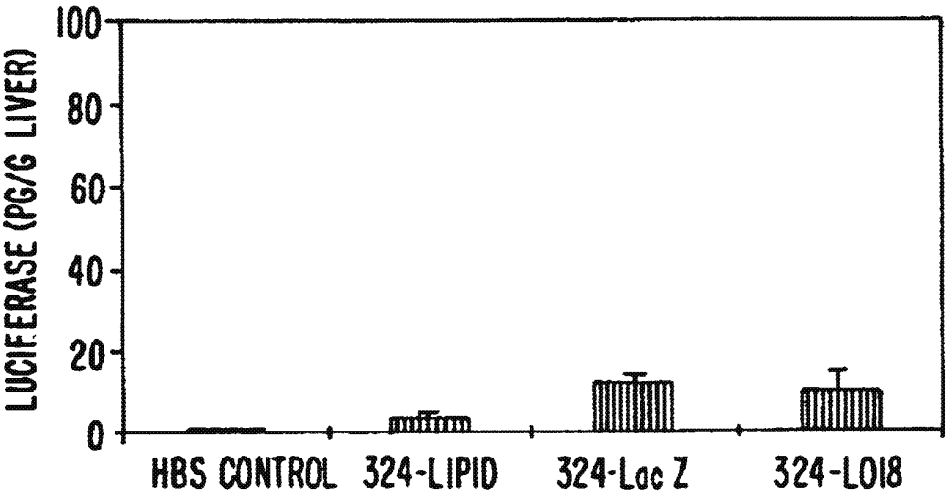

LUCIFERASE (PG/G LIVER)
100
80
60
40
20
0
HBS CONTROL
324-LIPID
324-Lac Z
324-LO18

FIG. 33.

METHODS FOR ENCAPSULATING NUCLEIC ACIDS IN LIPID BILAYERS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority under 119(e) to U.S. Provisional Patent Application Serial No. 60/063,473, filed on Oct. 10, 1997, which is hereby incoporated by reference.

FIELD OF THE INVENTION

This invention relates to lipid-based formulations for nucleic acid delivery to cells, methods for the preparation of such formulations and, in particular, to lipid encapsulated plasmids. The compositions are safe and practical for clinical use.

BACKGROUND OF THE INVENTION

Gene therapy is an area of current interest which involves the introduction of genetic material into a cell to facilitate expression of a deficient protein. Plasmid DNA has been encapsulated or complexed with lipid-based carriers by a number of methods including reverse phase evaporation (Fraley, et al., *J. Biol. Chem.*, 255:10431–10435 (1980); Soriano, et al., *Proc. Natl. Acad. Sci. USA*, 80:7128–7131 (1983); Nakanishi, et al., *Exper. Cell Res.*, 159:399–409 (1985); Nandi, et al., *J. Biol. Chem.*, 261:16722–16726 (1986); and Alino, et al., *Biochem. Biophys. Res. Commun.*, 192:174–181 (1993)); Ca^{2+} EDTA chelation (Szelei, et al., *Biochem. J.*, 259:549–553 (1989)); detergent dialysis (Wang, et al., *Proc. Natl. Acad. Sci. USA*, 84:7851–7855 (1987)); lipid hydration (Lurquin, *Nucleic Acids Res.*, 6:3773–3784 (1979); Yagi, et al., *Biochem. Mol. Biol. International*, 32:167–171 (1994)); ether injection (Fraley, et al., *Proc. Natl. Acad. Sci.*, 76:3348–3352 (1979); Nicolau, et al., *Biochem. Biophys. Res. Comm.*, 108:982–986 (1982)); and sonication (Jay, et al., *Bioconj. Chem.*, 6:187–194 (1987) and Puyal, et al., *Eur. J. Biochem.*, 228:697–703 (1993)).

Reverse phase techniques typically encapsulate only about 10 to 20% of DNA in solution and the final DNA to lipid ratio is quite low. For example, Nakanishi, et al. (*Exper. Cell Res.*, 159:399–409 (1985)) reported a final DNA to lipid ratio of 1.5 μg DNA to 2.5 mg lipid, while Soriano, et al. (*Proc. Natl. Acad. Sci. USA*, 80:7128–7131 (1983)) reported a DNA to lipid ratio of about 14 μg DNA to 60 μmol of lipids. The maximum theoretical encapsulation efficiency expected by reverse phase is only about 40%. Other methods, such as rehydration of freeze dried vesicles with DNA, have been shown to yield trapping efficiencies between 30 and 40% (Baru, et al., *Gene*, 161:143–150 (1995)). Others have sought to increase the entrapment of DNA by the inclusion of cationic lipids in the lipid suspension (Stavridis, et al., 1986; Puyal, et al., *Eur. J. Biochem.*, 228:697–703 (1995)), or by rendering the DNA positively charged by 10 coating it with basic proteins such as lysozymes (Jay, et al., *Proc. Natl. Acad. Sci. USA*, 84:1978–1980 (1987)). Although trapping efficiencies as high as 50% were achieved by the lysozyme method, the amount of DNA loaded per mg of lipid was low (5 μg/mg lipid) and the largest DNA molecule tested was only 1 kb. Trapping efficiencies as high as 60–90% were achieved by Puyal, et al. (*Eur. J. Biochem.*, 228:697–703 (1995)) with a higher DNA to lipid ratio (13 μg/μmole lipid) using a 6.3 kb ssDNA (M13 phage). The major drawback of this technique and the one described by Jay, et al., *Bioconj. Chem.*, 6:187–194 (1987)) is that sonication was used. Sonication of DNA typically leads to some degradation of the lipid vesicle.

Detergent dialysis is a method of encapsulation which has no deleterious effects on the DNA. Wang, et al., *Proc. Natl. Acad. Sci. USA*, 84:7851–7855 (1987) applied a detergent dialysis technique followed by extrusion through a 0.2 μm polycarbonate filter. A 4.6 kb plasmid was entrapped in vesicles approximately 200 nm in diameter with a trapping efficiency of about 14–17%, giving a DNA to lipid ratio of about 26 μg DNA to 10 μmole lipid.

Ideally, a delivery vehicle for a nucleic acid or plasmid will have the following characteristics: a) small enough and long lived enough to distribute from local injection sites when given intravenously, b) capable of carrying a large amount of DNA per particle to enable transfection of all sizes of genes and to reduce the volume of injection, c) homogeneous, d) reproducible, e) protective of DNA from extracellular degradation and f) capable of transfecting target cells in such a way that the DNA is not digested intracellularly.

The present invention provides such compositions and methods for their preparation and use.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides compositions which are nucleic acid (e.g., plasmid)-lipid compositions. In these compositions, a nucleic acid (e.g., plasmid or an antisense molecule) is encapsulated in a self-assembling lipid vesicle in an amount of from about 20 μg nucleic acid/mg lipid to about 400 μg nucleic acid/mg lipid. The lipid vesicle will typically be a liposome or lipid particle (a bilayer vehicle coating the plasmid and having little or no aqueous interior). The lipid vesicle can be prepared from a wide variety of lipids or combinations of lipids. The compositions can also include targeting groups and modified lipids (e.g., ATTA-lipids, gangliosides, such as ganglioside G_{M1}), PEG-lipids, such as PEG-ceramides, and lipids having reactive functional groups for the attachment of targeting groups or circulation stabilizers). Preferably, the lipid vesicles will comprise cationic lipids and fusogenic lipids. Additionally, the nucleic acid (e.g., plasmid)-lipid compositions described herein can be prepared having a narrow size distribution (typically 50 nm to about 150 nm) without the use of sizing methods, such as extrusion and sonication methods.

In another aspect, the present invention provides methods for the encapsulation of nucleic acids, antisense, ribozymes and, particularly, plasmids in a lipid bilayer carrier. Such methods are related to a detergent dialysis method using cationic lipids of any desired concentration in combination with a dialysis buffer of an ionic strength (salt concentration, type of ions) specific for the given cationic lipid concentration. With the dialysis buffer of appropriate ionic strength, the methods provide encapsulation of 40–80% of the nucleic acid solution. The compositions above, and those formed by the methods described below, exhibit preferably less than about 30% degradation, more preferably, less than about 15% degradation and, even more preferably, less than about 5% degradation when digested with 0.1 to 10 U and, more preferably, 1 U of a nuclease after 30 minutes at 37° C.

In particular, the invention provides a method for encapsulating a nucleic acid in a lipid bilayer carrier, comprising:

(a) combining a nucleic acid with a lipid-detergent mixture comprising an aggregation-preventing agent (e.g., an ATTA-lipid, a PEG-lipid, such as a PEG-ceramide, a ganglioside, etc.) in an amount of about 5 mol % to about 20 mol %, cationic lipids in an amount of about 0.5 mol % to about 50 mol % by weight, neutral or

fusogenic lipids in an amount of from about 30 mol % to about 70 mol % and a detergent, to provide a nucleic acid-lipid-detergent mixture; and

(b) dialyzing the nucleic acid-lipid-detergent mixture against a buffered salt solution and to encapsulate the nucleic acid in a lipid bilayer carrier. In these methods, the ionic strength (salt concentration) is adjusted for the cationic lipid concentration used in the lipid mixture and when necessary for the polynucleotide selected for encapsulation to entrap from about 40% to about 80% of the nucleic acid for any given concentration of cationic lipid.

In another aspect, the present invention provides methods for introducing nucleic acids into cells and for inhibiting tumor growth in cells using the lipid-nucleic acid formulations described above.

Other features, objects and advantages of the invention and its preferred embodiments will become apparent from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** provides an illustration of the phase properties of lipids.

FIG. **2** illustrates the conical form of one fusogenic lipid and further illustrates how fusion with another membrane can occur.

FIG. **3** provides an illustration of the detergent dialysis procedure for entrapping nucleic acids in fusogenic lipid vesicles.

FIG. **4** illustrates the structures of three PEG-Ceramide conjugates and also provides half-times for their dissociation from a lipid vesicle.

FIG. **5**. Encapsulation of pINEXL018 plasmid using DODAC/DOPE/PEG-Cer-C8 (30:55:15 mol %) by detergent dialysis in citrate buffer. Effect of varying NaCl concentration with constant citrate concentration (100 mM Na citrate, 5 mM HEPES, pH:7.2) is illustrated. The encapsulation efficiency and polydispersity, χ^2 (a measure of formulation homogeneity), of formulations are plotted as functions of NaCl. Successful formulations are indicated by the high encapsulation and low χ^2.

FIG. **6**. Encapsulation of pINEX L018 plasmid using DODAC/DOPE/PEG-Cer-C8 system by detergent dialysis in citrate buffer containing constant NaCl concentration (150 mM) and 5 mM HEPES, pH:7.2. The relationship between varying DODAC mol % and the optimal citrate concentration and the effect upon encapsulation efficiency is demonstrated. Each point represents a formulation of acceptable size and encapsulation efficiency. The optimal range of citrate and DODAC concentrations are indicated by the solid line. Typically, those preparations prepared below the optimal citrate concentration have large sizes or aggregate, and those formulations prepared above the optimal citrate concentration have low encapsulation efficiencies (0–30%).

FIG. **7**. Effect of DODAC concentration on plasmid encapsulation. In this study, the effect of small (1 mol %) changes of DODAC concentration were tested at constant lipid (10 mg/mL), plasmid (400 μg/mL) and buffer concentrations. Encapsulation efficiency dropped significantly with a decrease in DODAC concentration, indicating that care must be taken to precisely deliver DODAC at a given NaCl concentration. pINEXL002 was formulated in 150 mM $NaPO_4$, 175 mM NaCl, pH 7.4, and pINEXP005 was formulated in 150 mM $NaPO_4$, 150 mM NaCl pH 7.4.

FIG. **8**. Encapsulation of pINEXP005. Effect of varying NaCl with constant $NaPO_4$ concentration. The relationship between varying the salt concentration on the encapsulation of plasmid over a range of INEX TCS DODAC concentrations is illustrated. Negative NaCl concentrations indicate where the buffer concentration was decreased to an extent where no NaCl was included in the dialysis buffer, and the phosphate buffer concentration alone was decreased to achieve encapsulation. Formulations were prepared containing 10 mg/mL total lipid, 400 μg/mL plasmid DNA. In each 1.0 ml formulation, the PEG-C8 concentration was maintained at 15 mol %, the DODAC concentration was varied as indicated and the balance of the lipid was DOPE. At each DODAC concentration, formulations were dialyzed against a range of buffer salt concentrations. This study demonstrates that a range of encapsulation efficiencies can be achieved by adjusting the NaCl concentration in a phosphate buffer. Thus, the association of the DNA with the lipid particles can be regulated.

FIG. **9**. Encapsulation of pINEXL018. Effect of varying $NaPO_4$ and total lipid concentration. The effect of varying the lipid and phosphate buffer concentration on the encapsulation of plasmid over a range of INEX TCS DODAC concentrations is demonstrated. Formulations were prepared containing either 5 or 10 mg/mL total lipid, 400 μg/mL plasmid DNA. As in FIG. **3**, the formulations were prepared containing 15 mol % PEG-C8, the range of indicated DODAC concentrations and the balance of lipid made up with DODAC. In this study, the concentration of the dialysis phosphate buffer alone could be decreased to achieve encapsulation of a plasmid over this range of DODAC concentrations.

FIG. **10**. Effect of lipid concentration on the encapsulation of pINEXP005 in INEX 351. Formulations were prepared in 1 mL formulations containing 200 μg/mL plasmid and total lipids ranging from 1.25 mg/mL to 10 mg/mL. INEX 351 indicates a formulation containing 42.5 mol % DODAC, 42.5 mol % DOPE and 15 mol % PEG-C8. The formulations were dialyzed against 150 mM $NaPO_4$, 150 mM NaCl, pH 7.4. This study demonstrates that an increase in lipid concentration increases the extent of plasmid concentration. Thus, sufficient total lipid is required for loading of the plasmid into the particles.

FIG. **11**. Effect of plasmid concentration on encapsulation efficiency in INEX 351 particles were prepared in 1 mL formulations containing 5 mg/mL total lipid and the concentration of the plasmids ranged from 100 to 1000 μg/mL. Formulations containing pINEXP005 and pINEXL002 were dialyzed against 150 mM $NaPO_4$, pH 7.4 containing 150 mM and 175 mM NaCl, respectively. This study demonstrates that with increasing plasmid concentration, there is a decrease in the encapsulation efficiency. Thus, as seen in FIG. **5**, sufficient lipid is required in order to have significant loading of the plasmid into particles.

FIG. **12**. Sucrose density gradient isolation of an INEX TCS. Panel A: Separation of TCS formulation loaded with plasmid (lower band) from empty vesicles (upper bands) on the gradient after 12 hr centrifugation at 36,000 rpm (SW 41 Ti Rotor). Panel B: Removal of empty liposomes (non-DNA associated). Panel C: Removal of the DNA-loaded TCS from the gradient. Empty vesicles are removed from the TCS formulation using sucrose gradient isolation after the nonencapsulated plasmids are removed on a column of DEAE-Sepharose. In this preparation, a formulation containing 24 mol % DODAC (200 μg DNA/10 mg plasmid) was loaded onto a typical sucrose gradient which contained (from bottom to top) 10% (2 mL), 5% (4 mL), and 2.5% (3 mL) sucrose in 20 mM HEPES buffered saline. The gradient was centrifuged for 14 hr at room temp. In this case, the

DNA loaded TCS were removed using a syringe; however, fractions can be removed from the top of the gradient.

FIG. **13**. Distribution of lipid and DNA for a typical TCS containing 21 mol % DODAC after centrifugation on a sucrose density gradient for 5 hrs at 36000 rpm at 20° C. using a Beckman ultracentrifuge with a SW41 Ti rotor. Fractions of 2 mL were removed from top to bottom and assayed for DNA and lipid concentration. Upper panel: distribution of ^{3}H-labelled DNA. Lower panel: distribution of ^{14}C-labelled lipid on the sucrose gradient.

FIG. **14**. Electron-microscopy (EM) of various INEX TCS formulations. (A) Freeze-fracture EM of a formulation containing 20 mol % DODAC. (B) Freeze-fracture EM of a formulation containing 42.5 mol % DODAC formulation.

FIG. **15**. Serum stability assay of INEX TCS. Sepharose CL-4B gel filtration chromatography after treatment with 80% normal mouse serum at 37° C. for 60 minutes. Upper panel: free DNA vs. TCS containing 21 mol % DODAC (unencapsulated DNA removed). Lower panel: TCS containing 21 mol % DODAC after isolation on a sucrose density gradient. TCS when not incubated with serum, or incubated but protected from serum degradation was eluded at the void volume (fractions 5–8). DNA when degraded by serum was eluded in later fractions (fractions #10–20). The results showed that DNA was protected 74% and 84% in the cleaned TCS (by DEAE column) and the isolated TCS respectively.

FIG. **16**. Serum and DNAse stability of plasmid, Free, in complexes (INEX100.3, complexes prepared by mixing DODAC/DOPE liposomes with pINEXL018) and encapsulated in INEX TCS. Agarose gel electrophoresis of pINEXL018 phenol-chloroform extracted after treatment with 80% normal mouse serum. Lanes 1–4: Free pINEXL018; Lanes 5–8: pINEXL018 encapsulated 42.5% DODAC containing TCS; Lanes 9–12: DOPE:DODAC complexed-pINEXL018. This study illustrates that the encapsulated DNA remains intact after treatment with serum and DNAse, while demonstrating that complexed DNA is not as nuclease stable.

FIG. **17**. Effect of DODAC concentration in TCS on the transfection of COS-7 cells in culture using isolated TCSs prepared with pINEXL018 and DODAC/DOPE/PEG-Cer-C8 by the detergent dialysis method using citrate buffer. Cells (40,000/well) were seeded in 24 well plates 24 hr before transfection. The dose was 1.0 μg/well and the luciferase activity was assayed at 48 hr time point (n=3). INEX100.3 represents complexes prepared by mixing DODAC/DOPE liposomes with pINEXL018.

FIG. **18**. Effect of DODAC concentration in TCS on the transfection of Hep-G2 cells in culture using isolated TCSs prepared with pINEXL018 and DODAC/DOPE/PEG-Cer-C8 by the detergent dialysis method using citrate buffer. Cells (40,000/well) were seeded in 24 well plates 24 hr before transfection. The dose was 0.3 μg/well and the luciferase activity was assayed at 48 hr time point (n=3). INEX100.3 represents complexes prepared by mixing DODAC/DOPE liposomes with pINEXL018.

FIG. **19**. Effect of dose on the transfection of Hep-G2 cells in culture in isolated TCS prepared with pINEXL018 and DODAC/DOPE/PEG-Cer-C8 by the detergent dialysis method using citrate buffer. Cells (40,000/well) were seeded in 24 well plates 24 hr before transfection. The doses were 0.05, 0.3 & 0.7 μg/well and the luciferase activity was assayed at 48 hr time point (n=3). INEXice represents complexes prepared by mixing DODAC/DOPE liposomes with pINEXL018.

FIG. **20**. In vitro transfection study. Effect of the dose and DODAC concentrations of TCS prepared by phosphate buffer dialysis on the transfection and viability of COS-7 cells in culture. INEX TCS formulations containing 42.5, 38, 34 and 30 mol % DODAC (expressed as DOPE/DODAC ratios 50/50, 55/45, 60/40 and 63/35, respectively) were incubated with 35,000 cells/well at doses of 0.05, 0.1, 0.5 μg DNA. After 24 hr incubation, the cells were resuspended, lysed and measured for viability and luciferase activity (expressed as relative luminescence units). This study demonstrates that decreasing the DODAC concentration in non-isolated TCS increases the transfection activity and decreases the relative toxicity of the formulation.

FIG. **21**. Time course of transfection activity of TCS prepared by the phosphate dialysis method. COS-7 cells were incubated with 0.5 μg non-isolated TCS under the conditions described in FIG. **14**. After incubation for 24, 48 and 72 hours, transfection was measured as luciferase activity. This study illustrates that in vitro transfection increases for up to 48 hr and is sustained for well over 72 hr. As observed in FIG. **14**, transfection activity increased with decreasing DODAC concentration.

FIG. **22**. Effect of sucrose density gradient isolation of TCS prepared by the phosphate dialysis method on the transfection and viability of COS-7 cells in culture. COS-7 cells were incubated with both isolated and nonisolated TCS at 0.05, 0.1, 0.5 and 1.0 μg of DNA. TCS contained DODAC concentrations of 20, 24, 30 and 42.5 mol %. After 24 hours the cell viability and luciferase activity was measured. In total, FIGS. **14–16** demonstrate that TCS mediated transfection is dose dependent. Maximal in vitro transfection activity is obtained at 30 mol % DODAC. Decreasing the TCS DODAC concentration reduces toxicity. Removal of empty TCS by sucrose density gradient isolation also decreases the toxicity of the formulations and increases the in vitro transfection activity.

FIG. **23**. In vivo, intraperitoneal transfection study for TCS containing various DODAC concentrations in TCS prepared by detergent dialysis in citrate buffer after isolation on transfection of B16 i.p. tumors. The TCSs were composed of pINEXL018/DODAC/DOPE/PEG-Cer-C8. TCS (30 μg DNA/500 μl/mouse) formulations were injected i.p. into mice 7 days after tumor seeding. Tumors were removed from mice 24 hours after treatment and were assayed for luciferase activity.

FIG. **24**. Effect of time on the transfection of i.p. B16 tumors. Isolated TCS (30 μg DNA/500 μl/mouse) of various DODAC concentrations prepared by detergent dialysis in citrate buffer were tested for luciferase transfection activity 24 and 48 hours after injection. The TCSs were composed of pINEXL018/DODAC/DOPE/PEG-Cer-C8.

FIG. **25**. Comparison of the transfection activity of isolated vs. non-isolated TCS formulations. TCS containing DODAC concentrations of 42.5 mol % 30 mol %, 24 mol % and 20 mol % DODAC prepared by phosphate dialysis were injected i.p. at 30 μg DNA into mice 7 days after tumor seeding. Tumors removed from mice 24 hours after administration were assayed for luciferase activity. This study demonstrates that these TCS formulations transfect in vivo as well as in vitro. In addition, removal of the empty liposomes by sucrose density gradient isolation results in increased transfection activity.

FIG. **26**. The effect of DODAC TCS concentration on the transfection of mouse spleens after i.p. administration. TCS formulations described in FIG. **19** were injected i.p. at 30 μg DNA into mice 7 days after tumor seeding. Spleens removed

from the tumor bearing mice 24 hours after administration were assayed for luciferase activity.

FIG. **27**. The effect of DODAC TCS concentration on the transfection of mouse liver after i.p. administration. TCS formulations described in FIG. **19** were injected i.p. at 30 μg DNA into mice 7 days after tumor seeding. Livers removed from the tumor bearing mice 24 hours after administration were assayed for luciferase activity. The studies described in FIGS. **21** and **22** demonstrate that it is possible to transfect normal organs as well as tumor tissue after i.p. administration of TCS formulation.

FIG. **28**. TCS toxicity study was carried out by monitoring the plasma aspartate aminotransferase (AST) levels after i.p. administration of TCS containing various concentrations of DODAC. Isolated TCS composed of pINEXL018/DODAC/DOPE/PEG-Cer-C8 prepared by detergent dialysis in citrate buffer were injected i.p. into mice 7 days after tumor seeding. Blood was removed from mice 24 hours after administration and was assayed for AST activity. This study demonstrates that there is little significant tissue or organ damage associated with TCS formulations administered by the i.p. route.

FIG. **29**. Effect of repeated i.v. administration of INEX324-LacZ on serum levels of anti-β-gal antibodies. Mice received three i.v. injections of INEX324-LacZ and the level of antibodies against transgene product was measured using capture ELISA assay.

FIG. **30**. Effect of DNA concentration on the expression of β-galactosidase by BHK-21 cells in vitro. Cells were incubated with either INEX324-luc or INEX324-LacZ for 24 h and expression of β-galactosidase was determined by FACS using the FDG assay.

FIG. **31**. Time course of luciferase expression after i.v. administration of INEX324-luc formulation. Mice were given a single i.v. injection of INEX324-luc (100 μg DNA) and the expression of luciferase was determined 12 h and 24 h post-injection.

FIG. **32**. Transfection of the spleen after intravenous administration of INEX324-Luc formulation. Mice received three consecutive injections of INEX324-LacZ or INEX324 lipid, followed by a single injection of INEX324-Luc. Spleens were harvested 12 h after INEX324-Luc administration and the levels of luciferase were assayed.

FIG. **33**. Transfection of the liver after intravenous administration of INEX324-Luc formulation. Mice received three consecutive injections of INEX324-LacZ or 324 lipid, followed by single injection of INEX324-Luc. Livers were harvested 12 h after INEX324-Luc administration and the levels of luciferase were assayed.

DETAILED DESCRIPTION OF THE INVENTION

CONTENTS

I. Glossary

The following abbreviations are used herein: DC-Chol, 3β-(N-(N',N'-dimethylaminoethane)carbamoyl)cholesterol (see, Gao, et al., *Biochem. Biophys. Res. Comm.*, 179:280–285 (1991)); DDAB, N,N-distearyl-N,N-dimethylammonium bromide; DMRIE, N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide; DODAC, N,N-dioleyl-N,N-dimethylammonium chloride (see, commonly owned patent application U.S. Ser. No. 08/316,399, incorporated herein by reference); DOGS, diheptadecylamidoglycyl spermidine; DOPE, 1,2-sn-dioleoylphoshatidyethanolamine; DOSPA, N-(1-(2,3-dioleyloxy)propyl)-N-(2-(sperminecarboxamido) ethyl)-N,N-dimethylammonium trifluoroacetate; DOTAP, N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride; DOTMA, N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride; EPC, egg phosphatidylcholine; RT, room temperature; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES buffered saline (150 mM NaCl and 20 mM HEPES); PEG-Cer-C_{20}, 1-O-(2'-(ω-methoxypolyethyleneglycol)succinoyl)-2-N-arachidoyl-sphingosine; PEG-Cer-C_{14}, 1-O-(2'-(ω-methoxypolyethyleneglycol)succinoyl)-2-N-myristoyl-sphingosine; PBS, phosphate-buffered saline; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; OGP, n-octyl β-D-glycopyranoside (Sigma Chemical Co., St. Louis, Mo.); POPC, palmitoyl oleoyl phosphatidylcholine (Northern Lipids, Vancouver, BC); QELS, quasielastic light scattering; TBE, 89 mM Tris-borate with 2 mM EDTA; and EDTA, Ethylenediaminetetraacetic acid (Fisher Scientific, Fair Lawn, N.J.);

The term "acyl" refers to a radical produced from an organic acid by removal of the hydroxyl group. Examples of acyl radicals include acetyl, pentanoyl, palmitoyl, stearoyl, myristoyl, caproyl and oleoyl.

The term "lipid" refers to any fatty acid derivative which is capable of forming a bilayer such that a hydrophobic portion of the lipid material orients toward the bilayer while a hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of phosphato, carboxylic, sulfato, amino, sulfhydryl, nitro, and other like groups. Hydrophobicity could be conferred by the inclusion of groups that include, but are not limited to, long chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic or heterocyclic group(s). Preferred lipids are phosphoglycerides and sphingolipids, representative examples of which include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidyl-ethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoyl-phosphatidylcholine or dilinoleoylphosphatidylcholine could be used. Other compounds lacking in phosphorus, such as sphingolipid and glycosphingolipid families are also within the group designated as lipid. Additionally, the amphipathic lipids described above may be mixed with other lipids including triglycerides and sterols.

The term "cationic lipid" refers to any of a number of lipid species which carry a net positive charge at physiological pH or have a protonatable group and are positively charged at pH lower than the pK_a. Such lipids include, but are not limited to, DODAC, DOTMA, DOGS, DDAB, DOTAP, DC-Chol, DMRIE and amino lipids. The term "amino lipids" is meant to include lipids with an amino head group (including alkylamino or dialkylamino group) which is protonated to form a cationic lipid below its pK_a. Commercial preparations of cationic liposomes prepared from cat-

ionic lipids are generally not useful unless the liposomes are first disrupted to provide lipid mixtures. The compositions and methods described herein use lipids and lipid mixtures in a self-assembling process which occurs in the presence of a plasmid or other nucleic acid.

The terms "transfection" and "transformation" are used herein interchangeably, and refer to the introduction of polyanionic materials, particularly nucleic acids and plasmids, into cells. The term "lipofection" refers to the introduction of such materials using liposome or lipid-based complexes. The polyanionic materials can be in the form of DNA or RNA which is linked to expression vectors to facilitate gene expression after entry into the cell. The plasmids used in the present invention include DNA having coding sequences for structural proteins, receptors and hormones, as well as transcriptional and translational regulatory elements (i.e., promoters, enhancers, terminators and signal sequences) and vectors. Methods of incorporating particular nucleic acids into expression vectors are well known to those of skill in the art, but are described in detail in, for example, Sambrook, et al., *Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1–3, Cold Spring Harbor Laboratory, (1989) or *Current Protocols in Molecular Biology*, F. Ausubel, et al., ed. Greene Publishing and Wiley-Interscience, New York (1987), both of which are incorporated herein by reference.

The term "contacting" is used herein interchangeably with the following: combined with, added to, mixed with, passed over, incubated with, flowed over, etc. Moreover, the compounds of present invention can be "administered" by any conventional method such as, for example, parenteral, oral, topical and inhalation routes as described herein.

"An amount sufficient," "an effective amount," or "therapeutically effective amount" refer to an amount of a compound or composition effective to depress, suppress or regress cell growth or result in amelioration of symptoms associated with cancerous diseases. The desired result can be either a subjective relief of a symptom(s) or an objectively identifiable improvement in the recipient of the dosage, a decrease in tumor size, a decrease in the rate of growth of cancer cells as noted by the clinician or other qualified observer.

The terms "treating cancer," "therapy," and the like refer generally to any improvement in the mammal having the cancer wherein the improvement can be ascribed to treatment with the compounds and compositions of the present invention. The improvement can be either subjective or objective. For example, if the mammal is human, the patient may note improved vigor or vitality or decreased pain as subjective symptoms of improvement or response to therapy. Alternatively, the clinician may notice decrease in tumor size or tumor burden based on physical exam, laboratory parameters, tumor markers or radiographic findings. Some laboratory signs that the clinician may observe for response to therapy include normalization of tests such as white blood cell count, red blood cell count, platelet count, erythrocyte sedimentation rate, and various enzyme levels. Additionally, the clinician may observe a decrease in a detectable tumor marker. Alternatively, other tests can be used to evaluate objective improvement such as sonograms, nuclear magnetic resonance testing and positron emissions testing.

"Inhibiting the growth of tumor cells" can be evaluated by any accepted method of measuring whether growth of the tumor cells has been slowed or diminished. This includes direct observation and indirect evaluation such as subjective symptoms or objective signs as discussed above.

"Expression vectors," "cloning vectors," or "vectors" are often plasmids or other nucleic acid molecules that are able to replicate in a chosen host cell. Expression vectors may replicate autonomously, or they may replicate by being inserted into the genome of the host cell, by methods well known in the art. Vectors that replicate autonomously will have an origin of replication or autonomous replicating sequence (ARS) that is functional in the chosen host cell(s). Often, it is desirable for a vector to be usable in more than one host cell, e.g., in *E. coli* for cloning and construction, and in a mammalian cell for expression.

H. General—Plasmid-Lipid Compositions

The present invention derives from the discovery that nucleic acid (e.g., plasmid) can be encapsulated in lipid bilayer carriers in an amount significantly above that which has been previously demonstrated. In particular, the present invention provides lipid-plasmid compositions in which nucleic acids are encapsulated in self-assembling lipid vesicles in an amount of from about 5 μg to about 800 μg per milligram of lipid, preferably in an amount of from about 40 μg to about 400 μg per milligram of lipid and, more preferably, in an amount of from about 100 μg to about 400 μg per milligram of lipid. Additionally, the nucleic acid-lipid compositions which are described herein, form in a self-assembling process to yield particles having a narrow distribution of sizes (e.g., 50 nm to about 150 nm). The precise size of the compositions formed will depend on several factors including, for example, the choice of lipids and the size of the nucleic acid that is encapsulated. However, the size distribution is relatively narrow and is achieved without harsh sizing steps such as, for example, extrusion or sonication. Still further, the compositions described herein are nuclease resistant and can be concentrated in an aqueous solution without the formation of aggregate complexes. As used herein, the term "nuclease resistant" when used to describe a nucleic acid-lipid composition refers to a composition in which the nucleic acid portion is less than about 30% degraded, more preferably, less than about 15% degraded and, even more preferably, less than about 5% degraded when the composition is incubated with 0.1 to 10 U and, more preferably, 1 U of a nuclease (e.g., DNAse or normal serum) after 30 minutes at 37° C.

More particularly, it has now been discovered that encapsulation efficiency in detergent dialysis methods is dependent on the lipid composition as well as the dialysis buffer which is used in forming the lipid bilayer carriers. Optimal lipid bilayer carriers can now be constructed depending on the encapsulate (e.g., plasmid, antisense, ribozyme or other polyanionic therapeutic agent), the environment for transfection (e.g., diagnostics or in vivo or in vitro transfection) and other factors such as desired circulation lifetimes and fusogenic properties. Accordingly, particular lipid compositions can now be selected to exhibit certain circulation and targeting characteristics and formulated by control of salt concentrations to increase the amounts of plasmid or other nucleic acid which are encapsulated.

The unique detergent dialysis method by which the present compositions are prepared yields DNA to lipid ratios in excess of 20 μg DNA to 1 mg lipid. In some embodiments, the lipid coated DNA particle will have DNA to lipid ratios in excess of 200 μg DNA to 1 mg lipid.

Plasmids which are useful for the instant compositions are typically nucleotide polymers which are to be administered to a subject for the purpose of repairing or enhancing the expression of a cellular protein. Accordingly, the nucleotide

polymers can be polymers of nucleic acids including genomic DNA, cDNA, or mRNA. Still further, the plasmids can encode promoter regions, operator regions, structural regions, etc. The plasmids are preferably double-stranded DNA or DNA-RNA hybrids. Examples of double-stranded DNA include, but are not limited to, structural genes, genes including operator control and termination regions, and self-replicating systems such as plasmid DNA.

Multiple genetic sequences can also be used in the present compositions. Thus, the sequences for different proteins can be located on one strand or plasmid. Promoter, enhancer, stress or chemically-regulated promoters, antibiotic-sensitive or nutrient-sensitive regions, as well as therapeutic protein encoding sequences, can be included as required. Nonencoding sequences can be also be present to the extent they are necessary to achieve appropriate expression.

Plasmids used in the present method can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries or prepared by synthetic methods. The compositions of the present invention can be prepared from plasmids of essentially any size. In preferred embodiments, the plasmid is from about 2 kilobases to about 15 kilobases, more preferably from about 4 kilobases to about 10 kilobases.

In some embodiments, the plasmid will be replaced with other nucleic acids (e.g., single-stranded DNA or RNA, antisense, ribozymes or nucleic acids). When nucleic acids other than plasmids are used, the nucleic acids can contain nucleic acid analogs, for example, the antisense derivatives described in a review by Stein, et al., *Science* 261:1004–1011 (1993) and in U.S. Pat. Nos. 5,264,423 and 5,276,019, the disclosures of which are incorporated herein by reference.

Single-stranded nucleic acids include antisense oligonucleotides (complementary to DNA and RNA), ribozymes and triplex-forming oligonucleotides. In order to have prolonged activity, the single-stranded nucleic acids will preferably have some or all of the nucleotide linkages substituted with stable, nonphosphodiester linkages, including, for example, phosphorothioate, phosphorodithioate, phophoroselenate, or O-alkyl phosphotriester linkages.

The nucleic acids used in the present invention will also include those nucleic acids in which modifications have been made in one or more sugar moieties and/or in one or more of the pyrimidine or purine bases. Examples of sugar modifications include replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, azido groups or functionalized as ethers or esters. Additionally, the entire sugar can be replaced with sterically and electronically similar structures, including aza-sugars and carbocyclic sugar analogs. Modifications in the purine or pyrimidine base moiety include, for example, alkylated purines and pyrimidines, acylated purines or pyrimidines, or other heterocyclic substitutes known to those of skill in the art.

Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers, et al., U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage, et al., *Tetrahedron Lett.*, 22:1859–1862 (1981); Matteucci, et al., *J. Am. Chem. Soc.*, 103:3185–3191 (1981); Caruthers, et al., *Genetic Engineering*, 4:1–17 (1982); Jones, chapter 2, Atkinson, et al., chapter 3, and Sproat, et al., chapter 4, in *Oligonucleotide Synthesis: A Practical Approach*, Gait (ed.), IRL Press, Washington D.C. (1984); Froehler, et al., *Tetrahedron Lett.*, 27:469–472 (1986); Froehler, et al., *Nucleic Acids Res.*, 14:5399–5407 (1986); Sinha, et al. *Tetrahedron Lett.*, 24:5843–5846 (1983); and Sinha, et al., *Nucl. Acids Res.*, 12:4539–4557 (1984) which are incorporated herein by reference.

Lipids which are useful in the present invention can be any of a variety of lipids including both neutral lipids and charged lipids. Carrier systems having desirable properties can be prepared using appropriate combinations of lipids, targeting groups and circulation enhancers. Additionally, the compositions provided herein can be in the form of liposomes or lipid particles, preferably lipid particles. As used herein, the term "lipid particle" refers to a lipid bilayer carrier which "coats" a nucleic acid and has little or no aqueous interior. More particularly, the term is used to describe a self-assembling lipid bilayer carrier in which a portion of the interior layer comprises cationic lipids which form ionic bonds or ion-pairs with negative charges on the nucleic acid (e.g., a plasmid phosphodiester backbone). The interior layer can also comprise neutral or fusogenic lipids and, in some embodiments, negatively charged lipids. The outer layer of the particle will typically comprise mixtures of lipids oriented in a tail-to-tail fashion (as in liposomes) with the hydrophobic tails of the interior layer. The polar head groups present on the lipids of the outer layer will form the external surface of the particle.

Selection of suitable lipids for use with plasmids will typically involve consideration of the lipid's bilayer-forming capabilities, bilayer-stabilizing capabilities and fusogenic properties. The capabilities, or properties, of a lipid can often be estimated based on the physical shape of the lipid. For example, lipids can be classified according to the three basic structures which lipids can form (see, FIG. **1**). Lipids which form micelles typically have large headgroup cross-sectional areas in relation to that of the lipid tail or hydrophobic region. Examples of these lipids are detergents, such as n-octyl β-D-glycopyranoside (OGP), and lysolipids, such as lysophosphatidylcholine (lysoPC). Bilayer-forming lipids or bilayer-stabilizing lipids are typically those which are cylindrical in shape (e.g., DOPC, DOPS, and DODAC). Lipids which form an inverted micelle (the precursor to the hexagonal II phase) have larger tails than heads (e.g., DOPE, see, FIG. **2**). Inverted micelles cannot exist in aqueous solution so they must be solubilized in the membrane and form long tube structures called hexagonal II phase (H_{II}, phase). The H_{II} phase is thought to be a precursor to fusion of two adjacent membranes. For this reason, DOPE is a powerful membrane fusogen (otherwise referred to as a fusogenic lipid).

Fusogenic lipids such as DOPE, lysolipids and free fatty acids can be accommodated in a bilayer configuration with the appropriate quantities of bilayer-forming lipids. For example, about 20 mol % DOPC will stabilize DOPE in a bilayer. Alternatively, about 30% DODAC (a less effective bilayer-forming lipid) will stabilize DOPE in a bilayer, while only about 10% or perhaps less of PEG-Ceramide is necessary to stabilize DOPE in a bilayer. Similarly, non-micelle forming lipids can be stabilized within micelles with the appropriate quantities of micelle forming lipids such as detergents (e.g., OGP). As the detergent is removed by dialysis, the micelle becomes unstable and becomes a bilayer if enough bilayer-forming lipid is present (FIG. **3**). Similarly, a bilayer stabilized by PEG-Ceramide will become unstable once the PEG-Ceramide exchanges out of the outer monolayer.

Considering lipid properties, as noted above, the compositions of the present invention are optimized for the delivery of nucleic acid (e.g., plasmids) to cells. In particular, lipid-plasmid compositions are provided in which the carriers (lipid portions) are composed of at least two types of lipids including (i) fusogenic, nonbilayer forming lipids, and (ii) bilayer-forming or -stabilizing lipids. Preferably, the compositions will further comprise (iii) an aggregation-preventing agent (e.g., PEG-lipids, ATTA-lipids, gangliosides, etc.). In particularly preferred embodiments, the compositions will comprise cationic lipids (as the bilayer-forming or -stabilizing lipids), fusogenic lipids and PEG-lipids.

Cationic lipids which are useful in the present compositions include, for example, DODAC, DOTMA, DDAB, DOTAP, DC-Chol and DMRIE. These lipids and related analogs, which are also useful in the present invention, have been described in co-pending U.S. Ser. No. 08/316,399; U.S. Pat. Nos. 5,208,036, 5,264,618, 5,279,833 and 5,283,185, the disclosures of which are incorporated herein by reference. Particularly preferred within this group is DODAC.

Fusogenic lipids which are useful in the present invention include, for example, DOPE, lysolipids and free fatty acids. Each of these lipids (or lipid groups) can be accommodated in a bilayer configuration with an appropriate quantity of bilayer-forming or bilayer-stabilizing lipids (e.g., DOPC, DODAC, ATTA-lipids, PEG-lipids, such as PEG-Ceramides, etc.). Preferably, the fusogenic lipid is DOPE, or a related phosphatidylethanolamine having two attached fatty acyl chains, preferably unsaturated fatty acyl chains.

Preferably, the lipid-nucleic acid compositions of the present invention contain an aggregation-preventing agent, i.e., a compound (or mixture of compounds) that prevents aggregation during formulation of the lipid-nucleic acid compositions. In addition, such aggregation-preventing agents can also serve as cloaking agents, which help to reduce elimination of the lipid-nucleic acid compositions by the host immune system. These agents can also be targeting agents that help the lipid-nucleic acid formulations to accumulate in the area of the disease or target site. These agents can also be compounds that improve features of the formulation, such as leakiness, longevity in circulation, reduction in toxicity, encapsulation efficiency, etc. Examples of suitable aggregation-preventing agents, include but are not limited to, ATTA-lipid conjugates, such as those disclosed in U.S. patent application Ser. No. 08/996,783, filed Dec. 23, 1997, and U.S. patent application Ser. No. 60/073,852, Feb. 2, 1998; PEG-lipid conjugates, such as those disclosed in U.S. patent application Ser. Nos. 08/486214, 08/316407, and 08/485608; and gangliosides, e.g., G_{M1}, such as those disclosed in U.S. Pat. No. 4,837,028, the teachings of all of which are incorporated herein by reference. Examples of these components and others that can usefully be included in the formulations of the invention are known to and used by those skilled in the art.

In a preferred embodiment, PEG-modified lipids are incorporated into the compositions of the present invention as the aggregation-preventing agent. The use of a PEG-modified lipid positions bulky PEG groups on the surface of the liposome or lipid carrier and prevents binding of DNA to the outside of the carrier (thereby inhibiting cross-linking and aggregation of the lipid carrier). The use of a PEG-ceramide is often preferred and has the additional advantages of stabilizing membrane bilayers and lengthening circulation lifetimes. Additionally, PEG-ceramides can be prepared with different lipid tail lengths to control the lifetime of the PEG-ceramide in the lipid bilayer. In this manner, "programmable" release can be accomplished which results in the control of lipid carrier fusion. For example, PEG-ceramides having C_{20}-acyl groups attached to the ceramide moiety will diffuse out of a lipid bilayer carrier with a half-life of 22 hours (see, FIG. **4**). PEG-ceramides having C_{14}- and C_8-acyl groups will diffuse out of the same carrier with half-lives of 10 minutes and less than 1 minute, respectively. As a result, selection of lipid tail length provides a composition in which the bilayer becomes destabilized (and thus fusogenic) at a known rate. Though less preferred, other PEG-lipids or lipid-polyoxyethylene conjugates are useful in the present compositions. Examples of suitable PEG-modified lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-modified diacylglycerols and dialkylglycerols, PEG-modified dialkylamines and PEG-modified 1,2-diacyloxypropan-3-amines. Particularly preferred are PEG-ceramide conjugates (e.g., PEG-Cer-C_8, PEG-Cer-C_{14} or PEG-Cer-C_{20}) which are described in co-pending U.S. Ser. No. 08/486,214, now U.S. Pat. No. 5,820,873, incorporated herein by reference.

In one group of particularly preferred embodiments, the compositions comprise a nucleic acid (e.g., plasmid), DODAC, DOPE and an aggregation-preventing agent (e.g., ATTA-lipids, PEG-lipids, such as PEG-Ceramides), more preferably with the plasmid being encapsulated in an amount of from about 30 μg to about 400 μg per milligram of lipid. Still further preferred are those embodiments in which DODAC is present in an amount of from about 5 mol % to about 50 mol %, DOPE is present in an amount of from about 30 mol % to about 70 mol %, and the aggregation-preventing agent (e.g., PEG-Ceramide) is present in an amount of about 5 mol % to about 20 mol %.

The compositions of the present invention can be prepared by the methods described below to provide compositions which are about 50 nm to about 100 nm in size. One of skill in the art will understand that the size of the compositions can be larger or smaller depending of the size of the plasmid which is encapsulated. Thus, for larger plasmids, the size distribution will typically be from about 80 nm to about 180 nm and, more preferably, from about 50 nm to about 150 nm and, more preferably from about 50 nm to about 90 nm. Additionally, the methods described below result in encapsulation of about 40% to 80% of the plasmids in solution. Surprisingly, compositions having the above properties can be prepared by a detergent dialysis method via manipulation of the salt concentration present in the formulation mixture.

III. Methods of Encapsulating Nucleic Acids in a Lipid Bilayer Carrier

In another aspect, the present invention provides methods for the encapsulation of nucleic acids, preferably plasmids, in a lipid bilayer carrier. The plasmids or nucleic acids present in the compositions formed by these methods exhibit preferably less than about 30% degradation, more preferably, less than about 15% degradation and, even more preferably, less than about 5% degradation when subjected to standard nucleases, such as DNase or normal serum nuclease.

The methods for encapsulating a nucleic acid or plasmid in a lipid bilayer carrier, comprise:

(a) combining the nucleic acid, i.e., antisense, ribozyme or plasmid, with a lipid-detergent mixture, the lipid-detergent mixture comprising a lipid mixture of an aggregation-preventing agent (e.g., a PEG-ceramide) in an amount of about 5 mol % to about 20 mol %, cationic lipids in an amount of about 0.5 mol % to about

50 mol %, and neutral or, alternatively, fusogenic lipids in an amount of from about 30 mol % to about 70 mol % and a detergent, to provide a nucleic acid-lipid-detergent mixture; and

(b) dialyzing the nucleic acid-lipid-detergent mixture against a buffered salt solution to remove the detergent and to encapsulate the nucleic acid in a lipid bilayer carrier. In these methods, the salt concentration of the buffered salt solution is adjusted depending on the cationic lipid concentration in the lipid mixture to encapsulate from about 40% to about 80% of the nucleic acid.

In one group of embodiments, the methods further comprise the following step:

(c) removing substantially all of the unencapsulated nucleic acids to provide a purified lipid-bilayer-nucleic acid composition having from about 20 μg to about 400 μg of nucleic acid per about 1 mg of lipid.

The plasmids, antisense, ribozyme or nucleic acids, cationic lipids, fusogenic lipids and aggregation-preventing agent (e.g., ATTA-lipids, PEG-lipids, etc.) that are useful in the present invention are those which have been described above. In preferred embodiments, the amount of cationic lipid is from about 5 mol % to about 50 mol % by weight, more preferably about 10 mol % to about 40 mol % by weight, and most preferably about 20 mol % to about 40 mol % by weight. In a preferred embodiment, the amount of cationic lipids is 10 mol %, 11 mol %, 12 mol %, 13 mol %, 14 mol %, 15 mol %, 16 mol %, 17 mol %, 18 mol %, 19 mol %, 20 mol %, 21 mol %, 22 mol %, 23 mol %, 24 mol %, 25 mol %, 26 mol %, 27 mol %, 28 mol %, 29 mol %, 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, 35 mol %, 36 mol %, 37 mol %, 38 mol %, 39 mol %, 40 mol %, 41 mol %, 42 mol %, 43 mol %, 44 mol %, 45 mol %, 46 mol %, 47 mol %, 48 mol %, 49 mol % or 50 mol %.

Similarly, the amount of aggregation-preventing agent (e.g., PEG-Lipid) can vary from about 1 mol % to about 25 mol % and, more preferably, from about 5 mol % to about 20 mol %. For instance, the amount of PEG-ceramide can preferably vary from about 5 mol % to about 20 mol %, depending on the nature of the PEG-ceramide (e.g., PEG-Cer-C_8, PEG-Cer-C_{14} or PEG-Cer-C_{20}), or the combination of PEG-ceramides used. Selection of the amounts of each can provide compositions in which the fusogenic properties are programmable (i.e., become fusogenic within a predetermined timeframe, depending on the rate at which the PEG-ceramide diffuses out of the composition).

A nucleic acid-lipid-detergent mixture is formed by combining the nucleic acid or plasmid with a lipid-detergent mixture. The lipid-detergent mixture is a combination of aggregation-preventing agent (e.g., ATTA-modified lipids, PEG-modified lipids, such as PEG-ceramides), cationic lipids, neutral or fusogenic lipids and a detergent. The detergent is preferably an aqueous solution of a neutral detergent having a critical micelle concentration of 15–300 mM and, more preferably, 20–50 mM. Examples of suitable detergents include, for example, N,N'-((octanoylimino)-bis-(trimethylene))-bis-(D-gluconamide) (BIGCHAP); BRIJ 35; Deoxy-BIGCHAP; dodecylpoly(ethylene glycol) ether; Tween 20; Tween 40; Tween 60; Tween 80; Tween 85; Mega 8; Mega 9; Zwittergent® 3–08; Zwittergent® 3–10; Triton X-405; hexyl-, heptyl-, octyl- and nonyl-β-D-glucopyranoside; and heptylthioglucopyranoside; with octyl β-D-glucopyranoside being the most preferred. The concentration of detergent in the detergent solution is typically about 100 mM to about 2 M, preferably about 200 mM to about 1.5 M.

The lipid-detergent mixture and nucleic acids typically be combined to produce a charge ratio (+/−) of about 1:1 to about 20:1, preferably in a ratio of about 3:1 to about 15:1. Additionally, the overall concentration of nucleic acid in solution will typically be from about 25 μg/mL to about 1 mg/mL, preferably from about 25–500 μg/mL and, more preferably, from about 100–300 μ/mL. The combination of nucleic acids and lipids in detergent solution is kept, typically at room temperature, for a period of time which is sufficient for complete mixing to occur. While not intending to be bound by any particular theory, it is believed that coated complexes form in which the negative charges of the nucleic acid are paired with positively charged lipids. Excess lipids complete the formation of a bilayer surrounding and encapsulating the nucleic acids. In other embodiments, the nucleic acids and lipid-detergent mixture can be combined and warmed to temperatures of up to about 37° C. For those embodiments in which temperature-sensitive plasmids are used, the mixtures or coated complexes can be formed at lower temperatures, typically down to about 4° C.

The resulting nucleic acid-lipid-detergent mixture is then subjected to dialysis against a buffered salt solution to remove the detergent from the mixture. The removal of the detergent results in the completed formation of a lipid-bilayer which surrounds the nucleic acids or plasmid providing serum-stable nucleic acid-lipid particles which have a size of from about 50 nm to about 150 nm. The particles thus formed do not aggregate.

The buffered salt solution which is used in the dialysis step will typically be a solution of alkali or alkaline earth halides (e.g., NaCl, KCl, and the like), phosphates (e.g., sodium or potassium phosphate), citrates (e.g., sodium citrate) or combinations thereof. The buffer which is used will typically be HEPES or an equivalent buffer. In particularly preferred embodiments, the buffered salt solution is a HEPES-buffered NaCl solution.

Changes in the salt concentration of the dialysis buffer requires significant changes in the lipid composition for efficient encapsulation in the above process. More particularly, the encapsulation efficiency, particle size and the amount of cationic lipid which is used to achieve optimum loading is altered upon changing the salt concentration in the dialysis buffer.

For example, a dialysis buffer containing 150 mM NaCl provides optimal loading of a plasmid in compositions of about 6 mol % DODAC (by weight). By adding citrate to the dialysis buffer, the amounts of DODAC which are used to achieve optimal encapsulation of a plasmid are increased, while maintaining a narrow distribution of particle sizes.

Still higher levels of DODAC (or other suitable cationic lipids) can be used and provide high levels of plasmid encapsulation (typically greater than 30% encapsulation with plasmid/lipid ratios of >20 μg plasmid/mg lipid). For example, a dialysis buffer of 150 mM NaCl and 150 mM sodium phosphate is useful for compositions of about 40–45 mol % DODAC.

Once the plasmid-lipid compositions have formed (typically as particles), any unencapsulated plasmid or empty liposomes can be removed by ion-exchange chromatography or gel filtration, respectively, and any empty liposomes can be removed by density gradient centrifugation using techniques which are well known in the art.

IV. Pharmaceutical Preparations

The nucleic acid (e.g., plasmid)-lipid compositions of the present invention can be administered either alone or in mixture with a physiologically-acceptable carrier (such as

physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice.

Pharmaceutical compositions comprising the nucleic acid (e.g., plasmid)-lipid compositions (e.g., in particle or liposome form) of the invention are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, etc. These compositions may be sterilized by conventional, well known sterilization techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. Additionally, the particle suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

The concentration of particles in the pharmaceutical formulations can vary widely, i.e., from less than about 0.05%, usually at or at least about 2–5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, particles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration. For diagnosis, the amount of particles administered will depend upon the particular label used, the disease state being diagnosed and the judgement of the clinician but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight.

As noted above, it is often desirable to include polyethylene glycol (PEG), PEG-lipids (e.g., PEG-ceramides), ATTA-lipids, or ganglioside G_{M1}-modified lipids to the particles. Addition of such components prevents particle aggregation and provides a means for increasing circulation lifetime and increasing the delivery of the plasmid-lipid particles to the target tissues. Typically, the concentration of the PEG, PEG-lipids (e.g., PEG-ceramide), ATTA-lipids or G_{M1}-modified lipids in the particle will be about 1–25 mol %, preferably about 5–20 mol %.

Overall particle charge is also an important determinant in particle clearance from the blood, with negatively charged complexes being taken up more rapidly by the reticuloendothelial system (Juliano, *Biochem. Biophys. Res. Commun.* 63:651 (1975)) and thus having shorter half-lives in the bloodstream. Particles with prolonged circulation half-lives are typically desirable for therapeutic and diagnostic uses. For instance, particles which can be maintained from 8, 12, or up to 24 hours in the bloodstream are particularly preferred.

In another example of their use, the nucleic acid-lipid particles can be incorporated into a broad range of topical dosage forms including but not limited to gels, oils, emulsions and the like. For instance, a suspension containing the plasmid-lipid particles can be formulated and administered as topical creams, pastes, ointments, gels, lotions and the like.

The present invention also provides nucleic acid-lipid particles in kit form. The kit will typically be comprised of a container which is compartmentalized for holding the various elements of the kit. The kit will contain the compositions of the present inventions, preferably in dehydrated form, with instructions for their rehydration and administration. In still other embodiments, the particles and/or compositions comprising the particles will have a targeting moiety attached to the surface of the particle. Methods of attaching targeting moieties (e.g., antibodies, proteins) to lipids (such as those used in the present particles) are known to those of skill in the art.

Dosage for the nucleic acid-lipid particle formulations will depend on the ratio of nucleic acid to lipid and the administrating physician's opinion based on age, weight, and condition of the patient.

V. Administration of Nucleic Acid-Lipid Particle Formulations

The serum-stable nucleic acid-lipid compositions particles of the present invention are useful for the introduction of plasmids into cells. Accordingly, the present invention also provides methods for introducing a plasmid into a cell. The methods are carried out in vitro or in vivo by first forming the particles or compositions as described above, then contacting the particles with the cells for a period of time sufficient for transfection to occur.

The particles of the present invention can be adsorbed to almost any cell type. Once adsorbed, the particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the nucleic acid portion of the particle can take place via any one of these pathways. In particular, when fusion takes place, the particle membrane is integrated into the cell membrane and the contents of the particle combine with the intracellular fluid. Contact between the cells and the plasmid-lipid particles, when carried out in vitro, will take place in a biologically compatible medium. The concentration of particles can vary widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. Treatment of the cells with the plasmid-lipid particles will generally be carried out at physiological temperatures (about 37° C.) for periods of time of from about 1 to 6 hours, preferably of from about 2 to 4 hours. For in vitro applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

In one group of preferred embodiments, a nucleic acid-lipid particle suspension is added to 60–80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/mL, more preferably about 2×10^4 cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/mL, more preferably about 0.1 μg/mL.

Typical applications include using well known transfection procedures to provide intracellular delivery of DNA or MRNA sequences which code for therapeutically useful

polypeptides. However, the compositions can also be used for the delivery of the expressed gene product or protein itself. In this manner, therapy is provided for genetic diseases by supplying deficient or absent gene products (i.e., for Duchenne's dystrophy, see, Kunkel, et al., *Brit. Med. Bull.* 45(3):630–643 (1989), and for cystic fibrosis, see, Goodfellow, *Nature*, 341:102–103 (1989)). Other uses for the compositions of the present invention include introduction of antisense oligonucleotides in cells (see, Bennett, et al., *Mol. Pharm.*, 41:1023–1033 (1992)).

Alternatively, the compositions of the present invention can also be used for the transfection of cells in vivo, using methods which are known to those of skill in the art. In particular, Zhu, et al., *Science*, 261:209–211 (1993), incorporated herein by reference, describes the intravenous delivery of cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT) expression plasmid using DOTMA-DOPE complexes. Hyde, et al., *Nature*, 362:250–256 (1993), incorporated herein by reference, describes the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway and to alveoli in the lung of mice, using liposomes. Brigham, et al., *Am. J. Med. Sci.*, 298:278–281 (1989), incorporated herein by reference, describes the in vivo transfection of lungs of mice with a functioning prokaryotic gene encoding the intracellular enzyme, chloramphenicol acetyltransferase (CAT).

For in vivo administration, the pharmaceutical compositions are preferably administered parenterally, i.e., intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For example, see, Stadler, et al., U.S. Pat. No. 5,286,634, which is incorporated herein by reference. Intracellular nucleic acid delivery has also been discussed in Straubringer, et al., METHODS IN ENZYMOLOGY, Academic Press, New York, 101:512–527 (1983); Mannino, et al., *Biotechniques*, 6:682–690 (1988); Nicolau, et al., *Crit. Rev. Ther. Drug Carrier Syst.*, 6:239–271 (1989), and Behr, *Acc. Chem. Res.*, 26:274–278 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, Rahman, et al., U.S. Pat. No. 3,993,754; Sears, U.S. Pat. No. 4,145,410; Papahadjopoulos, et al., U.S. Pat. No. 4,235,871; Schneider, U.S. Pat. No. 4,224,179; Lenk, et al., U.S. Pat. No. 4,522,803; and Fountain, et al., U.S. Pat. No. 4,588,578.

In other methods, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical", it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures which include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera, or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

The nucleic acid-lipid particles can also be administered in an aerosol inhaled into the lungs (see, Brigham, et al., *Am. J. Sci.*, 298(4):278–281 (1989)) or by direct injection at the site of disease (Culver, HUMAN GENE THERAPY, Mary-Ann Liebert, Inc., Publishers, New York. pp.70–71 (1994)).

In accordance with the above administration methods, the compositions of the present invention can be used to inhibit tumor cell growth, the method comprising contacting the tumor cell with an effective amount of a lipid-nucleic acid composition of the present invention. Tumor cells include, but are not limited to, lung, colon, breast, ovarian, prostate and hepatic tumor cells as well as squamous cell carcinomas. In a presently preferred embodiment, the tumor cells are present in a mammalian subject. Mammalian subjects include, but are not limited to, humans, laboratory animals, domestic pets and farm animals. Preferred hosts include humans, nonhuman primates, dogs, cats, cattle, horses and sheep. In a further preferred embodiment, the above method further comprises the step of observing for a reduction in the growth of the tumor cells.

In another embodiment, the present invention provides a method of treating cancer, the method comprising administering to a mammalian subject having cancer a therapeutically effective amount of a lipid-nucleic acid composition of the present invention. The compositions of the present invention are useful for treating a wide variety of cancers. Such cancers include, by way of example and not limitation, carcinomas such as pharynx, colon, rectal, pancreatic, stomach, liver, lung, breast, skin, prostate, ovary, cervical, uterine and bladder cancers; leukemias; lymphomas; gliomas; retinoblastomas; and sarcomas. Moreover, in accordance with the above method, mammalian subjects include, but are not limited to, humans, laboratory animals, domestic pets and farm animals.

Lipid-nucleic acid compositions suitable for use in the methods of the present invention can readily be identified using in vitro and in vivo screening assays. Such assays may screen for the ability of a particular composition to inhibit tumor cell growth or to abolish tumorigenicity of malignant cells in vitro or in vivo. For instance, tumor cell lines can be exposed to varying concentrations of a composition of interest, and the viability of the cells can be measured at set time points using the alamar Blue® assay (commercially available from BioSource, International of Camarillo, Calif.). When alamar Blue dye is added to the culture medium, the dye is reduced by cellular mitochondrial enzymes yielding a soluble product with substantially enhanced fluorescence. This fluorescence can be measured with a fluorimeter, whereby the signal is directly proportional to the cell number. Using this information, IC_{50} (concentration of composition lethal to 50% of a cell culture as compared to a control culture) values for the compositions of interest can be readily be calculated.

As will be appreciated by the skilled artisan, many varieties of tumor cell cultures and cell lines can be used to screen for activity including, but not limited to, MDA MB 231 (breast), MCF-7 (breast), MDA MB 468 (breast), Siha (squamous cell carcinoma), A549 (nonsmall cell lung), HL-60 (leukemia) Ovcar-3 (ovarian), etc. Of course, other in vitro and/or in vivo assays to screen for anti-tumor and/or anti-cancer activity known to and used by the skilled artisan can also be employed to identify effective compositions useful in the methods of the present invention.

VII. EXAMPLE 1

Formulation of Lipid-Nucleic Acid Compositions

A. Materials and Methods

1. Materials

N,N-dioleyl-N,N-dimethyl ammonium chloride (DODAC), monomethoxy polyethylene2000 glycol succinate-(C8:0-ceramide) (PEG-Ceramide-C_8), pACN53, pINEXP005, pINEXL002 and pINEX018 plasmids and pL002 (luciferase) plasmid were manufactured and supplied by INEX Pharmaceuticals Corp. Dioleyl-phosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, British Columbia, Canada). Picogreen dsDNA quantitation reagent was obtained from Molecular Probes (Eugene, Oreg.). Dialysis buffers were prepared from commercially available reagents (HEPES, NaCl, dibasic sodium phosphate, monobasic sodium phosphate, trisodium citrate) by standard methods. Octyl-β-D-glucoside (OGP), Spectrapor dialysis tubing and ACS or higher grade reagents were obtained from VWR Scientific, Fisher Scientific or Sigma Chemical Company.

2. Method of Formulation

A variety of formulations were prepared using the procedures outlined below. In a first method, a formulation of plasmid with DOPE:DODAC:PEG-Ceramide-C_8 (42.5:42.5:15 mol %) was prepared. In other methods, the PEG-Ceramide was held constant and formulations were prepared by altering the amounts of DODAC present.

a. DOPE:DODAC:PEG-Ceramide-C_8 (42.5:42.5:15 mol %)

In a preparation containing 5 mg/ml total lipid, the concentration of each lipid at the above mol % quantities are DOPE (1.69 mg/ml), DODAC (1.315 mg/ml) and PEG-Ceramide-C8 (2.005 mg/ml) based on molecular weights calculated at 744, 582 and 2515, respectively. Each of these can be dissolved stock solutions using absolute ethanol, 2:1 chloroform:methanol or 9:1 benzene:methanol. If stock solutions of >20 mg/ml are required, the latter two solvent mixtures are not suitable. Lipids prepared in benzene:methanol have the added advantage that they can be lyophilized (freeze-dried) to a fluffy powder. A dried film of the above lipids are prepared in glass test-tubes (or in round bottomed flasks when prepared in large scale). The combined lipids are dried under a stream of nitrogen (small scale) or in a rotary evaporator (large scale) followed by incubation in vacuo (<100 microns Hg) for at least 2 hours at room temperature. Alternatively, benzene:methanol solutions may be freeze-dried directly. A 1 M solution of OGP (100 μl) is added to each tube containing dried lipid. The plasmid suspension (typically 200–300 μl at 1 mg/mL in TRIS-EDTA buffer) is added to the lipid film. The suspension is made up to 1.0 ml with dialysis buffer, and the suspension is mixed by vortexing until the lipid film is dissolved and a clear solution is formed. Alternatively, the plasmid may be added after the lipid film is dissolved, if desired. The tube is allowed to stand for approximately 30 minutes at room temperature and then the contents are loaded into prepared dialysis bags. Dialysis conditions are found to vary slightly with the quality and type of the plasmid. However, at this DODAC concentration optimum formulations are obtained with 150 mM $NaPO_4$, pH 7.4 with 150 to 175 mM NaCl. The formulations are dialyzed against 2 changes of 2 L of the appropriate buffer (per 1 to 10 ml of formulation).

b. Varying DODAC and DOPE amounts

Lipid mixtures of DODAC/DOPE/PEG-Cer-C8 containing 15 mol % of PEG-Cer-C8 were used for all samples. The amount of DODAC and DOPE were varied to reach desired mole % concentrations. For formulations containing greater than 30 mol % DODAC, the total lipid concentration is typically 5 mg/mL. Formulations containing 30 mol % DODAC and less are prepared at 10 mg/mL total lipid. Five or ten mg of lipid mixture of DODAC/DOPE/PEG-Cer-C8 was dissolved in ethanol or organic solvent (MeOH/$CHCl_3$: 1/1). The solvent was removed by gas N_2 and then dried under vacuum for at least 3 hrs. The lipid mixture is dried to a film in a glass tube or flask as described above. The detergent suspension is prepared as described above. Where DODAC concentrations are below 42.5 mol % (and DOPE increased correspondingly), the buffer and buffer salt concentrations must be adjusted accordingly to allow optimum encapsulation and the selection of specific buffer concentrations is described in the examples below (see, Table 1).

3. Method of Removing Unencapsulated Plasmid

In order to remove unencapsulated plasmid DNA and to determine the absolute recovery of encapsulated plasmid the formulations are *cleaned* by running through a short column (typically 3 cm×1 cm or larger) of DEAE Sepharose which had been pre-equilibrated in either HBS or the respective dialysis buffer for the formulation (i.e., with 150 mM sodium phosphate (NaPO4), 150 mM NaCl, pH 7.4). After running through the column the preparations are normally dialyzed against HBS and concentrated in the dialysis bag using Aquacide II (Calbiochem) to a desired plasmid concentration.

4. Method of Determining Percent Encapsulation

For the determination of percent encapsulation a −/+ Triton X-100 method was used. Typically, aliquots of the formulation taken directly from dialysis were diluted 1:400 in HBS and 2 μL of Picogreen reagent were added to 1 mL of the diluted samples. The fluorescence was measured at 495 nm (excitation) and 525 nm (emission), both in the absence and presence of 10 μL, 10% Triton-X100. The percent encapsulation was calculated as:

$$1-\text{Fluorescence}-\text{Triton}/\text{Fluorescence}+\text{Triton})\times 100.$$

For the determination of absolute plasmid DNA concentration, aliquots of formulation were measured for fluorescence as above in the presence of Triton X100 and compared to standard plasmid concentrations identically prepared.

The following examples are offered solely for the purposes of illustration, and are intended neither to limit nor to define the invention. In each of these examples, the term "DNA" or "plasmid" refers to the plasmid pCMV4-CAT.

5. Method for Separation of Empty Liposomes from Plasmid Containing Vesicles

a. Sucrose Density Gradient Isolation.

Sucrose density gradients are used for the removal of lipid which is not associated with plasmid. The particular gradient used varies with the DODAC concentration as formulations containing the highest DODAC concentrations are the least dense. Gradients are formed by layering decreasing concentrations of HBS-sucrose (W/V) solutions in Beckman (13.2 mL) ultraclear ultracentrifuge tubes above one another. This process is simplified if the solutions are prechilled (at 4° C.) to increase the viscosity of the solutions and using a short Pasteur pipette where the tip has been bent upward (done under a gentle flame). Two useful gradients have been employed. For INEX 351 formulations (42.5 mol % DODAC), a gradient of 5% (3 ml):2.5% (5 ml):1% (2 ml) sucrose (w/v in 20 mM HBS) has been employed. The formulation has been found to settle on the 2.5%–5% sucrose interface. For formulations containing 20–30 mol % DODAC, a gradient containing 10% (2 ml): 5% (2 ml):2.5% (4 ml):1% (2 ml) sucrose has been employed. The plasmic containing TCS has been found to settle on the 5%–10% interface. With either gradient, the formulations are centrifuged at 36,000 rpm in a Beckman SW41 Ti rotor at 25° C. for 7–14 hours. A half deceleration speed has not been found to disturb the bands which form. In every case, 3–4 diffuse bands are observed in the upper portion of the gradient, while a narrower band is generally observed at the lower

interface of each gradient. The lower band normally contains 90% of the applied DNA and is removed by piercing the centrifuge tube with a needle and removing the band using a syringe (3 ml normally adequate). Excellent separation of the desired band has been accomplished with starting plasmid DNA containing up to 400 μg of plasmid per tube. After removal from the gradient, the formulation is dialyzed against 2×21 HBS at 4° C., and the resulting preparation is analyzed for DNA content and particle size.

B. Experiments

1. Formulation: Detergent Dialysis

The ionic strength and the counter-ion concentration of the dialysis buffer required for efficient encapsulation of plasmid DNA are critical and there is a direct relationship between the ionic strength and the cationic lipid concentration used in the formulation. Both the ionic strength and the counter-ion concentration can be adjusted by varying the salt concentration and/or the type of salt ions in the buffer. The ionic strength/counter-ion concentration necessary for efficient encapsulation increases with increasing cationic lipid concentration in the formulation. Phosphate and citrate, respectively, are used as counter-ions to compete with the charges on the polynucleotide for interaction with the cationic charge on the lipid headgroup. When the ionic strength (salt concentration) is too high, it results in vesicle formation with little or no encapsulation, and when it is below the optimum concentration, there is formation of lipid/DNA complexes and of aggregates. An indication for aggregation is a wide size distribution (high polydispersity) of the particles/vesicles formed (see, FIG. **5**). Therefore, the optimum salt concentration needs to be determined for each desired cationic lipid concentration in the formulation. The optimum salt concentration required in the buffer for efficient encapsulation with different concentrations of cationic lipid is summarized in Table 1. Examples for two salt combinations, NaCl with citrate and NaCl with phosphate, are illustrated (FIGS. **5** through **9**). FIG. **5** shows the effect of the NaCl concentration in the citrate/NaCl buffer on the encapsulation efficiency at a given cationic lipid concentration. FIG. **6** shows the relationship between the cationic lipid concentration (DODAC) and the citrate concentration in the buffer to obtain efficient encapsulation. NaCl/citrate was suitable for formulations with DODAC concentration of up to 30 mol %, while NaCl/phosphate could be used over the entire DODAC concentration range tested.

TABLE 1

Characterization of representative large scale TCS formulations.

DODAC concentration	Buffer	Encapsulation efficiency	Nicomp particle size (nm)[a]
7%	0.0 mM citrate, 150 mM NaCl,	80.5%	37 ± 18
17.1%	40.0 mM citrate, 150 mM NaCl,	38.5%	39 ± 20
22.2%	70.0 mM citrate, 150 mM NaCl,	53.5%	43 ± 22
32.0%	105 mM citrate, 150 mM NaCl, Phosphate	51.0%	53 ± 35
20%	105 mM $NaPO_4$	63.%	178
24%	130 mM $NaPO_4$	50.7%	250
30%	150 mM $NaPO_4$	56.8%	109
42.5%	150 mM $NaPO_4$, 130 mM NaCl	49%	131

[a]Nicomp analysis of mean particle size, gaussian dist., volume weighting, before DEAE cleaning and isolation

FIGS. **7** through **11** represent examples where NaCl/phosphate buffers are applied. The effect of a small change in DODAC (cationic lipid) concentration on the encapsulation efficiency is shown in FIG. **7**. The % encapsulation as a function of NaCl concentration is presented in FIG. **8** for four different DODAC concentrations. The NaCl concentration required increased with increasing DODAC concentration. Similarly, the phosphate concentration was adjusted to obtain good encapsulation at different DODAC concentrations (see, FIG. **9**). An additional effect on the encapsulation efficiency was observed by the lipid and polynucleotide concentration, respectively (see, FIGS. **9**, **10** and **11**).

The particles/vesicles containing polynucleotides can be separated from empty vesicles by density gradient centrifugation. DNA containing particles accumulate at a higher sucrose concentration (i.e., lower band) than the empty vesicles (i.e., upper two bands) (see, FIGS. **12** and **13**). FIG. **13** shows the results of lipid and DNA analysis of the different gradient fractions, with DNA exclusively in the lower band. The size distribution of the particles/vesicles in the lower band is very narrow (small number for polydispersity, χ^2) and the mean diameter increases slightly with increasing DODAC concentration from about 65 to 94 nm (see, Table 2). The particles isolated from formulations with different DODAC concentrations had a similar lipid/DNA ratio (see, Table 3). The homogeneous size distribution of the isolated particles was also seen by electron microscopy (see, FIG. **14**).

TABLE 2

Effect of Isolation on Particle Size Parameters

	Particle Size Parameters			
	Before Isolation		After Isolation	
Formulation % DODAC	Mean diameter (nm)	x^2	Mean diameter (nm)	x^2
Citrate				
7%	37 ± 18	0.92	101 ± 11	0.36
17.1%	39 ± 20	2.7	96 ± 20	0.2
22.2%	43 ± 22	2.6	92 ± 25	0.1
32.0%	53 ± 35	23	114 ± 57 ±	1.7
Phosphate				
20%	178	22.9	64.0	0.3
24%	250	78.6	77.2	0.2
30%	109	1.77	89.3	0.18
42.5%	131	2.96	93.8	0.31

TABLE 3

Lipid/DNA ratio of TCS after isolation.

	Total Lipid/DNA ratio (mg/mg)	
Mol % DODAC	Before Sucrose Gradient Isolation	After Sucrose Gradient Isolation
Citrate		
17.1%	30.3	15.9
22.2%	45.0	14.0
23.0%	56.4	16.2
Phosphate		
20	48.8	14.8
24	n.d.	16.6
30	66.3	18.5
42.5	53.2	13.5

n.d. - not determined

The DNA inside the particles was largely protected from serum nucleases and DNase (see, FIGS. **15** and **16**). Formulations were incubated in either DNase or serum, and then separated by gel chromatography. FIG. **15** illustrates the separation profile from Sepharose CL-4B gel filtration chromatography for free plasmid DNA after serum

incubation, and for a formulation with 21 mol % DODAC before sucrose density gradient isolation (see, FIG. **15**A) and after the isolation (see, FIG. **15**B). DNA cleaved by nucleases eluted in fractions >7, while the encapsulated intact DNA eluted together with the lipids in the exclusion volume fractions 4–7. The integrity of the encapsulated DNA was characterized further by electrophoresis and, as illustrated in FIG. **16**, the plasmid remained intact.

2. Transfection: In Vitro

The transfection activity and toxicity of formulations containing various concentrations of the cationic lipid DODAC were tested in vitro in COS-7 and Hep-G2 cells (see, FIGS. **17** through **22**). Luciferase plasmid was formulated and expression of luciferase was determined at times indicated. Cell viability was used as an indication for toxicity. The transfection efficiency was determined as a function of DODAC concentration used in the formulation (see, FIGS. **17** through **22**). Furthermore, transfection activity was evaluated as a function of the DNA dose applied (see, FIGS. **19** and **20**) and as a function of time (see, FIG. **21**). There is limited transfection activity with formulations containing less than 16–18 mol % DODAC. The best transfection activities are obtained with DODAC concentration in the range of 20–30 mol % in the formulations. The cell viability indicates that toxicity increases significantly with preparations containing >30 mol % DODAC. The toxic effect of the preparation particularly for formulations with high DODAC concentrations can be reduced greatly by removal of the empty vesicles by gradient centrifugation. Furthermore, these isolated preparations showed a significant increase in the transfection activity (see, FIG. **22**).

3. Transfection: In Vivo

A murine tumor model was chosen to determine the transfection activity in vivo. Mice (C57) were injected by the intra peritoneal (i.p.) route with 100,000 B16 tumor cells. Formulations were administered i.p. on day 7 of B16 tumor growth. After 24 hours (unless indicated otherwise), the animals were sacrificed and the tumors, liver and spleen were analyzed for luciferase expression. The tumor transfection activity was dependent on the concentration of DODAC in the formulation (see, FIGS. **23** and **25**). The highest luciferase activity was observed from formulations with 20 to 30 mol % DODAC. Isolated preparations consistently showed increased transfection activity when compared to nonisolated preparations for the same DNA dose (see, FIG. **25**). The luciferase expression in tumors was higher 24 hours after injection than after 48 hours (see, FIG. **24**). Luciferase activity was also observed in liver (see, FIG. **27**) and in spleen (see, FIG. **26**). None of the formulations with the different DODAC concentrations showed significant liver toxicity following i.p. administration as based on the AST levels in the plasma (see, FIG. **28**).

VII. EXAMPLE 2

Immunological Effect of Repeated Injections of Lipid-Nucleic Acid Compositions

A. Materials and Methods

A formulation of plasmid with DOPE:DODAC:PEG-Ceramide-C8 (61:24:15 mol %) (INEX324) was prepared using the methods of Example 1. The formulations were prepared containing pCMV(3 and pINEXL018. They were isolated and assayed as described in Example 1.

1. Administration of INEX324 to Balb/c Mice.

Mice, 12 per group, were injected intravenously (i.v.) with TCS formulations as described below. In this study four injections were given at intervals of seven days over a period of five weeks (Table 4). Group A was injected with INEX324 expressing LacZ reporter gene on day 0, 7 and 14 (150 μl per mouse of INEX324-LacZ, 75 μg DNA). Two weeks after the last LacZ injection, mice were injected with INEX324-Luc (150 μl per injection, 80 μg DNA). To evaluate the immunogenicity of INEX324 lipid, Group B received three injections of INEX324 empty vesicles (150 μl per mouse) and one injection of INEX324-Luc two weeks later. Group C served as a base-line control, mice were given 150 μl of diluent at each time point. Mice from Group D received three diluent injections, followed by a single injection of INEX324-Luc, and served as a positive control for luciferase expression. Three mice from each group were sacrificed at 18 hours after each injection, or 24 hours prior to the following injection. Blood was collected and the serum samples were analyzed for the presence of antibodies specific for β-galactosidase using an ELISA assay. Spleens were harvested and splenocytes were processed for various immunological assays: carrier specific, β-gal, LacZ, or mitogen induced clonal expansion and cytokine (IL-2 and IL-4) release. Splenocytes were monitored for expression, of differentiation (CD4, CD8, CD22, CD11b) and activation (CD86, MHC-II, Ly-6A/E, CD54 and CD25) markers. The remaining mice from all groups were sacrificed 12 h following the last injection with INEX324-Luc. At this point, the organs (lung, liver and spleen) were fast frozen in liquid nitrogen and assayed for luciferase expression.

TABLE 4

		Treatment Schedule			
Group	Formulation	day 0 1st injection	Day 7 2nd injection	day 14 3rd injection	day 28 4th injection
A	324/LacZ	324/LacZ	324/LacZ	324/LacZ	324/Luc
B	324 lipid	324 lipid	324 lipid	324 lipid	324/Luc
C	Diluent	Diluent	Diluent	Diluent	Diluent
D	Diluent	Diluent	Diluent	Diluent	324/Luc

2. Flow Cytometry Analysis.

Splenocytes from all groups were analyzed for expression of differentiation and activation markers. Spleen cells (1×10^6) were stained with appropriate PE-conjugated antibodies (anti-CD22, anti-CD4, anti-CD8, anti-CD11b, anti-CD54, anti-CD86, anti-Ly6A/E and anti-CD25) and phenotypic analysis was performed on a FACSsort flow cytometer (Becton Dickinson, San Jose, Calif.). Splenocytes were analyzed either after 18 h and 6 days after in vivo treatment.

3. Cell Proliferation Assay.

Unseparated spleen cells were tested in vitro for their ability to proliferate upon re-stimulation with the empty INEX324 vesicles, INEX324-LacZ formulation, LacZ, transgene product (r-β-galactosidase), or with polyclonal activators for T (Concanavalin A, Con A) and β cells (Lipopolysaccharide, LPS). Single-cell suspensions of lymphocytes were prepared from whole spleens by grinding the spleens using the frosted ends of sterile glass slides in RPMI media containing 10% FBS. The suspension was allowed to settle standing on ice in a 15 ml polypropylene culture tube. An isolated cell suspension was separated from debris by removing the supernatant and the cell number was quantified using a Coulter counter (Coulter Instruments, Miami, Fla.). Aliquots of cell suspensions (100 μl, 5×10^6/ml) in the above media were placed into 96-well plates along with the equal volumes of various appropriate stimuli. Cells were labeled with ^{3}H-thymidine for 48 h, and after 3 days incubation, they were harvested. The levels of incorporated radioactivity were measured in a scintillation counter. ^{3}H-thymidine incorporation is expressed total ^{3}H-incorporation (DPMs), or as a mean percentage (±SD) of media control and plotted versus lipid concentration values.

4. Measurement of Cytokine Release.

Splenocytes (1×10^6 cells/ml) were either nonstimulated or cultured in the presence of various concentrations of

either empty INEX324 vesicles, or INEX324-LacZ formulation at different times after culture initiation (24 h and 48 h). The levels of cytokine in the cell culture supernatant (Interleukin-2 and Interleukin-4) were determined by an ELISA assay (below).

5. Cytokine ELISA Assays.

Cytokine-specific ELISA assays were performed using the protocol and specific anti-interleukin antibodies provided by reagent mini-kit (Endogen, Woburn MA). Briefly, Immuno-module (F8-maxisorb) 96-well plates were coated overnight with anti-IL-2, or anti-IL-4 antibody. Plates were washed with PBS-Tween 20 (0.05%) and blocked with PBS-Tween 20-BSA (2%) for 1 hour at room temperature. Supernatant samples and standards (diluted in blocking buffer) were added and allowed to incubate overnight. Plates were washed and biotinylated anti-IL-2 or anti-IL-4 antibody was added. After 2 hours incubation, plates were washed and HRP-Extravidin, followed by TMB, was added to each well. Plates were read on a plate reader at OD_{450nm}. The amount of released cytokine was determined by comparing the O.D. of test supernatants to a standard curve of serially diluted cytokine standards.

6. ELISA for Detection of β-gal-specific Antibodies.

β-gal specific antibodies in the serum were measured using an ELISA assay. Immuno-module (F8-maxisorb) 96-well plates were coated overnight with r-β-gal (10 μg/ml, 100 μl/well) diluted in bicarbonate buffer (pH 9.6). Plates were washed with PBS-Tween 20 (0.05%) and blocked with PBS-Tween 20-BSA (1%) for 30 minutes. Serum samples and standard anti-β-gal IgM and IgG, diluted in blocking buffer, were added to the wells and allowed to incubate overnight. Plates were washed and biotinylated anti-mouse IgG antibody was added. After 2 hours incubation, plates were washed and HRP-Extravidin, followed by TMB substrate, was added to each well. The development of a colored reaction product was quantified on plate reader at OD_{450}. The amount of IgG was determined by comparing OD of test serums to a standard curve of serially diluted antibodies as standards.

7. In Vivo Gene Expression.

Balb/c mice were given 3 intravenous injections of INEX324-LacZ or empty INEX324 vesicles on days 0, 7 and 14. After two additional weeks, mice from all groups (except for the diluent treated group) received one injection of INEX324-Luc (80 μg of DNA) and 12 h later the organs (liver, lung, spleen) were collected and assayed for luciferase activity. A standard assay for the determination of luciferase from the tissue samples was employed. Tissue homogenization was performed using a FastPrep Instrument (FastPrep™ FP120 Instrument, Bio 101) using supplied tubes and beads (FastDNA tubes with MS Matrix). Tissues were homogenized in Cell Culture Lysis Reagent (1×CCLR, Promega) supplemented with BSA (1 mg/ml). FastPrep Instrument settings: speed—5; time—8 sec twice. Samples were transferred to new microcentrifuge tubes and briefly centrifuged (2 min., 10,000 rpm) to remove debris. The luciferase assay was performed on luminometer (Dynatech Microlite™ ML3000) using a 96-well microlite plate. A set of purified luciferase standard solutions was prepared (Firefly luciferase) by serially diluting 1 μg/μl luciferase in 1×CCLR supplemented with BSA (1 mg/ml). For the standard curve, luciferase protein was diluted in a control tissue homogenate to compensate for quenching and 20 μl aliquots (in duplicates) were assayed for each sample/standard. Settings for Luminometer: grade—medium; delay time—2 sec; integrate time—10 sec; substrate—100 μl (Luciferase Assay System, Promega). The results were converted to pg of luciferase protein/g of tissue.

8. In Vitro Transfection of BHK Cells.

BHK-21 cells were plated at a density of 1×10^6 cells (in 10 ml media) per 75 cm^2 tissue culture flasks. The following day, when the cells were 60–70% confluent, the media was aspirated and replaced with 4.8 ml of fresh culture media 2–3 hours prior to transfection. INEX324-LacZ (12.5, 6.2 or 3.1 μg of DNA per 1×10^6 cells) was added to the culture media (total volume did not exceeded more than 200 μl) and particles were allowed to remain in contact with the cells for the next 24 hours (at 37° C., 5% CO_2). Separate flasks for appropriate transfection controls: untreated cells, lipid only, plasmid only, and gene unrelated plasmid/particles (i.e., INEX324-Luc) were included. The cell viability and gene expression assays were performed 24 hours post-transfection.

9. FACS Assay for in Vitro Detection of Transgene β-galactosidase.

Single cell suspensions from transfected cell monolayers were prepared by mechanical dissociation. Dissociated cells were transfered into polystyrene tubes, and stained for the presence of the transgene product, β-galactosidase, using the FDG assay. In this assay, aliquots containing 10^6 cells were pelleted and re-suspended in 100 μl of staining medium in a 6 ml polystyrene FACS tube and were incubated for 10 min. at 37° C. for 10 minutes. The FDG reagent was diluted into 2 ml of distilled water and was pre-warmed to 37° C. for no more than 10 minutes. A 100 μl aliquot of the FDG solution was added to each sample. The suspensions were vortexed and incubated an additional minute at 37° C. After incubation, 1.8 ml ice-cold staining medium was added to each tube and the tubes were placed on ice in darkness. Each sample was read within 5 min. after staining using the FACSort flow cytometer.

B. Results and Discussion

1. Analysis of Differentiation Markers.

Spleen cells analyzed for the expression of differentiation markers were isolated, stained and assayed either 18 h, or six days following INEX324-LacZ administration. The analysis of differentiation markers 18 h after in vivo treatment shows that there are no appreciable differences in the frequency of cells expressing CD22 (B cells) and CD11b (macrophages) markers between the groups (Table 5). The percentage of $CD4^+$ cells and $CD8^+$ cells within the spleen cell population from INEX324-LacZ treated group was approximately 40% higher compared with diluent or lipid treated group, however, alteration in the ratio of $CD4^+$ and $CD8^+$ was not observed. Spleen cells were processed for the expression of the same set of differentiation markers six days following last injection. Examination of the spleen cell population from INEX324-LacZ treated group revealed changes in phenotypic profile of spleen cells: a moderate decrease in the percentage of T cells (15%) and a significant 30% decrease of $CD22^+$ cells. Furthermore, INEX324-LacZ treatment resulted in a five-fold increase of macrophage number as defined by expression of CD11b antigen, compared with diluent treated mice and those that received the empty liposome. It might be that repeated administration of lipid/DNA induces alteration in the pool of splenic cells, evidenced by the decreased number of lymphocytes subsets, T and B cells, and increased the number of macrophages. On the other hand, it is possible that the number of T and B cells is not decreased and these changes are due to the increased spleen cellularity in this group (40% increase compared with control groups).

A possible explanation for the observed increased spleen cellularity (also observed as splenomegaly) is an infiltration of monocytes into the spleen. In this case, the promoted infiltration of monocytes (and consequently an increased number of CD11b cells), would make the relative contribution of other cell subpopulations within unfractionated spleen samples appear relatively lower (Table 5). In addition, an increased number of macrophages suggests that INEX324 lipid is not toxic. Macrophages have an innate

capacity for nonspecific phagocytosis of large quantities of foreign particles such as liposomes. Following internalization and processing of phagocytosed liposomes, their survival depends on the toxicity of the liposomal constituents. In the present study, repeated administration of INEX324 lipid is not accompanied with a decrease in $CD11b^+$ cells, on the contrary, their number is significantly increased and this strongly indicates that INEX324 lipid and INEX324/DNA are not toxic, even following repeated systemic administration.

TABLE 5

Expression of differentiation markers by spleen cells treated in vivo

	After 18 hours				After 6 days			
Formulation	CD4	CD8	CD22	CD11b	CD4	CD8	CD22	CD11b
Diluent	22.38	9.47	57.64	6.02	25.76	11.33	57.88	4.19
INEX324 lipid control	19.65	9.32	57.03	5.32	25.57	11.62	57.51	5.33
INEX324-LacZ	30.76	13.51	52.71	6.31	21.85	9.65	42.58	25.10

Expression of differentiation markers is quantified as the percentage of positive cells in the unseparated splenic suspension.

2. Expression of Activation Markers.

The expression of activation markers was analyzed either 18 h, or six days after the last injection in vivo. The results show that there was a significant difference in their expression that was both time and treatment dependent (Table 6). The expression of activation markers was up-regulated in spleen cells from animals injected with INEX324-LacZ compared with the untreated and control lipid treated groups 18 h following treatment. As up-regulated expression of activation markers is always associated and positively correlates with the activation of immune cells, these results indicate that only lipid/DNA administration results in activation of spleen cells. However, when splenocytes were analyzed six days following the third injection, expression of activation markers in lipid/DNA treated group, returned to normal level and there was no difference between the groups. These results clearly show that activation is not induced by the repeated administration of the INEX324 TCS. In addition, activation is only detected as a transient increase in mice injected with INEX324-LacZ and is probably associated with the inflammatory properties of DODAC in the INEX324 formulation combined with potential immunogenicity of plasmid DNA.

TABLE 6

Expression of activation markers by spleen cells treated in vivo

	After 18 hours				After 18 hours After 6 days			
Formulation	CD54	CD86	MHC-II	Ly6A/E	CD54	CD86	MHC-II	Ly6A/E
Diluent	40.14	1.27	53.97	1.49	6.18	0.76	53.16	1.20
324 lipid	39.87	2.14	55.83	2.38	7.06	0.99	53.03	2.13
324-LacZ	57.53	6.74	62.62	84.96	7.19	1.27	52.85	5.73

Expression of activation markers is quantified as the percentage of positive cells in the unseparated splenic suspension.

3. Influence of Repeated Administration of INEX324/DNA on Cell Proliferation.

Unseparated spleen cells, from all experimental groups, were tested in vitro for their ability to proliferate upon re-stimulation with empty INEX324 vesicles, INEX324-LacZ-formulation, LacZ (naked DNA), with transgene product (r-β-galactosidase), or with polyclonal activators for T (Con A) and B (LPS) cells. The immunogenicity of INEX324 lipid and INEX324-LacZ formulation was evaluated using three concentrations of lipid (0.005, 0.01 and 0.02 ng/ml) and three different encapsulated DNA concentrations (0.01, 0.05 and 0.1 μg/ml). At the same time, the mitogenicity of the same formulation was determined in vitro using spleen cells from control mice (diluent treated group). If in vivo treatment with control lipid, or with lipid/DNA formulation, had induced an immune stimulation, than a clone of memory cells would be generated, and in vitro restimulation of those cells with the same immunogen would result in augmented cell proliferation. The results show that when mice were primed with lipid or with lipid/DNA formulations, secondary responses to the same formulations in vitro were practically undetectable, indicative of minimal immunostimulation (Table 7).

Similar results were obtained upon in vitro stimulation of splenocytes from control mice with the same formulations, regardless of the concentrations used for stimulation none of them induced measurable mitogenic response. These results indicate that lipid and lipid/DNA formulations are not immunogenic in vivo and are not mitogenic in vitro. Stimulation of splenocytes from lipid/DNA treated group with plasmid DNA (LacZ) induced minimal, but detectable response that was not significantly different compared to those obtained from control and lipid-treated group in vitro. Stimulation of splenocytes from all experimental groups with r-β-Gal did not induce any differences in proliferative response that would correspond with the treatment in vivo. When splenocytes from all three groups were stimulated in vitro with polyclonal activator LPS (mitogen for B-cells and macrophages), again there were no measurable differences in the magnitude of proliferative response corresponding to the in vivo treatment (Table 4.). However, repeated administration of INEX324 into Balb/c mice resulted in a moderate (16%) decrease and INEX324/DNA caused a marked

(40%) decrease in ConA-induced proliferation compared with diluent injected mice. ConA is a T cell mitogen, and this result indicates that repeated administration of INEX324/DNA is accompanied by a down-regulation of the splenic T cell proliferative response. The factors involved in this suppression are not completely understood, but are consistent with the reported results regarding the ability of increased number of $CD11b^+$ cells to inhibit the proliferation of T cells in unseparated spleen cell population (Ostro MJ, TomBD/Six HR Liposomes and Immunobiology, Elsevier NH Inc., p225–239,(1980); Jaffe et al., Molecular Medicine 2, (6) 692–701, (1996)). The INEX324/DNA induced splenomegaly, increased spleen cellullarity, and five-fold increase of macrophage number observed in this study are consistent with this mechanism.

TABLE 7

Cell proliferation and cytokine release following in vitro stimulation of spleen cells.

In vivo treatment	In vitro stimulation	^{3}H-incorporation DPMs	Cytokine release IL-2	(OD_{450}) IL-4
Diluent	Diluent	1674	0.127	0.151
Diluent	324 empty vesicles	9673	0.073	0.097
Diluent	324-LacZ	9051	0.089	0.090
Diluent	LacZ	3352	0.082	0.095
Diluent	r-β-gal	18336	0.059	0.103
Diluent	ConA	215542	0.615	0.138
Diluent	LPS	35676	—	—
324 empty vesicles	324 empty vesicles	2447	0.083	0.099
324 empty vesicles	324-LacZ	6375	0.089	0.081
324 empty vesicles	LacZ	3798	0.116	0.098
324 empty vesicles	r-β-gal	16401	0.077	0.097
324 empty vesicles	ConA	182435	0.569	0.165
324 empty vesicles	LPS	33090	—	—
324-LacZ	324-LacZ	16959	0.103	0.076
324-LacZ	324 empty vesicles	9025	0.099	0.082
324-LacZ	LacZ	7520	0.134	0.089
324-LacZ	r-β-gal	13345	0.125	0.087
324-LacZ	ConA	125450	0.350	0.157
324-LacZ	LPS	41639	—	—

4. Influence of Repeated Administration of INEX324-DNA on Cytokine Release.

Splenocytes were nonstimulated, or cultured in the presence of either lipid, or various concentrations of lipid/DNA formulation, DNA, r-β-Gal, or Con A. Culture supernatants were collected at different times after culture initiation (after 24 h or 48 h), and the levels of released cytokines, IL-2 and IL-4, were measured (Table 4). Following in vivo treatment, there was no increased release of Th1 and Th2 cytokines from splenocytes of INEX324 and INEX324/DNA treated mice. TCS treated splenocytes produced IL-2 and IL-4 in levels similar to splenocytes from mice that received diluent only. Following in vivo treatment, there was also no difference in cytokine release pattern between control and lipid treated group, compared to group treated with lipid/DNA formulation. In addition, the splenocytes from all experimental groups released similar insignificant amounts of IL-2 and IL-4 after in vitro stimulation with LacZ and r-β-gal. In response to stimulation with T-cell mitogen (with a suboptimal concentration of ConA) however, unfractionated spleen cells obtained from lipid and lipid/DNA treated mice secreted markedly reduced amounts of IL-2. Splenocytes from lipid treated mice produced 25% less, and from lipid/DNA treated mice 40% less IL-2 compared with group of mice receiving diluent only (Table 4.). The decreased amounts of IL-2 release correlate with down-regulated ability of T cells to proliferate following stimulation with ConA. These results suggest that various subsets of cells of the immune system might be differentially affected by in vivo treatment with INEX324 and INEX324/DNA.

5. Effect of Repeated INEX324/DNA Administration on Production of β-gal Specific Antibodies.

Serum samples were assayed for presence of antibodies against the transgene protein (β-gal) following third injection of lipid/LacZ formulation. Results indicate that in vivo treatment with INEX324-LacZ formulations did not elicit measurable amounts of IgM antibodies, following first injection, and IgG antibodies, after three consecutive i.v. injections (FIG. **29**). The amount of IgM and IgG antibodies assayed on the same level as in a control and lipid treated group. The absence of detectable levels of anti-transgene IgG antibodies suggests that although there is measurable expression of transgene protein, the immune response against the expressed transgene protein has not been elicited.

6. Gene Expression in Vitro.

Transfectability of the INEX324-LacZ formulation was tested in vitro. BHK cells were transduced in vitro using various concentrations (3.1, 6.2 and 12.5 μg per 1×10^6 cells) of INEX324-LacZ and 24 hours later the expression of β-gal was assayed by FACS. The FDG assay was developed to evaluate the establishement of stable transformed cell lines (constititively express β-gal protein) and to test the quality of pINEXLacZ plasmids. When complexes (INEX100 series TCS) were used for in vitro transfection of various cell lines, in most cases transduced cells were 60–80% positive for the expression of β-gal protein. pINEXLacZ was encapsulated in 302 and 303 vesicles and used for transfection in vitro, but the expression of the transgene protein was not detected, even though expression can be detected in vivo. Using INEX324 to encapsulate pINEXLacZ, for the first time we were able to measure gene expression in vitro. INEX324 lipid and INEX324-Luc formulation were used as negative controls. Transfection of BHK cells with INEX324-LacZ was very efficient: 85% of cells transfected with 12.5 μg of DNA and 40% of cells transfected with 6.2 μg of DNA expressed transgene β-gal protein (FIG. **30**.).

7. Gene Expression in Vivo

A small-scale in vivo experiment was performed in order to determine the time course and the magnitude of gene expression following systemic (i.v.) administration of INEX324-Luc formulation. Balb/c mice (three per group) were given a single i.v. injection of INEX324-Luc (100 μg of DNA per mouse) and the expression of luciferase was determined either 12 h or 24 h post-injection. The organs (lung, liver and spleen) from both INEX324-Luc injected mice and diluent treated controls, were processed the same time and luciferase expression was evaluated. The results demonstrate that a single administration of INEX324-Luc resulted in significant gene expression in the spleen. Luciferase expression in the liver was measurable, but at a much lower level. No expression was detected in the lung. The time course of transgene expression was highest at 12 hours post injection and is still measurable at lower levels 24 hours post-injection (FIG. **31**.).

In the next study, Balb/c mice were given 3 intravenous injections of INEX324-LacZ or empty 324 vesicles on day 0, 7 and 14. Two weeks later the administration was repeated with INEX324-Luc (80 μg of DNA per mouse) and 12 h later the organs (liver, lung and spleen) were collected and assayed for luciferase activity. The results show that there was a significant luciferase expression in the spleen from all experimental groups (FIG. **32**).

Previous in vivo treatment with INEX324 empty vesicles or INEX324-LacZ formulation, did not result in decreased

gene expression in the spleen, luciferase expression in both groups assayed at levels similar to that from control group. Although the level of gene expression in the liver was much lower, it demonstrates the same pattern as in the spleen confirming that previous in vivo treatment did not compromise the ability of TCS to deliver gene of interest (FIG. **33**.). There was no measurable gene expression in the lungs from any of the groups tested (results not shown).

VIII. CONCLUSION

As discussed above, in accordance with one of its aspects, the present invention provides compositions and methods for preparing serum-stable nucleic acid (e.g., plasmid)-lipid particles which are useful for the transfection of cells, both in vitro and in vivo.

Still further, nucleic acids (e.g., plasmid DNA) can now be formulated using a variety of lipids to provide compositions having extremely high plasmid/lipid ratios. The process can be performed in a predictable manner, by generating theoretical curves for any set of lipids, whereby accurate predictions of the salt concentration necessary to achieve a serum stable formulation can be made.

Still further, nucleic acids (e.g., plasmid DNA) can be formulated using a variety of lipids to provide compositions that can be administered in repeat doses without eliciting an immune response, while still maintaining gene expression.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

What is claimed is:

1. A method of encapsulating a nucleic acid in a lipid bilayer carrier, said method comprising:

(a) combining said nucleic acid with a lipid-detergent mixture, said lipid-detergent mixture comprising a lipid mixture of an aggregation-preventing agent in an amount of about 5 mol % to about 20 mol %, a cationic lipid in an amount of about 0.5 mol % to about 50 mol %, and a flisogenic lipid and a detergent, to provide a nucleic acid-lipid detergent mixture; and

(b) dialyzing said nucleic acid-lipid-detergent mixture against a buffered salt solution to remove said detergent and to encapsulate said nucleic acid in a lipid bilayer carrier and provide a lipid bilayer-nucleic acid composition, wherein said buffered salt solution has an ionic strength sufficient to encapsulate of from about 40% to about 80% of said nucleic acid.

2. The method in accordance with claim **1**, further comprising

(c) removing substantially all of the unencapsulated nucleic acids to provide a purified lipid bilayer-nucleic acid composition having from about 20 μg to about 400 μg of nucleic acid per about 1 mg of lipid.

3. The method in accordance with claim **1**, wherein said nucleic acid is a plasmid.

4. The method in accordance with claim **1**, wherein said detergent is octylglucoside.

5. The method in accordance with claim **1**, wherein said cationic lipid is DODAC.

6. The method in accordance with claim **1**, wherein said aggregation-preventing agent is a member selected from the group consisting of gangliosides, ATTA-lipids and PEG-lipids.

7. The method in accordance with claim **6**, wherein said aggregation-preventing agent is a PEG-lipid.

8. The method in accordance with claim **7**, wherein said PEG-lipid is a PEG-ceramide.

9. The method in accordance with claim **8**, wherein said PEG-ceramide is selected from the group consisting of PEG-Cer-C8, PEG-Cer-C 14 and PEG-Cer-C20.

10. The method in accordance with claim **1**, wherein said buffered salt solution is HEPES-buffered NaCl solution.

11. The method in accordance with claim **1**, wherein said buffered salt solution is a citrate solution.

12. The method in accordance with claim **1**, wherein said buffered salt solution contains about 150 mM NaCl.

13. The method in accordance with claim **1**, wherein about 50% to about 70% of the initial concentration of said nucleic acid becomes encapsulated.

14. The method in accordance with claim **8**, wherein said cationic lipid is DODAC, said PEG-ceramide is selected from the group consisting of PEG-Cer-C8, PEG-Cer-C14 and PEG-Cer-C20, said fusogenic lipid comprises DOPE and said buffered salt solution comprises NaCl and sodium phosphate.

15. The method in accordance with claim **1**, wherein the lipid bilayer carrier-encapsulated nucleic acid formed has a mean particle diameter of from about 50 nm to about 150 nm in the absence of extrusion or sonication.

16. The method in accordance with claim **1**, wherein the lipid bilayer carrier-encapsulated nucleic acid formed has a mean particle diameter of from about 50 nm to about 90 nm in the absence of extrusion or sonication.

17. A method for introducing a nucleic acid into a cell, said method comprising:

(a) preparing a lipid-nucleic acid composition according to claim **1**; and

(b) contacting said cell with said lipid-nucleic acid composition for a period of time sufficient to introduce said nucleic acid into said cell.

18. The method in accordance with claim **17**, wherein said cell is a spleen cell.

19. The method in accordance with claim **17**, wherein the efficiency of transfection is not diminished by repeat doses administered within 2 weeks.

* * * * *

JOINT APPENDIX 46

Gene Therapy (1999) 6, 1438–1447

http://www.stockton-press.co.uk/gt

Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties

YP Zhang[1], L Sekirov[1], EG Saravolac[1], JJ Wheeler[1], P Tardi[1], K Clow[1], E Leng[1], R Sun[1], PR Cullis[1,2] and P Scherrer[1,2]

[1]*INEX Pharmaceuticals Corporation, Burnaby, BC; and* [2]*Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada*

Previous work (Wheeler et al, Gene Therapy 1999; **6***: 271–281) has shown that plasmid DNA can be entrapped in 'stabilized plasmid-lipid particles' (SPLP) containing the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE), low levels (5–10 mol%) of cationic lipid, and stabilized by a polyethyleneglycol (PEG) coating. The PEG moieties are attached to a ceramide anchor containing an arachidoyl acyl group (PEG-CerC_{20}). These SPLP exhibit low transfection potencies in vitro, due in part to the long residence time of the PEG-CerC_{20} on the SPLP surface. In this work we employed SPLP stabilized by PEG attached to ceramide containing an octanoyl acyl group (PEG-CerC_8), which is able to quickly exchange out of the SPLP, to develop systems that give rise to optimized in vitro and in vivo (regional) transfection. A particular objective was to achieve cationic lipid contents that give rise to maximum transfection levels. It is shown that by performing the dialysis procedure in the presence of increasing concentrations of citrate, SPLP containing up to 30 mol% of the cationic lipid dioleoydimethylammonium chloride (DODAC) could be generated. The SPLP produced could be isolated from empty vesicles by sucrose density gradient centrifugation, and exhibited a narrow size distribution (62 ± 8 nm, as determined by freeze–fracture electron microscopy) and a high plasmid-to-lipid ratio of 65 μg/μmol (corresponding to one plasmid per particle) regardless of the DODAC content. It was found that isolated SPLP containing 20–24 mol% DODAC resulted in optimum transfection of COS-7 and HepG2 cells in vitro, with luciferase expression levels comparable to those achieved for plasmid DNA–cationic lipid complexes. In vivo studies employing an intraperitoneal B16 tumor model and intraperitoneal administration of SPLP also demonstrated maximum luciferase expression for DODAC contents of 20–24 mol% and significantly improved gene expression in tumor tissue as compared with complexes. We conclude that SPLP stabilized by PEG-CerC_8 and containing 20–24 mol% cationic lipid are attractive alternatives to plasmid DNA–cationic lipid complexes for regional gene therapy applications.*

Keywords: *non-viral gene delivery; cationic lipid; detergent dialysis; cancer gene therapy; plasmid encapsulation; liposomes*

Introduction

Non-viral gene delivery systems offer many potential advantages over viral vectors, including avoidance of concerns related to viral immunogenicity and reversion to the infectious phenotype. In addition, well-defined synthetic gene delivery systems offer possible advantages with respect to vector manufacture and characterization. However, the most commonly employed non-viral systems, plasmid DNA–cationic lipid complexes[1–3] formed by adding plasmid to liposomes containing cationic lipids, are not well-defined systems. These complexes are often unstable, are susceptible to aggregation and exhibit transfection potencies that can be sample- and time-dependent. In addition, complexes do not have general applicability. For example, whereas complexes usually exhibit reasonable transfection potency *in vitro*,[4–7] these large systems are generally cleared rapidly following intravenous injection, which limits potential transfection sites to 'first-pass' organs such as the lung, liver and spleen.[8–12] Furthermore, DNA–cationic lipid complexes can also produce toxic side-effects *in vitro*[13] and *in vivo*.[14]

Efforts in our laboratory have focused on the development of small, well-defined lipid-based plasmid carrier systems with general applicability, where the plasmid is fully encapsulated within a lipid envelope. Initial studies have shown that plasmid can be entrapped in 'stabilized plasmid-lipid particles' (SPLP) through a detergent dialysis procedure, resulting in particles of approximately 70 nm in diameter containing one plasmid per particle.[15] These systems contain the fusogenic lipid dioleoylphosphatidylethanolamine (DOPE) and a small amount of cationic lipid, and are stabilized in aqueous media by the presence of a polyethylene glycol (PEG) coating. It has also been established that the *in vitro* transfection potency of SPLP is dependent on the hydrophobic ceramide (Cer) group anchoring the PEG polymer to the SPLP, where ceramide groups containing shorter acyl groups exhibit improved transfection properties.[15] This has been attributed to the ability of PEG-Cer molecules with shorter acyl groups to dissociate from the SPLP, thereby destabil-

Correspondence: PR Cullis, INEX Pharmaceuticals Corporation, 100–8900 Glenlyon Parkway, Burnaby, BC V5J 5J8, Canada
Received 17 November 1998; accepted 15 April 1999

izing the particle and improving association with and uptake into target cells. In this regard, it is well established that the presence of a PEG coating can inhibit association and fusion between large unilamellar vesicles (LUVs)[16,17] and between LUVs and biological membranes.[18]

This work focuses on the development of SPLP that exhibit optimized transfection properties *in vitro* and that have regional application *in vivo*. This application requires that the PEG coating rapidly dissociates from the carrier, therefore, SPLP have been constructed employing PEG-Cer molecules containing an octanoyl group (PEG-$CerC_8$). Previous work has shown that PEG-$CerC_8$ molecules exhibit half-times ($t_{1/2}$) for dissociation from LUV (in the presence of 'acceptor' LUV) of less than 1.2 min,[18] whereas PEG-$CerC_{14}$ and PEG-$CerC_{20}$ exhibit $t_{1/2}$ values of 1.2 h and greater than 13 days, respectively.[15] Particular attention has been paid to developing SPLP containing optimized amounts of cationic lipid. The previously described SPLP protocol was limited to low (5–10 mol%) cationic lipid contents in order to achieve efficient plasmid encapsulation.[15] In this work, it is shown that SPLP containing up to 30 mol% cationic lipid can be generated by including increasing amounts of citrate in the dialysis medium, and that optimum *in vitro* transfection potencies are obtained at approximately 24 mol% cationic lipid contents. The transfection potencies observed for regional transfection of peritoneal tumors employing these optimized SPLP are superior to those observed using plasmid DNA–cationic lipid complexes.

Results

Plasmid can be encapsulated in SPLP containing high levels of cationic lipid by employing citrate in the dialysis medium

Previous work has shown that plasmid can be encapsulated in SPLP with the lipid composition DOPE, dioleoyldimethylammonium chloride (DODAC) and PEG-$CerC_{20}$ (84:6:10; mol:mol:mol) by a detergent dialysis procedure employing octylglucoside.[15] Under the conditions used, the cationic lipid content was critical, as plasmid encapsulation levels approaching 70% could be achieved for DODAC contents of 6–7 mol%. Little encapsulation was obtained at lower DODAC levels, however, and at DODAC contents above 9% significant aggregation problems were encountered. Similar behavior was observed here for SPLP containing DOPE/DODAC/PEG-$CerC_8$ (78:7:15; mol:mol:mol). Following dialysis against HBS (5 mm HEPES, 150 mm NaCl, pH 7.4), small (diameter $<$70 nm) vesicles exhibiting high encapsulation efficiencies of 80% were formed (see Table 1). The degree of plasmid encapsulation was estimated by determining the relative accessibility of a DNA interchelating fluorescent dye (PicoGreen) to the plasmid in the presence and absence of Triton ×-100, as described in Materials and methods. DODAC contents above 10 mol% caused aggregation of the formulation during dialysis.

It has been proposed that SPLP form when plasmids interact with lipid structures exhibiting an appropriate surface charge during the detergent dialysis process.[15] Therefore, it was reasoned that encapsulation into SPLP with higher cationic lipid content might be possible if the ionic strength of the dialysis medium was raised to shield the surface charge on the lipid structures. Initial experiments employed higher NaCl concentrations ranging from 0.15 to 1.0 m NaCl (with 10 mm HEPES, pH 7.2). Increasing the NaCl concentration to 0.5 m was sufficient to prevent aggregation for a formulation containing 10 mol% DODAC (results not shown), but was not effective in a formulation containing 20 mol% DODAC, where aggregation persisted even up to 1 m NaCl. Polyvalent anionic counter-ions such as citrate may be expected to produce stronger shielding effects. A study of plasmid encapsulation as a function of citrate concentration was performed for a lipid mixture composed of DODAC/DOPE/PEG-$CerC_8$ (20:65:15; mol:mol:mol) and the pCMVLuc plasmid. As shown in Figure 1, at concentrations up to 60 mm citrate the dialyzed samples contained large (diameter $>$150 nm) and polydisperse (χ^2 $>$3.0) particles. However, small, monodisperse particles (82 ± 40 nm diameter as measured by quasi-elastic light scattering; QELS) exhibiting high encapsulation efficiencies of 50–70% were formed when the dialysis medium contained 65–80 mm citrate. Increasing the citrate concentration further also resulted in formation of small particles, but the encapsulation efficiency decreased dramatically.

The results shown in Figure 1 suggest two criteria for determining the optimum citrate concentration for plasmid encapsulation: (1) formation of monodisperse (χ^2 $<$3.0) particles with diameter smaller than 100 nm; and (2) an encapsulation efficiency greater than 50%. Studies to determine citrate concentrations that satisfy these criteria over a range of DODAC concentrations were performed, and the results are summarized in Figure 2. The citrate concentration range giving rise to particles with a diameter smaller than 100 nm and with encapsulation efficiencies of 50% or higher is represented by the solid circles. Higher citrate concentrations give rise to low plasmid encapsulation efficiencies of 30% or less, whereas citrate concentrations below the optimum levels resulted in large, polydisperse aggregates (χ^2 $>$5). The plasmid encapsulation efficiencies that could be achieved at optimum citrate concentrations for formulations containing up to 30 mol% DODAC are summarized in Table 1.

It was not possible to obtain satisfactory formulations for preparations containing 30 mol% DODAC by varying the citrate concentration. Aggregation persisted in formulations dialyzed in 70–90 mm citrate buffer, while at higher concentrations the plasmid encapsulation was less than 25%. Improved results were achieved, however, by leaving the citrate concentration constant at 100 mm and varying the NaCl concentration. Decreasing the NaCl concentration from 150 mm to 120–140 mm resulted in an increase in the plasmid encapsulation efficiency to 55–70% (Figure 3) while the particle size remained small ($<$100 nm diameter).

Lipid composition, plasmid-to-lipid ratio and size of isolated SPLP

Previous work has shown that SPLP formulations containing 7 mol% DODAC contain empty vesicles and unencapsulated plasmid in addition to SPLP, and that the free plasmid can be removed by passage over a DEAE column, whereas the removal of empty vesicles requires an additional density gradient centrifugation step.[15] In this study it was found that similar procedures could be

Table 1 Physical properties of SPLP formulations containing 7–30 mol% DODAC

DODAC content (mol%)[a]	*After detergent dialysis*			*After isolation by density gradient centrifugation*	
	Diameter (nm)[b]	χ^2 [b]	*Encapsulation efficiency (%)*[c]	*Diameter*	χ^2 [b]
7	64 ± 28	1.0	80.5	101 ± 11	0.4
12	68 ± 29	1.1	77.5	91 ± 29	0.6
16	75 ± 34	2.9	68.2	95 ± 21	0.3
20	82 ± 41	0.6	65.9	99 ± 22	0.3
24	97 ± 60	1.7	69.4	94 ± 30	0.9
28	101 ± 59	1.7	47.5	ND	ND
30	81 ± 48	ND	54.4	115 ± 57	1.7

[a]DODAC content in SPLP formulations composed of DODAC/DOPE/PEG-CerC_8 (x:85-x:15; mol:mol:mol).
[b]Mean diameter (± standard deviation) and polydispersity (χ^2) of the SPLP formulations after dialysis as measured by QELS using the volume-weighted vesicle mode.
[c]Plasmid encapsulation efficiency determined by the PicoGreen assay.
ND, not determined.

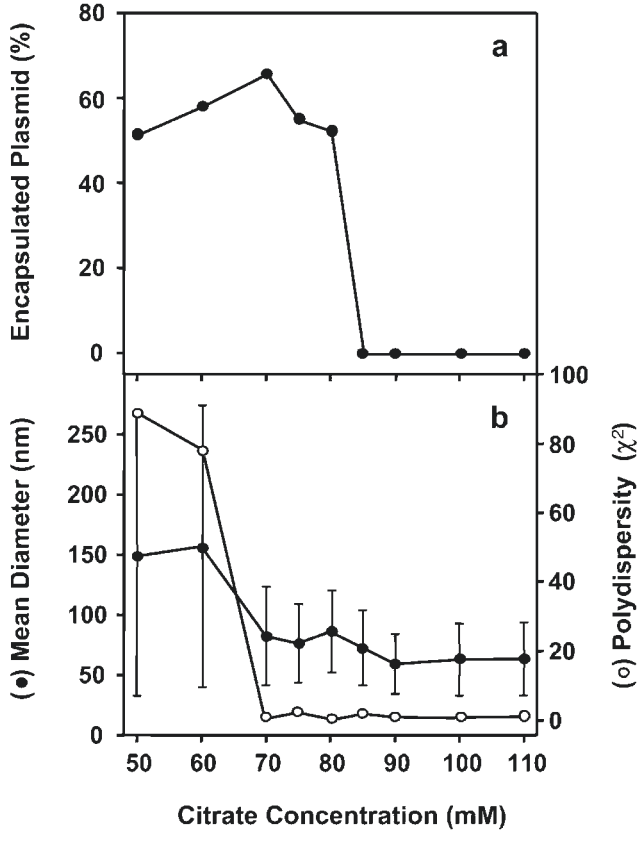


Figure 1 *Plasmid DNA can be entrapped in SPLP containing high levels of DODAC by raising the citrate concentration present during detergent dialysis. (a) The effect of varying the citrate concentration on plasmid encapsulation efficiency following detergent dialysis, as determined by the PicoGreen fluorescence assay (see Materials and methods). (b) The effect of citrate on the diameter (●) and polydispersity, χ^2 (○), of the formulations following detergent dialysis as measured by QELS (volume-weighted vesicle mode). Formulations were composed of DODAC/DOPE/PEG-CerC_8 (20:65:15; mol:mol:mol) and pCMVLuc (10 mg lipid and 200 μg plasmid per ml), and were prepared by detergent dialysis as described under Materials and methods.*

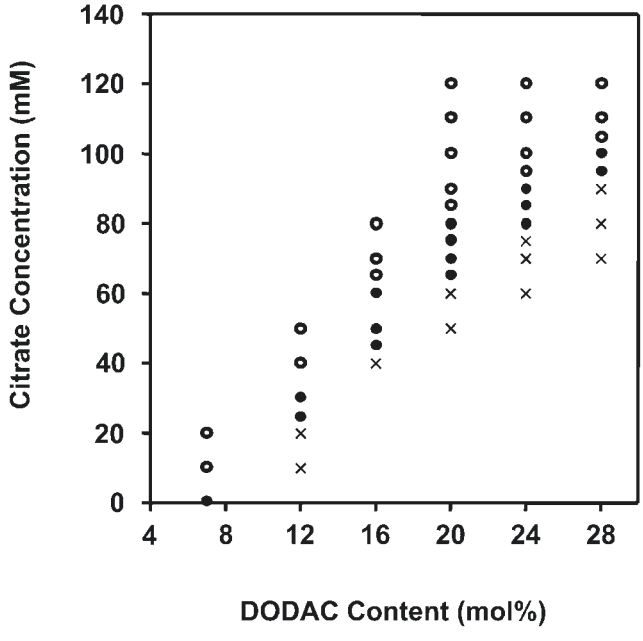


Figure 2 *Determination of the optimal citrate concentration range as a function of SPLP DODAC content to achieve maximum plasmid encapsulation in combination with minimum aggregation. Formulations were composed of DODAC/DOPE/PEG-CerC_8 (x:85-x:15; mol:mol:mol) and pCMVLuc (10 mg lipid and 100–200 μg plasmid per ml) and were prepared by detergent dialysis where the dialysate contained the indicated sodium citrate concentrations as well as 150 mm NaCl, 10 mm HEPES (pH 7.2). The solid circles (●) indicate formulations that exhibited plasmid encapsulation efficiencies greater than 50% and a small, monodisperse size distribution as determined by QELS (diameter <100 nm, χ^2 <3). The open circles (○) indicate formulations that exhibited plasmid encapsulation efficiencies of less than 40% in combination with a small, monodisperse size distribution (diameter <100 nm, χ^2 <3). The crosses (x) indicate polydisperse formulations with large size distributions (diameter >100 nm, χ^2 >3).*

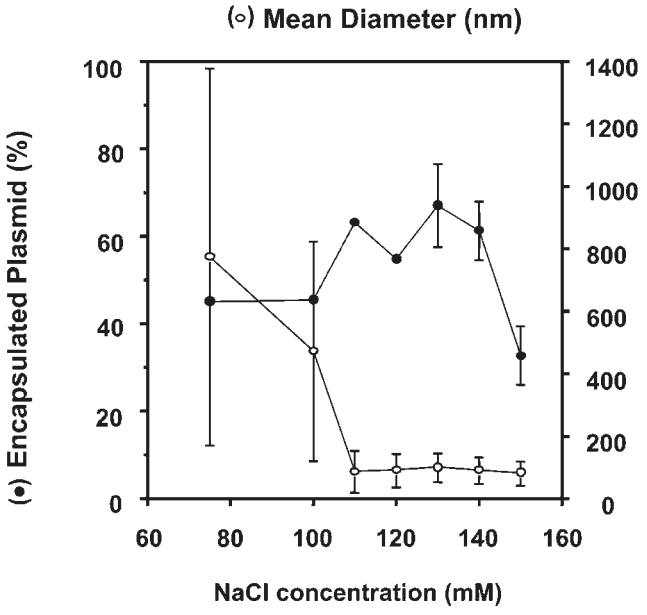


Figure 3 *Plasmid encapsulation in SPLP at a given DODAC content and citrate concentration can be optimized by varying the NaCl concentration. The formulations were composed of DOPE/DODAC/PEG-CerC$_8$ (55:30:15; mol:mol:mol) and pCMVLuc (10 mg lipid and 200 μg plasmid per ml), and were prepared by detergent dialysis where the dialysate contained varying amounts of NaCl and 100 mm sodium citrate, 10 mm HEPES (pH 7.2). The closed circles (●) indicate the plasmid encapsulation efficiencies determined employing the PicoGreen fluorescence assay. The open circles (○) indicate the mean diameter (± standard deviation) of the formulations following dialysis, as measured by QELS (volume-weighted vesicle mode).*

applied to SPLP formulations containing PEG-CerC$_8$ and higher cationic lipid contents. Nearly 90% of the plasmid loaded on to the gradient was recovered in the fraction containing the SPLP (located at the 5–10% interface of the density gradient), whereas less than 20% of the total lipid was associated with this fraction. The lipid composition of purified SPLP was found to be similar to the initial lipid composition, with a slight enrichment (1–3%) in the DODAC content. Importantly, regardless of the initial plasmid-to-lipid ratio or relative DODAC content, the final plasmid-to-lipid ratios in these purified SPLP were consistently in the range of 65 μg/μmol, which corresponds to one plasmid per SPLP.[15]

Particle size and morphology were characterized by QELS and by freeze–fracture electron microscopy techniques. The isolated SPLP (Table 1) were highly monodisperse ($\chi^2 < 1$) and had a consistent mean diameter of 90–110 nm with a narrow size distribution (standard deviation of approximately 20–30%) as determined by QELS (volume-weighted vesicle mode). Note, the mean diameter of isolated SPLP detected by QELS in 'solid-particle' mode was 70–90 nm. The empty vesicle fraction following density centrifugation contained very small particles with diameters of 30–60 nm. The morphology and size distribution of isolated SPLP containing 21 mol% DODAC as determined by freeze–fracture electron microscopy revealed highly uniform spheres of 62 ± 8 nm in diameter as shown in Figure 4. The morphology of these SPLP is similar to that observed for bilayer LUV systems,[19] consistent with an SPLP structure consisting of plasmid trapped within a lipid bilayer. Similar results

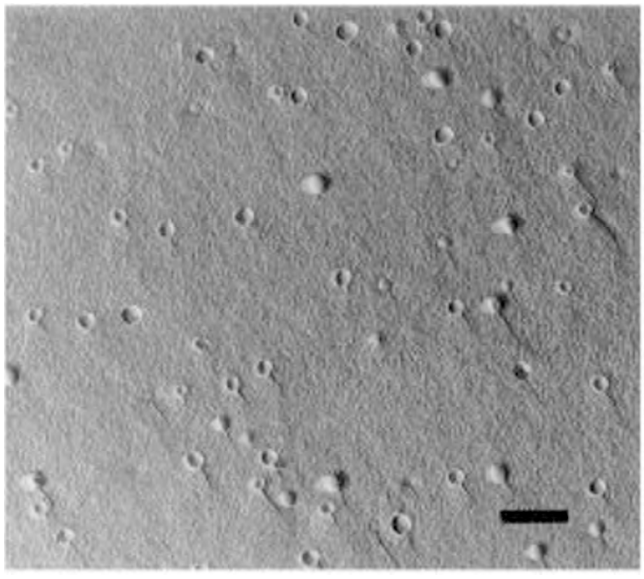


Figure 4 *Freeze–fracture electron micrograph of isolated SPLP. An SPLP formulation composed of DOPE/DODAC/PEG-CerC$_8$ (64:21:15; mol:mol:mol) and plasmid (pCMVLuc) was prepared as indicated in the caption for Figure 1 and non-encapsulated plasmid was removed by DEAE column chromatography. SPLP were separated from empty vesicles on a discontinuous sucrose density gradient. The gradient consisted of 2 ml 10% sucrose, 6 ml 5% sucrose, 1 ml 1% sucrose and was centrifuged at 160 000 g for 12 h. The bar indicates 200 nm. For details of sample preparation and electron microscopy, see Materials and methods.*

were observed for a SPLP formulation containing 15 mol% DODAC (data not shown). Freeze–fracture studies on the empty lipid fraction revealed small vesicles less than 40 nm in diameter.

SPLP containing high cationic lipid contents are stable against serum degradation and storage

SPLP stability in serum provides a rigorous test, as serum proteins associate with lipid vesicles,[20] resulting in leakage and potential exposure of encapsulated plasmid to serum nucleases. Isolated SPLP (DODAC/DOPE/PEG-CerC$_8$; 21:64:15, mol:mol:mol) containing ^{3}H-pCMVLuc were incubated in 90% serum for 1 h at 37°C and passed over a size exclusion chromatography column. Over 85% of the plasmid in SPLP was intact and eluted in the void volume (Figure 5). This may be compared to the behavior of free plasmid, which was completely degraded in serum and the plasmid fragments eluted in the included volume.

The stability of the isolated SPLP during storage was assessed by monitoring size and plasmid encapsulation. No significant changes in size and plasmid encapsulation were observed for the two SPLP formulations tested (containing 15 mol% and 20 mol% DODAC) during storage at 4°C for a period of 5 months (results not shown).

SPLP contain a trapped aqueous volume

The volume of one encapsulated plasmid is not sufficient to fill the interior volume of a particle of approximately 70 nm diameter, suggesting that SPLP contain an interior aqueous volume.[15] It is of interest to measure this entrapped volume directly. In this regard, the measurement of the trapped volume of liposomes usually employs a radiolabeled membrane-impermeable marker,

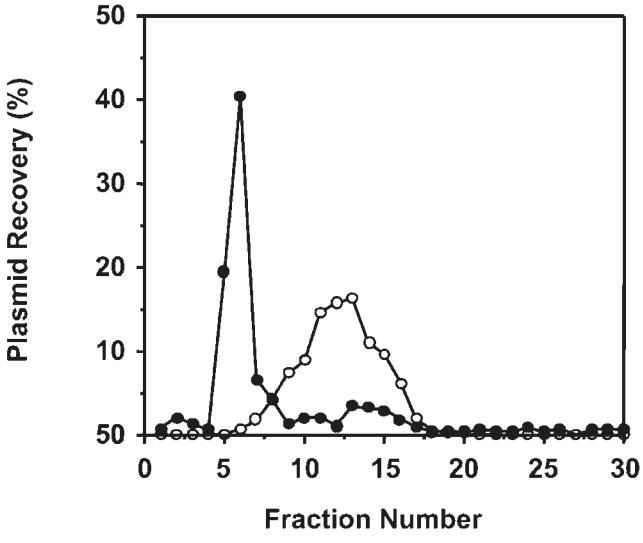


Figure 5 *Plasmid in SPLP containing 21 mol% DODAC is protected from serum nuclease degradation. An SPLP formulation composed of DOPE/DODAC/PEG-CerC$_8$ (64:21:15; mol:mol:mol) and plasmid (pCMVLuc) was prepared as indicated in the caption for Figure 1. The non-encapsulated plasmid was removed by DEAE column chromatography and the SPLP isolated by sucrose density gradient centrifugation. Free plasmid and SPLP-encapsulated plasmid was incubated in 90% mouse serum for 1 h at 37°C. The sample was then loaded on to a 5 ml Sepharose CL-4B gel filtration column and eluted with HBS. Fractions (0.5 ml) were analyzed for ^{3}H-labelled plasmid by scintillation counting. The open circles represent the elution profile of the 'free plasmid' sample and the closed circles the SPLP-encapsulated plasmid preparation.*

such as inulin or sucrose.[21,22] Before using sucrose to measure the trapped volume of SPLP, it was important to show that sucrose would not leak out during the time required to isolate the particles. The release rate of ^{14}C-sucrose from LUV with the same lipid composition as the SPLP was therefore measured as indicated in Materials and methods. Two LUV formulations composed of DODAC/DOPE/PEG-CerC$_8$, containing 7:78:15 and 20:65:15 molar ratios, respectively, were employed. The measured half-life for sucrose retention at 20°C was found to be in the range of 300–700 h (results not shown). The time required for sucrose density gradient centrifugation was 10–20 h, thus allowing ^{14}C-sucrose to be used as a membrane-impermeable marker to determine the trapped volumes of SPLP. Trapped volumes of 2.2 and 2.0 μl/μmol lipid were obtained following the procedures detailed in Materials and methods for the isolated SPLP formulations containing 7 and 20 mol% DODAC, respectively.

Influence of cationic lipid content on the transfection potency of SPLP in vitro

The effect of cationic lipid content in the SPLP on *in vitro* transfection was investigated in COS-7 and HepG2 cell lines using SPLP formulations that had been purified by DEAE chromatography only and SPLP isolated by both DEAE chromatography and sucrose density gradient centrifugation. The luciferase activities detected in COS-7 cells following transfection are shown in Figure 6. Luciferase expression was dramatically increased in cells incubated with the isolated SPLP (Figure 6b), as compared with SPLP that had not undergone density gradient centrifugation (Figure 6a). In addition, the transfection activity was strongly dependent on the DODAC content in the SPLP. Luciferase activity was low for isolated SPLP containing 7–8 mol% DODAC but increased substantially to reach a plateau value between 20 and 24 mol% DODAC. Luciferase expression remained essentially unchanged for further increases in DODAC content. Importantly, the luciferase activities detected for isolated SPLP containing high DODAC levels are comparable with those obtained for the plasmid–lipid complexes formed with pCMVLuc and DODAC/DOPE (1:1; mol:mol) liposomes. Similar results were also observed for the HepG2 cell line (results not shown). The charge ratio of 1.5:1 (+/–) used to form plasmid DNA–cationic lipid complexes resulted in optimal transfection in these cell lines.

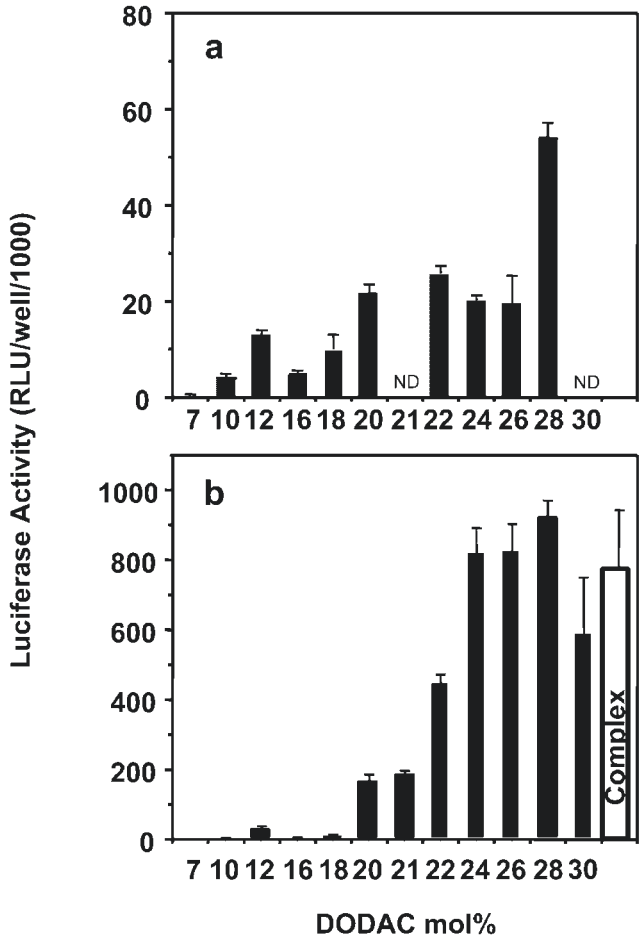


Figure 6 *Effect of DODAC content in SPLP on transfection activity in vitro. Transfection activity is shown with SPLP before (a) and after (b) isolation by density gradient centrifugation. Note the different y-axis scales for panels a and b. Plasmid (pCMVLuc) was encapsulated in SPLP containing 7–30 mol% DODAC, as described in the caption for Figure 2. SPLP isolation by density gradient centrifugation was conducted as described in the caption for Figure 4. SPLP were added to COS-7 cells (1 μg plasmid per well) and then incubated for 24 h. The luciferase activity was determined as described in Materials and methods. The 'complex' bar illustrates the transfection activity achieved with complexes of pCMVLuc and DODAC/DOPE (1:1; mol:mol) LUV at a charge ratio of 1.5:1 (+/–). ND, not determined.*

Influence of cationic lipid content on SPLP transfection potency in vivo

The ability of SPLP containing different levels of DODAC to transfect a tumor following regional injection *in vivo*

was evaluated in an intraperitoneal (i.p.) tumor model. B16BL-6 tumor cells were seeded in the peritoneal cavity of C57BL/6 mice. After 7 days isolated SPLP containing pCMVLuc were injected intraperitoneally at a dose of 30 μg plasmid per mouse. The tumors were collected after 24 h and assayed for luciferase activity. The mean luciferase expression was found to increase with increasing DODAC content in the SPLP (Figure 7) as was observed in the *in vitro* studies. The highest levels of luciferase expression, approximately 1.1 ng/g tumor, were observed for SPLP with DODAC concentrations ranging between 20 and 30 mol%. In this model system, luciferase expression at 24 h was 10 times higher than that observed with DOPE:DODAC (1:1; mol:mol) LUVs complexed with pCMVluc. These complexes were constructed at a 3:1 cationic lipid-to-DNA charge ratio, which was optimal for *in vivo* transfection in this model. Also, expression following transfection with complexes followed a different time-course than that observed for transfection with SPLP, and maximum transfection levels of 100–200 pg luciferase per gram of tumor were observed 12 h after injection with the complex system. For SPLP the highest expression levels were observed 24 h following transfection as compared with 12 and 48 h.

As an indicator for systemic toxicity of SPLP, aspartate aminotransferase (AST) levels in the serum were analyzed 24 h after i.p. injection of the preparations. It was found that administration of SPLP containing 6 to 30 mol% DODAC at a lipid dose of 120 mg/kg did not result in significantly elevated enzyme levels (data not shown).

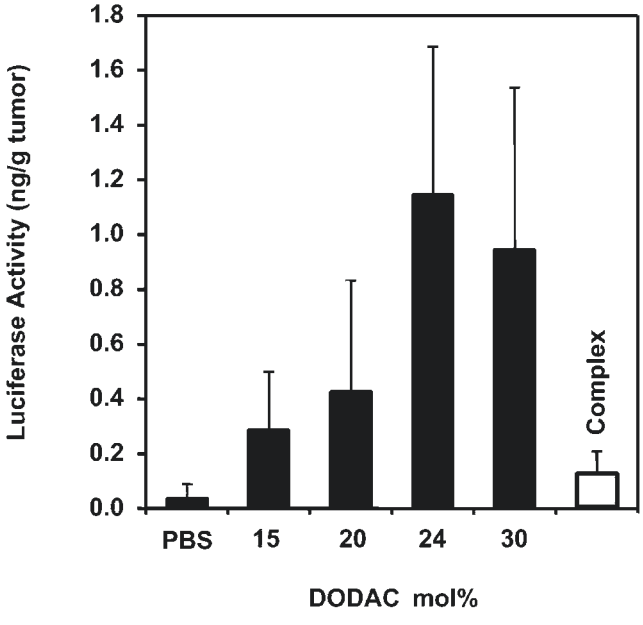


Figure 7 *Transfection of B16 tumors in vivo following regional (i.p.) injection of isolated SPLP is dependent on the DODAC concentration in SPLP. B16BL-6 cells (100 000) were seeded in C57BL/6 mice i.p 7 days before i.p. administration of the different formulations (30 μg plasmid in 500 μl). Tumors were collected 24 h after transfection and assayed for luciferase activity (n = 4). The 'complex' bar shows the transfection activity obtained with cationic lipid–plasmid DNA complexes formed with pCMVLuc and DODAC/DOPE (1:1; mol:mol) LUVs at a charge ratio of 3:1 (+/–).*

Discussion

This study demonstrates that SPLP with a dissociable PEG coating and elevated levels of the cationic lipid DODAC exhibit improved transfection potency *in vitro* and *in vivo*. There are three interesting aspects of these results. The first concerns the improved transfection properties of SPLP containing PEG-CerC_8 molecules that can rapidly dissociate from the carrier. The second concerns the mechanism whereby citrate facilitates formation of SPLP with higher cationic lipid contents and the *in vitro* and *in vivo* transfection potency of SPLP with optimized cationic lipid contents. The final point relates to the small, stable, well defined nature of SPLP. We discuss these aspects in turn.

The physical properties of the SPLP described here differ significantly from the properties of the SPLP described previously.[15] A major difference concerns the PEG-CerC_8 molecule used to stabilize the SPLP. In the previous work SPLP containing PEG-CerC_{20} were generated and it was shown that these SPLP exhibited poor transfection properties *in vitro*. Improved transfection potency could be achieved by using PEG-CerC_{14} as the stabilizing component. This improvement was attributed to the ability of the PEG-CerC_{14} to diffuse away from the SPLP due to the much faster dissociation rate of PEG-CerC_{14} ($t_{1/2}$ = 1.2 h) as compared with PEG-CerC_{20} ($t_{1/2} \geqslant 13$ days), thus rendering the particle more able to interact with target cells. It has been shown elsewhere that the presence of a PEG coating can inhibit interaction and fusion between lipid vesicles.[16,17] The SPLP employed in the present study contained PEG-CerC_8 as the stabilizing agent, which exhibits a considerably faster dissociation rate ($t_{1/2} \leqslant 1.2$ min), thus maximizing the *in vitro* transfection potency required for the *in vitro* studies performed here. It should be noted that these systems would not be suitable for intravenous delivery with the aim of reaching sites of disease such as distal tumor sites, as they will be highly unstable following interaction with biological fluids. More stable SPLP containing PEG-CerC_{20} which diffuses away over much longer times, are better suited to such applications.[23]

With regard to the mechanism whereby citrate facilitates formation of SPLP with higher cationic lipid contents, previous work conducted at a fixed ionic strength showed that the plasmid entrapment efficiency was a sensitive function of the cationic lipid content, with maximum entrapment in the range of 5–10 mol% DODAC.[15] It was proposed that plasmid first interacts with macromolecular structures composed of DOPE, DODAC and PEG-Cer that are formed as intermediates during the dialysis process, to produce SPLP. The formation of structures such as cylindrical micelles and lamellar sheets during the formation of lipid vesicles by detergent dialysis is well established.[24–26] The results presented here indicate that by using the appropriate amount of citrate to shield the positive charge on the lipid structures containing higher amounts of cationic lipid, the affinity of the plasmid for these intermediates can be reduced to levels compatible with good entrapment. Within this model citrate concentrations below the optimum range do not result in adequate shielding of the positively charged lipid structures formed during dialysis, resulting in crosslinking by plasmids and aggregate formation. At citrate concentrations above the optimum

range on the other hand, the positive charge on the lipid structures is shielded to the extent that interaction with plasmid is inhibited, resulting in little or no entrapment. At the critical citrate concentrations, the shielded charge on the lipid structure is just sufficient to bind plasmid, and encapsulation can then proceed as outlined previously.[15]

A major finding of this study is that SPLP containing 24 mol% DODAC and stabilized by PEG-CerC_8 exhibit *in vitro* and *in vivo* transfection properties that are comparable to, or better than, those observed for corresponding plasmid DNA–cationic lipid complexes. There are two factors that contribute to this enhanced activity. The first, as previously discussed, is related to the rapid dissociation of the PEG coating. The second factor that clearly plays a major role is the elevated cationic lipid content. It is possible that higher levels of cationic lipid result in enhanced association with, and uptake into, nearby cells. In this regard it has been noted that cellular uptake of plasmid delivered by SPLP stabilized by PEG-CerC_8 and containing 6 mol% DODAC is less than 3% of that delivered by DODAC/DOPE (1:1) complexes.[27] Alternatively, it is possible that the higher levels of cationic lipid contribute to the endosomolytic activity required to facilitate intracellular delivery of the encapsulated plasmid. A feature that is of particular interest concerns the dramatically enhanced transfection activity of SPLP following isolation by density gradient centrifugation as compared with the SPLP that contain empty vesicles (Figure 6). These results suggest that cells have limited uptake capacity and that saturation of this uptake by empty vesicles as compared with SPLP containing plasmid seriously compromises transfection activity.

The final topic of discussion concerns the well-defined nature of the SPLP system. Aside from the differences arising from the cationic lipid content and the ability of the PEG-Cer to dissociate, the physical characteristics of the SPLP generated here are remarkably similar to the SPLP generated employing 6% DODAC and PEG-CerC_{20}. The high plasmid-to-lipid ratio of 65 μg/μmol lipid, which corresponds to one plasmid per SPLP, is maintained, as is the size of the SPLP at a diameter of approximately 65 nm. The additional finding that SPLP contain a trapped volume is consistent with previous work[15] indicating that the interior volume of an SPLP is considerably larger than the volume taken up by the entrapped plasmid. The measured trapped volumes of 2 μl/μmol lipid correspond to an SPLP diameter of 66 nm, assuming an area per lipid molecule of 0.6 mm^2. This is in good agreement with the SPLP diameter measured by freeze–fracture electron microscopy (62 ± 8 nm). The fact that the SPLP system has an appreciable interior trapped volume clearly offers interesting opportunities for co-encapsulating agents to improve transfection properties along with the plasmid DNA.

The well-defined structure and stability of SPLP contrasts strongly with the properties of cationic lipid–plasmid DNA complexes. The purified SPLP exhibit a uniform size of approximately 70 nm and contain one plasmid per particle, with the plasmid fully encapsulated within a lipid envelope. As emphasized previously, complexes usually exhibit sizes greater than 200 nm diameter, contain an indeterminate number of plasmids per complex and do not fully protect associated plasmids from the external environment. In addition, SPLP exhibit certain practical advantages with regard to manufacturing and stability. Due to their small size, SPLP can be readily sterilized by passage through a 0.22 micron filter. Further, SPLP can be concentrated to plasmid concentrations greater than 1 mg/ml[15] and are also highly stable during extended storage. The preliminary studies performed here indicate that SPLP exhibit no significant changes in size or plasmid encapsulation during storage at 4°C for at least 5 months. Again, it is well recognized that complexes exhibit increased instability at plasmid concentrations of 100 μg/ml or greater. In our hands, DODAC-containing complexes prepared at a charge ratio of 3:1 could not be prepared at plasmid concentrations greater than 375 μg/ml (L Akhong, unpublished results). It should be noted that complexes are generally so unstable during extended storage that most laboratory and clinical applications employ a 'two-vial' approach, where the plasmid and cationic lipid vesicles are mixed together immediately before administration.

In summary, the results presented in this investigation illustrate that the SPLP system produced by detergent dialysis represents a highly flexible platform technology for construction of non-viral gene delivery vectors. It is demonstrated that SPLP can be stabilized by PEG-Cer molecules that freely dissociate, thus reducing or eliminating the inhibitory effects of a PEG coating on cell association. It is also shown that SPLP can be constructed to contain high amounts of cationic lipid if the dialysis medium contains high levels of a citrate buffer. SPLP containing approximately 24 mol% of the cationic lipid DODAC, together with the dissociable PEG coating, give rise to gene transfer vectors that exhibit *in vitro* transfection potencies that are comparable to those achieved for cationic lipid–plasmid DNA complexes. Further, these SPLP give rise to superior tumor transfection levels in a regional tumor model.

Materials and methods

Materials

Dioleoyldimethylammonium chloride (DODAC) was kindly provided by Dr S Ansell (Inex Pharmaceuticals, Burnaby, BC, Canada) and 1-O-[2′-(ω-methoxypolyethyleneglycol) succinoyl]-2-N-octanoylsphingosine (PEG-CerC_8) was synthesized as described elsewhere.[28] Dioleylphosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, BC, Canada). Dialysis tubing (SpectraPor 5, 12 000–14 000 MWCO) was obtained from Spectrum Medical Industries (Laguna Hills, CA, USA). The PicoGreen reagent was purchased from Molecular Probes (Eugene, OR, USA). Mouse serum was obtained from CelarLane (Mississauga, ON, Canada). ^{14}C-cholesteryl hexadecyl ether (^{14}C-CHE) was obtained from Mandel Scientific (Guelph, ON, Canada). Triton X-100, DEAE-Sepharose CL-6B and octyl-β-d-glucopyranoside (OGP) was obtained from Sigma (St Louis, MO, USA). ^{14}C-sucrose was obtained from NEN-Dupont (Markham, ON, Canada). The luciferase assay kit was obtained from Promega (Madison, WI, USA). The pCMVLuc plasmid (5650 bp, coding for the luciferase reporter gene) was provided by Dr P Tam (Inex Pharmaceuticals). The gene was under the control of the human CMV immediate–early promoter-enhancer element. The ^{3}H-pCMVLuc plasmid was produced as

previously described.[29,30] The cell lines COS-7 (African green monkey kidney, SV40 transformed, ATCC CRL-1651), HepG2 (human hepatocellular carcinoma, ATCC HB-8065), were obtained from the American Tissue Culture Collection. The B16BL-6 mouse melanoma cell line was obtained from Frederick Cancer Research Center (Frederick, MD, USA).

Encapsulation of plasmid DNA

Lipid dispersions were prepared from stock solutions of DODAC, DOPE and PEG-CerC_8 in chloroform/methanol (2/1; vol/vol). Appropriate amounts of lipid were transferred into a glass test tube and solvent was removed under a stream of N_2 gas followed by storage under vacuum for 3–5 h. An aqueous solution of OGP (0.2 ml of a 1 m solution for 10 mg lipid) was then added to the lipid film. The mixture was vortexed until the lipid film was dissolved and the solution became clear. An appropriate amount of pCMVLuc plasmid solution (typically 100–400 μg for 10 mg lipid) and citrate buffer was then added, to achieve a final lipid concentration of 10 mg/ml and a final OGP concentration of 200 mm. The solution was normally optically clear at this point; if not, 50–100 μl of the 1 m OGP solution was added. This mixture was incubated at room temperature for 1 h and then dialyzed for 2 days against 4 liter of citrate buffer containing the indicated concentration of sodium citrate as well as 5 mm HEPES and 150 mm NaCl (pH 7.2), with two changes of buffer daily. Non-encapsulated plasmid was removed by anion exchange chromatography on a DEAE-Sepharose column (1 × 5 cm).

Evaluation of plasmid encapsulation by the PicoGreen fluorescence assay

Plasmid encapsulation was evaluated by measuring the accessibility of the DNA-interchelating dye PicoGreen to plasmid at an excitation wavelength of 485 nm and emission wavelength of 525 nm (Aminco Bowman Series 2 Luminescence Spectrometer, SLM-Aminco, Urbana, IL, USA). Typically, 4 μl of PicoGreen was added to 1 ml of sample containing 0.2–0.8 μg plasmid. Plasmid encapsulation was calculated as $E(\%) = (I_o - I)/I_o \times 100$ where I and I_o refer to the fluorescence intensities before and after the addition of Triton X-100 (final concentration 0.4%, v/v). Fluorescence intensities in the absence of PicoGreen were used as background references. The plasmid contents of formulations after DEAE column chromatography and after isolation by sucrose density gradient centrifugation were determined either by the PicoGreen fluorescent assay and/or by using ^{3}H-labelled plasmid.

Isolation of SPLP by sucrose density gradient centrifugation

Isolation of SPLP from empty vesicles was carried out by sucrose density gradient centrifugation. The gradient was formed with 2 ml of 10% sucrose, 6 ml of 2.5% sucrose (for formulations containing 22–30 mol% DODAC) or 5.0% sucrose (for formulations containing 7–22 mol% DODAC) and 1 ml of 1% sucrose in HBS layered consecutively from bottom to top. The gradients were centrifuged at 160 000 *g* for 12–20 h. The lipid-encapsulated plasmid banded tightly at the lower interface of the gradient, and the empty vesicles formed a broad band near the top of the gradient. The gradient was either separated into aliquots (1 ml) or the band containing SPLP was carefully isolated by pipetting, depending on the purpose of the experiment. The lipid and plasmid concentrations of the fractions were evaluated by HPLC analysis and the PicoGreen fluorescent assay, respectively, or by scintillation counting when ^{14}C-CHE and/or ^{3}H-plasmid was used.

Measurement of size distribution

The size distributions were measured by quasi-elastic light scattering (QELS) and freeze–fracture electron microscopy. QELS measurements employed a Nicomp 370 Sub-Micron particle sizer (Santa Barbara, CA, USA) operated in the volume-weighted vesicle mode. Sample polydispersity was evaluated by the goodness-of-fit parameter χ^2, where values of $\chi^2 < 3$ indicate monodisperse formulations (according to specification by the manufacturer). Freeze–fracture electron microscopy was performed employing a Balzers Freeze-Etching system, BAF 400D (Balzers, Liechtenstein). Samples were cryofixed in the presence of 25% glycerol by plunging them into liquid freon 22. The fractured surface was shadowed unidirectionally with platinum/carbon (45°) and coated with carbon (90°) immediately after fracturing. Replicas were analyzed using a JEOL Model JEM 1200 EX electron microscope (Soquelec, Montreal, QC, Canada).

HPLC analysis of lipid composition

Lipid compositions were analyzed by HPLC (Beckman System Gold 128) using an Ultrasphere cyanopropyl column (2 × 15 mm, 5 μm). A ternary mobile phase consisting of two solvents (solvent A: 99.95% $CHCl_3$ 0.05% TFA; solvent B: 90% isopropanol, 9.95% water, 0.05% TFA) was employed. Separation was performed at a flow rate of 0.3 ml/min, and a gradient of 0–90% solvent B was applied in 9 min. Elution with 90% solvent B was maintained for 1 min and then the mobile phase was cycled back to 100% solvent A.

Trapped volume measurements

The trapped volume of isolated SPLP was determined using ^{14}C-sucrose as a marker for the internal volume.[21,22] A first step was to examine sucrose retention in vesicles with the SPLP lipid composition. This study employed 100 nm diameter LUV prepared by the freeze–thaw extrusion method.[19] Briefly, 10 mg of the appropriate lipid mixture was hydrated in 1 ml of citrate buffer containing 10 μCi of ^{14}C-sucrose and 5% (w/v) sucrose. After five freeze–thaw cycles, the sample was extruded 10 times through a filter of 100 nm pore size using an Extruder (Lipex, Vancouver, BC, Canada). Untrapped ^{14}C-sucrose was removed by passing the sample through a Sephadex G-50 spin column. The sample was then dialyzed (12–14 000 MWCO) against an HBS solution containing 5% (w/v) sucrose. Aliquots were removed at various time-points and the specific activity determined. The SPLP formulations used for trapped volume determinations were prepared as described above, except that 20 μCi of ^{14}C-sucrose was included in the detergent dialysis solution. The solution was then dialyzed in 150 ml of citrate buffer in the presence of 20 g SM-2 Bio-Beads (BioRad, Hercutes, CA, USA). The Bio-Beads were replaced with fresh ones after 12 h and the sample was dialyzed for an additional 12 h. The lipid-plasmid formulations were then purified and isolated using DEAE column chromatography and sucrose density gradient procedures as described above.

Assay for serum stability of SPLP

A 50 μl aliquot of SPLP containing ^{3}H-plasmid and ^{14}C-CHE was mixed with 450 μl of normal mouse serum. After incubation at 37°C for 2 h the mixture was loaded on to a Sepharose CL-4B gel filtration column and eluted with HBS (pH 7.2). Fractions of 0.5 ml were collected and the lipid and plasmid concentrations assayed by scintillation counting.

In vitro and in vivo transfection

One day before transfection, COS-7 cells were plated in 24-well plates at 45 000 cells per well. At the time of transfection, cells were 70% confluent. Formulations of up to 50 μl containing 0.2–1.0 μg plasmid were added to each well (in triplicate). After 24 h incubation the cells were lysed in 200 μl 0.1% Triton X-100, 250 mm NaH_2PO_4 pH 7.4 and 20 μl of the lysate was assayed for luciferase expression as previously described.[15] *In vivo* transfection was measured using an intraperitoneal B16BL-6 tumor model. Female C57BL/6 mice were injected intraperitoneally with 100 000 B16BL-6 tumor cells. Seven days following tumor seeding, SPLP formulations containing 30 μg plasmid in 500 μl were injected into the peritoneal cavity. Tumors were collected after 24 h, fast-frozen in liquid nitrogen and stored at –70°C before being assayed for luciferase. Tissue homogenization was performed with a FastPrep instrument using supplied tubes and beads. Tissues were homogenized in Cell Culture Lysis Reagent (CCLR; Promega), supplemented with BSA (1 mg/ml). Samples were then centrifuged for 2 min at 10 000 *g* to remove debris.

Luciferase assay

Luciferase assays were performed using the Promega Luciferase Assay reagent kit (Promega E1501) according to the manufacturer's instructions. Cell lysates were assayed for luciferase activity using a 96-well microplate luminometer (Dynex Technologies ML 3000). Luciferase (firefly luciferase, Sigma) standard solutions were prepared by serially diluting 1 μg/μl luciferase in CCLR supplemented with BSA (1 mg/ml). To determine the luciferase activity in tissue, the standard curve employed was obtained by diluting luciferase standard in control tissue homogenate to compensate for quenching.

Assay for aspartate aminotransferase

Plasma from mice was recovered 24 h following injection of SPLP into the peritoneal cavity and assayed immediately for aspartate aminotransferase activity using commercially available kits (Sigma).

Acknowledgements

We thank Drs Z Wang and S Ansell (Inex Pharmaceuticals) for providing PEG-ceramides and DODAC, respectively. We also acknowledge Dr K Wong (Department of Biochemistry and Molecular Biology, University of British Columbia) for performing the freeze–fracture electron microscopy studies and J Thompson and C Giesbrecht (Inex Pharmaceuticals) for providing plasmid.

References

1 Felgner PL *et al*. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* 1987; **84**: 7413–7417.
2 Gao X, Huang L. Cationic liposome-mediated gene transfer. *Gene Therapy* 1995; **2**: 710–722.
3 Chonn A, Cullis PR. Recent advances in liposome technologies and their applications for systemic gene delivery. *Adv Drug Del Rev* 1998; **30**: 73–83.
4 Hofland HEJ, Shephard L, Sullivan SM. Formation of stable cationic lipid/DNA complexes for gene transfer. *Proc Natl Acad Sci USA* 1996; **93**: 7305–7309.
5 Felgner JH *et al*. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J Biol Chem* 1994; **269**: 2550–2561.
6 Zabner J *et al*. Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem* 1995; **270**: 18997–19007.
7 Gao X, Huang L. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem Biophys Res Comm* 1991; **179**: 280–285.
8 Hong K, Zheng W, Baker A, Papahadjopoulos D. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient *in vivo* gene delivery. *FEBS Lett* 1997; **400**: 233–237.
9 Thierry AR *et al*. Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. *Proc Natl Acad Sci USA* 1995; **92**: 9742–9746.
10 Hofland HJ *et al*. *In vivo* gene transfer by intravenous administration of stable cationic lipid/DNA complex. *Pharmaceut Res* 1997; **14**: 742–749.
11 Templeton NS *et al*. Improved DNA:liposome complexes for increased systemic delivery and gene expression. *Nature Biotech* 1997; **15**: 647–652.
12 Huang L, Li S. Liposomal gene delivery: a complex package. *Nature Biotech* 1997; **15**: 620–621.
13 Harrison GS *et al*. Optimization of gene transfer using cationic lipids in cell lines and primary human $CD4^+$ and $CD34^+$ hematopoietic cells. *Biotechniques* 1995; **19**: 816–823.
14 Li S, Huang L. *In vivo* gene transfer via intravenous administration of cationic lipid-protamine–DNA (LPD) complexes. *Gene Therapy* 1997; **4**: 891–900.
15 Wheeler JJ *et al*. Stabilized plasmid-lipid particles: construction and characterization. *Gene Therapy* 1999; **6**: 271–281.
16 Holland JW, Cullis PR, Madden TD. Poly(ethleneglycol)-lipid conjugates promote bilayer formation in mixtures of non-bilayer forming lipids. *Biochemistry* 1996; **35**: 2610–2617.
17 Holland JW, Hui C, Cullis PR, Madden TD. Poly (ethyleneglycol)-lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. *Biochemistry* 1996; **35**: 2618–2624.
18 Holland JW *et al*. Programmable fusion between cationic liposomes and a eucaryotic plasma membrane. *J Biol Chem* 1999; (submitted).
19 Hope MJ, Bally MB, Webb G, Cullis PR. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim Biophys Acta* 1985; **812**: 55–65.
20 Chonn A, Semple SC, Cullis PR. Association of blood proteins with large unilamellar liposomes *in vivo*. Relation to circulation lifetimes. *J Biol Chem* 1992; **267**: 18759–18765.
21 Mayer LD, Hope MJ, Cullis PR, Janoff AS. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. *Biochim Biophys Acta* 1985; **817**: 193–196.
22 Mui BLS, Cullis PR, Evans EA, Madden TD. Osmotic properties of large unilamellar vesicles prepared by extrusion. *Biophys J* 1993; **64**: 443–453.
23 Monck M *et al*. Stabilized plasmid-lipid particles for systemic gene therapy. *Gene Therapy* (submitted).

24 Ollivon M, Eildelman O, Blumenthal R, Walter A. Micelle–vesicle transition of egg phosphatidylcholine and octyl glucoside. *Biochemistry* 1988; **27**: 1695–1703.
25 Szoka F Jr, Papahadjopoulos D. Comparative properties and methods of preparation of lipid vesicles (liposomes). *Ann Rev Biophys Bioeng* 1980; **9**: 467–508.
26 Vinson PK, Talmon Y, Walter A. Vesicle–micelle transition of phosphatidylcholine and octyl glucoside elucidated by cryo-transmission electron microscopy. *Biophys J* 1989; **56**: 669–681.
27 Mok KWC, Lam AMI, Cullis PR. Stabilized plasmid-lipid particles: factors influencing DNA entrapment and transfection properties. *Biochim Biophys Acta* (in press).
28 Webb MS *et al*. Comparison of different hydrophobic anchors conjugated to poly(ethyleneglycol): effects on the pharmacokinetics of liposomal vincristine. *Biochim Biophys Acta* 1998; **1372**: 272–282.
29 Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 1979; **7**: 1513–1522.
30 Sambrook J, Fritsch EF, Maniatis T. Extraction and purification of plasmid DNA. In: Ford N, Nolan C, Ferguson M (eds). *Molecular Cloning*, 2nd edn. Cold Spring Harbor Laboratory Press: New York, 1989, pp 1.38–1.39.

JOINT APPENDIX 47

Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Controlled Release 107 (2005) 276–287
journal of controlled release
www.elsevier.com/locate/jconrel

Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids

James Heyes [1], Lorne Palmer [1], Kaz Bremner, Ian MacLachlan *

Protiva Biotherapeutics Inc., 100-3480 Gilmore Way, Burnaby, B.C., Canada V5G-4Y1

Received 17 January 2005; accepted 15 June 2005
Available online 28 July 2005

Abstract

An analogous series of cationic lipids (1,2-distearyloxy-*N,N*-dimethyl-3-aminopropane (DSDMA), 1,2-dioleyloxy-*N,N*-dimethyl-3-aminopropane (DODMA), 1,2-dilinoleyloxy-*N,N*-dimethyl-3-aminopropane (DLinDMA) and 1,2-dilinolenyloxy-*N,N*-dimethyl-3-aminopropane (DLenDMA)) possessing 0, 1, 2 or 3 double bonds per alkyl chain respectively, was synthesized to determine the correlation between lipid saturation, fusogenicity and efficiency of intracellular nucleic acid delivery. ^{31}P-NMR analysis suggests that as saturation increases, from 2 to 0 double bonds, lamellar (L_α) to reversed hexagonal (H_{II}) phase transition temperature increases, indicating decreasing fusogenicity. This trend is largely reflected by the efficiency of gene silencing observed in vitro when the lipids are formulated as Stable Nucleic Acid Lipid Particles (SNALPs) encapsulating small inhibitory RNA (siRNA). Uptake experiments suggest that despite their lower gene silencing efficiency, the less fusogenic particles are more readily internalized by cells. Microscopic visualization of fluorescently labelled siRNA uptake was supported by quantitative data acquired using radiolabelled preparations. Since electrostatic binding is a precursor to uptake, the pKa of each cationic lipid was determined. The results support a transfection model in which endosomal release, mediated by fusion with the endosomal membrane, results in cytoplasmic translocation of the nucleic acid payload.

Keywords: siRNA delivery; RNA interference; Cationic lipid; Phase transition temperature and SNALP

1. Introduction

RNA interference (RNAi) is a recently discovered gene-silencing tool. Small interfering RNA (siRNA) are short, double stranded RNA molecules that, in the presence of endogenous RNA-Induced Silencing Complex (RISC) unwind and bind to specific sequences of messenger RNA (mRNA) subsequently mediating the destruction of the target mRNA by endogenous cellular machinery. In this way, RNAi has the potential to selectively inhibit the expression of disease-associated genes in humans.

Stabilized Plasmid Lipid Particles (SPLP), consisting of a unilamellar lipid bi-layer encapsulating a single copy of plasmid DNA, have been reported

* Corresponding author. Tel.: +1 604 630 5064.
E-mail address: ian@protivabio.com (I. MacLachlan).
[1] The first two authors contributed equally to this work.

0168-3659/$ - see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.jconrel.2005.06.014

as a novel method of systemic nucleic acid delivery [1–5]. Originally developed using a detergent dialysis technique, they are now prepared more quickly and uniformly using the spontaneous vesicle formation method of Jeffs et al [6]. This method has been adapted to encapsulate other nucleic acids such as duplex siRNA molecules. The resulting particles, referred to as Stabilized Nucleic Acid Lipid Particles (SNALP), exhibit the stability, small size (<200 nm) and low surface charge required for systemic delivery [7].

The SNALP bi-layer contains a mixture of cationic and fusogenic lipids that enable the cellular uptake and endosomal release of the particle's contents. SNALP also contain a diffusible poly (ethylene glycol)-lipid conjugate (PEG-lipid) that provides a neutral, hydrophilic coating to the particle's exterior. PEG-lipids both stabilize the particle during the formulation process and shield the cationic bi-layer, preventing rapid systemic clearance. Upon administration, the PEG-lipid conjugate dissociates from the SNALP at a rate determined by the chemistry of the PEG-lipid anchor, transforming the particle into a transfection-competent entity [1]. Cellular uptake of non-viral transfection reagents occurs primarily via endocytosis. Escape from the endosome is known to be a limiting step when using lipidic systems to deliver nucleic acids. Fusogenic systems readily overcome this barrier since they promote the breakdown of the endosomal membrane leading to cytoplasmic translocation of their nucleic acid payloads. Lipidic systems are most fusogenic when arranged in the reversed hexagonal phase (H_{II}), as opposed to the more stable bi-layer forming lamellar phase (L_{α}). A low phase transition temperature between the two states indicates a lower activation energy for the formation of the fusogenic H_{II} phase. Because of the highly defined nature of the SNALP particle, with its fully encapsulated nucleic acid payload and well-characterized mechanism of intracellular delivery, SNALP make an ideal system for examining the role of individual lipid components in the pharmacology of non-viral vector systems. In this work we explore the role of bi-layer fusogenicity in uptake, endosomal escape, and gene-silencing efficiency of SNALP.

Fusogenicity is considered to contribute to cytoplasmic delivery of nucleic acids [8–11]. It has been shown, using gold-labeled particles, that upon uptake via endocytosis the majority of lipoplex remain localized in the endosome, failing to escape to the cytoplasm [12]. The incorporation of fusogenic lipids such as dioleoylphosphatidylethanolamine (DOPE) improves the efficiency of endosomal release by encouraging fusion events between the liposomal and endosomal bi-layers [12–14]. The resulting disruption to the endosomal bi-layer aids in the escape of the therapeutic nucleic acid to the cytoplasm. In the case of a fully encapsulated system, fusion is expected to result in cytoplasmic translocation of the nucleic acid payload.

The fusogenic nature of DOPE-containing bi-layers is thought to be due to their polymorphic nature. Upon formulation, most lipids adopt the bi-layer-forming lamellar phase (L_{α}). DOPE however, has a tendency to form the inverse hexagonal phase (H_{II}) [14]. Using video microscopy, Koltover et al. studied the interaction of lipoplexes with giant anionic vesicles (G-vesicles), a model for the endosomal membrane [15]. They demonstrated that while lipoplex comprised of lipids in the L_{α} phase would simply attach themselves stably to the surface of the G-vesicles, H_{II} phase forming lipoplex rapidly fused with model endosomes. Lipids that adopt the H_{II} phase are therefore regarded as 'fusogenic'.

Other researchers have noted that the degree of saturation of a lipid hydrophobic domain affects its ability to adopt the H_{II} phase [16–19]. All have reported a trend whereby an increasing number of double bonds corresponds with an increasing propensity to form the non-bi-layer phase. It might therefore be possible to increase the tendency of the SNALP bi-layer to form the fusogenic H_{II} phase by decreasing the degree of saturation in the lipid hydrophobic domain of the cationic lipid component. Using the SNALP lipid DODMA (containing a single double bond per lipid chain) as a starting point, we synthesized a homologous series of lipids with 0, 1, 2 or 3 double bonds and studied the effect of these changes on physicochemical properties of bi-layers into which they were incorporated.

The lipids were incorporated in SNALP containing anti-luciferase siRNA and assessed for both uptake efficiency and their ability to inhibit luciferase expression in stably transfected Neuro 2A cells. The surface pKa was investigated using a toluene nitrosulphonic

acid (TNS) assay. The relative influence of each lipid on phase transition temperature (T_c) was also studied, using ^{31}P-NMR analysis.

2. Materials and methods

2.1. Materials

DPPS, 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). TNS was obtained from Sigma-Aldrich Canada (Oakville, ON). RiboGreen was obtained from Molecular Probes (Eugene, OR). The alkyl mesylates were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA). siRNA (anti-luciferase and mismatch control) was purchased from Dharmacon (Lafayette, CO, USA). The anti-luciferase sense sequence was 5′-G.A.U.U.A.U.G.U.C.C.G.-G.U.U.A.U.G.U.A.U.U-3′. The anti-luciferase antisense sequence was 5′-U.A.C.A.U.A.A.C.C.G.G.A.C.A.U.A.A.U.C.U.U-3′. All other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.2. Synthesis of DSDMA and DODMA

DSDMA and DODMA were synthesized using the respective alkyl bromides with methodology derived from that of a DOTMA precursor [20]. 3-(Dimethylamino)-1,2-propanediol (714 mg, 6 mmol) and 95% sodium hydride (NaH, 1.26 g, 50 mmol) were stirred in benzene (30 mL) under argon for 30 min. The correct (either oleyl or stearyl) alkyl bromide (5.0 g, 15 mmol) was added and the reaction refluxed under argon for 18 h. The reaction mixture was then cooled in an ice bath while quenching via the slow addition of ethanol. Following dilution with a further 150 mL of benzene, the mixture was washed with distilled water (2×150 mL) and brine (150 mL), using ethanol (~20 mL) to aid phase separation if necessary. The organic phase was dried over magnesium sulphate and evaporated. The crude product was purified on a silica gel (Kiesel Gel 60) column eluted with chloroform containing 0–5% methanol. Column fractions were analyzed by thin layer chromatography (TLC) (silica gel, chloroform/methanol 9:1 v/v, visualized with molybdate) and fractions containing pure product (R_f=0.5) were pooled and concentrated. The product was decolorized by stirring for 30 min in a suspension of activated charcoal (1 g) in ethanol (75 mL) at 60 °C. The charcoal was removed by filtration through Celite, and the ethanol solution concentrated to typically yield 2.4 g (65%) of pure product. ^{1}H-NMR (DSDMA): δ_H 3.65 – 3.32 (m, 7H, OCH, $3 \times OCH_2$), 2.45 – 2.31 (m, 2H, NCH_2), 2.27 (s, 6H, $2 \times NCH_3$), 1.61 – 1.45 (m, 4H, OCH_2CH_2), 1.40 – 1.17 (m, 60H, $H_{stearyl}$), 0.86 (t, 6H, CH_2CH_3). ^{1}H-NMR (DODMA): δ_H 5.4 – 5.27 (m, 4H, $2 \times CH=CH$), 3.65 – 3.35 (m, 7H, OCH, $3 \times OCH_2$), 2.47 – 2.33 (m, 2H, NCH_2), 2.28 (s, 6H, $2 \times NCH_3$), 2.06 – 1.94 (m, 8H, $4 \times CH_2CH=CH$), 1.61 – 1.50 (m, 4H, OCH_2CH_2), 1.38 – 1.20 (m, 48H, H_{oleyl}), 0.88 (t, 6H, CH_2CH_3).

2.3. Synthesis of DLinDMA and DLenDMA

The DLinDMA and DLenDMA were synthesized similarly to the DSDMA and DODMA, but used the alkyl mesylates instead of alky bromides. The general synthetic protocol was identical for those of DSDMA and DODMA, substituting the alkyl mesylates for the bromides in the same molar ratios. The activated charcoal decolorization step was omitted, since the products here contain conjugated double bonds and activated charcoal is expected to adsorb compounds containing such features. Yields were typically 2.0 g (55%). ^{1}H-NMR (DLinDMA): δ_H 5.43 – 5.27 (m, 8H, $4 \times CH=CH$), 3.65 – 3.35 (m, 7H, OCH, $3 \times OCH_2$), 2.77 (t, 4H, $=CHCH_2CH=$), 2.47 – 2.33 (m, 2H, NCH_2), 2.28 (s, 6H, $2 \times NCH_3$), 2.05 (q, 8H, $4 \times CH_2CH_2CH=$), 1.62 – 1.50(m, 4H, OCH_2CH_2), 1.40 – 1.22 (m, 32H, $H_{linoleyl}$), 0.89 (t, 6H, CH_2CH_3). ^{1}H-NMR (DLenDMA): δ_H 5.44 – 5.27 (m, 8H, $4 \times CH=CH$), 3.62 – 3.48 (m, 7H, OCH, $3 \times OCH_2$), 2.80 (t, 4H, $=CHCH_2CH=$), 2.43 – 2.32 (m, 2H, NCH_2), 2.26 (s, 6H, $2 \times NCH_3$), 2.12 – 1.99 (m, 8H, $4 \times CH_{2/3}CH_2CH=$), 1.61 – 1.51 (m, 4H, OCH_2CH_2), 1.40 – 1.22 (m, 20H, $H_{linolenyl}$), 0.98 (t, 6H, CH_2CH_3).

2.4. Synthesis of PEG2000-C-DMA

PEG-C-DMA was synthesized as follows. In brief, a C_{14} lipid anchor was prepared by first alkylating the hydroxyl groups of 3-allyloxypro-

pane-1,2-diol with myristyl bromide. The allyl group was subsequently removed via palladium catalysis, resulting in the C_{14} hydroxyl lipid. The hydroxyl group was converted to the primary amine by mesylation and amination to yield 1,2-dimyristyloxypropyl-3-amine, the lipid anchor. Conjugation with PEG was effected by treating monomethoxy poly(ethylene glycol) (average molecular weight 2000) with an excess of diphosgene to form the chloroformate. Addition of the C_{14} amine lipid anchor and stirring overnight yielded PEG_{2000}-C-DMA, referred to here as PEG-C-DMA.

2.5. SNALP Preparation

SNALP with a lipid composition of DSPC:Chol:-PEG-C-DMA:Cationic Lipid (20:48:2:30 molar percent) were prepared using the spontaneous vesicle formation by ethanol dilution method [6]. The samples were diafiltered against 100 mL of PBS (20 wash volumes) using a cross flow ultrafiltration cartridge (Amersham Biosciences, Piscataway, NJ) and sterile filtered through Acrodisc 0.8/0.2 μm syringe filters (Pall Corp., Ann Arbor, MI). The siRNA concentration of final samples was determined using the RiboGreen assay and a siRNA standard curve. Particle size and polydispersity was determined using a Malvern Instruments Zetasizer 3000HSA (Malvern, UK). Nucleic acid encapsulation was determined using a RiboGreen assay, comparing fluorescence in the presence and absence of Triton X-100. RiboGreen fluorescence was measured using a Varian Eclipse Spectrofluorometer (Varian Inc) with λ_{ex}=500 nm, λ_{em}=525 nm.

2.6. TNS Assay

20 μM of SNALP lipid and 6 μM of TNS were mixed in a fluorescence cuvette in 2mL of 20 mM sodium phosphate, 25 mM citrate, 20 mM ammonium acetate and 150 mM NaCl, at a pH that was varied from 4.5 to 9.5. Fluorescence was determined at each pH using a Varian Eclipse Spectrofluorometer (Varian Inc) with settings of λ_{ex}=322 nm, λ_{em}=431 nm. Fluorescence for each system at the various pH values was then normalized to the value at pH 4.5. The pKa values are the point at which 50% of the molecules present are charged. By assuming that minimum fluorescence represents zero charge, and maximum fluorescence represents 100% charge, pKa can be estimated by measuring the pH at the point exactly half way between the values of minimum and maximum charge.

2.7. ^{31}P Nuclear magnetic resonance spectroscopy

Multilamellar vesicles (MLV) were prepared comprising DPPS and cationic lipid at a molar ratio of 1:1. This was accomplished by drying the lipids from chloroform solution, transferring to 10 mm NMR tubes, and hydrating in 1.5 mL of 10 mM sodium citrate, pH 4. Free induction decays (FIDs) corresponding to 1000 scans were obtained with a 3.0 μs, 60° pulse with a 1 s interpulse delay and a spectral width of 25 000 Hz. A gated two-level proton decoupling was used to ensure sufficient decoupling with minimum sample heating. An exponential multiplication corresponding to 50 Hz of line broadening was applied to the FIDs prior to Fourier transformation. The sample temperature (+/− 1 °C) was regulated using a Bruker B-VT1000 variable temperature unit. Chemical shifts were referenced to 85% phosphoric acid as an external standard.

2.8. In Vitro Transfection

Cells were cultured in MEM (Invitrogen) containing 10% fetal bovine serum (FBS) (CanSera) and 0.25 mg/mL G418 (Invitrogen). Neuro2A-G cells (Neuro2A cells stably transfected to express luciferase [21]) were plated at a concentration of 4×10^4 cells per well in 24-well plates and grown overnight. Cells were treated with SNALP at doses of 0.0625 – 1.0 μg/mL nucleic acid (AntiLuc Active or Mismatch Control) and incubated for 48 h at 37 °C and 5% CO_2. Cells were then washed with PBS and lysed with 200 μL 250mM sodium phosphate containing 0.1% Triton X-100. The luciferase activity for each well was determined using Luciferase Reagent (Promega) and a standard luciferase protein (Roche). The luminescence for each was measured using a Berthold MicroLumat-Plus LB96V plate luminometer. The resulting luciferase activity was then normalized for the amount of protein using the Micro BCA assay kit (Pierce). Luciferase knockdown relative to a control was then determined for each system.

2.9. Cellular uptake

SNALP were prepared incorporating the non-exchangeable tritium-labeled lipid cholesteryl hexadecyl ether (^{3}H-CHE) (11.1 μCi/ μmol total lipid) [22]. Neuro2A cells (ATCC, VA, USA) were plated in 12 well plates at 1.6×10^5 cells per well in minimal essential media. The following day, media was removed and replaced with media containing radiolabelled SNALP at 0.5 μg/mL nucleic acid. After 24 h, the media and unincorporated SNALP were removed, adherent cells gently washed 4 times with PBS, and then lysed with 600 μL Lysis Buffer (250 mM phosphate with 0.1% Triton X-100). The resulting cell lysate (500 μL) was added to glass scintillation vials containing 5 mL Picofluor 40 (Perkin Elmer) and ^{3}H-CHE was determined using a Beckman LS6500 scintillation counter (Beckman Instruments). The protein content of cell lysates was determined using the Micro BCA assay (Pierce). Uptake was expressed as a percentage of the total amount of activity applied to the cells per mg of cellular protein.

2.10. Uptake of SNALP containing Cy3-labeled siRNA

SNALP were formulated as previously described, but using siRNA labelled with the fluorophore Cy3 (Cy3-siRNA was a gift of Sirna Therapeutics Inc, Boulder, CO). The encapsulation, siRNA concentration, and particle size were determined as described.

For the uptake study, 8×10^4 Neuro2A-G cells were grown overnight on 4-well chamber slides (BD Falcon, Mississauga, ON) in MEM containing 0.25 mg/mL G418. DSDMA, DODMA, DLinDMA, and DLenDMA SNALP containing Cy3-siRNA, as well as naked Cy3-siRNA and unlabeled DSDMA SNALP were placed on the cells at 0.5 μg/mL siRNA. After a 4 h incubation with the transfection media, the cells were washed with PBS, then with MEM containing G418 and finally with PBS once more. The cells were then fixed in a 4% paraformaldehyde solution in PBS for 10 min at room temperature. The cells were washed with PBS and stained with 300 nM DAPI (Molecular Probes, Eugene, OR) in PBS for 5 min. The cells were washed with PBS, the mounting media ProLong Gold Antifade Reagent (Molecular Probes, Eugene, OR) applied and a cover slip added. The cells were viewed using an Olympus BX60 Microscope modified for fluorescence capabilities. Cy3 fluorescence within the cells was visualized using a rhodamine cube set (Microgen Optics, Redding, CA) and the DAPI fluorescence was visualized using a DAPI cube set (Carsen Group, Markham, ON). Digital pictures were captured using an Olympus DP70 camera system. Pictures of the cells were taken at exposure times of 1/4 s when examining Cy3 fluorescence and 1/80 s when examining DAPI fluorescence.

3. Results

3.1. Formulation characteristics of unsaturated lipids are uniform and reproducible

SNALP containing the various cationic lipids were prepared as described and encapsulated RNA and particle size assessed (Table 1). The three unsaturated cationic lipids resulted in formulations that were approximately the same size (132 – 140 nm). Polydispersity of all formulations was low, indicating a narrow distribution of particle size. RNA encapsulation in the final particles was 84– 85% of the total. Attempts to encapsulate siRNA in SNALP using the saturated lipid DSDMA resulted in the formation of slightly larger particles (180 nm) with encapsulation of 67%.

3.2. pKa of cationic lipids is influenced by saturation

The apparent pKa of the cationic lipids was determined as described in Materials and Methods. Lipid pK_a correlated with degree of saturation with

Table 1
Physical properties of SNALP formulations

Cationic lipid	Percentage Encapsulation	Diameter (nm)	Polydispersity
DSDMA	67 ± 3	182 ± 11	0.15 ± 0.03
DODMA	84 ± 1	137 ± 4	0.12 ± 0.01
DLinDMA	84 ± 3	140 ± 6	0.11 ± 0.02
DLenDMA	85 ± 1	132 ± 7	0.13 ± 0.03

'Percentage Encapsulation' is determined using the RiboGreen fluorescence assay to measure the amount of encapsulated nucleic acid relative to the total nucleic acid present. Particle diameter and polydispersity was measured using a Malvern Zetasizer. Values are the mean of 3 separate experiments, the error is standard deviation.

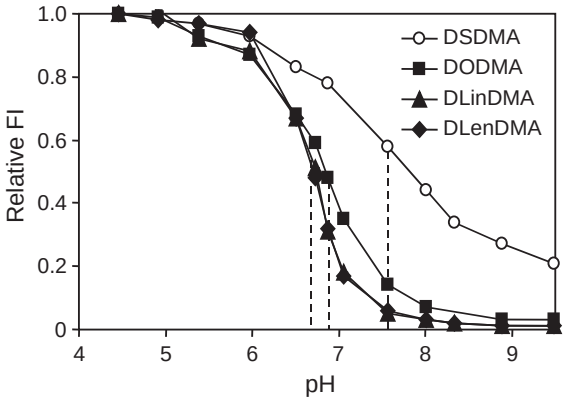


Fig. 1. Assay to determine the apparent pKa of the cationic lipid incorporated in SNALP. An increase in TNS fluorescence correlates with an increase of positive charge. pKa is defined as the point at which 50% of the molecules are charged, halfway between the upper (completely charged) and lower (completely uncharged) limits of each curve. Error bars represent standard deviation, $n=3$.

DSDMA, DODMA, DLinDMA, and DLenDMA exhibiting pK_as of 7.6, 7.0, 6.7, and 6.7, respectively (Fig. 1).

3.3. The bilayer-to-hexagonal phase transition temperature increases with alkyl chain saturation

^{31}P-NMR studies have previously shown that above certain temperatures (the Phase Transition Temperature, T_c), lipids may adopt the fusogenic H_{II} phase [23,24]. A higher temperature required to convert a bilayer (L_α phase) to the H_{II} phase indicates a less fusogenic bilayer. MLV were prepared using the anionic lipid DPPS in a 1:1 molar ratio with each cationic lipid. The ^{31}P-NMR spectra of the MLV were measured at various temperatures. As can be seen in Fig. 2A, for the DSDMA/DPPS system, the bilayer pattern occurs from temperatures of 30 to 50 °C (a high-field peak with a low-field shoulder). Therefore, DSDMA would appear to have very little

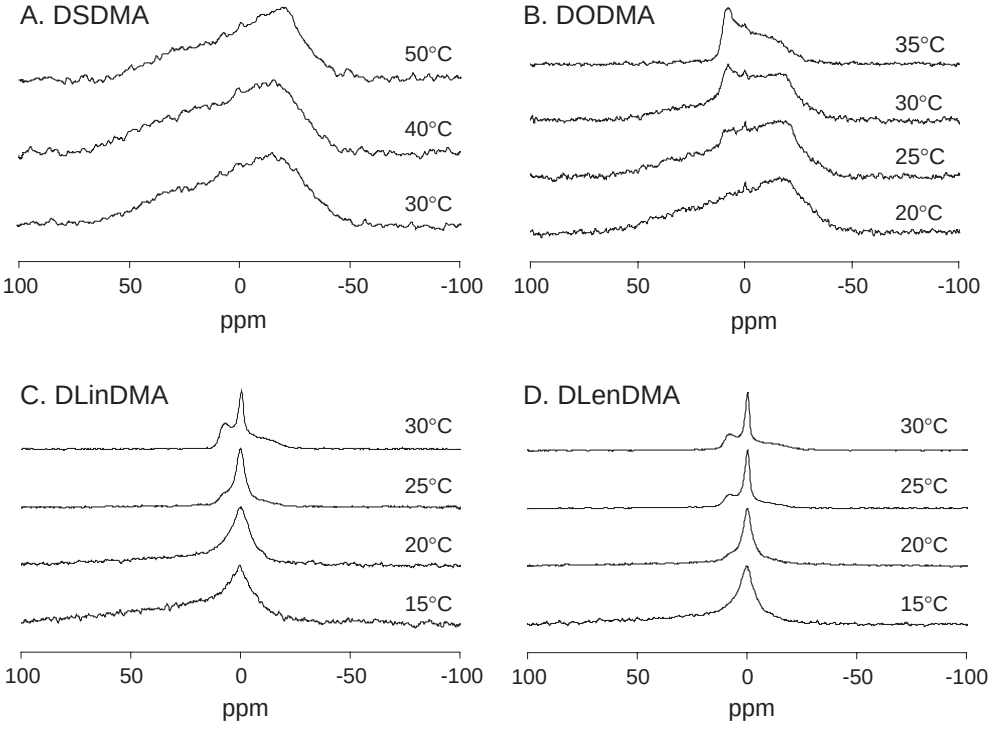


Fig. 2. ^{31}P-NMR analysis to determine the effect of unsaturation on phase transition temperature. Lipids were incorporated into MLV with the negatively charged lipid DPPS, in a 1:1 ratios. At lower temperatures, bi-layer patterns are observed (a high-field peak with a low-field shoulder). As temperature increases, a low-field peak with a high-field shoulder is observed, indicative of the inverted hexagonal phase transition. DSDMA-containing bilayers have a higher phase transition temperature and are accordingly less fusogenic.

ability to form H_{II} phases in conjunction with the anionic lipid. The cationic lipid with a single double bond, DODMA, possesses a transition temperature between 30 and 35 °C (Fig. 2B). The DLinDMA (2 double bonds) and DLenDMA (3 double bonds) systems exhibit somewhat similar transition temperatures between 20 and 25 °C (Fig. 2C, D). It should be noted that the central, isotropic peak seen in traces 3C and 3D does not represent the phase transition temperature but rather results from small phospholipid vesicles that are also present in the preparation. The shift in lineshape asymmetry from a high-field peak/low-field shoulder (bi-layer phase, lower temperatures) to low-field peak/high-field shoulder (inverted hexagonal phase, higher temperatures) is an indication of phase transition. This is exhibited most clearly in trace 3B (DODMA). Based on these results it may be assumed that the fusogenicity of these systems increases in the following order: DSDMA << DODMA < DLinDMA = DLenDMA.

3.4. SNALP containing unsaturated cationic lipids show increased gene-silencing activity

The ability of SNALP containing each of the four cationic lipids to effect gene silencing in stably transfected Neuro2A cells was evaluated (Fig. 3). It was found that, as hypothesized, knockdown efficiency corresponded to the ability of lipids to form the fusogenic inverted hexagonal phase. Formulations comprising the saturated lipid DSDMA demonstrated no activity. As unsaturation in the lipid's alkyl chain increased, so did the capacity for RNA interference, with DLinDMA particles yielding an 80% knockdown in gene expression. ^{31}P-NMR established DLinDMA as having the lowest phase transition temperature in the series and accordingly, being the most fusogenic lipid. Particles comprising DLenDMA, the most unsaturated lipid, were slightly less efficient than those containing DLinDMA. All results were found to be significant by *t*-Test ($P < 0.05$ at siRNA concentration of 0.5 μg/mL, and $P < 0.01$ at siRNA concentration of 1.0 μg/mL).

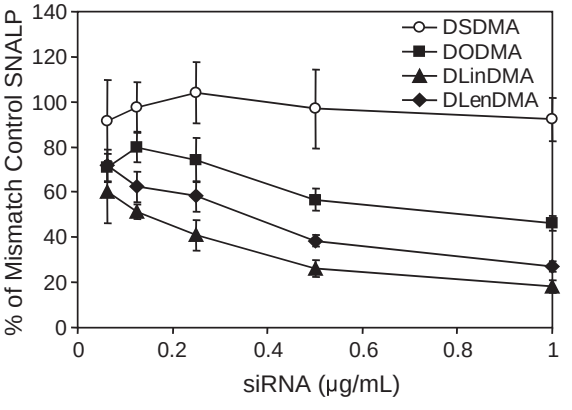


Fig. 3. SNALP mediated gene-silencing in vitro. Neuro2A cells stably transfected to express the luciferase were treated with SNALP containing anti-luciferase siRNA for 48 h. Gene-silencing efficiency was evaluated by comparing the remaining luciferase activity in these cells to that remaining in cells treated with control SNALP containing mismatch siRNA. Error bars represent standard deviation, $n = 3$.

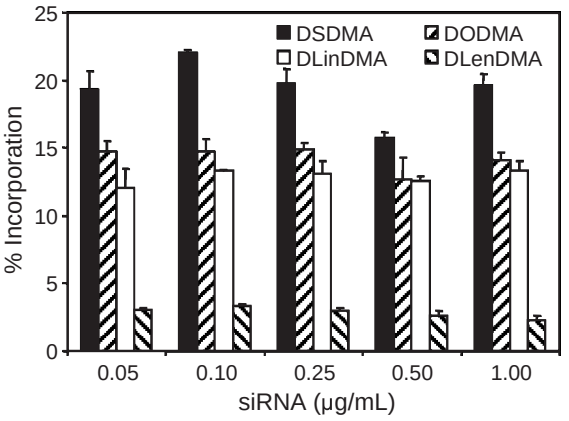


Fig. 4. Cellular uptake. Neuro2A cells were treated with SNALP containing ^{3}H-labeled CHE for 24 h. The cells were washed to remove unincorporated SNALP prior to determination of ^{3}H-CHE. Uptake is expressed as a percentage of the total activity applied to the cells. Cellular uptake is shown to increase with increasing cationic lipid saturation. Error bars represent standard deviation, $n = 3$.

3.5. SNALP uptake is not rate limiting for gene-silencing efficiency

The extent to which formulations are taken up by cells was measured with SNALP incorporating ^{3}H-labeled CHE [22]. After exposing cells to SNALP formulations for 24 h, cells were rinsed, lysed and ^{3}H-CHE uptake determined (Fig. 4). Uptake of each individual formulation was independent of SNALP concentration, with DSDMA particles exhibiting the greatest degree of uptake. SNALP uptake was ob-

served to decrease with decreasing saturation the DLenDMA formulation appearing particularly limited in this respect. These results are contrary to our expectations, based on the gene silencing results, where the DSDMA formulation is found to be least effective. They suggest that cellular uptake does not limit the gene silencing ability of SNALP, but that endosomal escape, mediated by a fusion event with the endosomal membrane is critical in SNALP mediated nucleic acid delivery. Analysis by *t*-test found all results to be significant ($P<0.05$), apart from the difference between DODMA and DLinDMA at concentrations of 0.10, 0.50 and 1.00 μg/mL.

3.6. Visualization of the uptake process

The uptake process was examined further with the use of fluorescently labelled SNALP. Neuro2A-G cells were treated with formulations containing Cy3-labeled siRNA for 4 h. After washing and fixing, cell nuclei were stained (blue) with the fluorescent marker DAPI, to more accurately determine the location of the fluorescently labelled siRNA (Fig. 5). In keeping with the results of the ^{3}H-CHE uptake experiment, it can be seen that the DSDMA formulation is clearly the most efficient at delivering siRNA to cells. The Cy3 fluorescence (red) is most intense in cells treated with DSDMA containing SNALP. Again, in agreement with the radiolabelled uptake study, as the degree of saturation of the cationic lipid increases, cellular uptake of Cy3 labelled siRNA increases. Again, Cy3 fluorescence is extremely faint for the DLenDMA formulation, indicating poor uptake. Negative controls treated with either naked Cy3-labeled siRNA or unlabeled SNALP revealing no cell associated Cy3 fluorescence.

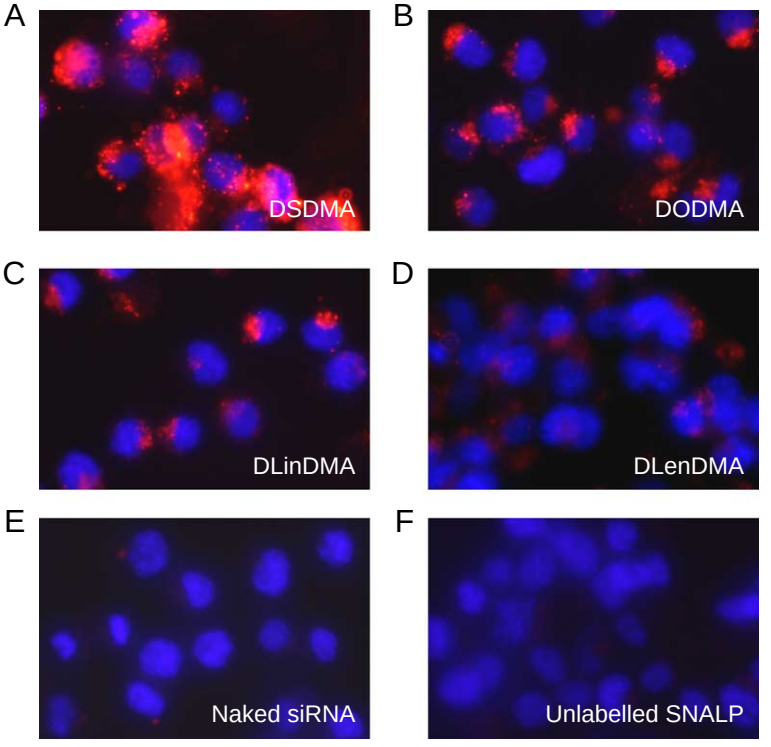


Fig. 5. Fluorescent microscopy of SNALP mediated uptake of Cy3 labeled siRNA. SNALP labeled with the fluorophore Cy3 were applied to cells and incubated for 4 h. After washing and fixing, fluorescence microscopy indicates that siRNA uptake, as measured by Cy3 fluorescence, correlates with cationic lipid saturation. Cell nuclei were stained with the fluorophore DAPI (blue). Unlabeled SNALP and naked Cy3-siRNA were used as negative controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Several obstacles beset delivery of genetic drugs. These include the ability to safely deliver the therapeutic to the target cell population, internalization by the cell, endosomal escape and protection from nuclease degradation. Stable Nucleic Acid Lipid Particles (SNALP) are a nucleic acid delivery platform described previously that has shown potential for overcoming these obstacles both in vitro and in vivo [1–5,25]. A diffusible outer PEG layer affords the particles an increased half-life in the bloodstream. This, combined with the particle's small, uniform sizes (~130 – 140 nm), allows them to accumulate passively in solid tumors via the fenestrated epithelia characteristic of such disease sites [1,3].

The model for SNALP mediated nucleic acid delivery suggests that following the accumulation of SNALP in the tumor's interstitial volume, the PEG layer slowly diffuses from the particles and the particle is taken up by endocytosis [12]. Following internalization by the cell, the particle must now either escape the endosome and release its payload into the cytoplasm, or face degradation in the lysosome. The physico-chemical properties of the lipid bi-layer will largely determine how effectively the nucleic acid payload is delivered to the cytoplasm. Fusion events between the SNALP and endosomal bi-layers will either promote their destabilization or facilitate direct translocation of the nucleic acid payload to the cytoplasm.

Fusion events are more likely to occur when lipids are able to adopt the non-bi-layer, inverted hexagonal (H_{II}) phase [14]. Increasing unsaturation in a lipid's alkyl chains has been reported to increase their H_{II} phase forming ability [16–19]. In this study we show that the transfection efficiency of SNALP can be improved by increasing the unsaturation of the cationic lipid's hydrophobic domain and thus, increasing the fusogenicity of the system.

Other researchers have examined the impact of lipid saturation on transfection efficiency, usually in the context of other studies examining the effects of lipid chain length on plasmid DNA delivery. Examination of C18:0 (oleyl) and C18:1 (stearyl) analogues suggests that the oleyl (C18:1) lipids are more effective when used to deliver plasmid DNA [9,26,27]. However, while these works suggest this result is related to the transition temperature/fusogenicity of the lipids in question, they have not examined this relationship directly (e.g. with ^{31}P-NMR).

Our study utilizes a series of four lipids of the *same* alkyl chain length (C18) modified with a systematic addition of double bonds. Further, as successive double bonds are added across the series, the position of existing double bonds within the alkyl chain is retained. The position of double bonds with lipid alkyl chains has previously been noted to have a dramatic effect on bi-layer transition temperature [28,29].

The four lipids examined in this work are DSDMA, DODMA, DLinDMA, and DLenDMA (Fig. 6). These lipids possess a protonatable tertiary amine head group, C18 alkyl chains, ether linkages between the head group and alkyl chains, and 0 to 3 double bonds respectively. DODMA is of interest for nucleic acid delivery since it possesses several desirable characteristics [6]. As well as utilizing the more stable ether linkages, its head group is pH titratable. At physiological pH the lipid is charge neutral, affording SNALP longer systemic circulation time. Inside the endosome however, the more acidic environment results in head group protonation, resulting in a cationic charge that facilitates fusion with the anionic lipid containing endosomal bi-layer. We therefore used DODMA as a starting point for the design of an analogous series of lipids.

PEG-C-DMA is a PEG-lipid modeled after the PEG-ceramides and PEG-diacylglycerols described previously [1,4]. However, unlike these other lipids it possesses no ester bonds, making it more stable in aqueous media. Based on the observation that PEG-lipids with smaller anchors are more effective for in vitro work [1,2,4], we employed a short (C_{14}) hydrophobic anchor when synthesizing PEG-C-DMA. Used in conjunction with the unsaturated cationic lipids (DODMA, DLinDMA and DLenDMA), PEG-C-DMA gave SNALP particles within the desired size range (130 – 140 nm, Table 1). When combined with the saturated lipid DSDMA, resulting particles were slightly larger (180 nm) and less efficient in their nucleic acid encapsulation. The increased size could be due to the larger pKa value observed for this formulation (Fig 1). DSDMA containing particles with a more pronounced positive charge are more likely to interact with small amounts of negatively

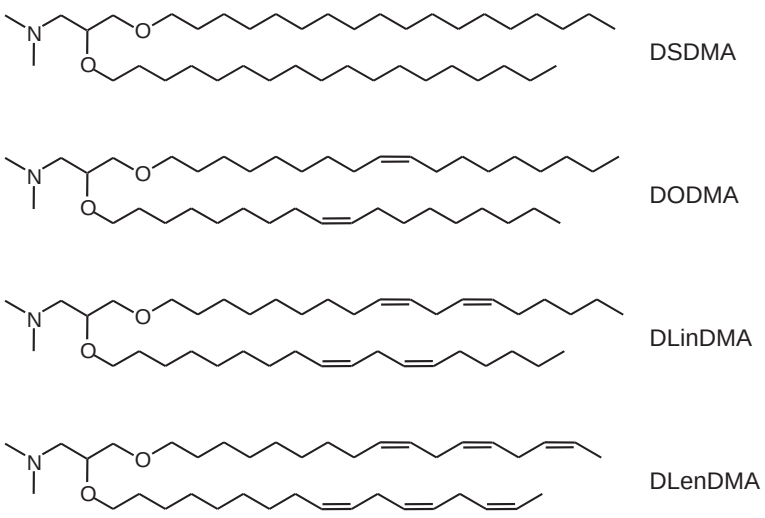


Fig. 6. Chemical structures of the cationic lipids incorporated into SNALP. Lipids were synthesized to study the effect of saturation on SNALP potency. The series of C18 lipids has a stepwise increase in the number of double bonds in the alkyl domains.

charged, unencapsulated nucleic acid present in SNALP preparations, thereby increasing the chance of particle aggregation. DSDMA was also observed to have a much higher phase transition temperature than the other lipids described in this study (Fig. 2), which would be expected to result in a more rigid bi-layer.

Our determination of lipid pKa utilized TNS, a negatively charged indicator of membrane potential [30]. TNS is electrostatically attracted to positively charged membranes. Subsequent adsorption to the lipid membrane results in the immediate environment of the TNS becoming more lipophilic, removing the water molecules that otherwise quench TNS fluorescence. Since positively charged membranes more readily absorb TNS, TNS fluorescence is an indicator of positive membrane surface charge. The surface pKa values of each SNALP formulation were determined by varying the local pH in the presence of TNS. In Fig. 1, it can be seen that formulations containing unsaturated lipids have similar pKa values (6.7 – 7.0) suggesting that the particles are charge neutral at physiological pH but become positively charged at endosomal pH. This is desirable as it promotes lipid exchange between the SNALP and the anionic components of the endosomal membrane. The increased frequency of fusion events destabilizes the endosomal bilayer, leading to more effective delivery of siRNA to the cytoplasm [23,31]. The saturated lipid DSDMA, however, generates particles with a higher pKa of approximately 7.6. SNALP particles containing DSDMA would be expected to possess some charge at physiological pH.

The significance of saturation with respect to phase transition temperature was investigated using ^{31}P-NMR. Lipid polymorphism in anionic phospholipid/cationic lipid mixtures has been examined by others using this technique, facilitated by the presence of a phosphate group in the phospholipid [23,24]. The shape of the NMR trace varies depending on the arrangement of the lipids. A bilayer structure yields a high field peak with a low field shoulder. However, above the Phase Transition Temperature, (T_c), lipids adopt a fusogenic H_{II} phase, indicated by a reversed pattern with the peak appearing on the low field side. By noting the temperature at which this occurs (Fig. 2), the relative ease with which the lipids form the H_{II} phase, their 'fusogenicity', can be determined. MLV containing the saturated lipid DSDMA showed no appreciable sign of adopting the H_{II} phase, even at temperatures as high as 50 °C. However DODMA (1 double bond per alkyl chain) containing MLV exhibit a phase transition temperature between 30 and 35 °C. The presence of a 2nd double bond (DLinDMA) reduced the T_C still further to between 20 and 25 °C, while incorporation of a 3rd double bond (DLenDMA) has

little further effect. We hypothesized that the fusogenicity and thus potency with respect to nucleic acid delivery of the corresponding SNALP would demonstrate a similar hierarchy (DSDMA << DODMA < DLinDMA = DLenDMA).

The results of luciferase silencing experiments, using SNALP to deliver siRNA directed against the luciferase gene, largely supported the ^{31}P-NMR data (Fig. 3). Cells were treated with SNALP containing each of the four cationic lipids. After 48 h, SNALP containing DSDMA, which was shown to be poorly fusogenic by NMR, had no effect on luciferase gene expression. In contrast, the unsaturated lipid formulations, which are more amenable to H_{II} phase formation, resulted in significant silencing of the luciferase gene. Further, the extent of silencing corresponds with the propensity for each cationic lipid to form the fusogenic H_{II} phase. DLinDMA, the most fusogenic lipid with the lowest apparent phase transition temperature, yielded the greatest knockdown when incorporated in SNALP, with luciferase expression only 21% that of the untreated control. This was followed by the DLenDMA formulation (32%), and DODMA (54%). The close correspondence between knockdown efficiency and the H_{II} phase forming ability of the cationic lipids observed suggests that the two are intrinsically linked.

Investigating the efficiency of SNALP uptake by incorporation of radiolabelled markers yields further interesting observations (Fig. 4). It might be expected that SNALP uptake would be related to the pKa of the cationic lipid component; the more positively charged particles having a greater affinity for the negatively charged cell surface and subsequently greater uptake. This hypothesis is borne out by the results of this study. The DSDMA containing formulation, possessing the highest pKa (~7.6) is clearly taken up most readily, followed by the DODMA and DLinDMA formulations. Curiously, the uptake of the DLenDMA formulation is limited when compared to that of the DLinDMA formulation given that the pKa of these particles are identical. This suggests that another attribute of these lipids, other than pKa, affects cellular uptake. This finding is unlikely to be related to differences in particle stability, since time-course studies confirm that the rate of DLenDMA formulation uptake is constant over the 24h period, suggesting that the formulation remains intact in tissue culture media.

These results suggest that endocytosis is not rate-limiting in gene-silencing in vitro when using encapsulated siRNA. In fact, differences in cellular uptake appear to have remarkably little impact on formulation potency. DLinDMA and DLenDMA formulations, while similar in their ability to inhibit gene expression, are very different in the extent to which they are taken up by cells. Conversely, the DSDMA formulation has almost no capacity for effecting RNA interference, yet it is clearly taken up by cells quite readily. The data suggest that the events which have the greatest effect on the efficiency of gene-silencing occur once the siRNA has been internalized by the cell.

5. Conclusion

In summary, we have synthesized a homologous series of cationic lipids with incremental degrees of saturation. We show that the degree of saturation of cationic lipids affects lipid pKa, fusogenicity, cellular uptake and gene silencing ability when used to encapsulate and deliver siRNA. Remarkably, more fusogenic cationic lipids are more potent mediators of RNAi, in spite of their reduced efficiency at mediating internalization by the cell. This highlights the relative importance of endosomal release of nucleic acid payloads. This knowledge can be used to enhance the efficacy of other lipidic nucleic acid delivery systems and should be considered in the design of delivery systems for small molecule drugs.

Acknowledgements

The authors wish to thank Dr Kim Wong of the University of British Columbia for assistance with and discussion of ^{31}P-NMR results. They also wish to thank Dr. Lloyd Jeffs for helpful discussions regarding the intracellular delivery process.

References

[1] E. Ambegia, S. Ansell, P. Cullis, J. Heyes, L. Palmer, I. MacLachlan, Stabilized plasmid–lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression, Biochim. Biophys. Acta, Biomembr. 1669 (2005) 155–163.

[2] K.W.C. Mok, A.M.I. Lam, P.R. Cullis, Stabilized plasmid-lipid particles: factors influencing plasmid entrapment and transfection properties, Biochim. Biophys. Acta, Biomembr. 1419 (1999) 137–150.
[3] P. Tam, M. Monck, D. Lee, O. Ludkovski, E. Leng, K. Clow, H. Stark, P. Scherrer, R.W. Graham, P.R. Cullis, Stabilized plasmid lipid particles for systemic gene therapy, Gene Ther. 7 (2000) 1867–1874.
[4] J.J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R.W. Graham, Y.P. Zhang, M.J. Hope, P. Scherrer, P.R. Cullis, Stabilized plasmid-lipid particles: construction and characterization, Gene Ther. 6 (1999) 271–281.
[5] Y.P. Zhang, L. Sekirov, E.G. Saravolac, J.J. Wheeler, P. Tardi, K. Clow, E. Leng, R. Sun, P.R. Cullis, P. Scherrer, Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties, Gene Ther. 6 (1999) 1438–1447.
[6] L.B. Jeffs, L. Palmer, E. Ambegia, C. Giesbrecht, S. Ewanick, I. MacLachlan, A scalable, extrusion free method for efficient liposomal encapsulation of plasmid DNA, Pharm. Res. 22 (2005) 362–372.
[7] I. MacLachlan, P. Cullis, R.W. Graham, Progress towards a synthetic virus for systemic gene therapy, Curr. Opin. Mol. Ther. 1 (1999) 252–259.
[8] H. Farhood, R. Bottega, R.M. Epand, L. Huang, Effect of cationic cholesterol derivatives on gene-transfer and protein-kinase-C activity, Biochim. Biophys. Acta 1111 (1992) 239–246.
[9] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, J. Biol. Chem. 269 (1994) 2550–2561.
[10] J.Y. Legendre, F.C. Szoka, Delivery of plasmid DNA into mammalian-cell lines using ph-sensitive liposomes— comparison with cationic liposomes, Pharm. Res. 9 (1992) 1235–1242.
[11] X.H. Zhou, L. Huang, Dna transfection mediated by cationic liposomes containing lipopolylysine- characterization and mechanism of action, Biochim. Biophys. Acta, Biomembr. 1189 (1994) 195–203.
[12] J. Zabner, A.J. Fasbender, T. Moninger, K.A. Poellinger, M.J. Welsh, Cellular and molecular barriers to gene transfer by a cationic lipid, J. Biol. Chem. 270 (1995) 18997–19007.
[13] H. Farhood, N. Serbina, L. Huang, The role of dioleoyl phosphatidylethanolamine in cationic liposome-mediated gene-transfer, Biochim. Biophys. Acta, Biomembr. 1235 (1995) 289–295.
[14] D.C. Litzinger, L. Huang, Phosphatidylethanolamine liposomes— drug delivery, gene-transfer and immunodiagnostic applications, Biochim. Biophys. Acta 1113 (1992) 201–227.
[15] I. Koltover, T. Salditt, J.O. Radler, C.R. Safinya, An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery, Science 281 (1998) 78–81.
[16] M.B. Sankaram, G.L. Powell, D. Marsh, Effect of acyl chain composition on salt-induced lamellar to inverted hexagonal phase-transitions in cardiolipin, Biochim. Biophys. Acta 980 (1989) 389–392.
[17] J.A. Szule, N.L. Fuller, R.P. Rand, The effects of acyl chain length and saturation of diacylglycerols and phosphatidylcholines on membrane monolayer curvature, Biophys. J. 83 (2002) 977–984.
[18] C.J. Dekker, W. Vankessel, J.P.G. Klomp, J. Pieters, B. Dekruijff, Synthesis and polymorphic phase-behavior of poly-unsaturated phosphatidylcholines and phosphatidylethanolamines, Chem. Phys. Lipids 33 (1983) 93–106.
[19] R.M. Epand, R.F. Epand, N. Ahmed, R. Chen, Promotion of hexagonal phase formation and lipid mixing by fatty-acids with varying degrees of unsaturation, Chem. Phys. Lipids 57 (1991) 75–80.
[20] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Lipofection — a highly efficient, lipid-mediated DNA-transfection procedure, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 7413–7417.
[21] R.E. Kingston, Current Protocols in Molecular Biology, vol. 2, John Wiley and Sons, Inc., 1997, pp. 9.1.4–9.1.9.
[22] M. Bally, L. Mayer, M. Hope, R. Nayar, Liposome Technology, vol. III, CRC Press, 1993, pp. 27–41.
[23] I.M. Hafez, N. Maurer, P. Cullis, On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids, Gene Ther. 8 (2001) 1188–1196.
[24] J. Gaucheron, T. Wong, E.F. Wong, N. Maurer, P.R. Cullis, Synthesis and properties of novel tetraalkyl cationic lipids, Bioconjug. Chem. 13 (2002) 671–675.
[25] J.S. Choi, J.A. MacKay, F.C. Szoka, Low-pH-sensitive PEG-stabilized plasmid-lipid nanoparticles: preparation and characterization, Bioconjug. Chem. 14 (2003) 420–429.
[26] F. Liu, H. Qi, L. Huang, D. Liu, Factors controlling the efficiency of cationic lipid-mediated transfection in vivo via intravenous administration, Gene Ther. 4 (1997) 517–523.
[27] J.A. Heyes, D. Niculescu-Duvaz, R.G. Cooper, C.J. Springer, Synthesis of novel cationic lipids: effect of structural modification on the efficiency of gene transfer, J. Med. Chem. 45 (2002) 99–114.
[28] R.M. Epand, N. Fuller, R.P. Rand, Role of the position of unsaturation on the phase behavior and intrinsic curvature of phosphatidylethanolamines, Biophys. J. 71 (1996) 1806–1810.
[29] G.Q. Wang, S.S. Li, H.N. Lin, E.E. Brumbaugh, C.H. Huang, Effects of various numbers and positions of cis double bonds in the *sn*-2 acyl chain of phosphatidylethanolamine on the chain-melting temperature, J. Biol. Chem. 274 (1999) 12289–12299.
[30] A. Bailey, P. Cullis, Modulation of membrane fusion by asymmetric transbilayer distributions of amino lipids, Biochemistry 33 (1994) 12573–12580.
[31] Y.H. Xu, F.C. Szoka, Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection, Biochemistry 35 (1996) 5616–5623.

JOINT APPENDIX 48

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K

A2

(11) International Publication Number: **WO 00/03683**

(43) International Publication Date: 27 January 2000 (27.01.00)

(21) International Application Number: PCT/US99/16166

(22) International Filing Date: 16 July 1999 (16.07.99)

(30) Priority Data:
60/093,481 20 July 1998 (20.07.98) US

(71) Applicant *(for all designated States except US)*: INEX PHARMACEUTICALS CORPORATION [US/US]; Glenyon Business Park, 100–8900 Glenlyon Parkway, Burnaby, British Columbia V6K 3S4 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BOEY, Anthony [CA/CA]; 419–2551 Parkview Lane, Port Coquitlam, British Columbia V3C 6J8 (CA). CHEN, Tao [CA/CA]; 9–7711 Minorn Boulevard, Richmond, British Columbia V64 1Z3 (CA). LENG, Esther [CA/CA]; 14136 88th Avenue, Surrey, British Columbia V3W 3L6 (CA). TARD, Paul, G. [CA/CA]; 19081 Sundale Court, Surrey, British Columbia V3S 7M6 (CA). WHEELER, Jeffrey, J. [US/CA]; 6179 17A Street, Surrey, British Columbia V34 8G9 (CA). SCHERRER, Peter [CH/CA]; 2664 Birch, Vancouver, British Columbia V6H 2T5 (CA). GRAHAM, Roger [CA/CA]; 2638 West 7th Avenue, Vancouver, British Columbia V6K 1Z1 (CA).

(74) Agents: SNYDER, Joseph, R. et al.; Townsend and Townsend and Crew LLP, Two Embarcadero Center, 8th floor, San Francisco, CA 94111–3834 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: LIPOSOMAL ENCAPSULATED NUCLEIC ACID–COMPLEXES

(57) Abstract

This invention relates to liposomes which are useful for the introduction of nucleic acids into cells. The liposomes of the present invention entrap a condensing agent–nucleic acid complex and are suitable for nucleic acid–transfer delivery vehicles in clinical use. In addition, methods of transfecting a cell with a nucleic acid using the liposomes of the present invention are also disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

LIPOSOMAL ENCAPSULATED NUCLEIC ACID-COMPLEXES

FIELD OF THE INVENTION

This invention relates to liposomes that are useful for introducing nucleic acids into cells. More particularly, the liposomes of the present invention entrap a condensing agent-nucleic acid complex and, thus, they are useful as nucleic acid-transfer delivery vehicles in clinical use.

BACKGROUND OF THE INVENTION

The introduction of foreign genes and other molecules into cells is of great interest to molecular biologists. One reason to introduce genetic material into cells is to express an encoded protein. Gene transfer involves delivering nucleic acids to target cells and then transferring the nucleic acid across the cell membrane in a form that can function in a therapeutic manner. Of the many methods used to facilitate entry of DNA into eukaryotic cells, liposomes are among the most efficacious and have found extensive use as DNA carriers in transfection experiments. Cationic lipids are known to bind to polynucleotides and to facilitate their intracellular delivery into mammalian cells. Nucleic acid is negatively charged and when combined with a positively charged lipid, forms a complex that is suitable for formulation and cellular delivery. The use of cationic lipid carriers for transfection is well established.

Other gene transfer methods under study include viral vectors. Although viral vectors have the inherent ability to transport nucleic acids across cell membranes and, in some instances, integrate exogenous DNA into chromosomes, they can carry only limited amounts of DNA and pose several risks. One such risk involves the random integration of viral genetic sequences into patient chromosomes, potentially damaging the genome and possibly inducing a malignant transformation. Another risk is that the viral vector may revert to a pathogenic genotype either through mutation or genetic exchange with a wild type virus.

Limitations associated with viral gene delivery systems have spawned the development of nonviral gene transfer vectors. These nonviral systems generally consist of plasmid DNA complexed to a cationic agent, such as a lipid or polymer, to condense the nucleic acid and to facilitate its cellular uptake into the cell membrane. One of the obstacles to gene expression is the degradation of the DNA in route to the nucleus within

the cytoplasm. In this respect, polycations have been used extensively to overcome this obstacle and improve gene expression. These cationic polymers include antibiotics, such as gramicidin S, dendrimers or cascade polymers or cationically modified albumin. In addition, spermidine has been shown to condense DNA and improve transfection of cell cultures. These condensing agents protect the DNA from degradation by endonucleases and restriction enzymes. The positive charge on these polymers is also expected to boost the transfection capability of the complexes.

Other polycationic polymers that are useful as condensing agents because of their affinity to electrostatically bind nucleic acids include polylysine, polyarginine and polyornithine. The polycation polyethylenimine (PEI), which is a highly-branched polymer, has been shown to be a highly efficient gene delivery agent. In this regard, PEI condenses nucleic acid into a highly compact form and offers good protection from various nucleases. It has been reported that the gene transfer with these complexes was boosted up to a 1000-fold, under certain conditions. Clearly, polycations like PEI have a clear advantage over the lipid/nucleic acid complexes in this respect.

However, one significant drawback of polycationic-nucleic acid complexes, such as a PEI-nucleic acid complex, is the toxicity associated with *in vivo* gene delivery via the use of such complexes. When PEI is condensed with nucleic acid at higher ratios, the complexes become toxic. At lower ratios (~2), transfection is reduced significantly. If these highly transfecting particles are to be used *in vivo* for transfection, the toxicity must be reduced to tolerable levels.

As such, there exists a need to design condensing agent-nucleic acid complexes that are effective for facilitating intracellular delivery of genetic material, but that will reduce the associated cellular toxicity. The present invention fulfills this and other needs.

SUMMARY OF THE INVENTION

In one aspect, the present invention relates to a liposome having (a) a lipid; and (b) an encapsulated condensing agent-nucleic acid complex. In certain preferred aspects, the liposomes of the present invention further comprise (c) a bilayer stabilizing component. The bilayer stabilizing component can be reversibly associated with the liposome. Such liposomes are extremely advantageous because they offer good protection to the nucleic acid from various nucleases that tend to degrade nucleic acid that is not protected by encapsulation. Moreover, in many instances, gene transfer with these complexes is increased up to a 1000-fold. In addition, using the encapsulating formulations of the present invention the toxicity of the condensing agents are reduced to tolerable levels.

Condensing agents suitable for use in the present invention include, but are not limited to, polycationic polymers, such as polyethylenimine, polylysine, polyarginine and polyornithine. Other condensing agents that have an affinity for nucleic acid and that are suitable for use in the present invention include, but are not limited to, natural DNA-binding proteins of a polycationic nature, such as histones and protamines or analogues or fragments thereof. Other condensing agents suitable for use in the present invention include spermidine, spermine, polycations having two or more different positively charged amino acids or basic proteins.

Although numerous lipids can be used, the lipids used in the liposomes of the present invention are preferably non-cationic lipids. Such non-cationic lipids include, but are not limited to, ceramides, phosphatidylethanolamines, phosphatidylserines and mixtures thereof. In a presently preferred embodiment, the non-cationic lipids used are ceramides, dioleoylphosphatidylethanolamine, dioleoylphosphatidylserine and mixtures thereof.

In another aspect, the present invention relates to a method for encapsulating a condensing agent-nucleic acid complex in a liposome, the method comprising: adding a condensing agent solution into a nucleic acid solution to form a condensing agent-nucleic acid complex; and adding said condensing agent-nucleic acid complex to a lipid suspension to form an encapsulated condensing agent-nucleic acid complex. In a preferred embodiment, the method comprises:

(a) admixing a first condensing agent solution into a nucleic acid solution to form precondensed nucleic acid;

(b) adding the precondensed nucleic acid into a second condensing agent solution to form a condensing agent-nucleic acid complex;

(c) dialyzing the condensing agent-nucleic acid complex to form a concentrated condensing agent-nucleic acid complex;

(d) adding the concentrated condensing agent-nucleic acid complex to a lipid suspension containing detergent; and

(e) removing the detergent from the lipid suspension to form an encapsulated condensing agent-nucleic acid complex in the liposome.

In this method, the first and second condensing agents can be the same or different.

In yet another aspect, the present invention relates to a method of transfecting a cell with a nucleic acid, the method comprises contacting the cell with a liposome having (a) a lipid; and (b) an encapsulated condensing agent-nucleic acid complex. In certain preferred embodiments, the liposomes of this method further comprise (c) a bilayer stabilizing component. The bilayer stabilizing component can be reversibly associated with the liposome.

In still yet another aspect, this invention relates to the treatment of a disease involving the transfection of a cell with nucleic acid and the introduction into cells of antisense nucleotides, as well as the stable transfection of a cell with DNA engineered to become incorporated into the genome of the living cell.

Other features, objects and advantages of the invention and its preferred embodiments will become apparent from the detailed description which follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the construction of uniformed small size polyethylenimine-nucleic acid complex.

Figure 2 illustrates an effect of dextran sulfate on PEI-DNA complexes.

Figure 3 illustrates the titration of dextran sulfate to determine the minimal amount of dextran sulfate required to completely expose DNA to picogreen.

Figure 4 illustrates a standard curve for quantifying DNA. At each data point, a standard amount of dextran sulfate is added, which is the same amount added to the random test samples of complexes.

Figure 5 illustrates that the relaxation or dissociation of the complexes is not an instantaneous event.

Figure 6 illustrates the extent of encapsulation when dextran sulfate is used in the assay to dissociate the nucleic acid from the PEI.

Figure 7 illustrates a titration of DOPS to optimize encapsulation efficiency.

Figure 8 illustrates a Guassian size distribution of a sample of lipid encapsulated PEI/DNA containing 8 mol% DOPS. The liposomes are typically around 75 to about 80 nm in diameter.

Figure 9 illustrates transfection of Cos-7 cells with encapsulated PEI condensed DNA liposomes-dose response and time course.

Figure 10 illustrates transfection of various cell lines with encapsulated PEI condensed DNA Liposomes. LS-180 is derived from a 58 year old female patient with the Duke's type adenocarcinoma of the colon; SK-OV-3 is a human ovarian adenocarcinoma tumor taken from a 64 year old; U87 is human glioblastoma; COS-7 is kidney, fibroblast-like cell line established from CV-1 simian cells which were transformed by an origin-defective mutant of SV40; Lewis Lung is human lung carcinoma; and B16 is mouse melanoma.

Figure 11 illustrates an *in vitro* toxicity of encapsulated PEI condensed DNA liposomes in Cos-7 cell line.

Figure 12 illustrates concentration dependence of the condensing agent-nucleic acid complex on cell death.

Figure 13 illustrates *in vivo* gene expression of PEI condensed DNA liposomes in Lewis Lung tumor.

Figure 14 illustrates gene expression of encapsulated PEI condensed DNA in B16 i.p. tumor.

Figure 15 illustrates *in vitro* toxicity of pre and post purification of encapsulated PEI condensed DNA liposomes.

DESCRIPTION OF THE PREFERRED EMBODIMENT

A. Glossary

The term "lipid" refers to any suitable material resulting in a bilayer such that a hydrophobic portion of the lipid material orients toward the bilayer while a hydrophilic portion orients toward the aqueous phase. Amphipathic lipids have a hydrophilic portion and a hydrophobic portion. Hydrophilic characteristics derive from the presence of phosphato, carboxylic, sulfato, amino, sulfhydryl, nitro, and other like

groups. Hydrophobicity could be conferred by the inclusion of groups that include, but are not limited to, long chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic or heterocyclic group(s). Amphipathic compounds include, but are not limited to, phosphoglycerides and sphingolipids, representative examples of which include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine or dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid and glycosphingolipid families are also within the group designated as lipid. Additionally, the amphipathic lipids described above may be mixed with other lipids including triglycerides and sterols.

The term "neutral lipid" refers to any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, and cerebrosides.

The term "non-cationic lipid" refers to any neutral lipid as described above as well as anionic lipids. Preferred non-cationic lipids include phosphatidylethanol-amines, phosphatidylserines and ceramides. Examples of preferred anionic lipids include cardiolipin, diacylphosphatidylserine, diacylphosphatidic acid, N-succinyl-phosphatydylethanolamine (N-succinyl-PE), phosphatidic acid, phosphatidylinositol, phosphatidylglycerol and phosphatidyl ethylene glycol.

The term "cationic lipid" refers to any of a number of lipid species which carry a net positive charge at physiological pH. Such lipids include, but are not limited to, DODAC, DOTMA, DDAB, DOTAP, DC-Chol and DMRIE. Additionally, there are a number of commercial preparations of cationic lipids. These include, for example, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic lipids comprising DOGS in ethanol from Promega Corp., Madison, Wisconsin, USA).

The term "bilayer stabilizing component" as used herein refers to compounds (*e.g.*, lipids, polymers, *etc.*) that allow lipids adopting a non-lamellar phase under physiological conditions to be stabilized in a bilayer structure. The bilayer

stabilizing components are either bilayer forming themselves, or are of a complementary dynamic shape. The non-bilayer forming lipid is stabilized in the bilayer structure when it is associated with, *i.e.*, in the presence of, the bilayer stabilizing component. In certain embodiments the bilayer stabilizing component is capable of transferring out of the liposome, or of being chemically modified by endogenous systems such that, with time, it loses its ability to stabilize the lipid in a bilayer structure. When liposomal stability is lost, destabilized or decreased, fusion can occur. Fusion can result in the release of liposome payload into the target cell. Thus, in certain embodiments, the bilayer stabilizing component is, "reversibly associated" with the lipid and when it is associated with the lipid, the lipid is constrained to adopt the bilayer structure under conditions where it would otherwise adopt a non-lamellar phase. As such, the bilayer stabilizing components of the present invention is capable of stabilizing the lipid in a bilayer structure, yet is capable of exchanging out of the liposome, or of being chemically modified by endogenous systems so that, with time, they lose their ability to stabilize the lipid in a bilayer structure, thereby allowing the liposome to become fusogenic or release its payload.

In certain other embodiments, the bilayer stabilizing component does not transfer out of the liposome. In these embodiments, the liposome is non-fusogenic and the bilayer stabilizing component is not, "reversibly associated" with the lipid.

The term "transfection" as used herein, refers to the introduction of polyanionic materials, particularly nucleic acids, into cells. The term "lipofection" refers to the introduction of such materials in association with lipids. The polyanionic materials can be in the form of DNA or RNA which is linked to expression vectors to facilitate gene expression after entry into the cell. Thus, the polyanionic material used in the present invention is meant to include DNA having coding sequences for structural proteins, receptors and hormones, as well as transcriptional and translational regulatory elements (*i.e.*, promoters, enhancers, terminators and signal sequences) and vector sequences. Methods of incorporating particular nucleic acids into expression vectors are well known to those of skill in the art, but are described in detail in, for example, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) or *Current Protocols in Molecular Biology*, F. Ausubel *et al.*, ed. Greene Publishing and Wiley-Interscience, New York (1987), both of which are incorporated herein by reference.

"Expression vectors," "cloning vectors," or "vectors" are often plasmids or other nucleic acid molecules that are able to replicate in a chosen host cell. Expression vectors may replicate autonomously, or they may replicate by being inserted into the genome of the host cell, by methods well known in the art. Vectors that replicate autonomously will have an origin of replication or autonomous replicating sequence (ARS) that is functional in the chosen host cell(s). Often, it is desirable for a vector to be usable in more than one host cell, *e.g.*, in *E. coli* for cloning and construction, and in a mammalian cell for expression.

The term "encapsulation" as used herein when discussing amount of encapsulation, refers to the amount of condensing agent-nucleic acid complex that is unavailable to picogreen binding in a picogreen/dextran binding assay or that is nuclease resistant in a nuclease assay.

B. General

It has now been discovered that lipid encapsulation of a condensing agent-nucleic acid complex offers greater protection against enzymatic digestion and gives consistently higher gene expression than unencapsulated condensing agent-nucleic acid complexes. As such, in one aspect, the present invention relates to a liposome comprising (a) a lipid; and (b) an encapsulated condensing agent-nucleic acid complex. In certain preferred embodiments, the liposome further comprises (c) a bilayer stabilizing component. The bilayer stabilizing component can be reversibly associated with the liposome.

The condensing agents used in the liposomes of the present invention can be any compound that has the ability to complex and compact nucleic acids. The complex generally comprises at least one negatively charged nucleic acid and at least one positively charged polymer, the association between the nucleic acid and the cationic polymer being electrostatic in nature.

As such, the condensing agents suitable for use in the present invention include, but are not limited to, polycationic polymers, such as polyethylenimine, polylysine, polyarginine and polyornithine. Other condensing agents that have an affinity for nucleic acid and that are suitable for use in the present invention include, but are not limited to, natural DNA-binding proteins of a polycationic nature, such as histones and protamines or analogues or fragments thereof. Other condensing agents suitable for use in the present invention include polyamines including, but not limited to, spermidine and

spermine, polycations having two or more different positively charged amino acids or basic proteins. In a preferred embodiment, the condensing agent is a polycationic polymer. In another preferred embodiment, condensing agents other than cationic lipids are used. Those of skill in the art will be aware of other condensing agents suitable for use in the present invention.

A particularly preferred example of a polycationic polymer is polyethylenimine. Polyethylenimine, which is a polymeric substance wherein every third atom is an amino nitrogen that can be protonated, has the general formula:

$$(\text{-NH-CH}_2\ \text{CH}_2\text{-})_x[\text{-N}(\text{CH}_2\text{CH}_2\text{NH}_2)\text{CH}_2\text{CH}_2\text{-}]_y \qquad \text{I}$$

In formula I, x is approximately 2 times the value of y. Polyethylenimine is a highly branched material where the ratio of primary to secondary to tertiary nitrogens is about 1:2:1. The primary nitrogens equal the tertiary nitrogens because each branch point has a chain end. Polyethylenimine of various molecular weights can be used. Preferably, molecular weights between 0.8 kDa to about 800 kDa can be used. More preferably, a molecular weight of about 25 kDa can be used. It will be apparent to those of skill in the art that various molecular weight polymers of polyethylenimine will be suitable for use in the present invention. Polyethylenimine of various molecular weights is commercially available from Aldrich Chemical Co., (Milwaukee, Wisconsin).

The nucleic acids of this invention are typically nucleotide polymers having from 10 to 100,000 nucleotide monomers. The nucleic acids are administered to a subject for the purpose of repairing or enhancing the expression of a cellular protein. Additionally, the nucleic acid can carry a label, *e.g.*, radioactive label, fluorescent label or colorimetric label for the purpose of providing clinical diagnosis relating to the presence or absence of complementary nucleic acids. Accordingly, the nucleic acids, or nucleotide polymers, can be polymers of nucleic acids including genomic DNA, cDNA, mRNA or oligonucleotides containing nucleic acid analogs, for example, the antisense derivatives described in a review by Stein, *et al.*, *Science,* **261**:1004-1011 (1993) and in U.S. Patent Nos. 5,264,423 and 5,276,019, the disclosures of which are incorporated herein by reference. Still further, the nucleic acids can encode transcriptional and translational regulatory sequences including promoter sequences and enhancer sequences.

The nucleotide polymers can be single-stranded DNA or RNA, or double-stranded DNA or DNA-RNA hybrids. In addition, nucleic acid with chemically modified phosphodiester bonds (*e.g.*, thiophosphodiester) are also suitable. Examples of double-

stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems, such as plasmid DNA. In preferred embodiments, the nucleic acid is a plasmid.

Single-stranded nucleic acids include antisense oligonucleotides (complementary to DNA and RNA), ribozymes and triplex-forming oligonucleotides and oligonucleotides having modified chemical backbones. These modifications will preferably have some or all of the nucleotide linkages substituted with stable, non-phosphodiester linkages, including, but not limited to, phosphorothioate, phosphorodithioate, phosphoroselenate, or O-alkyl phosphotriester linkages.

The nucleic acids used in the present invention will also include those nucleic acids in which modifications have been made in one or more sugar moieties and/or in one or more of the pyrimidine or purine bases. Examples of sugar modifications include replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, azido groups or functionalized as ethers or esters. Additionally, the entire sugar may be replaced with sterically and electronically similar structures, including aza-sugars and carbocyclic sugar analogs. Modifications in the purine or pyrimidine base moiety include, for example, alkylated purines and pyrimidines, acylated purines or pyrimidines, or other heterocyclic substitutes known to those of skill in the art.

Multiple genetic sequences can be also be used in the present methods. Thus, the sequences for different proteins may be located on one strand or plasmid. Promoter, enhancer, stress or chemically-regulated promoters, antibiotic-sensitive or nutrient-sensitive regions, as well as therapeutic protein encoding sequences, may be included as required. Non-encoding sequences may be also be present, to the extent that they are necessary to achieve appropriate expression.

The nucleic acids used in the present method can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries or prepared by synthetic methods. Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. *See*, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers *et al.*, U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage *et al.*, *Tetrahedron Lett.* **22**:1859-1862 (1981); Matteucci *et al.*, *J. Am. Chem. Soc.* **103**:3185-3191 (1981); Caruthers *et al.*, *Genetic Engineering* **4**:1-17 (1982); Jones, chapter 2, Atkinson *et al.*, chapter 3, and Sproat *et al.*, chapter 4, in

Oligonucleotide Synthesis: A Practical Approach, Gait (ed.), IRL Press, Washington, D.C. (1984); Froehler *et al., Tetrahedron Lett.* **27**:469-472 (1986); Froehler *et al., Nucleic Acids Res.*, **14**:5399-5407 (1986); Sinha *et al. Tetrahedron Lett.* **24**:5843-5846 (1983); and Sinha *et al., Nucl. Acids Res.*, **12**:4539-4557 (1984), which are incorporated herein by reference.

In addition, the nucleic acids of this invention can be chosen from among the following:

(a) gene markers, such as luciferase gene, β-galactosidase gene, chloramphenicol acetyl transferase gene, genes bestowing the resistance to an antibiotic, such as hygromycin or neomycin;

(b) genes for therapeutic purposes, such as gene encoding low density lipoprotein receptors, deficient in the case of hypercholesterolomia (liver), coagulation factors: factors VII and IX, phenylalanine-hydroxylase (phenylketonuria), adenosine deaminase (ADA immunodeficiency), lysosomic enzymes, such as β-glucosidase in the case of Gaucher's disease, dystrophine and minidistriphine (myopathy), tyrosine hydroxylase (Parkinson), neuron growth factors (Alzheimer), CFTR cystic fibrosis transmembrane conductance regulator (mucoviscidose), alpha1-antitrypsin, nuclear factors: NF-KB, CII TA, cytokines and interleukines, TNF: tumor necrosis factor, thymidine kinase of the Herpes simplex virus, NO synthase, angiotensin II receptors, gene suppressors of tumors, such as the gene for the p53 protein, MHC proteins, major histocompatibility system, in particular HLA-B7, antioncogenes: p53, RB, cytosine desaminase, sense and antisense RNA; and

(c) genes with vaccine purposes: genes encoding viral antigens, for example, the nucleoprotein of the influenza virus.

Other suitable nucleic acid for use in the present invention will be readily apparent to those of skill in the art.

C. Preparation of the Condensing Agent-Nucleic Acid Complex

Nucleic acid condensation by polycations is a function of the nature and concentration of all ions present in the condensing media. The complexation is dependent, therefore, on pH, volume and salt concentration of the complexation medium. Cationic polymer condensation with the negatively charged nucleic acid is a cooperative process that can be modulated and even inhibited in high salt concentration.

In addition, it was previously noted in O. Boussif *et al.*, *Proc. Natl. Acad. Sci.* **92**:7297-7301 (1995), incorporated herein by reference, that for the cationic polymer polyethylenimine, the order of adding reagents influences the properties of the resulting particles. For instance, adding the PEI solution dropwise to the nucleic acid, *e.g.*, DNA, solution was 10-fold more efficient than adding nucleic acid to PEI.

The following method illustrates the preparation of a stock cationic polymeric solution using PEI as an example. The cationic polymer, *e.g.*, PEI, is dissolved in deionized water and neutralized to pH 7.4 with, for example, HCl. The neutralized solution is then filtered using a Millipore filter having a pore size of about 0.2 μm. In order to encapsulate the PEI/nucleic acid complex in a liposome (described hereinbelow), a small uniform particle size is critical. Large and heterogenic aggregates are a result of complexation of PEI and nucleic acid using either too high a nucleic acid concentration or solutions other than water.

An important criteria for the condensing agent-nucleic acid complex is the calculation of charge ratio. In one embodiment, the condensing agent-nucleic acid complex bears a net positive charge. A condensing agent-nucleic acid charge ratio of about 10:1 to about 2:1 is preferred, and a ratio of about 7:1 to about 4:1 is more preferred. For the PEI-nucleic acid complexes of this invention, an average mass per negative charge ratio of 325 dalton was used for plasmid DNA. The mass per positive charge for PEI was calculated to be 258 dalton. This assumes that one out of six PEI nitrogens is protonated under physiological conditions, and that the average mass per -CH_2CH_2NH- repeat nitrogen unit in PEI is 43.

In another embodiment, the condensing agent-nucleic acid complex is neutral. In this embodiment, the positive charge of the condensing agent is equal to the negative charge of the nucleic acid. This results in a neutral complex.

Thus, in another embodiment, the present invention relates to a method of condensing a nucleic acid with a condensing agent to give uniformed complexes having a typical size of between about 30 nm to about 60 nm. Using PEI as a typical cationic polymer, the method involves, first, precondensing the nucleic acid by the dropwise addition of a PEI solution (10 μg of stock PEI in 250 mL water) into a nucleic acid solution (100 μg/250 mL) while vortexing. Second, precondensed nucleic acid is saturated with excess PEI. Next, the PEI/nucleic acid complexes are concentrated by dialysis. Finally, the concentrated PEI-nucleic acid complexes are dialyzed overnight against HBS buffer to adjust the salt concentration to 150 mM. In the final step, other

buffers can be used. These buffers include, but are not limited to, PBS, sucrose, water or organic solvent in ethanol, with the ethanol not exceeding 60-70%.

The polyethylenimine:nucleic acid ratio in the complex is about 10:1 wt/wt to about 1.5:1 wt/wt, preferably about 6:1 wt/wt to about 1.5:1 wt/wt, and, more preferably, about 4:1 wt/wt.

It is possible to quantitate the amount of nucleic acid condensed to the polycationic polymer. For instance, at a PEI:nucleic acid wt/wt ratio of 4:1, the complex is tightly condensed and not readily accessible to nucleic acid quantification probes like picogreen. If the nucleic acid is free and not complexed to the condensing agent, picogreen will bind to the nucleic acid and its fluorescence allows quantification of the nucleic acid. To free the nucleic acid, the complex is treated with a polyanion polymer such as dextran sulfate. Heparin, or heparan sulfate, which will "open" up or relax the complex from its condensed state can also be used. This reaction takes typically 10-15 minutes to complete. Picogreen is then added to quantify the exposed nucleic acid. A nucleic acid standard curve is set up with the range between 0.2 μg to 1 μg (*see*, Fig 4.). At each point, a standard amount of dextran sulfate is added. This addition is to offset the quenching effect dextran sulfate has on the fluorescence readings of the picogreen. This quantity is the same amount used to dissociate a PEI/nucleic acid sample. In this way, the nucleic acid associated with the polycatonic polymer can be quantitated (*see*, Fig 2). In Figure 2, the clear bar in Samples 1 and 2 represents the picogreen fluorescence *i.e.*, background.

Figure 3 illustrates the titration of dextran sulfate to determine the minimal amount of dextran sulfate required to release the condensed PEI-nucleic acid complex and completely expose the complexed nucleic acid. This allows accurate quantification of the nucleic acid using a picogreen assay. At least three times more dextran sulfate than PEI is required to completely expose the DNA to picogreen. This represents a charge ratio for PEI/nucleic acid of approximately 5:1. Various charge ratios of PEI/nucleic acid complexes require differing amounts of dextran sulfate. In this manner, the charge ratio can be calculated.

D. Encapsulation of the Condensing Agent-Nucleic Acid Complex

In another embodiment, the present invention relates to a method for encapsulating a condensing agent-nucleic acid complex in a liposome, said method comprising: adding a condensing agent solution into a nucleic acid solution to form a

condensing agent-nucleic acid complex; and adding said condensing agent-nucleic acid complex to a lipid suspension to form an encapsulated condensing agent-nucleic acid complex. In a preferred embodiment, the method comprises:

(a) admixing a condensing agent solution into a nucleic acid solution to form precondensed nucleic acid;

(b) adding the precondensed nucleic acid into a condensing agent solution to form condensing agent-nucleic acid complex;

(c) dialyzing the condensed nucleic acid complex to form concentrated condensing agent-nucleic acid complex;

(d) adding said concentrated condensing agent-nucleic acid complex to a lipid suspension in a detergent; and

(e) removing said detergent from the lipid suspension to form an encapsulated condensing agent-nucleic acid complex in the liposome.

Liposomal encapsulation of the condensing agent-nucleic acid complex offers protection against enzymatic digestion and gives consistently higher gene expression than other transfer methods. To optimize the transfection capability of condensing agent-nucleic acid complexes, the overall charge of the complexes needs to be positive. Unfortunately, with large positive charge ratios, the complexes are toxic and do not last very long in circulation. Thus, it has now been discovered that encapsulation of the condensing agent-nucleic acid complexes in liposomes can reduce the toxicity level of the complexes down to acceptable values.

A variety of lipids can be used in the liposomes of the present invention. Preferably, non-cationic lipids are used. Such lipids include, but are not limited to, phosphatidylethanolamines, phosphatidylserines, ceramides and mixtures thereof. These include, for example, dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidylserine (DOPS) and mixtures thereof. Other examples of preferred anionic lipids suitable for use in the present invention include, but are not limited to, cardiolipin, diacylphosphatidic acid, N-succinyl-phosphatydylethanolamine (N-succinyl-PE), phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, phosphatidyl ethylene glycol and mixtures thereof.

Phosphatidylethanolamines and phosphatidylserines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of about C_6 to C_{24} are preferred. Fatty acids with carbon chain lengths in the range of about C_{14} to C_{20} are especially preferred. Phosphatidylethanolamines with mono- or di-unsaturated fatty acids

and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoylphosphatidylethanolamine (DMPE), dipalmitoylphosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE) and distearoylphosphatidyl-ethanolamine (DSPE). Dioleoylphosphatidylethanolamine is a preferred phosphatidylethanolamine. The preferred phosphatidylserine is dioleoylphosphatidylserine.

Ceramides suitable for use in accordance with the present invention are commercially available from a number of sources. In addition, ceramides can be isolated, for example, from egg or brain using well-known isolation techniques or, alternatively, they can be synthesized using the methods and techniques disclosed in U.S. Patent No. 5,820,873, issued October 13, 1998, the teachings of which are incorporated herein by reference. Using the synthetic routes set forth in the foregoing application, ceramides having saturated or unsaturated fatty acids with carbon chain lengths in the range of C_6 to C_{24} can be prepared. Preferred ceramides have acyl chain lengths of about C_{14} to about C_{20}.

Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to polyethyleneglycol to form the bilayer stabilizing component. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skill in the art.

Ceramides having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be coupled to polyethyleneglycol to form the bilayer stabilizing component. It will be apparent to those of skill in the art that in contrast to the phosphatidylethanolamines, ceramides have only one acyl group which can be readily varied in terms of its chain length and degree of saturation.

In addition, the liposome contains a bilayer stabilizing component. Examples of suitable bilayer stabilizing components include, but are not limited to, lipid, lipid-derivatives, detergents, polyethylene glycol (PEG), proteins, peptides, polyamide oilgomers, (*e.g.*, ATTA) and pH sensitive oilgomer (*e.g.*, PEAA). (*see,* U.S. Application Serial Nos. 08/996,783 filed December 23, 1997, 60/073,852 filed February 2, 1998, and 60/083,294, filed April 28, 1998, the teachings of which are incorporated herein by reference). In a presently preferred embodiment, the bilayer stabilizing component is polyethyleneglycol conjugated to, *i.e.*, coupled to, a phosphatidylethanolamine or

phosphatidylserine. In an equally preferred embodiment, the bilayer stabilizing component is polyethyleneglycol conjugated to a ceramide. Polyethyleneglycol can be conjugated to a phosphatidylethanolamine, phosphatidylserine or, alternatively, to a ceramide using standard coupling reactions known to and used by those of skill in the art. In addition, preformed polyethylene-glycol phosphatidylethanolamine conjugates are commercially available from Avanti Polar Lipids (Alabaster, Alabama).

Polyethyleneglycols of varying molecular weights can be used to form the bilayer stabilizing components of the present invention. Polyethyleneglycols of varying molecular weights are commercially available from a number of different sources or, alternatively, they can be synthesized using standard polymerization techniques well-known to those of skill in the art. In a presently preferred embodiment, the polyethylene glycol has a molecular weight ranging from about 550 to about 8500 daltons, and even more preferably from about 2000 to about 5000 daltons. Generally, it has been found that increasing the molecular weight of the polyethyleneglycol reduces the concentration of the bilayer stabilizing component required to achieve stabilization.

In addition to the foregoing, polyamide oilgomers, (*e.g.*, ATTA), pH sensitive oilgomers, (*e.g.*, PEAA), detergents, proteins and peptides can be used as bilayer stabilizing components. Detergents which can be used as bilayer stabilizing components include, but are not limited to, Triton X-100, deoxycholate, octylglucoside and lyso-phosphatidylcholine. Proteins which can be used as bilayer stabilizing components include, but are not limited to, glycophorin and cytochrome oxidase. Cleavage of the protein, by endogenous proteases, resulting in the loss of the bulky domain external to the bilayer would be expected to reduce the bilayer stabilizing ability of the protein. In addition, peptides which can be used as bilayer stabilizing components include, for example, the pentadecapeptide, alanine-(aminobutyric acid-alanine)$_{14}$. This peptide can be coupled, for example, to polyethyleneglycol, which would promote its transfer out of the bilayer. Alternatively, peptides such as cardiotoxin and melittin, both of which are known to induce non-lamellar phases in bilayers, can be coupled to PEG and might thereby be converted to bilayer stabilizers.

Typically, the bilayer stabilizing component is present at a concentration ranging from about 0.05 mole percent to about 50 mole percent. In a presently preferred embodiment, the bilayer stabilizing component is present at a concentration ranging from 0.05 mole percent to about 25 mole percent. In an even more preferred embodiment, the bilayer stabilizing component is present at a concentration ranging from 5 mole percent to

about 15 mole percent. One of ordinary skill in the art will appreciate that the concentration of the bilayer stabilizing component can be varied depending on the bilayer stabilizing component employed.

One method of encapsulating the complexes of this invention is by using detergent dialysis. Typically, the encapsulation of the complexes is accomplished by dissolving the lipids in a solvent and then drying the solution under a stream of nitrogen. Preferably the lipids are non-cationic lipids. More preferably, the lipids are DOPE, DOPS, PEG-ceramide and mixtures thereof. The ratio of lipid to nucleic acid is about 5:1 wt/wt to about 100:1 wt/wt, preferably about 10:1 wt/wt to about 50:1 wt/wt. Final total lipid concentration desired is about 10 mg/mL. A thin lipid film is achieved by including a mixing step, such as vortexing, in the drying procedure. Any remaining solvent is removed by freeze-drying.

The lipid film is then dissolved in a detergent, or alternatively, ethanol. The condensing agent-nucleic acid complex is then added, such as a PEI/nucleic acid complex having a nucleic acid concentration of about 50 μg/mL to about 1000 μg/mL. A nucleic acid concentration of about 400 μg/mL is preferred. The resulting mixture is then vortexed until it becomes clear and then dialyzed. This procedure results in a liposome encapsulating the condensing agent-nucleic acid complex. The method can be scaled up proportionately for use in larger preparations.

The detergents that are useful for encapsulating the condensing agent-nucleic acid complexes in the present invention are typically one or more neutral detergents or combinations of detergents and organic solvents. The detergents are preferably, N,N'-((octanoylimino)-bis-(trimethylene))-bis-(D-gluconamide) (BIGCHAP); BRIJ 35; Deoxy-BIGCHAP; dodecylpoly(ethylene glycol) ether; Tween 20; Tween 40; Tween 60; Tween 80; Tween 85; Triton X-405; hexyl-, heptyl-, octyl- and nonyl-β-D-glucopyranoside; with octyl β-D-glucopyranoside being the most preferred.

The organic solvents that are useful in combination with a detergent include, but are not limited to, chloroform, dichloromethane, diethylether, cyclohexane, cyclopentane, benzene, toluene, acetone, benzyl alcohol, methanol, or other aliphatic alcohols such as propanol, *iso*-propanol, butanol, *tert*-butanol, *iso*-butanol, pentanol and hexanol. The selection of an organic solvent will typically involve consideration of solvent polarity and the ease with which the solvent can be removed at the later stages of encapsulation. Accordingly, the preferred organic solvents used in conjunction with the

detergent are ethanol, dichloromethane, chloroform, methanol and diethyl ether with chloroform and methanol being the most preferred.

The solution of non-cationic lipids, bilayer stabilizing component and detergent is an aqueous solution. Contacting the condensing agent-nucleic acid complex with the solution of non-cationic lipids and detergent is typically accomplished by mixing together a first solution of nucleic acids and a second solution of the lipids and detergent. One of skill in the art will understand that this mixing can take place by any number of methods, for example, by mechanical means such as by using vortex mixers. Preferably, the nucleic acid solution is also a detergent solution.

In an alternative embodiment, a dehydration-rehydration method can be used to encapsulate the condensing agent-nucleic acid complex. In this method, a lipid mixture in a solvent is dried down and then rehydrated in a buffer containing the condensing agent-nucleic acid complex. Extrusion of the liposome follows the rehydration step. The dehydration-rehydration method generates lower encapsulation efficiency than the detergent dialysis technique described above.

In yet another embodiment, the reverse phase evaporation method can be employed to encapsulate the complex. In this method, the lipids are first dissolved in a solvent system or mixed solvent system. The condensing agent-nucleic acid complex is dissolved in water and then added to the lipid mixture. The solvent system is added until a single phase is observed. After excess solvent is removed by evaporation, the solution is extruded to yield encapsulated liposomes.

In still yet another embodiment, the ethanol injection method can be used for encapsulation of the complex. In this method, the lipids are dissolved in ethanol, or another suitable solvent, and dripped into a tube containing the condensing agent-nucleic acid complexes in water. The liposomes are formed immediately and the ethanol is dialyzed away to yield encapsulated liposomes.

The size of the liposomes of the present invention are about 20 nm to about 200 nm in diameter. More preferably, the liposomes of the present invention are about 50 nm to about 150 nm in diameter. In an especially preferred embodiment, the liposomes of the present invention are about 70 nm to about 80 nm in diameter.

The size distribution of the condensing agent-nucleic acid complexes and liposomes can be measured by quasielastic light scattering using a Nicomp Submicron Particle Sizer (Model 370) in the solid particle mode and vesicle mode, respectively.

To measure the encapsulation efficiency of the liposomes using the methods above, picogreen and dextran sulfate are used. The amount of unencapsulated complexes M_{uncap} (determined from fluorescence of picogreen) can then be quantified via the combination of dextran sulfate and picogreen. By adding Triton X-100, which completely breaks apart the liposomes, it is also possible to quantify the total DNA present, M_{tot}. The extent of encapsulation is then calculated via the formula:

$$\% \text{ Encapsulation} = (1 - M_{uncap}/ M_{tot.}) \times 100$$

Encapsulation efficiency is best described with reference to Figure 6. As shown in Figure 6, when picogreen is added to an unencapsulated complex, fluorescence is in the background. When dextran sulfate is added to an unencapsulated complex, there is an increase in fluorescence to 3.0. When Triton X-100 is added to break up the liposomes, a further jump in fluorescence occurs (*see,* Sample 1). The ratio of these peaks gives the extent of encapsulation. To remove any unencapsulated complex, a cation exchange column can be used.

In using methods of the present invention, it is possible to encapsulate about 30% to about 70% of the condensing agent-nucleic acid complex . Preferably, percent encapsulation is about 40% to about 70% and most preferably, percent encapsulation is about 50% to about 70%.

With reference to Figure 7, the titration of DOPS to optimize encapsulation efficiency is illustrated. In certain embodiments, a concentration of approximately 8-9 mol% of DOPS in the liposome gives the best entrapment of the complexes, in this case PEI/DNA. Shifting this point by more than 2% in concentration of DOPS drops the encapsulation efficiency dramatically.

E. Administration of Liposome-Entrapped Complexes

Following formation of the liposomal entrapped condensing agent-nucleic acid complexes, the liposomes can be used to transfect cells by contacting the cells to be transfected with the liposomes. The liposome-entrapped complexes can be adsorbed to almost any cell type. Once adsorbed, the liposomes can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, destabilized or fuse with the cells. Transfer or incorporation of the nucleic acid portion of the liposome can take place via any one of these pathways. In particular, when fusion takes place, the lipid bilayer membrane is integrated into the cell membrane and the contents of the bilayer combine with the intracellular fluid. Fusion of the liposome with the plasma membrane takes

place when the bilayer stabilizing component transfers out of the liposome and the bilayer stability is lost or decreased. Without being bound to any theory, polycationic mediated gene transfer is thought to involve DNA aggregation and binding of the resulting complex to anionic residues on the plasma membranes. To be efficient, the complex should bear a net positive charge.

Contact between the cells and the liposomal entrapped condensing agent-nucleic acid complexes, when carried out *in vitro*, will take place in a biologically compatible medium.

Treatment of the cells with the liposome-entrapped complex will generally be carried out at physiological temperatures (about 37°C) for periods of time ranging from about 1 to 48 hours, preferably from about 2 to 4 hours. For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

With reference to Figure 9, Cos-7 cells were transfected with 1 μg of encapsulated pINEX/L018 plasmid DNA complexed with PEI at the 1:4 w/w ratio. In a dose response and time course analyses, Figure 9 shows that transfection activity increased as DNA dose increased. The highest transfection activity was observed at 5 μg of DNA. Minimal transfection was seen at the 24 hour time point. The transfection activity continued to increase up to the 72 hour time point.

F. Pharmaceutical Preparations

The liposome-entrapped condensing agent-nucleic acid complexes of the present invention can be administered alone or in mixture with a physiologically acceptable carrier. Such carriers include, but are not limited to, physiological saline or phosphate buffer selected in accordance with the route of administration and standard pharmaceutical practice.

Pharmaceutical compositions comprising the liposome-entrapped condensing agent-nucleic acid complexes are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* In

compositions comprising saline or other salt containing carriers, the carrier is preferably added following liposome formation. Thus, after the liposome-entrapped complexes are formed, the liposome can be diluted into pharmaceutically acceptable carriers, such as normal saline. These compositions may be sterilized by conventional sterilization techniques. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, *etc.*

The concentration of the liposome-entrapped complexes in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2-5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. For diagnosis, the amount of liposome-entrapped complex administered will depend upon the particular label used, the disease state being diagnosed and the judgement of the clinician but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight.

In another example of their use, the liposomal entrapped condensing agent-nucleic acid complexes can be incorporated into a broad range of topical dosage forms including, but not limited to, gels, oils, emulsions and the like. For instance, the suspension containing the liposomal entrapped condensing agent-nucleic acid complexes can be formulated and administered as topical creams, pastes, ointments, gels, lotions and the like.

The present invention also provides liposome-entrapped condensing agent-nucleic acid complexes in kit form. The kit will typically be comprised of a container which is compartmentalized for holding the various elements of the kit. The kit will contain the liposomes of the present invention, with instructions for administration. In still other embodiments, the liposomal entrapped condensing agent-nucleic acid complexes will have a targeting moiety attached to the liposome. Methods of attaching

targeting moieties (*e.g.*, antibodies, proteins) to lipids (such as those used in the present invention) are known to those of skill in the art.

Dosage for liposome-entrapped condensing agent-nucleic acid complexes will depend on the ratio of nucleic acid to lipid and the administrating physician's opinion based on age, weight, and condition of the patient.

The invention will be described in greater detail by way of specific examples. The following examples are offered for illustration purposes only and are not intended to limit the invention in any manner.

G. Examples

I. Materials

The reporter gene plasmid used in all experiments was pINEX/L018 (5650 bp), which encodes firefly luciferase under the control of the human cytomegalovirus immediate-early enhancer/promoter. Plasmid DNA was prepared from *E. coli* DH5alpha by alkaline lysis followed by double banding on cesium chloride gradients. (*See*, Thierry A.R., *J. Liposome Research* 7:143-159 (1997), incorporated herein by reference.) PEI 25 kDa was purchased from Aldrich Chemical Co. (Milwaukee, WI). Ethidium bromide picogreen was obtained from Sigma Chemical Co. (St. Louis, MO). Purified firefly luciferase was purchased from Boehringer Mannheim (Germany). All lipids, with exception of the PEG-ceramide (made in-house), are obtained from Avanti Lipids. Fetal bovine serum was purchased from Intergen (New York, USA). All culture media were purchased from Stemcell Technology (Vancouver, BC). Other reagents in this study were from Sigma Chemical Co., and used without further purification.

II. Methods

a. Method for Preparing PEI/plasmid DNA complexes.

1.) PEI solution (1.6 mg PEI/mL water): 0.1 volume of the PEI solution was added dropwise, using a syringe fitted with a needle size 26G3/8 gauge, into a plasmid DNA solution (1 mg plasmid DNA/1.25 mL water) while vortexing. 2.) The precondensed PEI/DNA made in step 1, was then added dropwise, using a syringe fitted with a needle size 26G3/8 gauge, into PEI solution (390 g of stock PEI diluted in 500 mL water) while vortexing to form PEI/plasmid DNA complexes. The resulting surface charge of the PEI/DNA complex is a net positive charge. 3.) The PEI/plasmid DNA complex was then transferred into a dialysis bag (6000 - 8000 molecular weight cut-off,

Spectra-Por, Spectrum) and covered with a drying agent (polyethylene glycol, 10,000 molecular weight). 4.) The concentrated PEI/plasmid DNA complexes was dialyzed overnight against HBS (150 mM NaCl, 5 mM - 25 mM HEPES), pH 7.45 to adjust the NaCl concentration to 150 mM.

ii. Method for the Quantification of Plasmid DNA.

The PEI/plasmid DNA complexes were treated with a polyanion polymer such as dextran sulfate in water (other polymers such as heparin or heparan sulfate can also be used) where for every 4 μg PEI used, 40 μg of dextran sulfate is used. This reaction takes typically between 10 to 15 minutes to complete. 4 μl picogreen is then added to the PEI/plasmid DNA complex. A DNA standard curve is set up with a range running between 0.2 μg to 1.0 μg, where at each point, a standardized amount of dextran sulfate is added. This is to offset the quenching effect of the dextran sulfate on the fluorescence readings of the picogreen. This amount must also be the same as the amount used to dissociate the PEI/plasmid DNA sample.

iii. Protocol for Encapsulating the PEI/plasmid DNA Complexes in Lipids.

The lipids, DOPE (82 mol %), DOPS (8 mol %), and Peg-Ceramide(C20) (10 mol %), dissolved in chloroform, were first dried under a stream of nitrogen. Final total lipid concentration desired is 10 mg/mL. A thin lipid film is achieved by including vortexing in the drying procedure. Any remaining solvent was removed by further freeze-drying overnight. The dried-down lipid film is then removed from the lyophilizer and 200 μl of OGP (200 mM) was added. Hard vortexing, followed by warming at 65°C for 5 minutes intermittently, helps to dissolve the lipids in the detergent. When no apparent undissolved lipid can be seen, the PEI/DNA complexes in the DNA concentration of 400 μg/mL, is then added to the lipid suspension. The lipid:DNA w/w ratio used is 10mg:400 μg respectively. Anything above the concentration of 500μg/mL DNA was found to cause precipitation or flocculation to occur (final product has big particles which disappeared momentarily upon shaking). The resulting mixture is then vortexed until it becomes clear and then transferred into a Spectra-Por dialysis bag for dialysis. The dialysis buffer is made up of 5 mM Hepes, 150 mM NaCl and titrated with appropriate amounts NaOH to achieve a pH of approximately 7.45. The mixture is then dialyzed for 24 hours with buffer changes at every 4 hours.

iv. Method for Determining Encapsulation Efficiency of the Lipid Formulation.

To measure the encapsulation efficiency of lipid particles, picogreen and dextran sulfate were used. The amount of unencapsulated complexes M_{uncap} (determined from fluorescence of picogreen) could be quantified via the combination of dextran sulfate and picogreen. When Triton X-100 was added, which completely dissociates the lipid particles, the total DNA present, M_{tot} could be determined. The extent of encapsulation is then calculated using the formula:

$$\% \text{ Encapsulation} = (1 - M_{uncap}/ M_{tot}) \times 100$$

v. Protocol for treating the Encapsulated PEI/plasmid DNA Lipid Particles.

The cation gel, about 100 mL (Dowex-50W, Catalog No. 50X8-400, Sigma) is first placed in a volumetric flask (1000 mL capacity) containing approximately 500 mL of 0.5 M HCl, which acts as a proton reservoir. A magnetic stir bar was included in the flask and the mixture was stirred slowly on a stir-plate overnight. The following day, the gel is then loaded into a chromatographic column (GlassEcono-column, Catalog No. 737-1012, BioRad) to a height of about 5 cm. The gel is then washed with 10 volumes of distilled water to normalize the pH of the gel. After the wash, 100 mL of 5 M NaCl is used to wash off any remaining impurities on the gel. Finally, 10 column volumes of 150 mM NaCl HBS was used to equilibrate the column.

v. Method for Transfection of the PEI/plasmid DNA Lipid Particles Using Several Cell Lines *in vitro*.

Lewis Lung, SK-OV-3, LS180, Cos 7, B16 and U87 cells were seeded in 24-well plates (Falcon 3047) at a density of 4×10^4, 2×10^4, 4×10^4, 2×10^4, 4×10^4 and 4×10^4 cells/well in 1 mL of media with 10% fetal bovine serum. B16 and LS180 cells were grown in MEM + Earls Salts, Lewis Lung , Cos 7 and U87 cells were grown in DMEM with high glucose, and SK-OV-3 cells were grown in RPMI 1640 medium. Cells were incubated overnight to 70-80% confluent at the time of transfection. The encapsulated PEI condensed DNA lipid formulations were prepared as described above and were added to the appropriate wells in triplicate. The plates were agitated briefly then incubated at 37°C (5% CO_2) for 24 and 48 hours before assaying for gene expression.

Luciferase gene expression was measured with a luminometer (Dynatech Microlite TM ML3000 Microtiter) using 96-well plates (Catalog No. 011-010-7411,

Dynatech). Cells were rinsed once with PBS buffer, and were then lysed with 150 mL of lysis buffer (0.1% Triton X-100, 250 mM sodium phosphate buffer, pH 8.0) at room temperature for 10 - 15 minutes. Duplicate assays for 10 µl of cell lysate were performed. A standard curve was prepared using purified luciferase protein diluted into mock transfected cell lysate.

vi. Method for Measuring the Total Protein of Each *in vitro* Transfected Sample

Protein assay was performed by using the bicinchoninic acid (BCA) colorimetric method. In this assay, 10 µl of lysate was transferred to the individual wells of 96-well plate (Catalog No. 011 - 010-7411, Dynatech), 200 µl of Micro BCA working reagent was added to each well, mixed and incubated at 37°C for 2 hours. The amount of protein in each well was determined by comparison with BSA protein standard (1-16 µg/well) added to a series of duplicate wells on the same plate. The plate with samples and BSA protein standard was read at 570 nm in a microtiter plate reader (Dynatech MR5000) after allowing the color to develop.

vii Method of Evaluating the Level of Toxicity *in vitro*

Dilute 0.1% crystal violet reagent to 0.05% with 20% ethanol. Centrifuge plates at 1500 rpm for 10 minutes. Rinse plates 2 times with PBS buffer by pouring the buffer into the lid of a pipette tip box and submerging the plates. Change the PBS buffer between plates. Invert plates onto a stack of paper towels and gently pat to dry. Add 50-100 µl of 0.05% crystal violet to each well. Incubate plates at room temperature for 10 minutes. Rinse plates with tap water as described above for the PBS wash. Allow plates to dry on the bench top overnight. Add 100 µl of 100% methanol to each well. Read plates within 5 minutes of methanol addition using the plate reader at 570nm.

Cos-7 cells were seeded onto 96-well plates at a density of 2.5×10^3 in 200 µl of completed medium and incubated 72 hours to 90% confluence. different amount of encapsulated PEI condensed DNA formulations and PEI/DNA complex were added to the appropriate wells in triplicate. Stained the cells with crystal violet after 24 hours incubated.

viii Method for delivering the PEI/plasmid DNA lipid particles in the *in vivo* system

Female C57 mice were injected intraperiotenially (i.p.) with 1 x 10^5 B16 tumor cells. On day 7 of B 16 tumor growth, DNA doses of 75 (g luciferase plasmid/ formulation (encapsulated PEI/DNA lipid particles) were administered in a volume of 500 µl by intraperitoneal injection. Control animals were injected with the same volume of saline. Tumors were collected at different time points, fast frozen in liquid nitrogen and stored at -70°C until analysis. Individual tumors were homogenized using a FastPrep homogenizer (Bio101 inc.) for 5 seconds at speed setting of 5, loaded with a small bead (Catalog No. 6520-401/404, Bio 101), then a second bead and a certain amount of 1 x CCLR reagent (Cell Culture Lysis Reagent Catalog No. E1531, Promega) supplemented with 1 mg/mL BSA (Catalog No. A-2153, Sigma) was added to each tube. The homogenization was performed twice in the FastPrep instrument (FastPrep™ FP120 Instrument, Bio 101) using a speed setting of 5 for 6 seconds. The homogenate was transferred to fresh Eppendorf tubes and large tissue debris was pelleted to the bottom of the tube by brief centrifugation. 20 µl of homogenate and standard luciferase protein diluted with control tissue homogenate were assayed in duplicates. The results were converted to pg of luciferase protein per organ or gram of tumor.

ix. Method for Evaluating Toxicity in the *in vivo* system by Measuring Levels of Aminotransferase (AST/GOT) Activity in Serum.

1.) Add 10 mL of deionized water to a vial. Mix immediately several times by inversion (not shaking). Store reagent up to 16 hours at room temperature or up to 7 days refrigerated. 2.) Turn the UV lamp on at least one half an hour before analysis. Go to kinetics analysis and use the default program within the kinetics setting. Set spectrophotometer to 340 nm and the times as 30, 60, 90, and 120 seconds. Blank with water. 3.) Add 500 µl of reagent to the cuvette (make sure the reagent is at 25°C). 4.) Add 50 µl of the test serum and mix by pipetting up and down. 5.) Read the absorbance after 60 seconds. This is the initial value (inA). 6.) Read the absorbance 30 seconds after the inA reading. This reading is used to verify a linear reaction. 7.) Read the absorbance 60 seconds after the inA reading. This is the final reading (finA). 8.) Calculate the (A per minute by subtracting FinalA from InitialA. If (A per minute is greater than 0.280, dilute 1 part sample with 1 part isotonic saline and reassay. Multiply the results by 2 to compensate for the dilution. 9.) To calculate the

AST(U/L) = (A per minute x TV x 1000 = Total volume (0.55 mL)
6.22 x LP x SVTV

SV = Sample volume (0.55 mL)

6.22 = Millimolar absorptivity of NADH at 340 nm

LP = Light path (1.0)

1000 = Conversion of units per mL to units per liter

$$= \frac{\text{A per minute x 0.55 x 1000}}{\text{6.22 x 1.0 x 0.05}}$$

=(A per minute x 1786 (x 1.37 if values were determined at 25°C)

One unit of activity is defined as the amount of enzyme which produces 1 (mole of NAD per minute under the conditions of the assay procedure.

EXAMPLE 1

This example illustrates the effect of dextran sulfate on PEI/DNA complexes.

Figure 2 (sample 1) illustrates that when picogreen is added to a sample of the liposome formulation containing a PEI/DNA complex, the fluorescence reading is well in the background. It is clear that the DNA is well protected by the condensing agent PEI. When dextran sulfate is added, a significant jump in fluorescence results. The DNA is clearly not in the same condensed form, thus allowing access to picogreen. In sample 2, when Triton X100 is added, there is no significant change in the fluorescence reading *i.e.*, similar to background. This is an indication that Triton does not affect the complexes in any significant way. Upon addition of dextran sulfate, the fluorescence increases.

EXAMPLE 2

This example illustrates the use of dextran sulfate and the amount required for complex dissociation

As is illustrated in Figure 3, when the amount of dextran sulfate added is increased, the fluorescence also increases. This indicates that more and more DNA is made accessible to the picogreen. The increase in fluorescence eventually tapers off at some level and eventually the fluorescence signal is quenched by the excess dextran sulfate. The optimum dextran sulfate to PEI w/w ratio is around 6:1. It is noted that each point is Figure 3 includes an incubation period of approximately 15 minutes.

EXAMPLE 3

This example illustrates the relaxation time of the PEI-DNA complexes.

Figure 5 illustrates the time relaxation profile of PEI/DNA complexes under the effect of dextran sulfate. The graph shows clearly that the relaxation of the complexes is not an instantaneous event. The initial relaxation is rapid, slowing down eventually to final equilibration, as indicated by the picogreen fluorescence signal. These results indicate that for quantification purposes, the required amount of dextran sulfate must be added in advance to the sample and allowed to incubate for at least 15 minutes, to ensure that the relaxation process is complete.

EXAMPLE 4

This example illustrates the encapsulation efficiency of PEI/DNA complexes into liposomes.

As shown in Figure 7, to optimize the encapsulation efficiency, DOPS was titrated and it was found that between 8-9 mol% DOPS gives the best encapsulation at about 55%. This percent encapsulation was determined prior to column loading. Encapsulation efficiencies drop dramatically below this concentration, indicating the high sensitivity of the procedure on the negative surface-charge density. Note that this was obtained in a buffer with 150 mM NaCl concentration. Changing the NaCl concentration will change the amount of DOPS required to optimize encapsulation. For *in vitro* tests, about 8 mol % of DOPS was optimum.

EXAMPLE 5

This example illustrates the efficiency of transfection of the PEI/DNA complexes *in vitro*.

As shown in Figure 9, Cos-7 cells were transfected with 1 μg of encapsulated pINEX/L018 plasmid DNA complexed with PEI at a 1:4 w/w ratio. In this experiment, dose response and time course were analyzed. Figure 9 shows that activity increased as DNA dose increased. The highest transfection activity was observed at 5 μg of DNA. Minimal transfection was seen at the 24 hour time point. The transfection activity continued to increase up to the 72 hour time point.

EXAMPLE 6

This example illustrates the reduction of toxicity of the encapsulated PEI/DNA complexes.

As shown in Figure 10, a toxicity study was conducted of an encapsulated PEI/DNA complex with a dose response having a complex charge-ratio at 5.3. As an illustration, the time-point selected was 48 hours. The control was the cell line with no added components. The graph clearly shows that unencapsulated complexes shows significant toxicity beginning at the 1 μg dose. The encapsulated complexes showed no relative toxicity up to 2 μg DNA.

As shown in Figure 11, the comparison of toxicity between (1) liposomes not treated by a cation exchange column and (2) those which have been so treated is illustrated. The unencapsulated complexes contains 0.75 μg DNA, which is selected to be exactly what is known to be on the outside of the liposome sample. The toxicity of (1) is seen to be comparable to that of the unencapsulated complexes. Sample (2) shows dramatic reduction in toxicity, and can be attributed to the removal of the complexes by a cation exchange column.

Figure 12 illustrates *in vivo* toxicity of encapsulated PEI/DNA complexes. Encapsulated PEI/DNA with a dose of 75 μg DNA is injected into 4 mice and the enzyme (AST) levels were found to be comparable to that of the PBS control. At a dosage of about 4:1 w/w ratio of PEI/DNA, an unencapsulated sample would have been lethal to the mice. The encapsulation efficiency in this liposomal injection is close to 90%.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the invention has been described with reference to preferred embodiments and examples thereof, the scope of the present invention is not limited only to those described embodiments. As will be apparent to persons skilled in the art, modifications and adaptations to the above-described invention can be made without departing from the spirit and scope of the invention, which is defined and circumscribed by the appended claims.

WHAT IS CLAIMED IS:

1. A liposome comprising:

(a) a lipid; and

(b) a condensing agent-nucleic acid complex encapsulated in said liposome.

2. A liposome in accordance with claim **1**, further comprising:

(c) a bilayer stabilizing component associated with said liposome.

3. A liposome in accordance with claim **2**, wherein said bilayer stabilizing component is reversibly associated with said liposome.

4. A liposome in accordance with claim **1**, wherein said lipid comprises a non-cationic lipid.

5. A liposome in accordance with claim **4**, wherein said non-cationic lipid is a member selected from the group consisting of phosphatidylethanolamines, phosphatidylserines and mixtures thereof.

6. A liposome in accordance with claim **4**, wherein said non-cationic lipid is a member selected from the group consisting of cardiolipin, diacylphosphatidic acid, N-succinyl-phosphatydylethanolamine, phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, phosphatidyl ethylene glycol and mixtures thereof.

7. A liposome in accordance with claim **5**, wherein said non-cationic lipid is a member selected from the group consisting of dioleoylphosphatidylethanolamine, dioleoylphosphatidylserine and mixtures thereof.

8. A liposome in accordance with claim **1**, wherein said condensing agent is a member selected from the group consisting of polyethylenimine, polylysine, polyarginine, polyornithine, histones, protamines, polyamines, spermidine and spermine.

9. A liposome in accordance with claim **8**, wherein said condensing agent is polyethylenimine having a molecular weight of about 0.8 kDa to about 800 kDa.

10. A liposome in accordance with claim **9**, wherein said polyethylenimine has a molecular weight of about 10 kDa to about 50 kDa.

11. A liposome in accordance with claim **1**, wherein said condensing agent-nucleic acid complex is about 30 nm to about 60 nm in diameter.

12. A liposome in accordance with claim **1**, wherein said liposome is about 20 nm to about 200 nm in diameter.

13. A liposome in accordance with claim **12**, wherein said liposome is about 50 nm to about 150 nm in diameter.

14. A liposome in accordance with claim **12**, wherein said liposome is about 70 nm to about 80 nm in diameter.

15. A liposome in accordance with claim **2**, wherein said bilayer stabilizing component is a member selected from the group consisting of a lipid, a lipid-derivative, a detergent, a polyethylene glycol, a protein, a peptide, a polyamide oligomer, a pH sensitive polymer and a PEG-lipid.

16. A liposome in accordance with claim **15**, wherein said bilayer stabilizing component is a PEG-lipid.

17. A liposome in accordance with claim **16**, wherein said lipid of said PEG-lipid stabilizing component is a member selected from the group consisting of ceramides, phosphatidylethanolamines and phosphatidylserines.

18. A liposome in accordance with claim **17**, wherein said PEG-lipid is a PEG-ceramide.

19. A liposome in accordance with claim **18**, wherein said PEG-ceramide has an alkyl chain length of about C_6 to about C_{24}.

20. A liposome in accordance with claim **19**, wherein said PEG-ceramide has an alkyl chain length of about C_{14} to about C_{20}.

21. A liposome in accordance with claim **16**, wherein said PEG is a polyethylene glycol with an average molecular weight of about 550 to about 8500 daltons.

22. A liposome in accordance with claim **21**, wherein said PEG has an average molecular weight of about 2000 to about 5000 daltons.

23. A liposome in accordance with claim **9**, wherein said polyethylenimine:nucleic acid ratio in said condensing agent-nucleic acid complex is about 10:1 wt/wt to about 1.5:1 wt/wt.

24. A liposome in accordance with claim **23**, wherein said polyethylenimine:nucleic acid ratio in said condensing agent-nucleic acid complex is about 6:1 wt/wt to about 1.5:1 wt/wt.

25. A liposome in accordance with claim **23**, wherein said polyethylenimine:nucleic acid ratio in said condensing agent-nucleic acid complex is about 4:1 wt/wt.

26. A liposome in accordance with claim **1**, wherein said lipid:nucleic acid ratio in said liposome is about 5:1 wt/wt to about 100:1 wt/wt.

27. A liposome in accordance with claim **26**, wherein said lipid:nucleic acid weight ratio in said liposome is about 10:1 wt/wt to about 50:1 wt/wt.

28. A liposome in accordance with claim **16**, wherein said PEG-lipid comprises about 5 to about 15 mol% of the composition of said liposome.

29. A liposome in accordance with claim **18**, wherein said PEG-ceramide comprises about 5 to about 15 mol% of the composition of said liposome.

30. A liposome in accordance with claim **1**, wherein said encapsulated condensing agent-nucleic acid complex represents greater than about 30% encapsulation efficiency as determined using picogreen and dextran sulfate.

31. A liposome in accordance with claim **1**, wherein said encapsulated condensing agent-nucleic acid complex represents greater than about 40% encapsulation efficiency as determined using picogreen and dextran sulfate.

32. A method of transfecting a cell with a nucleic acid, said method comprising contacting said cell with a liposome comprising:

(a) a lipid; and

(b) a condensing agent-nucleic acid complex encapsulated in said liposome.

33. A method of transfecting a cell with a nucleic acid in accordance with claim **32**, wherein said liposome further comprises:

(c) a bilayer stabilizing component associated with said liposome.

34. A method of transfecting a cell with a nucleic acid in accordance with claim **33**, wherein said bilayer stabilizing component is reversibly associated with said liposome.

35. A method of transfecting a cell with a nucleic acid in accordance with claim **32**, wherein said lipid comprises a non-cationic lipid.

36. A method of transfecting a cell with a nucleic acid in accordance with claim **35**, wherein said non-cationic lipid is a member selected from the group consisting of phosphatidylethanolamines, phosphatidylserines and mixtures thereof.

37. A method of transfecting a cell with a nucleic acid in accordance with claim **35**, wherein said non-cationic lipid is a member selected from the group consisting cardiolipin, diacylphosphatidic acid, N-succinyl-phosphatydylethanolamine, phosphatidic acid, phosphatidylinositol, phosphatidylglycerol, phosphatidyl ethylene glycol and mixtures thereof.

38. A method of transfecting a cell with a nucleic acid in accordance with claim **36**, wherein said non-cationic lipid is a member selected from the group consisting of dioleoylphosphatidylethanolamine, dioleoylphosphatidylserine and mixtures thereof.

39. A method of transfecting a cell with a nucleic acid in accordance with claim **32**, wherein said condensing agent is a member selected from the group consisting of polyethylenimine, polylysine, polyarginine, polyornithine, histones, protamines, polyamines, spermidine and spermine.

40. A method of transfecting a cell with a nucleic acid in accordance with claim **39**, wherein said condensing agent is polyethylenimine having a molecular weight of about 10 kDa to about 50 kDa.

41. A method of transfecting a cell with a nucleic acid in accordance with claim **32**, wherein said condensing agent-nucleic acid complex is about 30 nm to about 60 nm in diameter.

42. A method of transfecting a cell with a nucleic acid in accordance with claim **32**, wherein said liposome is about 70 nm to about 80 nm in diameter.

43. A method of transfecting a cell with a nucleic acid in accordance with claim **33**, wherein said bilayer stabilizing component is a member selected from the group consisting of a lipid, a lipid-derivative, a detergent, a polyethylene glycol, a protein, a peptide, a polyamide oligomer, a pH sensitive polymer and a PEG-lipid.

44. A method of transfecting a cell with a nucleic acid in accordance with claim **43**, wherein said bilayer stabilizing agent is a PEG-lipid.

45. A method of transfecting a cell with a nucleic acid in accordance with claim **44**, wherein said lipid of said PEG-lipid stabilizing agent is a member selected from the group consisting of ceramides, phosphatidylethanolamines and phosphatidylserines.

46. A method of transfecting a nucleic acid into a cell in accordance with claim **45**, wherein said bilayer stabilizing agent is a PEG-ceramide.

47. A method of transfecting a nucleic acid into a cell in accordance with claim **46**, wherein said PEG-ceramide has an alkyl chain length of about C_6 to about C_{24}.

48. A method of transfecting a nucleic acid into a cell in accordance with claim **47**, wherein said PEG-ceramide has an alkyl chain length of about C_{14} to about C_{20}.

49. A method of transfecting a nucleic acid into a cell in accordance with claim **44**, wherein said PEG has an average molecular weight of about 550 to about 8500 daltons.

50. A method for transfecting a nucleic acid into a cell in accordance with claim **40**, wherein said polyethylenimine:nucleic acid ratio in said polyethylenimine-nucleic acid complex is about 10:1 wt/wt to about 1.5:1 wt/wt.

51. A method of transfecting a nucleic acid into a cell in accordance with claim **50**, wherein said polyethylenimine:nucleic acid ratio in said polyethylenimine nucleic acid complex is about 4:1 wt/wt.

52. A method for transfecting a nucleic acid into a cell in accordance with claim **32**, wherein said lipid:nucleic acid weight ratio in said liposome is about 10:1 to about 50:1.

53. A method for transfecting a nucleic acid into a cell in accordance with claim **44**, wherein said PEG-lipid comprises about 5 to about 15 mol% of the composition of said liposome.

54. A method for transfecting a nucleic acid into a cell in accordance with claim **46**, wherein said PEG-ceramide comprises about 5 to about 15 mol% of the composition of said liposome.

55. A method for encapsulating a condensing agent-nucleic acid complex in a liposome, said method comprising:

adding a condensing agent solution into a nucleic acid solution to form a condensing agent-nucleic acid complex; and

adding said condensing agent-nucleic acid complex to a lipid suspension to form an encapsulated condensing agent-nucleic acid complex.

56. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim **55**, wherein said condensing agent-nucleic acid complex is formed by admixing a first condensing agent to form a precondensed nucleic acid and then adding said precondensed nucleic acid into a second condensing agent solution to form said condensing agent-nucleic acid complex wherein said first and said second condensing agents are the same or different.

57. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim **55**, wherein said lipid suspension comprises a non-cationic lipid.

58. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim **55**, wherein said condensing agent-nucleic acid complex is about 30 nm to about 60 nm in diameter.

59. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim **55**, wherein said lipid suspension comprises a PEG-lipid.

60. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim **58**, wherein said PEG-lipid comprises a PEG-ceramide.

61. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim **58**, wherein said first condensing agent is polyethylenimine.

62. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim **55**, wherein said lipid:nucleic acid ratio in said liposome is about 10:1 wt/wt to about 50:1 wt/wt.

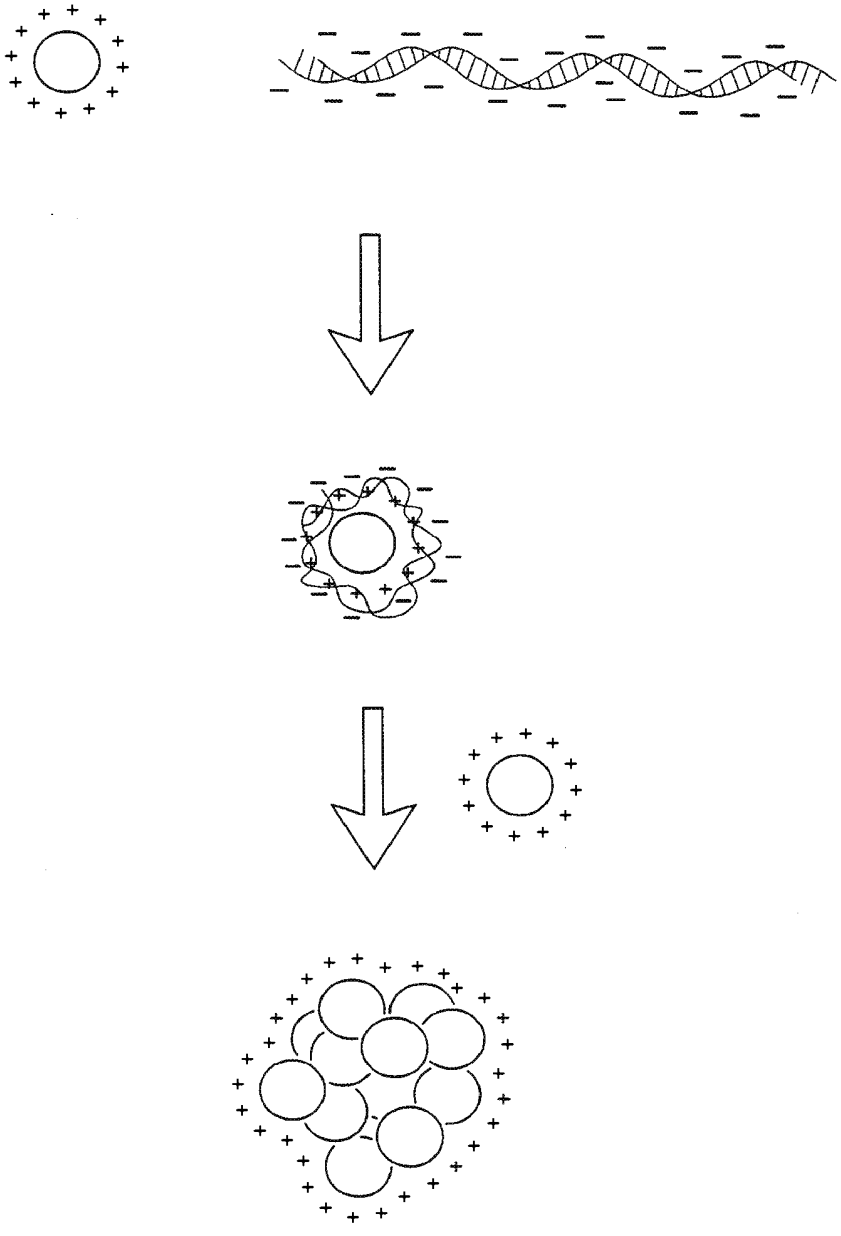
63. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim **58**, wherein said PEG-lipid comprises about 5 to about 15 mol% of the composition of said liposome.

64. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with 59, wherein said PEG-ceramide comprises about 5 to about 15 mol% of the composition of said liposome.

65. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 55, wherein step (c) employs a detergent dialysis.

66. A method for encapsulating a condensing agent-nucleic acid complex in a liposome in accordance with claim 55, wherein step (c) employs an ethanol injection.

Fig. 1



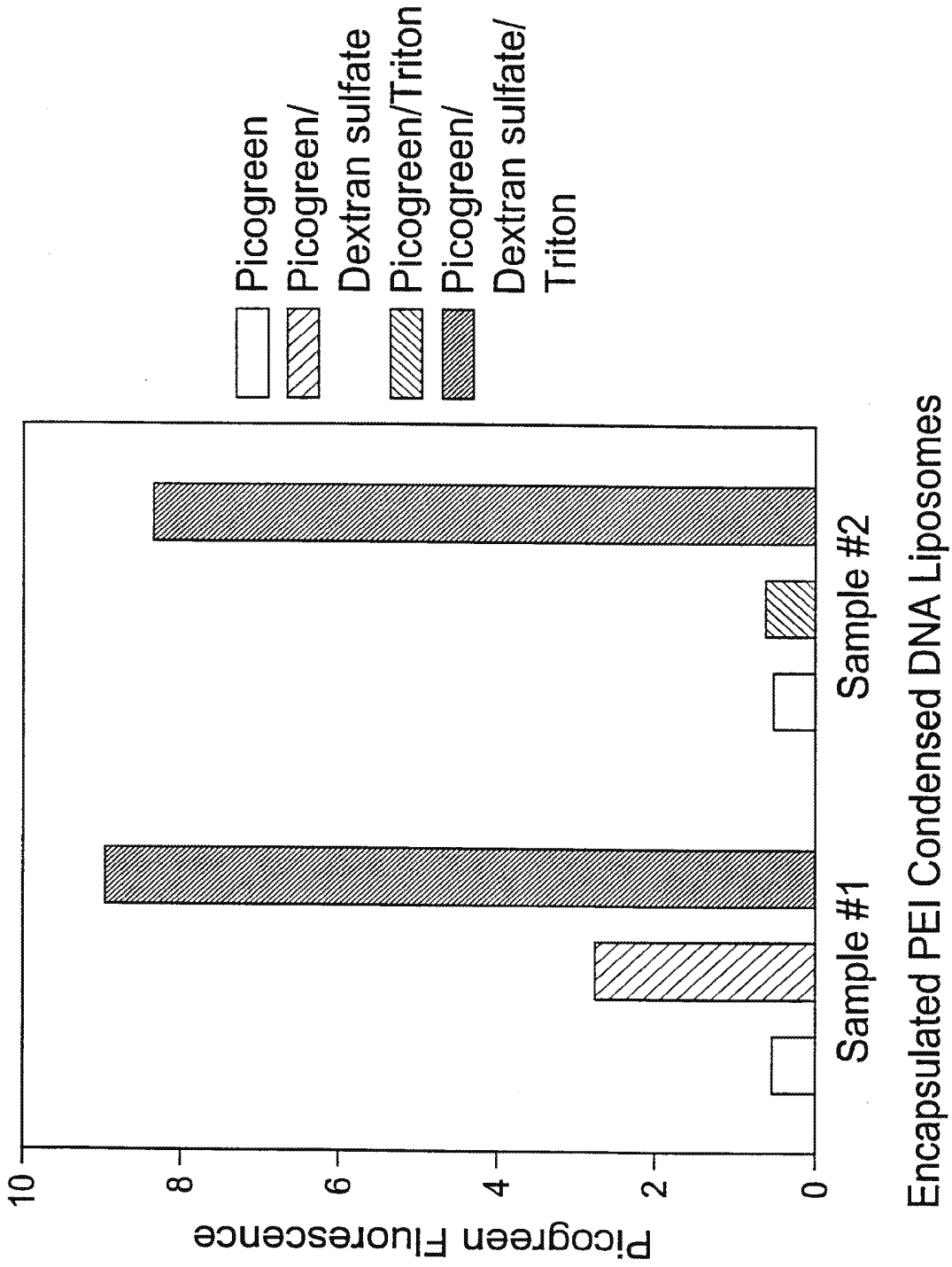

Picogreen
Picogreen/ Dextran sulfate
Picogreen/Triton
Picogreen/ Dextran sulfate/ Triton
Picogreen Fluorescence
0
2
4
6
8
10
Sample #1
Sample #2
Encapsulated PEI Condensed DNA Liposomes

Fig. 2

Fig. 3

Titration of Dextran Sulfate to Determine Threshold Amount Required to Unbind PEI Complexes

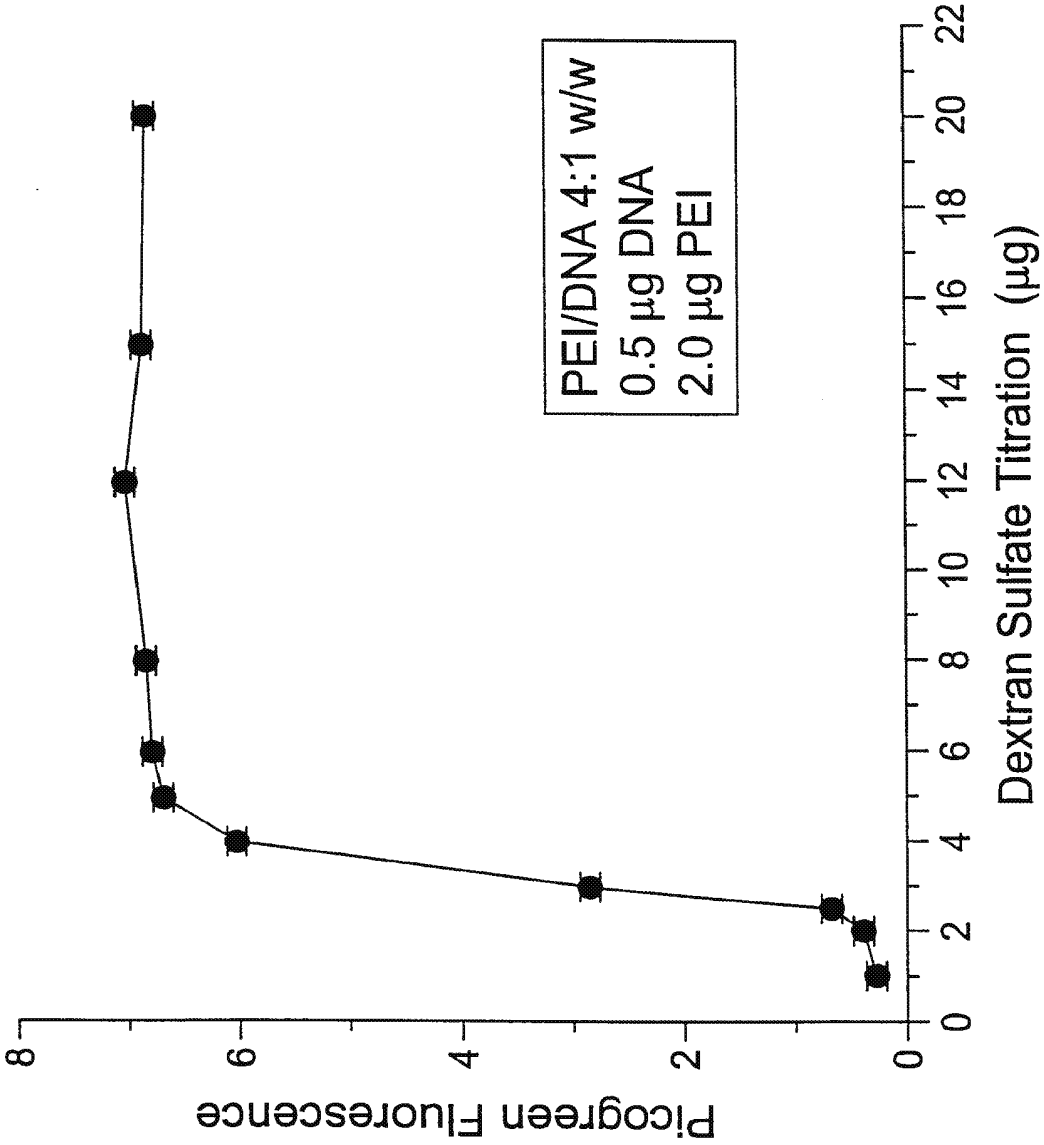


Fig. 4

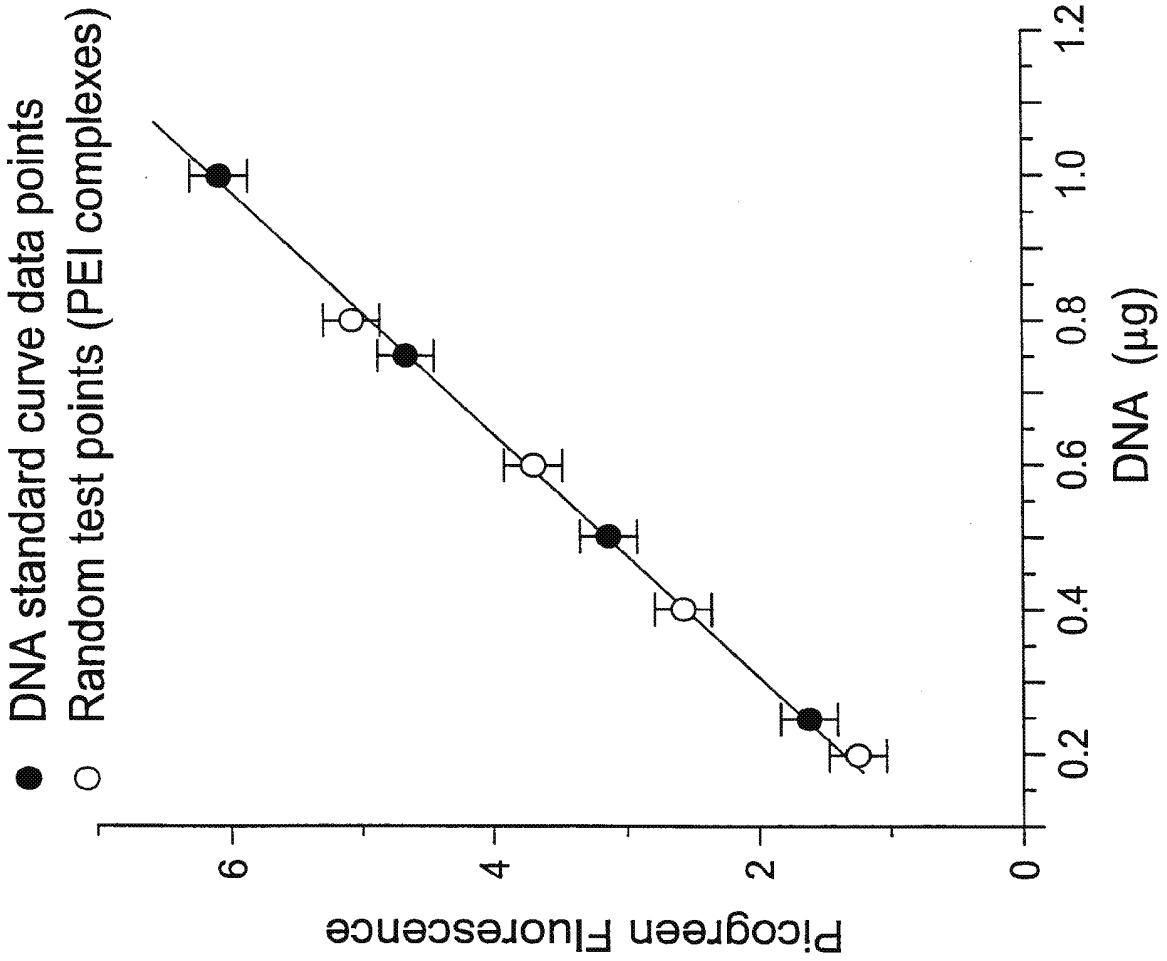
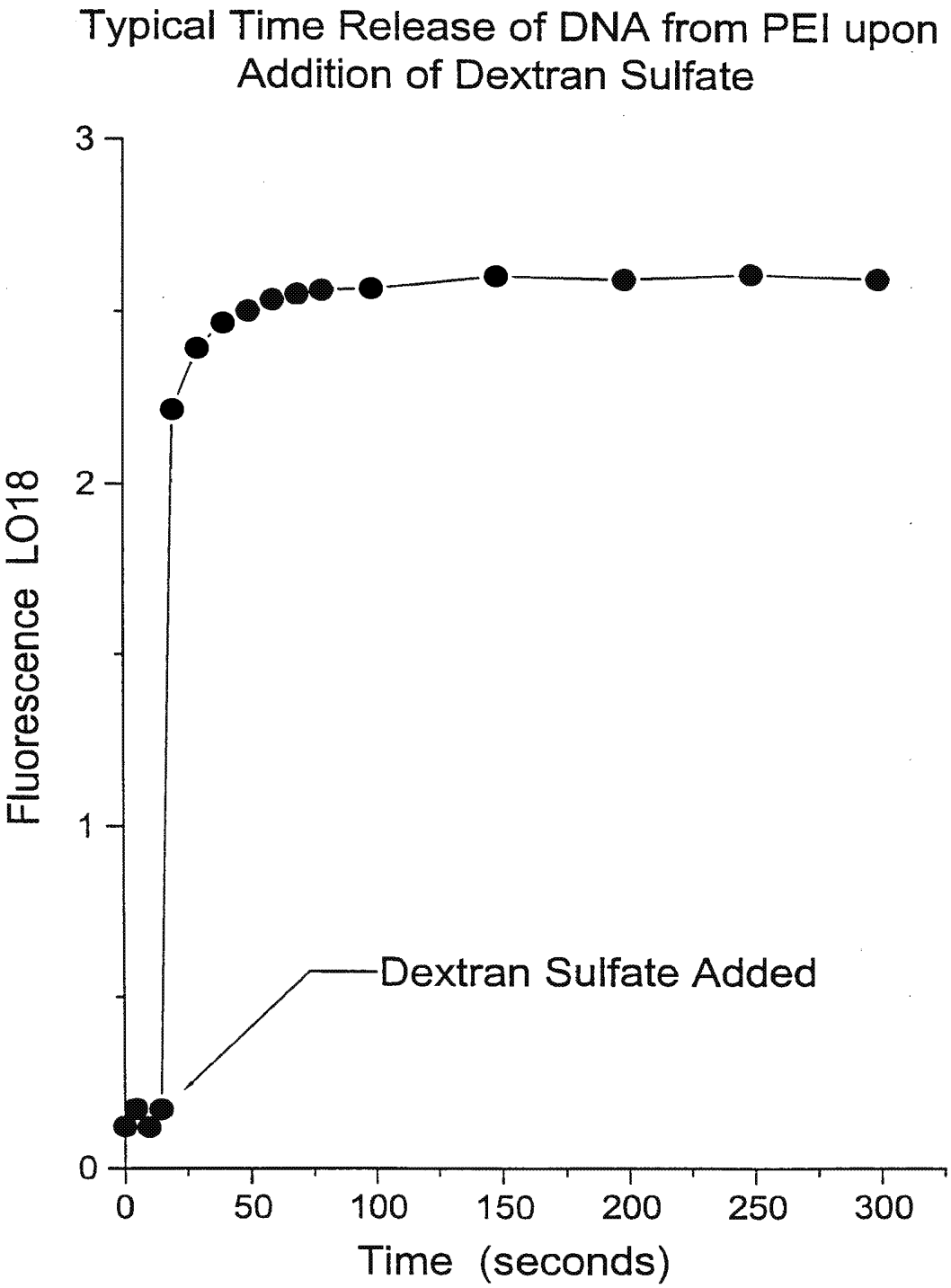

DNA standard curve data points
Random test points (PEI complexes)
Picogreen Fluorescence
0
2
4
6
0.2
0.4
0.6
0.8
1.0
1.2
DNA (µg)

Fig. 5


Typical Time Release of DNA from PEI upon Addition of Dextran Sulfate
Fluorescence LO18
0
1
2
3
0
50
100
150
200
250
300
Time (seconds)
Dextran Sulfate Added

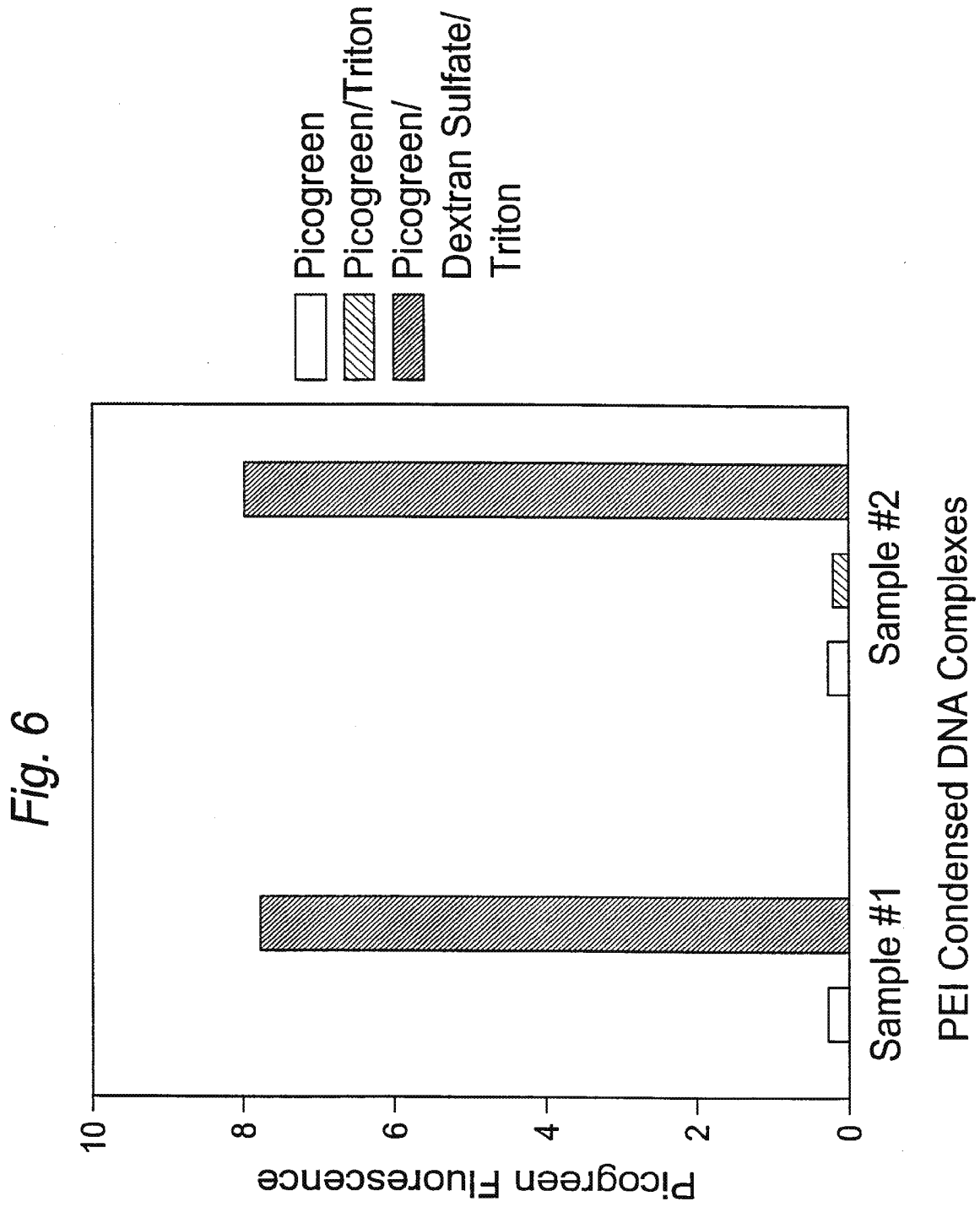

Picogreen
Picogreen/Triton
Picogreen/
Dextran Sulfate/
Triton
Picogreen Fluorescence
0
2
4
6
8
10
Sample #1
Sample #2
PEI Condensed DNA Complexes

Fig. 6

Fig. 7

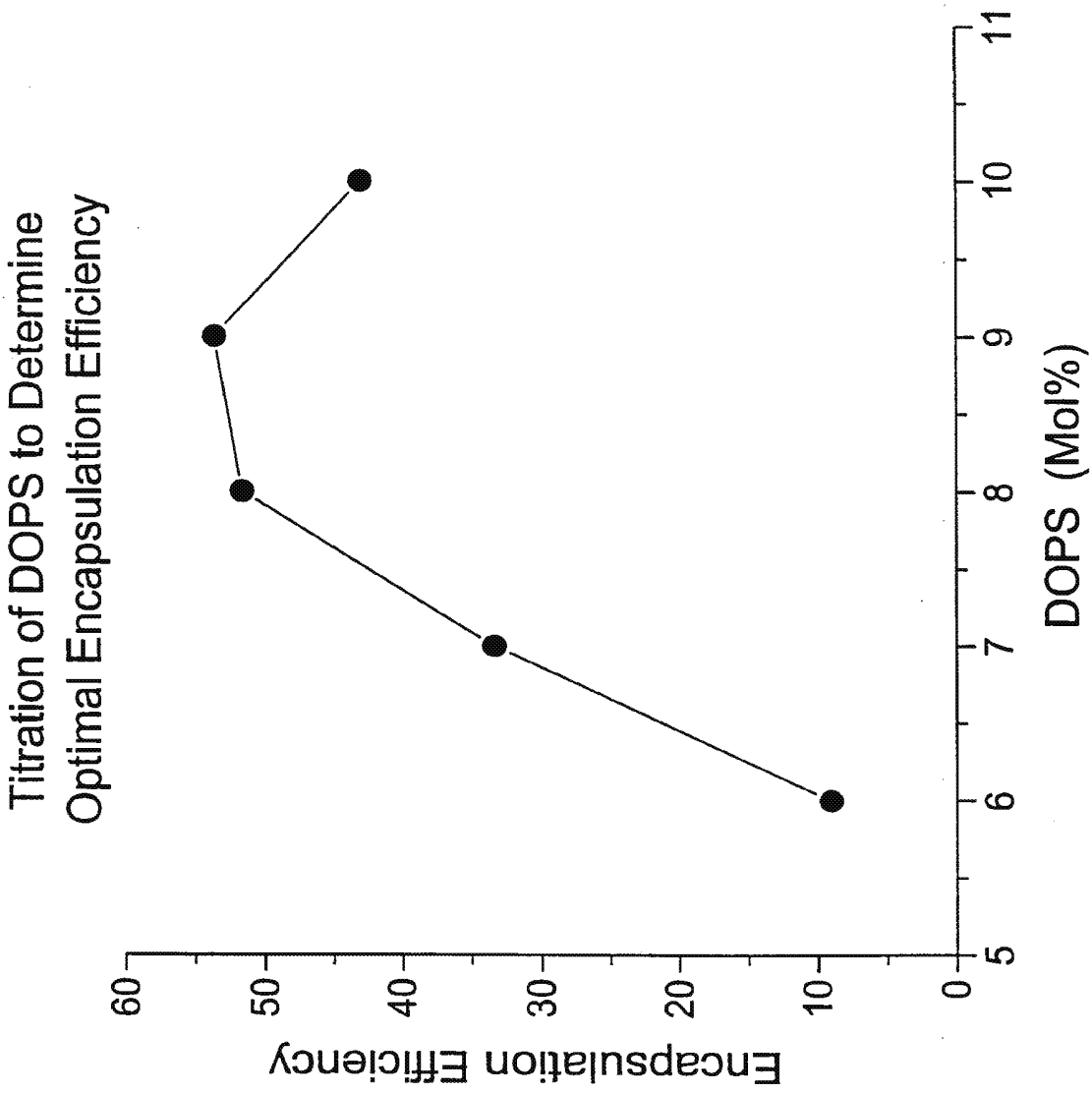

Titration of DOPS to Determine
Optimal Encapsulation Efficiency
Encapsulation Efficiency
0
10
20
30
40
50
60
5
6
7
8
9
10
11
DOPS (Mol%)

Fig. 8

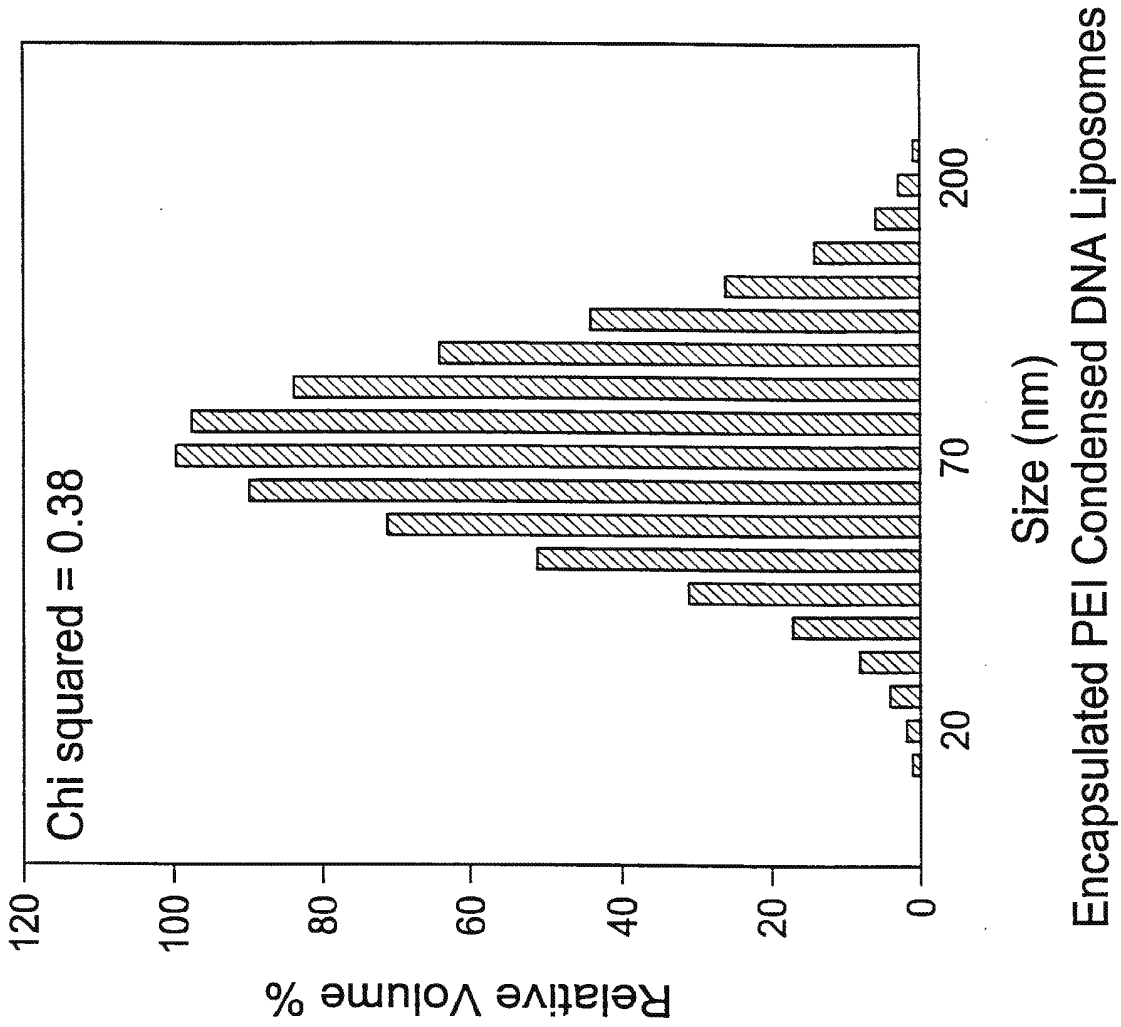

Encapsulated PEI Condensed DNA Liposomes
Chi squared = 0.38
Relative Volume %
120
100
80
60
40
20
0
20
70
200
Size (nm)

Fig. 9

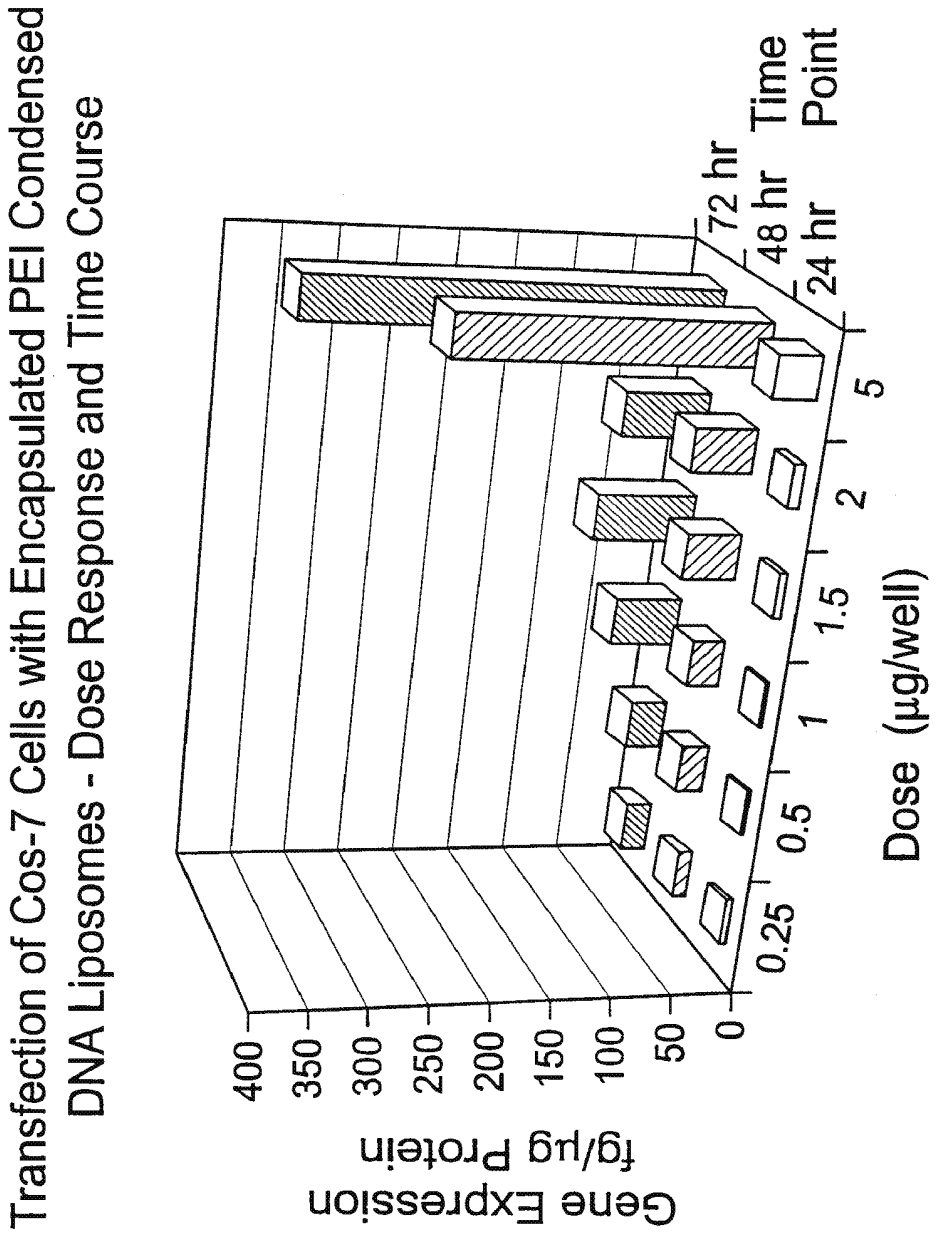


Fig. 10

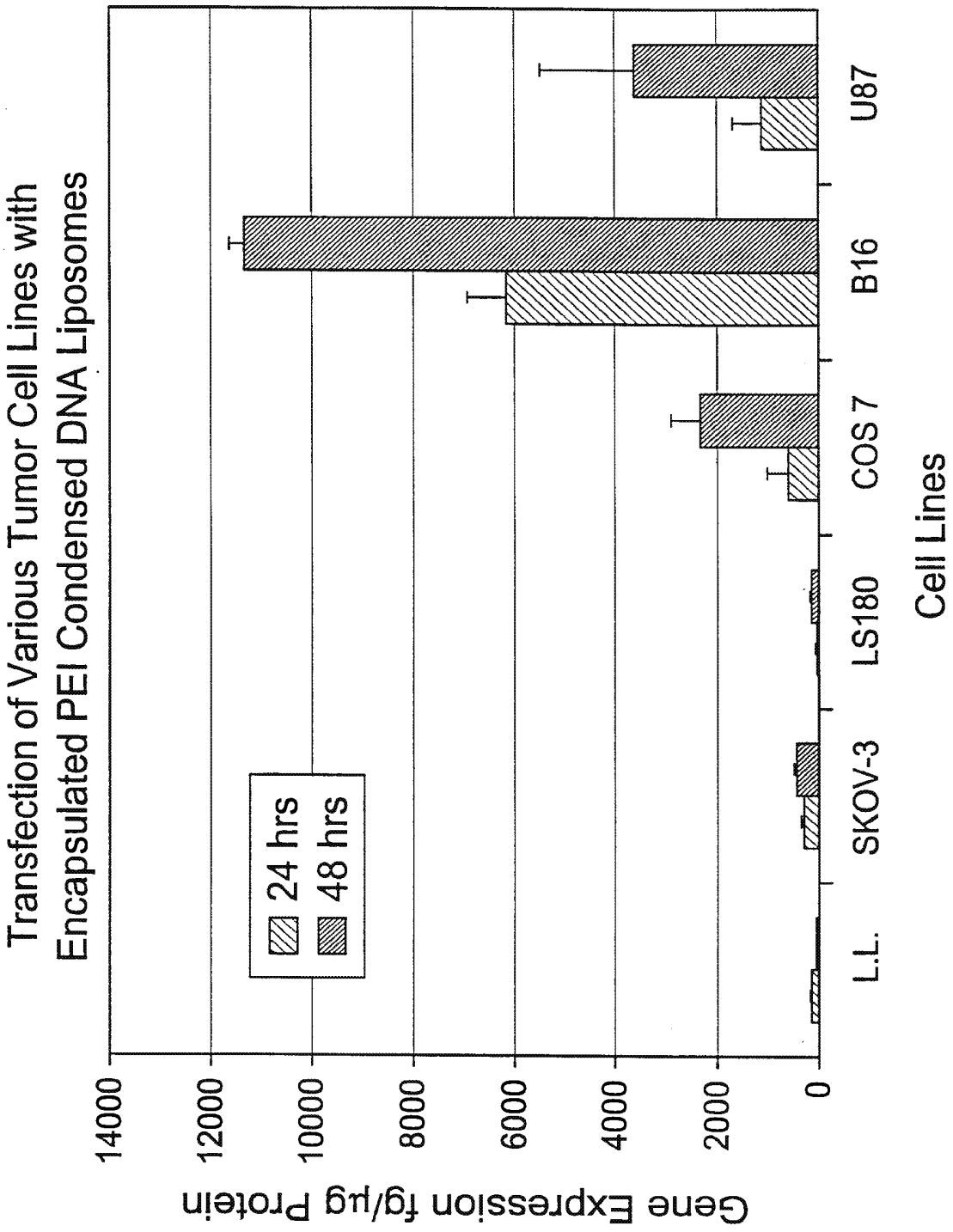

Transfection of Various Tumor Cell Lines with
Encapsulated PEI Condensed DNA Liposomes
Gene Expression fg/µg Protein
14000
12000
10000
8000
6000
4000
2000
0
24 hrs
48 hrs
L.L.
SKOV-3
LS180
COS 7
B16
U87
Cell Lines

Fig. 11

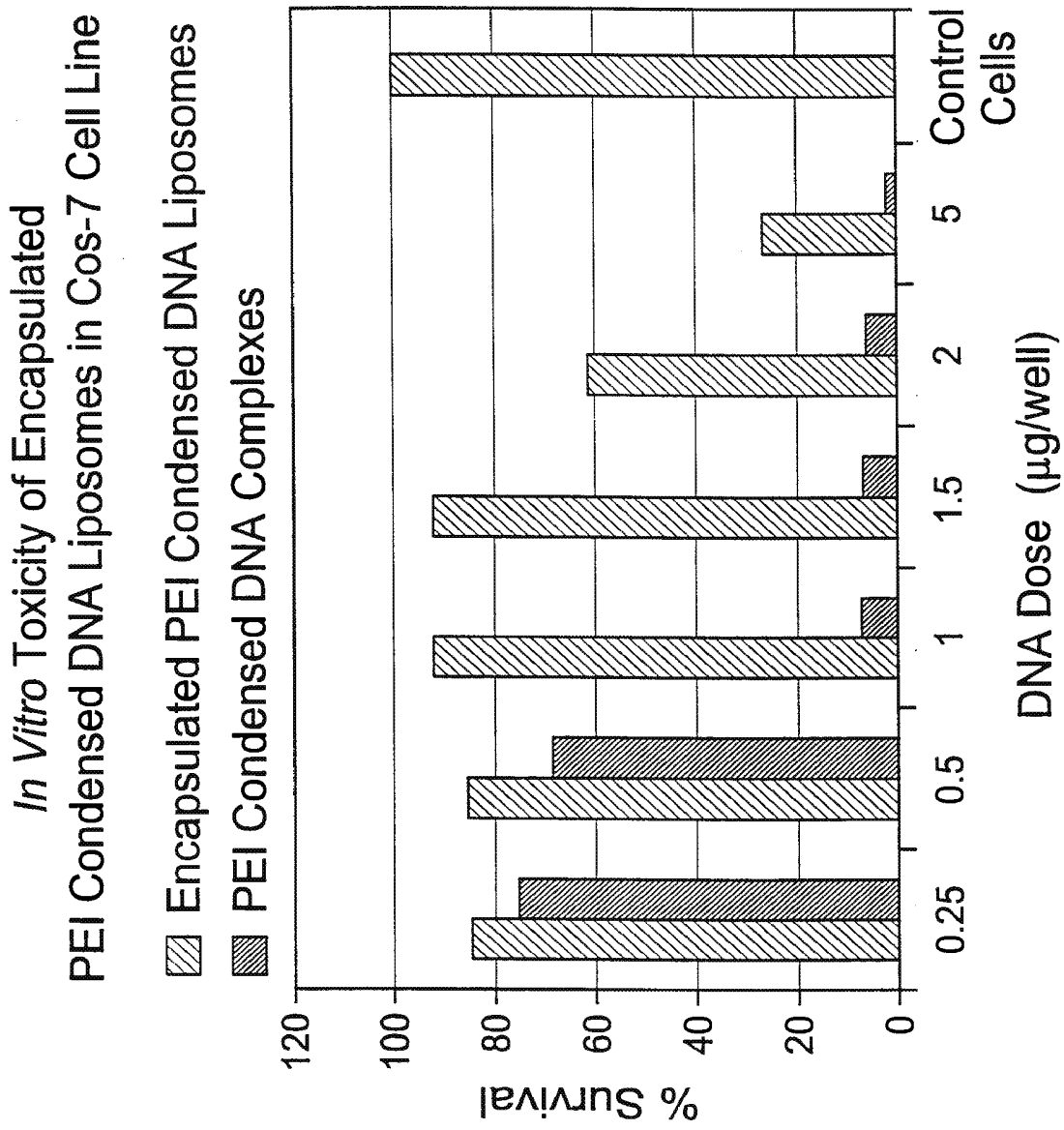

In Vitro Toxicity of Encapsulated
PEI Condensed DNA Liposomes in Cos-7 Cell Line
Encapsulated PEI Condensed DNA Liposomes
PEI Condensed DNA Complexes
% Survival
120
100
80
60
40
20
0
0.25
0.5
1
1.5
2
5
Control Cells
DNA Dose (µg/well)

Fig. 12

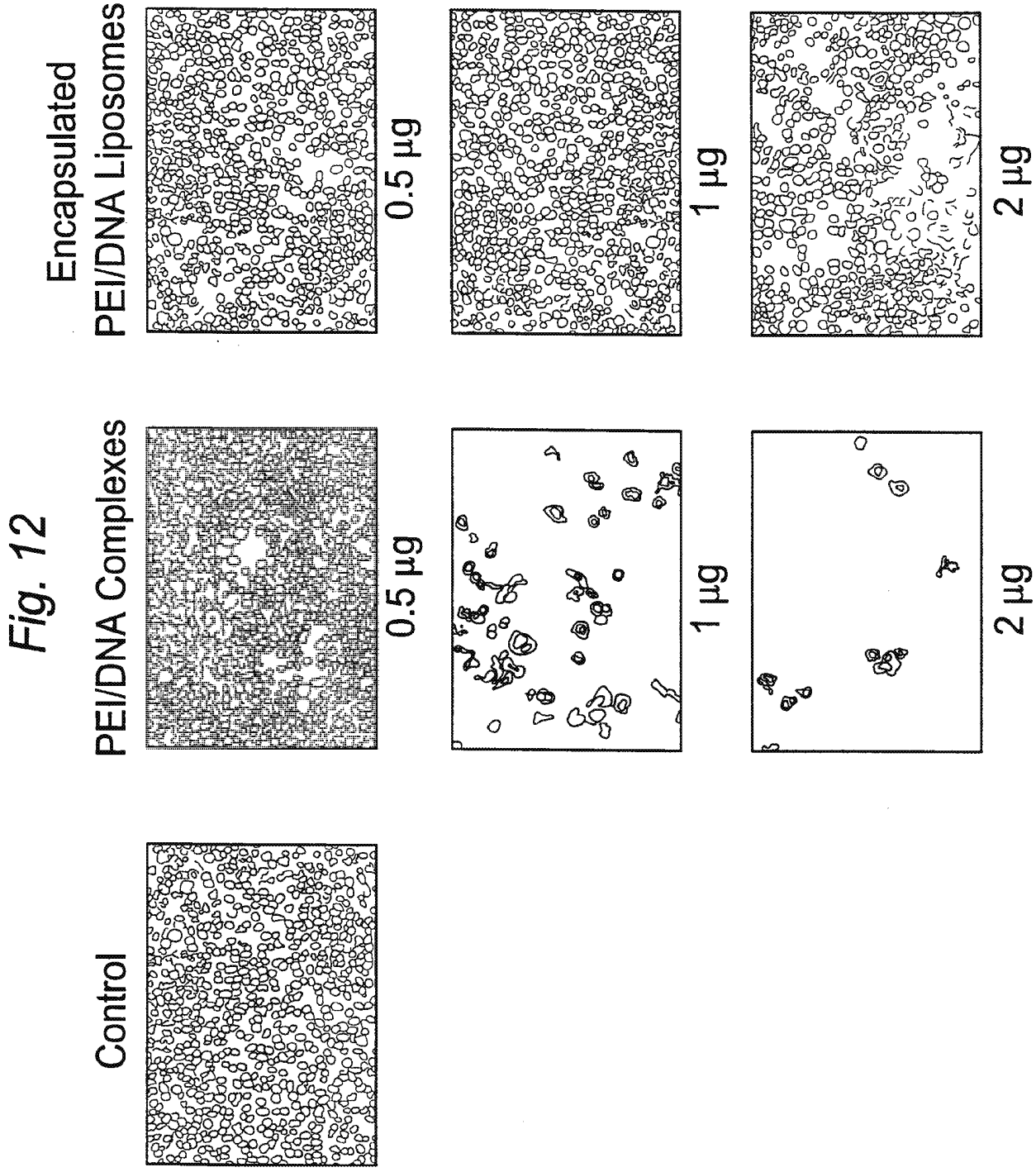

Encapsulated PEI/DNA Liposomes
0.5 µg
1 µg
2 µg
PEI/DNA Complexes
0.5 µg
1 µg
2 µg
Control

Fig. 13

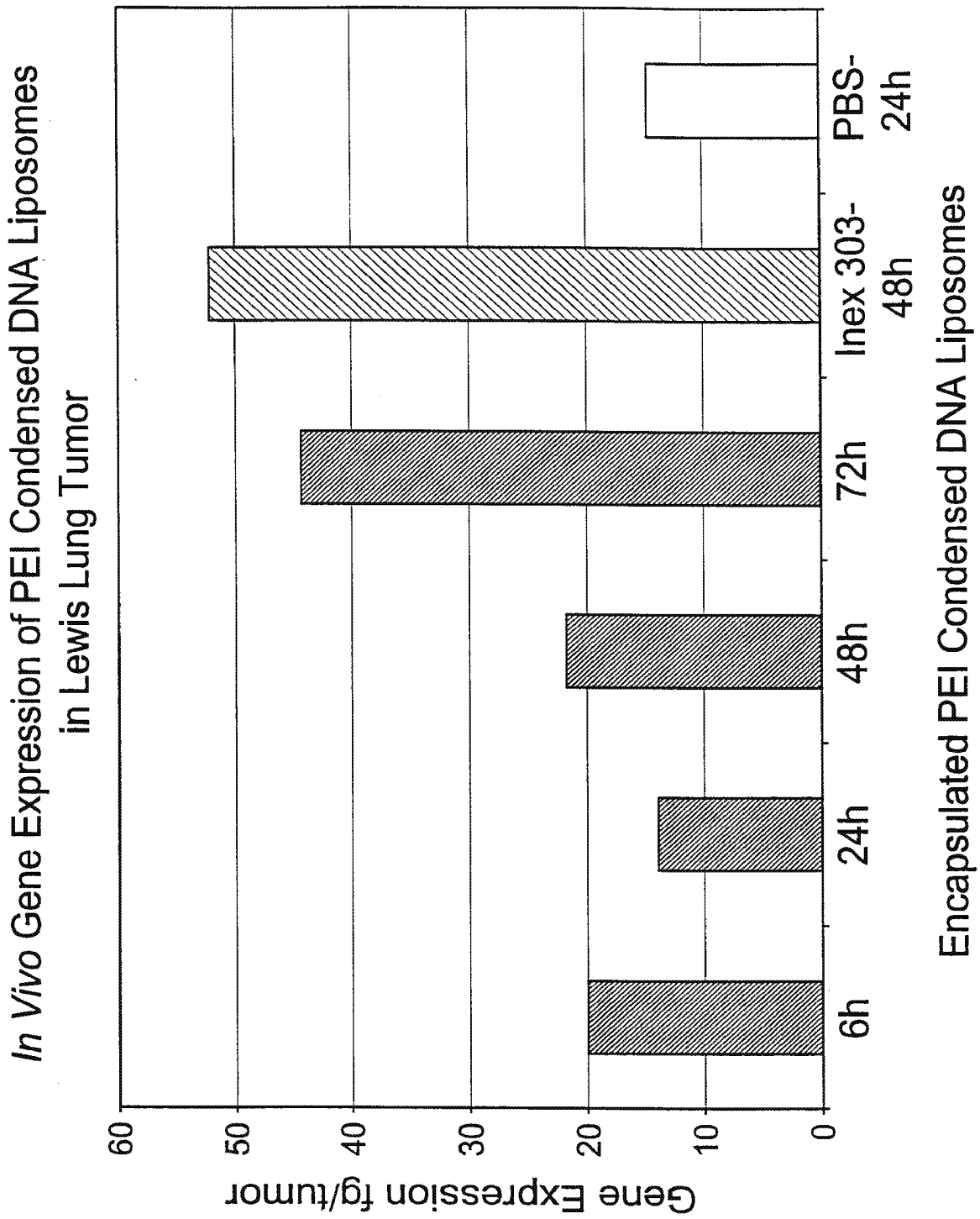

In Vivo Gene Expression of PEI Condensed DNA Liposomes
in Lewis Lung Tumor
Gene Expression fg/tumor
60
50
40
30
20
10
0
6h
24h
48h
72h
Inex 303-
48h
PBS-
24h
Encapsulated PEI Condensed DNA Liposomes

Fig. 14

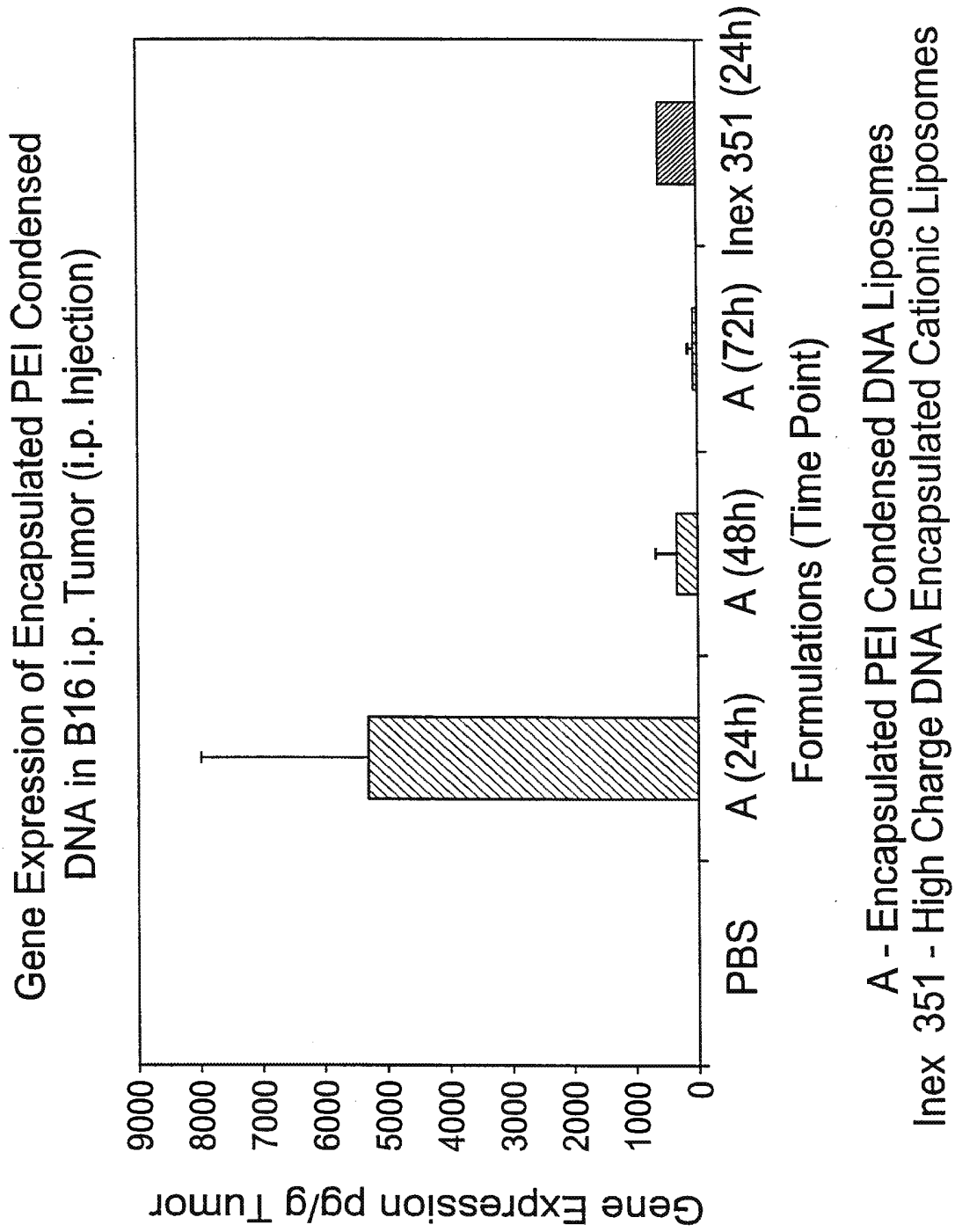
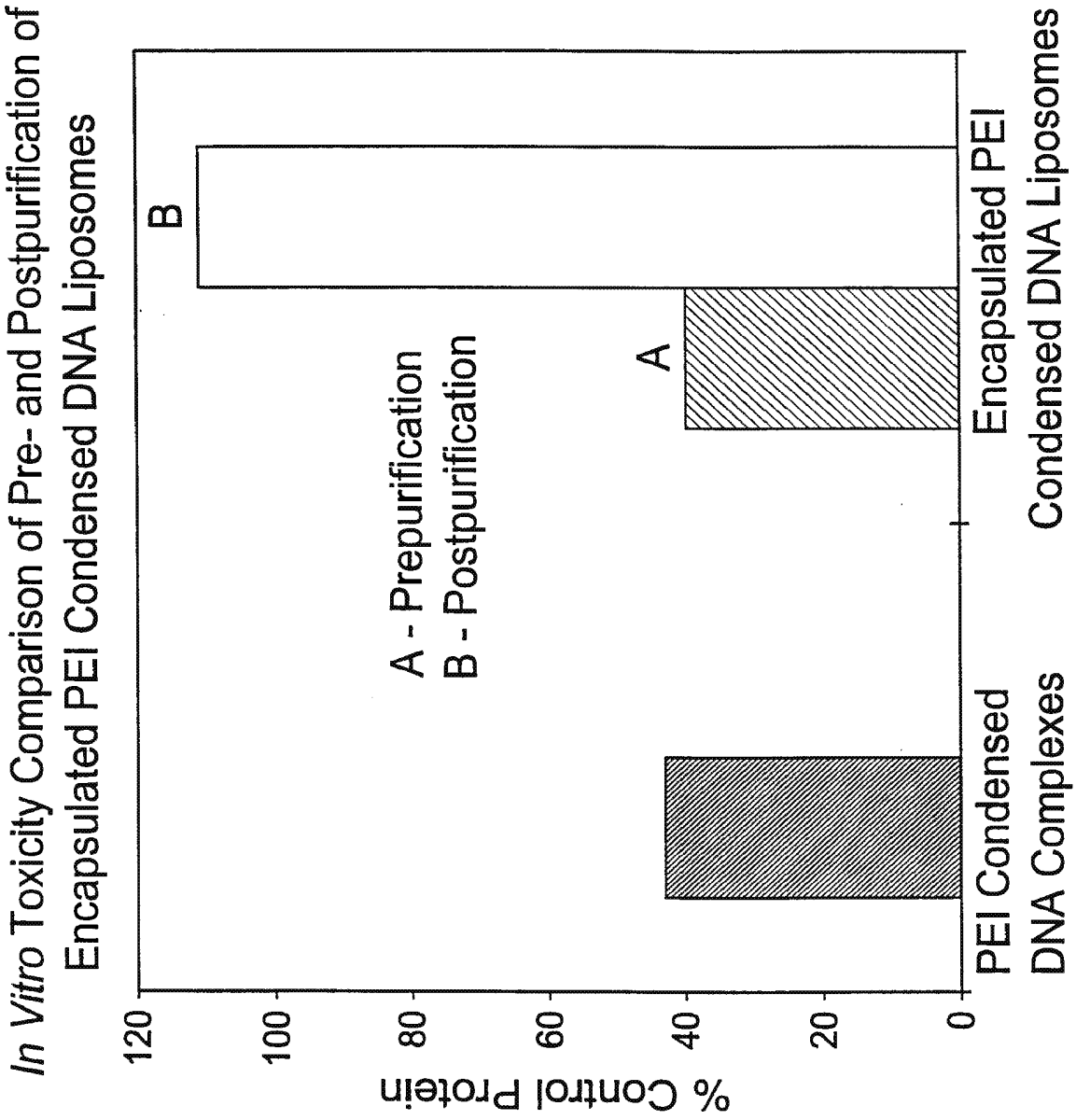


Fig. 15


In Vitro Toxicity Comparison of Pre- and Postpurification of Encapsulated PEI Condensed DNA Liposomes
% Control Protein
120
100
80
60
40
20
0
A - Prepurification
B - Postpurification
A
B
PEI Condensed DNA Complexes
Encapsulated PEI Condensed DNA Liposomes

JOINT APPENDIX 49

Gene Therapy (1999) 6, 271–281

http://www.stockton-press.co.uk/gt

Stabilized plasmid-lipid particles: construction and characterization

JJ Wheeler[1,2], L Palmer[3], M Ossanlou[1], I MacLachlan[1], RW Graham[1], YP Zhang[1], MJ Hope[1], P Scherrer[1] and PR Cullis[1,3]

[1]*Inex Pharmaceuticals Corporation, Burnaby; and* [3]*Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada*

A detergent dialysis procedure is described which allows encapsulation of plasmid DNA within a lipid envelope, where the resulting particle is stabilized in aqueous media by the presence of a poly(ethyleneglycol) (PEG) coating. These 'stabilized plasmid-lipid particles' (SPLP) exhibit an average size of 70 nm in diameter, contain one plasmid per particle and fully protect the encapsulated plasmid from digestion by serum nucleases and E. coli DNase I. Encapsulation is a sensitive function of cationic lipid content, with maximum entrapment observed at dioleoyldimethylammonium chloride (DODAC) contents of 5 to 10 mol%. The formulation process results in plasmid-trapping efficiencies of up to 70% and permits inclusion of 'fusigenic' lipids such as dioleoylphosphatidylethanolamine (DOPE). The in vitro transfection capabilities of SPLP are demonstrated to be strongly dependent on the length of the acyl chain contained in the ceramide group used to anchor the PEG polymer to the surface of the SPLP. Shorter acyl chain lengths result in a PEG coating which can dissociate from the SPLP surface, transforming the SPLP from a stable particle to a transfection-competent entity. It is suggested that SPLP may have utility as systemic gene delivery systems for gene therapy protocols.

Keywords: *plasmid encapsulation; nonviral gene delivery; intracellular delivery; gene therapy; liposomes*

Introduction

Currently available gene delivery systems for gene therapy protocols have limited utility for systemic applications. Viral systems, for example, are rapidly cleared from the circulation, limiting potential transfection sites to 'first-pass' organs such as the lungs, liver and spleen. In addition, these systems induce immune responses which compromise transfection resulting from subsequent injections. In the case of nonviral systems such as plasmid DNA–cationic lipid complexes (lipoplexes), the large size and positively charged character of these aggregates also result in rapid clearance, and the highest expression levels are again observed in first-pass organs, particularly the lungs.[1–4] Plasmid DNA–cationic lipid complexes can also result in toxic side-effects both *in vitro*[5] and *in vivo*.[6]

The need for a gene delivery system for treatment of systemic disease is obvious. For example, for cancer gene therapy there is a vital need to access metastatic disease sites, as well as primary tumors. Similar considerations apply to other systemic disorders, such as inflammatory diseases. The design features for lipid-based delivery systems that preferentially access such disease sites are increasingly clear. It is now generally recognized that preferential delivery of anticancer drugs to tumor sites following intravenous injection can be achieved by encapsulation of these drugs in large unilamellar vesicles (LUVs) exhibiting a small size (<100 nm diameter) and extended circulation lifetimes (circulation half-life in mice >5 h).[7–9] The accumulation of these drug delivery systems at disease sites, which includes sites of infection and inflammation as well as tumors, has been attributed to enhanced permeability of the local vasculature in diseased tissue.[10]

A gene delivery system containing an encapsulated plasmid for systemic applications should therefore be small (<100 nm diameter) and must exhibit extended circulation life-times to achieve enhanced delivery to disease sites. This requires a highly stable, serum-resistant plasmid-containing particle that does not interact with cells and other components of the vascular compartment. In order to maximize transfection after arrival at a disease site, however, the particle should interact readily with cells at the site, and should have the ability to destabilize cell membranes to promote intracellular delivery of the plasmid. In this work, we show that a straightforward detergent dialysis procedure can produce stabilized plasmid-lipid particles (SPLP) which satisfy the demands of plasmid encapsulation, small size and serum stability. Furthermore, we show that the transfection properties of these systems can be modulated by employing poly(ethyleneglycol) (PEG) coatings which can dissociate from the SPLP, transforming the particle from a stable particle to a transfection-competent entity.

Correspondence: P Scherrer, Inex Pharmaceuticals, 100–8900 Glenlyon Parkway, Burnaby, BC, Canada V5J 5J8
[2]*Present address: Kinetek Pharmaceuticals, 1779 West 75th Avenue, Vancouver, BC, V6P 6P2, Canada*
Received 24 April 1998; accepted 22 September 1998

Results

Entrapment of plasmid DNA into lipid particles by employing detergent dialysis

Previous work has shown that incubation of plasmid DNA with cationic lipids can result in a hydrophobic particle which is soluble in organic solvent.[11] It is of interest to determine whether this hydrophobic particle can be surrounded by an outer monolayer of lipid, which would then result in small, plasmid-containing particles stabilized in an aqueous medium. Detergent dialysis is a logical technique for achieving this, as the detergent may be expected to solubilize the hydrophobic plasmid DNA-cationic lipid particles. The addition of phospholipid and subsequent removal of detergent by dialysis could then result in the exchange of the solubilizing detergent with phospholipid, leaving particles which are stable in aqueous suspension.

Initial experiments employed the cationic lipid DODAC, the plasmid pCMVCAT, the non-ionic detergent octylglucopyranoside (OGP) and the bilayer-forming lipid palmitoyloleoylphosphatidylcholine (POPC). When DODAC was added to plasmid in distilled water, the formation of large (>1000 nm diameter) precipitates was observed. However, the subsequent addition of OGP (200 mm) resulted in solubilization of the precipitate, forming an optically clear suspension consistent with entrapment of hydrophobic plasmid DNA-cationic lipid particles within detergent micelles. This optically clear quality was maintained when POPC solubilized in OGP was added. However, during dialysis to substitute the detergent associated with the particles for POPC, extensive precipitation of the suspension was observed. A method to stabilize the plasmid-containing particles against aggregation and precipitation during the dialysis process was therefore required.

Previous studies have shown that a PEG coating can prevent aggregation of LUVs induced by covalent coupling of protein to the surface of the LUVs,[12] and can inhibit fusion between LUVs.[13] It was therefore of interest to determine whether the stabilizing properties of a PEG coating could prevent aggregation during dialysis. However, the use of the standard PEG-phosphatidylethanolamine (PEG-PE) was contraindicated because the PEG-PE molecule bears a net negative charge and could displace the cationic lipid from the plasmid, as has been noted for other negatively charged lipids.[14] As a result, PEG_{2000} was linked to ceramide as the hydrophobic anchor to produce a neutral molecule. Two ceramide anchors were synthesized which differed in the length of the ceramide acyl chain ($CerC_{14}$ and $CerC_{20}$). When 10 mol% PEG-$CerC_{20}$ was incorporated in the detergent mixture with POPC, DODAC and plasmid DNA, precipitation was not observed during detergent dialysis. Further, a proportion of the plasmid was encapsulated, as measured by recovery of DNA after elution on a DEAE-Sepharose CL-6B anion exchange column. As shown in Figure 1a, the encapsulation achieved is a sensitive function of the DODAC content, with encapsulation levels of 30% or higher at about 9% to 12% DODAC. It should be noted that addition of plasmid to preformed vesicles with the same lipid composition, followed by DEAE chromatography, resulted in complete plasmid retention on the column.

These results suggest that SPLP can be produced by detergent dialysis employing a POPC/DODAC/PEG-$CerC_{20}$ (79:11:10; mol:mol:mol) lipid mixture. However, it has been shown that when POPC is employed as a 'helper' lipid in plasmid DNA–cationic lipid complexes, very low transfection rates are observed, whereas when dioleoylphosphatidylethanolamine (DOPE) is present, much higher transfection rates are achieved.[15] The encapsulation properties of DOPE/DODAC/PEG-$CerC_{20}$ lipid mixtures were therefore investigated. As shown in Figure 1b, as the DODAC content was varied, an encapsulation profile for DOPE-containing systems similar to that obtained for the POPC-containing systems was

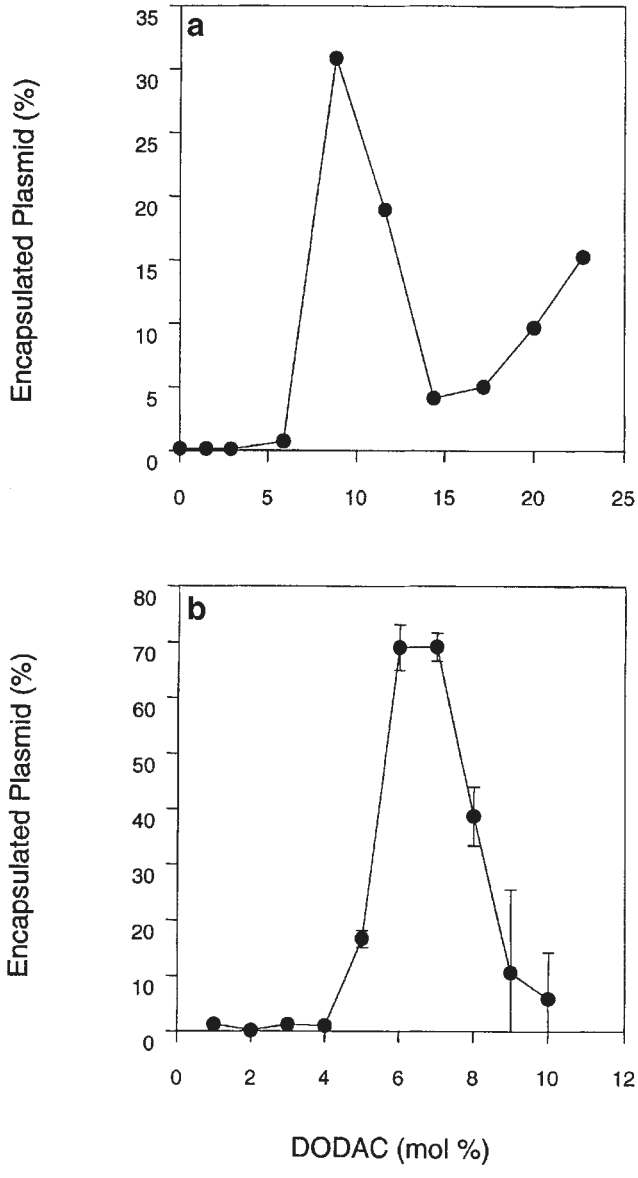


Figure 1 *Effect of DODAC concentration on the encapsulation efficiency of plasmid DNA (pCMVCAT) in SPLP. (a) Lipid composition POPC, DODAC and 10 mol% PEG-$CerC_{20}$. (b) Lipid composition DOPE, DODAC and 10 mol% PEG-$CerC_{20}$. Lipid (10 mg/ml total), dissolved in octylglucoside (0.2* m*), was mixed with plasmid DNA (50 μg/ml) in a total volume of 1 ml to form an optically clear solution. This was then placed in a dialysis tube (12–14 000 molecular weight cutoff) and dialyzed against HBS for 36 h at 20°C. Encapsulation efficiency was determined following removal of unencapsulated plasmid by anion exchange chromatography, as outlined in Materials and methods.*

observed. Significant differences are that maximum encapsulation was greater (approximately 70%) for the DOPE-containing system and that optimum encapsulation was observed at about 6 mol% DODAC, compared with approximately 9% DODAC for the POPC-containing particles. If PEG-CerC_{14} was substituted for PEG-CerC_{20} very similar plasmid encapsulation behavior was observed.

In subsequent experiments DOPE/DODAC/PEG-Cer formulations were employed containing 6 mol% DODAC. For this fixed DODAC content, some batch-to-batch variability of encapsulation efficiency (typically over the range 50–70%) was observed when different batches of plasmid were employed. This variability resulted from small changes (up to ±1 mol%) in the DODAC concentration required for maximum encapsulation efficiency for different plasmid batches. Other factors which may influence encapsulation efficiency include the amount of the plasmid present and the size of the plasmid. The plasmid (pCMVCAT) concentration was varied over the range 25 to 400 μg/ml employing the DOPE/DODAC/PEG-CerC_{20} (84:6:10; mol:mol:mol) lipid mixture at a fixed total lipid concentration of 10 mg/ml. Encapsulation efficiencies of more than 50% were achieved over this range (data not shown). In addition, at a plasmid concentration of 400 μg/ml, similar levels of entrapment were observed for plasmids of 4.49 and 10 kbp in length (data not shown).

It is important to show that the detergent dialysis process does not inhibit the transfection potential of the encapsulated plasmid. In order to test this, the plasmid was extracted from SPLP as described in Materials and methods. Characterization of the extracted DNA by agarose gel electrophoresis indicated no DNA degradation or plasmid relaxation relative to the starting material. Furthermore, the luciferase activity measured in cells following transfection (mediated by calcium phosphate) with plasmid extracted from SPLP was equivalent to the activity observed for plasmid which had not undergone encapsulation, with activities of 0.44 ± 0.15 ng and 0.35 ± 0.2 ng, respectively for 0.5 μg plasmid per well.

Plasmid DNA in stabilized plasmid-lipid particles is protected from DNase I and serum nucleases

It is important to demonstrate that the 'encapsulated' plasmid in the particles obtained by the detergent dialysis process is, in fact, fully protected from the external environment. As a first measure of protection, the ability of DNase I to digest plasmid DNA in DOPE-containing particles was examined. SPLP were prepared for the DOPE/DODAC/PEG-CerC_{20} (84:6:10; mol:mol:mol) lipid mixture and pCMVLuc (200 μg/ml). Protection of plasmid in the SPLP was compared to protection of plasmid in complexes with DODAC-DOPE (1:1; mol:mol) LUVs and to free plasmid. Samples containing 1 μg plasmid were exposed to 0, 100 and 1000 units of DNase I for 30 min at 37°C. After incubation the plasmid was isolated as described in Materials and methods and characterized by agarose gel electrophoresis. As shown in Figure 2, free plasmid is completely digested by incubation with both 100 and 1000 units of DNase I. The plasmid complexed with cationic LUVs is somewhat protected compared with free DNA when exposed to 100 units of DNase I, but is almost entirely digested by incubation with 1000 units. In contrast, plasmid DNA in the SPLP is digested only when detergent is added to disrupt the SPLP before incubation with DNase I.

A rigorous test of SPLP stability and protection of encapsulated plasmid involves incubation in serum. Serum contains a variety of nucleases, and serum proteins can rapidly associate with lipid systems,[16] resulting in enhanced leakage and rapid clearance of liposomal systems. The ability of serum nucleases to degrade plasmid is illustrated in Figure 3a. Intact pCMVCAT elutes in the void volume of the Sepharose CL-4B column, whereas after incubation with mouse serum (90%) at 37°C for 30 min the plasmid is degraded into fragments which elute in the included volume. The behavior of the DOPE/DODAC/PEG-CerC_{20} (84:6:10; mol:mol:mol) SPLP system where non-encapsulated plasmid has not been removed is shown in Figure 3b. In this particular preparation, 53% of the plasmid DNA elutes with the lipid in the void volume and 47% of the DNA, which

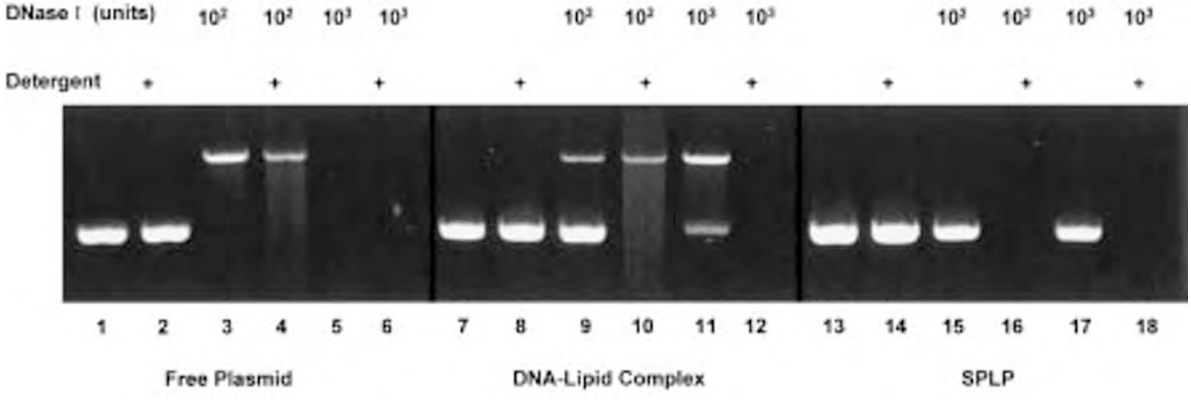


Figure 2 *Stability of free plasmid, plasmid encapsulated in SPLP and plasmid in plasmid DNA–cationic lipid complexes in the presence of DNase I. Each of the sample types was subjected to six different protocols, giving rise to six lanes for each sample. These protocols consisted of no exposure to DNase I or detergent (lanes 1, 7, 13), exposure to detergent alone (lanes 2, 8, 14), exposure to 100 and 1000 units of DNase I alone (lanes 3, 9, 15 with 100 units and lanes 5, 11, 17 with 1000 units) and exposure to both detergent and DNase I (lanes 4, 10, 16 with 100 units and lanes 6, 12, 18 with 1000 units). These experiments utilized 1 μg of plasmid DNA (pCMVLuc), 1% Triton X-100 and 100 or 1000 units of DNase I. These components were combined in a total volume of 100 μl of 5 mm HBS and 10 mm $MgCl_2$, and incubated for 30 min at 37°C before preparation for gel electrophoresis as outlined in Materials and methods. The plasmid DNA–cationic lipid complexes were prepared as indicated in Materials and methods and consisted of DODAC:DOPE (50:50; mol:mol) LUVs (100 nm diameter) complexed to plasmid at a 3:1 charge ratio (positive-to-negative).*

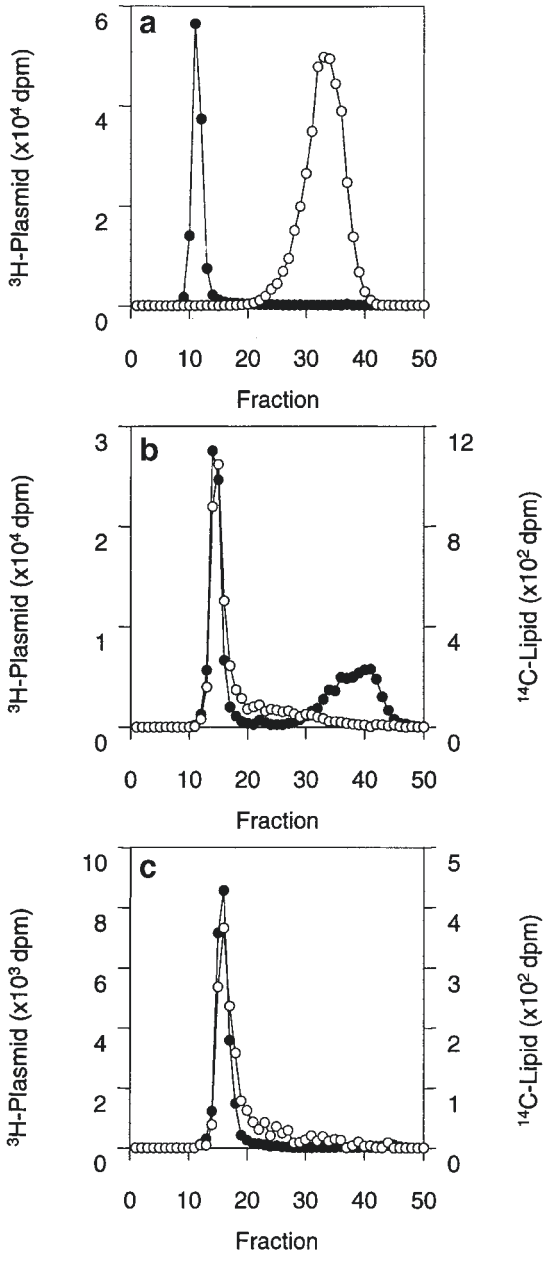


Figure 3 *Plasmid in SPLP is protected from serum nuclease cleavage. The stability of plasmid (pCMVCAT) in the free form or encapsulated in SPLP was determined in the presence of serum. The SPLP (DOPE/DODAC/PEG-CerC$_{20}$; 84:6:10; mol:mol:mol) were prepared as indicated in the legend to Figure 1 and contained ^{14}C-labeled CHE as a lipid marker. Samples with 5 μg of ^{3}H-labeled plasmid DNA were incubated in the presence of HBS or 90% mouse serum for 30 min at 37°C and eluted on a Sepharose CL-4B column equilibrated in HBS. (a) Elution profile of nucleic acid resulting from incubation of free plasmid in HBS (●) or 90 % mouse serum (○). (b) Elution profile of nucleic acid (●) and lipid (○) following incubation of SPLP in 90% mouse serum. (c) Elution profile of nucleic acid (●) and lipid (○) following incubation of SPLP with mouse serum where unencapsulated plasmid was removed by anion exchange chromatography before the serum treatment.*

represents degraded plasmid, elutes in the included volume. This indicates that 53% of the plasmid is encapsulated and protected from the external environment, in good agreement with a 55% trapping efficiency of this sample as determined by DEAE ion exchange chromatography.

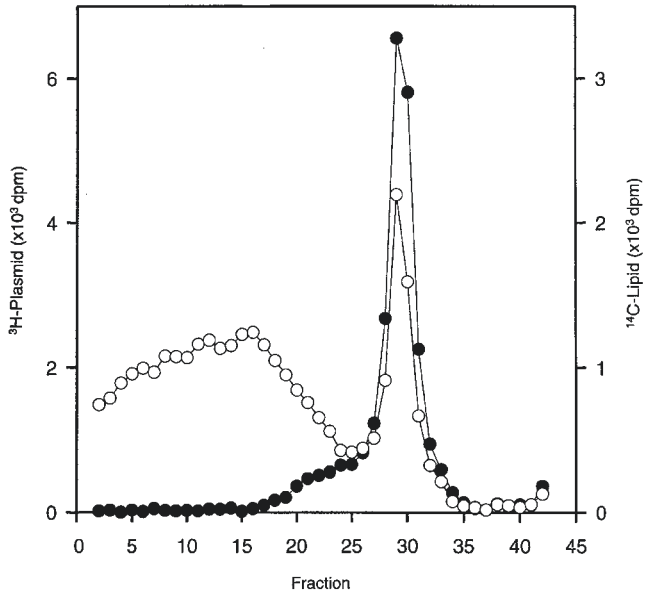


Figure 4 *Separation of SPLP from empty vesicles by discontinuous sucrose density gradient centrifugation. The solid circles indicate the behavior of the ^{3}H-labeled plasmid (pCMVLuc), whereas the open circles indicate the distribution of lipid as reported by the ^{14}C-labeled CHE lipid marker. SPLP (DOPE/DODAC/PEG-CerC$_{20}$; 84:6:10; mol:mol:mol) were prepared as indicated in the legend to Figure 1, and an aliquot (1.5 ml containing approximately 50 μg of ^{3}H-plasmid DNA) was applied to a discontinuous sucrose density gradient (3 ml 10% sucrose, 3 ml 2.5% sucrose, 3 ml 1% sucrose; all in HBS). The gradient was then centrifuged at 160 000 g for 2 h.*

A final test of the stability of the SPLP formulation is given in Figure 3c, which shows the elution profile of the DOPE / DODAC / PEG-CerC$_{20}$ (84:6:10; mol:mol:mol) SPLP system following removal of the external plasmid by DEAE chromatography and incubation in 90% mouse serum (30 min at 37°C). In this case more than 95% of plasmid applied to the column eluted in the void volume, demonstrating the stability and the plasmid protection properties of the SPLP formulation. It should also be noted that SPLP containing PEG-CerC$_{14}$, in place of PEG-CerC$_{20}$, exhibited similar plasmid protection properties.

Stabilized plasmid-lipid particles can be isolated by density centrifugation

The detergent dialysis process clearly results in plasmid-containing particles where the plasmid is protected from the external environment. However, it is likely that empty vesicles are also produced, as detergent dialysis of lipids (in the absence of plasmid) is well known to result in the formation of small lipid vesicles.[17] These empty vesicles may be expected to be less dense than SPLP. The density gradient profile of a DOPE / DODAC / PEG-CerC$_{20}$ (84:6:10; mol:mol:mol) SPLP preparation (plasmid-to-lipid ratio of 200 μg DNA to 10 mg lipid) was therefore examined employing sucrose density step gradient centrifugation. As shown in Figure 4, after centrifugation at 160 000 *g* for 2 h, the encapsulated DNA is present as a band which was localized at the 2.5% sucrose-10% sucrose interface in the step gradient. It is interesting to note that less than 10% of the total lipid (as assayed by the ^{3}H-CHE lipid marker) is associated with the plasmid DNA, which corresponds to 55% of the total DNA. The plasmid-to-lipid ratio in these

purified SPLP was determined (as indicated in Materials and methods) to be 62.5 μg plasmid per μmol lipid. It was found that SPLP generated by detergent dialysis and purified by density gradient centrifugation may be concentrated by dialysis against carboxymethyl cellulose to achieve plasmid concentrations of 1 mg/ml or higher.

Stabilized plasmid-lipid particles exhibit a narrow size distribution

The sizes of the empty lipid vesicles in the upper band and the isolated SPLP in the lower band of the sucrose density gradient were examined by quasi-elastic light scattering (QELS) and freeze–fracture electron microscopy techniques. As shown in Figure 5, the QELS analysis indicated that the mean diameter of the empty vesicles was approximately 44 nm ($\chi^2 = 0.48$), whereas the isolated SPLP were larger, with a mean diameter of 75 nm ($\chi^2 = 0.14$). Freeze–fracture electron microscopy studies gave similar results (Figure 6). A size analysis of the particles in these micrographs indicated a size of 36 ± 15 nm for the empty vesicles and 64 ± 9 nm for the isolated SPLP.

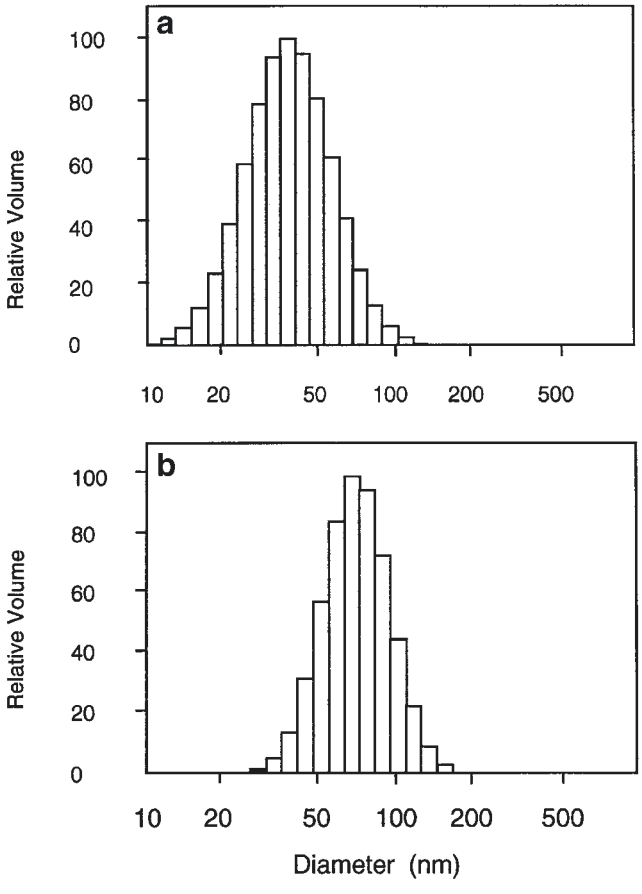


Figure 5 *Size distribution of SPLP and empty vesicles as determined by QELS. SPLP were prepared containing pCMVLuc as indicated in the legend to Figure 1, and separated from empty vesicles by discontinuous sucrose density gradient centrifugation. (a) Size distribution for empty vesicles (upper band). (b) Size distribution for SPLP (lower band). The sizes were determined by quasi-elastic light scattering using a Nicomp (Santa Barbara, CA, USA) model 370 sub-micron particle sizer operating in the solid particle mode.*

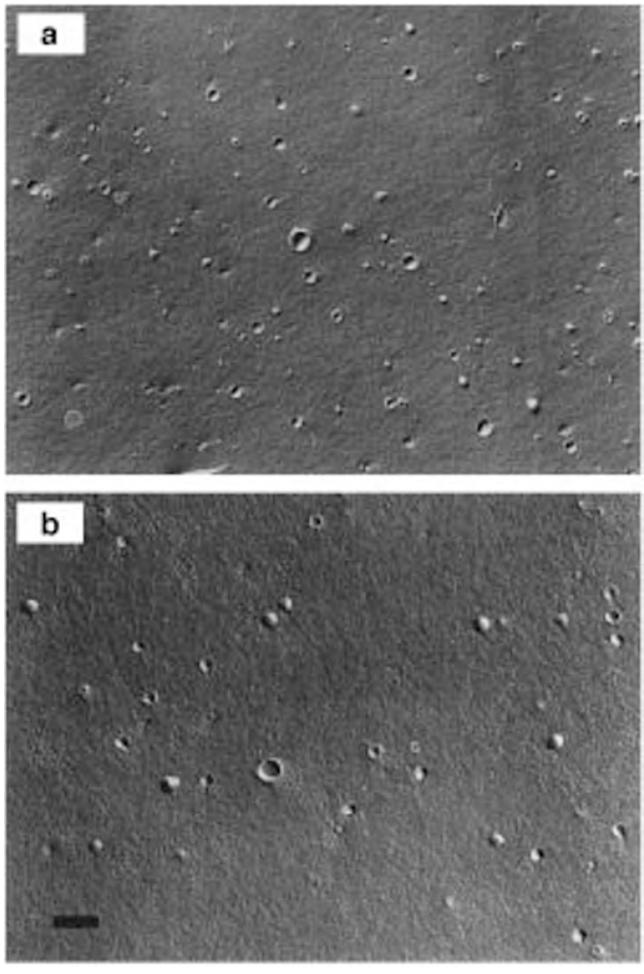


Figure 6 *Freeze–fracture electron microscopy of purified SPLP and empty vesicles. SPLP containing pCMVLuc were prepared as indicated in the legend to Figure 1 and separated into (a) empty vesicles and (b) SPLP employing discontinuous sucrose density gradient centrifugation. The bar indicates 200 nm. For details of sample preparation and electron microscopy, see Materials and methods.*

In vitro transfection properties of stabilized plasmid-lipid particles

SPLP consisting of DOPE/DODAC/PEG-CerC_{20} (84:6:10) containing pCMVLuc coding for the luciferase reporter gene were prepared for transfection studies. As shown in Figure 7, after incubation of these SPLP with COS-7 cells for 24 h, little if any transfection activity was observed. It is probable that the presence of the PEG coating on the SPLP inhibits the association and fusion of the SPLP with cells in the same manner that PEG coatings inhibit fusion between lipid vesicles,[13] and thus inhibit intracellular delivery of the encapsulated plasmid. In this regard, previous studies[13] on LUVs with PEG coatings attached to phosphatidylethanolamine (PE) anchors have demonstrated that, for PE anchors containing short acyl chains, the PEG-PE can rapidly exchange out of the LUV, rendering the LUVs increasingly able to interact and fuse with each other. The transfection properties of SPLP containing PEG-CerC_{20} were therefore compared to SPLP containing PEG-CerC_{14}, which has a shorter acyl chain. As shown in Figure 7, after incubation with COS-7 cells for 24 h, the SPLP containing PEG-CerC_{14} exhibits substantially higher levels of transfection compared with the

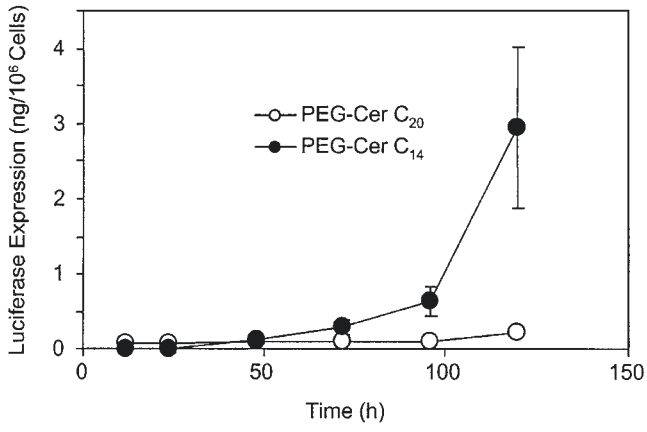


Figure 7 *Effect of PEG-Cer coating of SPLP on transfection activity in vitro. Plasmid (pCMVLuc) was encapsulated in SPLP (DOPE/DODAC/PEG-Cer; 84:6:10; mol/mol/mol) containing PEG-CerC$_{20}$ (○) or PEG-CerC$_{14}$ (●). Non-encapsulated plasmid was removed by anion exchange chromatography, as indicated in Materials and methods. The SPLP preparation (1 μg plasmid) was then added to COS-7 cells at a density of 2 x 10^4 per 24-well plate. The cells were incubated with the SPLP for the times indicated, and luciferase activity was measured as indicated in Materials and methods.*

system containing PEG-CerC$_{20}$. This is consistent with the ability of the PEG-CerC$_{14}$ coating to diffuse away from the SPLP surface. The SPLP containing either PEG-CerC$_{14}$ or PEG-CerC$_{20}$ exerted no apparent toxic effects on the cells as evaluated by monitoring protein content in the cell extract.

In order to determine whether the improved transfection properties of SPLP containing PEG-CerC$_{14}$ as compared with SPLP containing PEG-CerC$_{20}$ could be related to a faster dissociation rate from the SPLP surface, the dissociation rates at 37°C of radiolabeled PEG-CerC$_{14}$ and PEG-CerC$_{20}$ from 100 nm diameter large unilamellar vesicles (LUV) composed of egg phosphatidylcholine (EPC) were measured as indicated in Materials and methods. It should be noted that it is difficult to measure the dissociation rate of PEG-Cer from the surface of SPLP containing DOPE as the stability of these SPLP is dependent on the presence of the PEG-Cer coating. It was found that PEG-CerC$_{20}$ dissociated very slowly, with more than 90% remaining with the SPLP after 48 h incubation, corresponding to a half-time for dissociation of $t_{1/2} > 13$ days. In contrast, PEG-CerC$_{14}$ rapidly dissociated from the outer monolayer of the LUV with $t_{1/2} = 1.1 \pm 0.3$ h.

Discussion

This study presents a new method of encapsulating plasmid DNA in small, stable particulate systems that may find utility as gene delivery vehicles. Of particular interest are the relationship between properties of SPLP and other lipid-based systems containing plasmids, the structure of SPLP and the potential utility of SPLP with exchangeable PEG coatings. We discuss these areas in turn.

The SPLP protocol for plasmid entrapment allows trapping efficiencies of up to 70% and results in stable particles containing low levels of cationic lipids and high levels of fusigenic lipids, such as DOPE. These particles are small (<100 nm diameter), are resistant to external nucleases, exhibit high DNA-to-lipid ratios (62.5 μg/μmol) and can be concentrated to achieve high plasmid DNA concentrations (1 mg/ml). Furthermore, the detergent dialysis procedure is a gentle procedure that results in little, if any, plasmid degradation.

These features of SPLP contrast favorably with previous plasmid encapsulation procedures. Plasmid DNA has been encapsulated by a variety of methods, including reverse phase evaporation,[18–20] ether injection,[21,22] detergent dialysis in the absence of PEG stabilization,[20,21] lipid hydration and dehydration–rehydration techniques[25–27] and sonication,[28–30] among others. The characteristics of these protocols are summarized in Table 1. None of these procedures yields small, serum-stable particles at high plasmid concentrations and plasmid-to-lipid ratios in combination with high plasmid-encapsulation efficiencies. Trapping efficiencies comparable with the SPLP procedure can be achieved employing methods relying on sonication. However, sonication is a harsh technique which can shear nucleic acids.[31] Size ranges of 100 nm diameter or less can be achieved by reverse phase techniques; however, this requires an extrusion step through filters with 100 nm or smaller pore size which can often lead to significant loss of plasmid. Finally, it may be noted that the plasmid DNA-to-lipid ratios that can be achieved for SPLP are significantly higher than those achievable by any other encapsulation procedure.

With regard to the structure of SPLP, any model must take into account two important observations. First, SPLP form only at a critical cationic lipid content of approximately 6 mol%. At higher cationic lipid contents, aggregation is observed, whereas lower cationic lipid contents lead to little or no plasmid encapsulation. Second, purified SPLP exhibit a plasmid DNA-to-lipid ratio of 62.5 μg/μmol. For a 4.49 kbp (pCMVCAT) plasmid, this corresponds to a plasmid-to-particle ratio of 0.97 for an SPLP diameter of 70 nm (the average of the freeze–fracture electron microscopy and QELS results), assuming a lipid molecular area[32] of 0.67 nm^2 and an average nucleotide molecular weight of 330. It may therefore be concluded that SPLP contain one plasmid per particle.

The model that guided the construction of SPLP relied on the hypothesis that the plasmid combines with the cationic lipid to form a hydrophobic 'inverted micellar' structure that is stabilized in aqueous media by the detergent. In this model the addition of DOPE and PEG-Cer and subsequent dialysis results in deposition of a monolayer of DOPE and PEG-Cer around the hydrophobic intermediate, resulting in a stabilized plasmid-lipid particle. It is instructive to perform some simple calculations to see whether this model is consistent with experimental observations. In particular, if each negative charge on the plasmid has a cationic lipid associated with it, the total volume of each hydrophobic plasmid-cationic lipid particle can be calculated to be approximately 1.35 x 10^4 nm^3 for a 4.49 kbp plasmid. This calculation assumes that plasmid DNA has a density of 1.7 g/ml, the molecular weight of each base is 330, and that, as an upper limit, the volume per molecule of the cationic lipid is 1.5 nm^3, which is the volume of a liquid crystalline bilayer-forming lipid such as dioleoylphosphatidylcholine (lipid length 2.2 nm and area per molecule 0.67 nm^2).[32] Thus, if each SPLP contained one pCMVCAT plasmid completely neutralized by associated cationic lipid and arranged in a spherical conformation, the predicted diameter would

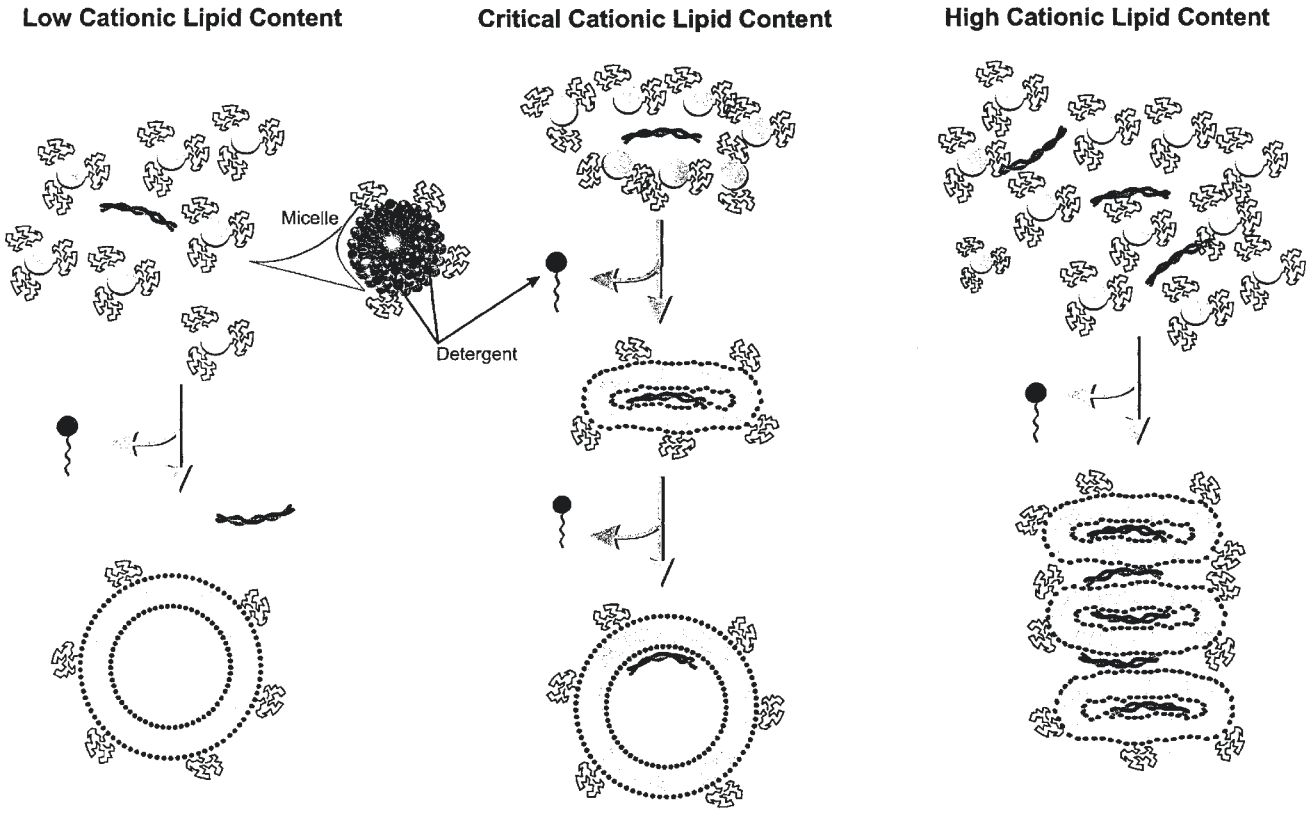


Figure 8 *Model of the formation and possible structure of SPLP. The first stage of dialysis is proposed to result in formation of macromolecular lipid intermediates, which may be in the form of lamellar sheets, cylindrical micelles or leaky vesicles.*[33,34] *If the cationic lipid content is too low (left panel), plasmid does not associate with these intermediates as dialysis proceeds, leading to formation of empty vesicles and free plasmid. At higher cationic lipid contents plasmid associates with the lipid intermediates, drawn here as a bilayer sheet wrapped around the plasmid. If the cationic lipid content is at a critical level the presence of the plasmid reduces the net positive surface charge of the lipid intermediate to the extent that further association of plasmid is inhibited. As dialysis proceeds further, additional lipid would be expected to condense on this structure, leading to formation of a vesicle containing encapsulated plasmid, as indicated. In addition, empty vesicles and free plasmid would be expected. At high cationic lipid contents (right panel), the surface charge on the lipid intermediate structures is so high that two or more plasmids can associate with a given membrane sheet, leading to the formation of large aggregates.*

be approximately 30 nm. The freeze–fracture electron microscopy results presented here indicate that SPLP containing the pCMVCAT plasmid exhibit a diameter of approximately 70 nm, and are therefore too large to be composed solely of a plasmid-lipid particle with no interior aqueous volume.

An alternative working model for SPLP formation and structure is shown in Figure 8. It is unlikely that plasmid associates directly with the micelles, as the presence of high levels of detergent may be expected to dilute the positive surface charge due to the cationic lipid to the extent that electrostatic association is reduced. A probable first step of the dialysis process is the formation of macromolecular lipid intermediates, which may include cylindrical micelles, lamellar sheets or leaky vesicles that form as detergent is removed. These structures have been observed as intermediates in the micelle to vesicle transition undergone by dispersions of egg phosphatidylcholine as detergent (OGP) is removed by dialysis.[33,34] These structures are represented in Figure 8 as lamellar sheets by way of example. As shown in the left panel of Figure 8, low concentrations of cationic lipid would result in little association of plasmid with these intermediate structures, which is consistent with little or no plasmid entrapment following detergent dialysis. At high concentrations of cationic lipid, intermediate structures may be expected to associate with the plasmid and, if the cationic lipid content is too high, plasmid-lipid-plasmid association could dominate as dialysis proceeds, leading to formation of aggregates (Figure 8, right panel).

If the cationic lipid content is at a critical level (Figure 8, central panel), the positive surface charge on the plasmid-associated intermediates will be reduced below that needed to associate with other plasmids, due to charge neutralization. This would mitigate against further aggregation. Further dialysis will result in fusion between intermediates eventually to produce empty vesicles or in fusion between intermediates and the plasmid-lipid particle. Fusion with the particle will result in the deposition of excess bilayer lipid, leading to the formation of an associated vesicle in the final SPLP. In the structure presented, the plasmid is associated with the inner monolayer of the vesicle that is produced as more lipid is deposited in the particle. It should be noted that the forces driving a partial removal of the plasmid lipid coat are not clear, and it is possible that the plasmid resides in a hydrophobic domain inside the particle.

The final area of discussion concerns the potential util-

Table 1 Procedures for encapsulating plasmid in lipid-based systems

Procedure	*Lipid composition*	*Length of DNA*	*Trapping efficiency*[a]	*DNA-to-lipid-ratio*[a]	*Diameter*
Reverse phase evaporation[18]	PS or PS:Chol (50:50)	SV40 DNA	30–50%	<4.2 μg/μmol	400 nm
Reverse phase evaporation[19]	PC:PS:Chol (40:10:50)	11.9 kbp plasmid	13–16%	0.23 μg/μmol	100 nm to 1 μm
Reverse phase evaporation[20]	PC:PS:Chol (50:10:40)	8.3 kbp, 14.2 kbp plasmid	10%	0.97 μg/μmol	ND
Reverse phase evaporation[41]	EPC:PS:Chol (40:10:50)	3.9 kbp plasmid	12%	0.38 μg/μmol	400 nm
Ether injection[21]	EPC:EPG (91:9)	3.9 kbp plasmid	2–6%	<1 μg/μmol	0.1–1.5 μm;
Ether injection[22]	PC:PS:Chol (40:10:50) PC:PG:Chol (40:10:50)	3.9 kbp plasmid	15%	15 μg/μmol	ND
Detergent dialysis[23]	EPC:Chol:stearylamine (43.5:5:43.5:13)	sonicated genomic DNA (250 000 mw)	11%	0.26 μg/μmol	50 nm
Detergent dialysis, extrusion[24]	DOPC:Chol:oleic acid or DOPE:Chol:oleic acid (40:40:20)	4.6 kbp plasmid	14–17%	2.25 μg/μmol	180 nm (DOPC) 290 nm (DOPE)
Lipid hydration[25]	EPC:Chol (65:35) or EPC	3.9 kbp, 13 kbp plasmid	ND	ND	0.5–7.5 μm
Dehydration–rehydration, extrusion (400 or 200 nm filters)[26]	Chol:EPC:PS (50:40:10)	ND	ND	0.83 μg/μmol (200 nm) 1.97 μg/μmol (400 nm)	142.5 nm (200 nm filter) 54.6 nm (400 nm filter, ultracentrifugation)
Dehydration–rehydration[27]	EPC	2.96 kbp, 7.25 kbp plasmid	35–40%	2.65–3.0 μg/μmol	1–2 μm
Sonication (in the presence of lysozyme)[28]	asolectin (soybean phospholipids)	1.0 kbp linear DNA	50%	0.08 μg/μmol	100–200 nm
Sonication[29]	EPC:Chol:lysine-DPPE (55:30:15)	6.3 kb ssDNA 1.0 kb dsRNA	60–95% ssDNA 80–90% dsRNA	13 μg/μmol ssDNA; 14 μg/μmol dsRNA	100–150 nm
Spermidine-condensed DNA, sonication, extrusion[30]	EPC:Chol:PS (40:50:10) EPC:Chol:EPA (40:50:10) or EPC:Chol:CL (50:40:10)	4.4 kbp, 7.2 kbp plasmid	46–52%	2.53–2.87 μg/μmol	400–500 nm
Ca^{2+}-EDTA entrapment of DNA–protein complexes[42]	PS:Chol (50:50)	42.1 kbp bacteriophage	52–59%	22 μg/μmol	ND
Freeze–thaw, extrusion[43]	POPC:DDAB (99:1)	3.4 kbp linear plasmid	17–50%	ND	80–120 nm
SPLP (this work)	DOPE:PEG-Cer:DODAC (84:10:6)	4.4–10 kbp plasmid	60–70%	62.5 μg/μmol	75 nm (QELS); 65 nm (freeze–fracture)

[a]Some values calculated based on presented data.
[b]ND, not determined.

ity of SPLP with exchangeable PEG coatings. As previously indicated, the SPLP system has been designed for systemic (intravenous) gene therapy applications. This places two potentially conflicting demands on the delivery system. First, the carrier must circulate long enough to achieve accumulation at disease sites, such as tumors, by taking advantage of the increased vascular permeability in these regions. Second, the carrier must be able to bind to target cells and to destabilize the plasma or endosomal membrane after arrival at the disease site in order to facilitate intracellular delivery of the enclosed plasmid. The first requirement implies a very stable carrier that does not interact with cells, whereas the second requirement necessitates a particle that can bind to cells and exhibit a membrane-destabilizing 'fusigenic' character.

PEG coatings that can dissociate from a carrier provide a potential solution to these demands. First, the presence of a PEG coating allows SPLP to be formed with a large proportion of DOPE in the outer monolayer. Previous work has shown that DOPE prefers the (non-bilayer) hexagonal H_{II} phase at temperatures above 10°C,[35] and that PEG lipids can stabilize DOPE in the bilayer organization.[36] Thus in the absence of the PEG-Cer the SPLP would be expected to be highly unstable and fusigenic. The detergent dialysis procedure therefore allows an intrinsically fusigenic plasmid-containing particle to be formed, where the stability of the particle is dependent on the presence of the PEG coating. As demonstrated here, these particles are stable in the presence of DNase I, as well as serum nucleases, consistent with an ability to protect encapsulated DNA in the circulation. In addition, the small size and presence of the PEG coating would be expected to promote the extended circulation life-times required to achieve preferential accumulation at disease sites such as tumors following intravenous administration.

The stability of the SPLP would, however, be expected to mitigate against uptake and intracellular delivery of the plasmid. The use of PEG coatings that dissociate from the SPLP after arrival at a disease site provides a potential solution to this problem. This is supported by the *in vitro* results presented here, which show that a PEG-$CerC_{20}$ coating, which has a long residence time in lipid bilayers, exhibits poor transfection properties, whereas improved transfection is observed for the SPLP containing a PEG-$CerC_{14}$ coating, which can dissociate from lipid bilayers more rapidly.

In summary, this study presents a method for encapsulating plasmid DNA in particulate systems that have the properties of small size, high plasmid-to-lipid ratio and high content of fusigenic lipid, and that can be concentrated to achieve high plasmid concentrations. These SPLP are stabilized by the presence of a PEG coating that can be designed to dissociate, thus increasing the transfection potency of the SPLP. It is expected that these systems will find utility as delivery systems for systemic gene therapy.

Materials and methods

Materials

Dioleoylphosphatidylethanolamine (DOPE) was obtained from Northern Lipids (Vancouver, BC, Canada). The lipids 1-O-(2′-(ω-methoxypolyethyleneglycol) succinoyl)-2-N-myristoylsphingosine (PEG-$CerC_{14}$) and 1-O-(2′-(ω-methoxypolyethyleneglycol) succinoyl)-2-N-arachidoylsphingosine (PEG-$CerC_{20}$) were synthesized as described elsewhere,[37] and dioleoyldimethylammonium chloride (DODAC) was kindly provided by Dr S Ansell (Inex Pharmaceuticals). Octylglucopyranoside (OGP), HEPES and NaCl were obtained from Sigma (St Louis, MO, USA). The plasmid pCMVCAT (4490 bp, coding for the chloramphenicol acyl transferase gene) was originally obtained from Dr K Brigham (Vanderbilt University, Nashville, TN, USA). The plasmid pCMVLuc (5650 bp, coding for the luciferase reporter gene) was provided by Dr P Tam (Inex Pharmaceuticals). All reporter genes were under the control of the human CMV immediate–early promoter-enhancer element. ^{3}H-cholesteryl hexadecyl ether (CHE) and ^{14}C-CHE were obtained from Mandel Scientific (Guelph, ON, Canada). Mouse serum was obtained from CedarLane (Mississauga, ON, Canada). Dialysis tubing (SpectraPor 12 000 to 14 000 mwco) was obtained from Fisher Scientific (Ottawa, ON, Canada), DEAE-Sepharose CL-6B column from Sigma, *E. coli* DNase I from Life Technologies (Mississauga, ON, Canada) and the luciferase assay kit from Promega (Madison, WI, USA).

Preparation of plasmids

Plasmid DNA was transformed into *E. coli* strain DH5α by electroporation. Plasmid DNA was then isolated from *E. coli* by alkaline lysis[38] followed by anion exchange chromatography (according to the manufacturer, Qiagen, Santa Clarita, CA, USA) or CsCl gradient centrifugation.[39] DNA was precipitated and dissolved in pyrogen-free water for formulation with lipids.

Radiolabeled ^{3}H-plasmid DNA was isolated from an *E. coli* JM101 strain bearing pCMVβ, pCMVCAT or pCMVLuc. Briefly, cultures were grown in supplemented minimal media (M9 salts with 0.1% thiamine, 1% glucose, 100 μg/ml ampicillin) to mid log phase. Ten mCi of 81.9 mCi/mmol tritiated thymidine (Mandel Scientific) was added, then the cultures were allowed to grow for a further 12–16 h. Plasmid DNA was isolated by alkaline lysis and anion exchange chromatography, as described above.

Encapsulation of plasmid DNA

Plasmid DNA (50–400 μg) was incubated with DODAC in 500 μl of 0.2 m octylglucoside, 150 mm NaCl, 5 mm HEPES pH 7.4 for 30 min at room temperature. The plasmid-DODAC mixture was then added to DOPE and PEG-$CerC_{14}$ or PEG-$CerC_{20}$ dissolved in 500 μl of 0.2 m OGP; 150 mm NaCl, 5 mm HEPES pH 7.4. The total lipid concentration was either 5 or 10 mg/ml with DOPE: DODAC:PEG-Cer at molar ratios of 84:6:10, unless otherwise indicated. The plasmid-lipid mixture was dialyzed against 5 mm HEPES in 150 mm NaCl pH 7.4 (HBS) for 36 to 48 h with two buffer changes. Non-encapsulated plasmid was removed by anion exchange chromatography on a DEAE-Sepharose CL-6B column (1 × 4 cm). To determine the encapsulation efficiency, a 50-μl aliquot of each sample was loaded on to a DEAE-Sepharose CL-6B column (1 ml) equilibrated with HBS. The column was eluted with HBS and the fractions were assessed for ^{3}H-plasmid and ^{14}C-lipid by scintillation counting.

Isolation of encapsulated plasmid by sucrose density gradient centrifugation

The fractions from the DEAE column containing co-eluting lipid and plasmid were pooled and equal volumes were applied to the top of a discontinuous sucrose gradient in 12.5 ml ultracentrifuge tubes. The gradient was formed with 3 ml each of 10% sucrose, 2.5% sucrose and 1% sucrose in HBS layered consecutively from bottom to top. The gradients were centrifuged at 160 000 *g* for 2 h at 20°C and separated into aliquots (250 μl) removed from top to bottom. The fractions were assayed for ^{3}H-plasmid and ^{14}C-CHE by dual-label scintillation counting. The lipid encapsulated plasmid DNA banded tightly at the interface between 2.5% and 10% sucrose, while the unassociated lipid was present as a smear from the top of the gradient to the interface between 1% and 2.5% sucrose. It was found that the isolated SPLP could be concentrated by dialysis against 500 000 molecular weight carboxymethyl cellulose (Aquacide II; Calbiochem, San Diego, CA, USA) in a 12 000–14 000 molecular weight cut-off dialysis tube. When the desired volume was reached, the formulation was transferred into a new dialysis bag and dialyzed overnight against HBS to adjust the NaCl concentration to 150 mm.

Freeze–fracture electron microscopy

Freeze–fracture was performed on a Balzers Freeze-Etching system, BAF 400D (Balzers, Lichtenstein). Samples were cryofixed in the presence of 25% glycerol by plunging them into liquid freon 22. The fractured surface was shadowed unidirectionally with platinum/carbon (45°) and coated with carbon (90°) immediately after fracturing. Replicas were analyzed using a Jeol model JEM 1200 EX electron microscope (Jeol, Montreal, QC, Canada).

Serum stability assay

SPLP formulations were assayed for serum stability in the presence of 90% mouse serum *in vitro*. A 50 μl aliquot was added to 450 μl mouse serum and incubated at 37°C for 30 min. The sample was then loaded on to a Sepharose CL-4B column and eluted with HBS, pH 7.4. The fractions were analyzed for ^{3}H-plasmid and the lipid label ^{14}C-CHE.

Determination of DNase I stability

Three sets of samples were exposed to DNase I digestion, including naked plasmid DNA, plasmid complexed with DOPE:DODAC vesicles and SPLP. Plasmid–cationic lipid complexes were prepared by mixing 500 μl plasmid (pCMVLuc, 0.5 mg/ml) in 5% glucose with 500 μl DODAC:DOPE (1:1) 100 nm diameter LUVs (0.9 mm lipid) prepared by the extrusion method[40] in 5% glucose. This corresponds to a lipid-to-DNA charge ratio (positive-to-negative) of 3. The resulting solution was incubated at room temperature for 30 min before DNase I treatment. For the DNase digestion, samples (free plasmid, plasmid–lipid complex, encapsulated plasmid) containing 1 μg of DNA were incubated with 0, 100 or 1000 units of DNase I in a total volume of 100 μl of 5 mm HEPES, 150 mm NaCl, 10 mm $MgCl_2$ pH 7.4 in the presence or absence of 1.0% Triton X-100. After incubation at 37°C for 30 min, the DNA was isolated by adding 500 μl of DNAzol (Life Technologies) followed by 1.0 ml of ethanol. The samples were centrifuged for 30 min at 20 000 *g* in a tabletop microfuge. The supernatant was decanted and the DNA pellet was washed twice with 80% ethanol and dried. The DNA was dissolved in 30 μl of TE buffer and analyzed by agarose (1.0%) gel electrophoresis in TAE buffer.

In vitro transfection

COS-7 cells and 293 cells were grown at 37°C, 5% CO_2 in complete media consisting of T75 flasks in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) and 10% fetal bovine serum (FBS; Intergen, Purchase, MA, USA). Transfections were performed in the presence of cell culture media when the cells were 60–70% confluent. The plasmid (pCMVLuc) formulations were diluted in complete medium to give 0.5 μg DNA/ml. The cells were incubated in the presence of the plasmid formulations for up to 120 h and assayed for luciferase activity. Calcium phosphate-mediated transfection with plasmid extracted from SPLP was performed as follows. Plasmid (0.1–1 μg) in 50 μl 0.25 m $CaCl_2$ was slowly added to 50 μl HBS, and the resulting precipitate was added to 293 cells. Following incubation for 2 days at 37°C, the luciferase activity was determined.

Luciferase assay

Luciferase assays were performed using the Promega Luciferase Assay System reagent kit (Promega E1501) according to the manufacturer's instructions. Cell lysates were assayed for luciferase activity using a Dynex Technologies ML3000 microplate luminometer (Dynex Technologies, Ghentilly, VT, USA). Luminescence readings were calibrated according to a standard curve obtained using a *Photinunus pyralis* luciferase standard (Boehringer Mannheim, Laval, QC, Canada; 634 409).

PEG-Cer dissociation rates

The dissociation rates of ^{3}H-PEG-CerC_{14} and ^{3}H-PEG-CerC_{20} from EPC LUV using EPC multilamellar vesicles (MLV) as a 'sink'. The LUV were prepared containing 10 mol% PEG-Cer and a trace of ^{14}C-CHE (^{3}H/^{14}C ratio approximately 5) by detergent dialysis as described above for SPLP. MLV were prepared by hydration of EPC in HBS (250 mg/ml) at 65°C. The MLV were washed five times in HBS by centrifugation (2 min at 12 000 *g*) to remove any small vesicles. LUV (1 mg lipid) were mixed with 125 mg MLV to give a final volume of 1.5 ml and incubated at 37°C. At different time intervals, 100 μl of the mixture were transferred into 0.5 ml ice-cold HBS and the MLV pelleted by centrifugation. The LUV in the supernatant were analyzed for ^{3}H-PEG-Cer and ^{14}C-CHE and the ^{3}H/^{14}C ratio plotted as a function of time.

Acknowledgements

Studies at UBC were supported by Inex and by the Natural Sciences and Engineering Research Council of Canada under the Collaborative Research and Development Program. Ian MacLachlan is a Postdoctoral Fellow of the Medical Research Council of Canada. We thank A Annuar, J Thompson and C Giesbrecht (Inex Pharmaceuticals) for provision of plasmid and Drs S Ansell and Z Wang (Inex Pharmaceuticals) for provision of DODAC and PEG-ceramides, respectively. We also thank Dr K Wong (Department of Biochemistry and Molecular Biology, UBC) for performing the freeze–fracture EM studies.

References

1 Huang L, Li S. Liposomal gene delivery: a complex package. *Nature Biotech* 1997; **15**: 620–621.
2 Templeton NS *et al*. Improved DNA:liposome complexes for increased systemic delivery and gene expression. *Nature Biotech* 1997; **15**: 647–652.
3 Hofland HJ *et al*. *In vivo* gene transfer by intravenous administration of stable cationic lipid / DNA complex. *Pharmaceut Res* 1997; **14**: 742–749.
4 Thierry AR *et al*. Systemic gene therapy: biodistribution and long-term expression of a transgene in mice. *Proc Natl Acad Sci USA* 1995; **92**: 9742–9746.
5 Harrison GS *et al*. Optimization of gene transfer using cationic lipids in cell lines and primary human $CD4^+$ and $CD34^+$ hematopoietic cells. *Biotechniques* 1995; **19**: 816–823.
6 Li S, Huang L. *In vivo* gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. *Gene Therapy* 1997; **4**: 891–900
7 Profitt RT *et al*. Liposomal blockade of the reticuloendothelial system: improved tumor imaging with small unilamellar vesicles. *Science* 1983; **220**: 502–505.
8 Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc Natl Acad Sci USA* 1988; **85**: 6949–6953.
9 Chonn A, Cullis PR. Recent advances in liposome drug delivery systems. *Curr Opin Biotech* 1995; **6**: 698–708.
10 Kohn S, Nagy JA, Dvorak HF, Dvorak AM. Pathways of macromolecular tracer transport across venules and small veins. Structural basis for the hyperpermeability of tumor blood vessels. *Lab Invet* 1992; **67**: 596–607.
11 Reimer DL *et al*. Formation of novel hydrophobic complexes between cationic lipids and plasmid DNA. *Biochemistry* 1995; **34**: 12877–12883.
12 Harasym TO *et al*. Poly(ethyleneglycol)-modified phospholipids prevent aggregation during covalent conjugation of proteins to liposomes. *Bioconj Chem* 1995; **6**: 187–194.
13 Holland JW, Hui C, Cullis PR, Madden TD. Poly (ethyleneglycol)-lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. *Biochemistry* 1996; **35**: 2618–2624.
14 Xu Y, Szoka FC. Mechanism of DNA release from cationic liposome / DNA complexes used in cell transfection. *Biochemistry* 1996; **35**: 5616–5623.
15 Farhood H, Serbina N, Huang L. The role of dioleoylphosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim Biophys Acta* 1995; **1235**: 289–295.
16 Chonn A, Semple SC, Cullis PR. Association of blood proteins with large unilamellar liposomes *in vivo*. Relation to circulation lifetimes. *J Biol Chem* 1992; **267**: 18759–18765.
17 Mimms LT *et al*. Phospholipid vesicle formation and transmembrane protein incorporation using octylglycoside. *Biochemistry* 1981; **20**: 833–840.
18 Fraley R, Subramani S, Berg P, Papahadjopoulos D. Introduction of liposome-encapsulated SV40 DNA into cells. *J Biol Chem* 1980; **255**: 10431–10435.
19 Soriano P *et al*. Targeted and nontargeted liposomes for *in vivo* transfer to rat liver cells of a plasmid containing the preproinsulin I gene. *Proc Natl Acad Sci USA* 1983; **80**: 7128–7131.
20 Nakanishi M *et al*. Efficient introduction of contents of liposomes into cells using HVJ (Sendai virus). *Exp Cell Res* 1985; **159**: 399–409.
21 Fraley RT, Fornari CS, Kaplan S. Entrapment of a bacterial plasmid in phospholipid vesicles: potential for gene therapy. *Proc Natl Acad Sci USA* 1979; **76**: 3348–3352.
22 Nicolau C, Rottem S. Expression of a β-lactamase activity in *Mycoplasma carpicolum* transfected with the liposome-encapsulated *E. coli* pBR32 plasmid. *Biochem Biophys Res Commun* 1982; **108**: 982–986.
23 Stavridis JC *et al*. Construction of transferrin-coated liposomes for *in vivo* transport of exogenous DNA to bone marrow erythroblasts in rabbits. *Exp Cell Res* 1986; **164**: 568–572.
24 Wang C-Y, Huang L. pH-sensitive immunoliposomes mediate target cell-specific delivery and controlled expression of a foreign gene in mouse. *Proc Natl Acad Sci USA* 1987; **84**: 7851–7855.
25 Lurquin PF. Entrapment of plasmid DNA by liposomes and their interactions with plant protoplasts. *Nucleic Acids Res* 1979; **6**: 3773–3784.
26 Alino SF *et al*. *In vivo* delivery of human alpha 1-antitrypsin gene to mouse hepatocytes by liposomes. *Biochem Biophys Res Commun* 1993; **192**: 174–181.
27 Baru M, Axelrod JH, Nur I. Liposome-encapsulated DNA-mediated gene transfer and synthesis of human factor IX in mice. *Gene* 1995; **161**: 143–150.
28 Jay DG, Gilbert W. Basic protein enhances the incorporation of DNA into lipid vesicles: model for the formation of primordial cells. *Proc Natl Acad Sci USA* 1987; **84**: 1978–1980.
29 Puyal C, Milhaud P, Bienvenue A, Philippot JR. A new cationic liposome encapsulating genetic material. A potential delivery system for polynucleotides. *Eur J Biochem* 1995; **228**: 697–703.
30 Ibanez M *et al*. Spermidine-condensed DNA and cone-shaped lipids improve delivery and expression of exogenous DNA transfer by liposomes. *Biochem Cell Biol* 1997; **74**: 633–643.
31 Ausubel F *et al* (eds). Preparation and analysis of DNA. In: *Short Protocols in Molecular Biology*, 3rd edn. John Wiley: New York, 1995, pp A1–A58.
32 King GI, Jacobs RE, White SH. Hexane dissolved in dioleoylleci-thin bilayers has a partial molar volume of approximately zero. *Biochem J* 1985; **24**: 4637–4645.
33 Ollivon M, Eidelman O, Blumenthal R, Walter A. Micelle-vesicle transition of egg phosphatidylcholine and octylglucoside. *Biochem J* 1988; **27**: 1695–1703.
34 Vinson PK, Talmon Y, Walter A. Vesicle-micelle transition of phosphatidylcholine and octylglucoside elucidated by cryo-transmission electron microscopy. *Biophys J* 1989; **56**: 669–681.
35 Cullis PR, de Kruijff B. The polymorphic phase behaviour of phosphatidylethanolamines of natural and synthetic origin. *Biochim Biophys Acta* 1978; **513**: 31–42.
36 Holland JW, Cullis, PR, Madden TD. Poly(ethyleneglycol)-lipid conjugates promote bilayer formation in mixtures of non-bilayer forming lipids. *Biochemistry* 1996; **35**: 2610–2617.
37 Webb MS *et al*. Comparison of different hydrophobic anchors conjugated to poly(ethyleneglycol): effects on the pharmacokinetics of liposomal vincristine. (submitted).
38 Birnboim HC, Doly J. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 1979; **7**: 1513–1522.
39 Sambrook J, Fritsch EF, Maniatis T. Extraction and purification of plasmid DNA. In: Ford N, Nolan C, Ferguson M (eds). *Molecular Cloning*, 2nd edn. Cold Spring Harbor Laboratory Press: New York, 1989, pp 1.38–1.39.
40 Hope MJ, Bally MB, Webb G, Cullis PR. Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size, trapped volume and ability to maintain a membrane potential. *Biochim Biophys Acta* 1985; **812**: 55–65.
41 Cudd A, Nicolau C. Intracellular fate of liposome-encapsulated DNA in mouse liver. Analysis using electron microscope autoradiography and subcellular fractionation. *Biochim Biophys Acta* 1985; **845**: 477–491.
42 Szelei J, Duda E. Entrapment of high molecular mass DNA molecules in liposomes for the genetic transformation of animal cells. *Biochem J* 1989; **259**: 549–553.
43 Monnard P-A, Oberholzer T, Luisi P. Entrapment of nucleic acids in liposomes. *Biochim Biophy Acta* 1997; **1329**: 39–50.

JOINT APPENDIX 50

Recent advances in liposomal drug-delivery systems

Arcadio Chonn and Pieter R Cullis

INEX Pharmaceuticals Corporation, Vancouver and The University of British Columbia, Vancouver, Canada

Liposomal drug-delivery systems have come of age in recent years, with several liposomal drugs currently in advanced clinical trials or already on the market. It is clear from numerous pre-clinical and clinical studies that drugs, such as antitumor drugs, packaged in liposomes exhibit reduced toxicities, while retaining, or gaining enhanced, efficacy. This results, in part, from altered pharmacokinetics, which lead to drug accumulation at disease sites, such as tumors, and reduced distribution to sensitive tissues. Fusogenic liposomal systems that are under development have the potential to deliver drugs intracellularly, and this is expected to markedly enhance therapeutic activity. Advances in liposome design are leading to new applications for the delivery of new biotechnology products, such as recombinant proteins, antisense oligonucleotides and cloned genes.

Current Opinion in Biotechnology 1995, 6:698–708

Introduction

Liposomes are microscopic spheres with an aqueous core surrounded by one or more outer shell(s) consisting of lipids arranged in a bilayer configuration. The potential use of liposomes as drug carriers was recognized more than 25 years ago [1] and, since that time, liposomes have been used in a broad range of pharmaceutical applications (Table 1). This review first highlights some of the key advances of the past decade in the design of liposomes for systemic delivery and then reviews the most recent literature involving specific applications of liposomal drug-delivery systems.

Liposome technology

Preparation of liposomes

Liposomes can be prepared by a variety of methods (extensively reviewed in [2,3]). In general, on the basis of size and lamellarity (number of bilayers present within a liposome), liposomes are classified into three categories: multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs), and small unilamellar vesicles (SUVs).

Drug loading

Drug loading can be achieved either passively (i.e. the drug is encapsulated during liposome formation) or actively (i.e. after liposome formation). Hydrophobic drugs, such as amphotericin B, taxol or annamycin, can be directly incorporated into liposomes during vesicle formation, and the extent of uptake and retention is governed by drug–lipid interactions. Trapping efficiencies of 100% are often achievable, but this is dependent on the solubility of the drug in the liposome membrane. Passive encapsulation of water-soluble drugs relies on the ability of liposomes to trap aqueous buffer containing a dissolved drug during vesicle formation. Trapping efficiencies (generally < 30%) are limited by the trapped volume contained in the liposomes and drug solubility. Alternatively, water-soluble drugs that have protonizable amine functions can be actively entrapped by employing pH gradients [4], which can result in trapping efficiencies approaching 100%.

Liposomes with prolonged circulation lifetimes

A significant advance in the development of liposomal drugs has come with the use of specialized lipids, such as monosialoganglioside G_{M1} or polyethylene glycol

Abbreviations

CFTR—cystic fibrosis transmembrane receptor; DOPE—dioleoylphosphatidyl ethanolamine; EGF—epidermal growth factor; FDA—Food and Drug Administration; HSV—herpes simpex virus; IL—interleukin; LUV—large unilamellar vesicle; MDR—multidrug resistance; MLV—multilamellar vesicle; MTP-PE—muramyl tripeptide phosphatidyl ethanolamine; PEG-PE—polyethylene glycol modified phosphatidyl ethanolamine; POPC—1-palmitoyl-2-oleoylphosphatidylcholine; SUV—small unilamellar vesicle.

 ISSN 0958-1669

Table 1. Liposomal drugs currently under development or on the market.

Product name (if any)	Drug	Company/institution	Phase of development
Conventional drugs			
ABLC (Abelcet)	Amphotericin B	The Liposome Company, Princeton, USA	Marketed in UK and Luxembourg. Awaiting approval for treatment of aspergillosis
AmBisome	Amphotericin B	NeXstar Pharmaceuticals Inc, Boulder, USA	Marketed in certain countries in Europe
Amphocil	Amphotericin B	Sequus Pharmaceuticals Inc, Menlo Park, USA	Awaiting FDA approval
Doxil (DOX-SL)	Doxorubicin		FDA-accelerated approval for treatment of Kaposi's sarcoma
TLC D-99	Doxorubicin	The Liposome Company Princeton, USA	Phase III
TLC C-53	Prostaglandin E_1	The Liposome Company Princeton, USA	Phase II
DaunoXome	Daunorubicin	NeXstar Pharmaceuticals Inc, Boulder, USA	Approval for treatment of Kaposi's sarcoma; in Phase II trials for breast cancer, small cell lung cancer, leukemia and lymphoma
AR-121	Nystatin	Argus Pharmaceuticals Inc, The Woodlands, Texas, USA	Phase II
Tretinoin (AR-623)	All-*trans* retinoic acid		Phase II (leukemia) and phase I (Kaposi's sarcoma)
–	Annamycin	Argus Pharmaceuticals Inc, The Woodlands, Texas, USA	Phase I
–	Vincristine	INEX Pharmaceuticals Corporation, Vancouver, Canada	Phase I
Proteins			
OncoLipin	IL-2	Oncotherapeutics, New Jersey, USA	Phase II (kidney cancer)
OncoVax	IL-3 and cancer tumor antigen		Phase I
Genes and antisense oligonucleotides			
Allovectin-7	pHLA-B7/b-2	Vical, San Diego and University of Michigan, Michigan, USA	Phase I completed
–	pHLA-B7/b-2	Mayo Clinic, Rochester, USA	Phase I
–	pHLA-B7/b-2	University of Chicago, Chicago, USA	Phase I
–	pHLA-B7/b-2	AZ Cancer Center, USA	Phase I
–	*CFTR* gene	Medical Research Council, UK	Phase I completed
–	pKCTR	University of Alabama, Alabama, USA	Phase I, pending FDA approval
–	pBMG-*neo*-hIL-2	University of Miami, Miami, USA	Phase I, pending FDA approval
–	pCMV4-AAT	Vanderbilt University, Nashville, USA	Phase I, pending FDA approval
–	pMP6-IL-2	Duke University, Durham, North Carolina, USA	Phase I, pending FDA approval

modified phosphatidyl ethanolamine (PEG-PE), that engender long circulation lifetimes when incorporated into liposomes [5–7]. Alternatively, the presence of entrapped cytotoxic drug can also lead to extended circulation times [8]. It has been demonstrated that increased circulation lifetimes enhance the opportunity for liposomes, administered systemically, to leave the vascular compartment and enter certain extravascular regions [9–11]. Tumors, for example, exhibit leaky blood vessels that have a reduced ability to retain circulating macromolecules [12,13]. Liposomes can extravasate in these regions, thus leading to preferential accumulation within tumors. Studies have now clearly demonstrated that long-circulating liposomes containing PEG-PE or cytotoxic drugs, such as doxorubicin, accumulate within these sites preferentially compared with conventional liposomes [9,11,14].

Targeted delivery

It is envisioned that the next generation of liposomal pharmaceuticals will consist of drug-loaded liposomes with surface-associated targeting information (Fig. 1). Site-directing targeting ligands, such as monoclonal antibodies, can be attached to liposomes by either covalent or non-covalent methods [15–17]. The advent of novel PEG-PE lipids that allow targeting ligands to

be conjugated at the distal ends of the PEG spacer has afforded both effective target binding *in vitro* and prolonged circulation times [18•,19–21].

To date, only two studies have demonstrated the improved therapeutic activity of liposomal drugs *in vivo* achieved through the use of antibody-mediated targeting [22,23], with both studies employing a monoclonal antibody against lung endothelial thrombomodulin (mAb 34A) and intravenously injected tumor cells. The use of immunoliposomes may be limited because of their potential immunogenicity [24].

In addition to antibodies, glycolipids (e.g. galactose [25] and mannose [26]), proteins (e.g. transferrin [27] and asialofetuin [28•]), and vitamins (e.g. folic acid [18•,29]) have been used to target specific cells via cell surface receptors.

Intracellular delivery

Liposomes can facilitate the intracellular delivery of drugs by fusing with the target cell. Alterations in the lipid composition can render liposomes pH sensitive, leading to enhanced fusogenic tendencies in low pH compartments such as endosomes [30]. The inclusion of lipids that are able to form non-bilayer phases, such as dioleoylphosphatidyl ethanolamine (DOPE), can promote destabilization of the bilayer, inducing fusion events. DOPE has been particularly useful for cationic liposomes complexed with plasmid DNA for gene delivery [31•,32].

Conventional drugs

A vast literature describes the feasibility of formulating a wide range of conventional drugs in liposomes, often resulting in enhanced therapeutic activity and/or reduced toxicity compared with the free drug. In general, altered pharmacokinetics for liposomal drugs can lead to enhanced drug bioavailability to specific target cells that reside in the circulation, or more importantly, to extravascular disease sites such as tumors. Recent

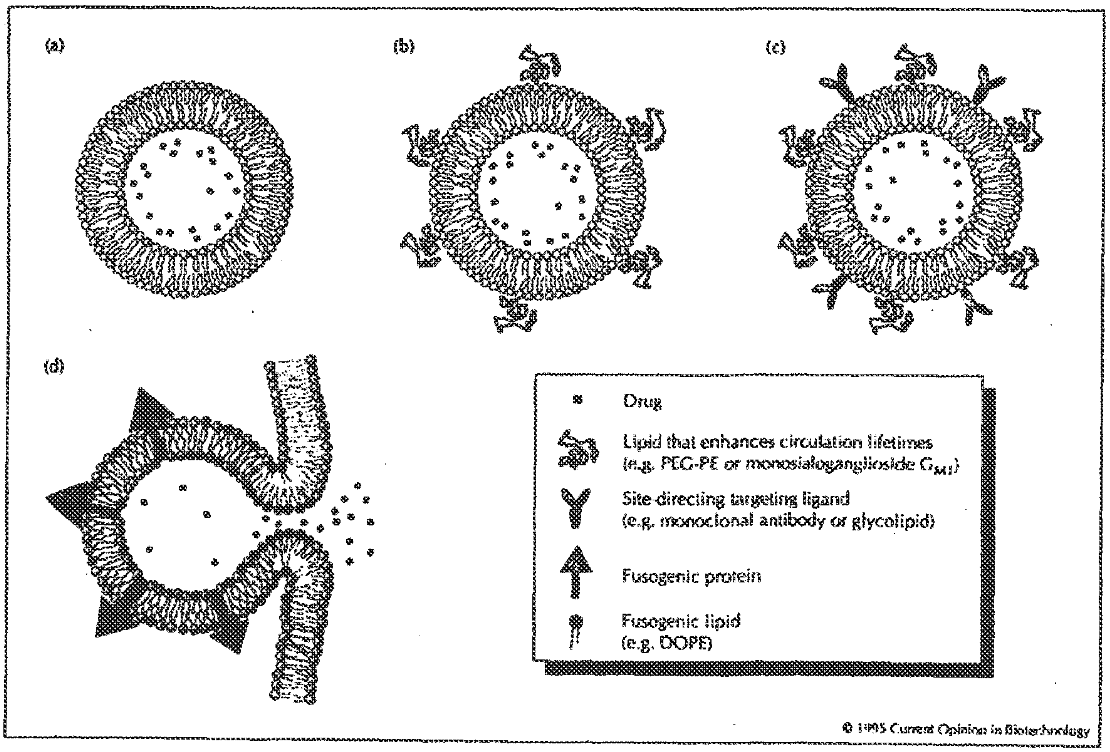


Fig. 1. Types of liposomal delivery. **(a)** Liposomes prepared from natural or synthetic phospholipids containing an encapsulated drug. This type of drug delivery reduces toxicity, maintains or enhances activity and facilitates accumulation in the disease site. **(b)** Conventional liposomes that incorporate lipids enhancing circulation lifetimes. Delivery in these molecules improves access to the disease site and reduces interaction with phagocytic cells of the reticulo-endothelial system. **(c)** Conventional liposomes with lipids that enhance circulation lifetimes and have surface-associated targeting information. Drug delivery using this type of liposome results in an improved therapeutic index and target cell specific delivery. **(d)** Fusogenic liposomes with DOPE or fusogenic *proteins*. This method allows intracellular drug delivery.

advances include liposomal formulations of all-*trans* retinoic acid [33,34] and daunorubicin [35–38], which has recently received Food and Drug Administration (FDA) approval as a first-line treatment of AIDS-related advanced Kaposi's sarcoma. Notable examples are given below.

Amphotericin B

Liposomal amphotericin B drugs are presently approved for sale in certain European countries and are nearing regulatory approval in North America. Acute toxicities associated with amphotericin B are markedly reduced with liposomal formulations, without losing broad-spectrum antifungal activity. Early studies on a variety of formulations of liposomal amphotericin B demonstrated the successful treatment of fungal infections in mice [39,40]. Recent studies have focused on understanding the possible mechanisms for reduced toxicities, which include altered pharmacokinetics [41,42] and increased association with high-density lipoproteins [43,44].

Although most applications involve an intravenous route of administration to treat systemic fungal infections, liposomal amphotericin B can also be administered in an aerosolized form, resulting in treatment of systemic *Candida albicans* or *Cryptococcus neoformans* infections in mice [45,46].

Doxorubicin

Phase III clinical trials on liposomal doxorubicin are ongoing. As demonstrated in several pre-clinical and clinical reports, the administration of liposomal doxorubicin significantly reduces drug-associated cardiotoxicity because cardiac uptake of liposome-encapsulated doxorubicin is substantially reduced compared with the free drug. A variety of liposome–doxorubicin formulations have been described. These include PEG-stabilized liposomes [14], as well as conventional egg phosphatidylcholine/cholesterol LUVs [8]. Recently described formulations that extend the circulation half-life of doxorubicin include dipalmitoylphosphatidylcholine/cholesterol (1:1) liposomes containing 10 mol% palmityl-D-glucuronide, a uronic acid derivative [47], and fluorinated liposomes [48]. Targeted liposomal doxorubicin systems have recently been described [29,49•]; however, their efficacy, compared with non-targeted systems *in vivo*, has yet to be determined.

Of the above types of formulation, reports of PEG-coated liposomal doxorubicin dominate the recent literature [50•,51–61]. Much attention has focused on the use of liposomal doxorubicin in the treatment of AIDS-related Kaposi's sarcoma [55–58,62]. In the prolonged use of liposomal doxorubicin for AIDS-related Kaposi's sarcoma, hand-foot syndrome may be a limiting toxicity [55]. With regard to liposomal doxorubicin-induced toxicities, a recent report indicates that the depletion and impairment of phagocytic activity of rat liver macrophages by liposomal doxorubicin can be substantial [63]. Whether this finding applies to humans remains to be seen. To date, severe hepatic toxicities have not been reported in any clinical trial.

The increase in therapeutic index of liposomal doxorubicin most likely results from the 'passive' targeting to tumor sites of liposomes, which because of the leaky vasculature, exhibit increased extravasation. This is particularly relevant for liposomes with long circulation lifetimes. At the tumor site, liposomes appear to act as a depot for slow release of drug. This model is supported by the findings of Suzuki *et al.* [64] indicating that liposomal doxorubicin remaining on the cell surface is more cytotoxic than endocytosed liposomal doxorubicin. Furthermore, several reports indicate that hyperthermia induces the release of doxorubicin from long-circulating liposomes and enhances their antitumor efficacy [59–61].

Natural or acquired resistance to doxorubicin may limit the clinical use of liposomal doxorubicin. Different ways of overcoming multidrug resistance, including the use of modulators that can inhibit drug efflux mediated by P-glycoprotein [65], have been explored and have proved effective in *in vitro* systems. Several successful attempts have also been described, at least *in vitro*, to overcome multidrug resistance by employing structurally different analogs of anthracyclines entrapped in liposomes [66,67•]. For instance, the non-cross-resistant anthracycline antibiotic, annamycin, formulated in dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol SUVs or MLVs, is more effective than doxorubicin against several tumor models, and multidrug resistance shows only partial crossresistance to annamycin, both *in vitro* and *in vivo* [67•].

Vincristine

The benefits of prolonged drug bioavailability as a result of administering the drug in a liposomal form is perhaps best exemplified by liposomal vincristine, an important anticancer drug effective against a wide variety of neoplasms. Vincristine is a cell cycle specific drug that arrests cell growth exclusively during metaphase by attaching to the growing ends of microtubules and inhibiting their assembly. As such, prolonged exposure of neoplastic cells to vincristine should greatly enhance its therapeutic index. Indeed, increased drug retention and increased circulation longevity, as achieved by encapsulating vincristine in distearoylphosphatidylcholine/cholesterol LUVs with an internal pH of 2.0, act synergistically to significantly enhance the circulation lifetime of encapsulated vincristine, the extent and duration of tumor exposure to vincristine, and ultimately, the therapeutic activity of vincristine [68•,69].

The development of a liposomal formulation of vincristine, employing sphingomyelin/cholesterol LUVs with an internal pH of 4.0 or 2.0 has recently been de-

scribed [70••]. This formulation displays significantly enhanced stability and antitumor properties compared with distearoylphosphatidylcholine/cholesterol LUV systems [70••]. Substantially increased vincristine accumulation, compared with the free drug, is observed in both peritoneal ascitic murine P388 tumors and subcutaneous solid A431 human xenograft tumors. In addition, a recent report of a liposomal vincristine formulation employing PEG-PE shows an enhanced therapeutic index for vincristine entrapped in liposomes against subcutaneously or intraperitoneally implanted P388 tumor cells [71].

As is the case for several toxic conventional drugs, liposomal vincristine exhibits reduced toxicity compared with the free drug [72]. Particularly notable is the greatly enhanced efficacy that can be achieved for liposomal vincristine compared with equivalent doses of the free drug. Liposomal vincristine is currently in clinical trials.

Proteins and peptides

The majority of current liposomal protein formulations are still in various preclinical research stages (recently reviewed in [73]), with one liposomal interleukin (IL)-2 drug entering a phase II clinical trial for kidney cancer. For the production of artificial blood substitutes, the use of liposomes to encapsulate hemoglobin is actively being investigated for the *in vivo* delivery of hemoglobin without many of the inherent toxicities associated with the delivery of the free molecule (recently reviewed in [74–76]). Another area of intense research is the application of liposomes exhibiting improved adjuvancy for vaccine development.

Immunomodulators: interleukins

The feasibility of formulating cytokines in MLVs [77–79] and in sterically stabilized SUVs [80,81•] has recently been demonstrated. These liposomal cytokines show great promise as immunoadjuvants for vaccine development. IL-2 encapsulated in sterically stabilized SUVs (65 nm in diameter) is significantly more effective than free IL-2 both in increasing leukocyte number in the blood and spleen and in triggering spleen lymphokine-activated killer-cell activity [81•]. Co-injection of phosphatidylcholine/cholesterol (1:1) MLVs containing IL-6 (50 000 U IL-6 mouse^{-1}) or 65 kDa heat-shock protein antigen (0.03 μg mouse^{-1} or 0.3 μg mouse^{-1}) significantly enhanced secondary antibody responses at antigen dosages where other adjuvants (e.g. Ribi or dimethyldioctadecylammonium-bromide) exhibit no adjuvant activity [79]. Liposomal formulations of IL-7 have been shown to enhance the immune responses of mice vaccinated with either alum-associated or liposome-formulated recombinant HIV envelope protein env-2-3SF2 [82]. Antibody titers resulting from vaccination with liposome-formulated antigen were higher than those with alum-associated antigen, and these antibody responses were enhanced by concurrent administration of IL-7 liposomes. In addition, immunogenicity of alum-associated herpes simplex virus (HSV) gD antigen can be enhanced by a recombinant IL-7 liposomal formulation, resulting in a significantly reduced severity and course of primary HSV-2 infection. The sustained release of IL-7, over a period of >6 days, contributes to the observed effects [83•].

Recent reports also indicate that unencapsulated cytokines, at relatively low doses, augment the therapeutic effects of liposomal reagents [84•,85]. For instance, unencapsulated recombinant IL-2 administered intraperitoneally (10 000 U day^{-1}), in combination with intravenously administered phosphatidylcholine/phosphatidylserine (1:1) MLVs containing a synthetic peptide derived from C-reactive protein (RS-83277), significantly inhibited tumor metastases and prolonged survival of C57Bl/6 mice bearing established pulmonary metastases of fibrosarcoma T241. The combination therapy was accompanied by an increase in the number of Thy1.2$^+$ cells in the lungs of RS-83277 MLV/IL-2 treated animals compared with those receiving RS-83277 MLVs alone.

Liposomal muramyl tripeptide

Muramyl tripeptide phosphatidyl ethanolamine (MTP-PE) is a synthetic lipophilic analog of muramyl dipeptide, the smallest component of a mycobacterium capable of stimulating the immune system. MTP-PE is a potent monocyte/macrophage activator and is currently under clinical investigation against metastatic melanoma and osteosarcoma (reviewed in [86]). Of major interest to the development of liposomal MTP-PE immunomodulators is the recent finding that repeated *per os* (oral) administration of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) MLVs containing a synthetic muramyl tripeptide, CGP 19835A (Ciba-Geigy, Basel, Switzerland), increased the tumoricidal activity of alveolar and peritoneal macrophages against renal cell carcinoma [87••]. Liposomes were rapidly absorbed in the intestine and reached the systemic circulation within 4 h, as determined by the biodistribution of radioactively labeled, or N-4-nitrobenzo-2-oxa-1,3-diazolephosphatidyl ethanolamine fluorescently labeled POPC-CGP 19835A liposomes. The mechanism of liposomal muramyl tripepride antitumor activity is linked to its activation of monocyte/macrophage tumoricidal function, as shown by several recent reports [88–90].

Antisense oligonucleotides, ribozymes and genes

Antisense molecules and ribozymes present interesting challenges for delivery systems. The efficacy of these

drugs is dependent on their ability to gain entry into cells in an intact form; however, they are particularly susceptible to degradation by nucleases in the biological milieu and usually cannot cross the target cell membrane. For example, in general, phosphodiester antisense oligonucleotides have been reported to have little or no inhibitory effect in culture because they are rapidly degraded in the culture medium. In addition, these molecules are highly charged and can activate the complement system, resulting in the generation of anaphylatoxins and other immunomodulators. The potential of liposomes to encapsulate antisense oligonucleotides or DNA, protecting them from nucleases and complement, represents a great advantage over other drug carriers, such as polymers or immunoconjugates. The further potential for fusogenic liposomes to promote intracellular delivery of these compounds is also of major importance. The application of liposomes to deliver antisense oligonucleotides, ribozymes and genes is an area of intense research.

Antisense oligonucleotides and ribozymes

Several reports demonstrate the feasibility of employing liposomal systems to deliver antisense oligonucleotides, with the accompanying significant enhancement of efficacy *in vitro* and *in vivo* [91,92,93•,94•]. Cellular uptake of fluorescently labeled oligonucleotides is significantly enhanced by cationic liposomes, as assessed by confocal laser scanning microscopy, flow cytofluorometry and laser-scanning microscopy. Intact oligonucleotides are found in the cytoplasm and nucleus only when they are delivered by cationic liposomes.

The overwhelming conclusion from studies to date is that liposomes are able to resolve the problems of extracellular degradation by nucleases and poor membrane permeability that are inherent for oligonucleotide drugs. This has been achieved using a variety of liposomal compositions, with the majority employing cationic lipids and DOPE [93•,95–97]. A recent report describes an extensive physicochemical study of the aggregation and fusion reactions that occur during the formation of oligonucleotide and cationic liposomal complexes in solution [98]. Furthermore, several approaches to encapsulate antisense oligonucleotides have been described. A probe sonication method employing phosphatidylcholine/cholesterol/dipalmitoylphosphatidyl ethanolamine covalently coupled to L-polylysine (5.5:3.0:1.5) has recently been shown to result in liposomes with a diameter of 110–140 nm and encapsulation efficiencies ranging from 55% to 100% depending on the oligonucleotide [99]. The use of immunoliposomes has also been described [100]. Aigner and Caroni [101•] report the use of liposomes composed of phosphatidylcholine/phosphatidylserine (10:1) and myelin proteins derived from adult rat spinal cord or sciatic nerve to deliver antisense oligonucleotides to dorsal root ganglion neurons. In addition, liposomes containing viral fusion proteins, derived from Sendai virus, have been used to promote fusion with target cells [94•]. Wang *et al.* [102••] describe the use of phosphatidylcholine/cholesterol (3:2) containing 0.5 mol% folate conjugated to PEG-distearoylphosphatidyl ethanolamine to deliver antisense oligonucleotides against human epidermal growth factor (EGF) (up to 2.0×10^7 molecules cell^{-1}) in a folate-specific manner, as free folic acid competes with EGF uptake.

An interesting approach to increase the association of antisense oligonucleotides with liposomes involves coupling antisense oligonucleotides to cholesterol via a reversible disulfide bond [103•,104–107]. Using this method, the association of oligonucleotides with immunoliposomes is improved by a factor of ~10. The capacity of modified oligonucleotides directed against the *tat* gene of HIV-1 to inhibit HIV-1 proliferation in acutely infected cells has been found to be the same as the unmodified oligonucleotide on an equimolar basis ($IC_{50} \approx 0.1$ μM) [103•].

To date, only a few papers have reported the use of cationic liposomes to deliver ribozymes, a class of RNA molecule that possesses enzymatic cleavage activity [108•,109,110•,111•]. Ribozymes, being RNA molecules, are highly susceptible to nuclease digestion. Their stability is markedly increased *in vitro* in the presence of cationic liposomes, with >30% remaining intact after a 60 min incubation in medium containing 10% fetal bovine serum. The feasibility of using a variety of cationic liposomes to deliver ribozymes into cultured cells *in vitro* has recently been described for ribozymes directed against leukocyte-type 12-lipoxygenase mRNA [108•], *bcr-abl* mRNA [110•], or multiple drug resistance (MDR)-1 mRNA [111•]. Liposome-mediated transfer of ribozymes against MDR-1 mRNA was shown to reverse the MDR phenotype of adriamycin-resistant and vindesine-resistant human pleural mesothelioma cell lines and restored sensitivity to chemotherapeutic drugs [111•]. As with antisense oligonucleotides, it is likely that liposomal systems will provide significant advantages to the delivery of ribozyme molecules *in vivo*. The development of such liposomal formulations is advancing rapidly.

Genes

Several reviews on the use of liposomes to deliver genes have appeared recently (see [112–114]; this issue, Cunliffe, Thatcher and Craig, pp 709–713). Although the utility of cationic liposomes in the delivery of reporter genes was noted in the early 1980s, we are only now beginning to characterize these systems and to understand the cellular processes that are required. For instance, the role of DOPE in mediating cytosolic delivery of plasmid DNA has now been elucidated [32,115]. It has been shown that the principal route of cationic liposome-mediated gene transfer occurs after endocytosis [116•]. Recent electron microscopy studies have attempted to reveal the structural features of plasmid

DNA–cationic liposomal complexes [117,118], which remain relatively poorly characterized. As with all liposomal drugs, well characterized liposomal systems will be the DNA carrier of choice. Procedures to produce well defined liposomal systems with encapsulated DNA, to protect the DNA from nuclease degradation, are at early stages of development.

The major barriers in the cellular processing of liposome–DNA complexes have recently been described [119••]. On average, COS-1 cells take up 3×10^5 plasmids after 6 h of incubation in the presence of *N*-[1-(2,3-dimyristyloxy)propyl]- *N*,*N*-dimethyl- *N*-(2-hydroxyethyl) ammonium bromide/DOPE–DNA complexes; however, after 24 h, the majority of the DNA–lipid complexes aggregate into large perinuclear complexes, with only a small amount of DNA in the cytoplasm of most cells. Another important factor is that the lipid and DNA must dissociate before transcription can occur. The maturation of liposomes as a viable systemic gene delivery vehicle *in vivo* will thus require the following steps: first, liposomes should be targeted to endocytic receptors in order to enhance the rate of endocytosis; second, fusion processes (mediated by lipids or proteins) should be optimized in order to enable efficient escape from the endosome and entry into the cytoplasm; and third, cytoplasmic stability and nuclear targeting of the plasmids should be enhanced.

Results from a phase I clinical study on cationic liposome-mediated cystic fibrosis transmembrane regulator (*CFTR*) gene transfer to the nasal epithelium of patients with cystic fibrosis has recently been reported [120•]. No adverse clinical effects were observed from cationic liposome-mediated gene transfer to nasal epithelia.

Conclusions

After three decades of development, liposomes are fulfilling their promise as a drug delivery vehicle with general applications. Liposomal drugs exhibit reduced toxicities and retain, or gain enhanced, efficacy compared with their free counterparts. Liposomes that allow enhanced drug delivery to disease sites, by virtue of long circulation residence times, are now achieving clinical acceptance. Also at hand are liposomes that promote targeting to particular diseased cells within the disease site. Finally, liposomes are showing particular promise as intracellular delivery systems for proteins/peptides, antisense molecules, ribozymes and DNA. The development of liposomes that can be administered systemically and exhibit targeted and fusogenic properties appears to be increasingly within our grasp.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:
- • of special interest
- •• of outstanding interest

1. Sessa G, Weissmann G: Phospholipid spherules (liposomes) as a model for biological membranes. *J Lipid Res* 1968, 9:310–318.
2. Gregoriadis G: Liposome preparation and related techniques. In *Liposome Technology*, vol 1, edn 2. Edited by Gregoriadis G. Boca Raton: CRC Press; 1993:1–63.
3. Watwe RM, Bellare JR: Manufacture of liposomes: a review. *Curr Sci* 1995, 68:715–724.
4. Mayer LD, Madden TM, Bally MB, Cullis PR. pH gradient-mediated drug entrapment in liposomes. In *Liposome Technology*, vol 2, edn 2. Edited by Gregoriadis G. Boca Raton: CRC Press; 1993:27–44.
5. Allen TM, Chonn A: Large unilamellar liposomes with low uptake into the reticuloendothelial system. *FEBS Lett* 1987, 223:42–46.
6. Klibanov AL, Maruyama K, Torchilin VP, Huang L: Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett* 1990, 268:235–237.
7. Blume G, Cevc G: Liposomes for the sustained drug release *in vivo*. *Biochim Biophys Acta* 1990, 1029:91–97.
8. Bally MB, Nayar R, Masin D, Hope MJ, Cullis PR, Mayer LD: Liposomes with entrapped doxorubicin exhibit extended blood residence times. *Biochim Biophys Acta* 1990, 1023:133–139.
9. Gabizon A, Papahadjopoulos D: Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc Natl Acad Sci USA* 1988, 85:6949–6953.
10. Bakker-Woudenberg IAJM, Lokerse AF, Ten-Kate MT, Storm G: Enhanced localization of liposomes with prolonged blood circulation time in infected lung tissue. *Biochim Biophys Acta* 1992, 1138:318–326.
11. Wu NZ, Da D, Rudoll TL, Needham D, Whorton AR, Dewhirst MW: Increased microvascular permeability contributes to preferential accumulation of stealth liposomes in tumor tissue. *Cancer Res* 1993, 53:3765–3770.
12. Gerlowski LE, Jain RK: Microvascular permeability of normal and neoplastic tissues. *Microvasc Res* 1986, 31:288–305.
13. Dvorak HF, Nagy JA, Dvorak JT, Dvorak AM: Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. *Am J Pathol* 1988, 133:95–109.
14. Papahadjopoulos D, Allen TM, Gabizon A, Mayhew E, Matthay K, Huang SK, Lee KD, Woodle MC, Lasic DD, Redemann C, Martin FJ: Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. *Proc Natl Acad Sci USA* 1991, 88:11460–11464.
15. Allen TM: Long-circulating (sterically stabilized) liposomes for targeted drug delivery. *Trends Pharmacol Sci* 1994, 15:215–220.
16. Loughrey HC, Choi LS, Wong KF, Cullis PR, Bally MB: Preparation of streptavidin-liposomes for use in ligand specific targeting applications. In *Liposome Technology*, vol 3, edn 2. Edited by Gregoriadis G. Boca Raton: CRC Press; 1993:163–178.
17. Laukkanen ML, Alfthan K, Keinanen K: Functional immunoliposomes harboring a biosynthetically lipid-tagged single-chain antibody. *Biochemistry* 1994, 33:11664–11670.
18. • Lee RJ, Low PS: Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. *J Biol Chem* 1994, 269:3198–3204.

A study showing that liposomes can be efficiently targeted to receptor-bearing tumor cells when conjugated to folate via a long PEG-spacer.

19. Blume G, Cevc G, Crommelin MDJA, Bakker-Woudenberg IAJM, Kluft C, Storm G: Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. *Biochim Biophys Acta* 1993, 1149:180–184.

20. Maruyama K, Takizawa T, Yuda T, Kennel SJ, Huang L, Iwatsuru M: Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies. *Biochim Biophys Acta* 1995, 1234:74–80.

21. Allen TM, Brandeis E, Hansen CB, Kao GY, Zalipsky S: A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. *Biochim Biophys Acta* 1995, 1237:99–108.

22. Ahmad I, Longenecker M, Samuel J, Allen TM: Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice. *Cancer Res* 1993, 53:1484–1488.

23. Mori A, Kennel SJ, Van Borssum-Waalkes M, Scherphof GL, Huang L: Characterization of organ-specific immunoliposomes for delivery of 3',5'-O-dipalmitoyl-5-fluoro-2'-deoxyuridine in a mouse lung-metastasis model. *Cancer Chemother Pharmacol* 1995, 35:447–456.

24. Phillips NC, Dahman J: Immunogenicity of immunoliposomes: reactivity against species-specific IgG and liposomal phospholipids. *Immunol Lett* 1995, 45:149–152.

25. Van Berkel TJC, Kruijt JK, Spanjer HH, Kempen HJM, Scherphof GL: Targeting of liposomes with tris-galactoside-terminated cholesterol. In *Liposome Technology*, vol 3, edn 2. Edited by Gregoriadis G. Boca Raton: CRC Press; 1993:219–230.

26. Barratt G, Schuber F: Targeting of liposomes with mannose-terminated ligands. In *Liposome Technology*, vol 3, edn 2. Edited by Gregoriadis G. Boca Raton: CRC Press, 1993:199–218.

27. Stavridis JC, Deliconstantinos G, Psallidopoulos MC, Armenakas NA, Hadjiminas DJ, Hadjiminas J: Construction of transferrin-coated liposomes for *in vivo* transport of exogenous DNA to bone marrow erythroblasts in rabbits. *Exp Cell Res* 1986, 164:568–572.

28. • Hara T, Aramaki Y, Takada S, Koike K, Tsuchiya S: Receptor-mediated transfer of pSV2CAT DNA to a human hepatoblastoma cell line HepG2 using asialofetuin-labeled cationic liposomes. *Gene* 1995, 159:167–174.
Describes the dehydration/rehydration method for encapsulating plasmid DNA, 60% of which is protected from DNase I treatment. Uptake of asialofetuin-labeled cationic liposomes by HepG2 cells was competitively inhibited by free asialofetuin and was sensitive to cytochalasin B treatment. Transfection activity was significantly enhanced compared with transfection using non-labeled liposomes.

29. Lee RJ, Low PS: Folate-mediated tumor cell targeting of liposome-entrapped doxorubicin *in vitro*. *Biochim Biophys Acta* 1995, 1233:134–144.

30. Tari AM, Zhou F, Huang L: Two types of pH-sensitive immunoliposomes. In *Liposome Technology*, vol 3, edn 2. Edited by Gregoriadis G. Boca Raton: CRC Press; 1993:289–300.

31. • Felgner JH, Kumar R, Sridhar CN, Wheeler CJ, Tsai YJ, Border R, Ramsey P, Martin M, Felgner PL: Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *J Biol Chem* 1994, 269:2550–2561.
A comprehensive study of structure/function relationships of cationic lipids and neutral lipids for optimization of cationic liposome-mediated gene transfer.

32. Farhood H, Serbina N, Huang L: The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim Biophys Acta* 1995, 1235:289–295.

33. Parthasarathy R, Sacks PG, Harris D, Brock H, Mehta K: Interaction of liposome-associated all-*trans*-retinoic acid with squamous carcinoma cells. *Cancer Chemother Pharmacol* 1994, 34:527–534.

34. Mehta K, Sadeghi T, McQueen T, Lopez-Berestein G: Liposome encapsulation circumvents the hepatic clearance mechanisms of all-*trans*-retinoic acid. *Leuk Res* 1994, 18:587–596.

35. Gill PS, Espina BM, Muggia F, Cabriales S, Tulpule A, Esplin JA, Liebman HA, Forssen E, Ross ME, Levine AM: Phase I/II clinical and pharmacokinetic evaluation of liposomal daunorubicin. *J Clin Oncol* 1995, 13:996–1003.

36. Gaughanone P, Chan K, Delaflor-Weiss E, Hanisch R, Jeffers S, Sharma O, Muggia F: Phase I and pharmacologic study of liposomal daunorubicin (DaunoXome). *Invest New Drugs* 1994, 12:103–110.

37. Eckardt JR, Campbell E, Burris HA, Weiss GR, Rodriguez G, Fields SM, Thurman AM, Peacock NW, Cobb P, Rothenberg ML: A phase II trial of DaunoXome, liposome-encapsulated daunorubicin, in patients with metastatic adenocarcinoma of the colon. *Am J Clin Oncol* 1994, 17:498–501.

38. Schurmann D, Dormann A, Grunewald T, Ruf B: Successful treatment of AIDS-related pulmonary Kaposi's sarcoma with liposomal daunorubicin. *Eur Respir J* 1994, 7:824–825.

39. Wasan KM, Lopez-Berestein G: The past, present, and future uses of liposomes in treating infectious diseases. *Immunopharmacol Immunotoxicol* 1995, 17:1–15.

40. Mitsutake K, Kohno S, Miyazaki Y, Noda T, Miyazaki H, Miyazaki T, Kaku M, Koga H, Hara K: *In vitro* and *in vivo* antifungal activities of liposomal amphotericin B, and amphotericin B lipid complex. *Mycopathologia* 1994, 128:13–17.

41. Heinemann V, Kahny B, Debus A, Wachholz K, Jehn U: Pharmacokinetics of liposomal amphotericin B (AmBisome) versus other lipid-based formulations. *Bone Marrow Transplant* 1994, 14:S8–S9.

42. Lee JW, Amantea MA, Francis PA, Navarro EE, Bahcer J, Pizzo PA, Walsh TJ: Pharmacokinetics and safety of a unilamellar liposomal formulation of amphotericin B (AmBisome) in rabbits. *Antimicrob Agents Chemother* 1994, 38:713–718.

43. Wasan KM, Morton RE, Rosenblum MG, Lopez-Berestein G: Decreased toxicity of liposomal amphotericin B due to association of amphotericin B with high-density lipoproteins: role of lipid transfer protein. *J Pharm Sci* 1994, 83:1006–1010.

44. Wasan KM, Rosenblum MG, Cheung L, Lopez-Berestein G: Influence of lipoproteins on renal cytotoxicity and antifungal activity of amphotericin B. *Antimicrob Agents Chemother* 1994, 38:223–227.

45. Gilbert BE, Wyde PR, Wilson SZ: Aerosolized liposomal amphotericin B for treatment of pulmonary and systemic *Cryptococcus neoformans* infections in mice. *Antimicrob Agents Chemother* 1992, 36:1466–1471.

46. Gilbert BE, Wyde PR, Lopez-Berestein G, Wilson SZ: Aerosolized amphotericin B liposomes for treatment of systemic *Candida* infections in mice. *Antimicrob Agents Chemother* 1994, 38:356–359.

47. Oku N, Doi K, Namba Y, Okada S: Therapeutic effect of adriamycin encapsulated in long-circulating liposomes on Meth-A-sarcoma-bearing mice. *Int J Cancer* 1994, 58:415–419.

48. Frezard F, Santaella C, Montisci MJ, Vierling P, Riess JG: Fluorinated phosphatidylcholine-based liposomes: H^+/Na^+ permeability, active doxorubicin encapsulation and stability in human serum. *Biochim Biophys Acta* 1994, 1194:61–68.

49. • Park JW, Hong K, Carter P, Asgari H, Guo LY, Keller GA, Wirth C, Shalaby R, Kotts C, Wood WI *et al.*: Development of anti-$p185^{HER2}$ immunoliposomes for cancer therapy. *Proc Natl Acad Sci USA* 1995, 92:1327–1331.
Describes the coupling to liposomes of anti-$p185^{HER2}$ antibodies, which retain their antiproliferative activity. Immunoliposomes are rapidly internalized via receptor-mediated endocytosis. Specific delivery of doxorubicin-loaded anti-$p185^{HER2}$ immunoliposomes to tumor cells *in vivo* was assessed 24 h after a single intraperitoneal injection, yielding a tumor:muscle ratio of 2.69 for doxorubicin levels on the basis of percentage injected dose per gramme of tissue.

50. Gabizon A, Catane R, Uziely B, Kaufman B, Safra T, Cohen R, Martin F, Huang A, Barenholz Y: Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. *Cancer Res* 1994, 54:987–992.

In this study, the pharmacokinetics in cancer patients of Doxil (doxorubicin encapsulated in PEG-containing liposomes) at two doses, 25 mg m^{-2} and 50 mg m^{-2}, was compared with free doxorubicin. With Doxil, ~30% of the injected dose was cleared from plasma, with an initial distribution half-life of 1–3 h. The rest of the Doxil was cleared slowly (second half-life of 45 h). Concomitant with the extended circulation times was an accumulation in malignant effusions, peaking between 3 days and 7 days. For free doxorubicin, the first half-life values were 0.07 h (25 mg m^{-2}) and 0.06 h (50 mg m^{-2}) and the second half-life values were 8.7 h (25 mg m^{-2}) and 10.4 h (50 mg m^{-2}).

51. Gabizon A, Isacson R, Libson E, Kaufman B, Uziely B, Catane, R, Ben-Dor CG, Rabello E, Cass Y, Peretz T *et al.*: Clinical studies of liposome-encapsulated doxorubicin. *Acta Oncol* 1994, 33:779–786.

52. Vaage J, Barbera-Guillem E, Abra R, Huang A, Working P: Tissue distribution and therapeutic effect of intravenous free or encapsulated liposomal doxorubicin on human prostate carcinoma xenografts. *Cancer* 1994, 73:1478–1484.

53. Vaage J, Donovan D, Loftus T, Abra R, Working P, Huang A: Chemoprevention and therapy of mouse mammary carcinomas with doxorubicin encapsulated in sterically stabilized liposomes. *Cancer* 1994, 73:2366–2371.

54. Vaage J, Donovan D, Loftus T, Uster P, Working P: Prophylaxis and therapy of mouse mammary carcinomas with doxorubicin and vincristine encapsulated in sterically stabilized liposomes. *Eur J Cancer* 1995, 31A:367–372.

55. Gordon KB, Tajuddin A, Guitan J, Kuzel TM, Eramo LR, VonRoenn J: Hand-foot syndrome associated with liposome-encapsulated doxorubicin therapy. *Cancer* 1995, 75:2169–2173.

56. Bogner JR, Kronawitter U, Rolinski B, Truckenbach K, Goebel FD: Liposomal doxorubicin in the treatment of advanced AIDS-related Kaposi sarcoma. *J Acquired Immune Deficiency Syndromes* 1994, 7:463–468.

57. Wagner D, Kern WV, Kern P: Liposomal doxorubicin in AIDS-related Kaposi's sarcoma: long-term experience. *Clin Invest* 1994, 72:417–423.

58. Harrison M, Tomlinson D, Stewart S: Liposomal-entrapped doxorubicin: an active agent in AIDS-related Kaposi's sarcoma. *J Clin Oncol* 1995, 13:914–920.

59. Huang SK, Stauffer PR, Hong K, Guo JW, Phillips TL, Huang A, Papahadjopoulos D: Liposomes and hyperthermia in mice: increased tumor uptake and therapeutic efficacy of doxorubicin in sterically stabilized liposomes. *Cancer Res* 1994, 54:2186–2191.

60. Unezaki S, Maruyama K, Takahashi N, Koyama M, Yuda T, Suginaka A, Iwasaru M: Enhanced delivery and antitumor activity of doxorubicin using long-circulating thermosensitive liposomes containing amphipathic polyethylene glycol in combination with local hyperthermia. *Pharm Res* 1994, 11:1180–1185.

61. Ning S, Macleod K, Abra RM, Huang AH, Hahn GM: Hyperthermia induces doxorubicin release from long-circulating liposomes and enhances their anti-tumor efficacy. *Int J Radiat Oncol Biol Phys* 1994, 29:827–834.

62. Gill PS, Rarick M, McCutchan JA, Slates L, Parker B, Muchmore E, Bernstein-Singer M, Akil B, Espina BM, Karilo M, Levine A: Systemic treatment of AIDS-related Kaposi's sarcoma: results of a randomized trial. *Am J Med* 1989, 87:57–61.

63. Daemen T, Hofstede G, Ten-Kate MT, Bakker-Woudenberg IAJM, Scherphof GL: Liposomal doxorubicin-induced toxicity: depletion and impairment of phagocytic activity of liver macrophages. *Int J Cancer* 1995, 61:716–721.

64. Suzuki S, Watanabe S, Uno S, Tanaka M, Masuko T, Hashimoto Y: Endocytosis does not necessarily augment the cytotoxicity of adriamycin encapsulated in immunoliposomes. *Biochim Biophys Acta* 1994, 1224:445–453.

65. Sela S, Husein SR, Pearson JW, Longo DL, Rahman A: Reversal of multidrug resistance in human colon cancer cells expressing the human *MDR1* gene by liposomes in combination with monoclonal antibody or verapamil. *J Natl Cancer Inst* 1995, 87:123–128.

66. Perez-Soler R, Ling YH, Zou Y, Priebe W: Cellular pharmacology of the partially non-cross-resistant anthracycline annamycin entrapped in liposomes in KB and KB-V1 cells. *Cancer Chemother Pharmacol* 1994, 34:109–118.

67. Zou Y, Ling H, Van NT, Priebe W, Perez-Soler R: Antitumor activity of free and liposome-entrapped annamycin, a lipophilic anthracycline antibiotic with non-cross-resistance properties. *Cancer Res* 1994, 54:1479–1484.

These authors assess the ability of annamycin to overcome multidrug resistance *in vitro* and *in vivo*. They report the following findings: first, MDR cell lines (KB-V1, 8226/Dox, P388/Dox and CEM/Vbl) display partial lack of cross-resistance to annamycin *in vitro*; second, liposomal annamycin is more effective than doxorubicin in the treatment of lung tumors, subcutaneous tumors, and tumors expressing the MDR phenotype *in vivo*; and third, MLVs are more effective than SUVs.

68. Boman NL, Masin D, Mayer LD, Cullis PR, Bally MB: Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors. *Cancer Res* 1994, 54:2830–2833.

In this report, use of a low internal pH and incorporation of ganglioside G_{M1} into the liposome acts synergistically to significantly enhance the circulation lifetime of encapsulated vincristine. Therapeutic activity of the resulting liposomal vincristine preparation is dramatically improved, resulting in cures in >50% of mice inoculated with P388 lymphocytic leukemia.

69. Mayer LD, Masin D, Nayar R, Boman NL, Bally MB: Pharmacology of liposomal vincristine in mice bearing L1210 ascitic and B16/BL6 solid tumours. *Br J Cancer* 1995, 71:482–488.

70. Webb MS, Harasym TO, Masin D, Bally MB, Mayer LD: Sphingomyelin–cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models. *Br J Cancer* 1995, 72:896–904.

Significantly enhanced stability and therapeutic activity is observed with a liposomal formulation that is composed of sphingomyelin/cholesterol, with an internal pH of 4.0 or 2.0. The improved circulation lifetime of vincristine in sphingomyelin/cholesterol liposomes correlates with increased vincristine accumulation in peritoneal ascitic murine P388 tumors and in subcutaneous solid A431 human xenograft tumors. Increased vincristine delivery to tumors is accompanied by increased antitumor efficacy with >50% cures in mice bearing ascitic P388 tumors. Furthermore, the time required for a 100% increase in the solid mass of human A431 xenograft tumors was delayed by >40 days compared with a delay of 7 days for free vincristine.

71. Allen TM, Newman MS, Woodle MC, Mayhew E, Uster PS: Pharmacokinetics and anti-tumor activity of vincristine encapsulated in sterically stabilized liposomes. *Int J Cancer* 1995, 62:199–204.

72. Mayer LD, Bally MB, Loughrey H, Masin D, Cullis PR: Liposomal vincristine preparations which exhibit decreased drug toxicity and increased activity against murine L1210 and P388 tumors. *Cancer Res* 1990, 50:575–579.

73. Weiner AL: Liposomes for protein delivery: selecting manufacture and development processes. *Immunomethods* 1994, 4:201–209.

74. Rudolph AS: Encapsulated hemoglobin: current issues and future goals. *Artif Cells Blood Substitute Immobil Biotechnol* 1994, 22:347–360.

75. Tsuchida E: Stabilized hemoglobin vesicles. *Artif Cells Blood Substitute Immobil Biotechnol* 1994, 22:467–477.

76. Zheng S, Zheng Y, Beissinger R: Efficacy, physical properties and pharmacokinetics of sterically-stabilized liposome-encapsulated hemoglobin. *Artif Cells Blood Substitute Immobil Biotechnol* 1994, 22:487–501.

77. Anderson PM, Hanson DC, Hasz DE, Halet MR, Blazar BR, Ochoa AC: Cytokines in liposomes: preliminary studies with IL-1, IL-2, IL-6, GM-CSF and interferon-γ. *Cytokine* 1994, 6:92–101.

78. Bergers JJ, Den Otter W, Dullens HF, Kerkvliet CT, Crommelin DJ: Interleukin-2-containing liposomes: interaction of interleukin-2 with liposomal bilayers and preliminary studies on application in cancer vaccines. *Pharm Res* 1993, 10:1715–1721.

79. Duits AJ, Van Puijenbroek A, Vermeulen H, Hofhuis FMA, Van de Winkel JGJ, Capel PJA: Immunoadjuvant activity of a liposomal IL-6 formulation. *Vaccine* 1993, 11:777–781.

80. Kedar E, Rutkowski Y, Braun E, Emanuel N, Barenholz Y: Delivery of cytokines by liposomes. I. Preparation and characterization of interleukin-2 encapsulated in long-circulating sterically stabilized liposomes. *J Immunother Emphasis Tumor Immunol* 1994, 16:47–59.

81. • Kedar E, Braun E, Rutkowski Y, Braun E, Emanuel N, Barenholz Y: Delivery of cytokines by liposomes. II. Interleukin-2 encapsulated in long-circulating sterically stabilized liposomes: immunomodulatory and anti-tumor activity in mice. *J Immunother Emphasis Tumor Immunol* 1994, 16:115–124.
Demonstrates the superior immunopotentiating activity of liposomal IL-2 compared with free IL-2 or PEG-modified IL-2. IL-2 formulated in long-circulating liposomes is shown to be more effective than free IL-2 both in increasing leukocyte number in the blood and spleen and in triggering spleen lymphokine-activated killer-cell activity. In mice with advanced metastatic carcinoma, survival was twofold to sixfold greater with liposomal IL-2 than with free IL-2.

82. Bui T, Dykers T, Hu SL, Faltynek CR, Ho RJ: Effect of MTP-PE liposomes and interleukin-7 on induction of antibody and cell-mediated immune responses to a recombinant HIV-envelope protein. *J Acquired Immune Deficiency Syndromes* 1994, 7:799–806.

83. • Bui T, Faltynek CR, Ho RJY: Biological response of recombinant interleukin-7 on herpes simplex virus infection in guinea-pigs. *Vaccine* 1994, 12:646–652.
An *in vivo* demonstration that co-administration of liposomal recombinant IL-7 enhances the immunogenicity of alum-associated HSV-gD antigen and can significantly reduce the severity and course of primary HSV-2 infection.

84. • Bama BP, Thomassen MJ, Maies M, Mederdrop SV, Tubbs RR, Chiang T, Zhou P, Yen-Lieberman B, Singh-Burgess S, Deodhar SD: Combination therapy with a synthetic peptide of C-reactive protein and interleukin 2: augmented survival and eradication of pulmonary metastases. *Cancer Immunol Immunother* 1994, 38:38–42.
Low doses of IL-2 can boost the therapeutic and immunomodulatory effects of C-reactive protein peptide RS-83277 MLVs in mice.

85. Bergers JJ, Den Otter W, Dullens, HF, De Groot JW, Steerenberg PA, Filius PM, Crommelin DJ: Effect of immunomodulators on specific tumor immunity induced by liposome-encapsulated tumor-associated antigens. *Int J Cancer* 1994, 56:721–726.

86. Kleinerman ES, Maeda M, Jaffe N: Liposome-encapsulated muramyl tripeptide: a new biologic response modifier for the treatment of osteosarcoma. *Cancer Treatment Res* 1993, 62:101–107.

87. •• Tanguay S, Bucana CD, Wilson MR, Fidler IJ, Von Eschenbach AC, Killion JJ: *In vivo* modulation of macrophage tumoricidal activity by oral administration of the liposome-encapsulated macrophage activator CGP 19835A. *Cancer Res* 1994, 54:5882–5888.
Following repeated *per os* (oral) feedings of a MLV formulation of CGP 19835A, a synthetic muramyl tripeptide, these authors observe systemic activation of tissue macrophages associated with both the release of cytokines and tumoricidal activity against syngeneic murine renal cell carcinoma cells.

88. Fox LE, King RR, Shi F, Kurzman ID, MacEwen EG, Kubilis PS: Induction of serum tumor necrosis factor-α and interleukin-6 activity by liposome-encapsulated muramyl tripeptide-phosphatidylethanolamine (L-MTP-PE) in normal cats. *Cancer Biother* 1994, 9:329–340.

89. Asano T, McWatters A, An T, Matsushima K, Kleinerman ES: Liposomal muramyl tripeptide up-regulates interleukin-1α, interleukin-1β, tumor necrosis factor-α, interleukin-6 and interleukin-8 gene expression in human monocytes. *J Pharmacol Exp Ther* 1994, 268:1032–1039.

90. Asano T, Matsushima K, Kleinerman ES: Liposome-encapsulated muramyl tripeptide up-regulates monocyte chemotactic and activating factor gene expression in human monocytes at the transcriptional and post-transcriptional levels. *Cancer Immunol Immunother* 1994, 38:16–22.

91. Wilhelm D, Schmit M, Hohl S, Senekowitsch R, Graeff H: Antisense inhibition of urokinase reduces spread of human ovarian cancer in mice. *Clin Exp Metastasis* 1995, 13:296–302.

92. Tari AM, Tucker SD, Deisseroth A, Lopez-Berestein G: Liposomal delivery of methylphosphonate antisense oligodeoxynucleotides in chronic myelogenous leukemia. *Blood* 1994, 84:601–607.

93. • Dean NK, McKay R: Inhibition of protein kinase C-α expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides. *Proc Natl Acad Sci USA* 1994, 91:11762–11766.
Demonstrates the utility of antisense oligonucleotides as specific inhibitors of gene expression *in vivo* after systemic administration of Lipofectin™-oligonucleotide complexes.

94. • Tomita N, Morishita R, Higaki J, Aoki M, Nakamura Y, Mikami H, Fukamizu A, Murakami K, Kaneda Y, Ogihara T: Transient decrease in high blood pressure by *in vivo* transfer of antisense oligodeoxynucleotides against rat angiotensinogen. *Hypertension* 1995, 26:131–136.
In vivo transfection of antisense oligonucleotides results in a transient decrease in plasma angiotensinogen levels in spontaneously hypertensive rats from 1 day to 7 days after injection.

95. Anazodo MI, Wainberg MA, Friesen AD, Wright JA: Sequence-specific inhibition of gene expression by a novel antisense oligodeoxynucleotide phosphorothioate directed against a nonregulatory region of the human immunodeficiency virus type 1 genome. *J Virol* 1995, 69:1794–1801.

96. Lappalainen K, Urtti A, Soderling E, Jaaskelainen I, Syrjanen K, Syrjanen S: Cationic liposomes mediated delivery of antisense oligonucleotides targeted to HPV 16 E7 mRNA in CaSki cells. *Antiviral Res* 1994, 23:119–130.

97. Lappalainen K, Urtti A, Soderling E, Jaaskelainen I, Syrjanen K, Syrjanen S: Cationic liposomes improve stability and intracellular delivery of antisense oligonucleotides into CaSki cells. *Biochim Biophys Acta* 1994, 1196:201–208.

98. Jaaskelainen I, Monkkonen J, Urtti A: Oligonucleotide–cationic liposome interactions. A physicochemical study. *Biochim Biophys Acta* 1994, 1195:115–123.

99. Puyal C, Milhaud P, Bienvenue A, Philippot JR: A new cationic liposome encapsulating genetic material: a potential delivery system for polynucleotides. *Eur J Biochem* 1995, 228:697–703.

100. Zelphati O, Imbach JL, Signoret N, Zon G, Rayner B, Leserman L: Antisense oligonucleotides in solution or encapsulated in immunoliposomes inhibit replication of HIV-1 by several different mechanisms. *Nucleic Acids Res* 1994, 22:4307–4314.

101. • Aigner L, Caroni P: Absence of persistent spreading, branching and adhesion in GAP-43-depleted growth cones. *J Cell Biol* 1995, 128:647–660.
Describes the use of myelin-derived liposomes to deliver antisense oligonucleotides to growth-associated protein GAP-43 to study its biological function. The findings indicate that GAP-43 promotes f-actin accumulation, evoking morphogenic activity and resistance to retraction of the growth cone.

102. •• Wang S, Lee RJ, Cauchon G, Gorenstein DG, Low PS: Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. *Proc Natl Acad Sci USA* 1995, 92:3318–3322.
Convincingly demonstrates the benefits of targeted liposomal delivery of antisense oligonucleotides *in vitro*. Specific delivery to KB tumor cells of antisense oligonucleotides against human EGF receptor using

liposomes containing folate as a targeting ligand. Delivery (2×10^7 EGF receptor molecules cell^{-1}) was enhanced 10-fold compared with non-targeted liposomes, whereas uptake of free oligonucleotides was barely detectable. Cell uptake was inhibited by the presence of 1 mM free folate. Phosphorothioate oligonucleotides and native phosphodiester DNA, when delivered via this method, exhibited similar growth inhibition activities.

103. Zelphati O, Wagner E, Leserman L: Synthesis and anti-HIV activity of thiocholesteryl-coupled phosphodiester antisense oligonucleotides incorporated into immunoliposomes. *Antiviral Res* 1994, 25:13–25.

Investigates the feasibility of encapsulating cholesterol-coupled antisense oligonucleotides in immunoliposomes. The authors successfully synthesize 100 nm LUVs containing 80–160 oligonucleotide molecules per liposome (with encapsulation efficiencies of 20–30%). Oligonucleotides were protected from DNase I digestion. The capacity of the modified anti-HIV antisense oligonucleotides to inhibit the replication of HIV was the same on an equimolar basis as that of unmodified oligonucleotides, as demonstrated *in vitro* using an HIV proliferation assay in acutely infected CEM cells. Furthermore, the cholesteryl-modified oligonucleotides acquire the target specificity of the antibody on the liposome.

104. Shea RG, Marsters JC, Bischofberger N: Synthesis, hybridization properties and antiviral activity of lipid–oligodeoxynucleotide conjugates. *Nucleic Acids Res* 1990, 18:3777–3783.

105. Krieg AM, Tonkinson J, Matson S, Zhao Q, Saxon M, Zhang LM, Bhanja U, Yakubov L, Stein CA: Modification of antisense phosphodiester oligodeoxynucleotides by a 5′ cholesteryl moiety increases cellular association and improves efficacy. *Proc Natl Acad Sci USA* 1993, 90:1048–1052.

106. Boutorine AS, Gus'kova LV, Ivanova EM, Kobetz ND, Zarytova VF, Ryte AS, Yurchenko LV, Vlassov VV: Synthesis of alkylating oligonucleotide derivatives containing cholesterol or phenazinium residues at their 3′-terminus and their interaction with DNA within mammalian cells. *FEBS Lett* 1989, 254:129–132.

107. Kabanov AV, Vinogradov SV, Ovcharenko AV, Krivonos AV, Melik-Nubarov NS, Kiselev VI, Severin ES: A new class of antivirals: antisense oligonucleotides combined with a hydrophobic substituent effectively inhibit influenza virus reproduction and synthesis of virus-specific proteins in MDCK cells. *FEBS Lett* 1990, 259:327–330.

108. Gu JL, Veerapanane D, Rossi J, Natarajan R, Thomas L, Nadler J: Ribozyme-mediated inhibition of expression of leukocyte-type 12-lipoxygenase in porcine aortic vascular smooth muscle cells. *Circ Res* 1995, 77:14–20.

Describes the first chimeric hammerhead ribozyme active against an eicosanoid-generating mRNA. Efficient delivery into porcine aortic vascular smooth muscle cells is achieved by transfection with cationic liposomes.

109. Taylor NR, Kaplan BE, Swiderski P, Li H, Rossi JJ: Chimeric DNA–RNA hammerhead ribozymes have enhanced *in vitro* catalytic efficiency and increased stability *in vivo*. *Nucleic Acids Res* 1992, 20:4559–4565.

110. Leopold LH, Shore SK, Mewkirk TA, Reddy RMV, Reddy EP: Multi-unit ribozyme-mediated cleavage of *bcr-abl* mRNA in myeloid leukemias. *Blood* 1995, 85:2162–2170.

Transfer of multi-unit ribozymes against *bcr-abl* mRNA into murine myeloblasts transformed with the *bcr-abl* gene is compared using cationic liposomes and folic acid/polylysine liposomes.

111. Kiehntopf M, Brach MA, Licht T, Petschauer S, Karawajew L, Kirschning C, Herrmann F: Ribozyme-mediated cleavage of the MDR-1 transcript restores chemosensitivity in previously resistant cancer cells. *EMBO J* 1994, 13:4645–4652.

Demonstrates *in vitro* the feasibility of liposome-mediated transfer of hammerhead ribozymes directed against MDR-1 transcripts, reversing the MDR phenotype in human pleural mesothelioma cell lines that are vindesine or doxorubicin resistant.

112. Ledley FD: Non-viral gene therapy. *Curr Opin Biotechnol* 1994, 5:626–636.

113. Miller N, Vile R: Targeted vectors for gene therapy. *FASEB J* 1995, 9:190–199.

114. Smith JG, Walzem RL, German JB: Liposomes as agents of DNA transfer. *Biochim Biophys Acta* 1993, 1154:327–340.

115. Zhou X, Huang L: DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. *Biochim Biophys Acta* 1994, 1189:195–203.

116. Wrobel I, Collins D: Fusion of cationic liposomes with mammalian cells occurs after endocytosis. *Biochim Biophys Acta* 1995, 1235:296–304.

Using a lipid mixing assay, these authors demonstrate that binding to the cell surface is insufficient for fusion of the cationic liposome with the cell and that uptake into the endocytic pathway is required for fusion to occur.

117. Gustafsson J, Arvidson G, Karlsson G, Almgren M: Complexes between cationic liposomes and DNA visualized by cryo-TEM. *Biochim Biophys Acta* 1995, 1235:305–312.

118. Gershon H, Ghirlando R, Guttman SB, Minsky A: Mode of formation and structural features of DNA–cationic liposome complexes used for transfection. *Biochemistry* 1993, 32:7143–7151.

119. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ: Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem* 1995, 270:18997–19007.

Comprehensive study investigating the key cellular processes required for cationic lipid-mediated gene transfer.

120. Caplen NJ, Alton EWFW, Middleton PG, Dorin JR, Stevenson BJ, Gao X, Durham SR, Jeffery PK, Hodson ME, Coutelle C *et al.*: Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nature Med* 1995, 1:39–46.

First placebo-controlled trial of liposome-mediated *CFTR* cDNA transfer to patients with cystic fibrosis. The safety and efficacy of 300 mg per nostril of liposome-encapsulated *CFTR* is compared with an equivalent dose of liposome alone. No evidence of treatment-related toxicity, either clinically or histologically, was detected in any of the treated subjects.

A Chonn, INEX Pharmaceuticals Corporation, 1779 West 75th Avenue, Vancouver, British Columbia V6P 6P2, Canada.
E-mail: achonn@inexpharm.com
PR Cullis, Department of Biochemistry and Molecular Biology, The University of British Columbia, 2146 Health Sciences Mall, Vancouver, British Columbia V6T 1Z3, Canada and INEX Pharmaceuticals Corporation, 1779 West 75th Avenue, Vancouver, British Columbia V6P 6P2, Canada.
E-mail: pcullis@inexpharm.com

JOINT APPENDIX 51

ELSEVIER

Biochimica et Biophysica Acta 1419 (1999) 137–150

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Stabilized plasmid-lipid particles: factors influencing plasmid entrapment and transfection properties

Kenneth W.C. Mok [a], Angela M.I. Lam [a], Pieter R. Cullis [a,b,*]

[a] *Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, B.C. V6T 1Z3, Canada*

[b] *Inex Pharmaceuticals Corporation, 100-8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, B.C. V5J 5J8, Canada*

Received 19 January 1999; received in revised form 9 April 1999; accepted 13 April 1999

Abstract

Previous work has shown that plasmid DNA can be encapsulated in small 'stabilized plasmid-lipid particles' (SPLP) composed of 1,2-dioleyl-3-phosphatidylethanolamine (DOPE), the cationic lipid *N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride (DODAC) and poly(ethylene glycol) (PEG) conjugated ceramides (PEG-Cer), employing a detergent dialysis procedure. These SPLP have potential as vectors for in vivo gene therapy. This study is aimed at characterizing the influence of the cationic lipid and PEG-Cer species on SPLP formation and in vitro transfection properties. It is shown that the transfection potency of SPLP is sensitive to the cationic lipid species employed, the size of the PEG polymer incorporated in the PEG-ceramide and the length of the acyl chain contained in the ceramide anchor. With regard to the influence of cationic lipid, the transfection levels achieved were highest for SPLP containing *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*-dimethyl-*N*-cyanomethylammonium chloride (DODMA-AN) and lowest for SPLP containing 3-β-[*N*-(*N*′,*N*′-dimethylaminoethyl)carbamoyl]-cholesterol (DC-CHOL), according to the series DODMA-AN > *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) > DODAC > *N*,*N*-distearyl-*N*,*N*-dimethylammonium chloride (DSDAC) > DC-CHOL. Incorporation of short (PEG_{750}) PEG polymers in the PEG-ceramide components resulted in modest improvements in transfection levels over PEG_{2000} and PEG_{5000} polymers, however variation of the length of the acyl chain contained in the hydrophobic ceramide anchor from octanoyl (PEG-$CerC_8$) to myristoyl (PEG-$CerC_{14}$) to arachidoyl (PEG-$CerC_{20}$) had the most dramatic effects. Transfection levels achieved for SPLP containing PEG-$CerC_8$ were substantially larger than observed for SPLP containing PEG-$CerC_{14}$ or PEG-$CerC_{20}$, consistent with a requirement for the PEG-ceramide to dissociate from the SPLP surface for maximum transfection potency. It is also shown that the ability of SPLP to be accumulated into cells is

Abbreviations: β-gal, β-galactosidase; BHK, baby hamster kidney; CAT, chloramphenicol acetyltransferase; ^{14}C-CHE, ^{14}C-labeled cholesteryl hexadecyl ether; CMC, critical micellar concentration; CMV, cytomegalovirus; DC-CHOL, 3-β-[*N*-(*N*′,*N*′-dimethylaminoethyl)carbamoyl]-cholesterol; DEAE-Sepharose, diethylaminoethyl-Sepharose; DODAC, *N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride; DODMA-AN, *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*-dimethyl-*N*-cyanomethylammonium chloride; DOPE, 1,2-dioleoyl-3-phosphatidylethanolamine; DOTMA, *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; DSDAC, *N*,*N*-distearyl-*N*,*N*-dimethylammonium chloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; ^{3}H-pCMVCAT, ^{3}H-labeled pCMVCAT plasmid; LUVs, large unilamellar vesicle system; OGP, *n*-octyl-β-D-glucopyranoside; pCMVβgal, pCMVβgal plasmid; pCMVCAT, pCMVCAT plasmid; PEG-$CerC_8$, 1-*O*-(2′-(ω-methoxypolyethyleneglycol$_{(2000)}$)succinoyl)-2-*N*-octanoylsphingosine; PEG-$CerC_{14}$, 1-*O*-(2′-(ω-methoxypolyethyleneglycol$_{(2000)}$)succinoyl)-2-*N*-myristoylsphingosine; PEG-$CerC_{20}$, 1-*O*-(2′-(ω-methoxypolyethyleneglycol$_{(2000)}$)succinoyl)-2-*N*-arachidoylsphingosine; PEG_{750}-$CerC_{14}$, 1-*O*-(2′-(ω-methoxypolyethyleneglycol$_{(750)}$)succinoyl)-2-*N*-myristoylsphingosine; PEG_{5000}-$CerC_{14}$, 1-*O*-(2′-(ω-methoxypolyethyleneglycol$_{(5000)}$)succinoyl)-2-*N*-myristoylsphingosine; QELS, quasi-elastic light scattering

* Corresponding author. Fax: +1 (604) 8224843; E-mail: pieterc@interchange.ubc.ca

PII: S0005-2736(99)00059-0

a dominant factor influencing transfection potency, and that the transfection potency of SPLP that are accumulated is at least equivalent to that of cationic lipid-plasmid DNA complexes.

Keywords: Cationic lipid; Liposome; Poly(ethylene glycol); Gene therapy

1. Introduction

Plasmid DNA-cationic liposome complexes have considerable utility as non-viral gene transfer systems [1,2]. However, complexes are large, charged, heterogeneous structures that have limited utility in certain applications. For example, the large size and positive charge of complexes results in rapid clearance from the circulation following intravenous injection, largely limiting transfection to 'first pass' organs such as the lung, liver or spleen [1–6]. In this regard recent work employing a detergent dialysis technique [7] has shown that plasmid DNA can be encapsulated in 'stabilized plasmid-lipid particles' (SPLP) that are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. These particles are small (diameter approximately 70 nm), contain high (>80 mol%) levels of the 'fusogenic' lipid dioleylphosphatidylethanolamine (DOPE), low (<10 mol%) levels of the cationic lipid *N*,*N*-dioleyl-*N*,*N*-dimethylammonium chloride (DODAC), protect encapsulated plasmid from degradation by serum nucleases, and are well-defined and stable systems containing one plasmid per particle [7].

The transfection potency of SPLP as generated by Wheeler et al. [7] is limited. However, SPLP are flexible systems that can potentially be constructed from a variety of compounds, the choice of which may strongly affect transfection properties. Such compounds include the cationic lipid contained in the SPLP and the PEG coating surrounding the SPLP. For example, plasmid DNA-cationic lipid complexes which contain different cationic lipids can exhibit markedly different transfection properties both in vitro and in vivo [6,8–12]. Alternatively, it has been shown that the presence of PEG coatings on vesicles can dramatically inhibit intervesicular contact and fusion [13]; and the presence of PEG-PE in DNA-cationic lipid complexes can dramatically reduce in vivo transfection activity [14].

In this work we investigate the influence of different species of cationic lipid and PEG coatings on the formulation and in vitro transfection properties of SPLP. It is shown that whereas the SPLP formulation process is relatively independent of the (monovalent) cationic lipid species employed, the highest levels of expression are observed for *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*-dimethyl-*N*-cyanomethylammonium chloride (DODMA-AN). Alternatively, incorporation of shorter acyl groups in the ceramide which 'anchors' the PEG to the SPLP surface, which results in rapid dissociation of the PEG from the SPLP surface, dramatically improves transfection levels. Finally, it is shown that low levels of cellular uptake is a dominant parameter modulating the transfection potential of SPLP, and that the intrinsic transfection potency of SPLP that are taken up is at least equivalent to that exhibited by complexes.

2. Materials and methods

2.1. Lipids and chemicals

N,*N*-Dioleyl-*N*,*N*-dimethylammonium chloride (DODAC), *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*-dimethyl-*N*-cyanomethylammonium chloride (DODMA-AN), *N*,*N*-distearyl-*N*,*N*-dimethylammonium chloride (DSDAC), 3-β-[*N*-(*N*′,*N*′-dimethylaminoethyl)-carbamoyl]-cholesterol (DC-CHOL) were obtained from Dr. S. Ansell (Inex Pharmaceuticals, Burnaby, B.C., Canada). 1-*O*-(2′-(ω-methoxypolyethyleneglycol$_{(2000)}$)succinoyl)-2-*N*-octanoylsphingosine (PEG-CerC$_8$), 1-*O*-(2′-(ω-methoxypolyethyleneglycol$_{(2000)}$)-succinoyl)-2-*N*-myristoylsphingosine (PEG-CerC$_{14}$), 1-*O*-(2′-(ω-methoxypolyethyleneglycol$_{(2000)}$)succinoyl)- 2-*N*-arachidoylsphingosine (PEG-CerC$_{20}$), 1-*O*-(2′-(ω-methoxypolyethyleneglycol$_{(750)}$)succinoyl)-2-*N*-myristoylsphingosine (PEG$_{750}$-CerC$_{14}$), 1-*O*-(2′-(ω-methoxypolyethyleneglycol$_{(5000)}$)succinoyl)-2-*N*-myristoylsphingosine (PEG$_{5000}$-CerC$_{14}$) were obtained from Dr. Z. Wang (Inex), and *N*-[2,3-(dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) was synthesized as described in previous

work [15]. 1,2-Dioleoyl-3-phosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). ^{14}C-labeled cholesteryl hexadecyl ether (^{14}C-CHE) was obtained from DuPont NEN Products (Boston, MA, USA). Spectra/Por 2 molecular-porous membrane tubing (MW 12 000–14 000) was purchased from VWR Scientific (Edmonton, Alta, Canada). Magnesium sulfate, β-mercaptoethanol, potassium acetate, potassium chloride, sodium acetate, sodium phosphate, sodium chloride, sodium hydroxide, and sucrose were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ampicillin, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), lithium chloride (LiCl), lysozyme, *n*-octyl-β-D-glucopyranoside (OGP), poly(ethylene glycol) (PEG_{8000}), sodium dodecyl sulfate, tris(hydroxymethyl)aminomethane (Tris), diethylaminoethyl (DEAE) Sepharose CL-6B anion exchanger, and Sepharose CL-4B resins were obtained from Sigma (St. Louis, MO, USA). Bacto-tryptone and bacto-yeast extract were purchased from Difco (Detroit, MI, USA). All organic solvents were purchased from Fisher Scientific (Nepeau, Ont., Canada). Agarose was purchased from Bio-Rad (Richmond, CA, USA). Aquacide II was purchased from Calbiochem (La Jolla, CA, USA). Glycerol and glucose were obtained from BDH (Toronto, Ont., Canada). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco BRL (Burlington, Ont., Canada). Chlorophenol red galactopyranoside (CPRG) was purchased from Boehringer Mannheim (Germany). Scintillation fluid (Ultima Gold grade) was obtained from Packard Instrument (Downers Grove, IL, USA). *Xho*I and *Hin*dIII restriction endonuclease and ribonuclease I 'A' from bovine pancreas (RNase) were purchased from Pharmacia Biotech (Uppsala, Sweden). BHK cells (BHK 21) were obtained from American Tissue Culture Collection (ATCC CCL-10). Distilled water was purified from Corning Mega-Pure MP-4S system.

2.2. Plasmid

pCMVCAT plasmid, initially obtained from Inex, and pCMVβgal plasmid, initially purchased from Clontech, were amplified in *Escherichia coli* (DH5α) with the selection of resistance to ampicillin, and were isolated by alkali lysis and purified by PEG precipitation as described elsewhere [15,16]. The purity of pCMVCAT and pCMVβgal was confirmed by 1% agarose gel electrophoresis with restriction endonuclease *Xho*I or *Hin*dIII digest, respectively. The plasmid concentration was determined by using standard phosphorus assays [17] and was expressed as phosphorus concentration of plasmid DNA. ^{3}H-labeled pCMVCAT was obtained from A. Annuar (Inex).

2.3. Preparation of vesicles

Mixtures of lipids dispensed in chloroform were dried under a stream of nitrogen gas with continuous vortex mixing. The residual solvent was removed under high vacuum for 2 h. The resulting lipid films were hydrated with 150 mM NaCl, 20 mM HEPES (pH 7.4) buffer and then freeze-thawed five times to produce multilamellar vesicle (MLV) systems. Large unilamellar vesicle system (LUVs) were obtained by extruding MLVs 10 times through two 100-nm pore size polycarbonate filters (Costar Nuclepore) under nitrogen at a pressure of 300–400 psi [18]. The size of the LUVs was checked by quasi-elastic light scattering (QELS) employing a Nicomp model 270 submicron particle sizer operating in the vesicle mode. Phosphorus assays were used to quantify phospholipid concentration [17].

2.4. Preparation of plasmid DNA-cationic lipid complexes

Plasmid DNA-cationic lipid complexes were formed by incubating appropriate amounts of preformed DOTMA/DOPE (1:1) LUVs with pCMVβgal to obtain the desired charge ratio (positive-to-negative) of 1.0 in 100 μl distilled water. The resulting mixture was incubated at room temperature for 20–30 min, and was then mixed with an equal volume of culture media (see below).

2.5. Preparation of stabilized plasmid-lipid particles (SPLP)

SPLP were prepared employing the method of Wheeler et al. [7] with modifications. Briefly, for

each preparation, appropriate amounts of cationic lipid dissolved in chloroform were dried under a stream of nitrogen gas. Similarly, appropriate amounts of DOPE, PEG-ceramide and trace amounts of ^{14}C-CHE (as the lipid marker) were mixed in chloroform and dried. Residual solvent was removed under high vacuum for 2 h. The resulting lipid films were hydrated separately. *n*-Octyl-β-D-glucopyranoside (OGP, 50 μl of a 1.0 M solution) was added to the dried cationic lipid followed by brief vortexing. Subsequently 200 μl of an aqueous solution containing appropriate amounts of NaCl, pCMVCAT and trace amounts of ^{3}H-pCMVCAT was added. Unless indicated otherwise, 25 μg pCMVCAT and 5.0 μmol total lipids in 0.5 ml total volume were used for each preparation in the entrapment studies. After thorough mixing, the clear homogenous mixture was incubated at room temperature for 30 min. A final concentration of 200 mM OGP in 150 mM NaCl was used for each preparation. The lipid-containing medium was made by adding 250 μl of 200 mM OGP in 150 mM NaCl to the dried DOPE/PEG-Cer/^{14}C-CHE. After mixing and incubating at room temperature for 30 min, a clear homogeneous mixture was obtained. The two mixtures were then mixed together and transferred into a 6 cm Spectra/Por 2 molecular-porous membrane tubing (MW 12 000–14 000) for dialysis for 40 h against two changes of a 150 mM NaCl, 5 mM HEPES (pH 7.4) buffer. The SPLP thus formed were then purified by DEAE anion exchange chromatography and sucrose density gradient centrifugation as indicated below, and characterized with respect to plasmid entrapment and size. All experiments involved triplicate samples. For the transfection studies, samples of SPLP containing pCMVβgal were prepared similarly with the exception that 400 μg pCMVβgal, 10 μmol total lipids, and a total volume of 1.0 ml were used initially.

2.6. Quantification of DNA entrapment and lipid recovery using anion exchange column chromatography

A column chromatography procedure employing DEAE-Sepharose CL-6B to remove free DNA was utilized [7]. The average of two 40 μl aliquots of the SPLP dialysate radioactivity was used as a reference for ^{3}H and ^{14}C radioactivity. The SPLP solution (100 μl of the dialysate) was applied to the DEAE-Sepharose column (diameter 1.0 cm, height 1.5 cm) and eluted using 150 mM NaCl, 20 mM HEPES (pH 7.4) buffer. Six fractions of 10 droplets each were collected in scintillation vials and were counted for ^{3}H and ^{14}C radioactivity. The percentage of recovery was obtained by comparing the total eluant radioactivity with the reference radioactivity after background correction. Thus the ^{3}H recovery represents the fraction of DNA associated with the SPLP and sequestered from the anion exchanger, whereas the ^{14}C recovery represents the fraction of lipid in the void volume. Free plasmid DNA bound to the column could be washed out using 10 ml of a 5.0 M NaCl solution. A Beckman LS3801 scintillation counter was used for all radioactivity measurements.

2.7. Purification of SPLP using sucrose density gradient centrifugation

All samples of SPLP used for transfection were further purified using sucrose density gradient centrifugation [7]. SPLP were prepared as outlined above with 400 μg pCMVβgal and 10 μmol total lipids in a total volume of 1.0 ml and free DNA was removed by passing through a DEAE column. The diluted eluant (3 ml) was transferred to a dialysis bag and briefly dried by placing Aquacide II around the bag. After the desired volume was reached, the contents were dialyzed overnight in 150 mM NaCl, 5 mM HEPES (pH 7.4) buffer. The concentrated eluant (800 μl) was then subjected to sucrose density gradient centrifugation (160 000×*g* for 2.5 h). The gradient was formed by loading 3.67 ml each of 1.0%, 2.5%, and 10% sucrose in 150 mM NaCl, 20 mM HEPES, pH 7.4 buffer into a centrifuge tube (Beckman Ultra-Clear Tubes) using a drawn out glass pipette. The crude SPLP preparation was then applied to the top of the gradient and centrifuged using a swinging bucket rotor (SW-41Ti) in an ultracentrifuge (Beckman L2-65B). Following centrifugation, a band of concentrated SPLP with high DNA content was observed at the interface between the 2.5% and 10% sucrose gradient levels. The fractionation profile was achieved by removing 250 μl fractions from the top of the gradient, and these fractions were counted for ^{3}H and ^{14}C radioactivity. An ali-

quot (100 μl) of the sample before density gradient separation was also counted as a reference. For the transfection studies, the band corresponding to the SPLP with high DNA content was isolated using needle suction. The resulting purified SPLP were concentrated by Aquacide II treatment as outlined above, and then dialyzed against 150 mM NaCl, 5 mM HEPES (pH 7.4) with one change of buffer. Quantification of DNA and lipid was performed by comparing the ^{3}H and ^{14}C radioactivity of the purified SPLP against the radioactivity of crude SPLP before sucrose density gradient. The overall recoveries of DNA and lipid were computed by multiplying the recovery from the DEAE column by the recovery from sucrose density gradient. For size analysis, purified SPLP were transferred into a 6×50 mm borosilicate glass tube and placed in a Nicomp Model 270 submicron particle sizer, using the particles mode of QELS.

2.8. Transfection studies employing BHK cells

The transfection protocol of Felgner et al. [11] was employed with certain modifications. Purified SPLP were prepared as described above. Transfection studies employing SPLP were conducted on samples containing 2.0 μg pCMVβgal. This high amount of plasmid could be employed due to the low toxicity of SPLP. Complexes (charge ratio of 1.0) were formed by incubating DOTMA/DOPE (1:1) vesicles with appropriate amounts of pCMVβgal for 30 min before transfection, as outlined in previous work [15]. Transfection studies employing complexes used 0.5 μg plasmid. All transfection studies were performed in triplicate. Standards of β-galactosidase (β-gal) were prepared by two-fold serial dilutions of 200 mU β-gal with 0.5% BSA in phosphate buffered saline (PBS) (pH 8.0). First, BHK 21 cells cultured in DMEM with 10% fetal bovine serum (FBS) and 100 units of penicillin and 100 μg streptomycin were plated onto a 96 well plate with 2×10^{4} cells per well. Wells used for the DNA standards were not plated with cells. The plate was then incubated for 20 h at 37°C with 5% CO_2. For each well containing the cells, appropriate amounts of complexes or SPLP were diluted with DMEM/FBS, and aliquots of 100 μl were used for transfection at 37°C, 5% CO_2 with incubation time of 4 or 24 h, respectively. For the 4 h transfection studies, the transfected media was removed, and 100 μl of DMEM/FBS was added. The cells were further incubated for 20 h at 37°C, 5% CO_2. After incubation, the incubated media was removed, and the cells were lysed by adding 50 μl of lysis buffer containing 0.1% Triton X-100 in 250 mM phosphate buffer (pH 8.0) and were freeze-thawed (−70°C) to ensure complete lysis. After thawing, aliquots of 10 μl of the lysis buffer was transferred to another 96 well plate for protein analysis. The remaining samples were assayed for β-gal activity. Briefly, 50 μl of PBS containing 0.5% BSA or 50 μl of appropriate β-gal standard (0–100 mU) was added. Color development was induced by adding 150 μl of the substrate buffer containing chlorophenol red galactopyranoside (CPRG) (1 mg/ml), 60 mM Na_2HPO_4, 1 M $MgSO_4$, 10 mM KCl, and 50 mM β-mercaptoethanol. For the protein assay, 50 μl of BSA standard (0–20 μg) was prepared by serial dilution in lysis buffer. An additional 40 μl of lysis buffer was added to the 10 μl sample aliquot. The BCA reaction mixture was prepared according to established protocols from Pierce (Rockford, IL, USA) prior to the assay, and 100 μl of this mixture was added to each well, including the protein standards. The plate was incubated at 37°C for 2 h or until the color was developed. The amounts of β-gal and protein were quantified against the standard, after volume adjustment, and the β-gal activity was expressed as mU of β-gal per mg of protein. All absorbance readings were measured at 540 nm using a Microplate Autoreader EL-309 (Bio-Tek Instruments). For the transfection of SPLP formed with different PEG-Cer, 1×10^{5} BHK cells were plated in a 24 well plate. Aliquots of 60 μl and 10 μl were used for the BSA and BCA assay, respectively.

2.9. Cellular uptake studies of plasmid DNA

BHK cells were plated at a density of 5×10^{5} cells per 25 cm^2 T-flask the day before transfection. For each transfection, complexes containing 2 μg pCMVβgal at a charge ratio of 1.0 were formed by incubating DOTMA/DOPE (1:1) LUVs with pCMVβgal for 20–30 min at room temperature before transfection. Purified SPLP samples (DOPE/DOTMA/PEG_{2000}-$CerC_8$, 73:7:20) containing 2 μg pCMVβgal were also used for each transfection.

Both plasmid DNA-cationic lipid complexes and SPLP samples were made up to a final volume of 2 ml with DMEM containing 10% FBS before applying to BHK cells. For the cells transfected with complexes, the transfection medium was replaced with complete DMEM after the 4 h incubation time point. Cells transfected with the SPLP samples were incubated in the transfection medium until the specific time point. All transfection studies were performed in triplicate. The cellular uptake kinetics of the plasmid DNA were analyzed by terminating the transfection process at 4, 8, 24, and 48 h. At each time point, the transfection medium was removed. The cells were washed twice with PBS, and then treated with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4Na from Gibco BRL). The cells were then washed with an isotonic buffer (250 mM sucrose, 50 mM HEPES, pH 7.2, 3 mM $MgCl_2$) and were centrifuged at 2000 rpm for 2 min in a Sorvall MC 12 V centrifuge. The resulting cell pellets were resuspended in the isotonic buffer, and the number of cells was counted using a hemacytometer. Then, the cells were centrifuged at 2000 rpm for 2 min, and the pellets were treated with the lysis buffer (10 mM Tris, pH 7.5, 0.5% SDS, 1 mM EDTA) containing Pronase E (1 mg/ml) at 37°C overnight.

2.10. Southern blot analysis of delivered plasmid DNA

Genomic DNA was isolated from the BHK cells transfected with DNA-cationic lipid complexes and SPLP [16]. Briefly, the cell lysates were extracted twice with phenol/chloroform (1:1), then the DNA was precipitated with 95% ethanol and resuspended in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 200 μl per 1×10^6 cells. The recovery of total genomic DNA was determined by measuring the absorbance at 260 nm of the resuspended samples. Genomic DNA (5 μg) from each sample was loaded into a 1% agarose gel with a set of plasmid DNA standards (0–5 ng). After size fractionation, the agarose gel was incubated in a denaturing buffer (1.5 M NaCl, 0.5 N NaOH) for 1 h, and then in a neutralizing solution (1.5 M NaCl, 1 M Tris, pH 7.4) for 45 min. The DNA fragments were transferred to a nylon membrane by capillary blotting overnight with 3 M NaCl, 0.3 M sodium citrate at pH 7.0. The nylon membrane was then baked at 80°C for 1 h prior to the hybridization procedure. A ^{32}P-labeled plasmid DNA probe was prepared using the ^{T7}QuickPrime kit (Pharmacia Biotech) with *Bam*HI cut pCMVβgal. This probe was then added to the DNA blot and allowed to hybridize overnight at 68°C. The blot was washed 3 times with 300 mM NaCl, 30 mM sodium citrate (pH 7.0) containing 0.1% SDS and once with 30 mM NaCl, 3 mM sodium citrate (pH 7.0) containing 0.1% SDS. The blot was then exposed for 2–4 h on a PhosphoImager screen and subsequently scanned (Molecular Dynamics-PhosphoImager SI). The amount of plasmid DNA taken up into cells was normalized by dividing the total plasmid DNA (pg) recovered by the total genomic DNA (μg).

3. Results

3.1. Influence of cationic lipid species on formation of SPLP

Previous work has shown that incubation of plasmid DNA with the lipid mixture DOPE, PEG_{2000}-$CerC_{14}$ and the cationic lipid DODAC (84:10:6; molar ratios) in the presence of OGP followed by dialysis results in the formation of SPLP that are capable of low levels of transfection in vitro [7]. It is of interest to determine whether SPLP can be formed using other cationic lipids, and whether this influences transfection potency. Here we characterize SPLP formation and plasmid entrapment achieved using DOTMA, DODMA-AN, DSDAC, DODAC, and DC-CHOL (for structures see Fig. 1) using a total of 5.0 μmol lipid and 25 μg pCMVCAT plasmid. In these systems the PEG-$CerC_{14}$ content was held constant at 10 mol% of total lipid, and the cationic lipid content varied over the 0–20 mol% range. Plasmid entrapment was assayed employing the DEAE anion exchange column procedure detailed Section 2. As shown in Fig. 2, optimum entrapment levels of approximately 60% were achieved for each of the cationic lipids used, the only difference being that this maximum entrapment was observed at slightly different cationic lipid content depending on the cationic lipid species. The optimal lipid composition (DOPE/cationic lipid/PEG-$CerC_{14}$; molar ratios) for plasmid encapsulation was 83:7:10 for DOTMA and DOD-

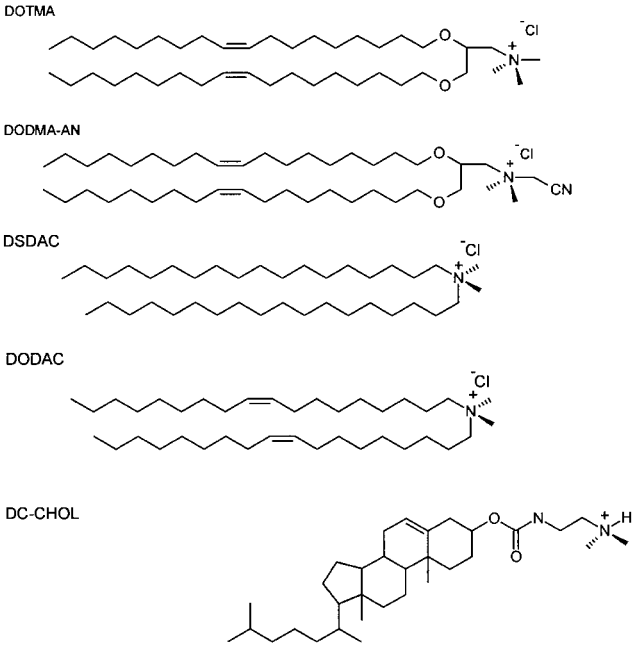


Fig. 1. Structures of the cationic lipids used in this study.

MA-AN, 82.5:7.5:10 for DSDAC and DODAC, and 81:9:10 for DC-CHOL. These SPLP exhibited particle sizes of 90 ± 20 nm as measured by QELS.

3.2. Influence of the PEG polymer anchor on formation of SPLP

The PEG coating of SPLP is likely to inhibit association with cells, thus reducing transfection efficiency. Previous work has shown that SPLP constructed from PEG-CerC_{20} did not result in appreciable transfection in vitro, whereas limited transfection was observed for SPLP containing PEG-CerC_{14} [7]. This was attributed to an ability of the PEG-CerC_{14} to dissociate from the SPLP surface. Here, we also examine the formulation and transfection properties of SPLP containing PEG_{2000} polymers linked to ceramide anchors containing octanoyl acyl groups (PEG-CerC_8). Results for SPLP containing PEG-CerC_{14} and PEG-CerC_{20} are also presented for comparison (for structures see Fig. 3). Preliminary experiments suggested that more than 10 mol% of the PEG-CerC_8 was required to achieve optimal plasmid entrapment and, therefore, the entrapment profile was generated as a function of PEG-ceramide content rather than cationic lipid content. The detergent dialysis protocol was then applied

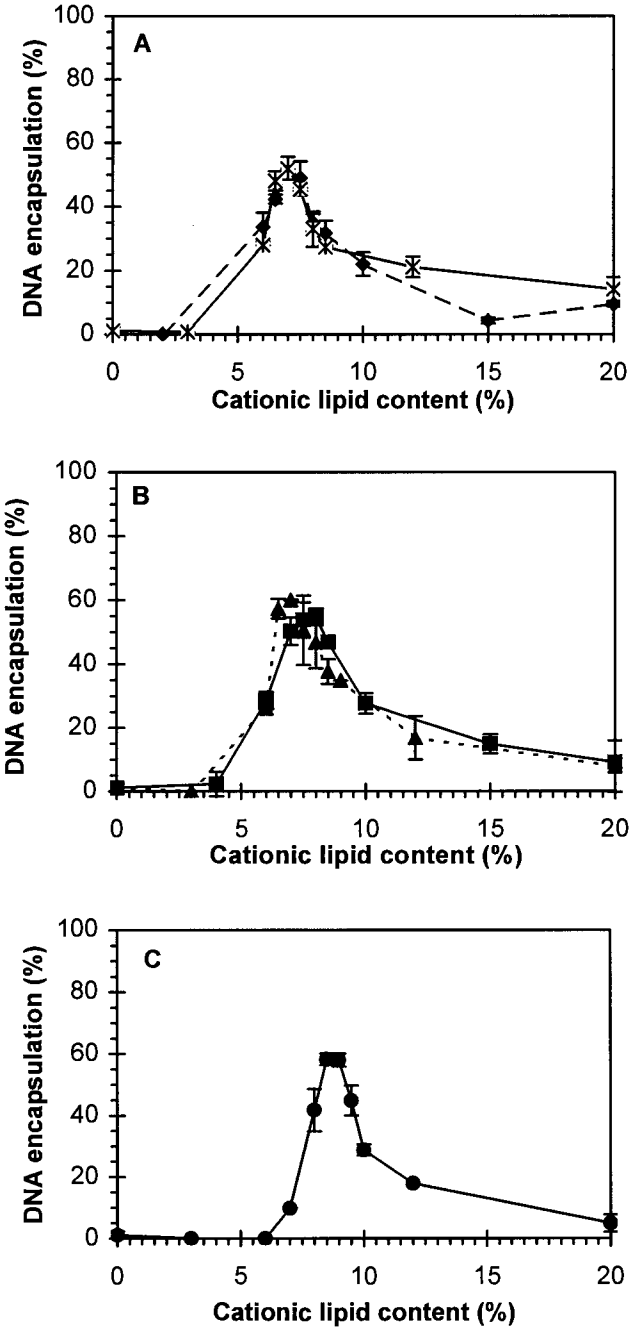
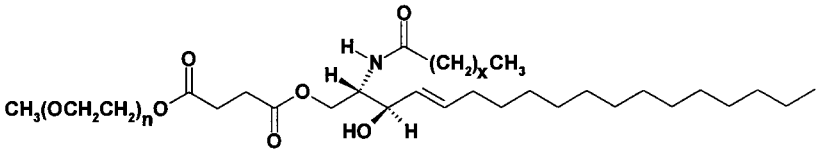


Fig. 2. Plasmid encapsulation efficiency for the SPLP detergent dialysis procedure as a function of cationic lipid content for a variety of cationic lipids: (A) DOTMA (♦), DODMA-AN (∗), (B) DSDAC (▲), DODAC (■), and (C) DC-CHOL (●). SPLP were prepared as described in Section 2, employing 25 μg pCMVCAT plasmid with 5 μmol total lipid consisting of 10 mol% PEG-CerC_{14}, x mol% cationic lipid, and $90-x$ mol% DOPE. Encapsulation was assayed by measuring DNA recovery after passage of the dialysate through a DEAE Sepharose CL-6B column (see Section 2). The average and standard deviation from three individual experiments are shown.



PEG_{2000}	$Cer\ C_8$	n = 45; x = 6
PEG_{2000}	$Cer\ C_{14}$	n = 45; x = 12
PEG_{2000}	$Cer\ C_{20}$	n = 45; x = 18
PEG_{750}	$Cer\ C_{14}$	n = 17; x = 12
PEG_{5000}	$Cer\ C_{14}$	n = 114; x = 12

Fig. 3. Structures of poly(ethylene glycol)-ceramides (PEG-Cer) used in this study.

to systems containing 25 μg pCMVCAT and 5.0 μmol total lipids where the cationic lipid content was maintained at 7.5 mol% DODAC. As shown in Fig. 4, maximum entrapment levels are observed at approximately 20 mol% PEG-$CerC_8$, whereas the maximum plasmid entrapment for the PEG-$CerC_{14}$ and PEG-$CerC_{20}$ systems are in the range of 10–12 mol% of the PEG-Cer.

3.3. Influence of the PEG anchor on transfection properties of SPLP

The transfection properties of SPLP were investigated employing SPLP with entrapped pCMVβgal to allow a convenient assay for transfection. Further, the transfection protocol involved using purified SPLP, where the empty vesicles produced during the detergent dialysis procedure were removed by density gradient centrifugation as detailed in Section 2. The initial set of experiments was designed to ascertain appropriate transfection conditions for the SPLP (DOPE/DOTMA/PEG-$CerC_8$; 73:7:20; molar ratios) system. As shown in Fig. 5A, protocols employing SPLP containing 0.5 μg pCMVβgal gave little or no transfection; however significant transfection was observed for SPLP containing 1.0 or 2.0 μg of plasmid DNA at transfection times of 24 h or longer. Thus, a 24 h incubation time and 2.0 μg plasmid DNA was utilized for the standardized SPLP transfection protocol in subsequent experiments. It is of interest to compare the transfection properties of SPLP with that achieved employing plasmid DNA-cationic lipid complexes. As shown in Fig. 5B, transfection of BHK cells employing pCMVβgal-DOTMA/DOPE (1:1) complexes gave transfection levels which are approximately an order of magnitude higher than observed for the SPLP preparation. In addition, good transfection activity was observed at low (0.5 μg pCMVβgal) plasmid levels and at short (4 h) incubation times.

In order to characterize the influence of the ceramide anchor on the transfection properties, SPLP containing pCMVβgal were generated by using 400 μg pCMVβgal and 10 μmol lipid mixtures. Lipid compositions of DOPE/DOTMA/PEG-Cer (83.5:6.5:10; molar ratios) using the amide chain lengths of C_8, C_{14}, and C_{20} were prepared. These systems were then purified to remove empty vesicles employing the density centrifugation protocol, and the transfection properties of the purified SPLP containing PEG-$CerC_8$, PEG-$CerC_{14}$ and PEG-$CerC_{20}$ in BHK cells under standard transfection conditions (2 μg pCMVβgal; 24 h incubation) are illustrated in Fig. 6. A correlation between the transfection activities and the length of the ceramide anchor is observed. SPLP formed with PEG-$CerC_8$ give rise to 30-fold higher transfection activity than systems formed with PEG-$CerC_{14}$, which in turn results in 8-fold higher transfection activity than SPLP formed with PEG-$CerC_{20}$.

3.4. Influence of cationic lipid species on transfection properties of SPLP

The transfection properties of SPLP containing the different cationic lipids with 20% PEG-$CerC_8$ are il-

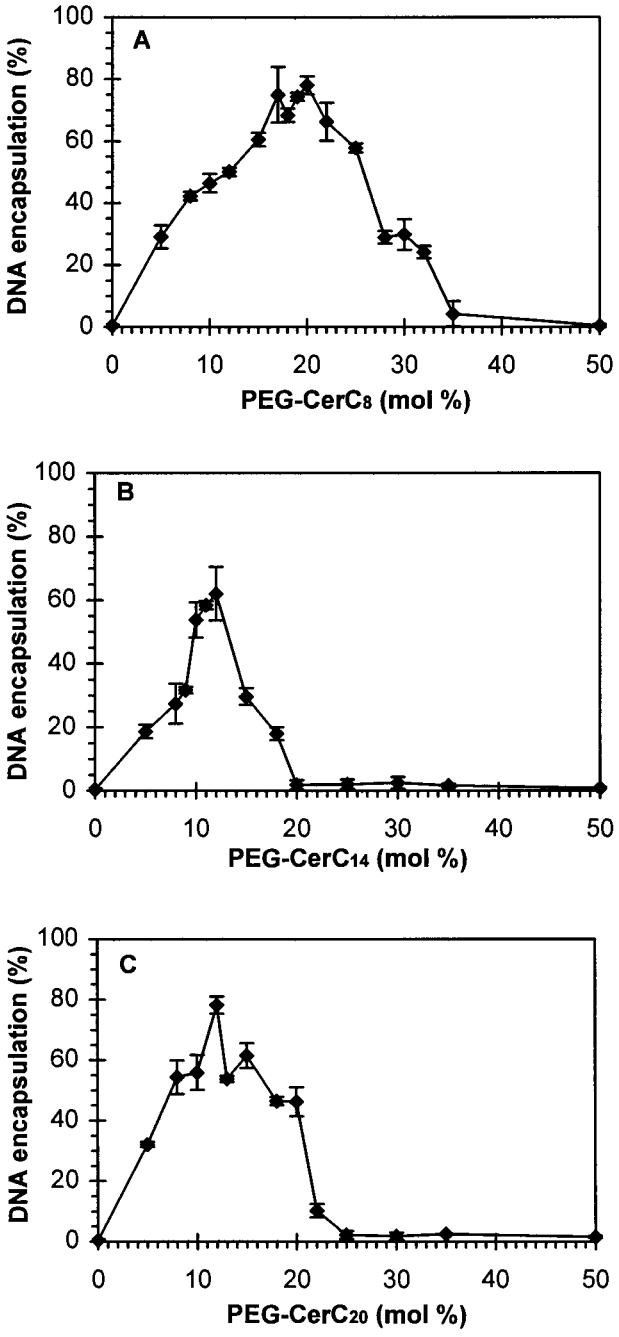


Fig. 4. Plasmid encapsulation efficiency for the SPLP detergent dialysis procedure utilizing (A) PEG-CerC_8, (B) PEG-CerC_{14} and (C) PEG-CerC_{20}. SPLP were prepared as described in Section 2 employing 25 μg pCMVCAT formulated with 5.0 μmol total lipids containing 7.5 mol% DODAC, x mol% PEG-Cer, and 92.5−x mol% DOPE. Encapsulation was assayed by measuring plasmid recovery after passage of the dialysate through a DEAE Sepharose CL-6B column. The average and standard deviations calculated from three individual experiments are shown.

lustrated in Fig. 7. The standard SPLP transfection protocol (2.0 μg pCMVβgal; 24 h incubation time) was utilized. It may be observed that the inclusion of different cationic lipids in SPLP did lead to different transfection activity, leading to the transfection potency profile of DODMA-AN > DOTMA > DODAC > DSDAC > DC-CHOL.

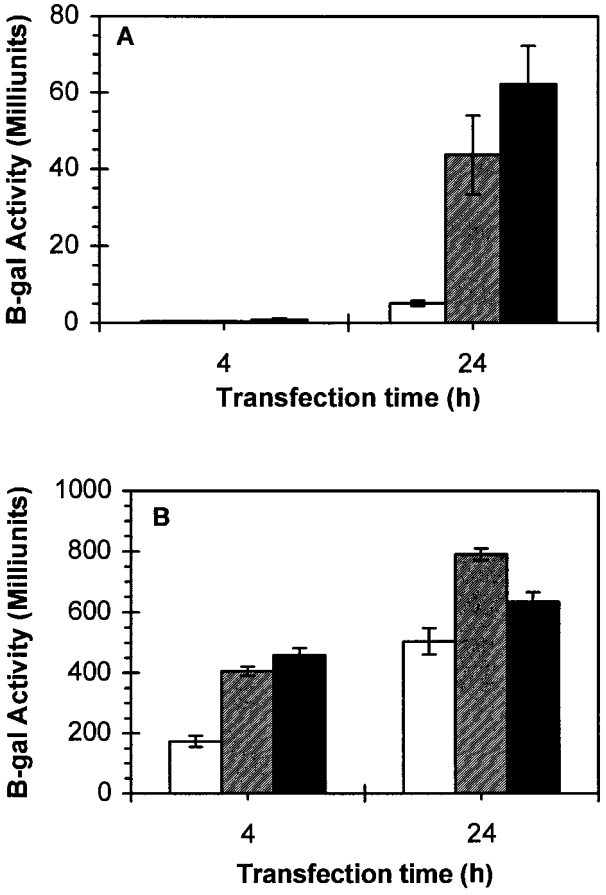


Fig. 5. Transfection properties of SPLP and plasmid DNA-cationic lipid complexes. (A) β-Gal expression in BHK cells resulting from incubation with SPLP containing 0.25 μg pCMVβgal (open bars), 1.0 μg pCMVβgal (shaded bars), and 2.0 μg pCMVβgal (solid bars) for 4 and 24 h is shown. (B) β-Gal expression in BHK cells resulting from incubation with complexes containing 0.25 μg pCMVβgal (open bars), 0.50 μg pCMVβgal (shaded bars), and 1.0 μg pCMVβgal (solid bars) for 4 and 24 h. SPLP (DOPE/DOTMA/PEG-CerC_8; 73:7:20; molar ratios) and plasmid DNA-cationic lipid complexes (DOTMA/DOPE (1:1)-pCMVβgal; charge ratio of 1.0) were prepared from pCMVβgal as described in Section 2. SPLP were purified employing the discontinuous sucrose density gradient centrifugation protocol. The average and standard deviation from triplicates are shown.

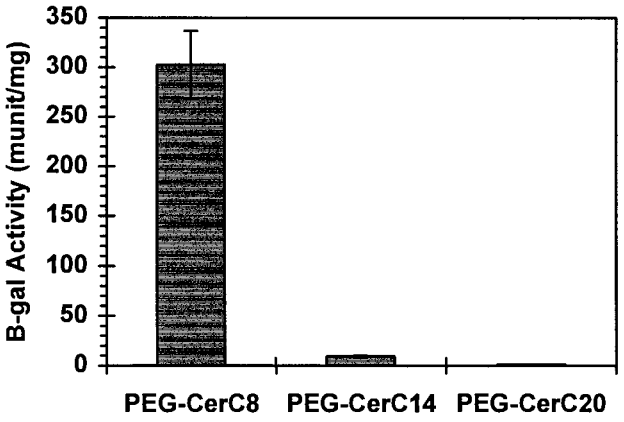


Fig. 6. Transfection properties of SPLP containing PEG-CerC_8, PEG-CerC_{14} and PEG-CerC_{20}. BHK cells were transfected with SPLP composed of DOPE/DOTMA/PEG-Cer (83.5:6.5:10; molar ratios) and 2.0 μg pCMVβgal for 24 h as outlined in Section 2. SPLP were purified employing the discontinuous sucrose density gradient centrifugation protocol. The average and standard deviation from triplicates are shown.

3.5. *Influence of PEG polymer length on formation and transfection properties of SPLP*

An alternative approach to improve the transfection potency of SPLP is to reduce the length of the PEG polymer associated with the ceramide anchor. This was investigated for PEG-CerC_{14} molecules containing PEG_{750}, PEG_{2000} and PEG_{5000}. Relatively poor entrapment levels in the range of 40% were achieved for the PEG_{750}-CerC_{14} and PEG_{5000}-CerC_{14} systems as compared to nearly 60% for the PEG_{2000}-CerC_{14} containing system (data not shown).

Transfection studies were performed on SPLP formed initially with 400 μg pCMVβgal and 10 μmol total lipids composed of DOPE, DOTMA, and PEG-CerC_{14} (83.5:6.5:10; molar ratios) for all the PEG species. These preparations were then purified employing the density centrifugation procedure and used to transfect BHK cells according to the standard protocol. The transfection properties of these SPLP containing PEG_{750}-CerC_{14}, PEG_{2000}-CerC_{14} and PEG_{5000}-CerC_{14} are illustrated in Fig. 8. SPLP containing PEG_{750} have slightly higher in vitro transfection potency than systems formed with PEG_{2000} or PEG_{5000}.

3.6. *Comparison of intracellular delivery of plasmid by SPLP and complexes*

The results to this point indicate that SPLP containing DODMA-AN, PEG anchors with shorter amide chains and PEG coatings composed of shorter

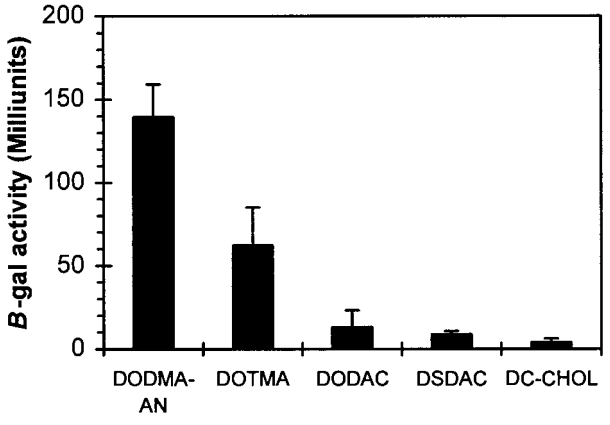


Fig. 7. Transfection properties of SPLP formed with the cationic lipids DODMA-AN, DOTMA, DODAC, DSDAC, and DC-CHOL and transfection achieved employing DOTMA-DOPE complexes. SPLP composed of cationic lipid (DODMA-AN and DOTMA, 7.0 mol%; DODAC and DSDAC, 7.5 mol%; DC-CHOL, 9.0 mol%), DOPE, and 20 mol% PEG-CerC_8 containing 2.0 μg pCMVβgal, and pCMVβgal-DOTMA/DOPE (1:1) complexes (charge ratio of 1.0; 0.5 μg pCMVβgal) were incubated with BHK cells and transfection assayed at 24 h. The average and standard deviation from triplicates are shown.

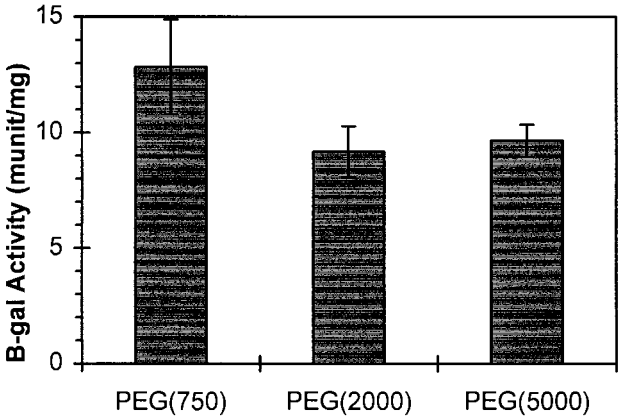
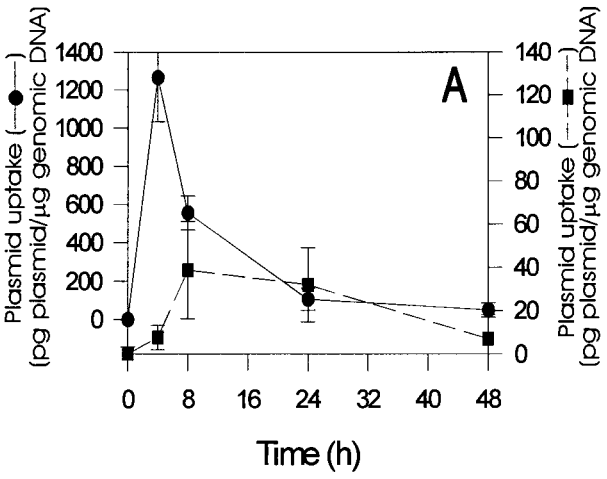


Fig. 8. Transfection properties of SPLP containing PEG-ceramides in which the size of the PEG polymer is varied (PEG_{750}, PEG_{2000} and PEG_{5000}). β-Gal expression in BHK cells was assayed following incubation with SPLP containing 2.0 μg pCMVβgal for 24 h. SPLP (DOPE/DOTMA/PEG-CerC_{14}; 83.5:6.5:10; molar ratios) containing pCMVβgal were prepared as indicated in Section 2. SPLP were purified employing the discontinuous sucrose density gradient centrifugation protocol. The average and standard deviation from triplicates are shown.

PEG polymers result in improved transfection of BHK cells in vitro. However, in all cases the transfection levels achieved are substantially lower than those observed for plasmid DNA-cationic lipid complexes. This may result from a reduced affinity of SPLP for cells due to the presence of the PEG coating on the SPLP and the much reduced positive charge on the SPLP as compared to complexes. Both of these effects may act to substantially reduce the amount of plasmid that is delivered to the cell. In order to determine whether this could account for the reduced transfection potency of SPLP as compared to complexes, the time dependent cellular uptake of pCMVβgal in both lipid-based DNA carriers was investigated. SPLP were formed from 400 μg pCMVβgal and 10 μmol total lipids composed of DOPE/DOTMA/PEG-CerC_8 (73:7:20; molar ratios) and were purified by density centrifugation. Purified SPLP samples (2 μg pCMVβgal) and DNA-cationic lipid complexes (DOTMA/DOPE (1:1)-pCMVβgal; charge ratio of 1.0; 2.0 μg pCMVβgal) were transfected as outlined in Section 2. Plasmid DNA delivered by the complexes demonstrate rapid, high cellular uptake and subsequent degradation with maximum plasmid levels at 4 h; whereas SPLP yield maximum plasmid delivery at a 8–24 h incubation (Fig. 9A). It may be noted that the maximum level of plasmid delivered by complexes is more than 30 times that delivered by SPLP.

The integrity of the delivered plasmid DNA over time is illustrated in Fig. 9B. Although the complexes delivered much more plasmid, this plasmid was degraded more rapidly than that delivered by the SPLP systems. This is indicated by the smears observed in the DNA delivered by the complexes as shown in the agarose gel electrophoretic pattern (Fig. 9B). This indicates that plasmid DNA entrapped within the particles is not as susceptible to degradation by cellular enzymes as compared to plasmid DNA associated with complexes.



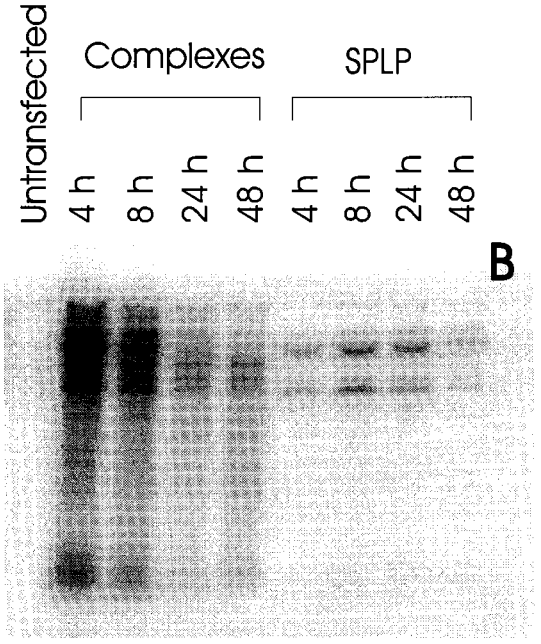


Fig. 9. Southern blot analysis of intracellular delivery of plasmid DNA by SPLP and plasmid DNA-cationic lipid complexes. SPLP composed of DOPE/DOTMA/PEG-CerC_8 (73:7:20; molar ratios) and complexes composed of DOTMA/DOPE (1:1)-pCMVβgal (charge ratio of 1.0) were used. DNA transfection and cellular uptake studies were performed as described in Section 2. Comparison of the cellular uptake of plasmid DNA using DNA-cationic lipid complexes (●) and SPLP (■) is shown in panel A. Southern blot analysis of the integrity of the plasmid DNA delivered by the two lipid-based DNA carriers is shown in panel B. The average and standard deviation from triplicates are shown.

4. Discussion

This work was aimed at characterizing factors that influence the transfection potency of stabilized plasmid-lipid particles with the aim of improving transgene expression. It is shown that three factors that can modulate SPLP transfection efficacy are the species of cationic lipid employed, the size of the PEG polymer coating the SPLP and the length of the acyl

chain contained in the ceramide 'anchor'. These factors are discussed in turn, followed by a discussion of the implications of the observation that the low levels of SPLP uptake into cells may be the primary parameter limiting transgene expression.

With regard to the influence of different cationic lipids, the results presented here demonstrate that plasmid encapsulation employing the detergent dialysis process is relatively independent of the species of monovalent cationic lipid employed. Although it is difficult to discern any definite trends, DODMA-AN and DOTMA appear to provide the maximum entrapment at the lowest cationic lipid content (~7%), followed by DODAC and DSDAC (~7.5%) and DC-CHOL (~9%). Interestingly, SPLP composed of DODMA-AN and DOTMA exhibit significantly higher transfection potencies than SPLP containing DODAC, DSDAC or DC-CHOL (Fig. 7). These results could be taken to suggest that cationic lipids with the greatest affinity for plasmid DNA under the conditions of detergent dialysis lead to the most potent transfection systems. In any event, it is clear that the species of cationic lipid does influence the transfection capability of the resulting SPLP, with DODMA-AN resulting in the highest transfection levels and DC-CHOL the lowest, with DODAC and DSDAC giving rise to intermediate transfection levels.

The second factor which clearly plays a major role in modulating the transfection potency of SPLP is the length of the acyl chain contained in the hydrophobic ceramide group which anchors the PEG coating to the SPLP surface. Previous work has shown that inclusion of PEG-CerC_{14} in SPLP results in enhanced expression in vitro as compared to SPLP containing PEG-CerC_{20}, however the levels of gene expression were low in all cases [7]. The results presented here show that 30-fold higher transfection levels can be achieved for SPLP containing PEG-CerC_8 as compared to PEG-CerC_{14} (Fig. 6). This improved transfection ability presumably reflects a faster leaving rate for the shorter chain PEG-ceramides from the SPLP surface in the sequence PEG-CerC_8 > PEG-CerC_{14} > PEG-CerC_{20}, leaving the SPLP surface less shielded by PEG for the C_8 containing system. This effect is fully consistent with previous work showing that the half-time ($t_{1/2}$) for dissociation of PEG-CerC_8 from LUV is less than 1.2 min [19], whereas PEG-CerC_{14} and PEG-CerC_{20} exhibit $t_{1/2}$ values of 1.2 h and greater than 13 days, respectively [7]. It is also consistent with the observation that fusion between LUVs can be inhibited by the presence of a PEG coating anchored to PE molecules, and that fusogenicity could be restored by using PE anchors with short acyl chains [13]. Other workers have shown that the transfection potency of plasmid DNA-cationic lipid complexes is significantly reduced by the presence of PEG-PE [14]. The ability of the PEG coating on SPLP to inhibit transfection can arise due to reduced binding and therefore reduced uptake into target cells, or reduced efficiency in fusing with the endosomal membrane in order to achieve intracellular delivery of the plasmid.

The formulation properties of SPLP containing PEG-CerC_8 are clearly different from the properties of SPLP containing longer chain ceramides, in that optimum encapsulation is achieved at ~20 mol% PEG-CerC_8 as compared to ~10 mol% for SPLP containing PEG-CerC_{14} or PEG-CerC_{20} (Fig. 4). It is possible that the partition coefficient of the shorter chain PEG-CerC_8 for the SPLP outer monolayer is reduced, resulting in a requirement for a higher concentration of the PEG-CerC_8 to achieve adequate membrane levels for stabilization.

The third parameter, the length of the PEG polymer, appears to influence the formulation properties of the SPLP more than the transfection potency. The transfection potency of SPLP containing PEG_{750}, PEG_{2000} and PEG_{5000} coupled to CerC_{14} are similar (Fig. 8), however the formulation properties differ significantly. In particular, the maximum efficiency for plasmid encapsulation that could be achieved for SPLP containing PEG_{750} or PEG_{5000} was ~40%, as compared to ~60% for the PEG_{2000} system (data not shown). Reduced plasmid entrapment for shorter PEG polymers would be expected due to the reduced steric stabilizing capacity of the shorter PEG polymers. The reason for the poorer entrapment for the SPLP containing PEG_{5000}-ceramide is not currently understood. It may result from a higher CMC of the PEG_{5000}-ceramide due to the larger size of the polar region, or to the interference of the longer PEG polymer with the association of plasmid with the lipid intermediates generated during formation of SPLP [7]. The fact that SPLP containing PEG-ceramides with shorter PEG polymers do not

exhibit significantly higher transfection potencies presumably reflects the fact that the presence of PEG-ceramides which are sufficient to stabilize formation of the SPLP during the detergent dialysis process are also sufficient to inhibit uptake into cells.

The final area of discussion concerns the observation that SPLP exhibit much lower levels of accumulation into target cells as compared to plasmid DNA-cationic lipid complexes, and that the plasmid delivered by SPLP remains intact in the cell for a longer time following cellular uptake. With regard to the first point, when BHK cells are presented with equivalent amounts of plasmid DNA in either the complex form or in SPLP (DOPE/DOTMA/PEG-CerC_8; 73:7:20; molar ratios) form, the maximum amount of plasmid that is delivered into the cells by the SPLP is less than 3% of the maximum amount delivered by the plasmid DNA-cationic lipid complexes. Thus even though the in vitro transfection potency of the complexes (DOTMA/DOPE (1:1)-pCMVβgal; charge ratio of 1.0; 2 μg pCMVβgal) is at least 10-fold higher than the SPLP (DOPE/DOTMA/PEG-CerC_8; 73:7:20; 2 μg pCMVβgal; 24 h transfection), the reduced potency of the SPLP can be attributed to a 30-fold or more reduction in plasmid uptake as compared to complexes. This reduced plasmid uptake likely results from a low affinity of the SPLP particle for the cell membrane as compared to complexes, although breakdown of SPLP-associated plasmid could give similar results. The fact that previous studies [7] show SPLP plasmid to be highly stable and the results presented here showing that plasmid delivered to cells in SPLP is broken down more slowly than plasmid in complexes suggests that a low affinity of the SPLP for the cell is the dominant factor. In any event, the fact that the reduced potency of SPLP as compared to complexes can be attributed to low cellular uptake argues strongly that SPLP exhibit an intrinsic ability to transfect cells following uptake that is at least comparable to complexes. These results also suggest that the most direct way to improve the transfection properties of SPLP is to enhance cellular uptake. This could be accomplished in a number of ways, including incorporation of external targeting ligands to promote cell association and uptake.

The second observation that plasmid delivered to cells by SPLP is broken down at a much slower rate than plasmid delivered in complexes presumably reflects the resistance of the SPLP particle to breakdown by intracellular factors. It also points out the potential for more stable particles such as SPLP to extend the duration of transfection resulting from transfection by non-viral gene delivery systems.

In summary, this investigation characterizes factors that regulate the transfection potency of stabilized plasmid-lipid particles. It is shown that the transfection potency is sensitive to both the cationic lipid species and the species of PEG-ceramide employed to construct the SPLP. Improved transfection activity can be achieved by the use of the cationic lipid DODMA-AN, PEG-ceramides incorporating smaller PEG polymers and, most importantly, the use of PEG-ceramides containing shorter acyl groups in the ceramide anchor. Further, it is shown that the dominant factor leading to lower levels of transfection by SPLP is the low level of SPLP accumulation by the target cells. These observations point the way to achieve plasmid delivery systems that exhibit enhanced levels of gene expression for in vivo gene therapy.

Acknowledgements

This work was supported by Inex Pharmaceuticals Corporation and the Natural Sciences and Engineering Research Council of Canada under the Collaborative Research and Development Program. We thank L. Palmer from our laboratory and Dr. P. Scherrer from Inex Pharmaceuticals Corporation for technical consultation on SPLP preparation. We would also like to thank Dr. S. Ansell from Inex for the generous supply of different cationic lipids, Dr. Z. Wang for the supply of different PEG-Cer, and A. Annuar for the supply of ^{3}H-pCMVCAT.

References

[1] N. Zhu, D. Liggitt, Y. Liu, R. Debs, Systemic gene expression after intravenous DNA delivery into adult mice, Science 261 (1993) 209–211.

[2] R.I. Mahato, K. Kawabata, T. Nomura, Y. Takakura, M. Hashida, Physicochemical and pharmacokinetic characteristics of plasmid DNA/cationic liposome complexes, J. Pharmacol. Sci. 84 (1995) 1267–1271.

[3] I. Solodin, C.S. Brown, M.S. Bruno, C.-Y. Chow, E.-H. Jang, R.J. Debs, T.D. Heath, A novel series of amphiphilic imidazolinium compounds for in vitro and in vivo gene delivery, Biochemistry 34 (1995) 13537–13544.
[4] D.C. Litzinger, J.M. Brown, I. Wala, S.A. Kaufman, G.Y. Van, C.L. Farrell, D. Collins, Fate of cationic liposomes and their complex with oligonucleotide in vivo, Biochim. Biophys. Acta 1281 (1996) 139–149.
[5] Y. Liu, L.C. Mounkes, H.D. Liggit, C.S. Brown, I. Solodin, T.D. Heath, R.J. Debs, Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery, Nature Biotechnol. 15 (1997) 167–173.
[6] N.S. Templeton, D.D. Lasic, P.M. Frederik, H.H. Strey, D.D. Roberts, G.N. Pavlakis, Improved DNA:liposome complexes for increased systemic delivery and gene expression, Nature Biotechnol. 15 (1997) 647–652.
[7] J.J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R. Graham, P. Scherrer, M.J. Hope, P.R. Cullis, Stabilized plasmid-lipid particles: construction and characterization, Gene Ther. 6 (1999) 271–281.
[8] X. Gao, L. Huang, A novel cationic liposome reagent for efficient transfection of mammalian cells, Biochem. Biophys. Res. Commun. 179 (1991) 280–285.
[9] J.K. Rose, L. Buonocore, M.A. Whitt, A new cationic liposome reagent mediating nearly quantitative transfection of animal cells, BioTechniques 10 (1991) 520–525.
[10] W.R. Jarnagin, R.J. Debs, S.-S. Wang, D.M. Bissell, Cationic lipid-mediated transfection of liver cells in primary culture, Nucleic Acids Res. 20 (1992) 4205–4211.
[11] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, J. Biol. Chem. 269 (1994) 2550–2561.
[12] N.K. Egilmez, Y. Iwanuma, R.B. Bankert, Evaluation and optimization of different cationic liposome formulations for in vivo gene transfer, Biochem. Biophys. Res. Commun. 221 (1996) 169–173.
[13] J.W. Holland, C. Hui, P.R. Cullis, T.D. Madden, Poly(ethylene glycol)-lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine, Biochemistry 35 (1996) 2618–2624.
[14] K. Hong, W. Zheng, A. Baker, D. Papahadjopoulos, Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery, FEBS Lett. 400 (1997) 233–237.
[15] K.W.C. Mok, P.R. Cullis, Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA, Biophys. J. 73 (1997) 2534–2545.
[16] J. Sambrook, E.F. Fritsch, T. Maniatis, Extraction and purification of plasmid DNA, in: C. Nolan (Ed.), Molecular Cloning: A Laboratory Manual, Vol. I, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, pp. 1.21–1.52, 5.28–5.32, and 6.3–6.19.
[17] C.H. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–379.
[18] L.D. Mayer, M.J. Hope, P.R. Cullis, Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim. Biophys. Acta 858 (1986) 161–168.
[19] J.W. Holland, M.A. Monck, L.S. Choi, P.R. Cullis, T.D. Madden, Programmable fusion between cationic liposomes and a eukaryotic plasma membrane, J. Biol. Chem. (1999) (submitted).

JOINT APPENDIX 52

US006287591B1

(12) **United States Patent**
Semple et al.

(10) **Patent No.: US 6,287,591 B1**
(45) **Date of Patent: Sep. 11, 2001**

(54) **CHARGED THERAPEUTIC AGENTS ENCAPSULATED IN LIPID PARTICLES CONTAINING FOUR LIPID COMPONENTS**

(75) Inventors: **Sean C. Semple**, Vancouver; **Sandra K. Klimuk**, N. Vancouver; **Troy Harasym**, Vancouver; **Michael J. Hope**, Vancouver; **Steven M. Ansell**, Vancouver; **Pieter Cullis**, Vancouver; **Peter Scherrer**, Vancouver; **Dan Debeyer**, Vancouver, all of (CA)

(73) Assignee: **Inex Pharmaceuticals Corp.**, Burnaby (CA)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/078,954**

(22) Filed: **May 14, 1998**

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/856,374, filed on May 14, 1997, now abandoned.

(51) **Int. Cl.**[7] **A61K 9/127**; A61K 31/70; C12N 11/02; C12N 15/88; C07H 21/00

(52) **U.S. Cl.** **424/450**; 428/402.2; 435/177; 435/458; 514/44; 536/22.1

(58) **Field of Search** 424/450; 435/174, 435/177, 458; 428/402.2; 514/44; 536/22.1, 23.1

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,264,618		11/1993	Felgner et al.	560/224
5,552,155		9/1996	Bailey et al.	424/450
5,595,756		1/1997	Bally et al.	424/450
5,965,542	*	10/1999	Wasan et al.	514/44

FOREIGN PATENT DOCUMENTS

WO 95/27478
A1 10/1995 (WO) .

(List continued on next page.)

OTHER PUBLICATIONS

Needham et al., J. Liposome Res. 2: 411–430 (1992).*

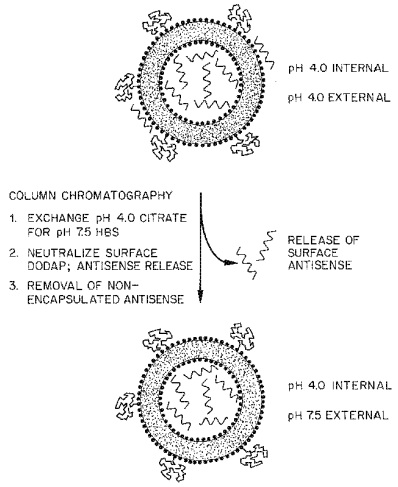
(List continued on next page.)

Primary Examiner—David M. Naff
(74) *Attorney, Agent, or Firm*—Oppedahl & Larson LLP

(57) **ABSTRACT**

Lipid-therapeutic agent particles are prepared containing a charged therapeutic agent encapsulated in lipid portion containing at least two lipid components including a protonatable or deprotonatable lipid such as an amino lipid and a lipid that prevents particle aggregation during lipid-therapeutic agent particle formation such as a PEG-modified or polyamide oligomer-modified lipid. Other lipid components may also be present and these include a neutral lipid such as DSPC, POPC, DOPE or SM, and a sterol such as Chol. The therapeutic agent is encapsulated by combining a mixture of the lipids with a buffered aqueous solution of a charged therapeutic agent to form an intermediate mixture containing lipid-encapsulated therapeutic agent particles, and changing the pH of the intermediate mixture to neutralize at least some surface charges on the particles. The method permits high ratios of therapeutic agent to lipid and encapsulation efficiencies in excess of 50%. The method is particularly useful for preparing lipid-encapsulated nucleic acids such as an antisense polyanionic nucleic acid having exclusively phosphodiester linkages. The encapsulated nucleic acid can be contacted with a cell to introduce the nucleic acid into the cell such as for treatment or prevention of a disease characterized by aberrant expression of a gene. A pharmaceutical composition may be prepared containing the lipid-encapsulated therapeutic agent particles and a carrier.

72 Claims, 17 Drawing Sheets



FOREIGN PATENT DOCUMENTS

WO 96/10390	4/1996	(WO) .
WO 96/10391	4/1996	(WO) .
WO 96/18372 A2	6/1996	(WO) .
WO 96/40964	12/1996	(WO) .
WO 97/07784	3/1997	(WO) .
WO 97/46223 A1	12/1997	(WO) .
WO 97/46671	12/1997	(WO) .

OTHER PUBLICATIONS

Li et al., J. Liposome Res. 7: 411–430 (1997).*

Zelphati, O. and Szoka, F.C., Jr., "Liposomes as a carrier for intracellular delivery of antisense oligonucleotides: a real or magic bullet?", Journal of controlled Release 41:99–119, 1996.

Tomita, N. et al. Transient Decrease in High Blood pressure by In Vivo Transfer of Antisense Oligodeoxynucleotides Against Rat Angiotensinogen', Hypertension 26(1): 131–136, Jul. 1995.

Litzinger, D.C., "Limitations of Cationic Liposomes for Antisense Oligonucleotide Delivery In Vivo", Journal of Liposome Research 7(1):51–61, 1997.

Wielbo, D., et al., "Inhibition of Hypertension by Peripheral Administration of Antisense Oligodeoxynucleotides", Hypertension 28(1): 147–151, Jul. 1996.

Thierry, A.R., et al., "Lipsomal Delivery as a New Approach to Transport Antisense Oligonucleotides", in Gene Regulation:Giology of Antisense RNA and DNA, ed. R.P. Erickson and J.G. Izant, Raven Press, Ltd. New York, pp. 147–161, 1992.

Akhtar, S. and R.L. Juliano, "Liposome delivery of antisense oligonucleotides: adsorption and efflux characteristics of phosphorothioate oligodeoxynucleotides", Journal of Controlled Release 22(1): 47–56, Sep. 1992.

Zelphati, O., et al., "Inhibition of HIV–1 Replication in Cultured Cells with Antisense Oligonucleotides Encapsulated in Immunoliposomes", Antisense Research and Development, 3:323–338, 1993.

* cited by examiner

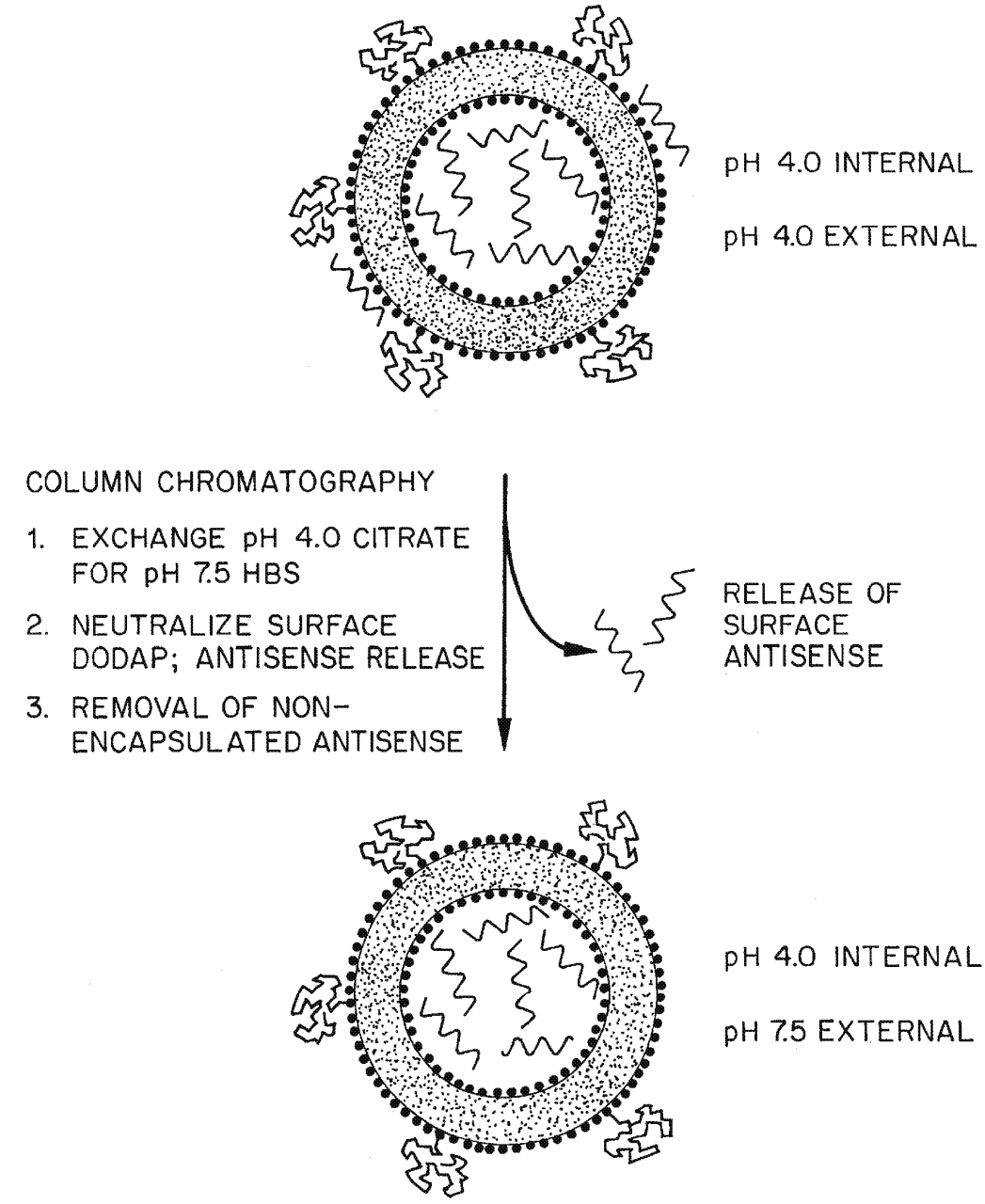
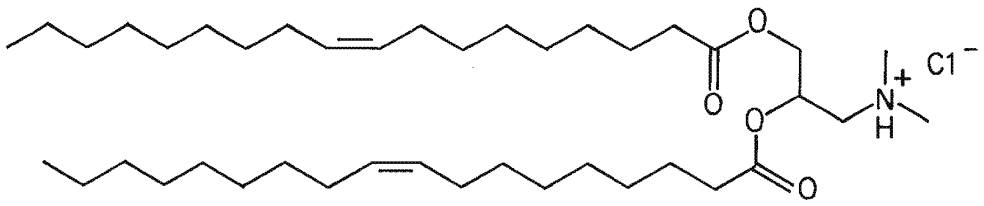

pH 4.0 INTERNAL
pH 4.0 EXTERNAL
COLUMN CHROMATOGRAPHY
1. EXCHANGE pH 4.0 CITRATE FOR pH 7.5 HBS
2. NEUTRALIZE SURFACE DODAP; ANTISENSE RELEASE
3. REMOVAL OF NON-ENCAPSULATED ANTISENSE
RELEASE OF SURFACE ANTISENSE
pH 4.0 INTERNAL
pH 7.5 EXTERNAL

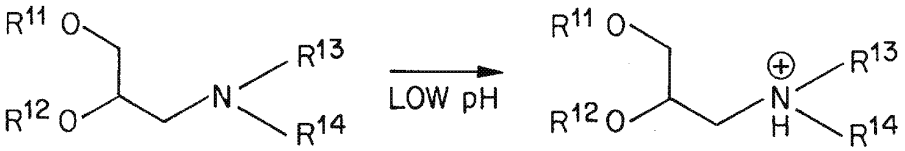
FIG. 1

DODAP: AL-1



OTHER AMINO LIPIDS:

R^1 AND/OR R^2 ARE H, ALKYL OR FATTY ALKYL GROUPS
R^3 IS H, LOWER ALKYL.

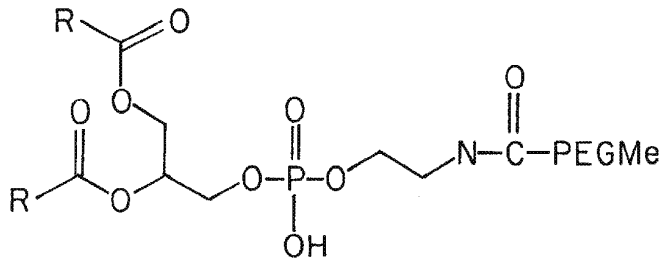


R^{11} AND/OR R^{12} ARE LOWER ALKYL/LOWER ACYL, FATTY ALKYL, FATTY ACYL.

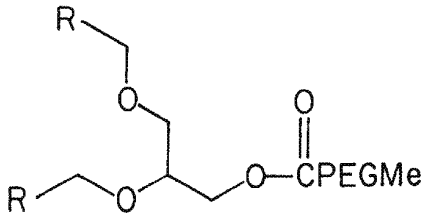
(AT LEAST ONE OF R^{11} OR R^{12} IS A LONG CHAIN ALKYL OR ACYL GROUP)

R^{13} AND R^{14} ARE EACH H, LOWER ALKYL.

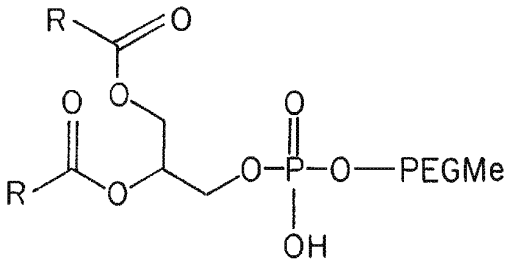
FIG. 2A



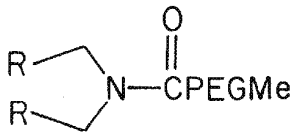
A



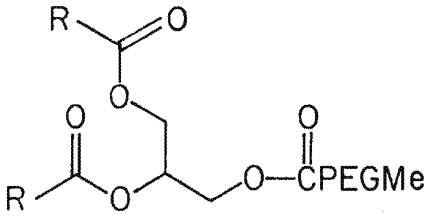
D



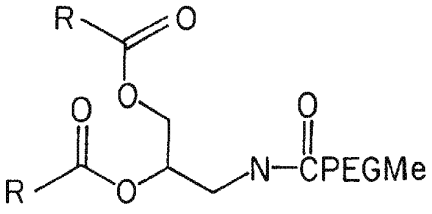
B



E



C



F

FIG. 2B

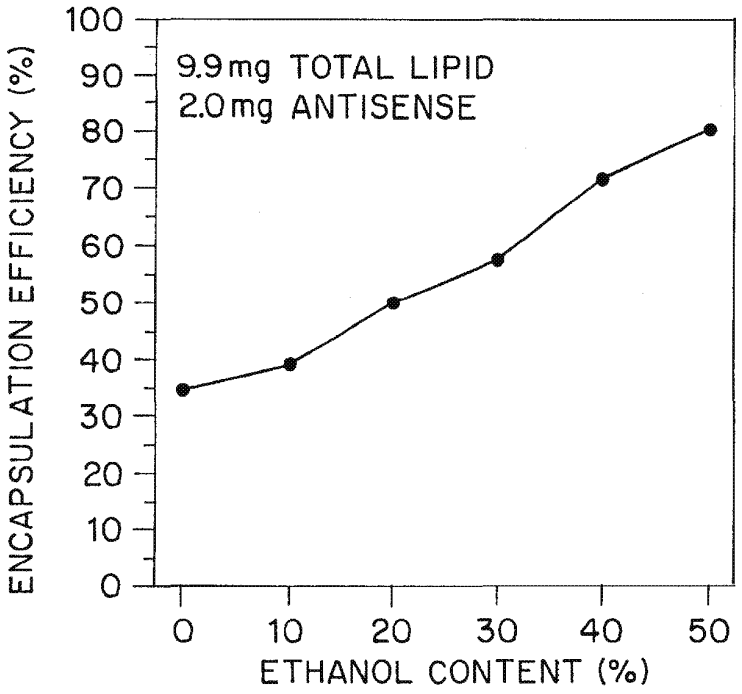

9.9mg TOTAL LIPID
2.0mg ANTISENSE
ENCAPSULATION EFFICIENCY (%)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
ETHANOL CONTENT (%)

FIG. 3

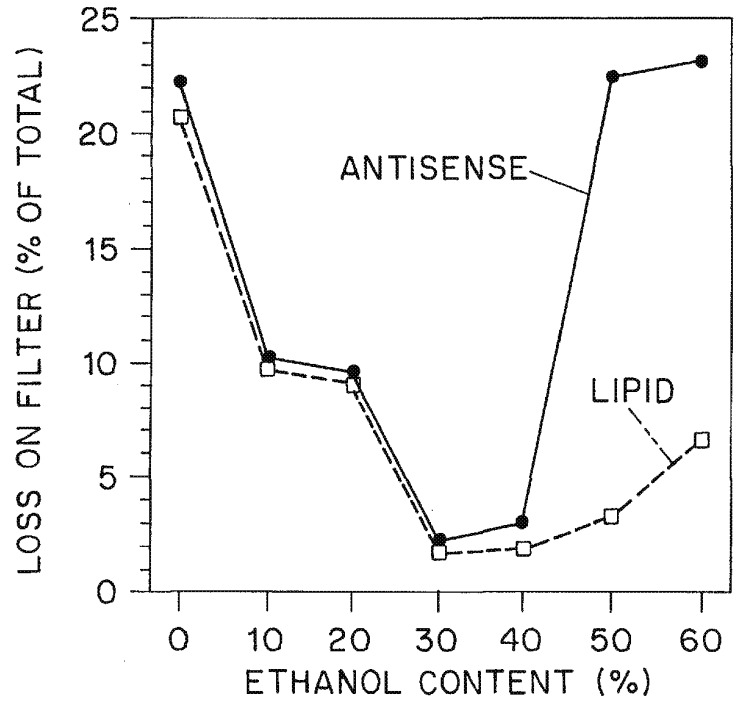

ANTISENSE
LIPID
LOSS ON FILTER (% OF TOTAL)
25
20
15
10
5
0
0
10
20
30
40
50
60
ETHANOL CONTENT (%)

FIG. 4

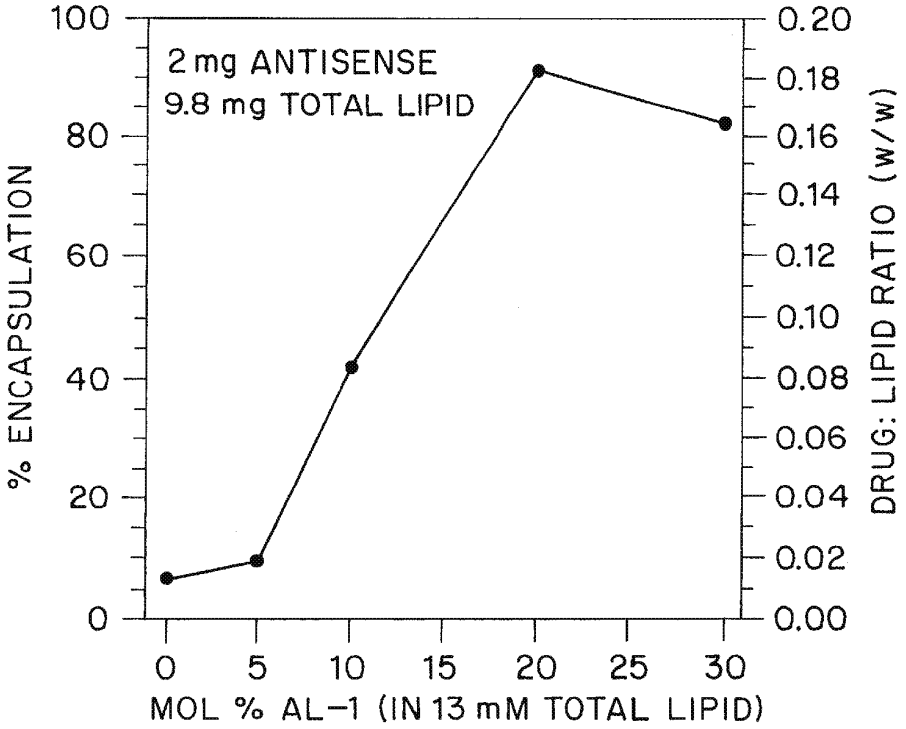

2 mg ANTISENSE
9.8 mg TOTAL LIPID
% ENCAPSULATION
DRUG: LIPID RATIO (W/W)
MOL % AL-1 (IN 13 mM TOTAL LIPID)
0
20
40
60
80
100
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
0.20
0
5
10
15
20
25
30

FIG. 5

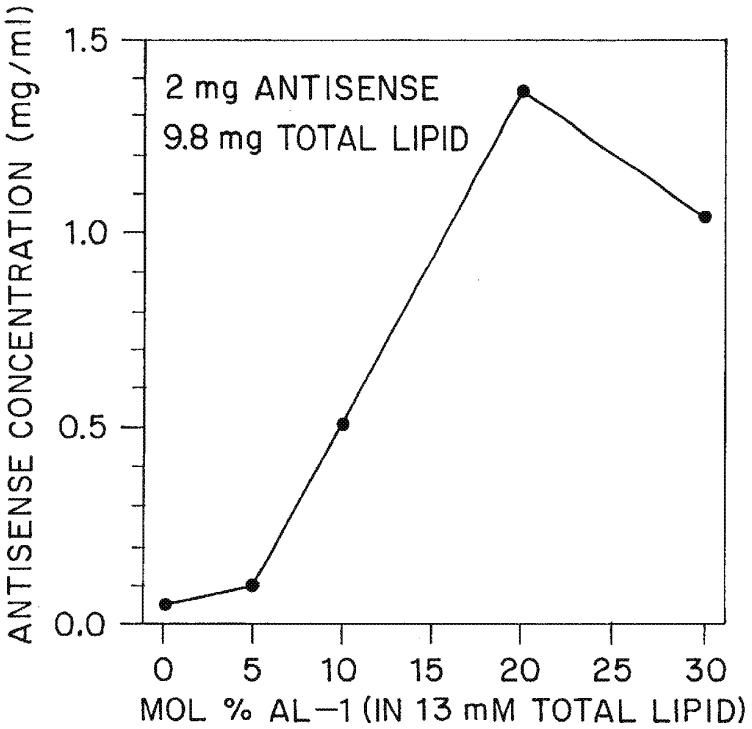
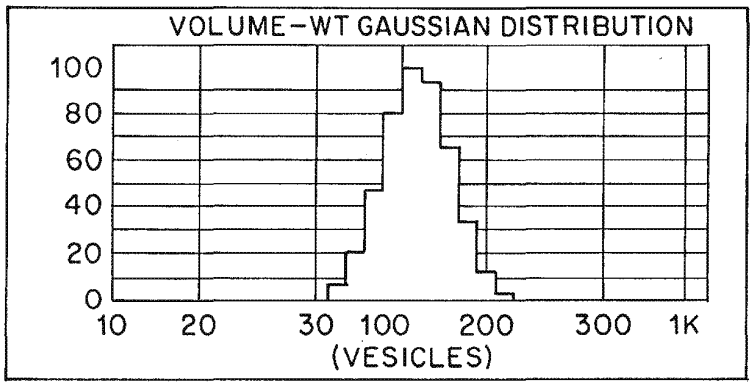

2 mg ANTISENSE
9.8 mg TOTAL LIPID
ANTISENSE CONCENTRATION (mg/ml)
MOL % AL-1 (IN 13 mM TOTAL LIPID)
0.0
0.5
1.0
1.5
0
5
10
15
20
25
30

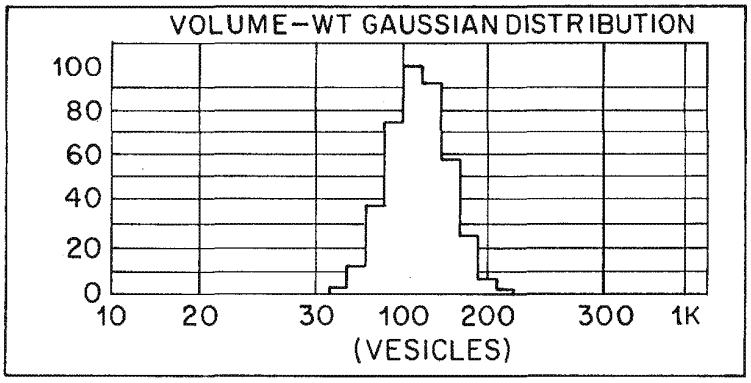
FIG. 6

IMMEDIATELY AFTER REMOVAL OF FREE ANTISENSE



VOLUME WEIGHTING:
MEAN DIAMETER = 119.3 nm
STD DEVIATION = 32.2 nm (27.0 %)
CUMULATIVE RESULTS:
25 % OF DISTRIBUTION < 88.60 nm
50 % OF DISTRIBUTION < 106.74 nm
75 % OF DISTRIBUTION < 127.93 nm
90 % OF DISTRIBUTION < 151.04 nm
99 % OF DISTRIBUTION < 199.22 nm

AFTER 2 MONTH STORAGE AT 4°C



VOLUME WEIGHTING:
MEAN DIAMETER = 114.2 nm
STD DEVIATION = 27.8 nm (24.3 %)
CUMULATIVE RESULTS:
25 % OF DISTRIBUTION < 86.96 nm
50 % OF DISTRIBUTION < 102.86 nm
75 % OF DISTRIBUTION < 121.31 nm
90 % OF DISTRIBUTION < 140.78 nm
99 % OF DISTRIBUTION < 183.74 nm

FIG. 7

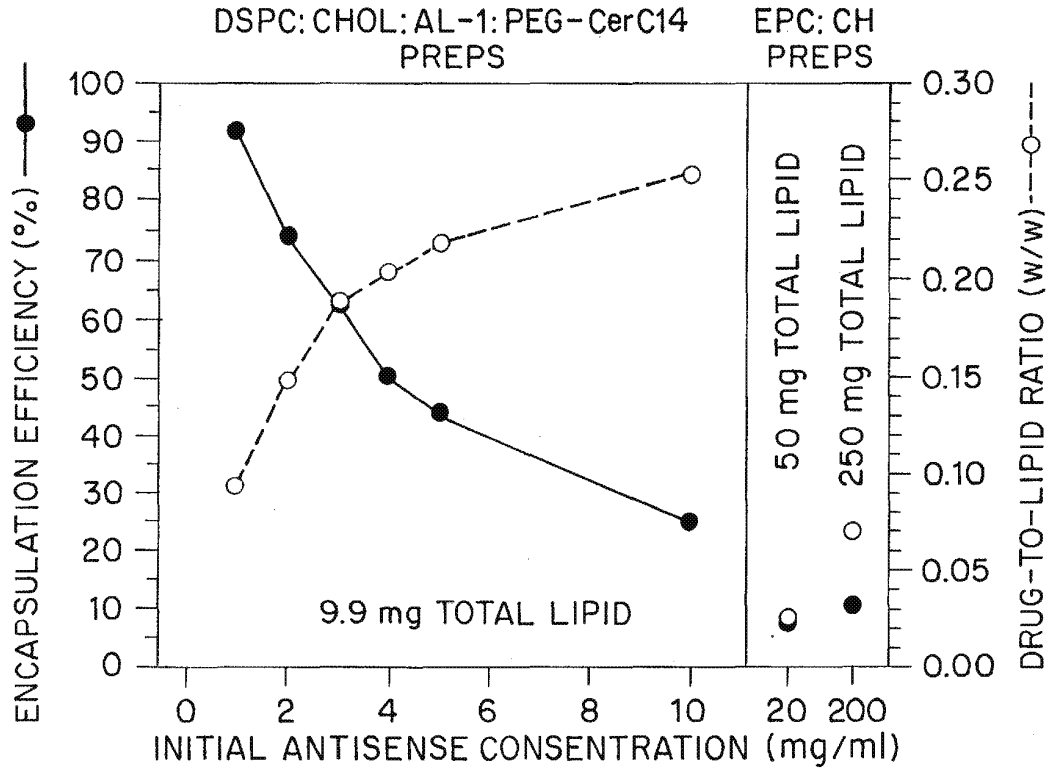

DSPC: CHOL: AL-1: PEG-CerC14
PREPS
EPC: CH
PREPS
ENCAPSULATION EFFICIENCY (%)
DRUG-TO-LIPID RATIO (w/w)
50 mg TOTAL LIPID
250 mg TOTAL LIPID
9.9 mg TOTAL LIPID
INITIAL ANTISENSE CONSENTRATION (mg/ml)

FIG. 8

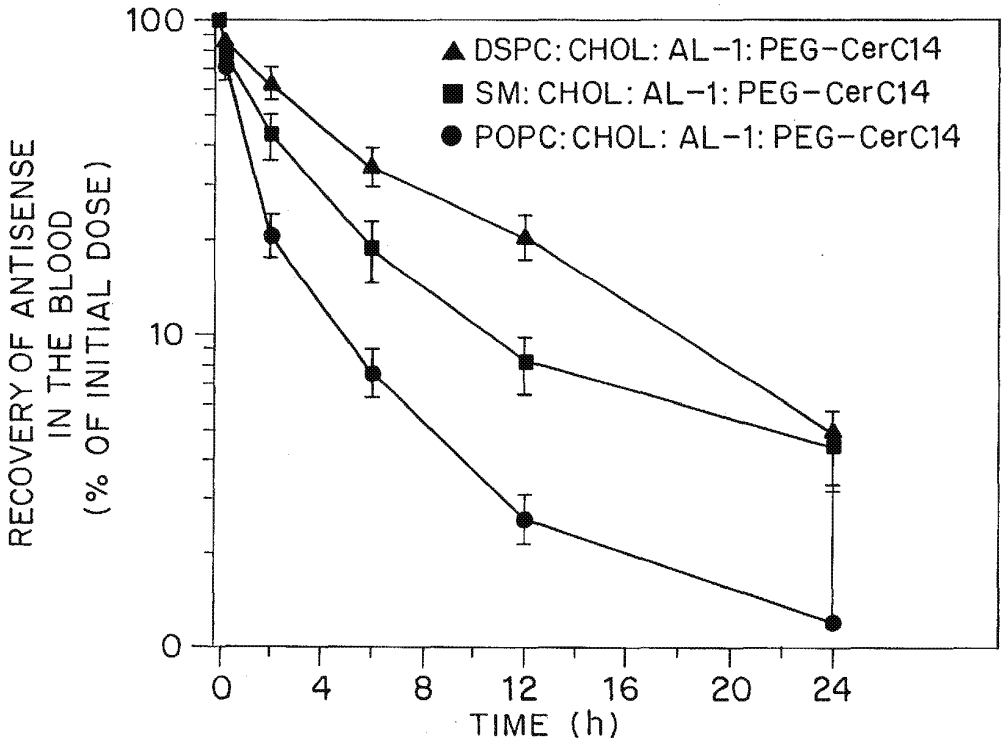

DSPC: CHOL: AL-1: PEG-CerC14
SM: CHOL: AL-1: PEG-CerC14
POPC: CHOL: AL-1: PEG-CerC14
RECOVERY OF ANTISENSE IN THE BLOOD (% OF INITIAL DOSE)
TIME (h)

FIG. 9

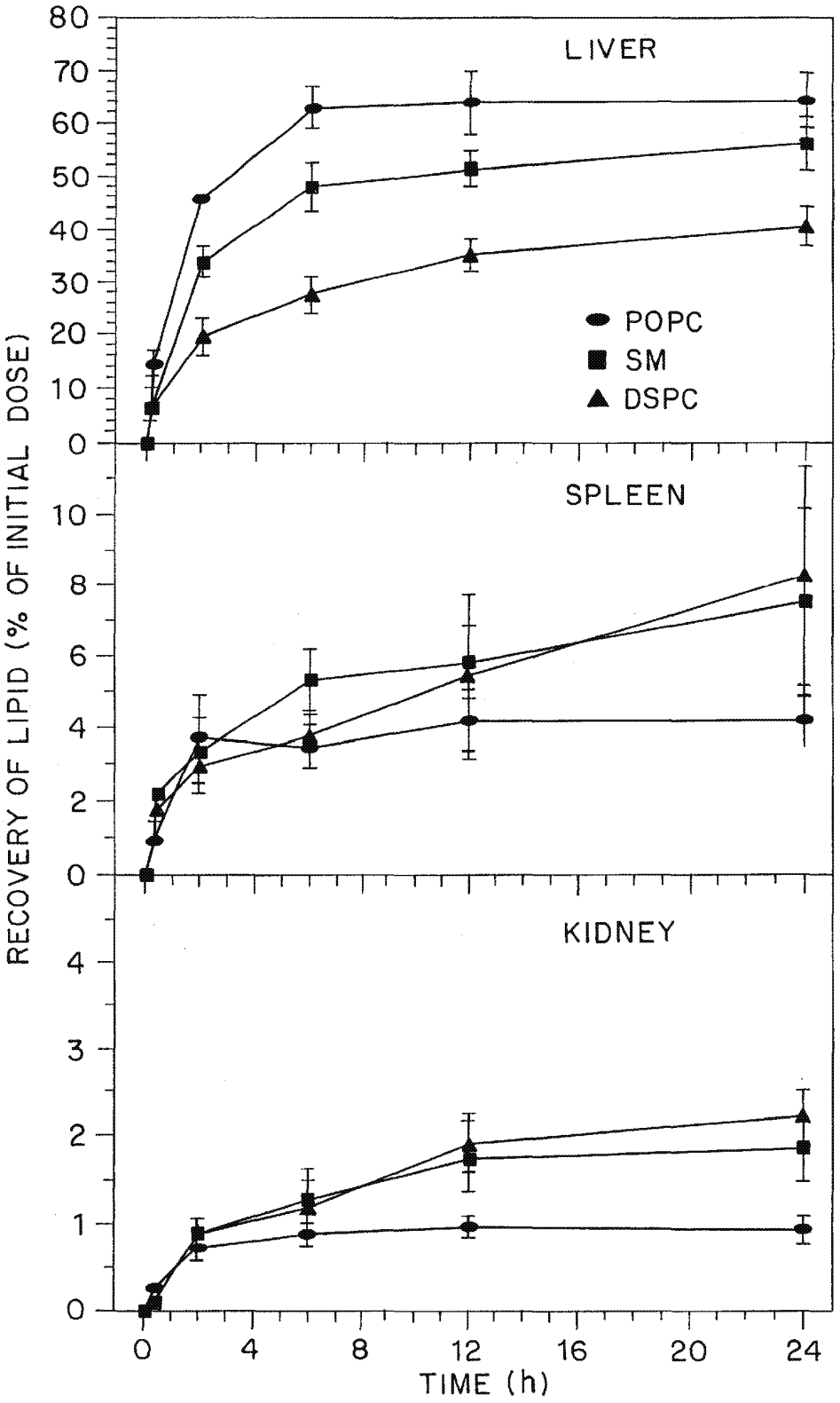

LIVER
SPLEEN
KIDNEY
POPC
SM
DSPC
RECOVERY OF LIPID (% OF INITIAL DOSE)
TIME (h)
0
4
8
12
16
20
24

FIG. 10

9/17

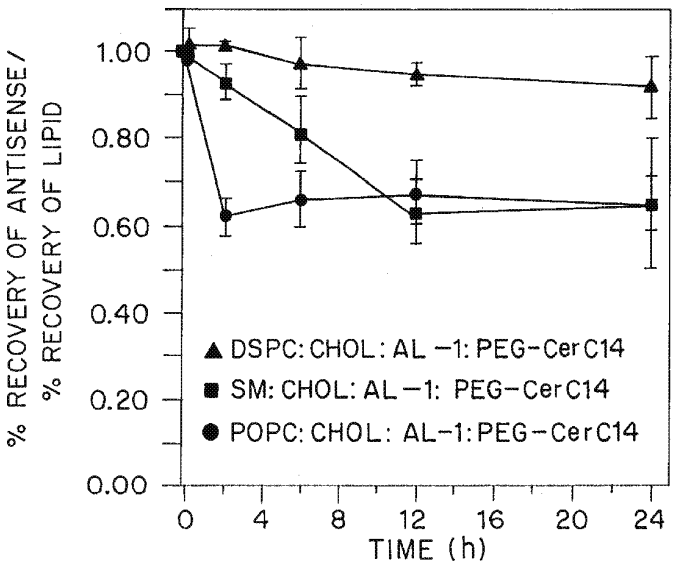


FIG. 11

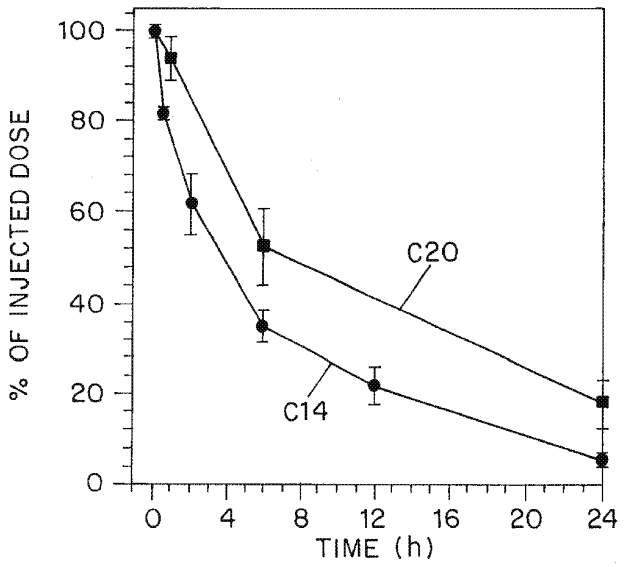


FIG. 12

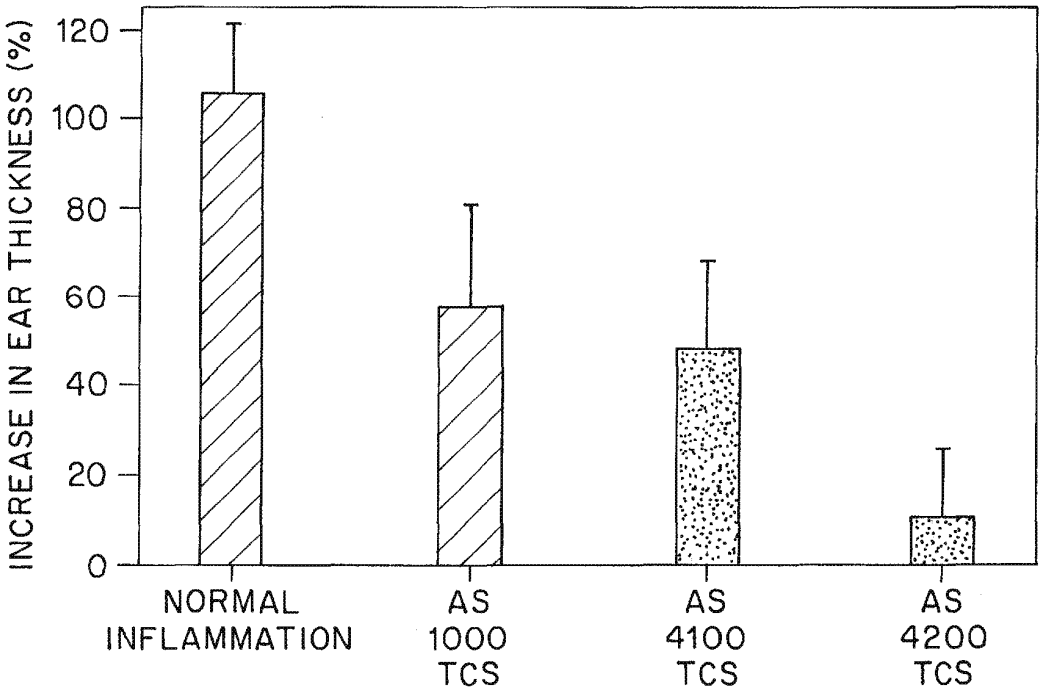

INCREASE IN EAR THICKNESS (%)
120
100
80
60
40
20
0
NORMAL INFLAMMATION
AS 1000 TCS
AS 4100 TCS
AS 4200 TCS

FIG. 13

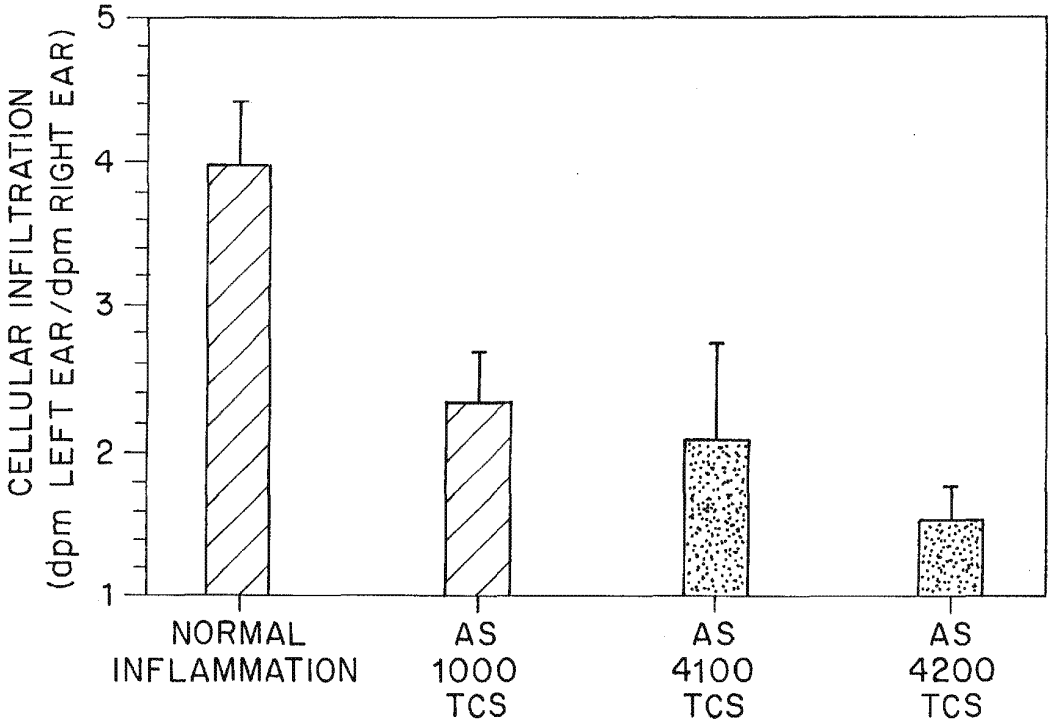

CELLULAR INFILTRATION (dpm LEFT EAR/dpm RIGHT EAR)
5
4
3
2
1
NORMAL INFLAMMATION
AS 1000 TCS
AS 4100 TCS
AS 4200 TCS

FIG. 14

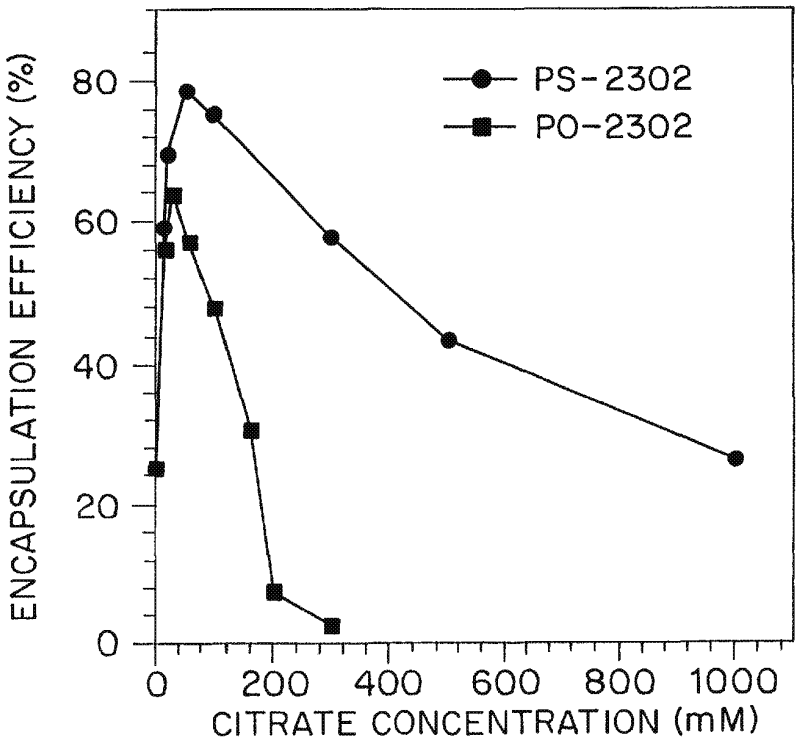

ENCAPSULATION EFFICIENCY (%)
80
60
40
20
0
PS-2302
PO-2302
0
200
400
600
800
1000
CITRATE CONCENTRATION (mM)

FIG. 15

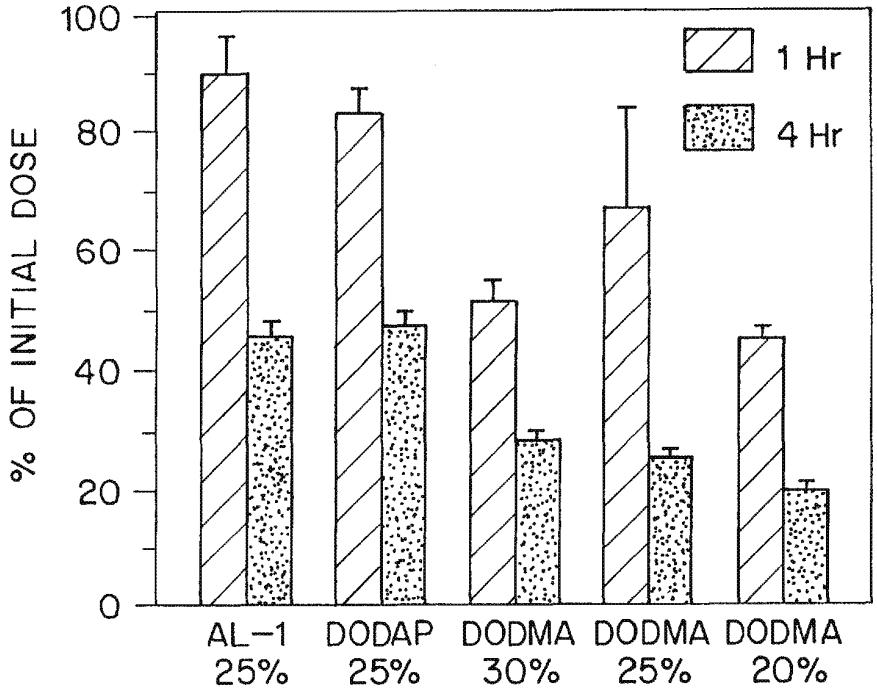
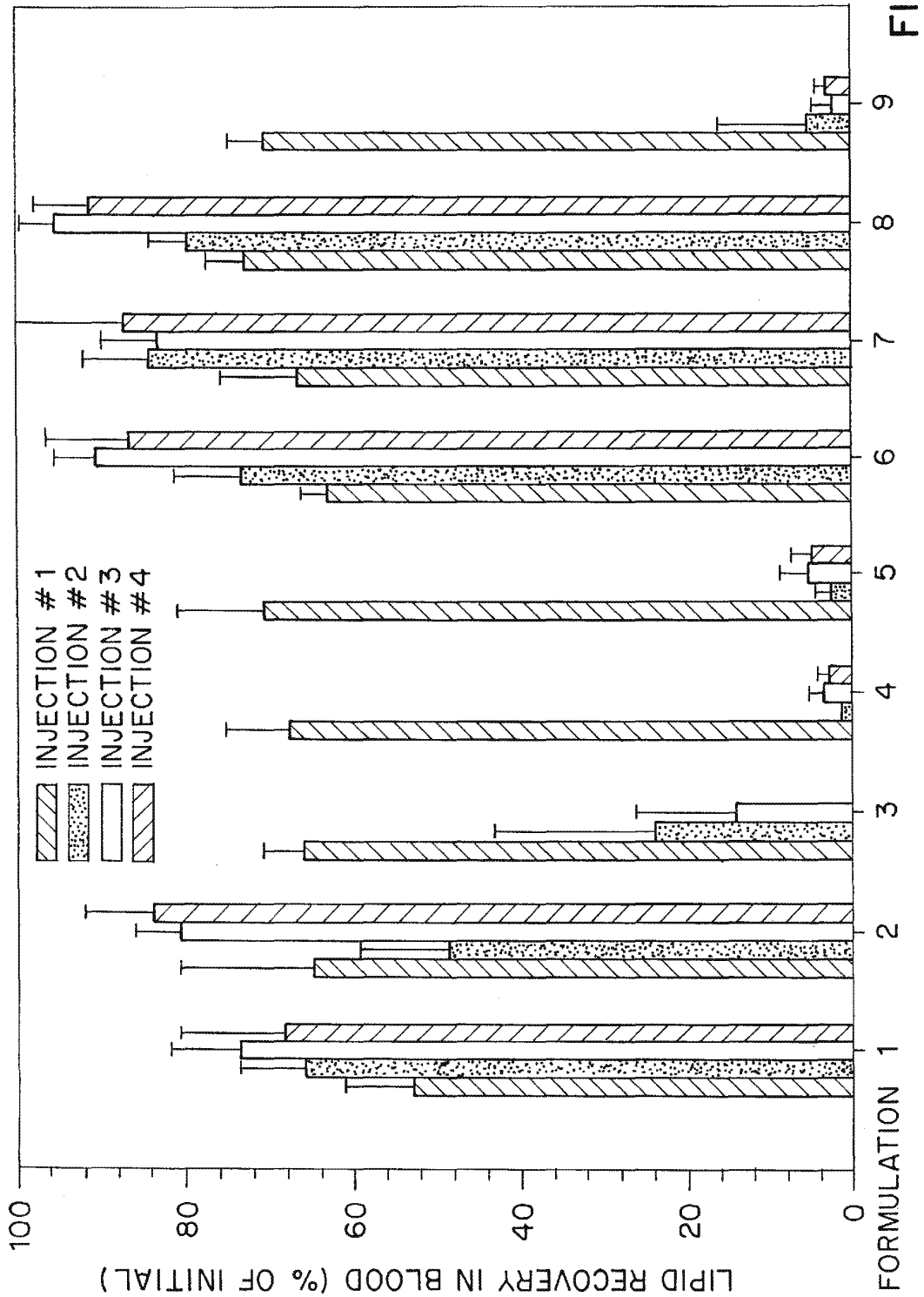

% OF INITIAL DOSE
100
80
60
40
20
0
1 Hr
4 Hr
AL-1 25%
DODAP 25%
DODMA 30%
DODMA 25%
DODMA 20%

FIG. 16

FIG. 17


INJECTION #1
INJECTION #2
INJECTION #3
INJECTION #4
LIPID RECOVERY IN BLOOD (% OF INITIAL)
100
80
60
40
20
0
FORMULATION 1
2
3
4
5
6
7
8
9

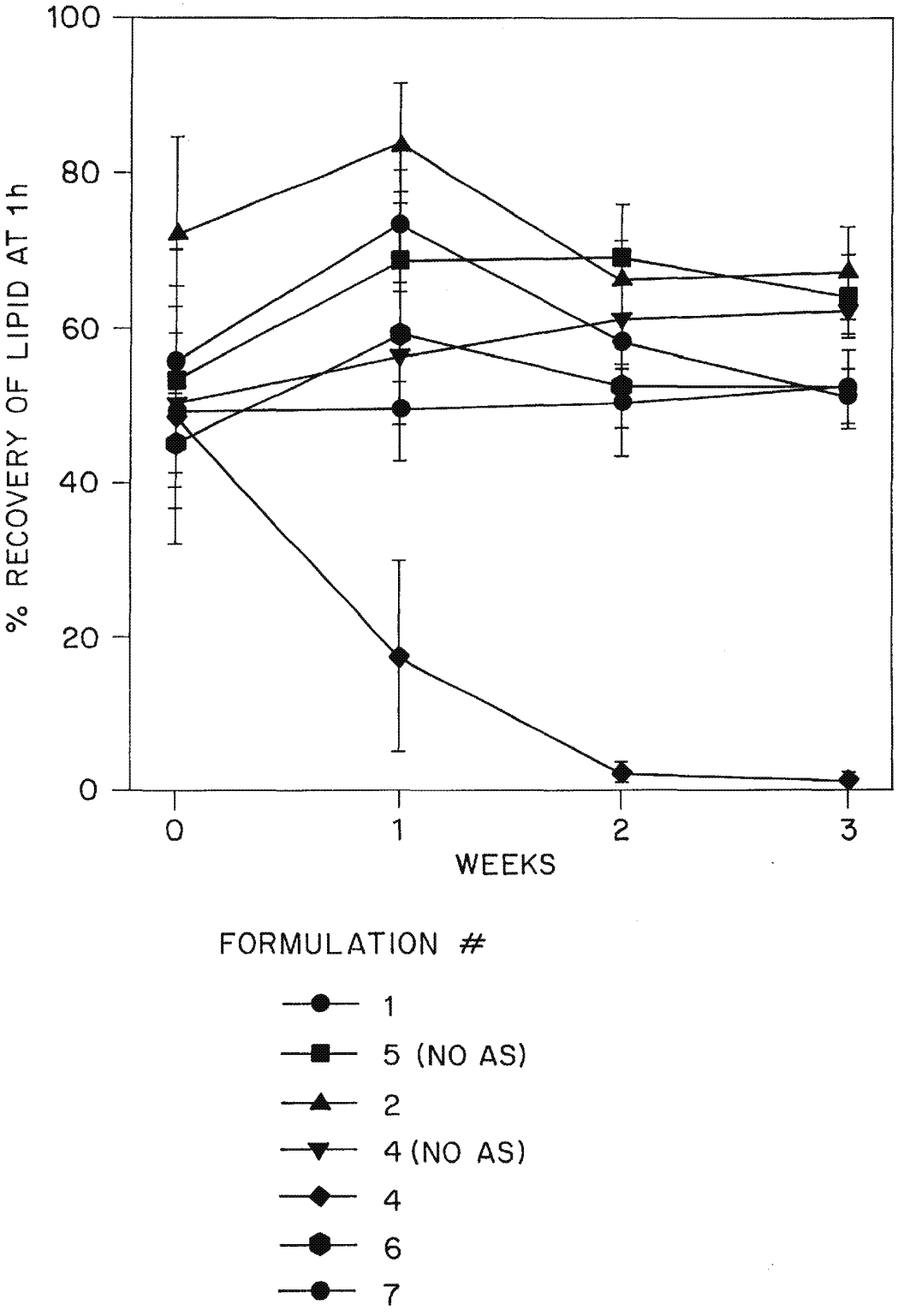

100
80
60
40
20
0
% RECOVERY OF LIPID AT 1h
0
1
2
3
WEEKS
FORMULATION #
1
5 (NO AS)
2
4 (NO AS)
4
6
7

FIG. 18

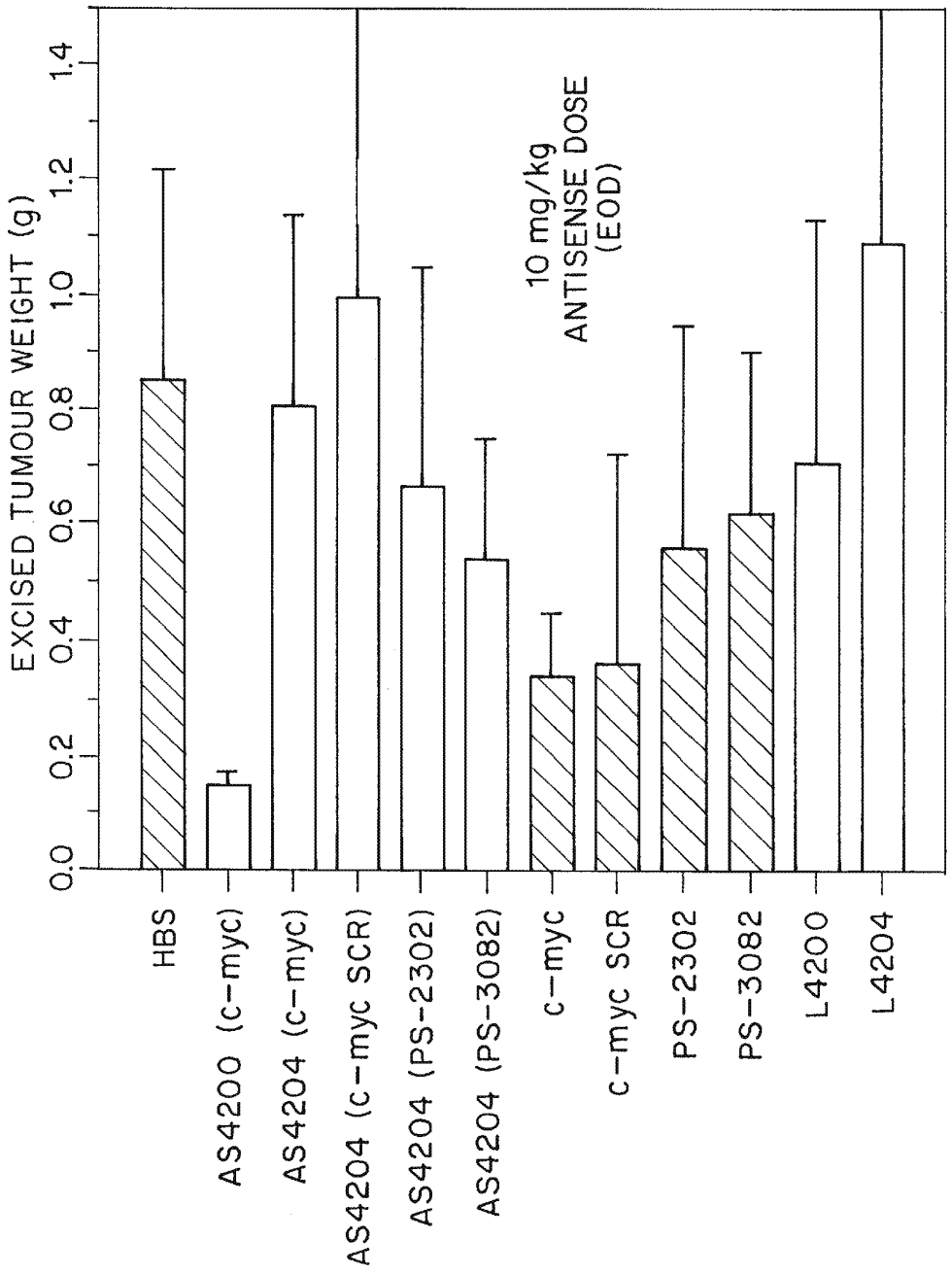

EXCISED TUMOUR WEIGHT (g)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
10 mg/kg
ANTISENSE DOSE
(EOD)
HBS
AS4200 (c-myc)
AS4204 (c-myc)
AS4204 (c-myc SCR)
AS4204 (PS-2302)
AS4204 (PS-3082)
c-myc
c-myc SCR
PS-2302
PS-3082
L4200
L4204

FIG. 19

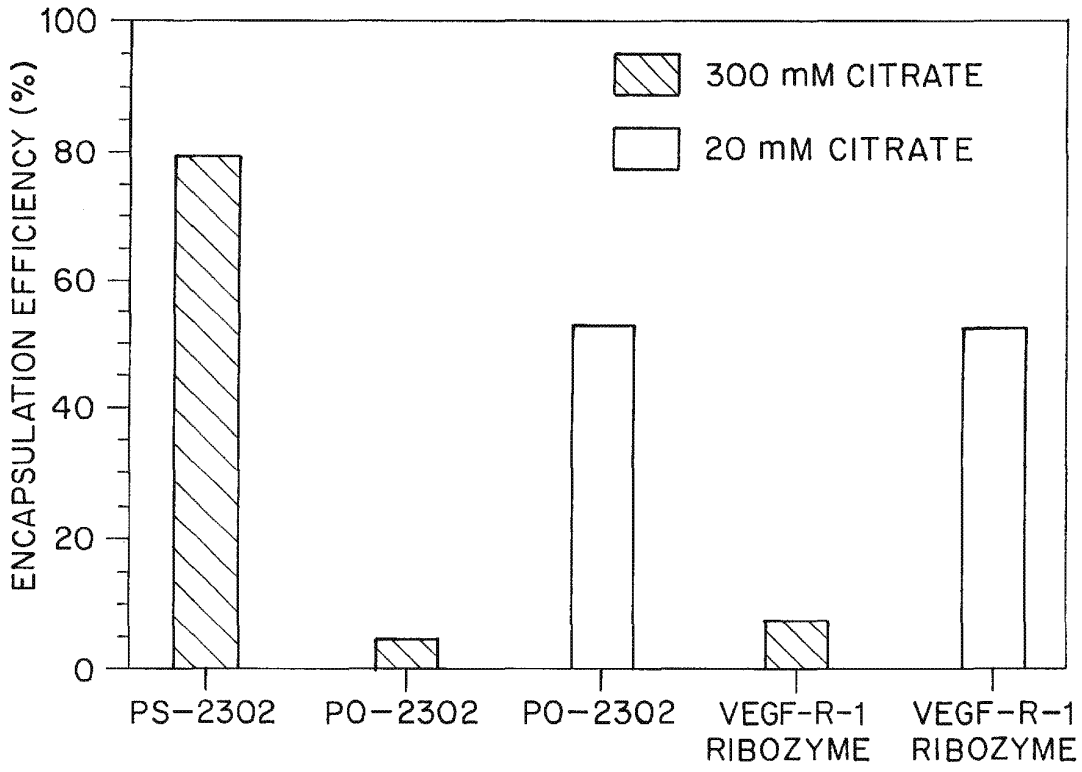

100
80
60
40
20
0
ENCAPSULATION EFFICIENCY (%)
300 mM CITRATE
20 mM CITRATE
PS-2302
PO-2302
PO-2302
VEGF-R-1 RIBOZYME
VEGF-R-1 RIBOZYME

FIG. 20

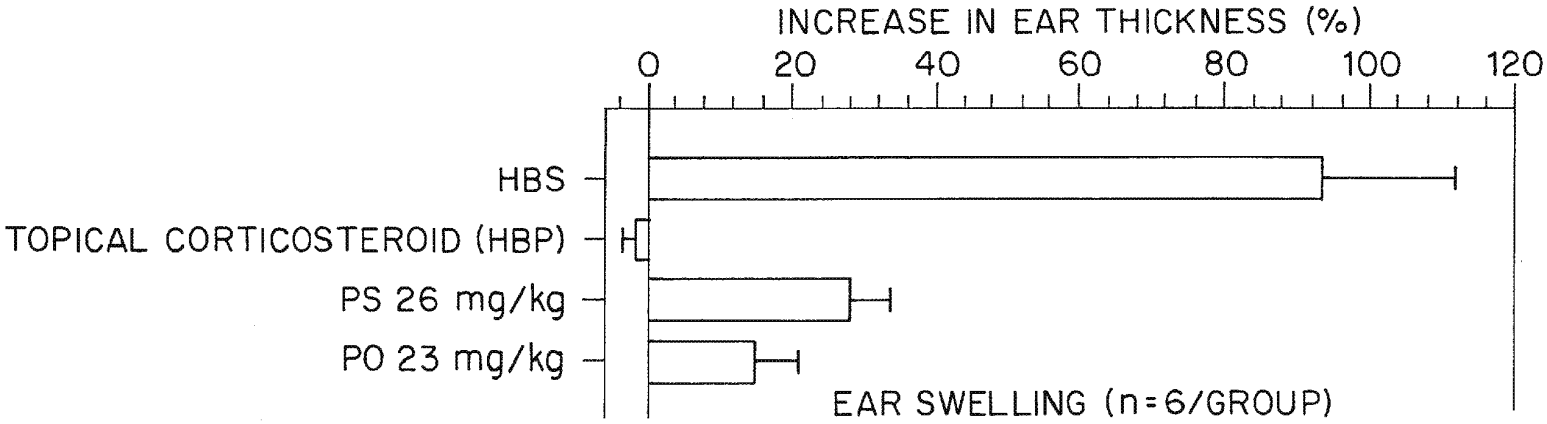


FIG. 21

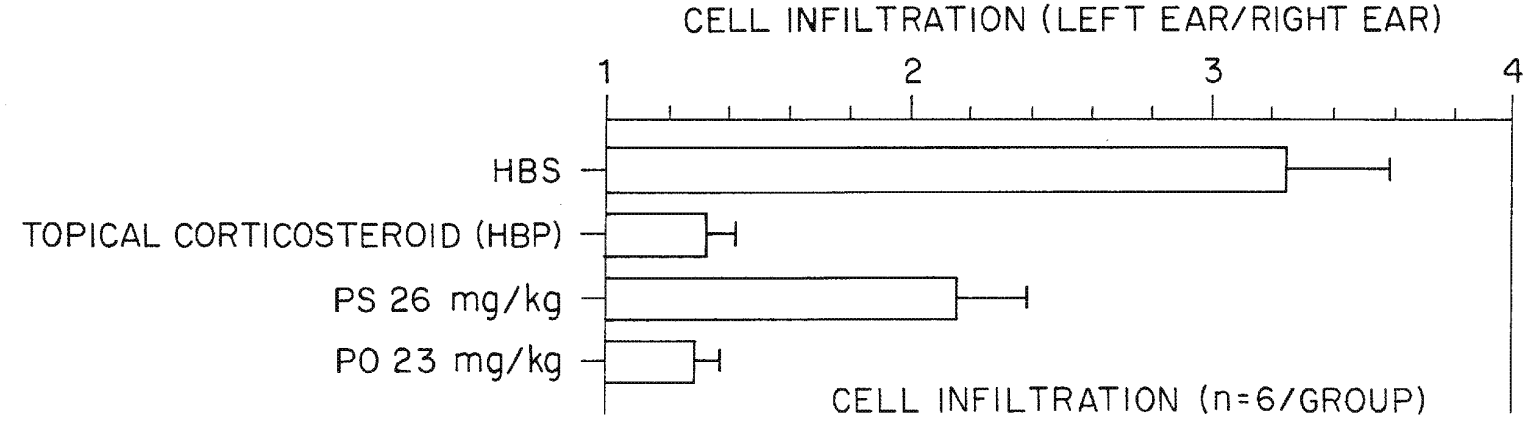


FIG. 22

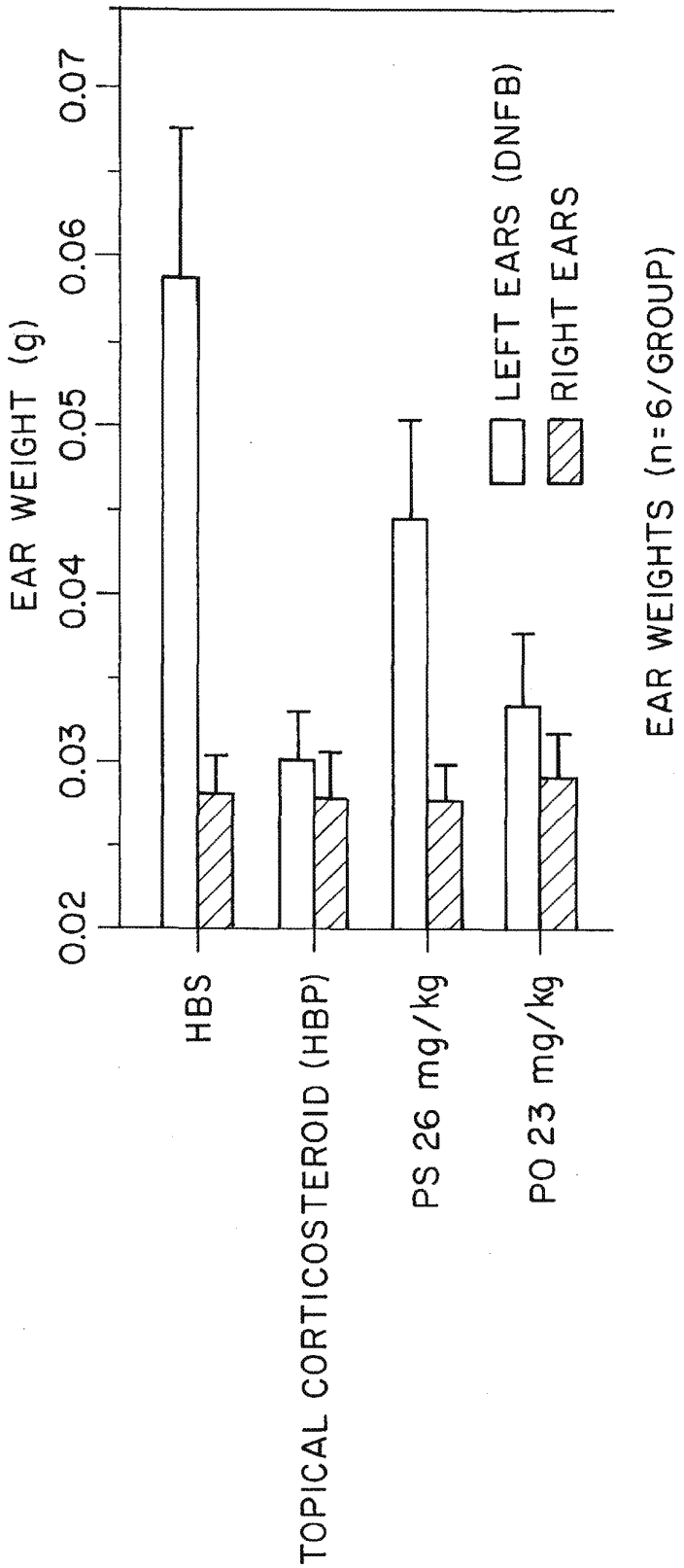

EAR WEIGHT (g)
0.02
0.03
0.04
0.05
0.06
0.07
LEFT EARS (DNFB)
RIGHT EARS
EAR WEIGHTS (n=6/GROUP)
HBS
TOPICAL CORTICOSTEROID (HBP)
PS 26 mg/kg
PO 23 mg/kg

FIG. 23

CHARGED THERAPEUTIC AGENTS ENCAPSULATED IN LIPID PARTICLES CONTAINING FOUR LIPID COMPONENTS

This application is a continuation-in-part of U.S. patent application Ser. No. 08/856,374 filed May 14, 1997, now abandoned, which is incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to compositions comprising a combination of a lipid and a therapeutic agent, particularly to lipid-nucleic acid compositions, for in vivo therapeutic use. In these compositions the therapeutic agent is encapsulated and protected from degradation and clearance in serum. Additionally, the invention provides methods of making the compositions, as well as methods of introducing the nucleic acids into cells using the compositions and treating disease conditions.

BACKGROUND OF THE INVENTION

Therapeutic oligonucleotides, such as antisense oligonucleotides or ribozymes, are short segments of DNA that have been designed to hybridize to a sequence on a specific mRNA. The resulting complex can down-regulate protein production by several mechanisms, including inhibition of mRNA translation into protein and/or by enhancement of RNase H degradation of the mRNA transcripts. Consequently, therapeutic oligonucleotides have tremendous potential for specificity of action (i.e. the down-regulation of a specific disease-related protein). To date, these compounds have shown promise in several in vitro and in vivo models, including models of inflammatory disease, cancer, and HIV (reviewed in Agrawal, *Trends in Biotech.* 14:376–387 (1996)). Antisense can also effect cellular activity by hybridizing specifically with chromosomal DNA. Advanced human clinical assessments of several antisense drugs are currently underway. Targets for these drugs include the genes or RNA products of c-myc, ICAM-1, and infectious disease organisms such as cytomegalovirus, and HIV-1.

One well known problem with the use of therapeutic oligonucleotides having a phosphodiester internucleotide linkage is its very short half-life in the presence of serum or within cells. (Zelphati, O et al. 1993. Inhibition of HIV-1 Replication in Cultured Cells with Antisense Oligonucleotides Encapsulated in Immunoliposomes. Antisense. Res. Dev. 3:323–338; and Thierry, AR et al. pp147–161 in Gene Regulation: Biology of Antisense RNA and DNA (Eds. Erickson, RP and Izant, JG) 1992. Raven Press, NY). No clinical assessment currently employs the basic phosphodiester chemistry found in natural nucleic acids, because of these and other known problems.

This problem has been partially overcome by chemical modifications which reduce serum or intracellular degradation. Modifications have been tested at the internucleotide phosphodiester bridge (i.e. using phosphorothioate, methylphosphonate or phosphoramidate linkages), at the nucleotide base (i.e.5-propynyl-pyrimidines), or at the sugar (i.e. 2'-modified sugars) (Uhlmann E., et al. 1997. Antisense: Chemical Modifications. Encyclopedia of Cancer Vol. X. pp 64–81 Academic Press Inc.). Others have attempted to improve stability using 2'–5' sugar linkages (see U.S. Pat. No. 5,532,130). Other changes have been attempted. However, none of these solutions have proven entirely satisfactory, and in vivo free antisense still has only limited efficacy. Problems remain, such as in the limited ability of some antisense to cross cellular membranes (see, Vlassov, et al., *Biochim. Biophys. Acta* 1197:95–1082 (1994)) and in the problems associated with systemic toxicity, such as complement-mediated anaphylaxis, altered coagulatory properties, and cytopenia (Galbraith, et al., *Antisense Nucl. Acid Drug Des.* 4:201–206 (1994)). Further, as disclosed in U.S. patent application Ser. No. 08/657,753 and counterpart patent application WO 97/46671, both incorporated herein by reference, modified antisense is still highly charged, and clearance from the circulation still takes place within minutes.

To attempt to improve efficacy, investigators have also employed lipid-based carrier systems to deliver chemically modified or unmodified antisense. In Zelphati, O and Szoka, F. C. (1996) J. Contr. Rel. 41:99–119, the authors refer to the use of anionic (conventional) liposomes, pH sensitive liposomes, immunoliposomes, fusogenic liposomes and cationic lipid/antisense aggregates.

None of these compositions successfully deliver phosphodiester antisense for in vivo therapy. In another paper, Zelphati & Szoka note that antisense phosphodiester oligonucleotides associated with cationic lipids have not been active in cell culture in vitro; and that only one study has reported the activity of phosphodiester antisense oligonucleotides complexed to cationic lipids. The authors argue that these findings " . . . necessitate[ ] the use [of -sic] backbone-modified oligonucleotides that are relatively resistant to both intracellular and extracellular nucleases even if a carrier is used to deliver the oligonucleotide into the target cell". (1997. J. Lip. Res. 7(1):31–49 at 34). This finding is corroborated by Bennett, C F. (1995. Intracellular Delivery of Oligonucleotides with Cationic Liposomes. Chp 14 CRC Press) who states at p. 224 that "In contrast, we have been unable to demonstrate inhibition of gene expression by uniform phosphodiester oligodeoxynucleotides directed towards a number of cellular targets in the presence of cationic lipids."

Prior art lipid formulations of modified antisense are also largely ineffective in vivo. They have poor encapsulation efficiency (15% or less for passive encapsulation systems), poor drug to lipid ratios (3% or less by weight), high susceptibility to serum nucleases and rapid clearance from circulation (particularly in the case of cationic lipid/antisense aggregates made from DOTMA, trade-name LIPOFECTIN™), and/or large sized particles (greater than 100 nm), which make them unsuitable for systemic delivery to target sites. No successful in vivo efficacy studies of lipid-encapsulated (nuclease-resistant) modified antisense are known in the prior art.

Two references to unique lipid-antisense compositions that may be significantly nuclease resistant bear consideration. Firstly, the anionic liposome (LPDII) composition of Li, S. and Huang, L (1997. J. Lip. Res. 7(1) 63–75), which encapsulates poly-lysine coated antisense, are said to have 60–70% encapsulation efficiency, but suffer from a large size of around 200 nm and a low drug to lipid ratio of 8% by weight. The effect of these particles in vivo is unknown. Secondly, the Minimal Volume Entrapment (MVE) technique for cardiolipin (anionic) liposomes results in the reasonably high encapsulation efficiency of 45–65% but again the drug:lipid ratio remains very small, approximately 6.5% by weight (see U.S. Pat. No. 5,665,710 to Rahman et al.; Thierry A R, and Takle, G B. 1995, Liposomes as a Delivery System for Antisense and Ribozyme Compounds. in *Delivery Strategies for Antisense Oligonucleotide Therapeutics,* S. Akhtar, ed, CRC Press, Boca Raton, Fla., pp. 199–221; Thierry, A R et al. pp147–161 in Gene Regu-

lation: Biology of Antisense RNA and DNA (Eds. Erickson, R P and Izant, J G) 1992. Raven Press, NY). Note that U.S. Pat. No. 5,665,710 also discloses encapsulation efficiencies of 60–90% for tiny, medically useless amounts of antisense (0.1 ug), where the drug to lipid ratio must be very low.

It is an observation of the inventors that a wide variety of prior art lipid compositions used for conventional drugs could be tested for efficacy in the antisense field, but the improvement (over free antisense) for in vivo efficacy is not known. In this regard, it is noted that although lipid compositions assertedly for use as drug carriers were disclosed by Bailey and Cullis (U.S. Pat. No. 5,552,155; and (1994) Biochem. 33(42):12573–12580), they did not disclose formulations of any bioactive compounds with these lipids, and did not suggest their utility for high efficiency loading of polyanionic species.

What is needed in the art are improved lipid-therapeutic oligonucleotide compositions which are suitable for therapeutic use. Preferably these compositions would encapsulate nucleic acids with high-efficiency, have high drug:lipid ratios, be encapsulated and protected from degradation and clearance in serum, and/or be suitable for systemic delivery. The present invention provides such compositions, methods of making the compositions and methods of introducing nucleic acids into cells using the compositions and methods of treating diseases.

SUMMARY OF THE INVENTION

In accordance with the invention, charged therapeutic agents are packaged into lipid-encapsulated therapeutic agent particles using a method comprising the steps of:

(a) combining a mixture of lipids comprising at least a first lipid component and a second lipid component with a buffered aqueous solution of a charged therapeutic agent to form an intermediate mixture containing lipid-encapsulated therapeutic particles, and

(b) changing the pH of the intermediate mixture to neutralize at least some exterior surface charges on said lipid-nucleic acid particles to provide at least partially-surface neutralized lipid-encapsulated therapeutic agent particles. The first lipid component is selected from among lipids containing a protonatable or deprotonatable group that has a pKa such that the lipid is in a charged form at a first pH and a neutral form at a second pH. The buffered solution has a pH such that the first lipid component is in its charged form when in the buffered solution, and the first lipid component is further selected such that the charged form is cationic when the therapeutic agent is anionic in the buffered solution and anionic when the therapeutic agent is cationic in the buffered solution. The second lipid component being selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation. The method the invention is particularly useful for preparation of lipid-encapsulated nucleic acids, for example antisense nucleic acids or ribozyme.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** illustrates a neutralization step which releases surface-bound antisense from the lipid-nucleic acid compositions according to the present invention.

FIGS. **2**A and **2**B illustrate certain lipid components which are useful in the present inventive methods. FIG. **2**A illustrates several groups of amino lipids including the chemical structure of DODAP. FIG. **2**B illustrates groups of PEG-modified lipids.

FIG. **3** illustrates the influence of ethanol on the encapsulation of antisense oligodeoxynucleotides. The liposomal antisense compositions were prepared as described in the Examples, with the final concentrations of antisense and lipids being 2 mg/mL and 9.9 mg/mL, respectively. The final ethanol concentration in the preparations was varied between 0 and 60%, vol/vol. Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

FIG. **4** illustrates the influence of ethanol on lipid and antisense loss during extrusion. The liposomal antisense compositions were prepared as described for FIG. **3**. The samples were extruded ten times through three 100 nm filters as described in "Materials and Methods". After extrusion, the filters were analyzed for [^{3}H]-antisense and [^{14}C]-lipid radioactivity by standard scintillation counting techniques. Results were expressed as a percent of the total initial radioactivity.

FIG. **5** illustrates the influence of DODAP content on the encapsulation of antisense oligodeoxynucleotides. A 0.6 mL aliquot of a [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 100-(55+X):45:X:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The molar ratio of DODAP was varied between 0 and 30%. The molar ratio of DSPC was adjusted to compensate for the changes in DODAP content. Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

FIG. **6** illustrates the influence of DODAP content on the encapsulation of antisense oligodeoxynucleotides. Samples were identical to those prepared in FIG. **5**. In this instance, the amount of antisense associated with the lipid was assessed by a solvent extraction procedure as described in "Material and Methods". Antisense was extracted into a methanol:water aqueous phase, while the lipid was soluble in the organic (chloroform) phase. The aqueous phase was preserved and antisense concentration was determined by measuring the absorbance at 260 nm. This confirmed that the antisense was associated with the lipid vesicles, and that the [^{3}H]-label on the antisense had not exchanged to the lipid.

FIG. **7** illustrates the quasi-elastic light scattering analysis of encapsulated liposomal antisense. The size distribution of a liposomal preparation of antisense was determined by quasi-elastic light scattering (QELS) immediately after removal of the free antisense (A), and after storage of the preparation for 2 months at 4° C. (B), using a Nicomp Model 370 sub-micron particle sizer.

FIG. **8** illustrates the influence of the initial antisense concentration on antisense loading in DODAP vesicles. Varying final concentrations of a 20mer of [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) were mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio), 9.9 mg/mL (final concentration). Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

EPC:CHOL liposomes containing encapsulated antisense are included for comparison.

FIG. **9** illustrates the plasma clearance of encapsulated antisense. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20–25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

FIG. **10** illustrates the biodistribution of encapsulated antisense. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20–25 g) ICR mice at a lipid dose of 120 mg/kg. Mice were terminated by cervical dislocation and the organs were recovered and processed as described in "Materials and Methods". Lipid and antisense recoveries were determined by standard scintillation counting techniques.

FIG. **11** illustrates the differential release rates of antisense in plasma. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20–25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques. Release rates were determined by measuring the [3H]/[14C] ratio over time.

FIG. **12** illustrates the influence of PEG-acyl chain lengths on plasma clearance of encapsulated antisense. Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of DSPC:CHOL:DODAP:PEG-CerC14 or C20 (25:45:20:10). The formulation contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20–25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

FIG. **13** illustrates the enhanced efficacy of liposomal antisense containing DODAP—ear swelling. Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped PS 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4200). Ear swelling was measured at 24 hours after initiating inflammation using an engineer's micrometer.

FIG. **14** illustrates the enhanced efficacy of liposomal antisense containing DODAP—cellular infiltration. Mice received 10 μCi of [^{3}H]-methylthymidine, i.p., 24 hours before initiating inflammation. Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC:CHOL liposomes with entrapped PS 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS 3082 (identified as 4200). Cell infiltration was monitored by measuring the radioactivity in the "challenged ear" versus the non-treated ear. Results are expressed as the ratio of radioactivity in the left (challenged ear) versus right ear.

FIG. **15** shows asymmetric loading of lipid-encapsulated-nucleic acid particles in accordance with the invention.

FIG. **16** shows clearance of lipid-encapsulated antisense particles formulated with several amino lipids at different levels.

FIG. **17** shows blood levels of antisense-containing particles after repeat dosages.

FIG. **18** shows blood levels of antisense-containing particles after repeat dosages.

FIG. **19** illustrates results of a study on the in vivo efficacy of lipid-encapsulated antisense particles in accordance with the invention in a mouse tumor model.

FIG. **20** shows encapsulation efficiency results for lipid-encapsulated therapeutic agent particles in accordance with the invention.

FIG. **21** shows results for studies on the use of murine ICAM1 in an ear inflammation model.

FIG. **22** shows results for studies on the use of murine ICAM1 in an ear inflammation model.

FIG. **23** shows results for studies on the use of murine ICAM1 in an ear inflammation model.

DETAILED DESCRIPTION OF THE INVENTION

Contents

I. Glossary

Abbreviations and Definitions

The following abbreviations are used herein: ATTA, N-(ω-N'-acetoxy-octa(14'amino-3',6',9',12'-

tetraoxatetradecanoyl)); CHE, cholesteryl-hexadecylether; CHOL, cholesterol; DODAP or AL-1, 1,2-dioleoyloxy-3-dimethylaminopropane (and its protonated ammonium form); DODMA, N-(1-(2,3-Dioleoyloxy) propyl)-N,N,-dimethyl ammonium chloride; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; HBS, HEPES-buffered saline; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; MES, 2-(N-morpholino)ethane sulfonic acid; PS 3082, murine ICAM-1 phosphorothioate oligodeoxynucleotide having the sequence: TGCATC-CCCCAGGCCACCAT (SEQ ID No. 1); NaCl, sodium chloride; OLIGREEN™, a dye that becomes fluorescent when interacting with an oligonucleotide; PEG-CerC20, polyethylene glycol coupled to a ceramide derivative with 20 carbon acyl chain; POPC, palmitoyloleoylphophatidylcholine; SM, sphingomyelin; DOPE, 1,2-dioleoyl-sn-3-phosphoethanolamine.

"Lipid-therapeutic agent particle" means a particle comprising lipids and a charged (cationic or anionic) therapeutic agent. "Lipid-therapeutic nucleic acid particle" means a particle comprising a lipid and a therapeutic nucleic acid.

"Lipid-encapsulated therapeutic agent (nucleic acid) particle" means a lipid-therapeutic agent particle wherein less than 50% and preferably less than 10% of the therapeutic agent (nucleic acid) is detectable on the external surface of the particle or in the buffer external to the particle. In the case of nucleic acids, the amount of encapsulated versus unencapsulated nucleic acid can be assayed by fluorescence assays or nuclease assays as described herein. Comparable assays can be used for other types of therapeutic agents.

"Therapeutically effective amount" means an amount which provides a therapeutic benefit. For antisense oligonucleotide this means generally 0.5 to 50 mg/kg of body weight, but when delivered in a lipid particle formulation, a below-toxic amount of lipid must be used.

"Lipid exchange out of particle" and the rate of this exchange is fully explained in U.S. patent application Ser. No. 08/486,214, filed Jun. 7, 1995, now U.S. Pat. No. 5,820,873, and U.S. patent application Ser. No. 08/485,608, filed Jun. 7, 1995, now U.S. Pat. No. 5,885,613, and PCT Patent publications WO 96/10391 and WO 96/10392, which are all incorporated herein by reference. Lipid exchange into the surrounding medium is possible for lipids which are reversibly associated with the lipid particle membrane. Each lipid has a characteristic rate at which it will exchange put of a particle which depends on a variety of factors including acyl chain length, saturation, head group size, buffer composition and membrane composition.

"Disease site" is the site in an organism which demonstrates or is the source of a pathology. The disease site may be focused, as in a site of neoplasm or inflammation, or may be diffuse as in the case of a non-solid tumor. "Administration at a site which is distal to the disease site" means that delivery to the disease site will require some kind of systemic delivery, either by blood or lymph circulation, or other fluid movement inside the organism.

The term "transfection" as used herein, refers to the introduction of polyanionic materials, particularly nucleic acids, into cells. The polyanionic materials can be in the form of DNA or RNA which is linked to expression vectors to facilitate gene expression after entry into the cell. Thus the polyanionic material or nucleic acids used in the present invention is meant to include DNA having coding sequences for structural proteins, receptors and hormones, as well as transcriptional and translational regulatory elements (i.e., promoters, enhancers, terminators and signal sequences) and vector sequences. Methods of incorporating particular nucleic acids into expression vectors are well known to those of skill in the art, but are described in detail in, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1–3, Cold Spring Harbor Laboratory, (1989) or *Current Protocols in Molecular Biology,* F. Ausubel et al., ed. Greene Publishing and Wiley-Interscience, New York (1987), both of which are incorporated herein by reference.

The term "physiological pH" refers to pH levels conventionally encountered in serum or blood. In general, this will be in the range of pH 7.2 to 7.5. Preferred protonatable or deprotonatable lipids have a pKa such that they are substantially neutral at this pH, i.e., a pKa of about 4 to 7 in the case of an amino lipid.

II. General

The present invention relates to methods and compositions for producing lipid-encapsulated therapeutic agent particles in which charged therapeutic agents are encapsulated within a lipid layer. The invention is applicable to both anionic and cationic therapeutic agents, including polyanionic nucleic acids, polyanionic proteins or peptides, cytokines and heparin, and cationic proteins and peptides. The invention is principally demonstrated herein with reference to polyanionic nucleic acids as the therapeutic agent, which is a preferred embodiment, but the same principles can be readily extended to other polyanionic or to cationic therapeutic agents.

To evaluate the quality of a lipid/nucleic acid formulation the following criteria, among others, may be employed:

drug to lipid ratio;

encapsulation efficiency;

nuclease resistance/serum stability; and

particle size.

High drug to lipid rations, high encapsulation efficiency, good nuclease resistance and serum stability and controllable particle size, generally less than 200 nm in diameter are desirable In addition, the nature of the nucleic acid polymer is of significance, since the modification of nucleic acids in an effort to impart nuclease resistance adds to the cost of therapeutics while in many cases providing only limited resistance. The present invention provides lipid-nucleic acid particles and methods for preparing lipid-nucleic acid formulations which are far superior to the art according to these criteria.

Unless stated otherwise, these criteria are calculated in this specification as follows:

drug to lipid ratio: The amount of drug (therapeutic agent) in a defined volume of preparation divided by the amount of lipid in the same volume. This may be on a mole per mole basis or on a weight per weight basis, or on a weight per mole basis. For final, administration-ready formulations, the drug:lipid ratio is calculated after dialysis, chromatography and/or enzyme (e.g., nuclease) digestion has been employed to remove as much of the external therapeutic agent (e.g., nucleic acid) as possible. Drug:lipid ratio is a measure of potency of the formulation, although the highest possible drug:lipid ratio is not always the most potent formulation;

encapsulation efficiency: the drug to lipid ratio of the starting mixture divided by the drug to lipid ratio of the final, administration competent formulation. This is a measure of relative efficiency. For a measure of absolute efficiency, the total amount of therapeutic agent (nucleic acid) added to the starting mixture that ends up in the administration competent formulation, can also be calculated. The amount of lipid lost during the formulation process may also be calculated. Efficiency is a measure of the wastage and expense of the formulation;

nuclease resistance/serum stability: the ability of the formulation to protect the nucleic acid therapeutic agents from nuclease digestion either in an in vitro assay, or in circulation. Several standard assays are detailed in this specification. Encapsulated particles have much greater nuclease resistance and serum stability than lipid-antisense aggregates such as DOTMA/DOPE (LIPOFECTIN™) formulations; and

size: the size of the particles formed. Size distribution may be determined using quasi-elastic light scattering (QELS) on a Nicomp Model 370 sub-micron particle sizer. Particles under 200 mn are preferred for distribution to neo-vascularized (leaky) tissues, such as neoplasms and sites of inflammation.

The methods and composition of the invention make use of certain lipids which can be present in both a charged and an uncharged form. For example, amino lipids which are charged at a pH below the pK_a of the amino group and substantially neutral at a pH above the pk_a can be used in a two-step process. First, lipid vesicles can be formed at the lower pH with (cationic) amino lipids and other vesicle components in the presence of nucleic acids. In this manner the vesicles will encapsulate and entrap the nucleic acids. Second, the surface charge of the newly formed vesicles can be neutralized by increasing the pH of the medium to a level above the pK_a of the amino lipids present, i.e., to physiological pH or higher. Particularly advantageous aspects of this process include both the facile removal of any surface adsorbed nucleic acid and a resultant nucleic acid delivery vehicle which has a neutral surface. Liposomes or lipid particles having a neutral surface are expected to avoid rapid clearance from circulation and to avoid certain toxicities which are associated with cationic liposome preparations.

It is further noted that the vesicles formed in this manner provide formulations of uniform vesicle size with high content of nucleic acids. Additionally, the vesicles are not aggregate complexes, but rather are large unilamellar vesicles having a size range of from about 70 to about 200 nm, more preferably about 90 to about 130 nm.

Without intending to be bound by any particular theory, it is believed that the very high efficiency of nucleic acid encapsulation is a result of electrostatic interaction at low pH. FIG. **1** provides an illustration of the processes described herein. More particularly, this figure illustrates a lipid-nucleic acid composition of amino lipids and PEG-modified lipids having encapsulated antisense nucleic acid and surface-bound antisense nucleic acid. At acidic pH (shown as pH 4.0), the surface is charged and binds a portion of the antisense through electrostatic interactions. When the external acidic buffer is exchanged for a more neutral (pH 7.5, HBS) buffer, the surface of the lipid particle or liposome is neutralized, resulting in release of the antisense nucleic acid.

Encapsulation efficiency results in FIG. **15** show a further unexpected benefit of the invention. As shown in the figure, for both phosphorothioate (PS-2302) and phosphodiester (PO-2302) formulations it is possible to obtain encapsulation efficiencies—i.e., the amount of nucleic acid that ends up on the inside of the particle—that are greater than 50%. Phosphodiesters achieve well over 60%, and phosphorothioates can be at least up to 80% encapsulated. The asymmetry of loading is surprising, given that in the simplest model of loading large unilamellar vesicles (LUV's) the therapeutic agent (nucleic acid) would be equally likely to associate with cationic charges on the inside and outside of the particle. A 1:1 distribution (inside to outside) would suggest that the 50% on the outside should be removed upon neutralization of the outside surface charges, such that 50% efficiency would be the theoretical upper limit. Through some unclear mechanism, however, the invention surprisingly provides an active process whereby the majority of the therapeutic agent (nucleic acid) ends up protected on the inside of the particles.

III. Methods of Preparing Lipid/Therapeutic Agent (Nucleic Acid) Formulations

In view of the above, the present invention provides methods of preparing lipid/nucleic acid formulations. In the methods described herein, a mixture of lipids is combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid encapsulated in lipid particles wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 10 wt % to about 20 wt %. The intermediate mixture may optionally be sized to obtain lipid-encapsulated nucleic acid particles wherein the lipid portions are large unilamellar vesicles, preferably having a diameter of 70 to 200 nm, more preferably about 90 to 130 nm. The pH is then raised to neutralize at least a portion of the surface charges on the lipid-nucleic acid particles, thus providing an at least partially surface-neutralized lipid-encapsulated nucleic acid composition.

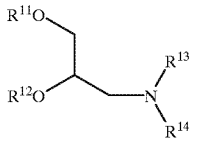
The mixture of lipids includes at least two lipid components: a first lipid component that is selected from among lipids which have a pKa such that the lipid is cationic at pH below the pKa and neutral at pH above the pKa, and a second lipid component that is selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation.

The first lipid component of is a lipid (or a mixture of lipid species with similar properties) which has at least one protonatable or deprotonatable group, such that the lipid is charged at a first pH (cationic or anionic, depending on the nature and pKa of the protonatable or deprotonatable group), and neutral at a second pH, preferably at physiological pH. It will of course be understood that the addition or removal of protons as a function of pH is an equilibrium process, and that the reference to a charged or a neutral lipid refers to the nature of the predominant species and does not require that all of the lipid be present in the charged or neutral form. Lipids which have more than one protonatable or deprotonatable group, or which are zwiterrionic are not excluded from use in the invention. Protonatable lipids are particularly useful as the first lipid component of the invention when the pKa of the protonatable group is in the range of about 4 to about 11. Most preferred is pKa of about 4 to about 7, because these lipids will be cationic at the lower pH formulation stage, while particles will be largely (though not completely) surface neutralized at physiological pH around pH 7.5. One of the benefits of this pKa is that at least some antisense stuck to the outside surface of the particle will lose its electrostatic interaction at physiological pH and be removed by simple dialysis; thus greatly reducing the particle's susceptibility to clearance.

Preferred lipids with a protonatable group for use as the first lipid component of the lipid mixture are amino lipids. As used herein, the term "amino lipid" is meant to include those lipids having one or two fatty acid or fatty alkyl chains and an amino head group (including an alkylamino or dialkylamino group) which is protonated to form a cationic lipid at physiological pH (see FIG. **2A**). In one group of embodiments, the amino lipid is a primary, secondary or tertiary amine represented by the formula:

in which R^1 is a C_{12} to C_{24} alkyl group which is branched or unbranched, and saturated or unsaturated. R^2 is hydrogen or a C_1 to C_{24} alkyl group which is also branched or unbranched, and saturated or unsaturated (when three or more carbons are present). R^3 is hydrogen or a C_1 to C_6 alkyl group. Examples of these amino lipids include, for example, stearylamine, oleylamine, dioleylamine, N-methyl-N,N-dioleylamine, and N,N-dimethyloleylamine.

In another group of embodiments, the amino lipid is a lipid in which the amino head group is attached to one or more fatty acid or fatty alkyl groups by a scaffold such as, for example, a glycerol or propanediol moiety. Illustrative of these amine lipids is the formula:



wherein at least one and preferably both of R^{11} and R^{12} is a C_{12} to C_{24} alkyl or acyl group which is branched or unbranched, saturated or unsaturated. In those embodiments in which only one of R^{11} or R^{12} is a long chain alkyl or acyl group, the other of R^{11} or R^{12} will be a hydrogen or lower alkyl or acyl group having from one to six carbon atoms. The remaining groups, R^{13} and R^{14} are typically hydrogen or C_1 to C_4 alkyl. In this group of embodiments, the amino lipid can be viewed as a derivative of 3-monoalkyl or dialkylamino-1,2-propanediol. An example of a suitable amino lipid is DODAP (1,2-dioleoyloxy-3-dimethylamino-propane, see FIG. **2**A). Other amino lipids would include those having alternative fatty acid groups and other dialkylamino groups, including those in which the alkyl substituents are different (e.g., N-ethyl-N-methylamino-, N-propyl-N-ethylamino- and the like). For those embodiments in which R^{11} and R^{12} are both long chain alkyl or acyl groups, they can be the same or different. In general, amino lipids having less saturated acyl chains are more easily sized, particularly when the complexes must be sized below about 0.3 microns, for purposes of filter sterilization. Amino lipids containing unsaturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are particularly preferred. Other scaffolds can also be used to separate the amino group and the fatty acid or fatty alkyl portion of the amino lipid. Suitable scaffolds are known to those of skill in the art.

Compounds that are related to DODAP that may be useful with this invention include: 1-oleoyl-2-hydroxy-3-N,N-dimethylamino propane; 1,2-diacyl-3-N,N-dimethylamino propane; and 1,2-didecanoyl-1-N,N-dimethylamino propane. Further, it is proposed that various modifications of the DODAP or DODMA headgroup, or any compound of the general formula: can be modified to obtain a suitable pKa. Suitable headgroup modifications that are useful in the instant invention include:

	R^1	R^2
1	H	H
2	H	CH_3
3	CH_3	CH_3
4	H	CH_2CH_3
5	CH_3	CH_2CH_3
6	CH_2CH_3	CH_2CH_3
7	H	CH_2CH_2OH
8	CH_3	CH_2CH_2OH
9	CH_2CH_3	CH_2CH_2OH
10	CH_2CH_2OH	CH_2CH_2OH
11*	H	$CH_2CH_2NH_2$
12*	CH_3	$CH_2CH_2NH_2$
13*	CH_2CH_3	$CH_2CH_2NH_2$
14*	CH_2CH_2OH	$CH_2CH_2NH_2$
15*	$CH_2CH_2NH_2$	$CH_2CH_2NH_2$

In other embodiments, the amino lipid can be a derivative of a naturally occurring amino lipid, for example, sphingosine. Suitable derivatives of sphingosine would include those having additional fatty acid chains attached to either of the pendent hydroxyl groups, as well as alkyl groups, preferably lower alkyl groups, attached to the amino functional group.

Other lipids which may be used as the first lipid component of the invention include phosphine lipids (although toxicity issues may limit their utility), and carboxylic acid lipid derivative. These generally have a pKa of about 5 and are therefore useful with cationic therapeutic agents.

The second lipid component is selected to improve the formulation process by reducing aggregation of the lipid particles during formation. This may result from steric stabilization of particles which prevents charge-induced aggregation during formation. Examples of suitable lipids for this purpose include polyethylene glycol (PEG)-modified lipids, monosialoganglioside Gm1, and polyamide oligomers ("PAO") such as ATTA (disclosed in U.S. patent application Ser. No. 60/073,852 assigned to the assignee of the instant invention and incorporated herein by reference). Other compounds with uncharged, hydrophilic, steric-barrier moieties, that prevent aggregation during formulation, like PEG, Gm1 or ATTA, can also be coupled to lipids for use as the second lipid component in the methods and compositions of the invention. Typically, the concentration of the second lipid component is about 1 to 15% (by mole percent of lipids).

Specific examples of PEG-modified lipids (or lipid-polyoxyethylene conjugates) that are useful in the present invention can have a variety of "anchoring" lipid portions to secure the PEG portion to the surface of the lipid vesicle. Examples of suitable PEG-modified lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid (see FIG. **2**B, structures A and B), PEG-modified diacylglycerols and dialkylglycerols (see FIG. **2**B, structures C and D), PEG-modified dialkylamines (FIG. **2**B, structure E) and PEG-modified 1,2-diacyloxypropan-3-amines (FIG. **2**B, structure F). Particularly preferred are PEG-ceramide conjugates (e.g., PEG-CerC14 or PEG-CerC20) which are described in co-pending U.S. Ser. No. 08/486,214, incorporated herein by reference.

In embodiments where a sterically-large moiety such as PEG or ATTA are conjugated to a lipid anchor, the selection of the lipid anchor depends on what type of association the conjugate is to have with the lipid particle. It is well known that mePEG(mw2000)-diastearoylphosphatidylethanolamine (PEG-DSPE) will remain associated with a liposome until the particle is

cleared from the circulation, possibly a matter of days. Other conjugates, such as PEG-CerC20 have similar staying capacity. PEG-CerC14, however, rapidly exchanges out of the formulation upon exposure to serum, with a $T_{1/2}$ less than 60 mins. in some assays. As illustrated in U.S. patent application Ser. No. 08/486,214 at least three characteristics influence the rate of exchange: length of acyl chain, saturation of acyl chain, and size of the steric-barrier head group. Compounds having suitable variations of these features may be useful for the invention.

It should be noted that aggregation preventing compounds do not necessarily require lipid conjugation to function properly. Free PEG or free ATTA in solution may be sufficient to prevent aggregation. If the particles are stable after formulation, the PEG or ATTA can be dialyzed away before administration to a patient.

In addition to the first and second lipid components, the lipid mixture may contain additional lipid species. These additional lipids may be, for example, neutral lipids or sterols.

Neutral lipids, when present in the lipid mixture, can be any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, and cerebrosides. The selection of neutral lipids for use in the complexes herein is generally guided by consideration of, e.g., liposome size and stability of the liposomes in the bloodstream. Preferably, the neutral lipid component is a lipid having two acyl groups, (i.e., diacylphosphatidylcholine and diacylphosphatidylethanolamine). Lipids having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In one group of embodiments, lipids containing saturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are preferred. In another group of embodiments, lipids with mono or diunsaturated fatty acids with carbon chain lengths in the range of C_{14} to C_{22} are used. Additionally, lipids having mixtures of saturated and unsaturated fatty acid chains can be used. Preferably, the neutral lipids used in the present invention are DOPE, DSPC, POPC, or any related phosphatidylcholine. The neutral lipids useful in the present invention may also be composed of sphingomyelin or phospholipids with other head groups, such as serine and inositol.

The sterol component of the lipid mixture, when present, can be any of those sterols conventionally used in the field of liposome, lipid vesicle or lipid particle preparation. A preferred sterol is cholesterol.

The mixture of lipids is typically a solution of lipids in an alcoholic solvent. Hydrophilic, low molecular weight water miscible alcohols with less than 10 carbon atoms, preferably less than 6 carbon atoms are preferred. Typical alcohols used in this invention are ethanol, methanol, propanol, butanol, pentanol and ethylene glycol and propylene glycol. Particularly preferred is ethanol. In most embodiments, the alcohol is used in the form in which it is commercially available. For example, ethanol can be used as absolute ethanol (100%), or as 95% ethanol, the remainder being water.

In one exemplary embodiment, the mixture of lipids is a mixture of amino lipids, neutral lipids (other than an amino lipid), a sterol (e.g., cholesterol) and a PEG-modified lipid (e.g., a PEG-ceramide) in an alcohol solvent. In preferred embodiments, the lipid mixture consists essentially of an amino lipid, a neutral lipid, cholesterol and a PEG-ceramide in alcohol, more preferably ethanol. In further preferred embodiments, the first solution consists of the above lipid mixture in molar ratios of about 10–35% amino lipid:25–45% neutral lipid:35–55% cholesterol:0.5–15% PEG-ceramnide. In still further preferred embodiments, the first solution consists essentially of DODAP, DSPC, Chol and PEG-CerC14, more preferably in a molar ratio of about 10–35% DODAP:25–45% DSPC:35–55% Chol:0.5–15% PEG-CerC14. In another group of preferred embodiments, the neutral lipid in these compositions is replaced with POPC or SM.

In accordance with the invention, the lipid mixture is combined with a buffered aqueous solution of charged therapeutic agent, preferably nucleic acids. The buffered aqueous solution of therapeutic agents (nucleic acids) which is combined with the lipid mixture is typically a solution in which the buffer has a pH of less than the pK_a of the protonatable lipid in the lipid mixture. As used herein, the term "nucleic acid" is meant to include any oligonucleotide or polynucleotide having from 10 to 100,000 nucleotide residues. Antisense and ribozyme oligonucleotides are particularly preferred. The term "antisense oligonucleotide" or simply "antisense" is meant to include oligonucleotides which are complementary to a targeted nucleic acid and which contain from about 10 to about 50 nucleotides, more preferably about 15 to about 30 nucleotides. The term also encompasses antisense sequences which may not be exactly complementary to the desired target gene. Thus, the invention can be utilized in instances where non-target specific-activities are found with antisense, or where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use.

The nucleic acid that is used in a lipid-nucleic acid particle according to this invention includes any form of nucleic acid that is known. Thus, the nucleic acid may be a modified nucleic acid of the type used previously to enhance nuclease resistance and serum stability. Surprisingly, however, acceptable therapeutic products can also be prepared using the method of the invention to formulate lipid-nucleic acid particles from nucleic acids which have no modification to the phosphodiester linkages of natural nucleic acid polymers, and the use of unmodified phosphodiester nucleic acids (i.e., nucleic acids in which all of the linkages are phosphodiester linkages) is a preferred embodiment of the invention. Still other nucleic acids which are useful in the present invention include, synthetic or pre-formed poly-RNA such as poly(IC) IC.

The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or DNA-RNA hybrids. Examples of double-stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems such as plasmid DNA. Single-stranded nucleic acids include antisense oligonucleotides (discussed above and complementary to DNA and RNA), ribozymes and triplex-forming oligonucleotides.

In order to increase stability, some single-stranded nucleic acids may have some or all of the nucleotide linkages substituted with stable, non-phosphodiester linkages, including, for example, phosphorothioate, phosphorodithioate, phosphoroselenate, boranophosphate, methylphosphonate, or O-alkyl phosphotriester linkages. Phosphorothioate nucleic acids (PS-oligos) are those oligonucleotides or polynucleotides in which one of the non-bridged oxygens of the internucleotide linkage has been replaced with sulfur. These PS-oligos are resistant to nuclease degradation, yet retain sequence-specific activity. Similarly, phosphorodithioate nucleic acids are those oligonucleotides or polynucleotides in which each of the non-

bridged oxygens of the internucleotide linkage have been replaced by a sulfur atom. These phosphorodithioate-oligos have also proven to be more nuclease resistant than the natural phosphodiester-linked form. Other useful nucleic acids derivatives include those nucleic acids molecules in which the bridging oxygen atoms (those forming the phosphoester linkages) have been replaced with —S—, —NH—, —CH_2— and the like. Preferably, the alterations to the antisense or other nucleic acids used will not completely affect the negative charges associated with the nucleic acids. Thus, the present invention contemplates the use of antisense and other nucleic acids in which a portion of the linkages are replaced with, for example, the neutral methyl phosphonate or phosphoramidate linkages. When neutral linkages are used, preferably less than 80% of the nucleic acid linkages are so substituted, more preferably less than 50%.

Those skilled in the art will realize that for in vivo utility, such as therapeutic efficacy, a reasonable rule of thumb is that if a thioated version of the sequence works in the free form, that encapsulated particles of the same sequence, of any chemistry, will also be efficacious. Encapsulated particles may also have a broader range of in vivo utilities, showing efficacy in conditions and models not known to be otherwise responsive to antisense therapy. Those skilled in the art know that applying this invention they may find old models which now respond to antisense therapy. Further, they may revisit discarded antisense sequences or chemistries and find efficacy by employing the invention.

Therapeutic antisense sequences (putatively target specific) known to work with this invention include the following:

Trivial Name:	Gene Target, Chemistry and Sequence	
PS-3082	murine ICAM-1 (Intracellular Adhesion Molecule-1) (phosphorothioate)	
	TGCATCCCCCAGGCCACCAT	(SEQ ID. No 1)
PO-3082	murine ICAM-1 (phosphodiester)	
	TGCATCCCCCAGGCCACCAT	(SEQ ID. No 1)
PS-2302	human ICAM-1 (phosphorothiote)	
	GCCCAAGCTGGCATCCGTCA	(SEQ ID. No 2)
PO-2302	human ICAM-1 (phosphodiester)	
	GCCCAAGCTGGCATCCGTCA	(SEQ ID. No 2)
PS-8997	human ICAM-1 (phosphorothioate)	
	GCCCAAGCTGGCATCCGTCA	(SEQ ID. No 2)
US3	human erb-B-2 gene (phosphodiester or phosphorothioate)	
	GGT GCT CAC TGC GGC	(SEQ ID. No 3)
LR-3280	human c-myc gene (phosphorothioate)	
	AACC GTT GAG GGG CAT	(SEQ ID. No 4)
Inx-6298	human c-myc gene (phosphodiester)	
	AAC GTT GAG GGG CAT	(SEQ ID. No 4)
Inx-6295	human c-myc gene (phosphodiester or phosphorothioate)	
	T AAC GTT GAG GGG CAT	(SEQ ID. No 5)
LR-3001	human c-myb gene (phosphodiester or phosphorothioate)	
	TAT GCT GTG CCG GGG TCT TCG GGC	(SEQ ID. No 6)
c-myb	human c-myb gene (phosphodiester or phosphorothioate)	
	GTG CCG GGG TCT TCG GGC	(SEQ ID. No 7)
IGF-1R	human IGF-1R (Insulin Growth Factor 1 - Receptor (phosphodiester or phosphorothioate)	
	GGA CCC TCC TCC GGA GCC	(SEQ ID. No 8)
LR-42	human IGF-1R (phosphodiester or phosphorothioate)	
	TCC TCC GGA GCC AGA CTT	(SEQ ID. No 9)
EGFR	human EGFR (Epidermal Growth Factor Receptor) (phosphodiester or phosphorothioate)	
	CCG TGG TCA TGC TCC	(SEQ ID. No 10)
VEGF	human VEGF (Vascular Endothelial Growth Factor) gene (phosphodiester or phosphorothioate)	
	CAG CCT GGC TCA CCG CCT TGG	(SEQ ID. No 11)
PS-4189	murine PKC-alpha (Phosphokinase C - alpha) gene (phosphodiester or phosphorothioate)	
	CAG CCA TGG TTC CCC CCA AC	(SEQ ID. No 12)
PS-3521	human PKC-alpha (phosphodiester or phosphorothioate)	
	GTT CTC GCT GGT GAG TTT CA	(SEQ ID. No 13)
Bcl-2	human bcl-2 gene (phosphodiester or phosphorothioate)	
	TCT CCC AgC gTg CgC CAT	(SEQ ID. No 14)
ATG-AS	human c-*raf*-1 protein kinase (phosphodiester or phosphorothioate)	
	GTG CTC CAT TGA TGC	(SEQ ID. No 15)
VEGF-R1	human VEGF-1 (Vascular Endothelial Growth Factor Receptor 1) ribozyme	
	GAG UU CUG AUG AGG CCG AAA GGC CGA AAG UCU G	(SEQ ID. No 16)

Using these sequences, the invention provides a method for the treatment of a diseases, including tumors, characterized by aberrant expression of a gene in a mammalian subject. The method comprises the steps of preparing a lipid-encapsulated therapeutic nucleic acid particle according to the methods as described herein, where the therapeutic nucleic acid component hybridizes specifically with the aberrantly expressed gene; and administering a therapeuti-

cally effective amount of the resulting particle to the mammalian subject. These sequences are, of course, only representative of the possible therapeutic oligonucleotide compounds that can be delivered using the invention. It is well known that, depending on the target gene, antisense that hybridizes to any part of the target gene, such as coding regions, introns, the 5' untranslated region (5'UTR), start of translation, or 3'UTR may have therapeutic utility. Therefore, the sequences listed above are only exemplary of antisense. Furthermore, all the alternative chemistries that have been proposed (i.e. see Background) can be tested with the invention to determine efficacy along with all types of ribozymes. In short, the compounds listed above represent the broad class of therapeutic 5–50 mer oligonucleotides of various chemistries which are useful with this invention. Other oligonucleotides which are useful include all those which have previously demonstrated efficacy in the free form.

While the invention is generally described and exemplified with regard to antisense oligonucleotides, other nucleic acids can be formulated and administered to a subject for the purpose of repairing or enhancing the expression of a cellular protein. Accordingly, the nucleic acid can be an expression vector, cloning vector or the like which is often a plasmid designed to be able to replicate in a chosen host cell. Expression vectors may replicate autonomously, or they may replicate by being inserted into the genome of the host cell, by methods well known in the art. Vectors that replicate autonomously will have an origin of replication or autonomous replicating sequence (ARS) that is functional in the chosen host cell(s). Often, it is desirable for a vector to be usable in more than one host cell, e.g., in *E. coli* for cloning and construction, and in a mammalian cell for expression.

Additionally, the nucleic acid can carry a label (e.g., radioactive label, fluorescent label or colorimetric label) for the purpose of providing clinical diagnosis relating to the presence or absence of complementary nucleic acids. Accordingly, the nucleic acids, or nucleotide polymers, can be polymers of nucleic acids including genomic DNA, cDNA, mRNA or oligonucleotides containing nucleic acid analogs, for example, the antisense derivatives described in a review by Stein, et al., *Science* 261:1004–1011 (1993) and in U.S. Pat. Nos. 5,264,423 and 5,276,019, the disclosures of which are incorporated herein by reference. Still further, the nucleic acids may encode transcriptional and translational regulatory sequences including promoter sequences and enhancer sequences.

The nucleic acids used in the present invention will also include those nucleic acids in which modifications have been made in one or more sugar moieties and/or in one or more of the pyrimidine or purine bases. Examples of sugar modifications include replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, azido groups or functionalized as ethers or esters. Additionally, the entire sugar may be replaced with sterically and electronically similar structures, including aza-sugars and carbocyclic sugar analogs. Modifications in the purine or pyrimidine base moiety include, for example, alkylated purines and pyrimidines, acylated purines or pyrimidines, or other heterocyclic substitutes known to those of skill in the art. As with the modifications to the phosphodiester linkages discussed above, any modifications to the sugar or the base moieties should also act to preserve at least a portion of the negative charge normally associated with the nucleic acid. In particular, modifications will preferably result in retention of at least 10% of the overall negative charge, more preferably over 50% of the negative charge and still more preferably over 80% of the negative charge associated with the nucleic acid.

The nucleic acids used in the present method can be isolated from natural sources, obtained from such sources as ATCC or GenBank libraries or prepared by synthetic methods. Synthetic nucleic acids can be prepared by a variety of solution or solid phase methods. Generally, solid phase synthesis is preferred. Detailed descriptions of the procedures for solid phase synthesis of nucleic acids by phosphite-triester, phosphotriester, and H-phosphonate chemistries are widely available. See, for example, Itakura, U.S. Pat. No. 4,401,796; Caruthers, et al., U.S. Pat. Nos. 4,458,066 and 4,500,707; Beaucage, et al., Tetrahedron Lett., 22:1859–1862 (1981); Matteucci, et al., *J. Am. Chem. Soc.,* 103:3185–3191 (1981); Caruthers, et al., *Genetic Engineering,* 4:1–17 (1982); Jones, chapter 2, Atkinson, et al., chapter 3, and Sproat, et al., chapter 4, in *Oligonucleotide Synthesis: A Practical Approach,* Gait (ed.), IRL Press, Washington D.C. (1984); Froehler, et al., *Tetrahedron Lett.,* 27:469–472 (1986); Froehler, et al., *Nucleic Acids Res.,* 14:5399–5407 (1986); Sinha, et al. *Tetrahedron Lett.,* 24:5843–5846 (1983); and Sinha, et al., *Nucl. Acids Res.,* 12:4539–4557 (1984) which are incorporated herein by reference.

As noted above, the solution of therapeutic agent (nucleic acids) comprises an aqueous buffer. Preferred buffers (in the case of anionic therapeutic agents) are those which provide a pH of less than the pK_a of the first lipid component. Examples of suitable buffers include citrate, phosphate, acetate, and MES. A particularly preferred buffer is citrate buffer. Preferred buffers will be in the range of 1–1000 mM of the anion, depending on the chemistry of the oligonucleotide being encapsulated, and optimization of buffer concentration may be significant to achieving high loading levels (See. FIGS. **15** and **20**). Alternatively, pure water acidified to pH 5–6 with chloride, sulfate or the like may be useful. In this case, it may be suitable to add 5% glucose, or another non-ionic solute which will balance the osmotic potential across the particle membrane when the particles are dialyzed to remove ethanol, increase the pH, or mixed with a pharmaceutically acceptable carrier such as normal saline. The amount of therapeutic agent (nucleic acid) in buffer can vary, but will typically be from about 0.01 mg/mL to about 200 mg/mL, more preferably from about 0.5 mg/mL to about 50 mg/mL.

The mixture of lipids and the buffered aqueous solution of therapeutic agent (nucleic acids) is combined to provide an intermediate mixture. The intermediate mixture is typically a mixture of lipid particles having encapsulated therapeutic agent (nucleic acids). Additionally, the intermediate mixture may also contain some portion of therapeutic agent (nucleic acids) which are attached to the surface of the lipid particles (liposomes or lipid vesicles) due to the ionic attraction of the negatively-charged nucleic acids and positively-charged lipids on the lipid particle surface (the amino lipids or other lipid making up the protonatable first lipid component are positively charged in a buffer having a pH of less than the pK_a of the protonatable group on the lipid). In one group of preferred embodiments, the mixture of lipids is an alcohol solution of lipids and the volumes of each of the solutions is adjusted so that upon combination, the resulting alcohol content is from about 20% by volume to about 45% by volume. The method of combining the mixtures can include any of a variety of processes, often depending upon the scale of formulation produced. For example, when the total volume is about 10–20 mL or less, the solutions can be combined in a test tube and stirred together using a vortex mixer. Large-scale processes can be carried out in suitable production scale glassware.

Optionally, the lipid-encapsulated therapeutic agent (nucleic acid) complexes which are produced by combining the lipid mixture and the buffered aqueous solution of therapeutic agents (nucleic acids) can be sized to achieve a desired size range and relatively narrow distribution of lipid particle sizes. Preferably, the compositions provided herein will be sized to a mean diameter of from about 70 to about 200 nm, more preferably about 90 to about 130 nm. Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size determination. For the methods herein, extrusion is used to obtain a uniform vesicle size.

Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the lipid-nucleic acid compositions which are formed can be used without any sizing.

The present invention further comprises a step of neutralizing at least some of the surface charges on the lipid portions of the lipid-nucleic acid compositions. By at least partially neutralizing the surface charges, unencapsulated antisense or other nucleic acid is freed from the lipid particle surface and can be removed from the composition using conventional techniques. Preferably, unencapsulated and surface adsorbed nucleic acids is removed from the resulting compositions through exchange of buffer solutions. For example, replacement of a citrate buffer (pH about 4.0, used for forming the compositions) with a HEPES-buffered saline (HBS pH about 7.5) solution, results in the neutralization of liposome surface and antisense release from the surface. The released antisense can then be removed via chromatography using standard methods, and then switched into a buffer with a pH above the pKa of the lipid used.

In other aspects, the present invention provides lipid-encapsulated nucleic acid compositions, preferably prepared by the methods recited above. Accordingly, preferred compositions are those having the lipid ratios and nucleic acid preferences noted above.

In still other aspects, the present invention contemplates reversed-charge methods in which the lipid portion of the complex contains certain anionic lipids and the component which is encapsulated is a positively charged therapeutic agent. One example of a positively charged agent is a positively charged peptide or protein. In essentially an identical manner, liposome-encapsulated protein is formed at a pH above the pKa of the anionic lipid, then the surface is neutralized by exchanging the buffer with a buffer of lower pH (which would also release surface-bound peptide or protein).

IV. Pharmaceutical Preparations

The lipid-nucleic acid compositions prepared by the above methods can be administered either alone or in mixture with a physiologically-acceptable carrier (such as physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice.

Pharmaceutical compositions comprising the lipid-nucleic acid compositions of the invention are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, e.g., water, buffered water, 0.9% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, etc. In compositions comprising saline or other salt containing carriers, the carrier is preferably added following lipid particle formation. Thus, after the lipid-nucleic acid compositions are formed, the compositions can be diluted into pharmaceutically acceptable carriers such as normal saline. The resulting pharmaceutical preparations may be sterilized by conventional, well known sterilization techniques. The aqueous solutions can then be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. Additionally, the lipidic suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as α-tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

The concentration of lipid-nucleic acid complexes in the pharmaceutical formulations can vary widely, i.e., from less than about 0.01%, usually at or at least about 0.05–5% to as much as 10 to 30% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, complexes composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration. In one group of embodiments, the nucleic acid will have an attached label and will be used for diagnosis (by indicating the presence of complementary nucleic acid). In this instance, the amount of complexes administered will depend upon the particular label used, the disease state being diagnosed and the judgement of the clinician but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight.

As noted above, the lipid-therapeutic agent (nucleic acid) compositions of the invention include polyethylene glycol (PEG)-modified phospholipids, PEG-ceramide, or ganglioside G_{M1}-modified lipids or other lipids effective to prevent or limit aggregation. Addition of such components does not merely prevent complex aggregation, however, it may also provides a means for increasing circulation lifetime and increasing the delivery of the lipid-nucleic acid composition to the target tissues.

The present invention also provides lipid-nucleic acid compositions in kit form. The kit will typically be comprised of a container which is compartmentalized for holding the

various elements of the kit. The kit will contain the compositions of the present inventions, preferably in dehydrated or concentrated form, with instructions for their rehydration or dilution and administration. In still other embodiments, the lipid-encapsulated-therapeutic agent (nucleic acid) particles will have a targeting moiety attached to the surface of the lipid particle. Methods of attaching targeting moieties (e.g., antibodies, proteins, small molecule mimetics, vitamins, oligosaccharides and hyaluronic acid) to lipids (such as those used in the present compositions) are known to those of skill in the art.

Dosage for the lipid-nucleic acid compositions will depend on the ratio of nucleic acid to lipid and the administrating physician's opinion based on age, weight, and condition of the patient.

V. Methods of Introducing Lipid-Encapsulated Therapeutic Agents into Cells

The lipid-therapeutic agent compositions of the invention can be used for introduction of those therapeutic agents into cells. In the case of nucleic acid-containing compositions, the composition of the invention are useful for the introduction of nucleic acids, preferably plasmids, antisense and ribozymes into cells. Accordingly, the present invention also provides methods for introducing a therapeutic agent such as a nucleic acid into a cell. The methods are carried out in vitro or in vivo by first forming the compositions as described above, then contacting the compositions with the target cells for a period of time sufficient for transfection to occur.

The compositions of the present invention can be adsorbed to almost any cell type. Once adsorbed, the complexes can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the nucleic acid portion of the complex can take place via any one of these pathways. In particular, when fusion takes place, the liposome membrane is integrated into the cell membrane and the contents of the liposome combine with the intracellular fluid. Contact between the cells and the lipid-nucleic acid compositions, when carried out in vitro, will take place in a biologically compatible medium. The concentration of compositions can vary widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. Treatment of the cells with the lipid-nucleic acid compositions will generally be carried out at physiological temperatures (about 37° C.) for periods of time of from about 1 to 6 hours, preferably of from about 2 to 4 hours. For in vitro applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

In one group of preferred embodiments, a lipid-nucleic acid particle suspension is added to 60–80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/mL, more preferably about 2×10^4 cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/mL, more preferably about 0.1 μg/mL.

Typical applications include using well known transfection procedures to provide intracellular delivery of DNA or MRNA sequences which code for therapeutically useful polypeptides. In this manner, therapy is provided for genetic diseases by supplying deficient or absent gene products (i.e., for Duchenne's dystrophy, see Kunkel, et al., *Brit. Med. Bull.* 45(3):630–643 (1989), and for cystic fibrosis, see Goodfellow, *Nature* 341:102–103 (1989)). Other uses for the compositions of the present invention include introduction of antisense oligonucleotides in cells (see, Bennett, et al., *Mol. Pharm.* 41:1023–1033 (1992)).

Alternatively, the compositions of the present invention can also be used for the transfection of cells in vivo, using methods which are known to those of skill in the art. In particular, Zhu, et al., *Science* 261:209–211 (1993), incorporated herein by reference, describes the intravenous delivery of cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT) expression plasmid using DOTMA-DOPE complexes. Hyde, et al., *Nature* 362:250–256 (1993), incorporated herein by reference, describes the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to epithelia of the airway and to alveoli in the lung of mice, using liposomes. Brigham, et al., *Am. J. Med. Sci.* 298:278–281 (1989), incorporated herein by reference, describes the in vivo transfection of lungs of mice with a functioning prokaryotic gene encoding the intracellular enzyme, chloramphenicol acetyltransferase (CAT). Thus, the compositions of the invention can be used in the treatment of infectious diseases.

For in vivo administration, the pharmaceutical compositions are preferably administered parenterally, i.e., intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. More preferably, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection. For example, see Stadler, et al., U.S. Pat. No. 5,286,634, which is incorporated herein by reference. Intracellular nucleic acid delivery has also been discussed in Straubringer, et al., METHODS IN ENZYMOLOGY, Academic Press, New York. 101:512–527 (1983); Mannino, et al., *Biotechniques* 6:682–690 (1988); Nicolau, et al., *Crit. Rev. Ther. Drug Carrier Syst.* 6:239–271 (1989), and Behr, *Acc. Chem. Res.* 26:274–278 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, Rahman et al., U.S. Pat. No. 3,993,754; Sears, U.S. Pat. No. 4,145,410; Papahadjopoulos et al., U.S. Pat. No. 4,235,871; Schneider, U.S. Pat. No. 4,224,179; Lenk et al., U.S. Pat. No. 4,522,803; and Fountain et al., U.S. Pat. No. 4,588,578.

In other methods, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical", it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures which include incising the skin of a patient and directly visualizing the underlying tissue to which the pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera, or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

The lipid-nucleic acid compositions can also be administered in an aerosol inhaled into the lungs (see, Brigham, et al., *Am. J. Sci.* 298(4):278–281 (1989)) or by direct injection

at the site of disease (Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70–71 (1994)).

The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

VI. Examples

Materials and Methods

Lipids

Distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), and palmitoyloleoylphosphatidylcholine (POPC) were purchased from Northern Lipids (Vancouver, Canada). 1,2-dioleoyloxy-3-dimethylammoniumpropane (DODAP or AL-1) was synthesized by Dr. Steven Ansell (Inex Pharmaceuticals) or, alternatively, was purchased from Avanti Polar Lipids. Cholesterol was purchased from Sigma Chemical Company (St. Louis, Mo., USA). PEG-ceramides were synthesized by Dr. Zhao Wang at Inex Pharmaceuticals Corp. using procedures described in PCT WO 96/40964, incorporated herein by reference. [^{3}H] or [^{14}C]-CHE was purchased from NEN (Boston, Mass., USA). All lipids were >99% pure.

Buffers and Solvents

Ethanol (95%), methanol, chloroform, citric acid, HEPES and NaCl were all purchased from commercial suppliers.

Synthesis and Purification of Phosphorothioate Antisense

PS 3082, a 20 mer phosphorothioate antisense oligodeoxynucleotide, was synthesized, purified and donated by ISIS Pharmaceuticals (Carlsbad, Calif., USA). The sequence for this oligo is: TGCATCCCCCAGGCCAC-CAT. (Seq ID No 1). The details of the synthesis and purification can be found elsewhere (see, Stepkowski, et al., *J. Immunol.* 153:5336–5346 (1994)).

Preparation of Liposomal Antisense

Lipid stock solutions were prepared in 95% ethanol at 20 mg/mL (PEG-Ceramides were prepared at 50 mg/mL). DSPC, CHOL, DODAP, PEG-CerC14 (25:45:20:10, molar ratio), 13 μmol total lipid, were added to a 13×100 mm test tube containing trace amounts of [^{14}C]-cholesterylhexadecylether. The final volume of the lipid mixture was 0.4 mL. In some experiments, SM or POPC was substituted for DSPC. A 20 mer antisense oligodeoxynucleotide, PS 3082 (2 mg), and trace amounts of [^{3}H]-PS 3082 were dissolved in 0.6 mL of 300 mM citric acid, pH 3.8 in a separate 13×100 mm test tube. The antisense solution was warmed to 65° C. and the lipids (in ethanol) were slowly added, mixing constantly. The resulting volume of the mixture was 1.0 mL and contained 13 μmol total lipid, 2 mg of antisense oligodeoxynucleotide, and 38% ethanol, vol/vol. The antisense-lipid mixture was subjected to 5 cycles of freezing (liquid nitrogen) and thawing (65 ° C.), and subsequently was passed 10X through three stacked 100 nm filters (Poretics) using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and pressure during extrusion were 65 ° C. and 300–400 psi (nitrogen), respectively. The extruded preparation was diluted with 1.0 mL of 300 mM citric acid, pH 3.8, reducing the ethanol content to 20%. The preparation was immediately applied to a gel filtration column. Alternatively, the extruded sample was dialyzed (12 000–14 000 MW cutoff; SpectraPor) against several liters of 300 mM citrate buffer, pH 3.8 for 3–4 hours to remove the excess ethanol. The sample was subsequently dialyzed against HBS, pH 7.5, for 12–18 hours to neutralize the DODAP and release any antisense that was associated with the surface of the vesicles. The free antisense was removed from the encapsulated liposomal antisense by gel exclusion chromatography as described below.

Gel Filtration Chromatography

A 20×2.5 cm glass column containing Biogel A15m, 100–200 mesh, was equilibrated in HEPES-buffered saline (HBS; 20 mM HEPES, 145 mM NaCl, pH 7.5). The 2.0 mL liposomal antisense preparation was applied to the column and allowed to drain into the gel bed under gravity. The column was eluted with HBS at a flow rate of 50 mL/hr. Column fractions (1.0 mL) were collected and analyzed for radioactivity using standard liquid scintillation counting techniques. The fractions were pooled based on the levels of [^{14}C]-CHE present in the fraction. The size distribution of the pooled liposomal antisense was determined using a NICOMP Model 370 Sub-micron particle sizer and was typically 110±30 nm.

Ion Exchange Chromatography

As an alternative to gel filtration chromatography, samples were sometimes dialyzed first in 300 mM citrate, pH 3.80, for 2–3 hours to remove residual ethanol, followed by at least a 12 hour dialysis in HBS, to exchange the external citrate for HBS and remove residual ethanol. The sample was applied to a 1.5×8 cm DEAE-Sepharose® column equilibrated in HBS. Free oligonucleotide binds to the DEAE with very high affinity. The peak containing the lipid was pooled, concentrated, and analyzed for antisense content, as described below.

Assessment of Antisense Encapsulation

Antisense encapsulation was typically assessed by dual label ([^{3}H]-antisense and [^{14}C]-lipid) liquid scintillation counting after gel filtration chromatography to separate the free and encapsulated antisense. Antisense encapsulation was evaluated by summing the total [^{3}H]-antisense radioactivity associated with the lipid peak and dividing by the total [^{3}H]-antisense radioactivity. Alternatively, the [^{3}H]/[^{14}C] ratio was determined before and after (i.e., in the pooled lipid peak) gel filtration chromatography. Antisense encapsulation was also assessed by measuring the absorbance of the sample at 260 nm, preceded by a Bligh and Dyer extraction of the antisense from the lipid, as described below.

Extraction of the Antisense

The antisense was extracted from the lipid according to the procedure outlined by Bligh and Dyer (Bligh, et al., *Can. J. Biochem. Physiol.* 37:911–917 (1959)). Briefly, up to 250 μL of aqueous sample was added to a 13×100 mm glass test tube, followed by the addition of 750 μL of chloroform-:methanol (1:2.1, vol/vol), 250 μL of chloroform, and 250 μL of distilled water. The sample was mixed after each addition. The sample was centrifuged for 10 min. at 3000 rpm, resulting in a clear two-phase separation. The aqueous phase (top) was removed into a new 13×100 mm test tube. An aliquot (500 μL) of this phase was diluted with 500 μL of distilled water, mixed, and the absorbance at 260 nm was assessed using a spectrophotometer. In some instances, the organic phase (bottom) was washed with 250 μL of methanol, centrifuged for 10 min. at 3000 rpm, and the upper phase removed and discarded. This was repeated 3 times. The washed organic phase was assessed for phospholipid content according to the method of Fiske and Subbarrow (Fiske, et al., *J. Biol. Chem.* 66:375–400 (1925)).

Oligreen Assay

A fluorescent dye binding assay for quantifying single stranded oligonucleotide in aqueous solutions was established using a Biolumin™ 960 fluorescent plate reader (Molecular Dynamics, Sunnyvale, Calif., USA). Briefly, aliquots of encapsulated oligonucleotide were diluted in

HEPES buffered saline (HBS; 20 mM HEPES, 145 mM NaCl, pH 7.5). A 10 μL aliquot of the diluted sample was added to 100 μL of a 1:200 dilution of Oligreen™ reagent, both with and without 0.1% of Triton X-100 detergent. An oligo standard curve was prepared with and without 0.1% Triton X-100 for quantification of encapsulated oligo. Fluorescence of the OLIGREEN™-antisense complex was measured using excitation and emission wavelengths of 485 nm and 520 nm, respectively. Surface associated antisense was determined by comparing the fluorescence measurements in the absence and presence of detergent.

Ear Inflammation Model and Efficacy Studies

Sensitization and Elicitation of Contact Sensitivity

Mice were sensitized by applying 25 μL of 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) in acetone:olive oil (4:1) to the shaved abdominal wall for two consecutive days. Four days after the second application, mice were challenged on the dorsal surface of the left ear with 10 μL of 0.2% DNFB in acetone:olive oil (4:1). Mice received no treatment on the contralateral (right) ear. In some cases, control mice received 10 μL of vehicle on the dorsal surface of the left ear.

Evaluation of Ear Swelling

Ear thickness was measured immediately prior to ear challenge, and at various time intervals after DNFB challenge, using an engineer's micrometer (Mitutoyo, Tokyo, Japan). Increases in ear thickness measurements were determined by subtracting the pre-challenge from post-challenge measurements.

The progression of ear inflammation over a 3 day period for ICR (outbred) mice is indicated in FIGS. **12** and **13**. Erythema was evident almost immediately after ear challenge and gradually declined in intensity over the remainder of the study. ICR mice exhibited peak ear thickness at 24 hours after the induction of ear inflammation. Maximal ear thickness measurements were found to be 170×10^{-4} inches, corresponding to a 70% increase in ear thickness. Although ear swelling gradually declines at 48 and 72 hours after inflammation initiation, ear measurements still have not returned to baseline thickness levels ($90–100\times10^{-4}$ inches).

The mouse in vivo experimental systems in this specification were selected in part because of their high degree of correlation to human disease conditions. The mouse ear inflammation model, which can be treated using methods and compositions of the invention, is well known to be an excellent model for human allergic contact dermatitis and other disease conditions. The control therapeutic used in this model is a corticosteroid which demonstrates efficacy both in the mouse model and in related human disease conditions.

The mouse B16 tumor model, a fast growing melanoma, which can be treated using methods and compositions of the invention, is a standard, widely used experimental system. This tumor model can be successfully treated using vinca alkaloids, such as vincristine or vinblastine, which are known to be efficacious against human tumors as well.

Treatments which demonstrate utility in the mouse models of this invention are excellent candidates for testing against human disease conditions, at similar dosages and administration modalities.

EXAMPLE 1

This example illustrates the effects of ethanol on the encapsulation of antisense.

A 20 mer of [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The final ethanol concentration in the preparations was varied between 0 and 60%, vol/vol. The samples were extruded ten times through three 100 nm filters as described in "Materials and Methods". The samples were dialyzed for 2–3 hours in 300 mM citrate buffer, pH 3.80, to remove a majority of the excess ethanol. The samples were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. This renders the majority of DODAP in the outer bilayer neutral, and will release any surface bound antisense. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

In another experiment, the formulations were prepared as described. After extrusion, the filters were analyzed for [^{3}H]-antisense and [^{14}C]-lipid radioactivity by standard scintillation counting techniques. Results were expressed as a percent of the total initial radioactivity.

FIG. **3** demonstrates the effects of ethanol on the encapsulation of antisense at pH 3.8. The encapsulation efficiency of phosphorothioate antisense increases in a near linear manner up to a final ethanol concentration of 50%, vol/vol. At an ethanol content greater than 50%, a large amount of aggregation/precipitation is observed. The effect of ethanol on vesicle formation can be further observed by monitoring both lipid and antisense loss on the filters during extrusion (FIG. **4**). At low ethanol contents, extrusion is slow and the proportion of lipid and antisense loss is the same, suggesting that the losses are due to the formation of large complexes which get trapped on the filter. At ethanol contents of 30 and 40%, extrusion is very quick and losses of both lipid and antisense are minimal. As the ethanol content is increased above 40%, the loss of antisense becomes disproportionally high relative to the lipid. This can be attributed to the insolubility of DNA in high concentrations of alcohol. Furthermore, in the presence of ethanol, PEG is required to prevent aggregation and fusion of the vesicles (results not shown).

EXAMPLE 2

This example illustrates the effects of DODAP on the encapsulation of antisense, and further illustrates the effect of initial antisense concentration on the compositions.

Having demonstrated that ethanol can greatly facilitate the preparation of lipid vesicles containing entrapped antisense, the next step was to examine the influence of DODAP (AL-1) content on the encapsulation of antisense (FIG. **5**). Accordingly, a 0.6 mL aliquot of a [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 100-(55+X):45:X:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The molar ratio of DODAP was varied between 0 and 30%. The molar ratio of DSPC was adjusted to compensate for the changes in DODAP content. The samples were extruded ten times through three 100 nm filters as described in "Materials and Methods", and were dialyzed for 2–3 hours in 300 mM citrate buffer, pH 3.80, to remove a majority of the excess ethanol. The samples were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-

encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity. As seen in FIG. **5**, antisense encapsulation increased significantly between 5–20% DODAP. At DODAP contents greater than 20–25%, extrusion of the vesicles became more difficult suggesting the formation of complexes. At DODAP concentration of 40 and 50%, extrusion of the lipid/antisense mixture took hours compared to minutes for a lipid composition containing 20% DODAP. To verify that the antisense was indeed associated with the lipid and that the observed encapsulation was not due to exchange of the [^{3}H]-label from the antisense onto the lipid, the antisense was extracted from the lipid using a Bligh and Dyer extraction. Using this technique, the antisense, which is soluble in the aqueous phase, was separated from the lipid (soluble in the organic phase) and quantified by measuring the absorbance at 260 nm (FIG. **6**). While this method can underestimate the antisense concentration, the technique substantiated that the observed association of antisense with the lipid was not an artifact.

In yet another experiment, varying concentrations of a 20 mer of [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) were mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio), 9.9 mg/mL (final concentration). The samples were extruded and dialyzed twice as described above. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods". Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity. EPC:CH liposomes containing encapsulated antisense are included for comparison.

Optimization of the drug:lipid ratio was accomplished by increasing the initial antisense concentration that was mixed with 9.8 mg total lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10) (FIG. **8**). Drug:lipidratios of up to 0.25, w/w, were obtained using 10 mg/mL of antisense in the preparation. However, the increased drug:lipid ratio was accompanied by a decrease in encapsulation efficiency, therefore a compromise must be made between optimizing the drug:lipid ratio and encapsulation efficiency. In comparison, antisense encapsulated by hydration of a dry lipid film (i.e. EPC:CHOL) in the absence of cationic lipid typically yields low encapsulation efficiencies (<12–15%) and drug:lipid ratios (<0.1, w/w). Consequently, significant quantities of antisense are wasted during the encapsulation procedure.

EXAMPLE 3

This example illustrates the properties of the liposomal antisense formulations provided in the Materials and Methods above.

The size distribution of a liposomal preparation of antisense was determined by quasi-elastic light scattering (QELS) immediately after removal of the free antisense (A), and after storage of the preparation for 2 months at 4° C. (B), using a Nicomp Model 370 sub-micron particle sizer. A 0.6 mL aliquot of a [^{3}H]-phosphorothioate antisense oligodeoxynucleotide (in 300 mM citrate buffer, pH 3.80) was mixed with 0.4 mL of a 95% ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The sample was extruded ten times through three 100 nm filters as described in "Materials and Methods", and dialyzed for 2–3 hours in 300 mM citrate buffer, pH 3.80, to remove a majority of the excess ethanol. The sample was switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-encapsulated antisense was then removed from the liposomal antisense by DEAE-sepharose chromatography as described in "Material and Methods".

The size distribution and storage stability of antisense preparations described herein is demonstrated in FIG. **7**. The size distribution of a standard DSPC:CHOL:DODAP:PEG-CerC14 (25:45:20:10) preparation containing a 2 mg/mL initial antisense concentration was analyzed immediately after column chromatography to remove any free antisense. A very homogenous distribution is observed after preparation (119±32 nm). This size distribution remained stable for at least 2 months after storage at 4° C. (119±32 nm).

EXAMPLE 4

This example illustrates the clearance pharmacokinetics, biodistribution and biological activity of an encapsulated murine ICAM-1 phosphorothioate antisense oligodeoxynucleotide.

4.1 Plasma Clearance

Encapsulated liposomal antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of X:CHOL:DODAP:PEG-CerC14 (25:45:20:10), where X represents either distearoylphosphatidylcholine (DSPC), sphingomyelin (SM), or palmitoyloleoylphosphatidylcholine (POPC). The formulations contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20–25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

The plasma clearance of three formulations, DSPC:CHOL:DODAP:PEG-CerC14, SM:CHOL:DODAP:PEG-CerC14, and POPC:CHOL:DODAP:PEG-CerC14, of encapsulated antisense were examined in inflamed ICR mice (FIG. **9**). The circulation time was longest for the DSPC version of the formulation.

4.2 Organ Accumulation

Liposomal antisense compositions were prepared and administered to mice as outlined in the preceding section. Mice were terminated by cervical dislocation and the organs were recovered and processed as described in "Materials and Methods". Lipid and antisense recoveries were determined by standard scintillation counting techniques.

Organ accumulation of the various formulations was typical of previously described liposome clearance patterns, with the RES organs, principally the liver and spleen, being responsible for the majority of clearance (FIG. **10**). One interesting observation is that the liver and spleen clearance account for only 40–45% of the total clearance of the "DSPC" formulation, suggesting that a significant population of vesicles is accumulating in another organ system or is being excreted.

4.3 Stability

Liposomal antisense compositions were prepared and administered to mice as outlined in the preceding section. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques. Release rates were determined by measuring the [3H]/[14C] ratio over time.

The stability of the formulations was also assessed by measuring the ratio of antisense and lipid recovery in the blood at various times (FIG. **11**). A ratio of 1.0 suggests that the antisense and the lipid are staying together in the circulation. The "DSPC" formulation showed little deviation from a ratio of 1.0 over 24 h, suggesting that it is very stable in the circulation. The "POPC" formulation dropped to a ratio of 0.6 after 2 h, while the ratio for the "SM" formulation decreased more slowly, reaching 0.6 after 12 h in the circulation. These results indicate that it may be possible to deliberately alter the antisense release rates by modifying the lipid composition.

4.4 PEG-Acyl Influence on Circulation Half-life of Single Dose of Thioate Antisense

Encapsulated lipid-encapsulated antisense was prepared using the ethanol-citrate procedure as described in "Material and Methods". Initial lipid and antisense concentrations were 9.9 and 2 mg/mL, respectively. Liposomal formulations were composed of DSPC:CHOL:DODAP:PEG-CerC14 or C20 (25:45:20:10). The formulation contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense and were injected (200 μL) intravenously via the lateral tail vein of female (20–25 g) ICR mice at a lipid dose of 120 mg/kg. Blood was recovered by cardiac puncture on anesthetized mice. Lipid and antisense recoveries were determined by standard scintillation counting techniques.

The influence of PEG-acyl chain length on clearance rates of a DSPC:CHOL:DODAP:PEG-Cer formulation was investigated using PEG-CerC14 and PEG-CerC20 (FIG. **12**). The inclusion of PEG-CerC20 in the formulation resulted in enhanced circulation times over the PEG-CerC14. This corresponds to in vitro data suggesting that the C14 version of the PEG is exchanged much more rapidly out of the vesicle than the C20 version.

4.5 In vivo Efficacy of Single Dose of Lipid Encapsulated ICAM-1 (Phosphorothioate) Antisense

The efficacy of PS- 3082 encapsulated in various lipid formulations containing DODAP was tested in an ear inflammation model using ICR mice.

Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC-:CHOL liposomes with entrapped PS-3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS-3082 (identified as AS 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS-3082 (identified as AS 4200). Ear swelling was measured at 24 hours after initiating inflammation using an engineer's micrometer.

Ear swelling measurements were made 24 hours after initiating inflammation in mice treated i.v. at the time of ear challenge with either HBS (control), PS-3082 encapsulated in EPC:CHOL vesicles (30 mg/kg dose of oligo), PS-3082 encapsulated in POPC:CHOL:DODAP:PEG-CerC14 vesicles (30 mg/kg dose of oligo), or PS-3082 encapsulated in DSPC:CHOL:DODAP:PEG-CerC14 vesicles (30 mg/kg dose of oligo) (FIG. **13**). The "DSPC" formulation resulted in the greatest efficacy, exhibiting only 10% increase in ear swelling over pre-challenge values. A similar trend was observed for cellular infiltration into the "challenged" ear versus the non-treated ear (FIG. **14**).

In another evaluation, mice received 10 μCi of [^{3}H]-methylthymidine, i.p., 24 hours before initiating inflammation. Inflamed mice were treated at the time of ear challenge with a 30 mg/kg i.v. dose of either HBS (no oligo), EPC-:CHOL liposomes with entrapped PS- 3082 (identified as AS 1000), POPC:CHOL:DODAP:PEG-CerC14 with entrapped PS- 3082 (identified as AS 4100), or DSPC:CHOL:DODAP:PEG-CerC14 with entrapped PS-3082 (identified as AS 4200). Cell infiltration was monitored by measuring the radioactivity in the "challenged ear" versus the non-treated ear. Results are expressed as the ratio of radioactivity in the left (challenged ear) versus right ear.

4.6 In vivo Efficacy of Single Dose of Lipid Encapsulated ICAM-1 (Phosphodiester) Antisense

This experiment demonstrates the in vivo efficacy of a phosphodiester antisense oligodeoxynucleotide encapsulated in lipid particles according to the invention. In specific, the phosphodiester was targeted to the ICAM- 1 gene in an ear inflammation model.

Group	Test Sample/Drug	Dose	Time Point
1	control inflammation - HBS	200 μl	24 hr
2	corticosteroid	200 μl	24 hr
3	empty vesicles	200 μl	24 hr
4	PS-3082	200 μl	24 hr
5	PO-3082	200 μl	24 hr

Antisense Sample Preparation: Antisense was encapsulated using the standard methods of Examples 5–9, using the phosphodiester modification. The phosphodiester formulation used 10–50 mM citrate (preferably 20 mM citrate), pH 4.0 instead of 300 mM citrate, pH 4.0 preferred for phosphorothioates. Empty vesicles consisted of lipid components only. Corticosteroid (either Halobetasol propionate 0.05% by weight (Westwood Squibb, Montreal) or Dexamethasone (50 ug dissolved in 4:1 acetone:olive oil)) was applied topically in a thin film to cover the surface of the ear 15 minutes after ear challenge.

Inflammation and Dosing: Mouse ear inflammation was induced using DNFB as described above in Materials and Methods. Female ICR mice (6–8 weeks old) received intravenous tail vein injections of antisense (200 μl). Antisense doses for the phosphorothioate and phosphodiester antisense were adjusted to be 20–30 mg/kg. 6 mice were tested with each formulation. Administration occurred 15 min. after the application of 0.2% DNFB to the mouse ear. Ear measurements were made on anaesthetized mice 24 hours after treatment (unless shown otherwise) and prior to termination. Mice are terminated by cervical dislocation and the ears are removed around the pinna. Ears are then weighted, digested (Solvable) and analyzed for radioactivity by liquid scintillation counting. Ears were analyzed for 1) Ear edema—based on the increase in ear thickness due to ear swelling. Calculated by subtracting pre-ear thickness values from post-ear thickness values FIG. **21**. 2) Cell infiltration—based on radioactivity accumulated in the inflamed (right) ear vs. the control (left) ear FIG. **22**; and 3) Ear weights—left ear versus right ear (measurement of edema) FIG. **23**.

Results: The controls consisting of buffer alone (HBS) or Empty Vesicles alone demonstrated no efficacity. Topical corticosteroid demonstrates its known excellent efficacity by reducing inflammation to below pre-challenge levels. Both the phosphorothioate and phosphodiester antisense show excellent efficacy through a systemic delivery administration, reducing the degree of inflammation by around 70% and 85%, respectively. Thus, it is possible to

administer the compositions of the invention at a site where the disease site is distal to the site of the injection.

4.7 In vivo Efficacy of US3 Antisense (Tumor Window Model)

In this example, the anti-tumor activity of lipid encapsulated US3, an antisense oligonucleotide directed at the erb-B-2 gene, has been demonstrated in an in vivo human breast tumor model.

The human breast carcinoma line MDA-MB-453 was implanted in a mouse tumor window model according to the method of Wu, N. Z., Da, D., Rudoll, T. L., Needham, D., Whorton, R. & Dewhirst, M. W. 1993. Increased microvascular permeability contributes to preferential accumulation of Stealth liposomes in tumor tissue. Cancer Research 53: 3765–3770; and Dewhirst, M. W., Tso, C. Y., Oliver, R., Gustafson, C. S., Secomb, T. W. & Gross, J. F. 1989. Morpholigic and hemodynamic comparison of tumor and healing normal tissue microvasculature. Int. J. Radiat. Oncol. Biol. Phys. 17: 91–99. See also Dewhirst, M W., and Needham, D. 1995. Extravasation of Stealth Liposomes into Tumors: Direct Measurement of Accumulation and Vascular Permeability using a Skin Flap Window Chamber. In Stealth Liposomes (Eds. Lasic, D. and Martin, F.) CRC Press.

The lipid-antisense formulation consists of disteroylphosphatidylcholine (DSPC, 25 mol %), cholesterol (Chol, 45 mol %), dioleoylphosphatidyldiaminopropane, (DODAP, or AL1, 20 mol %) and PEG-ceramide (C14 chain length, 10 mol %). For some experiments detailed below, proportions and constituents were altered, but the method of preparation remained the same. Lipids were dissolved in ethanol at 20 mg/ml (PEG-ceramide at 50 mg/ml). Routinely, 1 to 2 μCi ^{14}C-cholesterylhexadecylether was added as a lipid radiolabel. Lipids were mixed in the correct proportions in ethanol to a final concentration of 10 mg in 400 μl. The lipid mixture was then added dropwise to phosphorothioated antisense (US3: anti-human erb-B-2 GGT GCT CAC TGC GGC (SEQ ID. No 3) dissolved in 300 mM citrate buffer pH 4.0 (600 μl to make a final volume of 1 ml). The antisense was used at a variety of concentrations, but the optimum concentration for maximum encapsulation efficiency and drug:lipid ratio was determined to be 0.5 mg/ml final. During the addition, the solution becomes opaque. The DODAP is positively charged at pH 4.0 (pKa=6.53) and so attracts the negatively charged DNA molecules. The mixture was subjected to five cycles of freezing in liquid N_2 and thawing at 65° C. followed by extrusion through 100 nm filters ten times at 65 ° C.

After extrusion, two methods can be used for removal of the external antisense. Firstly, the liposomes are diluted 2:1 with citrate (to reduce ethanol content to 20%) then applied to a Bio-Gel A18M 100–200 mesh column equilibrated with HBS. The column profiles shown in this report were generated in this manner. Alternatively, the liposomes are dialysed 2 h against citrate to remove ethanol, the overnight against HBS to increase the external pH. The resulting mixture is then applied to a DEAE cation exchange column to remove external oligo. This method was the routine method used for sample preparation for in vivo studies. Antisense concentrations were routinely determined by A260 measurements. Lipid concentrations were determined by scintillation counting after spiking initial mixture with a known concentration of ^{3}H or ^{14}C cholesterylhexadecyl ether, or by HPLC. Encapsulation efficiency was determined by division of the final drug to lipid ratio by the initial drug to lipid ratio.

In vivo efficacy evaluation: When the tumor in the window has reached a diameter of 2–3 mm, treatment with free or TCS-encapsulated US3 oligonucleotide is initiated. Treatment consists of a 200 ul intravenous administration (tail vein) of either free US3 or TCS-encapsulated US3 on a 3 administrations/week schedule and an antisense dose of 10 mg/kg/administration. Tumor size is monitored 3 times per week by microscopy.

Results: The TCS-encapsulated US3 oligonucleotide was very effective at preventing the growth, or causing extensive size reduction, of the MDA-MB-453 human breast carcinoma in the window model. In contrast, unencapsulated oligonucleotide was ineffective at inhibiting tumor growth.

4.8 In vivo Clearance of Various Formulations Using Alternative Amino Lipids: DODAP or DODMA

Antisense particle formulations were prepared according to Example 2, with the following modifications: In assay#1 and #2, 25% AL-1 (hydrochloride salt of DODAP) and 25% free base DODAP were employed, respectively, with a concomitant reduction in the amount of DSPC. Assay#3, 4 and 5 employed 30%, 25% and 20% DODMA (free base (prepared at Inex Pharmaceuticals Corp., Burnaby BC)), respectively, again with a concomitant reduction of DSPC.

Both the encapsulation efficiency and in vivo clearance of the formulations were studied. There was no significant difference between the encapsulation or clearance of the free base or HCl salt of DODAP. Decreasing DODMA concentration (30, 25, 20%) severely decreased the encapsulation efficiency of PS-2302 (91%, 43%, 35%) and likewise the Drug/Lipid ratio of the resulting formulation.

In the clearance study outlined in FIG. **16**, DODMA formulations demonstrated slightly higher rates of clearance than 25% DODAP or AL-1, although all formulations appear to be retained in the circulation to a degree which is suitable for human therapeutics.

4.9 PEG-acyl Influence on Clearance Rate of Repeat Doses of Encapsulated EGF-R Phosphorothioate Antisense

Lipid-encapsulated antisense was prepared using the ethanol-citrate procedure as described above, with changes to molar ratios of components as indicated. Initial lipid and antisense concentrations were about 9.9 and 2 mg/mL, respectively. DODAP containing formulations had drug:lipid ratios of 0.15 (+/–) 0.05. Passive encapsulation systems had drug:lipid ratios of 0.03. Nine different liposomal formulations were prepared, using standard techniques, in the following molar ratios:

Formulation	DSPC (mol %)	Chol (mol %)	DODAP (mol %)	Steric Barrier Derivatized Lipid (name: mol %)	Antisense (EGF-R 2 mg/ml)
1	55	45	Nil	Nil	Empty
2	50	45	Nil	ATTA8-DSPE: 5	Empty
3	50	45	Nil	ATTA8-DSPE: 5	AS
4	20	45	30	ATTA8-DSPE: 5	AS
5	20	45	30	PEG-DSPE: 5	AS
6	25	45	25	PEG-CerC14: 5	Empty
7	25	45	25	PEG-CerC14: 5	AS
8	25	45	25	PEG-CerC20: 5	Empty
9	25	45	25	PEG-CerC20: 5	AS

Antisense ("AS") used was fully phosphorothioated EGFR (anti-human Epidermal Growth Factor Receptor) CCG TGG TCA TGC TCC (SEQ ID. No 10) (prepared by Hybridon, Inc.) PEG-CerC14 is PEG(mw2000)-Ceramide with 14 carbon acyl chain. PEG-CerC20 is PEG(mw2000)-Ceramide with 20 carbon acyl chain. PEG-DSPE is PEG(mw2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine ATTA8-DSPE is N-(ω-N'-acetoxy-octa(14'amino-3',6',9',12'-tetraoxatetradecanoyl))- 1,2-distearoyl-sn-glycero-3-

phosphoethanolamine (molec weight about 2660). Synthesis of ATTA8-DSPE is fully disclosed in U.S. Provisional Pat. Application Ser. No. 60/073,852, filed Dec. 23, 1997 and U.S. Provisional Pat. Application filed Feb. 2, 1998 both assigned to the assignee of the instant invention and incorporated herein by reference.

Each formulation contained a lipid label ([^{14}C]-cholesterylhexadecylether) and [^{3}H]-antisense, as described in Example 4.4, above. All samples were prepared in 300 mM citrate pH 4.0 containing 40% ethanol and extruded 10× through 100 nm filters. Formulations contained phosphorothioate antisense and lipid or empty lipid alone. Samples were dialyzed in HBS (20 mM Hepes, 145 mM NaCl, pH 7.45) to remove ethanol and citrate. Sample lipid concentrations were adjusted such that the injected lipid dose will be 1.8 μmol/mouse/week (5–10 mg AS per kg mouse/week). Samples were filtered (0.22 μm) prior to injection.

In this experiment female (20–25g) ICR mice (6–8 weeks old) were divided into 9 groups of 6, plus other control groups. Each group received four injections of the same formulation. All injections were 200 μL intravenous (via the lateral tail vein) at a lipid dose of 120 mg/kg. Mice were dosed every week for 3 weeks (4 injections). At 4 weeks, certain groups (treated with lipid and antisense) were given an injection of empty lipid carriers of varying composition to evaluate whether there is rapid clearance of the carrier in the absence of antisense. Blood (25 μl, pipettor) was collected 1 h post-injection each week for 3 weeks by tail nicks. Mice were weighed each week to estimate blood volume (8.0 ml whole blood/100 g body weight). Blood was placed in a glass scintillation vial containing 200 μl of 5% EDTA. Solvable (500 μl) was added and the blood was digested for 3 h at 65° C. Samples were decolorized by the addition of 100 μl 70% hydrogen peroxide. Samples were analyzed for radioactivity by liquid scintillation counting. At the end of 4 weeks, mice were terminated by CO_2 inhalation or cervical dislocation preceded by general anesthesia.

The results of this experiment are shown in FIG. **17**. For all formulations not containing antisense ("empty liposomes") repeat dosages demonstrated circulation times reasonably consistent with the first dosage. However, when antisense is used in the formulation, it was surprisingly found that the acyl chain length of the lipid derivatized to the steric barrier (i.e. ATTA or PEG) moiety demonstrates a profound effect on clearance rates. Repeat dosages of PEG-CerC20, PEG-DSPE and ATTA8-DSPE formulations are rapidly cleared from the circulation compared to the first dosage, whereas the PEG-CerC14 formulation is reasonably consistent with the first dosage.

Similar results are demonstrated in FIG. **18**. The formulations were identical to those of FIG. **17**, with the additional formulation of empty vesicles using the same lipids as formulations 4 and 5.

Without intending to be bound by any particular theory of action, it is suggested by these results that lipids like the PEG-CerC14 lipid, a lipid which exchanges out of the liposome membrane with a T1/2 on the order of minutes (i.e. 1–60 mins) in blood provides a tremendous benefit over lipids like PEG-CerC20, PEG-DSPE and ATTA8-DSPE which do not exchange out, where repeat dosing of a lipid-formulated compound, such as a therapeutic compound or diagnostic compound, is required. The mammalian blood clearance response may not recognize these as foreign antigens if the derivatized lipid is removed expeditiously from the liposome surface when in circulation. However, when the derivatized-lipid remains with the formulation for extended periods, a clearance response is invoked, which causes rapid clearance upon repeat dosing. This data suggests that any lipid derivatized with a steric barrier molecule that exchanges out of the liposome membrane faster than PEG-CerC20, PEG-DSPE or ATTA8-DSPE will be superior for use in repeat dosing. For example ATTA8-DMPE, or PEG-CerC8 to C18 all being exchangeable, will have improved circulation characteristics upon repeat administration.

Taken together, it will be evident to one skilled in the art, that on the basis of these teachings, any diagnostic or therapeutic agent that may be delivered in a lipid formulation comprising a steric-barrier derivatized lipid, such as a PEG-lipid or ATTA-lipid, should be tested with both a long and short acyl-chain anchors, in order to determine which formulation is best for repeat dosings.

Further, without intending to be bound by any theory of action, the invention herein may prove to be particularly useful when the bioactive agent being delivered is a non-cytotoxic agent. Cytotoxic agents kill those cells which clear long circulating (i.e. PEG-DSPE) liposomes. This ensures that repeat dosings will not be rapidly cleared, because the cells responsible (usually macrophages) do not survive. In these situations, the acyl-chain length may not be significant. However, where the bioactive agent is non-cytotoxic, such as in the case of antisense drugs (regardless of chemistry or target), plasmids, proteins, etc., and many conventional drugs, the invention will be useful for repeat dosing.

4.10 In vivo Efficacy of Repeat Doses of Encapsulated Phosphorothioate c-myc Antisense in an Oncology Model

In vivo efficacy of repeat injections of using formulations of the invention are shown in a mouse tumor system in FIG. **19**. This experiment demonstrated efficacy of the antisense formulated according to the invention in a human oncology model, and showed the importance of PEG-acyl chain length on the efficacy of repeat dosings.

Lipid-antisense particle formulation: Formulations were prepared as described in these Examples.

Formulation	DSPC (mol %)	Chol (mol %)	DODAP (mol %)	Steric Barrier Derivatized Lipid (name: mol %)	Antisense (c-myc 2 mg/ml)
HBS Buffer					Empty
AS4200 (c-myc)	25	45	25	PEG-CerC14: 5	LR-3280
AS4204 (c-myc)	25	45	25	PEG-CerC20: 5	LR-3280
AS4204 (c-myc SCR)	25	45	25	PEG-CerC20: 5	c-myc SCR
AS4204 (PS-2302)	25	45	25	PEG-CerC20: 5	PS-2302
A54204 (PS-3082)	25	45	25	PEG-CerC20: 5	PS-3208
c-myc					LR-3280
c-myc SCR					c-myc SCR
PS-2302					PS-2302
PS-3082					PS-3082
AS4200 (no antisense)	25	45	25	PEG-CerC14: 5	Empty
AS4204 (no antisense)	25	45	25	PEG-CerC20: 5	Empty

-continued

Formulation	DSPC (mol %)	Chol (mol %)	DODAP (mol %)	Steric Barrier Derivatized Lipid (name: mol %)	Antisense (c-myc 2 mg/ml)

Antisense used were:

LR-3280:	human c-myc gene (phosphorothioate) AAC GTT GAG GGG CAT	(SEQ ID. No 4)
c-myc SCR:	GAA CGG AGA CGG TTT	(SEQ ID. No 17)
PS-2302	human ICAM-1 (phosphorothioate) GCCCAAGCTGGCATCCGTCA	(SEQ ID. No 2)
PS-3082	murine ICAM-1 (Intracellular Adhesion Molecule-1) (phosphorothioate) TGCATCCCCCAGGCCACCAT	(SEQ ID. No 1)

Formulations were diluted in filtered HBS, pH 7.6 to achieve required antisense dose (i.e. lipid dose decreases as well). Samples were filtered (0.22 μm) prior to injection. External buffer was HBS (20 mM Hepes, 145 mM NaCl, pH 7.6). Free antisense was dissolved in HBS and adjusted to the required dose by A260 (Extinction coefficients: active and control c-myc=30.6, PS-2302=32.8, PS-3082=33.6).

Tumour Inoculum: B16/BL6 murine melanoma cells were maintained in culture in MEM media supplemented with 10% FBS. On day 0 of the study, 3×10^5 cells were injected sub-cutaneously (s.c.) into the dorsal flank (injection volume: 50 μl) of female C57BL/6 mice (20–23 g). Typically, 15% extra mice will be injected so non-spheroidal tumours or mice in which no tumours are observed can be excluded from the study. Tumours were allowed to grow for a period of 5–7 days until tumors reached 50–100 mm^3 prior to initiating treatments with test samples/controls.

Treatment: On the day of first treatment mice with acceptable tumours were randomly grouped with 5 animals per group. Treatment began when tumours were 50–100 mm^3. Mice were dosed every other day for a total of 7 doses. Administrations were via intravenous tail vein injections (200 μl). Initial drug:lipid ratio of formulation was 0.20 (w/w) and the final drug:lipid ratio (0.14) was held constant; consequently, the lipid concentration varied as samples were diluted to the desired antisense concentration. The antisense dose was 10 mg/kg.

Endpoints: Primary tumour volume was measured using calipers. Length (mm) and width (mm) measurements were made every other day (on non-injection days) for the duration of the study. Tumour height measurements (mm) were made when feasible. Tumour volumes were calculated using the following formulas:

$$\text{Tumour Volume } (mm^3)=(L\times W^2)/2 \quad \#1$$

$$\text{Tumour Volume } (mm^3)=(L\times W\times H)\times\pi/6 \quad \#2$$

Mice were euthanized when tumour volumes reach 10% of body weight or on the first signs of ulceration. Mouse weights were recorded every day during the dosing portion of the study. On termination, all tumours were excised, weighed, observed by FACS analysis or by Northern/Western analysis. Mice were euthanized by CO_2 inhalation or cervical dislocation preceded by general anesthesia.

Results: FIG. **9** shows weights of tumors excised and weighed at day 18 for all groups treated with antisense at 10 mg/kg/dose compared with empty lipid controls. Tumour sizes for the AS4200(c-myc) group exhibited the best efficacy and were very consistent with only small ranges in tumour volumes observed (285–451 mm^3). The group treated with free c-myc also resulted in smaller tumours but exhibited more variability in tumour volume (156–838 mm^3). The encapsulated c-myc controls (c-myc SCR/PS-2302/PS-3082), AS4204(c-myc), empty lipid carriers, and free antisense controls, however, showed no inhibitory effect on tumor volumes over the 18 days when compared to HBS controls.

c-myc expression in tumor tissue was also evaluated by FACS. A correlation between tumour size and c-myc protein expression was detected (data not shown).

To determine the importance of the stability of the PEG-polymers, PEG-acyl chain length was evaluated using formulations containing PEG-CerC14 and PEG-CerC20. Interestingly, the formulation containing the PEG-CerC20 (AS4204) showed no apparent efficacy at any of the doses studied. The PEG-CerC14 formulation (AS4200) showed a dose response. The difference observed between the PEG-CerC14 and PEG-CerC20 formulations may reflect the rapid clearance phenomenon that has been observed in other models.

To establish the tolerability of free and encapsulated antisense, mouse weights were measured on a daily basis during the treatment phase of the study. No significant changes in mouse weights for either free or encapsulated formulations were apparent over the course of the dosing phase or throughout the study.

EXAMPLE 5

This example illustrates a high efficiency formulation according to Example 2, but instead of phosphorothioate antisense, employing 1) a phosphodiester antisense compound having exclusively phosphodiester internucleotide linkages (PO-2302 anti-human ICAM-1 GCCCAAGCTG-GCATCCGTCA (SEQ ID. No 1)) prepared by Inex Pharmaceuticals (USA), Inc., Hayward Calif.) or 2) ribozyme molecule to VEGF-R-1 (human Vascular Endothelial Growth Factor Receptor 1) comprising a modified RNA sequence of GAG UUG CUG AUG AGG CCG AAA GGC CGA AAG UCU G (SEQ ID. No 16).

A 15 mer of [^{3}H]-phosphodiester antisense oligodeoxynucleotide (PO-2302) in citrate buffer, pH 3.80 (experiments ranged from 10–1000 mM citrate) was mixed with an ethanol solution of lipid (DSPC:CHOL:DODAP:PEG-CerC14; 25:45:20:10, molar ratio) at final concentrations of 2 mg/mL and 9.9 mg/mL, respectively. The final ethanol concentration in the 1 ml preparation was 38% vol/vol. The sample was extruded ten times through three 100 nm filters as described in "Materials and Methods". The sample was dialyzed for 2–3 hours in citrate buffer, pH 3.80 (same molarity as experiment), to remove a majority of the excess ethanol. The samples were switched to HEPES-buffered saline (HBS), pH 7.50, and dialyzed for a minimum of 12 hours to replace the external citrate buffer with HBS. Non-encapsulated antisense was removed either by this regular dialysis, tangential flow dialysis, or chromatography. Encapsulation was assessed either by analyzing the pre-column and post-column ratios of [^{3}H]-antisense and [^{14}C]-lipid or by determining the total pre-column and post-column [^{3}H]-antisense and [^{14}C]-lipid radioactivity.

FIG. **15** illustrates results. Encapsulation efficiency was over 50% across the 10–50 mM citrate range, and all final (administration ready) drug:lipid ratios were greater than 10% by weight. Parallel experiments varying citrate concentration were conducted with phosphorothioate antisense PS-2302. Results are also above 50% encapsulation, and in fact show a higher encapsulation efficiency than phosphodiesters, particularly at higher citrate concentrations.

This experiment was repeated using 20 mM citrate instead of 300 mM citrate to encapsulate the ribozyme molecule to VEGF-R-1 (human Vascular Endothelial Growth Factor Receptor 1) GAG UUG CUG AUG AGG CCG AAA GGC CGA AAG UCU G (SEQ ID. No 16). FIG. **20** shows the encapsulation efficiency of the ribozyme at was over 50%, approximately the same as the phosphodiester.

EXAMPLE 6

This example illustrates a high efficiency formulation as in Example 5, but replacing DODAP with an alternative protonatable lipid. Typically, the preparation for the alternative will be X:DSPC:CHOL:PEG-CerC14 at 20:25:45:10 molar ratio where X can be DODAC, OA, DODMA or any other lipid suitable for the invention.

Materials: distearoylphosphatidylcholine, DSPC; cholesterol, CHOL (both from Northern Lipids, Vancouver, BC); N,N-dioleyl-N,N-dimethylammonium chloride, DODAC; Oleylamine, OA (prepared by Steve Ansell, Inex); N-(1-(2,3-Dioleoyloxy) propyl)-N,N,-dimethyl ammonium chloride, DODMA(Avanti Polar Lipids, Alabaster AB, chloride salt prepared by Steve Ansell, INEX); poly(ethylene glycol)2000 coupled to a ceramide derivative with 14 carbon acyl chains, PEG-CerC14 (Zhou Wang, INEX Pharmaceuticals); 13×100 mm glass tube; filter sterilized 300 mM citrate buffer, pH 3.9–4.0 (use a 0.2 μm filter). Fully thioated c-myc antisense (INEX (USA), Hayward Calif.), Anhydrous Ethanol (Commercial Alcohols, Toronto, On), Citric acid, Monobasic Sodium phosphate, Dibasic Sodium phosphate, Sodium hydroxide, HEPES (BDH, Mississauga On). Deionized water, Chloroform, Methanol, Oligreen™ oligonucleotide reagent (Molecular Probes, Eugene Oreg.), Sodium chloride, Triton X-100, alcohol dehydrogenase reagent kit, (Sigma Chemical Co., St Louis Mo.),

Lipid stock solutions were made in 100% ethanol with the working concentrations of the lipids which is as follows:

DSPC, 20 mg/ml; CHOL, 20 mg/ml (not very soluble above this concentration); DODMA, 20 mg/ml; PEG-CerC14; 50 mg/ml.

To prepare stock solutions of antisense, the antisense molecules were dissolved in the filtered 300 mM citrate buffer at a concentration of 3.33 mg/ml. Lipids were mixed in the desired proportions in a 13×100 mm glass tube to achieve a final volume of 0.4 ml of lipids using 100% ethanol as listed in table 1, below:

TABLE 1

Proportional mixture of lipids in a 13 × 100 mm glass test tube.

Lipid	Mol %	M. Wt.	mg	μmol	Stock (mg/ml)	Vol of Stock (μl)
DODMA	20	652.6	1.69	2.60	20	84.5
DSPC	25	790	2.57	3.25	20	115
CHOL	45	386.7	2.26	5.85	20	113.1
PEG-CerC14	10	2600	3.38	1.30	50	67.6
	100		9.9	13.00		380.2

In a separate 13×100 mm glass tube, 0.6 ml of antisense at 3.33 mg/ml was added. The pH of this solution should be 3.9–4.0. (NOTE: the antisense concentration is NOT determined by weight but rather by measuring absorbance at 260 nm). The lipid mixture solution was warmed to 65° C. for about 2 minutes. The antisense tube was vortexed and during this time, using a Pasteur pipette, the lipids (in ethanol) were added slowly in a dropwise manner. The mixture will get "cloudy" and some bubbles may be observed due to the ethanol, but no aggregates should be present. The resulting volume of the antisense-lipid mixture was 1.0 ml with a 10 mg (13 umol) total lipid at 13 μmols, 2 mg of antisense, and 38% ethanol, vol/vol. It can be expected that the pH to rise to about 4.4.

The antisense-lipid mixture was subjected to 5 (five) cycles of freezing in liquid nitrogen and thawing at 65° C. in a waterbath. After each thaw, the mixture was vortexed briefly. Subsequently, the mixture was passed 10 times through three stacked 100 nm polytcarbonate filters (Poretics) or extruded using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and nitrogen pressure during extrusion were 65° C. and no more than 200 psi to 300 psi, respectively. Each pass should take no more than 2 minutes and is vortexed after each pass.

After extrusion, the mixture was dialyzed in a dialysis tubing (3500 Mwt cutoff; SpectraPor) for 1 hour in 300 mM citrate at pH 3.9–4.0, removing the ethanol. The mixture was transferred into 5 L of HBS buffer at pH 7.5 and allowed to further dialyze to a minimum of 12 hours, to neutralize the DODMA and release any surface bound antisense associated with the vesicles. Alternatively, tangential flow dialysis, ion exchange-chromatography or gel filtration chromatography can be used to process the extruded antisense-lipid mixture to an administration ready preparation.

EXAMPLE 7

This example illustrates a high efficiency formulation as in Example 5, but replacing DSPC with SM to generate a preparation of DODAP:SM:CHOL:PEG-CerC14 at 20:25:45:10 molar ratio. Antisense is processed with the formulation for a standard 1.0 ml volume, which can be scaled up proportionately as required.

Materials: Sphingomyelin SM; cholesterol, CHOL; dimethylaminopropane, DODAP; polyethylene glycol coupled to a ceramide derivative with 14 carbon acyl chains, PEG-CerC14; 13×100 mm glass tube; filter sterilized 300 mM citrate buffer, pH 3.9–4.0 (use a 0.2 μm filter).

Lipid stock solutions were made in 100% ethanol with the working concentrations of the lipids which is as follows:

SM, 20 mg/ml; CHOL, 20 mg/ml (not very soluble above this concentration); DODAP, 20 mg/ml; PEG-CerC14; 50 mg/ml.

To prepare stock solutions of antisense, the antisense molecules were dissolved in the filtered 300 mM citrate buffer at a concentration of 3.33 mg/ml. Lipids were mixed in the desired proportions in a 13×100 mm glass tube to achieve a final volume of 0.4 ml of lipids using 100% ethanol as listed in Table 2, below:

TABLE 2

Proportional mixture of lipids in a 13 × 100 mm glass test tube.

Lipid	Mol %	M. Wt.	mg	μmol	Stock (mg/ml)	Vol of Stock (μl)
DODAP	20	684.5	1.78	2.60	20	89.0
SM	25	703	2.30	3.27	20	115
CHOL	45	386.7	2.26	5.85	20	113.1
PEG-CerC14	10	2600	3.38	1.30	50	67.6
	100		9.72	13.02		384.7

In a separate 13×100 mm glass tube, 0.6 ml of antisense at 3.33 mg/ml was added. The pH of this solution should be 3.9–4.0. (NOTE: the antisense concentration is NOT determined by weight but rather by measuring absorbance at 260 nm). The lipid mixture solution was warmed to 65° C. for about 2 minutes. The antisense tube was vortexed and during this time, using a Pasteur pipette, the lipids (in ethanol) were added slowly in a dropwise manner. The mixture will get "cloudy" and some bubbles may be observed due to the ethanol, but no aggregates should be present. The resulting volume of the antisense-lipid mixture was 1.0 ml with a 10 mg (13 umol) total lipid at 13 μmols, 2 mg of antisense, and 38% ethanol, vol/vol. It can be expected that the pH to rise to about 4.4.

The antisense-lipid mixture was subjected to 5 (five) cycles of freezing in liquid nitrogen and thawing at 65° C. in a waterbath. After each thaw, the mixture was vortexed briefly. Subsequently, the mixture was passed 10 times through three stacked 100 nm polytcarbonate filters (Poretics) or extruded using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and nitrogen pressure during extrusion were 65° C. and no more than 200 psi to 300 psi, respectively. Each pass should take no more than 2 minutes and is vortexed after each pass.

After extrusion, the mixture was dialyzed in a dialysis tubing (3500 Mwt cutoff; SpectraPor) for 1 hour in 300 mM citrate at pH 3.9–4.0, removing the ethanol. The mixture was transferred into 5 L of HBS buffer at pH 7.5 and allowed to further dialyze to a minimum of 12 hours, to neutralize the DODAP and release any surface bound antisense associated with the vesicles. Alternatively, tangential flow dialysis, ion exchange-chromatography or gel filtration chromatography can be used to process the extruded antisense-lipid mixture to an administration ready preparation.

EXAMPLE 8

This example illustrates a high efficiency formulation as in Example 5, but replacing PEG-CerC14 with ATTA8-DSPE to prepare DODAP:DSPC:CHOL:ATTA8-DSPE at 40:10:45:5 molar ratio of antisense formulation.

Materials: distearoylphosphatidylcholine, DSPC; cholesterol, CHOL; dimethylaminopropane, DODAP; N-(ω-N'-acetoxy-octa(14'amino-3', 6', 9', 12'-tetraoxatetradecanoyl))-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, ATTA8-DSPE; 13×100 mm glass tube; filter sterilized 300 mM citrate buffer, pH 3.9–4.0 (use a 0.2 μm filter).

Lipid stock solutions were made in 100% ethanol with the working concentrations of the lipids which is as follows:

DSPC, 20 mg/ml; CHOL, 20 mg/m/l (not very soluble above this concentration); DODAP, 20 mg/ml; ATTA8-DSPE; 50 mg/ml.

To prepare stock solutions of antisense, the antisense molecules were dissolved in the filtered 300 mM citrate buffer at a concentration of 3.33 mg/ml. Lipids were mixed in the desired proportions in a 13×100 mm glass tube to achieve a final volume of 0.4 ml of lipids using 100% ethanol as listed in Table 3, below:

TABLE 3

Proportional mixture of lipids in a 13 × 100 mm glass test tube.

Lipid	Mol %	M. Wt.	mg	μmol	Stock (mg/ml)	Vol of Stock (μl)
DODAP	40	684.5	4.16	6.08	20	208
DSPC	10	790	1.2	1.52	20	60
CHOL	45	386.7	2.6	6.72	20	130
ATTA8-DSPE	5	2638	2.0	0.76	50	40
	100		10.26	15.1		438

In a separate 13×100 mm glass tube, 0.6 ml of antisense at 3.33 mg/ml was added. The pH of this solution should be 3.9–4.0. (NOTE: the antisense concentration is NOT determined by weight but rather by measuring the absorbance at 260 nm). The lipid mixture solution was warmed 65° C. for about 2 minutes. The antisense tube was vortexed and during this time, using a Pasteur pipette, the lipids (in ethanol) were added slowly in a dropwise manner. The mixture will get "cloudy" and some bubbles may be observed due to the ethanol, but no aggregates should be present. The resulting volume of the antisense-lipid mixture was 1.0 ml with a 10 mg (13 umol) total lipid at 13 μmols, 2 mg of antisense, and 38% ethanol, vol/vol. It can be expected that the pH to rise to about 4.4.

The antisense-lipid mixture was subjected to 5 (five) cycles of freezing in liquid nitrogen and thawing at 65° C. in a waterbath. After each thaw, the mixture was vortexed briefly. Subsequently, the mixture was passed 10 times through three stacked 100 nm polytcarbonate filters (Poretics) or extruded using a pressurized extruder apparatus with a thermobarrel attachment (Lipex Biomembranes). The temperature and nitrogen pressure during extrusion were 65° C. and no more than 200 psi to 300 psi, respectively. Each pass should take no more than 2 minutes and is vortexed after each pass.

After extrusion, the mixture was dialyzed in a dialysis tubing (3500 Mwt cutoff; SpectraPor) for 1 hour in 300 mM citrate at pH 3.9–4.0, removing the ethanol. The mixture was transferred into 5 L of HBS buffer at pH 7.5 and allowed to further dialyze to a minimum of 12 hours, to neutralize the DODAP and release any surface bound antisense associated with the vesicles. Alternatively, tangential flow dialysis, ion exchange-chromatography or gel filtration chromatography can be used to process the extruded antisense-lipid mixture to an administration ready preparation.

EXAMPLE 9

This example illustrates use of tangential flow dialysis to clean up a large scale (>50 ml) preparation of extruded antisense-lipid mixture to obtain an administration ready preparation. Tangential Flow Diafiltration has been shown to be useful in four functions in the formulation process 1) buffer exchange, 2) removal of ethanol, 3) removal of unencapsulated antisense and 4) concentration of the formulation. Using TF it is demonstrated that it is possible to efficiently exchange these components using only 10–15 sample volumes with a single buffer system at a very significant reduction in the process time.

Materials for Tangential Flow Dialysis: Microcross Sampler™ Tangential Flow column (Microgon, Laguna Hills, Calif.) Masterflex™ console drive and Easyload™ Pump head (Cole-Palmer, Vernon Hills Ill.), Extruder (Lipex Biomembranes, Vancouver BC), Polycarbonate membranes, 100 μm, (AMD Manufacturing, Mississauga On).

Antisense (c-myc) is prepared by dissolving in 300 mM Na Citrate buffer to a final concentration of 4.17 mg/ml for c-myc as verified by absorbance at 260 nm. The antisense stock solution is typically warmed to 65° C. for 2 minutes to dissolve and to remove secondary structure. AS4200 consists of DODAP:DSPC:CHOL:PEG-CER-14 at the percent mol ratio of25:20:45:10 and the lipids are aliquoted from stock solutions to a total concentration of 10 mg/0.400 ml in anhydrous ethanol. In this study 50–60 ml scale formulations were produced. Thus 20–24 ml of the ethanolic lipid solution is added dropwise, at room temperature, using a peristaltic pump at 1 ml/nin into 30–36 ml of the AS solution which is stirring in a 100 ml round bottom flask with a 2 cm magnet stir bar (Stirrer setting 2–3). After mixing, the lipid/antisense suspension was pipetted into a 100 ml extruder prepared with 2–3, 100 μm polycarbonate membranes and pre-equilibrated at 65° C. The suspension was extruded using ten passes at ~300 psi. After extrusion the formulation was processed using tangential flow diafiltration.

Tangential Flow Ultrafiltration. A 230 cm^2 Microcross tangential flow cartridge (50 kDa cut off) was attached to a Masterflex peristaltic pump, sample reservoir and buffer reservoir using Tygon tubing. The tubing length was adjusted so that the total circuit of tubing, pump and TF cartridge had a total dead volume of 30 ml. To this system a 60 ml sample reservoir was attached. The sample was loaded into the tubing and reservoir by running the peristaltic pump at a low speed. After loading, the system was closed and the pump speed gradually increased to the pump maximum (approx. 100 ml/min) until the initial TF cartridge inlet pressure was 12–15 psi and the outlet pressure was 8–11 psi. When the system pressure stabilized, both the filtrate outlet and the buffer reservoir were opened. Opening these valves allowed filtrate to flow out of the cartridge at ~10–15 ml/min while wash buffer (i.e. PBS, pH 7.5) was being collected. For a 50–60 ml formulation 700–900 ml of buffer was used to "wash" the sample. Fractions (10 ml) of the filtrate were collected for analysis of ethanol removal, pH, and antisense. After diafiltration was completed the wash buffer reservoir was closed and with the pump continuing to run, filtrate was allowed to flow, concentrating the sample, typically reducing the preparation volume to the tubing dead volume (30–35 ml). The sample was collected from the system and the tubing and column were washed with 15 ml wash buffer to remove any remaining formulation.

Antisense Quantification. Antisense concentration was normally determined by measuring absorbance at 260 nm as outlined in the current protocol. Briefly, antisense stock solutions were quantified by diluting 1:500 in MilliQ water and measuring absorbance. TF filtrate fractions were diluted 1:10 in MilliQ water and absorbance was measured. Antisense in suspension with lipids was measured by adding 10 μl of the suspension to 250 μl MilliQ water. A monophase was created by adding 750 μl $CHCl_3$/MeOH (2.1:1) and 100 μl MeOH. Immediately after vortexing the mixture the absorbance was measured at 260 nm. In each case the extinction coefficient for the given antisense was multiplied by the dilution factor to determine the antisense concentration.

Lipid Quantification. As outlined in the current protocol, 50 μl aliquots of the lipid/antisense suspension was diluted with 100 μl MilliQ water and submitted for analysis by HPLC. The percent encapsulation efficiency of the formulation is determined by dividing the Drug/Lipid ratio of the finished product by the initial Drug/Lipid ratio formed when the lipid and antisense stock solutions are mixed.

Ethanol Assay. Ethanol in the TF filtrate was determined using an alcohol dehydrogenase reagent kit supplied by Sigma Chemical Co.

DEAE Sephadex chromatography. A suspension of the processed formulation was loaded onto a 1×10 cm column of DEAE sephadex equilibrated in 20 mM PBS, pH 7.5. After eluting through the column the formulation was collected into a sterile falcon tube. The volume, antisense and lipid concentration were measured to determine recovery.

Particle Size. The particle size of the formulation was measured by QELS using a Nicomp Particle sizer, (Nicomp, Santa Barbara, Calif.) and particle sizes are reported in the particle mode with volume weighing.

Results of Large Scale Preparations:

Assay	Initial Lipid Content (mg/ml)	Initial Antisense Content (mg/ml)	Final Lipid Content (mg/ml)	Final Antisense Content (mg/ml)	Initial Drug:Lipid	Final Drug:Lipid	Encaps. Effic.
A	10.581	1.936	14.604	1.681	0.183	0.115	63%
B	8.727	2.284	7.926	1.008	0.262	0.127	48%
C	11.06	2.97	2.69	0.556	0.286	0.207	77%

EXAMPLE 10

Phosphodiester and phosphorothioate antisense oligonucleotides encapsulated according to the methods in Example 2 and 5–9 were examined for their relative susceptibility to nuclease digestion by serum or S1 nuclease. Protection of the phosphodiester-linked oligonucleotide was significantly higher in serum when encapsulated as opposed to the free, raising the $T_{1/2}$ of degradation from 10 mins to at least 8 h. Free phosphorothioate oligodeoxynucleotide showed significant breakdown in serum within 30 minutes, however encapsulated phosphorothioate oligodeoxynucleotide did not show any sign of degradation even after 24 h incubation in serum. In vivo data agrees with these findings, showing no sign of degradation of the encapsulated phosphorothioate antisense until 8 h.

As a positive control, the free phosphodiester and phosphorothioate antisense were subjected to very potent levels of S1 nuclease (100U/50 μg) (1U of S1 nuclease will digest 1 ug DNA per minute at 37° C.). The enzyme completely digested the free phosphodiester and phosphorothioate within seconds after its addition. The encapsulated phosphodiester under the same conditions was over 90% intact at 24 h, and the encapsulated phosphorothioate was fully intact at 24 h.

The experiments were conducted as described in the specification, or modified as follows.

S1 Nuclease Digestion. 50 μg aliquots containing free, encapsulated, or encapsulated +0.5% Triton X100 were aliquoted into 1.5 ml eppendorf tubes. To the tubes were added 10 μl 10×S1 nuclease buffer, dH2O (to make final volume 100 μl), and, just prior to digestion, 100U of S1 nuclease to each eppendorf tube. The tubes were sealed with parafilm and incubated at 55° C. A sample of the free, encapsulated, or encapsulated +0.5% Triton X100 not digested by nuclease (standard) was frozen in liquid nitrogen in an eppendorf tube and stored at −20° C. At each desired time point, an aliquot of each sample was collected, added to GDP buffer containing proteinase K (133 μg/ml) and immediately frozen in liquid nitrogen in order to stop the reaction. Once all of the time points were collected, the samples were incubated at 55° C. in a waterbath to activate proteinase K enabling it to denature any remaining S1 nuclease. Proteinase K digested samples were applied to polyacrylamide gels, described below, to assess levels of S1 nuclease degradation

Normal Murine/Human Serum Digestion. 50 μg of the free, encapsulated, or encapsulated +0.5% Triton X100 was aliquoted into 1.5 ml eppendorf tubes. To the tubes we added 45 μl normal murine/human serum, dH2O (to make final volume 50 μl), to each eppendorf tube. The tubes were sealed with parafilm and incubated at 37° C. A sample of the free, encapsulated, or encapsulated +0.5% Triton X100 not digested by nuclease (standard) was frozen in liquid nitrogen in an eppendorf tube and stored at −20° C. Aliquots were taken at various time points, added to GDP buffer containing proteinase K (133 μg/ml) and immediately frozen in liquid nitrogen to stop the reaction. Once all of the time points were collected, the samples were incubated at 55*C. in a waterbath to activate proteinase K enabling it to denature any remaining exonuclease. Proteinase K digested samples were applied to polyacrylamide gels to assess levels of exonuclease degradation Micrococcal Nuclease. An alternative standard nuclease assay not employed in the present experiment is the assay disclosed by Rahman et al. U.S. Pat. No. 5,665,710, wherein nucleic acid/lipid particles are incubated for 30 mins at 37° C. in presence of an excess of micrococcal nuclease in 1 mM $CaCl_2$.

Polyacrylamide Gel Electrophoresis (PAGE). Prepared 14 cm×16 cm×7.5 mm polyacrylamide (15% or 20%) gels in 7M urea and TBE. Approximately 300 ng of sample (at each time point) and standard were aliquoted into eppendorf tubes. An equivalent volume of 2× loading buffer was added to each sample. The samples were then heated in a waterbath to 90° C. for 3 min to reduce secondary structures and then applied to the gel. The loaded gel was electrophoresed at 600V for 10 min (to sharpen the band) and then at 300V for the duration of the gel. The gel was incubated in 1×SyberGreen I stain in TBE for a minimum of 15 min and then photographed while illuminated under UV light (3.5 sec exposure, 4.5 aperture).

VII. Conclusion

As discussed above, the present invention provides methods of preparing lipid-encapsulated therapeutic agent (nucleic acid) compositions in which the therapeutic agent (nucleic acid) portion is encapsulated in large unilamellar vesicles at a very high efficiency. Additionally, the invention provides compositions prepared by the method, as well as methods of introducing therapeutic agents (nucleic acids) into cells. The compositions are surprisingly efficient in transfecting cells, both in vivo and in vitro.

All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 17

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGCATCCCCC AGGCCACCAT 20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCCCAAGCTG GCATCCGTCA 20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGTGCTCACT GCGGC 15

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AACGTTGAGG GGCAT 15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TAACGTTGAG GGGCAT 16

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TATGCTGTGC CGGGGTCTTC GGGC 24

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTGCCGGGGT CTTCGGGC 18

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GGACCCTCCT CCGGAGCC 18

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TCCTCCGGAG CCAGACTT 18

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

-continued

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CCGTGGTCAT GCTCC 15

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CAGCCTGGCT CACCGCCTTG G 21

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CAGCCATGGT TCCCCCCAAC 20

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GTTCTCGCTG GTGAGTTTCA 20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

-continued

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TCTCCCAGCG TGCGCCAT 18

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GTGCTCCATT GATGC 15

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GAGUUGCUGA UGAGGCCGAA AGGCCGAAAG UCUG 34

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GAACGGAGAC GGTTT 15

What is claimed is:

1. A composition comprising lipid-therapeutic agent particles comprising a lipid portion and a charged therapeutic agent, said charged therapeutic agent being encapsulated in said lipid portion, wherein said lipid portion comprises a first lipid component, a second lipid component, a third lipid component, and a fourth lipid component, said first lipid component being selected from among lipids containing a protonatable or deprotonatable group that has a pKa such that the lipid is in a charged form at a first pH and a neutral form at a second pH, wherein the pKa of the first lipid component is in the range of from 4 to 11, and said first lipid component being further selected such that the charged form is cationic when the therapeutic agent is anionic and anionic when the therapeutic agent is cationic, said second lipid component being selected from among lipids that prevent particle aggregation during lipid-therapeutic agent particle formation and which exchange out of the lipid particle at a rate greater than PEG-CerC20, said third lipid component being a neutral lipid selected from the group consisting of DSPC, POPC, DOPE, and SM, and said fourth lipid component being Chol.

2. The composition according to claim **1**, wherein at least some of the protonatable or deprotonatable groups are disposed on the exterior surface, of the particles and at least some of these groups have been neutralized.

3. The composition according to claim 2, wherein the therapeutic agent is anionic.

4. The composition according to claim 3, wherein the therapeutic agent is a polyanionic nucleic acid.

5. The composition according to claim 4, wherein the polyanionic nucleic acid is an antisense nucleic acid.

6. The composition according to claim 4, wherein at least 50% of the polyanionic nucleic acid in the composition is encapsulated within the particle.

7. The composition of claim 4, wherein at least 90% of the polyanionic nucleic acid in the composition is encapsulated within the particle.

8. The composition of claim 4, wherein the polyanionic nucleic acid has exclusively phosphodiester linkages.

9. The composition of according to claim 3, wherein the first lipid component is an amino lipid.

10. The composition of claim 9, wherein the second lipid component is a polyethylene glycol-modified or polyamide oligomer-modified lipid.

11. The composition of claim 3, wherein the second lipid component is a polyethylene glycol-modified or polyamide oligomer-modified lipid.

12. A composition comprising lipid-therapeutic agent particles comprising a lipid portion and a charged therapeutic agent, said charged therapeutic agent being encapsulated in said lipid portion, wherein said lipid portion comprises a first lipid component, a second lipid component, a third lipid component and a fourth lipid component, said first lipid component being selected from among lipids containing a protonatable group that has a pKa such that the lipid is in a charged form at a first pH and a neutral form at a second pH, and said second lipid component being selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation, said third lipid component being a neutral lipid selected from the group consisting of DSPC, POPC, DOPE, and SM, and said fourth lipid component being Chol, said particles having a nucleic acid/lipid ratio of at least 10% by weight and a size of from about 70 to about 200 nm, wherein said therapeutic agent is a polyanionic nucleic acid, said nucleic acid having exclusively phophodiester linkages, and wherein at least some of said protonatable groups are disposed on the exterior surface of the particles and at least some of these groups have been neutralized.

13. The composition according to claim 12, wherein the nucleic acid is an antisense nucleic acid.

14. The composition of according to claim 12, wherein the first lipid component is an amino lipid.

15. The composition of claim 14, wherein the second lipid component is a polyethylene glycol-modified lipid.

16. The composition of claim 12, wherein the second lipid component is a polyethylene glycol-modified lipid.

17. The composition of claim 12, wherein the first lipid component is an amino lipid, and the second component is PEG-modified or polyamide oligomer-modified lipid, and wherein said lipids are present at molar percents of about 25–45% neutral lipid, about 35–55% cholesterol, about 10–40% amino lipid and about 0.5–15% PEG-modified or Polyamide oligomer-modified lipid.

18. The composition of claim 12, wherein said lipid portion comprises DODAP as the first lipid component, DSPC as the neutral lipid, and PEG-CerC14 as the second lipid component.

19. The composition of claim 18, wherein the lipid components are present in molar percents of about 25–45% DSPC, about 35–55% Chol, about 10–40% DODAP and about 0.5–15% PEG-CerC14.

20. The composition of claim 12, wherein said lipid portion comprises DODAP, POPC, Chol and PEG-CerC14.

21. The composition of claim 12, wherein said lipid comprises of DODAP, SM, Chol and PEG-CerC14.

22. The composition according to claim 12, wherein at least 50% of the nucleic acid in the composition is encapsulated within the particle.

23. The composition of claim 12, wherein at least 90% of the nucleic acid in the composition is encapsulated within the particle.

24. The composition of claim 12, wherein said nucleic acid is a ribozyme.

25. The composition of claim 14, wherein the second lipid component is a polyethylene glycol-modified or polyamide oligomer-modified lipid.

26. The composition of claim 12, wherein the second lipid component is a polyethylene glycol-modified or polyamide oligomer-modified lipid.

27. A method for preparation of a composition comprising lipid-encapsulated therapeutic agent particles, said method comprising the steps of:

(a) preparing a mixture of lipids comprising a first lipid component, a second lipid component, a third lipid component, and a fourth lipid component, and combining the mixture of lipids with a buffered aqueous solution of a charged therapeutic agent to form an intermediate mixture containing lipid-encapsulated therapeutic agent particles having exterior surface charges, wherein said first lipid component is selected from among lipids containing a protonatable or deprotonatable group that has a pKa such that the lipid is in a charged form at a first pH and a neutral form at a second pH, wherein the pKa of the first lipid component is in the range of from 4 to 11, said buffered solution having a pH such that the first lipid component is in its charged form when in the buffered solution, said first lipid component being further selected such that the charged form is cationic when the charged therapeutic agent is anionic in the buffered solution, and anionic when the charged therapeutic agent is cationic in the buffered solution, and said second lipid component being selected from among lipids that prevent particle aggregation during lipid-therapeutic agent particle formation, said third lipid component being a neutral lipid selected from the group consisting of DSPC, POPC, DOPE, and SM, and said fourth lipid component being Chol, and

(b) changing the pH of the intermediate mixture to neutralize at least some of the exterior surface charges on said lipid-encapsulated therapeutic agent particles to provide at least partially-surface neutralized lipid-encapsulated therapeutic agent particles.

28. The method of claim 27, wherein said composition consists essentially of lipid-nucleic acid particles, said particles having a size of from 70 nm to about 200 nm.

29. The method of claim 27, wherein said mixture of lipids in step (a) is a mixture of lipids in alcohol.

30. The method of claim 27, wherein the first lipid component is an amino lipid.

31. The method of claim 27, wherein the second lipid component is a polyethylene glycol-modified or polyamide oligomer-modified lipid.

32. The method of claim 31, wherein the second lipid component is a PEG-Ceramide.

33. The method of claim 31, wherein the first lipid component is an amino lipid.

34. The method of claim 27, wherein said lipid mixture comprises an amino lipid having a pKa of from about 5 to

about 11 as the first lipid component, and a PEG-modified or Polyamide oligomer-modified lipid as the second component.

35. The method of claim **34**, wherein said lipid mixture comprises in molar percents about 25–45% neutral lipid, about 35–55% Chol, about 10–40% amino lipid and about 0.5–15% PEG-Ceramide as the modified lipid.

36. The method of claim **27**, wherein said mixture of lipids comprises DODAP as the first lipid component, DSPC as the neutral lipid, and PEG-CerC14 as the second lipid component.

37. The method of claim **36**, wherein said lipids are present in molar percents of about 25–45% DSPC, about 35–55% Chol, about 10–40% DODAP and about 0.5–15% PEG-CerC14.

38. The method of claim **27**, wherein said mixture of lipids comprises DODAP as the first lipid component, POPC as the neutral lipid, and PEG-CerC14 as the second lipid component.

39. The method of claim **27**, wherein said mixture of lipids comprises DODAP as the first lipid component, SM as the neutral lipid, and PEG-CerC14 as the second lipid component.

40. The method of claim **27**, wherein the pH is changed in step (b) to physiological pH.

41. The method of claim **27**, wherein the step of changing the pH is performed using tangential flow dialysis.

42. A pharmaceutical composition comprising lipid-encapsulated therapeutic agent particles prepared according to claim **27** and a pharmaceutically acceptable carrier.

43. The method of claim **27**, wherein the therapeutic agent is a polyanionic nucleic acid.

44. A pharmaceutical composition comprising lipid-encapsulated therapeutic agent particles comprising a polyanionic nucleic acid as therapeutic agent prepared according to claim **43** and a pharmaceutically acceptable carrier.

45. The pharmaceutical composition according to claim **44**, wherein the polyanionic nucleic acid is an antisense nucleic acid.

46. A method for preparation of a composition comprising lipid encapsulated therapeutic agent particles, said method comprising the steps of:

(a) preparing a mixture of lipids comprising an amino lipid, a neutral lipid, a sterol, and a PEG-modified or polyamide oligomer-modified lipid, and combining the mixture of lipids with a buffered aqueous solution of a charged therapeutic agent to form an intermediate mixture containing lipid-encapsulated therapeutic agent particles having exterior surface charges, wherein the amino lipid is selected from among lipids containing a protonatable or deprotonatable group that has a pKa such that the amino lipid is in a charged form at a first pH and a neutral form at a second pH, and said buffered solution having a pH such that the amino lipid is in its charged form when in the buffered solution, said amino lipid being further selected such that the charged form is cationic, said PEG-modified or polyamide oligomer-modified lipid being selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation, said neutral lipid being selected from the group consisting of DSPC, POPC, DOPE, and SM, and said sterol being Chol, and said therapeutic agent being a polyanionic nucleic acid, and

(b) changing the pH of the intermediate mixture to neutralize at least some of the exterior surface charges on said lipid-encapsulated therapeutic agent particles to provide at least partially-surface neutralized lipid-encapsulated therapeutic agent particles.

47. The method of claim **46**, wherein said composition consists essentially of lipid-nucleic acid particles, said particles having a size of from 70 nm to about 200 nm.

48. The method of claim **46**, wherein said mixture of lipids in step (a) is a mixture of lipids in alcohol.

49. The method of claim **46**, wherein the second lipid component is a PEG-Ceramide.

50. The method of claim **46**, wherein said lipids are present in molar percents of about 25–45% neutral lipid, 35–55% Chol, 10–40% amino lipid and 0.5–15% PEG-modified or polyamide oligomer-modified lipid.

51. The method of claim **46**, wherein said mixture of lipids comprises DODAP as the amino lipid, DSPC as the neutral lipid, and PEG-CerC14 as the PEG-modified lipid.

52. The method of claim **51**, wherein said lipids are present in molar percents of about 25–45% DSPC, about 35–55% Chol, about 10–40% DODAP and about 0.5–15% PEG-CerC14.

53. The method of claim **46**, wherein said mixture of lipids comprises DODAP as the amino lipid, POPC as the neutral lipid, and PEG-CerC14 as the PEG-modified lipid.

54. The method of claim **46**, wherein said mixture of lipids comprises DODAP as the amino lipid, SM as the neutral lipid, and PEG-CerC14 as the PEG-modified lipid.

55. The method of claim **46**, wherein said polyanionic nucleic acid is an antisense nucleic acid.

56. The method of claim **55**, wherein said antisense nucleic acid contains linkages selected from the group consisting of phosphodiester, phosphorothioate, phosphorodithioate, boranophosphate, phosphoroselenate and amidate linkages.

57. The method of claim **46**, wherein said polyanionic nucleic acid contains exclusively phosphodiester linkages.

58. The method of claim **57**, wherein said polyanionic nucleic acid is an antisense nucleic acid.

59. The method of claim **57**, wherein the buffered solution comprises 10 to 50 mM citrate or phosphate buffer.

60. The method of claim **46**, wherein the polyanionic nucleic acid contains at least some phosphorothioate or phosphorodithioate linkages.

61. The method of claim **60**, wherein the buffered solution comprises 10 to 300 mM citrate or phosphate buffer.

62. The method of claim **46**, wherein said polyanionic nucleic acid is a ribozyme.

63. A method for introducing a polyanionic nucleic acid into a cell, comprising contacting a cell with a composition containing the polyanionic nucleic acid for a period of time sufficient to introduce the polyanionic nucleic acid into said cell, wherein the composition comprises lipid-encapsulated therapeutic agent particles containing the polyanionic nucleic acid and said composition is prepared according to the method of claim **46**.

64. A method for the treatment or prevention of a disease characterized by aberrant expression of a gene in a mammalian subject comprising,

preparing a lipid-encapsulated therapeutic agent particle, wherein the therapeutic agent is a polyanionic nucleic acid, according to the method of claim **46**, wherein the polyanionic nucleic acid hybridizes specifically with the aberrantly expressed gene; and

administering a therapeutically effective or prophylactic amount of the particle to the mammalian subject, whereby expression of the aberrantly expressed gene is reduced.

65. The method of claim **64**, wherein the gene is selected from among ICAM-1, c-myc, c-myb, ras, raf, erb-B-2, PKC-alpha, IGF-1R, EGFR, VEGF and VEGF-R-1.

66. The method of claim 64, wherein the disease is a tumor.

67. The method of claim 64, wherein the disease is characterized by inflammation.

68. The method of claim 64, wherein the disease is an infectious disease.

69. The method of claim 64, wherein the therapeutically effective amount of the particle is administered to the mammalian subject by intravenous injection.

70. The method of claim 69, wherein the therapeutically effective amount of the particle is administered to the mammalian subject by intravenous injection at an injection site, and wherein the disease is localized at a disease site distal to the injection site.

71. The method of claim 64, wherein the polyanionic nucleic acid comprises exclusively phosphodiester linkages.

72. A method of preventing expression of a disease-associated gene in a mammalian cell comprising,

preparing a lipid-encapsulated therapeutic agent particle, wherein the therapeutic agent is an antisense polyanionic nucleic acid, according to the method of claim 55; and

exposing the mammalian cell to the lipid-encapsulated therapeutic agent particle for a period of time sufficient for the therapeutic agent to enter the cell;

wherein the therapeutic agent has a sequence complementary to the disease-associated gene and reduces the production of the gene product of the disease-associated gene in the cell.

* * * * *

JOINT APPENDIX 53

US007901708B2

(12) **United States Patent**
MacLachlan et al.

(10) **Patent No.: US 7,901,708 B2**
(45) **Date of Patent: Mar. 8, 2011**

(54) **LIPOSOMAL APPARATUS AND MANUFACTURING METHODS**

(75) Inventors: **Ian MacLachlan**, Vancouver (CA); **Lloyd Brian Jeffs**, Delta (CA); **Lorne R. Palmer**, Vancouver (CA); **Cory Giesbrecht**, Vancouver (CA); **Noelle Giesbrecht**, legal representative, Vancouver (CA)

(73) Assignee: **Protiva Biotherapeutics, Inc.**, Burnaby, B.C. (CA)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1094 days.

(21) Appl. No.: **10/611,274**

(22) Filed: **Jun. 30, 2003**

(65) **Prior Publication Data**

US 2004/0142025 A1 Jul. 22, 2004

Related U.S. Application Data

(60) Provisional application No. 60/392,887, filed on Jun. 28, 2002.

(51) **Int. Cl.**

A61K 9/00	(2006.01)
A61K 9/127	(2006.01)
A61K 31/52	(2006.01)
A61K 31/519	(2006.01)
C07H 19/00	(2006.01)
C07H 19/06	(2006.01)
C07H 19/16	(2006.01)

(52) **U.S. Cl.** **424/450**; 514/263.1; 514/264.1

(58) **Field of Classification Search** 424/417, 424/420, 450, 9.321; 514/263.1, 264.1; 536/22.1, 536/23.1

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,687,661	A		8/1987	Kikuchi et al.
5,478,860	A		12/1995	Wheeler et al.
5,552,157	A	*	9/1996	Yagi et al. 424/450
5,653,996	A		8/1997	Hsu
5,885,613	A		3/1999	Holland et al.
5,976,567	A		11/1999	Wheeler et al.
5,981,501	A		11/1999	Wheeler et al.
6,093,348	A	*	7/2000	Kowalski et al. 252/363.5
6,835,395	B1		12/2004	Semple et al.
6,843,942	B2		1/2005	Katinger et al.
7,341,738	B2	*	3/2008	Semple et al. 424/450
7,641,915	B2		1/2010	Chen et al.
2003/0077829	A1		4/2003	MacLachlan
2003/0124033	A1		7/2003	Baker et al.
2004/0037874	A1		2/2004	Hong et al.
2006/0058249	A1		3/2006	Tong et al.
2008/0200417	A1	*	8/2008	Semple et al. 514/44
2009/0191259	A1	*	7/2009	Li et al. 424/450

FOREIGN PATENT DOCUMENTS

CA	2041075	A1	10/1991
CA	2427640	A1	5/2003
EP	0 055 576	A	7/1982
EP	1 203 614	A	5/2002
EP	1 203 614	A1	5/2002
EP	1 013 268	A	6/2002
IE	911350		4/1991
WO	WO 89/07929	A1	9/1989
WO	WO 91 16039	A	10/1991
WO	WO 91/16039	A1	10/1991
WO	WO 96/40964	A2	12/1996
WO	WO 00 29103	A	5/2000
WO	WO 01 05373	A	1/2001
WO	WO 02 43699	A	6/2002
WO	WO 2004/002453	A1	1/2004

OTHER PUBLICATIONS

Szoka et al. "Comparative Properties and Methods of Preparation of Lipid Vesicles (liposomes)," Ann. Rev. Biophys. Bioeng. 1980, 9, pp. 467-508.*

Batzri et al. "Single Bilayer Liposomes Prepared without Sonication," Biochimica et Biophysica Acts 1973, 298, pp. 1015-1019.*

Zelphati et al."Stable and Monodisperse Lipoplex Formulations for Gene Delivery", Gene Therapy, 1998, 5, pp. 1272-1282.*

Paul et al. "Effective expression of small interfering RNA in human cells," Nature Biotechnology, May 2002, vol. 20, pp. 505-508.*

Maurer et al. "Spontaneous Entrapment of Polynucleotides upon Electrostatic Interaction with Ethanol-Destabilized Cationic Liposomes," Biophysical Journal, May 2001, vol. 80, pp. 2310-2326.*

"Drug Transport in Antimicrobial and Anticancer Chemotherapy." G. Papadakou Ed.. CRC Press (1995).

Wheeler et al. "Stabilized plasmid-lipid particles: construction and characterization," Gene Ther., 1999, vol. 6, No. 2, pp. 271-281.

Semple et al. "Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures," Biochim. Biophys. Acta, 2001, vol. 1510, No. 1-2, pp. 152-166.

MacLachlan; Liposomal Formulations for Nucleic Acid Delivery; Antisense Drug Technologies, Second Edition; 2007; pp. 237-270.

Welter et al. "Simple Mixing Device to Reporducibly Prepare Catonic Lipid-DNA Complexes (Lipoplexes)", *BioTechniques*, Aug. 1999, vol. 27, pp. 286-290. Isele, Ute et al.: "Large-scale production of liposomes containing monomeric zinc phthalocyanine by controlled dilution of organic solvents"; J. Pharmaceutical Sciences, vol. 83, No. 11; pp. 1608-1616 (Nov. 1994).

Wagner et al. "The Crossflow Injection Technique: an Improvement of the Ethanol Injection Method," Journal of Liposome Research, Sep. 2002, vol. 12, No. 3, pp. 259-270.

First Action Inverview Pilot Program Pre-Interview Communication dated Aug. 27, 2010 issued in U.S. Appl. No. 11/495,150, filed Jul. 27, 2006. (4 pages).

* cited by examiner

Primary Examiner — James H Alstrum Acevedo
(74) *Attorney, Agent, or Firm* — Townsend and Townsend and Crew LLP

(57) **ABSTRACT**

The present invention provides apparatus and processes for producing liposomes. By providing a buffer solution in a first reservoir, and a lipid solution in a second reservoir, continuously diluting the lipid solution with the buffer solution in a mixing chamber produces a liposome. The lipid solution preferably comprises an organic solvent, such as a lower alkanol.

22 Claims, 15 Drawing Sheets

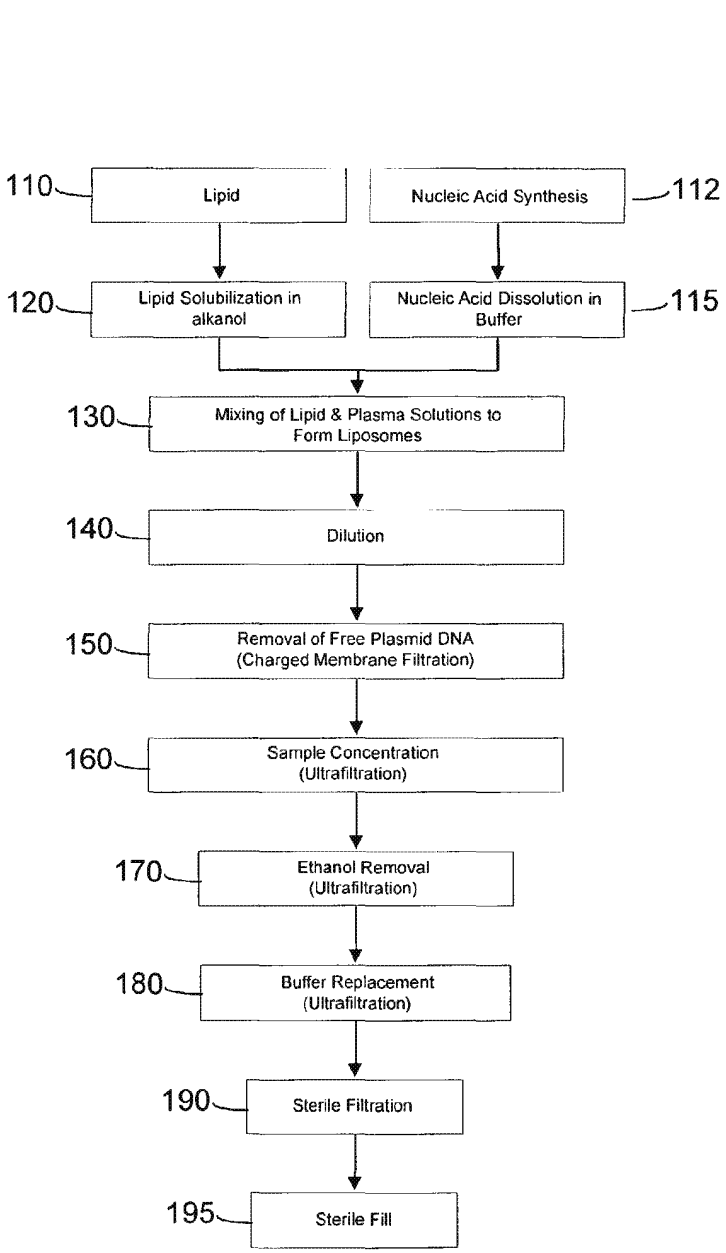

100
110
Lipid
Nucleic Acid Synthesis
112
120
Lipid Solubilization in alkanol
Nucleic Acid Dissolution in Buffer
115
130
Mixing of Lipid & Plasma Solutions to Form Liposomes
140
Dilution
150
Removal of Free Plasmid DNA (Charged Membrane Filtration)
160
Sample Concentration (Ultrafiltration)
170
Ethanol Removal (Ultrafiltration)
180
Buffer Replacement (Ultrafiltration)
190
Sterile Filtration
195
Sterile Fill

FIG. 1

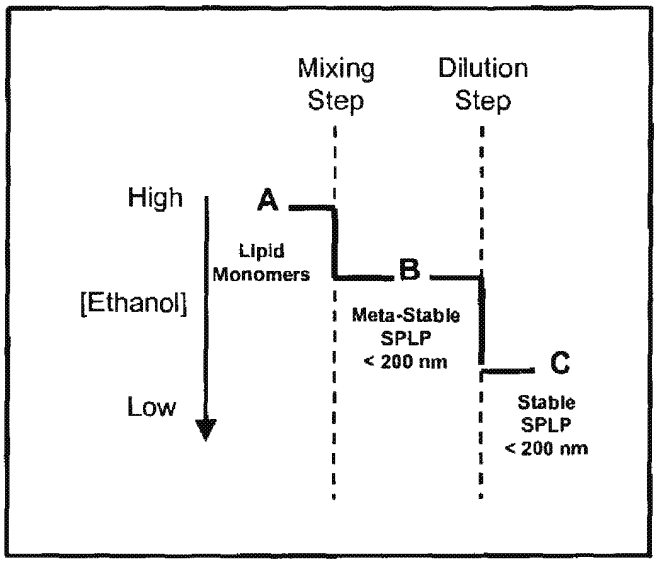

Mixing Step
Dilution Step
High
[Ethanol]
Low
A
Lipid Monomers
B
Meta-Stable SPLP < 200 nm
C
Stable SPLP < 200 nm

FIG. 2

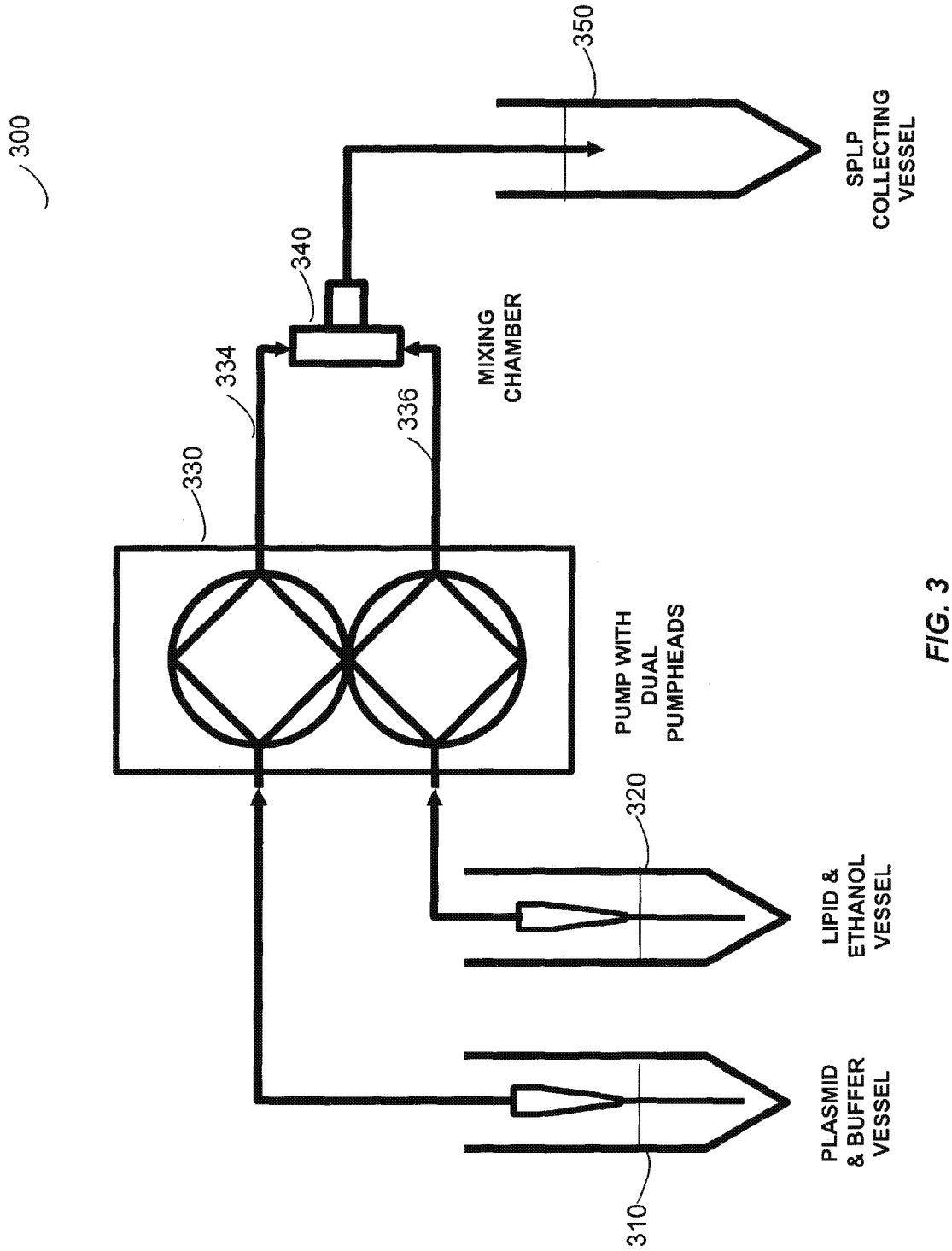

300
350
SPLP COLLECTING VESSEL
340
MIXING CHAMBER
334
336
330
PUMP WITH DUAL PUMPHEADS
320
LIPID & ETHANOL VESSEL
PLASMID & BUFFER VESSEL
310

FIG. 3

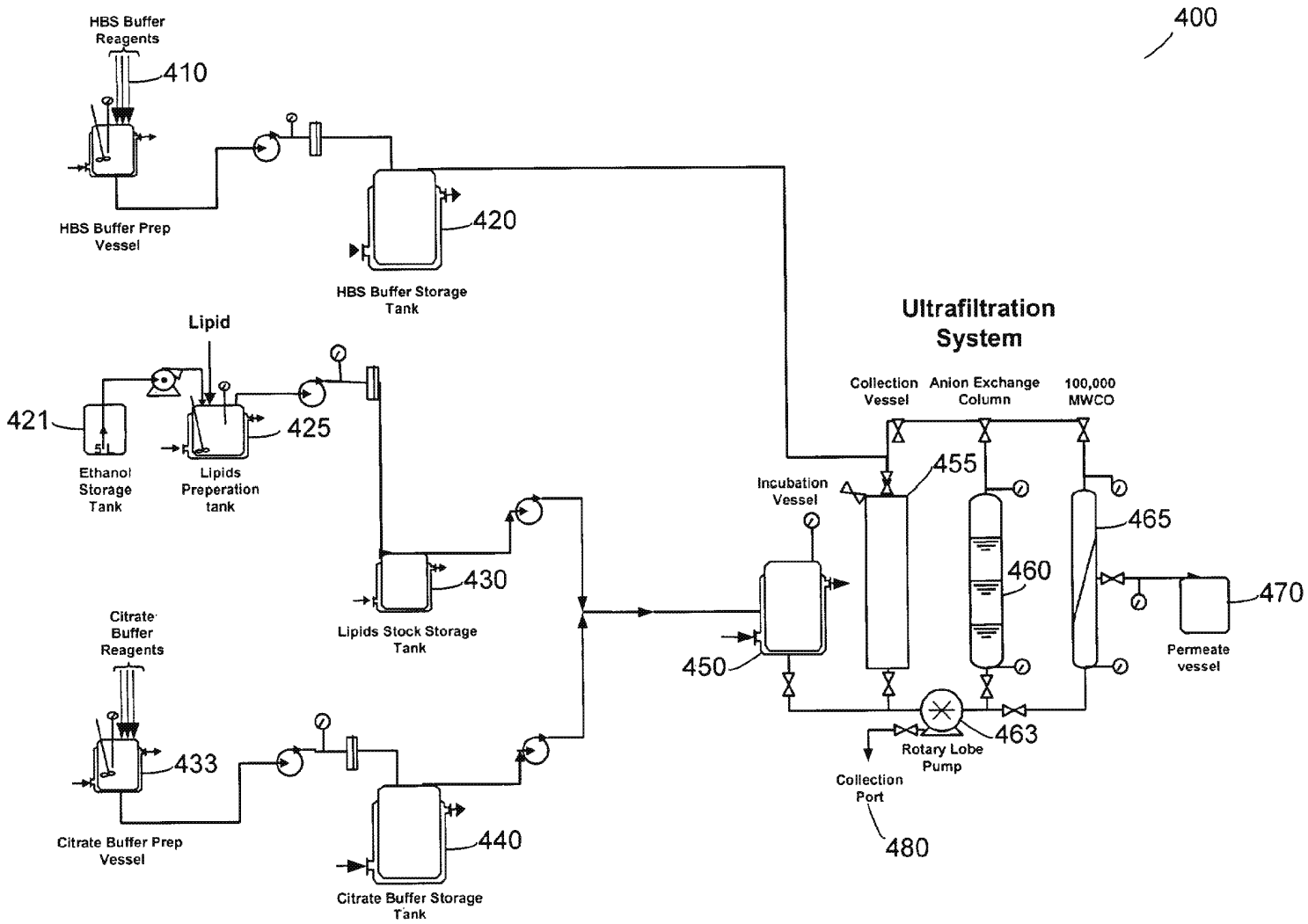

400
HBS Buffer Reagents
410
HBS Buffer Prep Vessel
420
HBS Buffer Storage Tank
Lipid
421
425
Ethanol Storage Tank
Lipids Preperation tank
430
Lipids Stock Storage Tank
Citrate Buffer Reagents
433
Citrate Buffer Prep Vessel
440
Citrate Buffer Storage Tank
Ultrafiltration System
Collection Vessel
Anion Exchange Column
100,000 MWCO
Incubation Vessel
450
455
460
465
470
Permeate vessel
463
Rotary Lobe Pump
Collection Port
480

FIG. 4

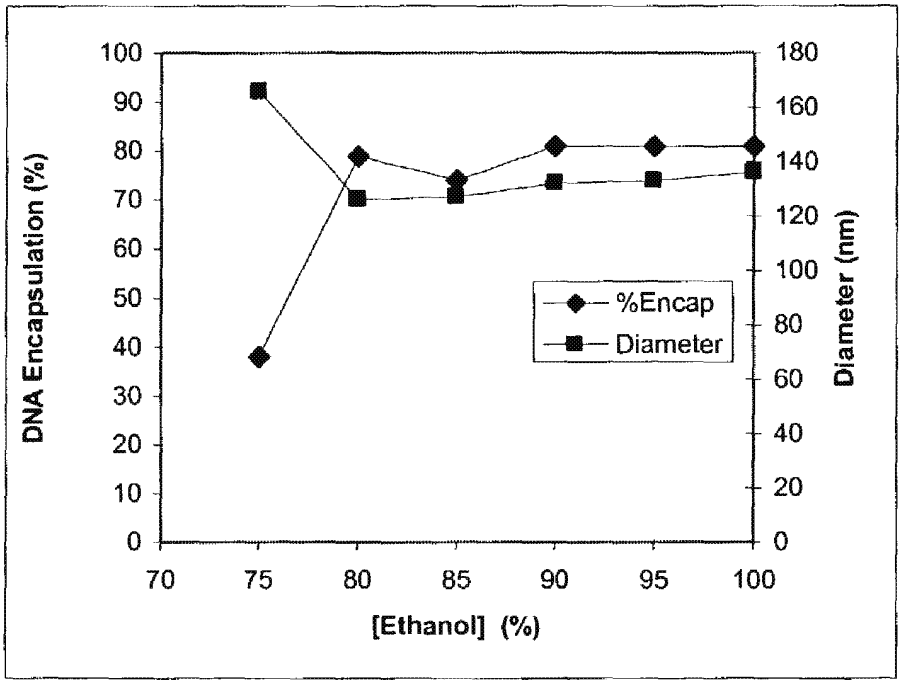

DNA Encapsulation (%)
Diameter (nm)
[Ethanol] (%)
%Encap
Diameter
100
90
80
70
60
50
40
30
20
10
0
180
160
140
120
100
80
60
40
20
0
70
75
80
85
90
95
100

FIG. 5

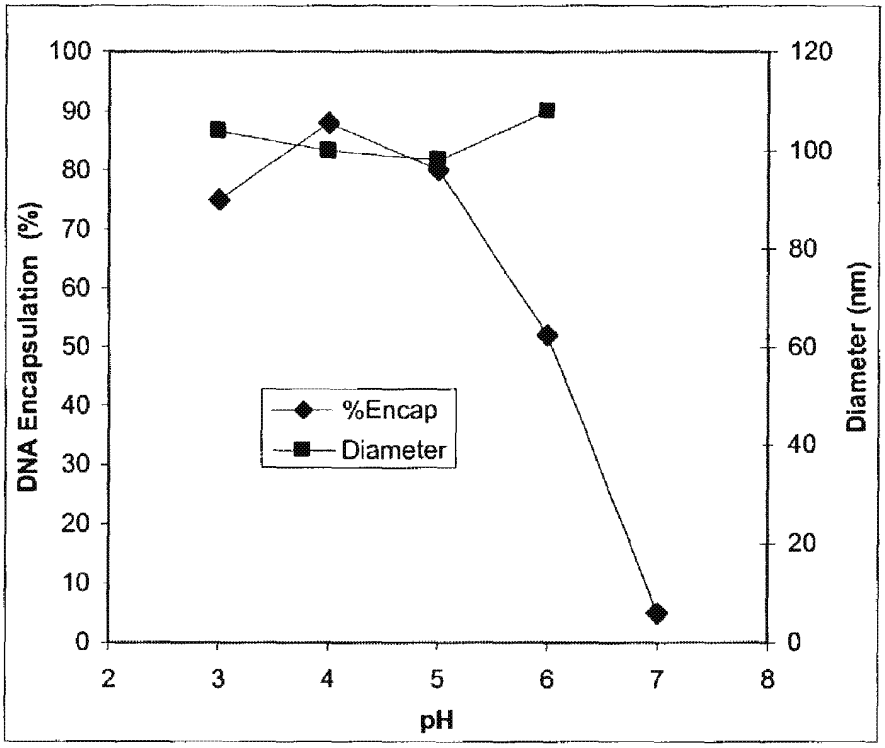

DNA Encapsulation (%)
Diameter (nm)
pH
%Encap
Diameter
100
90
80
70
60
50
40
30
20
10
0
120
100
80
60
40
20
0
2
3
4
5
6
7
8

FIG. 6

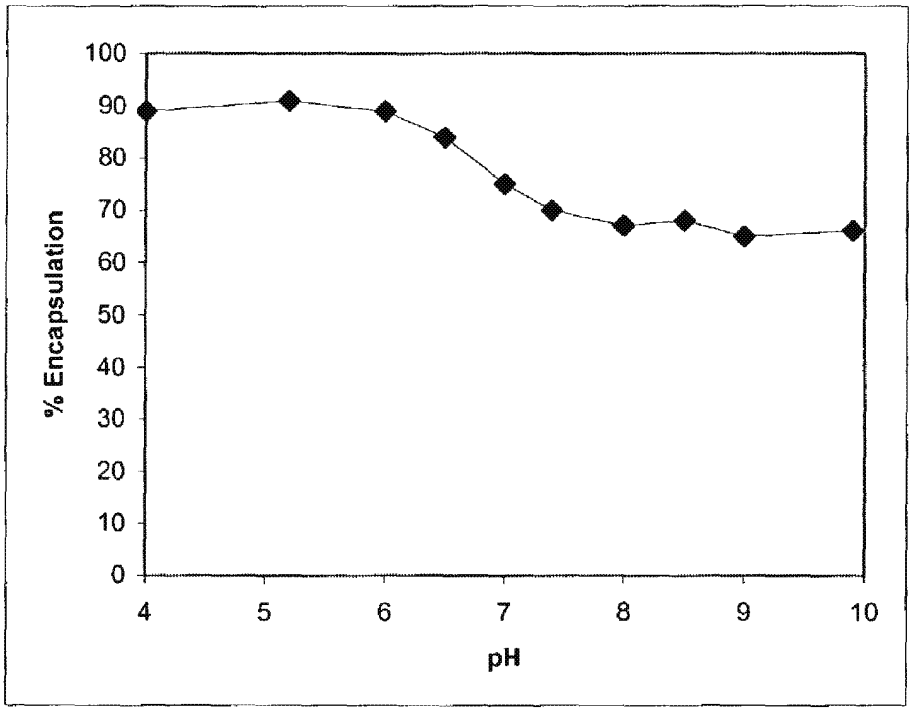


FIG. 7

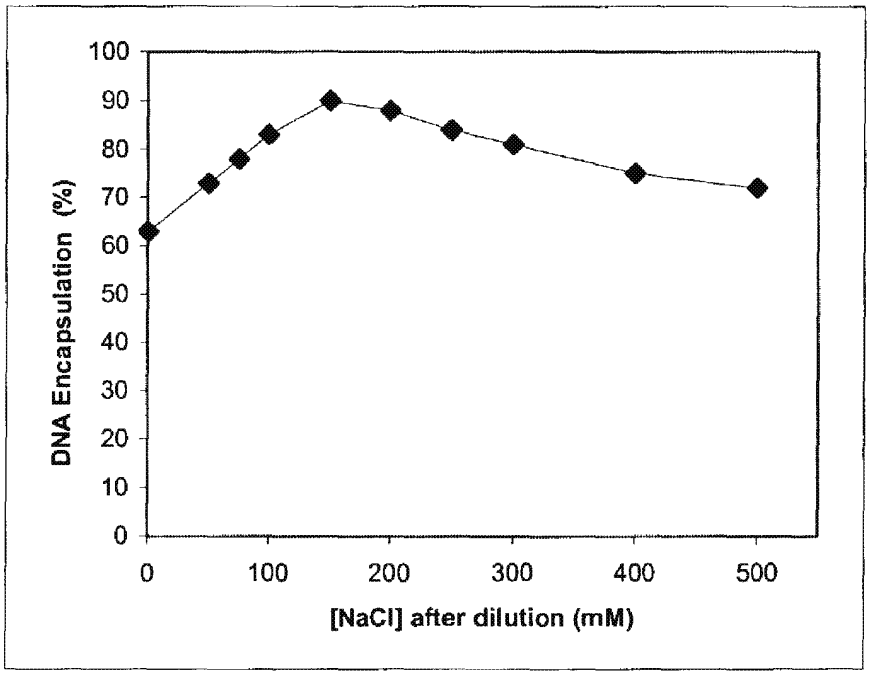

DNA Encapsulation (%)
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
[NaCl] after dilution (mM)

FIG. 8

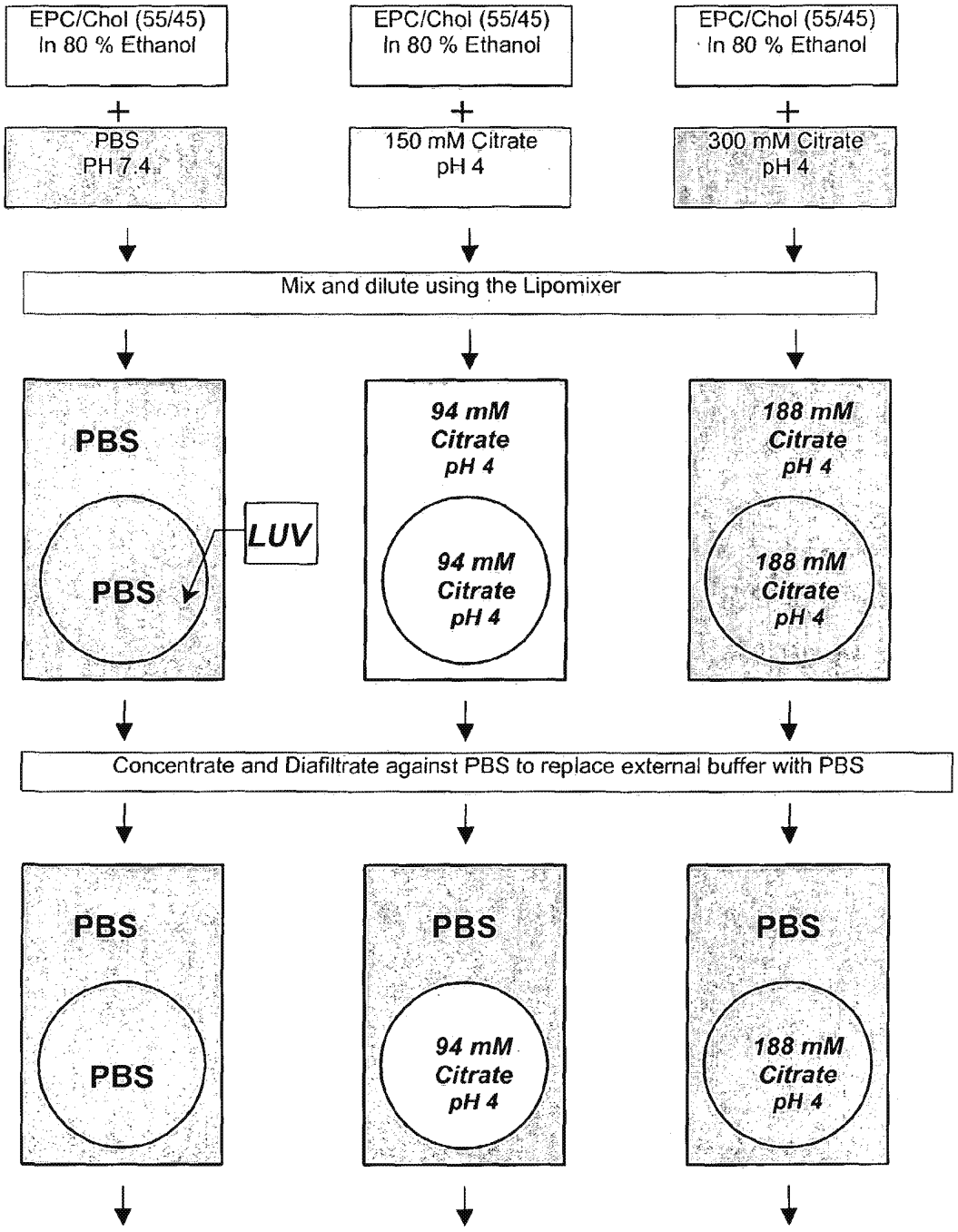

EPC/Chol (55/45) In 80 % Ethanol
EPC/Chol (55/45) In 80 % Ethanol
EPC/Chol (55/45) In 80 % Ethanol
+
PBS PH 7.4
+
150 mM Citrate pH 4
+
300 mM Citrate pH 4
Mix and dilute using the Lipomixer
PBS
PBS
LUV
94 mM Citrate pH 4
94 mM Citrate pH 4
188 mM Citrate pH 4
188 mM Citrate pH 4
Concentrate and Diafiltrate against PBS to replace external buffer with PBS
PBS
PBS
PBS
94 mM Citrate pH 4
PBS
188 mM Citrate pH 4

FIG. 9A

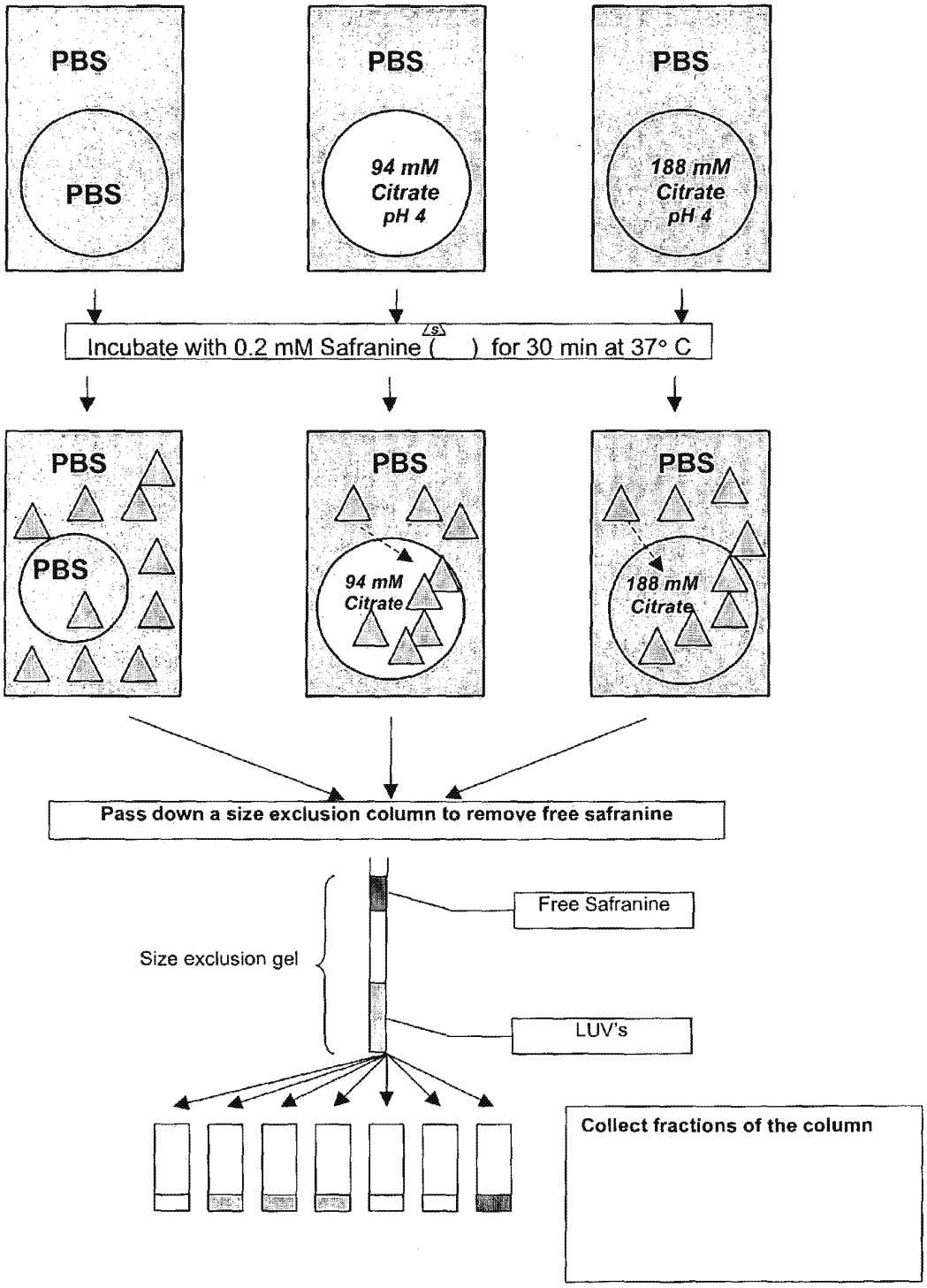

PBS
PBS
PBS
94 mM Citrate pH 4
PBS
188 mM Citrate pH 4
Incubate with 0.2 mM Safranine ( ) for 30 min at 37° C
PBS
PBS
PBS
94 mM Citrate
PBS
188 mM Citrate
Pass down a size exclusion column to remove free safranine
Size exclusion gel
Free Safranine
LUV's
Collect fractions of the column

FIG. 9B

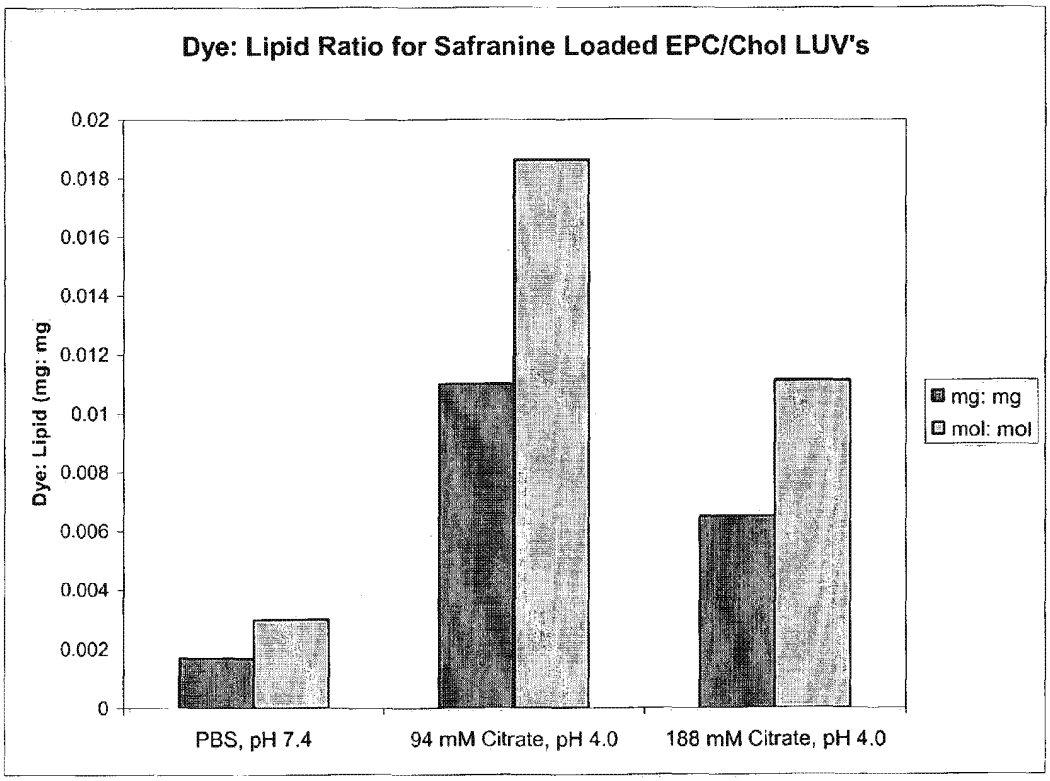
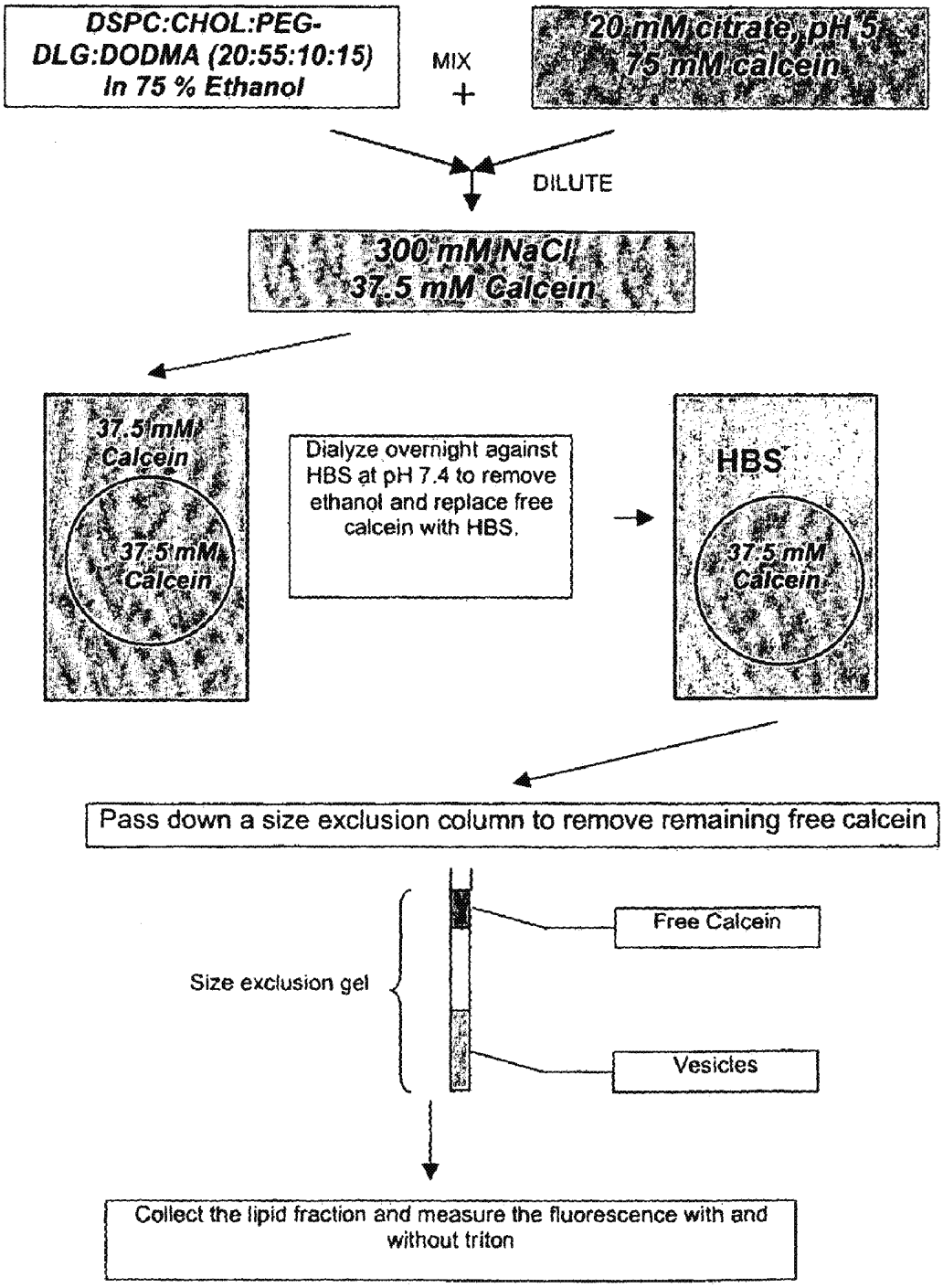

Dye: Lipid Ratio for Safranine Loaded EPC/Chol LUV's
Dye: Lipid (mg: mg
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
PBS, pH 7.4
94 mM Citrate, pH 4.0
188 mM Citrate, pH 4.0
mg: mg
mol: mol

FIG. 10



rF before Triton = 0.4 & rF after Triton = 4.0

Therefore, calcein is: (1) encapsulated within the vesicle
(2) self quenching at the current concentration

FIG. 11

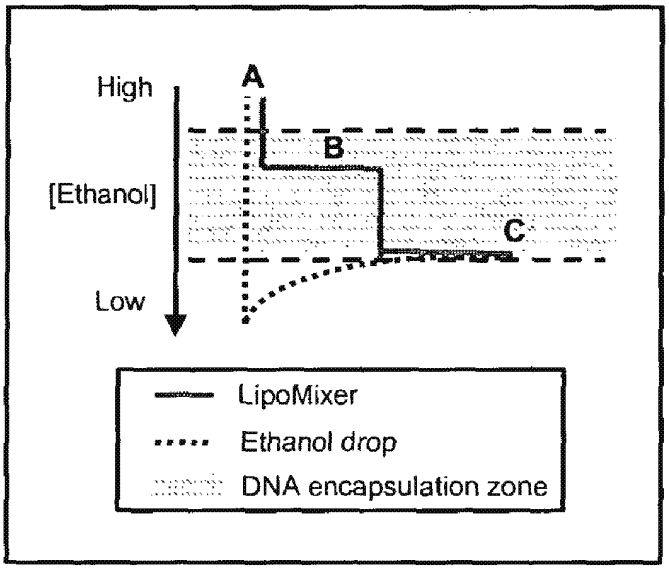

High
A
B
[Ethanol]
C
Low
LipoMixer
Ethanol drop
DNA encapsulation zone

FIG. 12

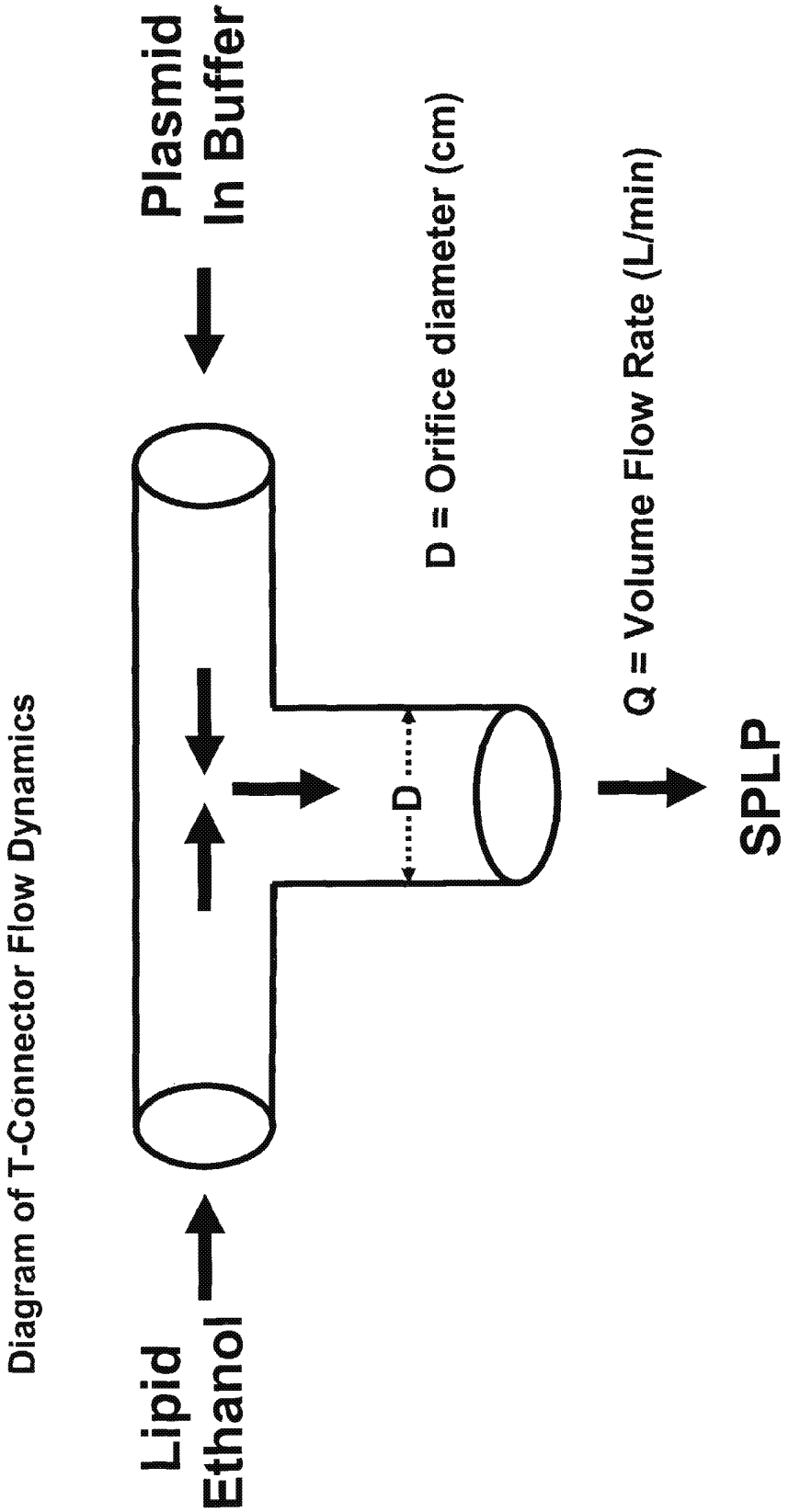


Diagram of T-Connector Flow Dynamics
Lipid
Ethanol
Plasmid
In Buffer
D = Orifice diameter (cm)
D
Q = Volume Flow Rate (L/min)
SPLP

FIGURE 13

Conditions and Properties for SPLP and Liposome Formation

Note: all samples prepared at 37°C

Sample Description	Q Flow rate (L/min)	D Orifice ID (cm)	Vesicle Size (nm)	Size Std Dev (nm)	$\chi 2$	Linear Velocity (m/s)	Shear Rate (s^{-1})	Reynolds number
SPLP Chol:DSPC:DODMA:PEG-DSG (55:20:15:10 mol ratio)	0.078	0.16	108	36	0.6	0.65	3233	496
SPLP Chol:DSPC:DODMA:PEG-DSG (55:20:15:10 mol ratio)	0.275	0.32	112	43	0.5	0.57	1430	878
Liposomes Chol:DSPC:DODMA:PEG-DSG (55:20:15:10 mol ratio)	0.1	0.32	127	43	1.8	0.21	518	318
Liposomes Chol:DSPC:DODMA:PEG-DSG (55:20:15:10 mol ratio)	0.4	0.32	112	14	0.3	0.83	2072	1272
Liposomes EPC:CHOL (55:45 mol ratio)	0.275	0.32	125	N/d	n/d	0.57	1430	887
Liposomes EPC:CHOL (55:45 mol ratio)	0.078	0.16	90	33	2.7	0.65	3233	503
PEI-SPLP Chol:DSPC:POPG:PEG-DSG (50:20:20:10 mol ratio)	0.078	0.16	108	N/d	N/d	0.65	3233	503

N/dN/d

N/d Not Determined.

FIGURE 14

LIPOSOMAL APPARATUS AND MANUFACTURING METHODS

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is a non-provisional application of, and claims priority to, U.S. Provisional Application Ser. No. 60/392,887, filed Jun. 28, 2002, which is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Many systems for administering active substances into cells are already known, such as liposomes, nanoparticles, polymer particles, immuno- and ligand-complexes and cyclodextrins (see, Drug Transport in antimicrobial and anticancer chemotherapy. G. Papadakou Ed., CRC Press, 1995). Liposomes are typically prepared in the laboratory by sonication, detergent dialysis, ethanol injection or dilution, French press extrusion, ether infusion, and reverse phase evaporation. Liposomes with multiple bilayers are known as multilamellar lipid vesicles (MLVs). MLVs are candidates for time release drugs because the fluids entrapped between layers are only released as each membrane degrades. Liposomes with a single bilayer are known as unilamellar lipid vesicles (UV). UVs may be made small (SUVs) or large (LUVs).

Some of the methods above for liposome production impose harsh or extreme conditions which can result in the denaturation of the phospholipid raw material and encapsulated drugs. In addition, these methods are not readily scalable for mass production of large volumes of liposomes. Further, lipid vesicle formation by conventional ethanol dilution, involves the injection or dropwise addition of lipid in an aqueous buffer. The resulting vesicles are typically heterogenous in size and contain a mixture of unilamellar and multilamellar vesicles.

Conventional liposomes are formulated to carry therapeutic agents either contained within the aqueous interior space (water-soluble drugs) or partitioned into the lipid bilayer(s) (water-insoluble drugs). Active agents which have short half-lives in the bloodstream are particularly suited to delivery via liposomes. Many anti-neoplastic agents, for example, are known to have a short half-life in the bloodstream such that their parenteral use is not feasible. However, the use of liposomes for site-specific delivery of active agents via the bloodstream is severely limited by the rapid clearance of liposomes from the blood by cells of the reticuloendothelial system (RES).

U.S. Pat. No. 5,478,860, which issued to Wheeler et al., on Dec. 26, 1995, and which is incorporated herein by reference, discloses microemulsion compositions for the delivery of hydrophobic compounds. Such compositions have a variety of uses. In one embodiment, the hydrophobic compounds are therapeutic agents including drugs. The patent also discloses methods for in vitro and in vivo delivery of hydrophobic compounds to cells.

PCT Publication WO01/05373 to Knopov, et al., which is incorporated by reference herein, discloses techniques for preparing lipid vesicles using an ethanol injection-type process with a static mixer that provides a turbulent environment (e.g., Reynolds numbers>2000). Therapeutic agents may then be loaded after vesicle formation

Despite the apparent advances of U.S. Pat. No. 5,478,860 and WO05373, there exists a need for processes and apparatus for formulating and producing lipid vesicles, and in particular lipid vesicles encapsulating a therapeutic agent such as nucleic acid. The present invention fulfills these and other needs.

BRIEF SUMMARY OF THE INVENTION

The present invention provides processes and apparatus for making lipid vesicles that optionally contain a therapeutic agent. The therapeutic agent can include, for example, a protein, a nucleic acid, an antisense nucleic acid, a drug, or the like. The present invention can be used to form lipid vesicles that contain encapsulated plasmid DNA or small molecule drugs. In one aspect, the lipid vesicles are prepared rapidly at low pressure and the approach is fully scalable. In certain preferred embodiments, the process does not involve a static mixer or specialized extrusion equipment.

As such, in one embodiment, the present invention provides a process for producing a liposome. The process typically includes providing an aqueous solution in a first reservoir, the first reservoir in fluid communication with an organic lipid solution in a second reservoir, and mixing the aqueous solution with the organic lipid solution, wherein the organic lipid solution undergoes a continuous stepwise dilution to produce a liposome.

In certain aspects, the aqueous solution such as a buffer, comprises a therapeutic product, such that the therapeutic product is encapsulated in the liposome. Suitable therapeutic products include, but are not limited to, a protein, a nucleic acid, an antisense nucleic acid, a ribozyme, tRNA, snRNA, siRNA (small interfering RNA), pre-condensed DNA, and an antigen. In certain preferred aspects, the therapeutic product is nucleic acid.

In another embodiment, the present invention provides a process for producing a liposome encapsulating a therapeutic product. The process typically includes providing an aqueous solution in a first reservoir, and providing an organic lipid solution in a second reservoir, wherein one of the aqueous solution and the organic lipid solution includes a therapeutic product. The process also typically includes mixing the aqueous solution with the organic lipid solution, wherein the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a liposome encapsulating the therapeutic product. In certain aspects, the therapeutic product is a nucleic acid included in the aqueous solution. In certain aspects, the therapeutic product is lipophilic and is included in the organic lipid solution. In certain aspects, the initial therapeutic product encapsulation efficiency is as high as about 90%.

In still yet another embodiment, the present invention provides apparatus for producing a liposome encapsulating a therapeutic product. The apparatus typically includes a first reservoir for holding an aqueous solution, and a second reservoir for holding an organic lipid solution, wherein one of the aqueous solution and the organic lipid solution includes a therapeutic product. the apparatus also typically includes a pump mechanism configured to pump the aqueous and the organic lipid solutions into a mixing region at substantially equal flow rates. In operation, the organic lipid solution mixes with the aqueous solution in the mixing region to substantially instantaneously form a therapeutic product encapsulated liposome.

These and other aspects will be more apparent when read with the accompanying drawings and detailed descriptions that follow.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** provides a flow diagram for a manufacturing process according to one embodiment of the present invention.

FIG. **2** provides a schematic of a process of making liposomes in one embodiment of the present invention.

FIG. **3** provides a schematic of an apparatus according to one embodiment of the present invention.

FIG. **4** provides a schematic of an apparatus having an ultrafiltration system according to one embodiment of the present invention.

FIG. **5** shows the effect of varying the ethanol concentration of the initial lipid solution on SPLP mean diameter and DNA encapsulation. DNA encapsulation efficiency and vesicle sizes determined after the dilution step.

FIG. **6** shows the effect of varying pH of the initial plasmid solution on SPLP mean diameter and DNA encapsulation. DNA encapsulation efficiency and vesicle sizes were determined after the dilution step.

FIG. **7** shows the effect of varying pH of the buffer used for the dilution step on pDNA encapsulation efficiency.

FIG. **8** shows the effect of varying the salt concentration of the buffer used for the dilution step on pDNA encapsulation efficiency.

FIG. **9**A-B shows a schematic process of making liposomes of the present invention.

FIG. **10** shows encapsulation of safranine in certain liposomes of the present invention.

FIG. **11** shows a schematic process of making liposomes of the present invention.

FIG. **12** illustrates a comparison between one embodiment of the present invention and an ethanol drop method for encapsulating pDNA.

FIG. **13** shows a T-connector and associated flow dynamics according to one embodiment.

FIG. **14** shows various parameters associated with flow in the T-connector of FIG. **13**.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

I. Definitions

The term "nucleic acid" refers to a polymer containing at least two nucleotides. "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

DNA may be in the form of antisense, plasmid DNA, parts of a plasmid DNA, pre-condensed DNA, product of a polymerase chain reaction (PCR), vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives of these groups. RNA may be in the form of oligonucleotide RNA, tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), antisense RNA, siRNA (small interfering RNA), ribozymes, chimeric sequences, or derivatives of these groups.

"Antisense" is a polynucleotide that interferes with the function of DNA and/or RNA. This may result in suppression of expression. Natural nucleic acids have a phosphate backbone, artificial nucleic acids may contain other types of backbones and bases. These include PNAs (peptide nucleic acids), phosphothionates, and other variants of the phosphate backbone of native nucleic acids. In addition, DNA and RNA may be single, double, triple, or quadruple stranded.

The term "gene" refers to a nucleic acid (e.g., DNA) sequence that comprises coding sequences necessary for the production of a polypeptide or precursor (e.g., herpes simplex virus). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (e.g., enzymatic activity, ligand binding, signal transduction, and the like) of the full-length or fragment are retained.

As used herein, the term "aqueous solution" refers to a composition comprising in whole, or in part, water.

As used herein, the term "organic lipid solution" refers to a composition comprising in whole, or in part, an organic solvent having a lipid.

The term "lipid" refers to a group of organic compounds that are esters of fatty acids and are characterized by being insoluble in water but soluble in many organic solvents. They are usually divided in at least three classes: (1) "simple lipids" which include fats and oils as well as waxes; (2) "compound lipids" which include phospholipids and glycolipids; (3) "derived lipids" such as steroids.

The term "amphipathic lipid" refers, in part, to any suitable material wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while a hydrophilic portion orients toward the aqueous phase. Amphipathic lipids are usually the major component of a lipid vesicle. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxy and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids and sphingolipids. Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine or dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid, glycosphingolipid families, diacylglycerols and β-acyloxyacids, are also within the group designated as amphipathic lipids. Additionally, the amphipathic lipid described above can be mixed with other lipids including triglycerides and sterols.

The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerol, cardiolipin, diacylphosphatidylserine, diacylphosphatidic acid, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, and other anionic modifying groups joined to neutral lipids.

The term "cationic lipid" refers to any of a number of lipid species which carry a net positive charge at a selective pH, such as physiological pH. Such lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTMA"); N,N-distearyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleoyloxy) propyl)-N,N,N-trimethylammonium chloride ("DOTAP"); 3-(N-(N',N'-dimethylaminoethane)carbamoyl)cholesterol ("DC-Chol") and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dim-

ethyl-N-hydroxyethyl ammonium bromide ("DMRIE"). Additionally, a number of commercial preparations of cationic lipids are available which can be used in the present invention. These include, for example, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and 1,2-dioleoyl-sn-3-phosphoethanolamine ("DOPE"), from GIBCO/BRL, Grand Island, N.Y., USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising N-(1-(2,3-dioleyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate ("DOSPA") and ("DOPE"), from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic lipids comprising dioctadecylamidoglycyl carboxyspermine ("DOGS") in ethanol from Promega Corp., Madison, Wis., USA). The following lipids are cationic and have a positive charge at below physiological pH: DODAP, DODMA, DMDMA and the like.

"Lipid vesicle" refers to any lipid composition that can be used to deliver a compound including, but not limited to, liposomes, wherein an aqueous volume is encapsulated by an amphipathic lipid bilayer; or wherein the lipids coat an interior comprising a large molecular component, such as a plasmid, with a reduced aqueous interior; or lipid aggregates or micelles, wherein the encapsulated component is contained within a relatively disordered lipid mixture.

As used herein, "lipid encapsulated" can refer to a lipid formulation which provides a compound with full encapsulation, partial encapsulation, or both.

As used herein, the term "SPLP" refers to a stable plasmid lipid particle. A SPLP represents a vesicle of lipids coating an interior comprising a nucleic acid such as a plasmid with a reduced aqueous interior.

II. General

The present invention provides processes and apparatus for making lipid vesicles. The processes can be used to make lipid vesicles possessing a wide range of lipid components including, but not limited to, cationic lipids, anionic lipids, neutral lipids, polyethylene glycol (PEG) lipids, hydrophilic polymer lipids, fusogenic lipids and sterols. Hydrophobic actives can be incorporated into the organic solvent (e.g., ethanol) with the lipid, and nucleic acid and hydrophilic actives can be added to an aqueous component. In certain aspects, the processes of the present invention can be used in preparing microemulsions where a lipid monolayer surrounds an oil-based core. In certain preferred aspects, the processes and apparatus are used in preparing lipid vesicles, or liposomes, wherein a therapeutic agent is encapsulated within a liposome coincident with liposome formation.

III. Processes of Making

FIG. **1** is an example of a representative flow chart **100** of a method of the present invention. This flow chart is merely an illustration and should not limit the scope of the claims herein. One of ordinary skill in the art will recognize other variations, modifications, and alternatives.

In one aspect, the present method provides a lipid solution **110** such as a clinical grade lipid synthesized under Good Manufacturing Practice (GMP), which is thereafter solubilized in an organic solution **120** (e.g., ethanol). Similarly, a therapeutic product, e.g., a therapeutic active agent such as nucleic acid **112** or other agent, is prepared under GMP. Thereafter, a therapeutic agent solution (e.g., plasmid DNA) **115** containing a buffer (e.g., citrate) is mixed with a lipid solution **120** solubilized in a lower alkanol to form a liposomal formulation **130**. In preferred aspects of the present invention, the therapeutic agent is "passively entrapped" in the liposome substantially coincident with formation of the liposome. However, those of skill in the art will realize that the processes and apparatus of the present invention are equally applicable to active entrapment or loading of the liposomes after formation of the vesicle.

According to the processes and apparatus of the present invention, the action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a liposome substantially instantaneously upon mixing. As used herein, the phrase "continuously diluting a lipid solution with a buffer solution" (and variations) generally means that the lipid solution is diluted sufficiently rapidly in an hydration process with sufficient force to effectuate vesicle generation. By mixing the aqueous solution with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution in the presence of the buffer (aqueous) solution to produce a liposome.

In the processes of the present invention, the organic lipid solution preferably includes an organic solvent, such as a lower alkanol. In one aspect, the liposomes are then diluted **140** with a buffer (e.g., citrate) to increase nucleic acid (e.g., plasmid) entrapment. Before sample concentration **160**, free therapeutic agent (e.g., nucleic acid) is removed by using, for example, an anion exchange cartridge **150**. Further, by using an ultrafiltration step **170** to remove the alkanol, the sample is concentrated (e.g., to about 0.9 mg/mL plasmid DNA), the alkanol is removed, and the buffer is replaced with a substitute buffer (e.g., with a saline buffer) **180**. Thereafter, the sample is filtered **190** and filled in vials **195**. The process will now be discussed in more detail herein below using the steps as set forth in FIG. **1**.

1. Lipid Solubilization and Therapeutic Agent Dissolution

In one embodiment, the liposome vesicles of the present processes are stable plasmid lipid particle (i.e., SPLP) formulations. Those of skill in the art will appreciate that the following description is for illustration purposes only. The processes of the present invention are applicable to a wide range of lipid vesicle types and sizes. These lipid vesicles include, but are not limited to, single bilayer lipid vesicles known as unilamellar lipid vesicles which can be made small (SUVs) or large (LUVs), as well as multilamellar lipid vesicles (MLVs). Further vesicles include, micelles, lipid-nucleic acid particles, virosomes, and the like. Those of skill in the art will know of other lipid vesicles for which the processes and apparatus of the present invention will be suitable.

The preferred size for liposomes made in accordance with the present processes and apparatus are between about 50-550 nm in diameter. In certain preferred aspects, the liposome preparation has a size distribution in which the mean size (e.g., diameter) is about 70 nm to about 300 nm, and more preferably the mean size is less than about 200 nm, such as about 150 nm or less (e.g., about 100 nm).

In certain aspects, the liposome formulation (e.g., SPLP formulation) of the present invention includes four lipid components: a phospholipid; cholesterol; a PEG-lipid; and a cationic lipid. In one preferred aspect, the phospholipid is DSPC, the PEG-lipid is PEG-DSG and the cationic lipid is DODMA. In one preferred aspect, the molar composition is about 20:45:10:25 DSPC:Chol:PEG-DSG:DODMA. In certain embodiments, the organic solvent concentration wherein the lipids are solubilized is about 45% v/v to about 90% v/v. In certain preferred aspects, the organic solvent is a lower alkanol. Suitable lower alkanols include, but are not limited to, metha-

nol, ethanol, propanol, butanol, pentanol, their isomers and combinations thereof. In one embodiment, the solvent is preferably ethanol with a volume of about 50-90% v/v. Preferably, the lipids occupy a volume of about 1 mL/g to about 5 mL/g.

The lipids are solubilized **120** using for example, an overhead stirrer at a suitable temperature. In one aspect, the total lipid concentration of the solution is about 15.1 mg/mL (20 mM). In certain preferred aspects, the therapeutic agent (e.g., nucleic acid) is included in an aqueous solution (e.g., buffer) and is diluted to a final concentration. In one preferred aspect, for example, the final concentration is about 0.9 mg/mL in citrate buffer, with a pH of about 4.0. In this instance, the volume of the plasmid solution is the same as the alkanol-lipid solution. In one embodiment, the preparation of the therapeutic agent (e.g., nucleic acid) solution is performed in a jacketed stainless steel vessel with an overhead mixer. The sample does not need to be heated to be prepared, although in certain instances it is at the same temperature as the lipid solution prior to lipid vesicle formation.

In one embodiment, the therapeutic agent is included in the lipid solution. In certain preferred aspects, the therapeutic agent in the lipid solution is lipophilic. Suitable lipophilic agents include taxol, taxol derivatives, including, for example, protax III and paclitaxol, lipophilic benzoporphyrins, verteporfin the lipid prodrug of foscarnet, 1-O-octadecyl-sn-glycerol-3-phosphonoformate (ODG-PFA), dioleoyl [3H]iododeoxyuridine ([3H]IDU-O12), lipid derivatized HIV protease inhibitory peptides such as iBOC-[L-Phe]-[D-beta-Na1]-Pip-[alpha-(OH)-Leu]-Val (7194) and other lipid derivatized drugs or prodrugs.

2. Liposome Formation

After the solutions, e.g., lipid solution **120** and aqueous therapeutic agent (e.g., nucleic acid) solution **115**, have been prepared, they are mixed together **130** using, for example, a peristaltic pump mixer. In one aspect, the solutions are pumped at substantially equal flow rates into a mixing environment. In certain aspects, the mixing environment includes a "T"-connector or mixing chamber. In this instance, it is preferred that the fluid lines, and hence fluid flows, meet in a narrow aperture within the "T"-connector as opposing flows at approximately 180° relative to each other. Other relative introduction angles may be used, such as for example between 27° and 90° and between 90° and 180°. Upon meeting and mixing of the solution flows in the mixing environment, lipid vesicles are substantially instantaneously formed. Lipid vesicles are formed when an organic solution including dissolved lipid and an aqueous solution (e.g., buffer) are simultaneously and continuously mixed. Advantageously, and surprisingly, by mixing the aqueous solution with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution to substantially instantaneously produce a liposome. The pump mechanism can be configured to provide equivalent or different flow rates of the lipid and aqueous solutions into the mixing environment which creates lipid vesicles in a high alkanol environment.

Advantageously, and surprisingly, the processes and apparatus for mixing of the lipid solution and the aqueous solution as taught herein provides for encapsulation of therapeutic agent in the formed liposome substantially coincident with liposome formation with an encapsulation efficiency of up to about 90%. Further processing steps as discussed herein can be used to further refine the encapsulation efficiency and concentration if desired.

In one preferred aspect, using the processes and apparatus of the present invention, it is possible to form lipid vesicles instantaneously in a continuous two-step process that is fully scaleable. In one aspect, lipid vesicles are formed having a mean diameter of less than about 200 nm, which do not require further size reduction by high-energy processes such as membrane extrusion, sonication or microfluidization.

In one embodiment, lipid vesicles form when lipids dissolved in an organic solvent (e.g., ethanol) are diluted in a stepwise manner by mixing with an aqueous solution (e.g., buffer). This controlled stepwise dilution is achieved by mixing the aqueous and lipid streams together in an aperture, such as a T-connector. The resultant lipid, solvent and solute concentrations can be kept constant throughout the vesicle formation process.

One embodiment of the inventive process is shown in FIG. **2**. In one aspect, using the processes of the present invention, a vesicle is prepared by a two-stage step-wise dilution without gradients. For example, in the first stepwise dilution, vesicles are formed in a high alkanol (e.g., ethanol) environment (e.g., about 30% to about 50% v/v ethanol). These vesicles can then be stabilized by lowering the alkanol (e.g., ethanol) concentration to less than or equal to about 25% v/v, such as about 17% v/v to about 25% v/v, in a stepwise manner. In preferred aspects, with therapeutic agent present in the aqueous solution, or in the lipid solution, the therapeutic agent is encapsulated coincident with liposome formation.

As shown in FIG. **2**, in one embodiment, lipids are initially dissolved in an alkanol environment of about 40% v/v to about 90% v/v, more preferably about 65% v/v to about 90% v/v, and most preferably about 80% v/v to about 90% v/v (A). Next, the lipid solution is diluted stepwise by mixing with an aqueous solution resulting in the formation of vesicles at an alkanol (e.g., ethanol) concentration of between about 37.5-50% (B). By mixing the aqueous solution with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution to produce a liposome. Further, lipid vesicles such as SPLPs (a lipid-particle) can be further stabilized by an additional stepwise dilution of the vesicles to an alkanol concentration of less than or equal to about 25%, preferably between about 19-25% (C).

In certain aspects, for both stepwise dilutions (A→B and B→C), the resulting ethanol, lipid and solute concentrations are kept at constant levels in the receiving vessel. At these higher ethanol concentrations following the initial mixing step, the rearrangement of lipid monomers into bilayers proceeds in a more orderly fashion compared to vesicles that are formed by dilution at lower ethanol concentrations. Without being bound by any particular theory, it is believed that these higher ethanol concentrations promote the association of nucleic acid with cationic lipids in the bilayers. In one preferred aspect, nucleic acid encapsulation occurs within a range of alkanol (e.g., ethanol) concentrations above 22%.

In certain aspects, after the lipid vesicles are formed, they are collected in another vessel, for example, a stainless steel vessel. In one aspect, the lipid vesicles are formed at a rate of about 60 to about 80 mL/min. In one aspect, after the mixing step **130**, the lipid concentration is about 1-10 mg/mL and the therapeutic agent (e.g., plasmid DNA) concentration is about 0.1-3 mg/mL. In certain preferred aspects, the lipid concentration is about 7.0 mg/mL and the therapeutic agent (e.g., plasmid DNA) concentration is about 0.4 mg/mL to give a DNA:lipid ratio of about 0.06 mg/mg. The buffer concentration is about 1-3 mM and the alkanol concentration is about 45% v/v to about 90% v/v. In preferred aspects, the buffer concentration is about 3 mM and the alkanol concentration is about 45% v/v to about 60% v/v.

3. Liposome Dilution

Turning back to FIG. **1**, after the mixing step **130**, the degree of therapeutic agent (e.g., nucleic acid) encapsulation

can be enhanced if the lipid vesicle suspension is optionally diluted **140** prior to removal of free plasmid. For example, prior to dilution step **140**, if the therapeutic agent entrapment is at about 30-40%, it can be increased to about 70-80% following incubation after the dilution step **140**. In step **140**, the liposome formulation is diluted to about 10% to about 40%, preferably about 20% alkanol, by mixing with an aqueous solution such as a buffer (e.g., 1:1 with citrate buffer, 100 mM NaCl, pH 4.0). Such further dilution is preferably accomplished with a buffer. In certain aspects, such further diluting the liposome solution is a continuous stepwise dilution. The diluted sample is then optionally allowed to incubate at room temperature.

4. Removal of Free Therapeutic Agent

After the optional dilution step **140**, about 70-80% or more of the therapeutic agent (e.g., nucleic acid) is entrapped within the lipid vesicle (e.g., SPLP) and the free therapeutic agent can be removed from the formulation **150**. In certain aspects, anion exchange chromatography is used. Advantageously, the use of an anion exchange resin results in a high dynamic nucleic acid removal capacity, is capable of single use, may be pre-sterilized and validated, and is fully scaleable. In addition, the method preferably results in removal of free therapeutic agent (e.g., nucleic acid such as approximately 25% of total plasmid). The volume of sample after chromatography is unchanged, and the therapeutic agent (e.g., nucleic acid) and lipid concentrations are about 0.64 and 14.4 mg/mL, respectively. At this point, the sample can be assayed for encapsulated therapeutic agent and adjusted to about 0.55 mg/mL.

5. Sample Concentration

In certain instances, the liposome solution is optionally concentrated about 2-6 fold, preferably about 4 fold, using for example, ultrafiltration **160** (e.g., tangential flow dialysis). In one embodiment, the sample is transferred to a feed reservoir of an ultrafiltration system and the buffer is removed. The buffer can be removed using various processes, such as by ultrafiltration. In one aspect, buffer is removed using cartridges packed with polysulfone hollow fibers, for example, having internal diameters of about 0.5 mm and a 30,000 nominal molecular weight cut-off (NMWC). The liposomes are retained within the hollow fibers and recirculated while the solvent and small molecules are removed from the formulation by passing through the pores of the hollow fibers. In this procedure, the filtrate is known as the permeate solution. On completion of the concentration step, the therapeutic agent (e.g., nucleic acid) and lipid concentrations increase to about 0.90 and 15.14 mg/mL, respectively. In one embodiment, the alkanol concentration remains unchanged, but the alkanol: lipid ratio decreases about four fold.

6. Alkanol Removal

In one embodiment, the concentrated formulation is then diafiltrated against about 5-15 volumes, preferably about 10 volumes, of aqueous solution (e.g., buffer) (e.g., citrate buffer pH 4.0 (25 mM citrate, 100 mM NaCl) to remove the alkanol **170**. The alkanol concentration at the completion of step **170** is less than about 1%. Preferably, lipid and therapeutic agent (e.g., nucleic acid) concentrations remain unchanged and the level of therapeutic agent entrapment also remains constant.

7. Buffer Replacement

After the alkanol has been removed, the aqueous solution (e.g., buffer) is then replaced by dialfiltration against another buffer **180** (e.g., against 10 volumes of saline 150 mM NaCl with 10 mM Hepes pH 7.4). Preferably, the ratio of concentrations of lipid to therapeutic agent (e.g., nucleic acid) remain unchanged and the level of nucleic acid entrapment is about constant. In certain instances, sample yield can be improved by rinsing the cartridge with buffer at about 10% volume of the concentrated sample. In certain aspects, this rinse is then added to the concentrated sample.

8. Sterile Filtration

In certain preferred embodiments, sterile filtration **190** of the sample at lipid concentrations of about 12-14 mg/mL can optionally be performed. In certain aspects, filtration is conducted at pressures below about 40 psi, using a capsule filter and a pressurized dispensing vessel with a heating jacket. Heating the sample slightly can improve the ease of filtration.

9. Sterile Fill

The sterile fill step **195** is performed using similar processes as for conventional liposomal formulations. The processes of the present invention result in about 50-60% of the input therapeutic agent (e.g., nucleic acid) in the final product. In certain preferred aspects, the therapeutic agent to lipid ratio of the final product is approximately 0.04 to 0.07.

IV. Therapeutic Agents

The lipid-based drug formulations and compositions of the present invention are useful for the systemic or local delivery of therapeutic agents or bioactive agents and are also useful in diagnostic assays. The following discussion refers generally to liposomes; however, it will be readily apparent to those of skill in the art that this same discussion is fully applicable to the other drug delivery systems of the present invention.

As described above, therapeutic agent is preferably incorporated into the lipid vesicle during formation of the vesicle. In one embodiment, hydrophobic actives can be incorporated into the organic solvent with the lipid, while nucleic acid and hydrophilic actives can be added to the aqueous component. In certain instances, the therapeutic agent includes one of a protein, a nucleic acid, an antisense nucleic acid, ribozymes, tRNA, snRNA, siRNA, pre-condensed DNA, an antigen and combinations thereof. In preferred aspects, the therapeutic agent is nucleic acid. The nucleic acid may encode a protein such as, for example, a herpes simplex virus, thymidine kinase (HSV-TK), a cytosine deaminase, a xanthine-guanine-nephosphoribosyl transferase, a p53, a purine nucleoside phosphorylase, a carboxylesterase, a deoxycytidine kinase, a nitroreductase, a thymidine phosphorylase, or cytochrome P450 2B 1.

In certain aspects, therapeutic agent is incorporated into the organic lipid component. In certain instances, the therapeutic agent is lipophilic. Suitable lipophilic agents include taxol, taxol derivatives, including, for example, protax III and Paclitaxol, lipophilic benzoporphyrins, verteporfin the lipid prodrug of foscarnet, 1-O-octadecyl-sn-glycerol-3-phosphonoformate (ODG-PFA), dioleoyl[3H]iododeoxyuridine ([3H] IDU-O12), lipid derivatized HIV protease inhibitory peptides such as iBOC-[L-Phe]-[D-beta-Na1]-Pip-[alpha-(OH)-Leu]-Val (7194) and other lipid derivatized drugs or prodrugs.

In another embodiment, the lipid vesicles of the present invention can be loaded with one or more therapeutic agents after formation of the vesicle. In certain aspects, the therapeutic agents which are administered using the present invention can be any of a variety of drugs which are selected to be an appropriate treatment for the disease to be treated. Often the drug is an antineoplastic agent, such as vincristine, doxorubicin, mitoxantrone, camptothecin, cisplatin, bleomycin, cyclophosphamide, methotrexate, streptozotocin, and the like. Especially preferred antitumor agents include, for example, actinomycin D, vincristine, vinblastine, cystine arabinoside, anthracyclines, alkylative agents, platinum compounds, antimetabolites, and nucleoside analogs, such as methotrexate and purine and pyrimidine analogs. It may also be desirable to deliver anti-infective agents to specific tissues by the present processes. The compositions of the present invention can also be used for the selective delivery of other drugs including, but not limited to, local anesthetics, e.g.,

dibucaine and chlorpromazine; beta-adrenergic blockers, e.g., propranolol, timolol and labetolol; antihypertensive agents, e.g., clonidine and hydralazine; antidepressants, e.g., imipramine, amitriptyline and doxepim; anti-conversants, e.g., phenyloin; antihistamines, e.g., diphenhydramine, chlorpheniramine and promethazine; antibiotic/antibacterial agents, e.g., gentamycin, ciprofloxacin, and cefoxitin; antifungal agents, e.g., miconazole, terconazole, econazole, isoconazole, butaconazole, clotrimazole, itraconazole, nystatin, naftifine and amphotericin B; antiparasitic agents, hormones, hormone antagonists, immunomodulators, neurotransmitter antagonists, antiglaucoma agents, vitamins, narcotics, and imaging agents.

V. Apparatus

In another embodiment, the present invention provides apparatus for carrying out the processes of the present invention. FIG. **3** is an example of a representative schematic of an apparatus **300** according to one embodiment of the present invention. This schematic is merely an illustration and should not limit the scope of the claims herein. One of ordinary skill in the art will recognize other variations, modifications, and alternatives.

In one embodiment, the apparatus of the present invention includes two reservoirs, an aqueous solution reservoir **310** and an organic solution reservoir **320**, for holding aqueous solution and organic solution, respectively. In certain aspects, the lipid vesicle formulations are prepared rapidly, at low pressure (e.g., <10 psi) and the apparatus and processes of the present invention are fully scaleable (e.g., 0.5 mL-5000 L). At a 1-L scale, lipid vesicles are formed at about 0.4-0.8 L/min. In certain preferred aspects, the apparatus do not use static mixers nor specialized extrusion equipment.

The mixing chamber **340** is, in one embodiment, a T-connector, having optional hose barbs, wherein fluid lines **334** and **336** impact each other at about 180°. The angle of mixing can also be changed, and lipid vesicles less than about 100 nm can be formed at angles of between about 27° and about 90° or even between 90° and 180°. In preferred aspects, lipid vesicles of well defined and reproducible mean diameters are prepared using substantially equal flow rates of the flow lines. In other aspects, lipid vesicles of well defined and reproducible mean diameters are prepared by changing the flow rate of the fluid lines, e.g., to ensure sufficient mixing in some cases. In preferred aspects, the variance between flow rates is less that 50%, more preferably less than about 25% and even more preferably less than about 5%.

FIG. **13** shows a T-connector and associated flow dynamics according to one embodiment. Examples of flow rates, and resulting shear rates and Reynolds numbers (turbulence measure) are shown in FIG. **14** and discussed in more detail hereafter in Example 8. In comparison with prior systems, the present invention provides non-turbulent flow and increased shear rates at much lower (and substantially equivalent) flow rates. For example, the present invention advantageously provides non-turbulent flow (N_{re}<2000) in the mixing environment with a shear rate between about 500/s and about 3300/s at a flow rate (both flow lines) of between about 0.075 and about 0.3 L/min.

Mixing of the two fluid components can be driven using, for example, a peristaltic pump **330**, a positive displacement pump, or by pressurizing both the lipid-ethanol and buffer vessels **320**, **310**. In one aspect, a Watson-Marlow 505Di/L pump fitted with a 505L pump head is used; silicone tubing (e.g., platinum cured with 3.2 mm ID, 2.4 mm wall thickness; available from Watson Marlow as catalog no. 913A032024) can be used for flow lines into a polypropylene or stainless steel T-connector (e.g., with a ⅛" ID). Lipid vesicles are typically formed at room temperature, but lipid vesicles may be formed at elevated temperatures according to the present invention. Unlike other existing approaches, there are no general requirements for buffer composition. In fact, the processes and apparatus of the present invention can formulate a lipid vesicle by mixing lipid in an alkanol with water. In certain aspects, the processes and apparatus of the present invention form lipid vesicles that are less than 200 nm in diameter.

When lipid vesicles are prepared containing plasmid DNA (such as SPLPs), the ratio of plasmid to cationic lipid and counter ions can be optimized. For refined formulations, 70-95% plasmid DNA ("pDNA") encapsulation after mixing, and ethanol removal steps is preferred. The level of pDNA encapsulation can be increased by diluting this initial SPLP formulation. Surprisingly, the processes and apparatus of the present invention provide an encapsulation efficiency, upon mixing the solutions (with therapeutic agent in one of the solution components) in the mixing environment, of up to about 90%. Further refinement, e.g., dilution, may be performed as discussed herein.

In certain aspects, liposome producing apparatus **300** of the present invention further includes a temperature control mechanism (not shown) for controlling the temperature of the reservoirs **310** and **320**. Preferably, fluid from the first reservoir **310** and the second reservoirs **320** flows into mixing chamber **340** simultaneously at separate apertures. Apparatus **300** further includes a collection reservoir **350** downstream of the mixing chamber for liposome collection. Moreover, in certain aspects, apparatus **300** further includes storage vessels upstream of either or both of the reservoirs **310** and **320**. Further, either or both of the reservoirs **310** and **320** are preferably jacketed stainless steel vessels equipped with an overhead mixer.

In another embodiment, the present invention provides an apparatus having an ultrafiltration system for carrying out the processes of the present invention. FIG. **4** is an example of a representative schematic of an apparatus **400** according to one embodiment of the present invention. This schematic is merely an illustration and should not limit the scope of the claims herein. One of ordinary skill in the art will recognize other variations, modifications, and alternatives.

In certain aspects, apparatus **400** includes a plurality of reservoirs and is equipped with an ultrafiltration system. An aqueous solution reservoir **440** and an organic solution reservoir **430** each have upstream preparation vesicles **433** and **425**, respectively. In one aspect, lipid preparation vessel **425** is optionally equipped with an alkanol storage vessel **421** in fluid communication therewith.

As shown in FIG. **4**, the ultrafiltration system includes an incubation vessel **450** in fluid communication with a collection vessel **455**, an exchange column **460** and a tangential flow ultrafiltration cartridge **465**. The ultrafiltration system optionally includes a permeate vessel **470**. In certain aspects, ultrafiltration is used to concentrate SPLP samples and then remove ethanol from the formulation by buffer replacement.

In one embodiment of operation, the diluted SPLPs are transferred to the feed reservoir of the ultrafiltration system. Concentration is performed by removing buffer and ethanol using, for example, cross flow cartridges **465** packed with polysulfone hollow fibers that possess internal diameters of about 0.5 mm and a 100,000 molecular weight cut-off (MWCO). The SPLPs are retained within the hollow fibers and re-circulated, whereas the ethanol and buffer components are removed from the formulation by passing through the pores of these hollow fibers. This filtrate is known as the permeate solution and is discarded via vessel **470**. After the SPLPs are concentrated to the desired plasmid concentration, the buffer in which the SPLPs are suspended is removed by

ultrafiltration and replaced by an equal volume of the final buffer. Ultrafiltration can be replaced with other methods such as conventional dialysis.

VI. Examples

Example 1

This Example illustrates various physical and chemical properties of SPLPs made in accordance with one embodiment of the present invention.

Table I the amount of ethanol, pDNA and lipid content in process steps according to the present invention.

TABLE I

STEP	% Initial Volume	[Ethanol] (%)	[pDNA] (mg/ml)	pDNA Recovery (%)	[Lipid] (mg/ml)	Lipid Recovery (%)
SPLP formation	100	45	0.45	95	7.6	95
Dilution	200	22.5	0.23	90	3.8	90
Concentration	50	22.5	0.90	90	15.1	90
Ethanol removal	50	<1%	0.90	90	15.1	90
Buffer replacement*	45	<0.1%	0.90	81	15.1	81
Free DNA Removal**	45	<0.1%	0.64 (0.55)	55	14.4 (12.4)	76
Sterile filtration & Vial fill***	49	<0.1%	0.50	50	11.1	68

*Estimate 10% total volume and SPLP loss after buffer replacement step.

**Assume that 75% of pDNA is encapsulated and all free DNA is removed. Estimate 5% loss of SPLP on anion exchange cartridge. At this step the sample will be assayed for encapsulated pDNA and adjusted to 0.55 mg/ml to anticipate loss of SPLP during the filtration step (concentrations after adjustment to 0.55 mg/ml pDNA shown in brackets).

***Assume a maximum 5% volume loss and up to 10% total SPLP loss.

Table II sets forth the plasmid specification made according to one aspect of the present invention.

TABLE II

Plasmid Specification

	Test	Specification
1.	Appearance	Clear, Colorless solution.
2.	Electrophoresis	Relative migration vs standard.
3.	Circular plasmid	>90%
4.	Potentiometric pH value	6.5-8.5
5.	Electrophoresis	RNA undetectable
6.	BCA protein assay	Undetectable
7.	Spectrometric A_{260}/A_{280}	1.7-2.0
8.	DNA hybridization assay	<1% *E. coli* DNA
9.	Sterility Testing	No growth observed in bacteriologic media
10.	LAL	<20 EU/mg.
11.	UV Absorbance	2.0-3.0 mg/mL.

Table III sets forth the SPLP specification made according to one aspect of the present invention.

TABLE III

	Test	Specification
1.	Appearance	Homogenous, opaque white solution
2.	pH	7.4 (6.0-8.5)
3.	Osmolality	320 mOsm/kg (290-500 mOsm/kg)
4.	Plasmid Content	0.5 mg/mL (0.25-1.0 mg/mL)
5.	DSPC Content	20 +/− 4.0 mol %
6.	DODMA Content	25 +/− 5.0 mol %
7.	PEG-DSG Content	10 +/− 2.0 mol %
8.	Cholesterol Content	45 +/− 5.0 mol %
9.	Particle size	Mean diameter 100 ± 25 nm
10.	Plasmid Encapsulation	>85%
11.	Plasmid Integrity	>80%
	Supercoiled	
	Nicked	<20%
	Linear	<2%
12.	LAL	<50 EU/mg DNA
13.	Sterility	Pass

Example 2

This Example illustrates various process parameters in one embodiment of the present invention.

In one SPLP embodiment, varying the initial ethanol concentration for lipid dissolution had little impact on either vesicle size or DNA encapsulation, providing that the ethanol concentration was high enough to ensure that none of the individual lipid components precipitated (see, FIG. **5**). Below 75% ethanol, lipids were not soluble even with heating to 55° C. Lipids dissolved in 75% ethanol at 55° C. formed SPLP with larger mean diameters and lower DNA encapsulation (see, FIG. **5**).

The initial DNA to lipid ratio has been varied from 0.048-0.081 mg DNA: mg lipid formulation and vesicles of similar size with 77-90% DNA encapsulation were formed.

SPLPs have been prepared at a pH range of about 3.5-6 for the initial mixing step and all formulations possessed mean particle diameters of less than 150 nm and DNA encapsulation efficiencies of greater than 50% (see, FIG. **6**). At higher pH, vesicles can also be prepared with similar vesicle sizes, but with lower DNA encapsulation efficiencies.

In certain aspects, mean vesicle diameters of empty vesicles prepared using one process of the present invention depend upon the salt concentration of the diluting buffer, (e.g., Sphingomyelin:cholestesterol vesicles, EPC:EPG vesicles). Varying the ionic conditions in the buffer, influences the tendency for a given lipid to arrange itself into bilayers and vesicles.

During the development of one SPLP formulation, it was found that both the pH and salt concentration of the diluting buffer had a significant effect on the DNA encapsulation efficiency. Naturally, diluting buffers with pH values lower than the pKa for the cationic lipid component (DODMA)

gave higher encapsulation values (FIG. **7**). Interestingly, a final salt concentration of 150 mM was also optimal for DNA encapsulation (FIG. **8**).

Example 3

This Example illustrates the use of one process of the present invention to make EPC and POPC vesicles.

POPC vesicles are useful as "sink" vesicles for membrane fusion assays. In particular, they can be used in excess to remove PEG lipids from other liposomes, thus destabilizing the other liposomes and allowing them to fuse with the desired membrane. EPC vesicles are useful for removing cholesterol from arterial plaques.

The vesicles were prepared at an initial ethanol concentration of 80%, and lipid concentration of 10 mM. After mixing and dilution, the ethanol concentration was 20%, and lipid concentration was 5 mM. The EPC formulation was mixed and diluted with PBS, and the POPC was mixed and diluted with HBS. Both preparations were concentrated and ethanol removed using an ultrafiltration cartridge, i.e., the EPC against PBS, and the POPC against HBS. Both preparations were then sterile filtered using 0.22 um syringe filters.

TABLE IV

EPC and POPC vesicle data

		Vesicle Size (nm)			Lipid Concentration
Sample	Lot Number	Diam	SD	Chi^2	mg/mL
POPC	25031302-02	125	62	7	22.0
EPC	25031302-01	89	39	9	18.2

Example 4

This Example illustrates the use of one process of the present invention to make EPC/Cholesterol vesicles with a pH gradient.

Unilamellar lipid vesicles (LUV) comprising EPC and Cholesterol have traditionally been prepared by hydrating lipid films to form multilamellar lipid vesicles (MLV) that have been subjected to vesicle size reduction using high-pressure extrusion. It is well known that these vesicles can be prepared with acidic aqueous interiors and a pH gradient across the lipid bilayer. Weakly basic lipophilic molecules have been shown to accumulate in these vesicles at high internal concentrations. Various drug-loaded liposomes that are currently in late stage clinical trials utilize this approach (e.g., Myocet: doxorubicin loaded vesicles).

In one aspect, safranine was used to determine whether such a pH gradient was present. Safranine is a lipophilic basic dye that has been used to study membrane pH gradients

EPC/Chol vesicles were prepared using the present processes and apparatus at an initial ethanol concentration of 80%, and lipid concentration of 10 mM (See FIG. **9**A-B). After mixing and dilution, the ethanol concentration was 20%, and lipid concentration was 5 mM. Three different formulations were prepared:

1. Mixed and diluted with PBS (control).
2. Mixed and diluted with 150 mM citrate (final citrate concentration is 94 mM).
3. Mixed and diluted with 300 mM citrate (final citrate concentration is 188 mM).

After mixing and dilution, each sample was concentrated and ethanol was removed using ultrafiltration. After the concentration step, each sample was diafiltrated against its diluting buffer to ensure that the acidic citrate buffer present within vesicles would not leak out during ethanol removal. All samples were finally formulated with an external buffer of phosphate-buffered saline at pH 7.4. After sterile filtration, the mean vesicle diameters of these formulations were very similar (90-92 nm) and possessed acceptable standard deviation and Chi squared values (Table V).

Following dialysis, the vesicles were assayed for lipid concentration using the Infinity cholesterol assay. Solutions were then prepared containing 5 mM lipid and 0.2 mM safranine obtained from a filtered 10 mM stock solution. The solutions were incubated at 37° C. for 30 minutes. A 500 ul aliquot of each incubated solution was then passed down a 2-mL Sepharose CL4B gel filtration column. The free dye was separated from the vesicles, and the lipid-containing fractions were collected and analyzed. The safranine concentration was determined by measuring the fluorescence of the samples at 516 nm excitation and 585 nm emission.

The vesicles with acidic interiors accumulated safranine, with the 94 mM citrate-containing vesicles showing the highest encapsulation. In contrast, the PBS control vesicles encapsulated very little safranine. The 188 nM citrate vesicles also encapsulated some safranine, but not as much as the 94 mM citrate-containing vesicles (See FIG. **10**).

TABLE V

Safranine-Loaded EPC/Chol Vesicles

	Safranine	Vesicle Size (nm)			Dye: Lipid Ratio	
Sample	Encapsulation	Diam	SD	Chi^2	Mg:mg	mol:mol
PBS Control	9%	90	33	2.7	0.002	0.003
94 mM Citrate	54%	92	41	1.7	0.011	0.019
188 mM Citrate	31%	91	35	4.8	0.007	0.011

Example 5

This Example illustrates the use of one process of the present invention to make sphingomyelin/cholesterol vesicles.

Sphingomyelin/cholesterol vesicles are desirable due to their durability and strength. These vesicles can also be used to encapsulate drugs using a pH gradient. However, these LUV have traditionally needed to be formed at temperatures greater than 65° C. and using high pressure extrusion. In order to form these vesicles with the lipomixer, a number of variables needed to be taken into consideration, such as ethanol concentration, lipid concentration, and the salt concentration of the mixing and dilution buffer.

The vesicles were formulated at a ratio of 55/45 SM/Chol (mol:mol), while the initial ethanol concentration after mixing varied from 50 to 25%. Dilution buffers tested included PBS, water, 10 mM citrate, 150 mM citrate, and 300 mM citrate. Final lipid concentrations ranged from 0.5 to 2.5 mM. The vesicles formulated in the presence of salt (i.e., using buffers) were 200-500 nm, indicating an MLV. Aliquots of these samples were dialyzed against both 150 mM citrate and water in an attempt to remove ethanol and stabilize the vesicles.

Example 6

This Example illustrates the use of one process of the present invention to prepare vesicles that passively encapsulation small molecules such as calcein.

Calcein is a fluorescent dye that is self-quenching at concentrations greater than 10 mM. Vesicles encapsulating calcein can be used in fusion assays to determine whether vesicles have fused together. Fusion decreases the internal calcein concentration, causing it to fluoresce. Vesicles were prepared with DSPC:CHOL:PEG-DLG:DODMA (20:55:10:15) at an ethanol concentration of 19% and 2 mM lipid after mixing and dilution (See FIG. **11**). Lipids dissolved in ethanol were mixed with a solution containing 20 mM citrate and 75 mM calcein, and then the resulting vesicles were diluted with 300 mM NaCl and 37.5 mM Calcein. The calcein was obtained from a 100 mM stock solution. The final calcein concentration in the vesicles was 37.5 mM.

After mixing and dilution the vesicles were dialyzed overnight against HBS to remove unencapsulated dye. This was unsuccessful at removing all of the free dye, so the vesicles were passed down a gel filtration column. The lipid fraction was collected and analyzed. It was found that the calcein was indeed self quenching at the concentration inside the vesicles. This is a clear demonstration that the processes and apparatus of the present invention can be used to prepare vesicles that passively encapsulate small molecules.

TABLE VI

Calcein-encapsulated vesicles

	Vesicle Size (nm)			Fluorescence	
Step	Diam	SD	Chi^2	$rF_{without\ triton}$	$rF_{with\ triton}$
Post Dilution	205	109	0.4	N/d	N/d
Post Dialysis	173	74	0.5	N/d	N/d
Post Gel Filtration	178	77	5.4	0.4	4.1

Example 7

This Example illustrates the use of one process of the present invention versus prior art methods.

With reference to FIG. **12**, lipids were dissolved in 90% ethanol (A) and diluted either: step-wise using an apparatus of the present invention to 45% (B) and 22.5% ethanol (C), represented by the solid line ("LipoMixer"); or added dropwise with into stirred buffer to a final ethanol concentration of 22.5% (C), represented by the dotted line. Even though the final ethanol concentrations for both preparations were the same, the SPLP formed according to the processes of the present invention had 85% DNA encapsulation whereas vesicles prepared by ethanol drop had only 5% DNA encapsulation.

Example 8

This example illustrates various conditions and properties for forming liposomes according to the present invention. It should be appreciated that other conditions and parameters may be used and that those used herein are merely exemplary.

With reference to FIGS. **13** and **14**, various flow rates (substantially equivalent for both lipid and aqueous solution flows) are modeled and analyzed to show various parameters such as shear rate and Reynolds number (N_{re}) and vesicle size. Parameters and conditions were determined at the outlet of the T-connector correcting for the density and viscosity of the resulting ethanol solution. Additional turbulence as a result of the two streams meeting one another in opposition has not been accounted for, nor has additional turbulence as a result of the streams having to turn a 90 degree corner.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

What is claimed is:

1. A process for producing a lipid vesicle encapsulating a nucleic acid within the lipid vesicle, said process comprising:

providing an aqueous solution including a nucleic acid in a first reservoir;

providing an organic lipid solution in a second reservoir, wherein the lipids present in said organic lipid solution are solubilized in a lower alkanol at a concentration of about 75% v/v to 100% v/v; and

mixing said organic lipid solution with said aqueous solution by introducing said organic lipid solution and said aqueous solution into a mixing environment at about equal flow rates;

wherein said mixing instantaneously produces a lipid vesicle encapsulating the nucleic acid within the lipid vesicle by diluting said lower alkanol to a concentration of between 45% v/v to about 60% v/v; and

wherein the mixing environment includes a T-connector, wherein the aqueous solution and the organic lipid solution are introduced into the T-connector as opposing flows at about 180° relative to each other and mixed within the T-connector.

2. The process of claim **1**, further comprising diluting said lipid vesicle with a buffer solution wherein said lipid vesicle undergoes a continuous stepwise dilution to further stabilize the lipid vesicle.

3. The process of claim **1**, wherein said lipid vesicle is in a solution having a pH of about 3.5 to about 8.0.

4. The process of claim **1**, wherein the lipid vesicle is in a solution having a pH of about 6 or lower, and wherein said lipid vesicle has a nucleic acid encapsulation efficiency of greater than 50%.

5. The process of claim **1**, wherein the lipid vesicle is in a solution having a pH of about 5 or lower, and wherein said lipid vesicle has a nucleic acid encapsulation efficiency of between about 80% and about 90%.

6. The process of claim **1**, wherein said lipid vesicle has a diameter of about 150 nm or less.

7. The process of claim **1**, wherein said lipid vesicle is in a solution having a salt concentration of about 100 mM to about 200 mM.

8. The process of claim **1**, wherein said nucleic acid is selected from the group consisting of a plasmid, an antisense polynucleotide, a ribozyme, tRNA, snRNA, siRNA, and precondensed DNA.

9. The process of claim **1**, wherein said nucleic acid comprises an siRNA.

10. The process of claim **1**, wherein said lower alkanol is selected from the group consisting of methanol, ethanol, propanol, butanol, pentanol, isomers thereof, and combinations thereof.

11. The process of claim **1**, wherein said lower alkanol comprises ethanol.

12. The process of claim **1**, wherein the lipids present in said organic lipid solution are solubilized in said lower alkanol at a concentration of between about 80% v/v to about 90% v/v.

13. The process of claim **1**, wherein the lipid vesicle is a liposome.

14. The process of claim 2, wherein said lipid vesicle is in a solution having a concentration of less than 25% or about 25% v/v of said lower alkanol after dilution.

15. The process of claim 1, wherein said lipid vesicle is produced at a flow rate of between about 0.075 L/min and about 0.3 L/min.

16. The process of claim 1, wherein said lipid vesicle is produced at a shear rate of between about 500/s and about 3300/s.

17. The process of claim 1, wherein one or both of the first and the second reservoirs are temperature controlled.

18. The process of claim 1, wherein one or both of the first and the second reservoirs include a jacketed stainless steel vessel with an overhead mixer.

19. The process of claim 1, wherein the T-connector is polypropylene or stainless steel.

20. The process of claim 1, wherein the lipids present in said organic lipid solution comprise a phospholipid, cholesterol, a PEG-lipid, and a cationic lipid.

21. The process of claim 2, further comprising concentrating said lipid vesicle by tangential flow ultrafiltration.

22. The process of claim 1, wherein the lipids present in said organic lipid solution are solubilized in said lower alkanol at a concentration of about 100% v/v.

* * * * *

JOINT APPENDIX 54

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 2 279 254 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
05.07.2017 Bulletin 2017/27

(21) Application number: **09731866.1**

(22) Date of filing: **15.04.2009**

(51) Int Cl.:
C12N 15/113 *(2010.01)* ***C12N 15/88*** *(2006.01)*
A61K 31/712 *(2006.01)* ***A61K 31/713*** *(2006.01)*
A61K 9/127 *(2006.01)* ***A61P 35/00*** *(2006.01)*

(86) International application number:
PCT/CA2009/000496

(87) International publication number:
WO 2009/127060 (22.10.2009 Gazette 2009/43)

(54) **NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY**

NEUE LIPIDFORMULIERUNGEN ZUR NUKLEINSÄUREZUFÜHRUNG

NOUVELLES FORMULATIONS LIPIDIQUES POUR L'ADMINISTRATION D'ACIDES NUCLÉIQUES

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK TR
Designated Extension States:
AL BA RS

(30) Priority: **15.04.2008 US 45228 P**

(43) Date of publication of application:
02.02.2011 Bulletin 2011/05

(73) Proprietor: **Protiva Biotherapeutics Inc.**
Burnaby, British Columbia V5J 5J8 (CA)

(72) Inventors:
- **MACLACHLAN, Ian**
  Mission, B.C. V4S 1E5 (CA)
- **YAWORSKI, Edward**
  Maple Ridge, B.C. V2X 9Z7 (CA)
- **LAM, Kieu**
  Surrey, B.C. V4N 5M7 (CA)
- **JEFFS, Lloyd B.**
  Delta, B.C. V4K 3B4 (CA)
- **PALMER, Lorne R.**
  Vancouver, B.C. V5S 2P2 (CA)

(74) Representative: **Campbell, Patrick John Henry**
J A Kemp
14 South Square
Gray's Inn
London WC1R 5JJ (GB)

(56) References cited:
WO-A1-2005/035764
WO-A1-2006/002538
WO-A1-2006/053430
WO-A1-2007/056861
WO-A2-2005/120152
US-A1- 2005 064 595
US-A1- 2006 008 910
US-A1- 2007 042 031

Printed by Jouve, 75001 PARIS (FR)

Description

BACKGROUND OF THE INVENTION

[0001] RNA interference (RNAi) is an evolutionarily conserved process in which recognition of double-stranded RNA (dsRNA) ultimately leads to posttranscriptional suppression of gene expression. This suppression is mediated by short dsRNA, also called small interfering RNA (siRNA), which induces specific degradation of mRNA through complementary base pairing. In several model systems, this natural response has been developed into a powerful tool for the investigation of gene function (*see, e.g.,* Elbashir et al., Genes Dev., 15:188-200 (2001); Hammond et al., Nat. Rev. Genet., 2:110-119 (2001)). More recently, it was discovered that introducing synthetic 21-nucleotide dsRNA duplexes into mammalian cells could efficiently silence gene expression.

[0002] Although the precise mechanism is still unclear, RNAi provides a potential new approach to downregulate or silence the transcription and translation of a gene of interest. For example, it is desirable to modulate (*e.g.*, reduce) the expression of certain genes for the treatment of neoplastic disorders such as cancer. It is also desirable to silence the expression of genes associated with liver diseases and disorders such as hepatitis. It is further desirable to reduce the expression of certain genes for the treatment of atherosclerosis and its manifestations, *e.g.,* hypercholesterolemia, myocardial infarction, and thrombosis.

[0003] A safe and effective nucleic acid delivery system is required for RNAi to be therapeutically useful. Viral vectors are relatively efficient gene delivery systems, but suffer from a variety of limitations, such as the potential for reversion to the wild-type as well as immune response concerns. As a result, nonviral gene delivery systems are receiving increasing attention (Worgall et al., Human Gene Therapy, 8:37 (1997); Peeters et al., Human Gene Therapy, 7:1693 (1996); Yei et al., Gene Therapy, 1:192 (1994); Hope et al., Molecular Membrane Biology, 15:1 (1998)). Furthermore, viral systems are rapidly cleared from the circulation, limiting transfection to "first-pass" organs such as the lungs, liver, and spleen. In addition, these systems induce immune responses that compromise delivery with subsequent injections.

[0004] Plasmid DNA-cationic liposome complexes are currently the most commonly employed nonviral gene delivery vehicles (Feigner, Scientific American, 276:102 (1997); Chonn et al., Current Opinion in Biotechnology, 6:698 (1995)). For instance, cationic liposome complexes made of an amphipathic compound, a neutral lipid, and a detergent for transfecting insect cells are disclosed in U.S. Patent No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Publication No. 20030073640.

[0005] Cationic liposome complexes are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects (Harrison et al., Biotechniques, 19:816 (1995); Li et al., The Gene, 4:891 (1997); Tam et al, Gene Ther., 7:1867 (2000)). As large, positively charged aggregates, lipoplexes are rapidly cleared when administered *in vivo,* with highest expression levels observed in first-pass organs, particularly the lungs (Huang et al., Nature Biotechnology, 15:620 (1997); Templeton et al., Nature Biotechnology, 15:647 (1997); Hofland et al., Pharmaceutical Research, 14:742 (1997)).

[0006] Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Patent No. 6,429,200. Anionic liposomes are disclosed in U.S. Patent Publication No. 20030026831. Polymer liposomes that incorporate dextrin or glycerol-phosphocholine polymers are disclosed in U.S. Patent Publication Nos. 20020081736 and 20030082103, respectively.

[0007] A gene delivery system containing an encapsulated nucleic acid for systemic delivery should be small (*i.e.*, less than about 100 nm diameter) and should remain intact in the circulation for an extended period of time in order to achieve delivery to affected tissues. This requires a highly stable, serum-resistant nucleic acid-containing particle that does not interact with cells and other components of the vascular compartment. The particle should also readily interact with target cells at a disease site in order to facilitate intracellular delivery of a desired nucleic acid.

[0008] Recent work has shown that nucleic acids can be encapsulated in small (*e.g.*, about 70 nm diameter) "stabilized plasmid-lipid particles" (SPLP) that consist of a single plasmid encapsulated within a bilayer lipid vesicle (Wheeler et al., Gene Therapy, 6:271 (1999)). These SPLPs typically contain the "fusogenic" lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid, and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. SPLPs have systemic application as they exhibit extended circulation lifetimes following intravenous (i.v.) injection, accumulate preferentially at distal tumor sites due to the enhanced vascular permeability in such regions, and can mediate transgene expression at these tumor sites. The levels of transgene expression observed at the tumor site following i.v. injection of SPLPs containing the luciferase marker gene are superior to the levels that can be achieved employing plasmid DNA-cationic liposome complexes (lipoplexes) or naked DNA.

[0009] Thus, there remains a strong need in the art for novel and more efficient methods and compositions for introducing nucleic acids such as siRNA into cells. In addition, there is a need in the art for methods of downregulating the expression of genes of interest to treat or prevent diseases and disorders such as cancer and atherosclerosis. The present invention addresses these and other needs.

[0010] U.S. Patent Publication No. 2005064595 discloses lipid encapsulated interfering RNA.

[0011] U.S. Patent Publication No 2006008910 discloses lipid encapsulated interfering RNA.

[0012] WO Patent Publication No. 2006053430 discloses siRNA silencing of apolipoprotein b. WO Patent Publication No 2007056861 discloses siRNA silencing of influenza virus gene expression.

[0013] WO Patent Publication No 2005035764 discloses autogene nucleic acids encoding a secretable RNA polymerase.

[0014] WO Patent Publication No 2006002538 discloses immunostimulatory siRNA molecules and uses therefor.

BRIEF SUMMARY OF THE INVENTION

[0015] Described herein are novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.*, for the treatment of a disease or disorder).

The present invention provides a nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from 50 mol % to 65 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising up to 49.5 mol % of the total lipid present in the particle and comprising a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle.

[0016] In preferred embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In other preferred embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0017] In one aspect, the present disclosure provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0018] More particularly, the present disclosure provides serum-stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (*e.g.,* one or more interfering RNA molecules such as siRNA, aiRNA, and/or miRNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP (*e.g.,* for the treatment of a disease or disorder).

[0019] In certain aspects, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) a nucleic acid (*e.g.,* an interfering RNA); (b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0020] In one preferred aspect, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This nucleic acid-lipid particle is generally referred to herein as the "1:62" formulation.

[0021] In a preferred embodiment, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:57" formulation.

[0022] The present invention also provides pharmaceutical compositions comprising a nucleic acid-lipid particle of the invention and a pharmaceutically acceptable carrier. The present invention also provides a method for introducing a nucleic acid into a cell, the method comprising: contacting the cell *in vitro* with a nucleic acid-lipid particle of the invention, optionally wherein the cell is a mammalian cell.

[0023] In another aspect, the present disclosure provides methods for introducing an active agent or therapeutic agent (*e.g.*, nucleic acid) into a cell, the method comprising contacting the cell with a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0024] The present invention also provides the nucleic acid-lipid particle of the invention for use in a method for the

in vivo delivery of a nucleic acid, the method comprising administering said nucleic acid-lipid particle to a mammalian subject.

[0025] In yet another aspect, the present disclosure provides methods for the *in vivo* delivery of an active agent or therapeutic agent (*e.g.*, nucleic acid), the method comprising administering to a mammalian subject a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

The present invention also provides a nucleic acid-lipid particle of the invention for use in a method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising administering a therapeutically effective amount of said nucleic acid-lipid particle to the mammalian subject, wherein the disease or disorder is optionally selected from the group consisting of a viral infection, a liver disease or disorder, and cancer.

[0026] In a further aspect, the present disclosure provides methods for treating a disease or disorder in a mammalian subject in need thereof, the method comprising administering to the mammalian subject a therapeutically effective amount of a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0027] Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028]

Figure 1 illustrates data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

Figure 2 illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

Figure 3 illustrates additional data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice. Each bar represents the group mean of five animals. Error bars indicate the standard deviation.

Figure 4 illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

Figure 5 illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

Figure 6 illustrates data demonstrating that the tolerability of 1:57 SNALP containing ApoB siRNA prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters.

Figure 7 illustrates data demonstrating that the efficacy of 1:57 SNALP containing ApoB siRNA prepared by gear pump was similar to the same SNALP prepared by syringe press.

Figure 8 illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

Figure 9 illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

Figure 10 illustrates data demonstrating that clinically significant liver enzyme elevations (3xULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

Figure 11 illustrates data demonstrating that the potency of the lower lipid:drug (L:D) 1:57 SNALP containing ApoB siRNA was as good as that of the higher L:D SNALP at the tested drug dosages.

Figure 12 illustrates data demonstrating that ApoB protein and total cholesterol levels were reduced to a similar extent by 1:57 SNALP containing ApoB siRNA at a 6:1 input L:D ratio (final ratio of 7:1) and 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

Figure 13 illustrates data demonstrating that a treatment regimen of 1:57 SNALP with siRNA targeting PLK-1 is well tolerated with no apparent signs of treatment related toxicity in mice bearing Hep3B liver tumors.

Figure 14 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA caused a significant increase in the survival of Hep3B tumor-bearing mice.

Figure 15 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA reduced PLK-1 mRNA levels by 50% in intrahepatic Hep3B tumors growing in mice 24 hours after SNALP administration.

Figure 16 illustrates data demonstrating that a specific cleavage product of PLK-1 mRNA was detectable by 5' RACE-PCR in mice treated with 1:57 SNALP containing PLK-1 siRNA. 10 μl PCR product/well were loaded onto a 1.5% agarose gel. Lane Nos.: (1) molecular weight (MW) marker; (2) PBS mouse 1; (3) PBS mouse 2; (4) PBS mouse 3; (5) Luc SNALP mouse 1; (6) Luc SNALP mouse 2; (7) PLK SNALP mouse 1; (8) PLK SNALP mouse 2; (9) PLK SNALP mouse 3; and (10) no template control.

Figure 17 illustrates data demonstrating that control (Luc) 1:57 SNALP-treated mice displayed normal mitoses in Hep3B tumors (top panels), whereas mice treated with 1:57 SNALP containing PLK-1 siRNA exhibited numerous

aberrant mitoses and tumor cell apoptosis in Hep3B tumors (bottom panels).

Figure 18 illustrates data demonstrating that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B subcutaneous (S.C.) tumors.

Figure 19 illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

Figure 20 illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

Figure 21 illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

Figure 22 illustrates data demonstrating the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0029] The present invention is based, in part, upon the surprising discovery that lipid particles comprising from about 50 mol % to about 85 mol % of a cationic lipid, from about 13 mol % to about 49.5 mol % of a non-cationic lipid, and from about 0.5 mol % to about 2 mol % of a lipid conjugate provide advantages when used for the *in vitro* or *in vivo* delivery of an active agent, such as a therapeutic nucleic acid (*e.g.,* an interfering RNA). In particular, as illustrated by the Examples herein, described herein are stable nucleic acid-lipid particles (SNALP) that advantageously impart increased activity of the encapsulated nucleic acid (*e.g.,* an interfering RNA such as siRNA) and improved tolerability of the formulations *in vivo,* resulting in a significant increase in the therapeutic index as compared to nucleic acid-lipid particle compositions previously described. Additionally, the SNALP described herein are stable in circulation, *e.g.,* resistant to degradation by nucleases in serum, and are substantially non-toxic to mammals such as humans. As a non-limiting example, Figure 3 of Example 4 shows that one SNALP embodiment of the invention ("1:57 SNALP") was more than 10 times as efficacious as compared to a nucleic acid-lipid particle previously described ("2:30 SNALP") in mediating target gene silencing at a 10-fold lower dose. Similarly, Figure 2 of Example 3 shows that the "1:57 SNALP" formulation was substantially more effective at silencing the expression of a target gene as compared to nucleic acid-lipid particles previously described ("2:40 SNALP").

[0030] In certain embodiments, the present disclosure provides improved compositions for the delivery of interfering RNA such as siRNA molecules. In particular, the Examples herein illustrate that the improved lipid particle formulations described herein are highly effective in downregulating the mRNA and/or protein levels of target genes. Furthermore, the Examples herein illustrate that the presence of certain molar ratios of lipid components results in improved or enhanced activity of these lipid particle formulations of the present disclosure. For instance, the "1:57 SNALP" and "1:62 SNALP" formulations described herein are particularly advantageous because they provide improved efficacy and tolerability *in vivo,* are serum-stable, are substantially non-toxic, are capable of accessing extravascular sites, and are capable of reaching target cell populations.

[0031] The lipid particles and compositions of the present disclosure may be used for a variety of purposes, including the delivery of associated or encapsulated therapeutic agents to cells, both *in vitro* and *in vivo.* Accordingly, the present disclosure provides methods for treating diseases or disorders in a subject in need thereof, by contacting the subject with a lipid particle described herein comprising one or more suitable therapeutic agents.

[0032] Various exemplary embodiments of the lipid particles of the present disclosure, as well as compositions and formulations comprising the same, and their use to deliver therapeutic agents and modulate target gene and protein expression, are described in further detail below.

II. Definitions

[0033] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0034] The term "interfering RNA" or "RNAi" or "interfering RNA sequence" refers to single-stranded RNA (*e.g.,* mature miRNA) or double-stranded RNA (*i.e.,* duplex RNA such as siRNA, aiRNA, or pre-miRNA) that is capable of reducing or inhibiting the expression of a target gene or sequence (*e.g.,* by mediating the degradation or inhibiting the translation of mRNAs which are complementary to the interfering RNA sequence) when the interfering RNA is in the same cell as the target gene or sequence. Interfering RNA thus refers to the single-stranded RNA that is complementary to a target mRNA sequence or to the double-stranded RNA formed by two complementary strands or by a single, self-complementary strand. Interfering RNA may have substantial or complete identity to the target gene or sequence, or may comprise a region of mismatch (*i.e.,* a mismatch motif). The sequence of the interfering RNA can correspond to the full-length target gene, or a subsequence thereof.

[0035] Interfering RNA includes "small-interfering RNA" or "siRNA," *e.g.,* interfering RNA of about 15-60, 15-50, or

15-40 (duplex) nucleotides in length, more typically about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.,* each complementary sequence of the double-stranded siRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, preferably about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded siRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, preferably about 18-22, 19-20, or 19-21 base pairs in length). siRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides and 5' phosphate termini. Examples of siRNA include, without limitation, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand; a double-stranded polynucleotide molecule assembled from a single stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions; and a circular single-stranded polynucleotide molecule with two or more loop structures and a stem having self-complementary sense and antisense regions, where the circular polynucleotide can be processed *in vivo* or *in vitro* to generate an active double-stranded siRNA molecule.

[0036] Preferably, siRNA are chemically synthesized. siRNA can also be generated by cleavage of longer dsRNA (*e.g.*, dsRNA greater than about 25 nucleotides in length) with the *E. coli* RNase III or Dicer. These enzymes process the dsRNA into biologically active siRNA (*see, e.g.,* Yang et al., Proc. Natl. Acad. Sci. USA, 99:9942-9947 (2002); Calegari et al., Proc. Natl. Acad. Sci. USA, 99:14236 (2002); Byrom et al., Ambion TechNotes, 10(1):4-6 (2003); Kawasaki et al., Nucleic Acids Res., 31:981-987 (2003); Knight et al., Science, 293:2269-2271 (2001); and Robertson et al., J. Biol. Chem., 243:82 (1968)). Preferably, dsRNA are at least 50 nucleotides to about 100, 200, 300, 400, or 500 nucleotides in length. A dsRNA may be as long as 1000, 1500, 2000, 5000 nucleotides in length, or longer. The dsRNA can encode for an entire gene transcript or a partial gene transcript. In certain instances, siRNA may be encoded by a plasmid (*e.g.*, transcribed as sequences that automatically fold into duplexes with hairpin loops).

[0037] As used herein, the term "mismatch motif" or "mismatch region" refers to a portion of an interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) sequence that does not have 100 % complementarity to its target sequence. An interfering RNA may have at least one, two, three, four, five, six, or more mismatch regions. The mismatch regions may be contiguous or may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides. The mismatch motifs or regions may comprise a single nucleotide or may comprise two, three, four, five, or more nucleotides.

[0038] An "effective amount" or "therapeutically effective amount" of an active agent or therapeutic agent such as an interfering RNA is an amount sufficient to produce the desired effect, *e.g.,* an inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of an interfering RNA. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with an interfering RNA relative to the control is about 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, *e.g.,* examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0039] By "decrease," "decreasing," "reduce," or "reducing" of an immune response by an interfering RNA is intended to mean a detectable decrease of an immune response to a given interfering RNA (*e.g.,* a modified interfering RNA). The amount of decrease of an immune response by a modified interfering RNA may be determined relative to the level of an immune response in the presence of an unmodified interfering RNA. A detectable decrease can be about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more lower than the immune response detected in the presence of the unmodified interfering RNA. A decrease in the immune response to interfering RNA is typically measured by a decrease in cytokine production (*e.g.*, IFNγ, IFNα, TNFα, IL-6, or IL-12) by a responder cell *in vitro* or a decrease in cytokine production in the sera of a mammalian subject after administration of the interfering RNA.

[0040] As used herein, the term "responder cell" refers to a cell, preferably a mammalian cell, that produces a detectable immune response when contacted with an immunostimulatory interfering RNA such as an unmodified siRNA. Exemplary responder cells include, *e.g.*, dendritic cells, macrophages, peripheral blood mononuclear cells (PBMCs), splenocytes, and the like. Detectable immune responses include, *e.g.*, production of cytokines or growth factors such as TNF-α, IFN-α, IFN-β, IFN-γ, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TGF, and combinations thereof.

[0041] "Substantial identity" refers to a sequence that hybridizes to a reference sequence under stringent conditions, or to a sequence that has a specified percent identity over a specified region of a reference sequence.

[0042] The phrase "stringent hybridization conditions" refers to conditions under which a nucleic acid will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal

melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0043] Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C-95°C for 30 sec.-2 min., an annealing phase lasting 30 sec.-2 min., and an extension phase of about 72°C for 1-2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, *e.g.,* in Innis et al., PCR Protocols, A Guide to Methods and Applications, Academic Press, Inc. N.Y. (1990).

[0044] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in IX SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous references, *e.g.,* Current Protocols in Molecular Biology, Ausubel *et al*., eds.

[0045] The terms "substantially identical" or "substantial identity," in the context of two or more nucleic acids, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (*i.e.,* at least about 60%, preferably at least about 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition, when the context indicates, also refers analogously to the complement of a sequence. Preferably, the substantial identity exists over a region that is at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length.

[0046] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0047] A "comparison window," as used herein, includes reference to a segment of any one of a number of contiguous positions selected from the group consisting of from about 5 to about 60, usually about 10 to about 45, more usually about 15 to about 30, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.,* by the local homology algorithm of Smith and Waterman, Adv. Appl. Math., 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch, J. Mol. Biol., 48:443 (1970), by the search for similarity method of Pearson and Lipman, Proc. Natl. Acad. Sci. USA, 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g.,* Current Protocols in Molecular Biology, Ausubel et al., eds. (1995 supplement)).

[0048] A preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., Nuc. Acids Res., 25:3389-3402 (1977) and Altschul et al., J. Mol. Biol., 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids described herein. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

[0049] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.,* Karlin and Altschul, Proc. Natl. Acad. Sci. USA, 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide sequences would occur by chance. For example, a nucleic acid is considered similar to a reference

sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0050] The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA and RNA. DNA may be in the form of, *e.g.*, antisense molecules, plasmid DNA, pre-condensed DNA, a PCR product, vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives and combinations of these groups. RNA may be in the form of siRNA, asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, tRNA, viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res., 19:5081 (1991); Ohtsuka et al., J. Biol. Chem., 260:2605-2608 (1985); Rossolini et al., Mol. Cell. Probes, 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

[0051] The term "gene" refers to a nucleic acid (*e.g.,* DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

[0052] "Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

[0053] The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are characterized by being insoluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

[0054] A "lipid particle" is used herein to refer to a lipid formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.,* an interfering RNA), to a target site of interest. In the lipid particle of the present disclosure, which is typically formed from a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of the particle, the active agent or therapeutic agent may be encapsulated in the lipid, thereby protecting the agent from enzymatic degradation.

[0055] As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a particle made from lipids (*e.g.,* a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of the particle), wherein the nucleic acid (*e.g.,* siRNA, aiRNA, miRNA, ssDNA, dsDNA, ssRNA, short hairpin RNA (shRNA), dsRNA, or a plasmid, including plasmids from which an interfering RNA is transcribed) is fully encapsulated within the lipid. As used herein, the term "SNALP" includes an SPLP, which is the term used to refer to a nucleic acid-lipid particle comprising a nucleic acid (*e.g.,* a plasmid) encapsulated within the lipid. SNALP and SPLP typically contain a cationic lipid, a non-cationic lipid, and a lipid conjugate (*e.g.*, a PEG-lipid conjugate). SNALP and SPLP are extremely useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (i.v.) injection, they can accumulate at distal sites (*e.g.,* sites physically separated from the administration site), and they can mediate expression of the transfected gene or silencing of target gene expression at these distal sites. SPLP include "pSPLP," which comprise an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683.

[0056] The lipid particles described herein (*e.g.*, SNALP) typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm, and are substantially non-toxic. In addition, nucleic acids, when present in the lipid particles, are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.,* U.S. Patent Publication Nos. 20040142025 and 20070042031.

[0057] As used herein, "lipid encapsulated" can refer to a lipid particle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.,* an interfering RNA), with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid is fully encapsulated in the lipid particle (*e.g.,* to form an SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle).

[0058] The term "lipid conjugate" refers to a conjugated lipid that inhibits aggregation of lipid particles. Such lipid conjugates include, but are not limited to, polyamide oligomers (*e.g.*, ATTA-lipid conjugates), PEG-lipid conjugates, such as PEG coupled to dialkyloxypropyls, PEG coupled to diacylglycerols, PEG coupled to cholesterol, PEG coupled to

phosphatidylethanolamines, PEG conjugated to ceramides (*see, e.g.,* U.S. Patent No. 5,885,613), cationic PEG lipids, and mixtures thereof. PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.,* non-ester containing linker moieties and ester-containing linker moieties. In preferred embodiments, non-ester containing linker moieties are used.

[0059] The term "amphipathic lipid" refers, in part, to any suitable material wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxyl, and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids.

[0060] Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethano lamine, dipalmitoylphosphatidylcho line, dio leoylphosphatidylcho line, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, are also within the group designated as amphipathic lipids. Additionally, the amphipathic lipids described above can be mixed with other lipids including triglycerides and sterols.

[0061] The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethano lamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides, and diacylglycerols.

[0062] The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

[0063] The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerols, cardiolipins, diacylphosphatidylserines, diacylphosphatidic acids, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethano lamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

[0064] The term "cationic lipid" refers to any of a number of lipid species that carry a net positive charge at a selected pH, such as physiological pH (*e.g.,* pH of about 7.0). It has been surprisingly found that cationic lipids comprising alkyl chains with multiple sites of unsaturation, *e.g.*, at least two or three sites of unsaturation, are particularly useful for forming lipid particles with increased membrane fluidity. A number of cationic lipids and related analogs, which are also useful in the present invention, have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390. Non-limiting examples of cationic lipids are described in detail herein. In some cases, the cationic lipids comprise a protonatable tertiary amine (*e.g.*, pH titratable) head group, C18 alkyl chains, ether linkages between the head group and alkyl chains, and 0 to 3 double bonds. Such lipids include, *e.g.*, DSDMA, DLinDMA, DLenDMA, and DODMA.

[0065] The term "hydrophobic lipid" refers to compounds having apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups optionally substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Suitable examples include, but are not limited to, diacylglycerol, dialkylglycerol, N-N-dialkylamino, 1,2-diacyloxy-3-aminopropane, and 1,2-dialkyl-3-aminopropane.

[0066] The term "fusogenic" refers to the ability of a lipid particle, such as a SNALP, to fuse with the membranes of a cell. The membranes can be either the plasma membrane or membranes surrounding organelles, *e.g.*, endosome, nucleus, *etc.*

[0067] As used herein, the term "aqueous solution" refers to a composition comprising in whole, or in part, water.

[0068] As used herein, the term "organic lipid solution" refers to a composition comprising in whole, or in part, an organic solvent having a lipid.

[0069] "Distal site," as used herein, refers to a physically separated site, which is not limited to an adjacent capillary bed, but includes sites broadly distributed throughout an organism.

[0070] "Serum-stable" in relation to nucleic acid-lipid particles such as SNALP means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNAse assay, or an RNAse assay.

[0071] "Systemic delivery," as used herein, refers to delivery of lipid particles that leads to a broad biodistribution of an active agent or therapeutic agent such as an interfering RNA within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. To obtain broad biodistribution generally requires a blood lifetime such that the agent is not rapidly degraded or cleared (such as by first pass organs (liver, lung, *etc*.) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration. Systemic delivery

of lipid particles can be by any means known in the art including, for example, intravenous, subcutaneous, and intraperitoneal. In a preferred embodiment, systemic delivery of lipid particles is by intravenous delivery.

[0072] "Local delivery," as used herein, refers to delivery of an active agent or therapeutic agent such as an interfering RNA directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site such as a tumor or other target site such as a site of inflammation or a target organ such as the liver, heart, pancreas, kidney, and the like.

[0073] The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

[0074] The term "cancer" refers to any member of a class of diseases characterized by the uncontrolled growth of aberrant cells. The term includes all known cancers and neoplastic conditions, whether characterized as malignant, benign, soft tissue, or solid, and cancers of all stages and grades including pre- and post-metastatic cancers. Examples of different types of cancer include, but are not limited to, lung cancer, colon cancer, rectal cancer, anal cancer, bile duct cancer, small intestine cancer, stomach (gastric) cancer, esophageal cancer; gallbladder cancer, liver cancer, pancreatic cancer, appendix cancer, breast cancer, ovarian cancer; cervical cancer, prostate cancer, renal cancer (*e.g.*, renal cell carcinoma), cancer of the central nervous system, glioblastoma, skin cancer, lymphomas, choriocarcinomas, head and neck cancers, osteogenic sarcomas, and blood cancers. Non-limiting examples of specific types of liver cancer include hepatocellular carcinoma (HCC), secondary liver cancer (*e.g.*, caused by metastasis of some other non-liver cancer cell type), and hepatoblastoma. As used herein, a "tumor" comprises one or more cancerous cells.

III. Description of the Embodiments

[0075] The present disclosure provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.,* for the treatment of a disease or disorder).

[0076] In one aspect, the present disclosure provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0077] In certain embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In certain other embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0078] In some embodiments, the active agent or therapeutic agent comprises a nucleic acid. In certain instances, the nucleic acid comprises an interfering RNA molecule such as, *e.g.,* an siRNA, aiRNA, miRNA, or mixtures thereof. In certain other instances, the nucleic acid comprises single-stranded or double-stranded DNA, RNA, or a DNA/RNA hybrid such as, *e.g.,* an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

[0079] In other embodiments, the active agent or therapeutic agent comprises a peptide or polypeptide. In certain instances, the peptide or polypeptide comprises an antibody such as, *e.g.,* a polyclonal antibody, a monoclonal antibody, an antibody fragment; a humanized antibody, a recombinant antibody, a recombinant human antibody, a Primatized™ antibody, or mixtures thereof. In certain other instances, the peptide or polypeptide comprises a cytokine, a growth factor, an apoptotic factor, a differentiation-inducing factor, a cell-surface receptor, a ligand, a hormone, a small molecule (*e.g.*, small organic molecule or compound), or mixtures thereof.

[0080] In preferred embodiments, the active agent or therapeutic agent comprises an siRNA. In one embodiment, the siRNA molecule comprises a double-stranded region of about 15 to about 60 nucleotides in length (*e.g.*, about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, or 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length). The siRNA molecules described herein are capable of silencing the expression of a target sequence *in vitro* and/or *in vivo.*

[0081] In some embodiments, the siRNA molecule comprises at least one modified nucleotide. In certain preferred embodiments, the siRNA molecule comprises one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides in the double-stranded region. In certain instances, the siRNA comprises from about 1% to about 100% (*e.g.*, about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region. In preferred embodiments, less than about 25% (*e.g.,* less than about 25%, 20%, 15%, 10%, or 5%) or from about 1% to about 25% (*e.g.,* from about 1%-25%, 5%-25%, 10%-25%, 15%-25%, 20%-25%, or 10%-20%) of the nucleotides in the double-stranded region comprise modified nucleotides.

[0082] In other embodiments, the siRNA molecule comprises modified nucleotides including, but not limited to, 2'-O-

methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, locked nucleic acid (LNA) nucleotides, and mixtures thereof. In preferred embodiments, the siRNA comprises 2'OMe nucleotides (*e.g.*, 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof. In certain instances, the siRNA does not comprise 2'OMe-cytosine nucleotides. In other embodiments, the siRNA comprises a hairpin loop structure.

[0083] The siRNA may comprise modified nucleotides in one strand (*i.e.,* sense or antisense) or both strands of the double-stranded region of the siRNA molecule. Preferably, uridine and/or guanosine nucleotides are modified at selective positions in the double-stranded region of the siRNA duplex. With regard to uridine nucleotide modifications, at least one, two, three, four, five, six, or more of the uridine nucleotides in the sense and/or antisense strand can be a modified uridine nucleotide such as a 2'OMe-uridine nucleotide. In some embodiments, every uridine nucleotide in the sense and/or antisense strand is a 2'OMe-uridine nucleotide. With regard to guanosine nucleotide modifications, at least one, two, three, four, five, six, or more of the guanosine nucleotides in the sense and/or antisense strand can be a modified guanosine nucleotide such as a 2'OMe-guanosine nucleotide. In some embodiments, every guanosine nucleotide in the sense and/or antisense strand is a 2'OMe-guanosine nucleotide.

[0084] In certain embodiments, at least one, two, three, four, five, six, seven, or more 5'-GU-3' motifs in an siRNA sequence may be modified, *e.g.,* by introducing mismatches to eliminate the 5'-GU-3' motifs and/or by introducing modified nucleotides such as 2'OMe nucleotides. The 5'-GU-3' motif can be in the sense strand, the antisense strand, or both strands of the siRNA sequence. The 5'-GU-3' motifs may be adjacent to each other or, alternatively, they may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides.

[0085] In some preferred embodiments, a modified siRNA molecule is less immunostimulatory than a corresponding unmodified siRNA sequence. In such embodiments, the modified siRNA molecule with reduced immunostimulatory properties advantageously retains RNAi activity against the target sequence. In another embodiment, the immunostimulatory properties of the modified siRNA molecule and its ability to silence target gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the siRNA sequence such as, *e.g.*, within the double-stranded region of the siRNA duplex. In certain instances, the modified siRNA is at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% less immunostimulatory than the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that the immunostimulatory properties of the modified siRNA molecule and the corresponding unmodified siRNA molecule can be determined by, for example, measuring INF-α and/or IL-6 levels from about two to about twelve hours after systemic administration in a mammal or transfection of a mammalian responder cell using an appropriate lipid-based delivery system (such as the SNALP delivery system disclosed herein).

[0086] In certain embodiments, a modified siRNA molecule has an IC_{50} (*i.e.,* half-maximal inhibitory concentration) less than or equal to ten-fold that of the corresponding unmodified siRNA (*i.e.,* the modified siRNA has an IC_{50} that is less than or equal to ten-times the IC_{50} of the corresponding unmodified siRNA). In other embodiments, the modified siRNA has an IC_{50} less than or equal to three-fold that of the corresponding unmodified siRNA sequence. In yet other embodiments, the modified siRNA has an IC_{50} less than or equal to two-fold that of the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that a dose-response curve can be generated and the IC_{50} values for the modified siRNA and the corresponding unmodified siRNA can be readily determined using methods known to those of skill in the art.

[0087] In yet another embodiment, a modified siRNA molecule is capable of silencing at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the expression of the target sequence relative to the corresponding unmodified siRNA sequence.

[0088] In some embodiments, the siRNA molecule does not comprise phosphate backbone modifications, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In other embodiments, the siRNA comprises one, two, three, four, or more phosphate backbone modifications, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise phosphate backbone modifications.

[0089] In further embodiments, the siRNA does not comprise 2'-deoxy nucleotides, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In yet further embodiments, the siRNA comprises one, two, three, four, or more 2'-deoxy nucleotides, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise 2'-deoxy nucleotides.

[0090] In certain instances, the nucleotide at the 3'-end of the double-stranded region in the sense and/or antisense strand is not a modified nucleotide. In certain other instances, the nucleotides near the 3'-end (*e.g.*, within one, two, three, or four nucleotides of the 3'-end) of the double-stranded region in the sense and/or antisense strand are not modified nucleotides.

[0091] The siRNA molecules described herein may have 3' overhangs of one, two, three, four, or more nucleotides on one or both sides of the double-stranded region, or may lack overhangs (*i.e.*, have blunt ends) on one or both sides of the double-stranded region. Preferably, the siRNA has 3' overhangs of two nucleotides on each side of the double-

stranded region. In certain instances, the 3' overhang on the antisense strand has complementarity to the target sequence and the 3' overhang on the sense strand has complementarity to a complementary strand of the target sequence. Alternatively, the 3' overhangs do not have complementarity to the target sequence or the complementary strand thereof. In some embodiments, the 3' overhangs comprise one, two, three, four, or more nucleotides such as 2'-deoxy (2'H) nucleotides. In certain preferred embodiments, the 3' overhangs comprise deoxythymidine (dT) and/or uridine nucleotides. In other embodiments, one or more of the nucleotides in the 3' overhangs on one or both sides of the double-stranded region comprise modified nucleotides. Non-limiting examples of modified nucleotides are described above and include 2'OMe nucleotides, 2'-deoxy-2'F nucleotides, 2'-deoxy nucleotides, 2'-O-2-MOE nucleotides, LNA nucleotides, and mixtures thereof. In preferred embodiments, one, two, three, four, or more nucleotides in the 3' overhangs present on the sense and/or antisense strand of the siRNA comprise 2'OMe nucleotides (*e.g.*, 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof.

[0092] The siRNA may comprise at least one or a cocktail (*e.g.,* at least two, three, four, five, six, seven, eight, nine, ten, or more) of unmodified and/or modified siRNA sequences that silence target gene expression. The cocktail of siRNA may comprise sequences which are directed to the same region or domain (*e.g.*, a "hot spot") and/or to different regions or domains of one or more target genes. In certain instances, one or more (*e.g.,* at least two, three, four, five, six, seven, eight, nine, ten, or more) modified siRNA that silence target gene expression are present in a cocktail. In certain other instances, one or more (*e.g.,* at least two, three, four, five, six, seven, eight, nine, ten, or more) unmodified siRNA sequences that silence target gene expression are present in a cocktail.

[0093] In some embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to the target sequence or a portion thereof. In other embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is 100% complementary to the target sequence or a portion thereof. In further embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that specifically hybridizes to the target sequence or a portion thereof.

[0094] In further embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the target sequence or a portion thereof. In additional embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is 100% identical to the target sequence or a portion thereof.

[0095] In the lipid particles described herein (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the cationic lipid may comprise, *e.g.*, one or more of the following: 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 - (N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane(CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), or mixtures thereof. In certain preferred embodiments, the cationic lipid is DLinDMA, DLin-K-C2-DMA ("XTC2"), or mixtures thereof.

[0096] The synthesis of cationic lipids such as DLin-K-C2-DMA ("XTC2"), DLin-K-C3-DMA, DLin-K-C4-DMA, DLin-K6-DMA, and DLin-K-MPZ, as well as additional cationic lipids, is described in U.S. Provisional Application No. 61/104,212, filed October 9, 2008. The synthesis of cationic lipids such as DLin-K-DMA, DLin-C-DAP, DLin-DAC, DLin-MA, DLinDAP, DLin-S-DMA, DLin-2-DMAP, DLin-TMA.Cl, DLin-TAP.Cl, DLin-MPZ, DLinAP, DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Application No. PCT/US08/88676, filed December 31, 2008.

The synthesis of cationic lipids such as CLinDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060240554.

[0097] In some embodiments described herein, the cationic lipid may comprise from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 50 mol % to about 60 mol % of the total lipid present in the particle.

[0098] In other embodiments, the cationic lipid may comprise from about 55 mol % to about 90 mol %, from about 55 mol % to about 85 mol %, from about 55 mol % to about 80 mol %, from about 55 mol % to about 75 mol %, from about 55 mol % to about 70 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0099] In yet other embodiments, the cationic lipid may comprise from about 60 mol % to about 90 mol %, from about 60 mol % to about 85 mol %, from about 60 mol % to about 80 mol %, from about 60 mol % to about 75 mol %, or from about 60 mol % to about 70 mol % of the total lipid present in the particle.

[0100] In still yet other embodiments, the cationic lipid may comprise from about 65 mol % to about 90 mol %, from about 65 mol % to about 85 mol %, from about 65 mol % to about 80 mol %, or from about 65 mol % to about 75 mol % of the total lipid present in the particle.

[0101] In further embodiments, the cationic lipid may comprise from about 70 mol % to about 90 mol %, from about 70 mol % to about 85 mol %, from about 70 mol % to about 80 mol %, from about 75 mol % to about 90 mol %, from about 75 mol % to about 85 mol %, or from about 80 mol % to about 90 mol % of the total lipid present in the particle.

[0102] In additional embodiments, the cationic lipid may comprise (at least) about 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0103] In the lipid particles of the present disclosure (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the non-cationic lipid may comprise, *e.g.*, one or more anionic lipids and/or neutral lipids. The non-cationic lipid may comprise one of the following neutral lipid components: (1) cholesterol or a derivative thereof; (2) a phospholipid; or (3) a mixture of a phospholipid and cholesterol or a derivative thereof.

[0104] Examples of cholesterol derivatives include, but are not limited to, cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof. The synthesis of cholesteryl-2'-hydroxyethyl ether is described herein.

[0105] The phospholipid may be a neutral lipid including, but not limited to, dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidyleth-anolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphati-dylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), egg phosphatidylcholine (EPC), and mix-tures thereof. In certain preferred embodiments, the phospholipid is DPPC, DSPC, or mixtures thereof.

[0106] In some embodiments described herein, the non-cationic lipid (*e.g.,* one or more phospholipids and/or choles-terol) may comprise from about 10 mol % to about 60 mol %, from about 15 mol % to about 60 mol %, from about 20 mol % to about 60 mol %, from about 25 mol % to about 60 mol %, from about 30 mol % to about 60 mol %, from about 10 mol % to about 55 mol %, from about 15 mol % to about 55 mol %, from about 20 mol % to about 55 mol %, from about 25 mol % to about 55 mol %, from about 30 mol % to about 55 mol %, from about 13 mol % to about 50 mol %, from about 15 mol % to about 50 mol % or from about 20 mol % to about 50 mol % of the total lipid present in the particle. When the non-cationic lipid is a mixture of a phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 50, or 60 mol % of the total lipid present in the particle.

[0107] In other embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 49.5 mol %, from about 13 mol % to about 49.5 mol %, from about 15 mol % to about 49.5 mol %, from about 20 mol % to about 49.5 mol %, from about 25 mol % to about 49.5 mol %, from about 30 mol % to about 49.5 mol %, from about 35 mol % to about 49.5 mol %, or from about 40 mol % to about 49.5 mol % of the total lipid present in the particle.

[0108] In yet other embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 45 mol %, from about 13 mol % to about 45 mol %, from about 15 mol % to about 45 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, or from about 35 mol % to about 45 mol % of the total lipid present in the particle.

[0109] In still yet other embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 40 mol %, from about 13 mol % to about 40 mol %, from about 15 mol % to about 40 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0110] In further embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 35 mol %, from about 13 mol % to about 35 mol %, from about 15 mol % to about 35 mol

%, from about 20 mol % to about 35 mol %, or from about 25 mol % to about 35 mol % of the total lipid present in the particle.

[0111] In yet further embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 30 mol %, from about 13 mol % to about 30 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 13 mol % to about 25 mol %, or from about 15 mol % to about 25 mol % of the total lipid present in the particle.

[0112] In additional embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise (at least) about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0113] In certain embodiments, the non-cationic lipid comprises cholesterol or a derivative thereof of from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle described herein may comprise cholesterol or a derivative thereof at about 37 mol % of the total lipid present in the particle. In other preferred embodiments, a phospholipid-free lipid particle described herein may comprise cholesterol or a derivative thereof of from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 32 mol % to about 45 mol %, from about 32 mol % to about 42 mol %, from about 32 mol % to about 40 mol %, from about 34 mol % to about 45 mol %, from about 34 mol % to about 42 mol %, from about 34 mol % to about 40 mol %, or about 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0114] In certain other preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 4 mol % to about 10 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 30 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 7 mol % and cholesterol at about 34 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 3 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 4 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 4 mol % to about 8 mol %, from about 5 mol % to about 12 mol %, from about 5 mol % to about 9 mol %, from about 6 mol % to about 12 mol %, from about 6 mol % to about 10 mol %, or about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 40 mol %, from about 25 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 28 mol % to about 40 mol %, from about 28 mol % to about 38 mol %, from about 30 mol % to about 38 mol %, from about 32 mol % to about 36 mol %, or about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0115] In further preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 20 mol % and cholesterol at about 20 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0116] In the lipid particles described herein (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the conjugated lipid that inhibits aggregation of particles may comprise, e.g., one or more of the following: a polyethyleneglycol (PEG)-lipid conjugate, a polyamide (ATTA)-lipid conjugate, a cationic-polymer-lipid conjugates (CPLs), or mixtures thereof. In one preferred embodiment, the nucleic acid-lipid particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate. In certain embodiments, the PEG-lipid conjugate or ATTA-lipid conjugate is used together with a CPL. The conjugated lipid that inhibits aggregation of particles may comprise a PEG-lipid including, *e.g.*, a PEG-diacylglycerol (DAG), a PEG dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or mixtures thereof. The PEG-DAA conjugate may be PEG-dilauryloxypropyl (C12), a PEG-dimyristyloxypropyl (C14), a PEG-dipalmityloxypropyl (C16), a PEG-distearyloxypropyl (C18), or mixtures thereof.

[0117] Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-*sn*3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed December 31, 2008. Yet additional PEG-lipid conjugates suitable for use in the invention include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol) (2KPEG-DMG). The synthesis of 2KPEG-DMG is described in U.S. Patent No. 7,404,969.

[0118] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.).* In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

[0119] In some embodiments, the conjugated lipid that inhibits aggregation of particles is a CPL that has the formula: A-W-Y, wherein A is a lipid moiety, W is a hydrophilic polymer, and Y is a polycationic moiety. W may be a polymer selected from the group consisting of polyethyleneglycol (PEG), polyamide, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, or combinations thereof, the polymer having a molecular weight of from about 250 to about 7000 daltons. In some embodiments, Y has at least 4 positive charges at a selected pH. In some embodiments, Y may be lysine, arginine, asparagine, glutamine, derivatives thereof, or combinations thereof.

[0120] In certain instances, the conjugated lipid that inhibits aggregation of particles (*e.g.*, PEG-lipid conjugate) may comprise from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 1 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, from about 1.4 mol % to about 1.5 mol %, or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0121] In the lipid particles described herein, the active agent or therapeutic agent may be fully encapsulated within the lipid portion of the particle, thereby protecting the active agent or therapeutic agent from enzymatic degradation. In preferred embodiments, a SNALP comprising a nucleic acid such as an interfering RNA (*e.g.*, siRNA) is fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the nucleic acid in the SNALP is not substantially degraded after exposure of the particle to a nuclease at 37°C for at least about 20, 30, 45, or 60 minutes. In certain other instances, the nucleic acid in the SNALP is not substantially degraded after incubation of the particle in serum at 37°C for at least about 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In other embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid such as siRNA) is complexed with the lipid portion of the particle. One of the benefits of the formulations described herein is that the lipid particle compositions are substantially non-toxic to mammals such as humans.

[0122] The term "fully encapsulated" indicates that the active agent or therapeutic agent in the lipid particle is not significantly degraded after exposure to serum or a nuclease or protease assay that would significantly degrade free DNA, RNA, or protein. In a fully encapsulated system, preferably less than about 25% of the active agent or therapeutic agent in the particle is degraded in a treatment that would normally degrade 100% of free active agent or therapeutic agent, more preferably less than about 10%, and most preferably less than about 5% of the active agent or therapeutic agent in the particle is degraded. In the context of nucleic acid therapeutic agents, full encapsulation may be determined by an Oligreen® assay. Oligreen® is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA or RNA in solution (available from Invitrogen Corporation; Carlsbad, CA). "Fully encapsulated" also indicates that the lipid particles are serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

[0123] In another aspect, the present disclosure provides a lipid particle (*e.g.*, SNALP) composition comprising a plurality of lipid particles. In preferred embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid) is fully encapsulated within the lipid portion of the lipid particles (*e.g.*, SNALP), such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, %, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the lipid particles (*e.g.*, SNALP) have the active agent or therapeutic agent encapsulated therein.

[0124] Typically, the lipid particles (*e.g.*, SNALP) described herein have a lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 1 to about 100. In some instances, the lipid:active agent (*e.g.*, lipid:nucleic acid)

ratio (mass/mass ratio) ranges from about 1 to about 50, from about 2 to about 25, from about 3 to about 20, from about 4 to about 15, or from about 5 to about 10. In preferred embodiments, the lipid particles described herein have a lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 5 to about 15, *e.g.,* about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 (or any fraction thereof or range therein).

[0125] Typically, the lipid particles (*e.g.*, SNALP) described herein have a mean diameter of from about 40 nm to about 150 nm. In preferred embodiments, the lipid particles (*e.g.*, SNALP) described herein have a mean diameter of from about 40 nm to about 130 nm, from about 40 nm to about 120 nm, from about 40 nm to about 100 nm, from about 50 nm to about 120 nm, from about 50 nm to about 100 nm, from about 60 nm to about 120 nm, from about 60 nm to about 110 nm, from about 60 nm to about 100 nm, from about 60 nm to about 90 nm, from about 60 nm to about 80 nm, from about 70 nm to about 120 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 70 nm to about 90 nm, from about 70 nm to about 80 nm, or less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm (or any fraction thereof or range therein).

[0126] In one specific embodiment of the present disclosure, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:62" formulation. In a preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is cholesterol, and the conjugated lipid is a PEG-DAA conjugate. Although these are preferred embodiments of the 1:62 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic lipids (including other cholesterol derivatives), and conjugated lipids can be used in the 1:62 formulation as described herein.

[0127] In a specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:57" formulation. In one preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 5 mol % to about 9 mol % of the total lipid present in the particle (*e.g.*, about 7.1 mol %) and the cholesterol (or cholesterol derivative) comprises from about 32 mol % to about 37 mol % of the total lipid present in the particle (*e.g.*, about 34.3 mol %), and the PEG-lipid is a PEG-DAA (*e.g.*, PEG-cDMA). In another preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.*, about 20 mol %) and the cholesterol (or cholesterol derivative) comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.*, about 20 mol %), and the PEG-lipid is a PEG-DAA (*e.g.*, PEG-cDMA). Although these are preferred embodiments of the 1:57 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic lipids (including other phospholipids and other cholesterol derivatives), and conjugated lipids can be used in the 1:57 formulation as described herein.

[0128] In preferred embodiments, the 1:62 SNALP formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-cDMA (or PEG-cDSA), about 61.5 mol % DLinDMA (or XTC2), and about 36.9 mol % cholesterol (or derivative thereof). In other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 7.1 mol % DPPC, and about 34.3 mol % cholesterol (or derivative thereof). In yet other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 20 mol % DPPC, and about 20 mol % cholesterol (or derivative thereof). It should be understood that these SNALP formulations are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the SNALP formulations may vary.

[0129] The present disclosure also provides a pharmaceutical composition comprising a lipid particle (*e.g.*, SNALP) described herein and a pharmaceutically acceptable carrier.

[0130] In a further aspect, the present disclosure provides a method for introducing one or more active agents or therapeutic agents (*e.g.*, nucleic acid) into a cell, comprising contacting the cell with a lipid particle (*e.g.*, SNALP) described herein. In one embodiment, the cell is in a mammal and the mammal is a human. In another embodiment, the present disclosure provides a method for the *in vivo* delivery of one or more active agents or therapeutic agents (*e.g.,* nucleic acid), comprising administering to a mammalian subject a lipid particle (*e.g.,* SNALP) described herein. In a preferred embodiment, the mode of administration includes, but is not limited to, oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. Preferably, the mammalian subject is a human.

[0131] In one embodiment, at least about 5%, 10%, 15%, 20%, or 25% of the total injected dose of the lipid particles (*e.g.*, SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In other embodiments, more than about 20%, 30%, 40% and as much as about 60%, 70% or 80% of the total injected dose of the lipid particles (*e.g.*, SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In certain instances, more than about 10% of a plurality of the particles is present in the plasma of a mammal about 1 hour after administration. In certain other instances, the presence of the lipid particles (*e.g.*, SNALP) is detectable at least about 1 hour after administration of the particle. In certain embodiments, the presence of an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is detectable in cells of the lung, liver, tumor, or at a site of inflammation at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is detectable at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In yet other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.,* siRNA) occurs preferentially in tumor cells or in cells at a site of inflammation. In further embodiments, the presence or effect of an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) in cells at a site proximal or distal to the site of administration or in cells of the lung, liver, or a tumor is detectable at about 12, 24, 48, 72, or 96 hours, or at about 6, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24, 26, or 28 days after administration. In additional embodiments, the lipid particles (*e.g.*, SNALP) are administered parenterally or intraperitoneally.

[0132] In some embodiments, the lipid particles (*e.g.*, SNALP) are particularly useful in methods for the therapeutic delivery of one or more nucleic acids comprising an interfering RNA sequence (*e.g.*, siRNA). In particular, it is an object of the present disclosure to provide *in vitro* and *in vivo* methods for treatment of a disease or disorder in a mammal (*e.g.,* a rodent such as a mouse or a primate such as a human, chimpanzee, or monkey) by downregulating or silencing the transcription and/or translation of one or more target nucleic acid sequences or genes of interest. As a non-limiting example, the methods described herein are useful for *in vivo* delivery of interfering RNA (*e.g.,* siRNA) to the liver and/or tumor of a mammalian subject. In certain embodiments, the disease or disorder is associated with expression and/or overexpression of a gene and expression or overexpression of the gene is reduced by the interfering RNA (*e.g.*, siRNA). In certain other embodiments, a therapeutically effective amount of the lipid particle (*e.g.*, SNALP) may be administered to the mammal. In some instances, an interfering RNA (*e.g.*, siRNA) is formulated into a SNALP, and the particles are administered to patients requiring such treatment. In other instances, cells are removed from a patient, the interfering RNA (*e.g.,* siRNA) is delivered *in vitro* (*e.g.,* using a SNALP described herein), and the cells are reinjected into the patient.

[0133] In an additional aspect, the present disclosure provides lipid particles (*e.g.*, SNALP) comprising asymmetrical interfering RNA (aiRNA) molecules that silence the expression of a target gene and methods of using such particles to silence target gene expression.

[0134] In one embodiment, the aiRNA molecule comprises a double-stranded (duplex) region of about 10 to about 25 (base paired) nucleotides in length, wherein the aiRNA molecule comprises an antisense strand comprising 5' and 3' overhangs, and wherein the aiRNA molecule is capable of silencing target gene expression.

[0135] In certain instances, the aiRNA molecule comprises a double-stranded (duplex) region of about 12-20, 12-19, 12-18, 13-17, or 14-17 (base paired) nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 (base paired) nucleotides in length. In certain other instances, the 5' and 3' overhangs on the antisense strand comprise sequences that are complementary to the target RNA sequence, and may optionally further comprise nontargeting sequences. In some embodiments, each of the 5' and 3' overhangs on the antisense strand comprises or consists of one, two, three, four, five, six, seven, or more nucleotides.

[0136] In other embodiments, the aiRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0137] In a related aspect, the present disclosure provides lipid particles (*e.g.*, SNALP) comprising microRNA (miRNA) molecules that silence the expression of a target gene and methods of using such compositions to silence target gene expression.

[0138] In one embodiment, the miRNA molecule comprises about 15 to about 60 nucleotides in length, wherein the miRNA molecule is capable of silencing target gene expression.

[0139] In certain instances, the miRNA molecule comprises about 15-50, 15-40, or 15-30 nucleotides in length, more typically about 15-25 or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In a preferred embodiment, the miRNA molecule is a mature miRNA molecule targeting an RNA sequence of interest.

[0140] In some embodiments, the miRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the

2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0141] As such, the lipid particles (*e.g.*, SNALP) are advantageous and suitable for use in the administration of active agents or therapeutic agents such as nucleic acid (*e.g.*, interfering RNA such as siRNA, aiRNA, and/or miRNA) to a subject (*e.g.,* a mammal such as a human) because they are stable in circulation, of a size required for pharmacodynamic behavior resulting in access to extravascular sites, and are capable of reaching target cell populations.

IV. Active Agents

[0142] Active agents (*e.g.*, therapeutic agents) include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be, *e.g.,* biological, physiological, and/or cosmetic. Active agents may be any type of molecule or compound including, but not limited to, nucleic acids, peptides, polypeptides, small molecules, and mixtures thereof. Non-limiting examples of nucleic acids include interfering RNA molecules (*e.g.*, siRNA, aiRNA, miRNA), antisense oligonucleotides, plasmids, ribozymes, immunostimulatory oligonucleotides, and mixtures thereof. Examples of peptides or polypeptides include, without limitation, antibodies (*e.g.*, polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, Primatized™ antibodies), cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell-surface receptors and their ligands, hormones, and mixtures thereof. Examples of small molecules include, but are not limited to, small organic molecules or compounds such as any conventional agent or drug known to those of skill in the art.

[0143] In some embodiments, the active agent is a therapeutic agent, or a salt or derivative thereof. Therapeutic agent derivatives may be therapeutically active themselves or they may be prodrugs, which become active upon further modification. Thus, in one embodiment, a therapeutic agent derivative retains some or all of the therapeutic activity as compared to the unmodified agent, while in another embodiment, a therapeutic agent derivative is a prodrug that lacks therapeutic activity, but becomes active upon further modification.

A. Nucleic Acids

[0144] In certain embodiments, lipid particles of the present disclosure are associated with a nucleic acid, resulting in a nucleic acid-lipid particle (*e.g.*, SNALP). In some embodiments, the nucleic acid is fully encapsulated in the lipid particle. As used herein, the term "nucleic acid" includes any oligonucleotide or polynucleotide, with fragments containing up to 60 nucleotides generally termed oligonucleotides, and longer fragments termed polynucleotides. In particular embodiments, oligonucleotides are from about 15 to about 60 nucleotides in length. Nucleic acid may be administered alone in the lipid particles described herein, or in combination (*e.g.*, co-administered) with lipid particles comprising peptides, polypeptides, or small molecules such as conventional drugs.

[0145] In the context of this invention, the terms "polynucleotide" and "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally-occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also include polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake, reduced immunogenicity, and increased stability in the presence of nucleases.

[0146] Oligonucleotides are generally classified as deoxyribooligonucleotides or ribooligonucleotides. A deoxyribooligonucleotide consists of a 5-carbon sugar called deoxyribose joined covalently to phosphate at the 5' and 3' carbons of this sugar to form an alternating, unbranched polymer. A ribooligonucleotide consists of a similar repeating structure where the 5-carbon sugar is ribose.

[0147] The nucleic acid that is present in a lipid-nucleic acid particle according to the present disclosure includes any form of nucleic acid that is known. The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Examples of double-stranded DNA are described herein and include, *e.g.*, structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA are described herein and include, *e.g.,* siRNA and other RNAi agents such as aiRNA and pre-miRNA. Single-stranded nucleic acids include, *e.g.,* antisense oligonucleotides, ribozymes, mature miRNA, and triplex-forming oligonucleotides.

[0148] Nucleic acids may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to about 100,000 nucleotide residues in length. In particular embodiments, oligonucleotides may range from about 10 to about 100 nucleotides in length. In various related embodiments, oligonucleotides, both single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 60 nucleotides, from about 15 to about 60 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, or from about 20 to about 30 nucleotides in length.

[0149] In particular embodiments, an oligonucleotide (or a strand thereof) specifically hybridizes to or is complementary

to a target polynucleotide sequence. The terms "specifically hybridizable" and "complementary" as used herein indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. In preferred embodiments, an oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target sequence interferes with the normal function of the target sequence to cause a loss of utility or expression therefrom, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.,* under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or, in the case of *in vitro* assays, under conditions in which the assays are conducted. Thus, the oligonucleotide may include 1, 2, 3, or more base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

1. siRNA

[0150] An siRNA component of a nucleic acid-lipid particle of the present invention is capable of silencing the expression of a target gene of interest. Each strand of the siRNA duplex is typically about 15 to about 60 nucleotides in length, preferably about 15 to about 30 nucleotides in length. In certain embodiments, the siRNA comprises at least one modified nucleotide. The modified siRNA is generally less immunostimulatory than a corresponding unmodified siRNA sequence and retains RNAi activity against the target gene of interest. In some embodiments, the modified siRNA contains at least one 2'OMe purine or pyrimidine nucleotide such as a 2'OMe-guanosine, 2'OMe-uridine, 2'OMe-adenosine, and/or 2'OMe-cytosine nucleotide. In preferred embodiments, one or more of the uridine and/or guanosine nucleotides are modified. The modified nucleotides can be present in one strand (*i.e.,* sense or antisense) or both strands of the siRNA. The siRNA sequences may have overhangs (*e.g.,* 3' or 5' overhangs as described in Elbashir et al., Genes Dev., 15:188 (2001) or Nykänen et al., Cell, 107:309 (2001)), or may lack overhangs (*i.e.,* have blunt ends).

[0151] The modified siRNA generally comprises from about 1% to about 100% (*e.g.*, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region of the siRNA duplex. In certain embodiments, one, two, three, four, five, six, seven, eight, nine, ten, or more of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0152] In some embodiments, less than about 25% (*e.g.,* less than about 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0153] In other embodiments, from about 1% to about 25% (*e.g.,* from about 1%-25%, 2%-25%, 3%-25%, 4%-25%, 5%-25%, 6%-25%, 7%-25%, 8%-25%, 9%-25%, 10%-25%, 11%-25%, 12%-25%, 13%-25%, 14%-25%, 15%-25%, 16%-25%, 17%-25%, 18%-25%, 19%-25%, 20%-25%, 21%-25%, 22%-25%, 23%-25%, 24%-25%, *etc.*) or from about 1% to about 20% (*e.g.,* from about 1%-20%, 2%-20%, 3%-20%, 4%-20%, 5%-20%, 6%-20%, 7%-20%, 8%-20%, 9%-20%, 10%-20%, 11%-20%, 12%-20%, 13%-20%, 14%-20%, 15%-20%, 16%-20%, 17%-20%, 18%-20%, 19%-20%, 1%-19%, 2%-19%, 3%-19%, 4%-19%, 5%-19%, 6%-19%, 7%-19%, 8%-19%, 9%-19%, 10%-19%, 11%-19%, 12%-19%, 13%-19%, 14%-19%, 15%-19%, 16%-19%, 17%-19%, 18%-19%, 1%-18%, 2%-18%, 3%-18%, 4%-18%, 5%-18%, 6%-18%, 7%-18%, 8%-18%, 9%-18%, 10%-18%, 11%-18%, 12%-18%, 13%-18%, 14%-18%, 15%-18%, 16%-18%, 17%-18%, 1%-17%, 2%-17%, 3%-17%, 4%-17%, 5%-17%, 6%-17%, 7%-17%, 8%-17%, 9%-17%, 10%-17%, 11%-17%, 12%-17%, 13%-17%, 14%-17%, 15%-17%, 16%-17%, 1%-16%, 2%-16%, 3%-16%, 4%-16%, 5%-16%, 6%-16%, 7%-16%, 8%-16%, 9%-16%, 10%-16%, 11%-16%, 12%-16%, 13%-16%, 14%-16%, 15%-16%, 1%-15%, 2%-15%, 3%-15%, 4%-15%, 5%-15%, 6%-15%, 7%-15%, 8%-15%, 9%-15%, 10%-15%, 11%-15%, 12%-15%, 13%-15%, 14%-15%, *etc.*) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0154] In further embodiments, *e.g.*, when one or both strands of the siRNA are selectively modified at uridine and/or guanosine nucleotides, the resulting modified siRNA can comprise less than about 30% modified nucleotides (*e.g.*, less than about 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% modified nucleotides) or from about 1% to about 30% modified nucleotides (*e.g.*, from about 1%-30%, 2%-30%, 3%-30%, 4%-30%, 5%-30%, 6%-30%, 7%-30%, 8%-30%, 9%-30%, 10%-30%, 11%-30%, 12%-30%, 13%-30%, 14%-30%, 15%-30%, 16%-30%, 17%-30%, 18%-30%, 19%-30%, 20%-30%, 21%-30%, 22%-30%, 23%-30%, 24%-30%, 25%-30%, 26%-30%, 27%-30%, 28%-30%, or 29%-30% modified nucleotides).

a. **Selection of siRNA Sequences**

[0155] Suitable siRNA sequences can be identified using any means known in the art. Typically, the methods described in Elbashir et al., Nature, 411:494-498 (2001) and Elbashir et al., EMBO J., 20:6877-6888 (2001) are combined with rational design rules set forth in Reynolds et al., Nature Biotech., 22(3):326-330 (2004).

[0156] Generally, the nucleotide sequence 3' of the AUG start codon of a transcript from the target gene of interest is scanned for dinucleotide sequences (*e.g.*, AA, NA, CC, GG, or UU, wherein N = C, G, or U) (*see, e.g.,* Elbashir et al., EMBO J., 20:6877-6888 (2001)). The nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences (*i.e.,* a target sequence or a sense strand sequence). Typically, the 19, 21, 23, 25, 27, 29, 31, 33, 35, or more nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences. In some embodiments, the dinucleotide sequence is an AA or NA sequence and the 19 nucleotides immediately 3' to the AA or NA dinucleotide are identified as potential siRNA sequences. siRNA sequences are usually spaced at different positions along the length of the target gene. To further enhance silencing efficiency of the siRNA sequences, potential siRNA sequences may be analyzed to identify sites that do not contain regions of homology to other coding sequences, *e.g.*, in the target cell or organism. For example, a suitable siRNA sequence of about 21 base pairs typically will not have more than 16-17 contiguous base pairs of homology to coding sequences in the target cell or organism. If the siRNA sequences are to be expressed from an RNA Pol III promoter, siRNA sequences lacking more than 4 contiguous A's or T's are selected.

[0157] Once a potential siRNA sequence has been identified, a complementary sequence (*i.e.*, an antisense strand sequence) can be designed. A potential siRNA sequence can also be analyzed using a variety of criteria known in the art. For example, to enhance their silencing efficiency, the siRNA sequences may be analyzed by a rational design algorithm to identify sequences that have one or more of the following features: (1) G/C content of about 25% to about 60% G/C; (2) at least 3 A/Us at positions 15-19 of the sense strand; (3) no internal repeats; (4) an A at position 19 of the sense strand; (5) an A at position 3 of the sense strand; (6) a U at position 10 of the sense strand; (7) no G/C at position 19 of the sense strand; and (8) no G at position 13 of the sense strand. siRNA design tools that incorporate algorithms that assign suitable values of each of these features and are useful for selection of siRNA can be found at, e.g., http://boz094.ust.hk/RNAi/siRNA. One of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may be selected for further analysis and testing as potential siRNA sequences.

[0158] Additionally, potential siRNA sequences with one or more of the following criteria can often be eliminated as siRNA: (1) sequences comprising a stretch of 4 or more of the same base in a row; (2) sequences comprising homopolymers of Gs (*i.e.,* to reduce possible non-specific effects due to structural characteristics of these polymers; (3) sequences comprising triple base motifs (*e.g.*, GGG, CCC, AAA, or TTT); (4) sequences comprising stretches of 7 or more G/Cs in a row; and (5) sequences comprising direct repeats of 4 or more bases within the candidates resulting in internal fold-back structures. However, one of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may still be selected for further analysis and testing as potential siRNA sequences.

[0159] In some embodiments, potential siRNA sequences may be further analyzed based on siRNA duplex asymmetry as described in, *e.g.,* Khvorova et al., Cell, 115:209-216 (2003); and Schwarz et al., Cell, 115:199-208 (2003). In other embodiments, potential siRNA sequences may be further analyzed based on secondary structure at the target site as described in, *e.g.,* Luo et al., Biophys. Res. Commun., 318:303-310 (2004). For example, secondary structure at the target site can be modeled using the Mfold algorithm (available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) to select siRNA sequences which favor accessibility at the target site where less secondary structure in the form of base-pairing and stem-loops is present.

[0160] Once a potential siRNA sequence has been identified, the sequence can be analyzed for the presence of any immunostimulatory properties, *e.g.,* using an *in vitro* cytokine assay or an *in vivo* animal model. Motifs in the sense and/or antisense strand of the siRNA sequence such as GU-rich motifs (*e.g.*, 5'-GU-3', 5'-UGU-3', 5'-GUGU-3', 5'-UGUGU-3', *etc.*) can also provide an indication of whether the sequence may be immunostimulatory. Once an siRNA molecule is found to be immunostimulatory, it can then be modified to decrease its immunostimulatory properties as described herein. As a non-limiting example, an siRNA sequence can be contacted with a mammalian responder cell under conditions such that the cell produces a detectable immune response to determine whether the siRNA is an immunostimulatory or a non-immunostimulatory siRNA. The mammalian responder cell may be from a naive mammal (*i.e.*, a mammal that has not previously been in contact with the gene product of the siRNA sequence). The mammalian responder cell may be, *e.g.,* a peripheral blood mononuclear cell (PBMC), a macrophage, and the like. The detectable immune response may comprise production of a cytokine or growth factor such as, *e.g.,* TNF-α, IFN-α, IFN-β, IFN-γ, IL-6, IL-12, or a combination thereof. An siRNA molecule identified as being immunostimulatory can then be modified to decrease its immunostimulatory properties by replacing at least one of the nucleotides on the sense and/or antisense strand with modified nucleotides. For example, less than about 30% *(e.g.,* less than about 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA duplex can be replaced with modified nucleotides such as 2'OMe nucleotides. The modified siRNA can then be contacted with a mammalian responder cell as described above to confirm that its immunostimulatory properties have been reduced or abrogated.

[0161] Suitable *in vitro* assays for detecting an immune response include, but are not limited to, the double monoclonal antibody sandwich immunoassay technique of David et al. (U.S. Patent No. 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide *et al.*, in Kirkham and Hunter, eds., Radioimmunoassay Methods, E. and S. Livingstone, Edinburgh (1970)); the "Western blot" method of Gordon et al. (U.S. Patent No. 4,452,901); immunoprecipitation of labeled

ligand (Brown et al., J. Biol. Chem., 255:4980-4983 (1980)); enzyme-linked immunosorbent assays (ELISA) as described, for example, by Raines et al., J. Biol. Chem., 257:5154-5160 (1982); immunocytochemical techniques, including the use of fluorochromes (Brooks et al., Clin. Exp. Immunol., 39:477 (1980)); and neutralization of activity (Bowen-Pope et al., Proc. Natl. Acad. Sci. USA, 81:2396-2400 (1984)). In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S. Patent Nos. 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876.

[0162] A non-limiting example of an *in vivo* model for detecting an immune response includes an *in vivo* mouse cytokine induction assay as described in, *e.g.,* Judge et al., Mol. Ther., 13:494-505 (2006). In certain embodiments, the assay that can be performed as follows: (1) siRNA can be administered by standard intravenous injection in the lateral tail vein; (2) blood can be collected by cardiac puncture about 6 hours after administration and processed as plasma for cytokine analysis; and (3) cytokines can be quantified using sandwich ELISA kits according to the manufacturer's instructions (*e.g*., mouse and human IFN-α (PBL Biomedical; Piscataway, NJ); human IL-6 and TNF-α (eBioscience; San Diego, CA); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences; San Diego, CA)).

[0163] Monoclonal antibodies that specifically bind cytokines and growth factors are commercially available from multiple sources and can be generated using methods known in the art *(see, e.g.,* Kohler et al., Nature, 256: 495-497 (1975) and Harlow and Lane, ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publication, New York (1999)). Generation of monoclonal antibodies has been previously described and can be accomplished by any means known in the art (Buhring et al., in Hybridoma, Vol. 10, No. 1, pp. 77-78 (1991)). In some methods, the monoclonal antibody is labeled (*e.g*., with any composition detectable by spectroscopic, photochemical, biochemical, electrical, optical, or chemical means) to facilitate detection.

b. Generating siRNA Molecules

[0164] siRNA can be provided in several forms including, *e.g*., as one or more isolated small-interfering RNA (siRNA) duplexes, as longer double-stranded RNA (dsRNA), or as siRNA or dsRNA transcribed from a transcriptional cassette in a DNA plasmid. The siRNA sequences may have overhangs (*e.g.,* 3' or 5' overhangs as described in Elbashir et al., Genes Dev., 15:188 (2001) or Nykänen et al., Cell, 107:309 (2001), or may lack overhangs (*i.e.,* to have blunt ends).

[0165] An RNA population can be used to provide long precursor RNAs, or long precursor RNAs that have substantial or complete identity to a selected target sequence can be used to make the siRNA. The RNAs can be isolated from cells or tissue, synthesized, and/or cloned according to methods well known to those of skill in the art. The RNA can be a mixed population (obtained from cells or tissue, transcribed from cDNA, subtracted, selected, *etc.),* or can represent a single target sequence. RNA can be naturally occurring (*e.g*., isolated from tissue or cell samples), synthesized *in vitro* (*e.g.,* using T7 or SP6 polymerase and PCR products or a cloned cDNA), or chemically synthesized.

[0166] To form a long dsRNA, for synthetic RNAs, the complement is also transcribed *in vitro* and hybridized to form a dsRNA. If a naturally occuring RNA population is used, the RNA complements are also provided (*e.g.,* to form dsRNA for digestion by *E. coli* RNAse III or Dicer), *e.g.,* by transcribing cDNAs corresponding to the RNA population, or by using RNA polymerases. The precursor RNAs are then hybridized to form double stranded RNAs for digestion. The dsRNAs can be directly administered to a subject or can be digested *in vitro* prior to administration.

[0167] Methods for isolating RNA, synthesizing RNA, hybridizing nucleic acids, making and screening cDNA libraries, and performing PCR are well known in the art (*see, e.g.,* Gubler and Hoffman, Gene, 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*), as are PCR methods (*see,* U.S. Patent Nos. 4,683,195 and 4,683,202; PCR Protocols: A Guide to Methods and Applications (Innis et al., eds, 1990)). Expression libraries are also well known to those of skill in the art. Additional basic texts disclosing the general methods of use in this invention include Sambrook et al., Molecular Cloning, A Laboratory Manual (2nd ed. 1989); Kriegler, Gene Transfer and Expression: A Laboratory Manual (1990); and Current Protocols in Molecular Biology (Ausubel et al., eds., 1994).

[0168] Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules described herein can be synthesized using any of a variety of techniques known in the art, such as those described in Usman et al., J. Am. Chem. Soc., 109:7845 (1987); Scaringe et al., Nucl. Acids Res., 18:5433 (1990); Wincott et al., Nucl. Acids Res., 23:2677-2684 (1995); and Wincott et al., Methods Mol. Bio., 74:59 (1997). The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example, small scale syntheses can be conducted on an Applied Biosystems synthesizer using a 0.2 μmol scale protocol. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer from Protogene (Palo Alto, CA). However, a larger or smaller scale of synthesis is also within the scope of this disclosure. Suitable reagents for oligonucleotide synthesis, methods for RNA deprotection, and methods for RNA purification are known to those of skill in the art.

[0169] siRNA molecules can also be synthesized via a tandem synthesis technique, wherein both strands are synthesized as a single continuous oligonucleotide fragment or strand separated by a cleavable linker that is subsequently cleaved to provide separate fragments or strands that hybridize to form the siRNA duplex. The linker can be a polynu-

cleotide linker or a non-nucleotide linker. The tandem synthesis of siRNA can be readily adapted to both multiwell/multiplate synthesis platforms as well as large scale synthesis platforms employing batch reactors, synthesis columns, and the like. Alternatively, siRNA molecules can be assembled from two distinct oligonucleotides, wherein one oligonucleotide comprises the sense strand and the other comprises the antisense strand of the siRNA. For example, each strand can be synthesized separately and joined together by hybridization or ligation following synthesis and/or deprotection. In certain other instances, siRNA molecules can be synthesized as a single continuous oligonucleotide fragment, where the self-complementary sense and antisense regions hybridize to form an siRNA duplex having hairpin secondary structure.

c. Modifying siRNA Sequences

[0170] In certain aspects, siRNA molecules comprise a duplex having two strands and at least one modified nucleotide in the double-stranded region, wherein each strand is about 15 to about 60 nucleotides in length. Advantageously, the modified siRNA is less immunostimulatory than a corresponding unmodified siRNA sequence, but retains the capability of silencing the expression of a target sequence. In preferred embodiments, the degree of chemical modifications introduced into the siRNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the siRNA and retention of RNAi activity. As a non-limiting example, an siRNA molecule that targets a gene of interest can be minimally modified (*e.g.,* less than about 30%, 25%, 20%, 15%, 10%, or 5% modified) at selective uridine and/or guanosine nucleotides within the siRNA duplex to eliminate the immune response generated by the siRNA while retaining its capability to silence target gene expression.

[0171] Examples of modified nucleotides suitable for use in the invention include, but are not limited to, ribonucleotides having a 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl group. Modified nucleotides having a Northern conformation such as those described in, *e.g.,* Saenger, Principles of Nucleic Acid Structure, Springer-Verlag Ed. (1984), are also suitable for use in siRNA molecules. Such modified nucleotides include, without limitation, locked nucleic acid (LNA) nucleotides (*e.g.*, 2'-O, 4'-C-methylene-(D-ribofuranosy nucleotides), 2'-O-(2-methoxyethyl) (MOE) nucleotides, 2'-methyl-thio-ethyl nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy-2'-chloro (2'Cl) nucleotides, and 2'-azido nucleotides. In certain instances, the siRNA molecules described herein include one or more G-clamp nucleotides. A G-clamp nucleotide refers to a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine nucleotide within a duplex (*see, e.g.,* Lin et al., J. Am. Chem. Soc., 120:8531-8532 (1998)). In addition, nucleotides having a nucleotide base analog such as, for example, C-phenyl, C-naphthyl, other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole (*see, e.g.,* Loakes, Nucl. Acids Res., 29:2437-2447 (2001)) can be incorporated into siRNA molecules.

[0172] In certain embodiments, siRNA molecules may further comprise one or more chemical modifications such as terminal cap moieties, phosphate backbone modifications, and the like. Examples of terminal cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4',5'-methylene nucleotides, 1-(β-D-erythrofuranosyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α-nucleotides, modified base nucleotides, *threo*-pentofuranosyl nucleotides, acyclic 3',4'-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3'-3'-inverted nucleotide moieties, 3'-3'-inverted abasic moieties, 3'-2'-inverted nucleotide moieties, 3'-2'-inverted abasic moieties, 5'-5'-inverted nucleotide moieties, 5'-5'-inverted abasic moieties, 3'-5'-inverted deoxy abasic moieties, 5'-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, 6-aminohexyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3'-phosphoramidate, 5'-phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 5'-amino, 3'-phosphorothioate, 5'-phosphorothioate, phosphorodithioate, and bridging or non-bridging methylphosphonate or 5'-mercapto moieties (*see, e.g.,* U.S. Patent No. 5,998,203; Beaucage et al., Tetrahedron 49:1925 (1993)). Non-limiting examples of phosphate backbone modifications (*i.e*., resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and alkylsilyl substitutions (*see, e.g.,* Hunziker et al., Nucleic Acid Analogues: Synthesis and Properties, in Modern Synthetic Methods, VCH, 331-417 (1995); Mesmaeker et al., Novel Backbone Replacements for Oligonucleotides, in Carbohydrate Modifications in Antisense Research, ACS, 24-39 (1994)). Such chemical modifications can occur at the 5'-end and/or 3'-end of the sense strand, antisense strand, or both strands of the siRNA.

[0173] In some embodiments, the sense and/or antisense strand of the siRNA molecule can further comprise a 3'-terminal overhang having about 1 to about 4 (*e.g.,* 1, 2, 3, or 4) 2'-deoxy ribonucleotides and/or any combination of modified and unmodified nucleotides. Additional examples of modified nucleotides and types of chemical modifications that can be introduced into siRNA molecules are described, *e.g*., in UK Patent No. GB 2,397,818 B and U.S. Patent Publication Nos. 20040192626, 20050282188, and 20070135372.

[0174] The siRNA molecules described herein can optionally comprise one or more non-nucleotides in one or both

strands of the siRNA. As used herein, the term "non-nucleotide" refers to any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base such as adenosine, guanine, cytosine, uracil, or thymine and therefore lacks a base at the 1'-position.

[0175] In other embodiments, chemical modification of the siRNA comprises attaching a conjugate to the siRNA molecule. The conjugate can be attached at the 5' and/or 3'-end of the sense and/or antisense strand of the siRNA via a covalent attachment such as, *e.g.,* a biodegradable linker. The conjugate can also be attached to the siRNA, *e.g.*, through a carbamate group or other linking group (*see, e.g.,* U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). In certain instances, the conjugate is a molecule that facilitates the delivery of the siRNA into a cell. Examples of conjugate molecules suitable for attachment to siRNA include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (*e.g.,* folic acid, folate analogs and derivatives thereof), sugars (*e.g.*, galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, *etc*.), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof (*see, e.g.,* U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Patent No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule, oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Yet other examples include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidininium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples include the hydrophobic group, membrane active compound, cell penetrating compound, cell targeting signal, interaction modifier, and steric stabilizer conjugate molecules described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described in U.S. Patent Publication No. 20050239739. The type of conjugate used and the extent of conjugation to the siRNA molecule can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the siRNA while retaining RNAi activity. As such, one skilled in the art can screen siRNA molecules having various conjugates attached thereto to identify ones having improved properties and full RNAi activity using any of a variety of well-known *in vitro* cell culture or *in vivo* animal models.

d. Target Genes

[0176] The siRNA component of the nucleic acid-lipid particles described herein can be used to downregulate or silence the translation (*i.e*., expression) of a gene of interest. Genes of interest include, but are not limited to, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders (*e.g*., liver diseases and disorders), genes associated with tumorigenesis and cell transformation (*e.g*., cancer), angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0177] Genes associated with viral infection and survival include those expressed by a virus in order to bind, enter, and replicate in a cell. Of particular interest are viral sequences associated with chronic viral diseases. Viral sequences of particular interest include sequences of Filoviruses such as Ebola virus and Marburg virus (*see, e.g.,* Geisbert et al., J. Infect. Dis., 193:1650-1657 (2006)); Arenaviruses such as Lassa virus, Junin virus, Machupo virus, Guanarito virus, and Sabia virus (Buchmeier et al., Arenaviridae: the viruses and their replication, In: FIELDS VIROLOGY, Knipe et al. (eds.), 4th ed., Lippincott-Raven, Philadelphia, (2001)); Influenza viruses such as Influenza A, B, and C viruses, (*see, e.g.,* Steinhauer et al., Annu Rev Genet., 36:305-332 (2002); and Neumann et al., J Gen Virol., 83:2635-2662 (2002)); Hepatitis viruses (*see, e.g.,* Hamasaki et al., FEBS Lett., 543:51 (2003); Yokota et al., EMBO Rep., 4:602 (2003); Schlomai et al., Hepatology, 37:764 (2003); Wilson et al., Proc. Natl. Acad. Sci. USA, 100:2783 (2003); Kapadia et al., Proc. Natl. Acad. Sci. USA, 100:2014 (2003); and FIELDS VIROLOGY, Knipe et al. (eds.), 4th ed., Lippincott-Raven, Philadelphia (2001)); Human Immunodeficiency Virus (HIV) (Banerjea et al., Mol. Ther., 8:62 (2003); Song et al., J. Virol., 77:7174 (2003); Stephenson, JAMA, 289:1494 (2003); Qin et al., Proc. Natl. Acad. Sci. USA, 100:183 (2003)); Herpes viruses (Jia et al., J. Virol., 77:3301 (2003)); and Human Papilloma Viruses (HPV) (Hall et al., J. Virol., 77:6066 (2003); Jiang et al., Oncogene, 21:6041 (2002)).

[0178] Exemplary Filovirus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding structural proteins (*e.g*., VP30, VP35, nucleoprotein (NP), polymerase protein (L-pol)) and membrane-associated proteins (*e.g*., VP40, glycoprotein (GP), VP24). Complete genome sequences for Ebola virus are set forth in, e.g., Genbank Accession Nos. NC_002549; AY769362; NC_006432; NC_004161; AY729654; AY354458; AY142960; AB050936; AF522874; AF499101; AF272001; and AF086833. Ebola virus VP24 sequences are set forth in, *e.g*., Genbank Accession Nos. U77385 and AY058897. Ebola virus L-pol sequences are set forth in, *e.g*., Genbank Accession No. X67110. Ebola virus VP40 sequences are set forth in, *e.g*., Genbank Accession No. AY058896. Ebola

virus NP sequences are set forth in, *e.g.*, Genbank Accession No. AY058895. Ebola virus GP sequences are set forth in, *e.g.*, Genbank Accession No. AY058898; Sanchez et al., Virus Res., 29:215-240 (1993); Will et al., J. Virol., 67:1203-1210 (1993); Volchkov et al., FEBS Lett., 305:181-184 (1992); and U.S. Patent No. 6,713,069. Additional Ebola virus sequences are set forth in, *e.g.*, Genbank Accession Nos. L11365 and X61274. Complete genome sequences for Marburg virus are set forth in, e.g., Genbank Accession Nos. NC_001608; AY430365; AY430366; and AY358025. Marburg virus GP sequences are set forth in, e.g., Genbank Accession Nos. AF005734; AF005733; and AF005732. Marburg virus VP35 sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005731 and AF005730. Additional Marburg virus sequences are set forth in, *e.g.*, Genbank Accession Nos. X64406; Z29337; AF005735; and Z12132. Non-limiting examples of siRNA molecules targeting Ebola virus and Marburg virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070135370.

[0179] Exemplary Influenza virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding nucleoprotein (NP), matrix proteins (M1 and M2), nonstructural proteins (NS1 and NS2), RNA polymerase (PA, PB1, PB2), neuraminidase (NA), and haemagglutinin (HA). Influenza A NP sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004522; AY818138; AB166863; AB188817; AB189046; AB189054; AB189062; AY646169; AY646177; AY651486; AY651493; AY651494; AY651495; AY651496; AY651497; AY651498; AY651499; AY651500; AY651501; AY651502; AY651503; AY651504; AY651505; AY651506; AY651507; AY651509; AY651528; AY770996; AY790308; AY818138; and AY818140. Influenza A PA sequences are set forth in, *e.g.*, Genbank Accession Nos. AY818132; AY790280; AY646171; AY818132;AY818133; AY646179; AY818134; AY551934; AY651613; AY651610; AY651620; AY651617; AY651600; AY651611; AY651606; AY651618; AY651608; AY651607; AY651605; AY651609; AY651615; AY651616; AY651640; AY651614; AY651612; AY651621; AY651619; AY770995; and AY724786. Non-limiting examples of siRNA molecules targeting Influenza virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070218122.

[0180] Exemplary hepatitis virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences involved in transcription and translation (*e.g.*, En1, En2, X, P) and nucleic acid sequences encoding structural proteins (*e.g.*, core proteins including C and C-related proteins, capsid and envelope proteins including S, M, and/or L proteins, or fragments thereof) (*see, e.g.*, FIELDS VIROLOGY, *supra*). Exemplary Hepatits C virus (HCV) nucleic acid sequences that can be silenced include, but are not limited to, the 5'-untranslated region (5'-UTR), the 3'-untranslated region (3'-UTR), the polyprotein translation initiation codon region, the internal ribosome entry site (IRES) sequence, and/or nucleic acid sequences encoding the core protein, the E1 protein, the E2 protein, the p7 protein, the NS2 protein, the NS3 protease/helicase, the NS4A protein, the NS4B protein, the NS5A protein, and/or the NS5B RNA-dependent RNA polymerase. HCV genome sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004102 (HCV genotype 1a), AJ238799 (HCV genotype 1b), NC_009823 (HCV genotype 2), NC_009824 (HCV genotype 3), NC_009825 (HCV genotype 4), NC_009826 (HCV genotype 5), and NC_009827 (HCV genotype 6). Hepatitis A virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001489; Hepatitis B virus nucleic acid sequences are set forth in, *e.g.,* Genbank Accession No. NC_003977; Hepatitis D virus nucleic acid sequence are set forth in, *e.g.*, Genbank Accession No. NC_001653; Hepatitis E virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001434; and Hepatitis G virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001710. Silencing of sequences that encode genes associated with viral infection and survival can conveniently be used in combination with the administration of conventional agents used to treat the viral condition. Non-limiting examples of siRNA molecules targeting hepatitis virus nucleic acid sequences include those described in U.S. Patent Publication Nos. 20060281175, 20050058982, and 20070149470; U.S. Patent No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed March 20, 2009.

[0181] Genes associated with metabolic diseases and disorders (*e.g.*, disorders in which the liver is the target and liver diseases and disorders) include, for example, genes expressed in dyslipidemia (*e.g.*, liver X receptors such as LXRα and LXRβ (Genback Accession No. NM_007121), farnesoid X receptors (FXR) (Genbank Accession No. NM_005123), sterol-regulatory element binding protein (SREBP), site-1 protease (SIP), 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG coenzyme-A reductase), apolipoprotein B (ApoB) (Genbank Accession No. NM_000384), apolipoprotein CIII (ApoC3) (Genbank Accession Nos. NM_000040 and NG_008949 REGION: 5001..8164), and apolipoprotein E (ApoE) (Genbank Accession Nos. NM_000041 and NG_007084 REGION: 5001..8612)); and diabetes *(e.g.,* glucose 6-phosphatase) *(see, e.g.,* Forman et al., Cell, 81:687 (1995); Seol et al., Mol. Endocrinol., 9:72 (1995), Zavacki et al., Proc. Natl. Acad. Sci. USA, 94:7909 (1997); Sakai et al., Cell, 85:1037-1046 (1996); Duncan et al., J. Biol. Chem., 272:12778-12785 (1997); Willy et al., Genes Dev., 9:1033-1045 (1995); Lehmann et al., J. Biol. Chem., 272:3137-3140 (1997); Janowski et al., Nature, 383:728-731 (1996); and Peet et al., Cell, 93:693-704 (1998)). One of skill in the art will appreciate that genes associated with metabolic diseases and disorders (*e.g.*, diseases and disorders in which the liver is a target and liver diseases and disorders) include genes that are expressed in the liver itself as well as and genes expressed in other organs and tissues. Silencing of sequences that encode genes associated with metabolic diseases and disorders can conveniently be used in combination with the administration of conventional agents used to treat the disease or disorder. Non-limiting examples of siRNA molecules targeting the ApoB gene include those described in U.S.

Patent Publication No. 20060134189. Non-limiting examples of siRNA molecules targeting the ApoC3 gene include those described in U.S. Provisional Application No. 61/147,235, filed January 26, 2009.

[0182] Examples of gene sequences associated with tumorigenesis and cell transformation *(e.g.,* cancer or other neoplasia) include mitotic kinesins such as Eg5 (KSP, KIF11; Genbank Accession No. NM_004523); serine/threonine kinases such as polo-like kinase 1 (PLK-1) (Genbank Accession No. NM_005030; Barr et al., Nat. Rev. Mol. Cell Biol., 5:429-440 (2004)); tyrosine kinases such as WEE1 (Genbank Accession Nos. NM_003390 and NM_001143976); inhibitors of apoptosis such as XIAP (Genbank Accession No. NM_001167); COP9 signalosome subunits such as CSN1, CSN2, CSN3, CSN4, CSN5 (JAB1; Genbank Accession No. NM_006837); CSN6, CSN7A, CSN7B, and CSN8; ubiquitin ligases such as COP1 (RFWD2; Genbank Accession Nos. NM_022457 and NM_001001740); and histone deacetylases such as HDAC1, HDAC2 (Genbank Accession No. NM_001527), HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, *etc.* Non-limiting examples of siRNA molecules targeting the Eg5 and XIAP genes include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007. Non-limiting examples of siRNA molecules targeting the PLK-1 gene include those described in U.S. Patent Publication Nos. 20050107316 and 20070265438; and U.S. Patent Application No. 12/343,342, filed December 23, 2008. Non-limiting examples of siRNA molecules targeting the CSN5 gene include those described in U.S. Provisional Application No. 61/045,251, filed April 15, 2008p.

[0183] Additional examples of gene sequences associated with tumorigenesis and cell transformation include translocation sequences such as MLL fusion genes, BCR-ABL (Wilda et al., Oncogene, 21:5716 (2002); Scherr et al., Blood, 101:1566 (2003)), TEL-AML1, EWS-FLI1, TLS-FUS, PAX3-FKHR, BCL-2, AML1-ETO, and AML1-MTG8 (Heidenreich et al., Blood, 101:3157 (2003)); overexpressed sequences such as multidrug resistance genes (Nieth et al., FEBS Lett., 545:144 (2003); Wu et al, Cancer Res. 63:1515 (2003)), cyclins (Li et al., Cancer Res., 63:3593 (2003); Zou et al., Genes Dev., 16:2923 (2002)), beta-catenin (Verma et al., Clin Cancer Res., 9:1291 (2003)), telomerase genes (Kosciolek et al., Mol Cancer Ther., 2:209 (2003)), c-MYC, N-MYC, BCL-2, growth factor receptors (*e.g.,* EGFR/ErbB1 (Genbank Accession Nos. NM_005228, NM_201282, NM_201283, and NM_201284; *see also,* Nagy et al. Exp. Cell Res., 285:39-49 (2003), ErbB2/HER-2 (Genbank Accession Nos. NM_004448 and NM_001005862), ErbB3 (Genbank Accession Nos. NM_001982 and NM_001005915), and ErbB4 (Genbank Accession Nos. NM_005235 and NM_001042599); and mutated sequences such as RAS (reviewed in Tuschl and Borkhardt, Mol. Interventions, 2:158 (2002)). Non-limiting examples of siRNA molecules targeting the EGFR gene include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007.

[0184] Silencing of sequences that encode DNA repair enzymes find use in combination with the administration of chemotherapeutic agents (Collis et al., Cancer Res., 63:1550 (2003)). Genes encoding proteins associated with tumor migration are also target sequences of interest, for example, integrins, selectins, and metalloproteinases. The foregoing examples are not exclusive. Those of skill in the art will understand that any whole or partial gene sequence that facilitates or promotes tumorigenesis or cell transformation, tumor growth, or tumor migration can be included as a template sequence.

[0185] Angiogenic genes are able to promote the formation of new vessels. Of particular interest is vascular endothelial growth factor (VEGF) (Reich et al., Mol. Vis., 9:210 (2003)) or VEGFR. siRNA sequences that target VEGFR are set forth in, *e.g.,* GB 2396864; U.S. Patent Publication No. 20040142895; and CA 2456444.

[0186] Anti-angiogenic genes are able to inhibit neovascularization. These genes are particularly useful for treating those cancers in which angiogenesis plays a role in the pathological development of the disease. Examples of anti-angiogenic genes include, but are not limited to, endostatin (*see, e.g.,* U.S. Patent No. 6,174,861), angiostatin (*see, e.g.,* U.S. Patent No. 5,639,725), and VEGFR2 (*see, e.g.,* Decaussin et al., J. Pathol., 188: 369-377 (1999)).

[0187] Immunomodulator genes are genes that modulate one or more immune responses. Examples of immunomodulator genes include, without limitation, cytokines such as growth factors (*e.g.*, TGF-α, TGF-□β, EGF, FGF, IGF, NGF, PDGF, CGF, GM-CSF, SCF, *etc.*), interleukins (*e.g.*, IL-2, IL-4, IL-12 (Hill et al., J. Immunol., 171:691 (2003)), IL-15, IL-18, IL-20, *etc.*), interferons (*e.g.*, IFN-α, IFN-β, IFN-γ, *etc.*) and TNF. Fas and Fas ligand genes are also immunomodulator target sequences of interest (Song et al., Nat. Med., 9:347 (2003)). Genes encoding secondary signaling molecules in hematopoietic and lymphoid cells are also included in the present disclosure, for example, Tec family kinases such as Bruton's tyrosine kinase (Btk) (Heinonen et al., FEBS Lett., 527:274 (2002)).

[0188] Cell receptor ligands include ligands that are able to bind to cell surface receptors (*e.g.*, insulin receptor, EPO receptor, G-protein coupled receptors, receptors with tyrosine kinase activity, cytokine receptors, growth factor receptors, *etc.*), to modulate (*e.g.*, inhibit, activate, *etc.*) the physiological pathway that the receptor is involved in (*e.g.*, glucose level modulation, blood cell development, mitogenesis, *etc.*). Examples of cell receptor ligands include, but are not limited to, cytokines, growth factors, interleukins, interferons, erythropoietin (EPO), insulin, glucagon, G-protein coupled receptor ligands, *etc.* Templates coding for an expansion of trinucleotide repeats (*e.g.*, CAG repeats) find use in silencing pathogenic sequences in neurodegenerative disorders caused by the expansion of trinucleotide repeats, such as spinobulbular muscular atrophy and Huntington's Disease (Caplen et al., Hum. Mol. Genet., 11:175 (2002)).

[0189] In addition to its utility in silencing the expression of any of the above-described genes for therapeutic purposes, the siRNA described herein are also useful in research and development applications as well as diagnostic, prophylactic,

prognostic, clinical, and other healthcare applications. As a non-limiting example, the siRNA can be used in target validation studies directed at testing whether a gene of interest has the potential to be a therapeutic target. The siRNA can also be used in target identification studies aimed at discovering genes as potential therapeutic targets.

2. aiRNA

[0190] Like siRNA, asymmetrical interfering RNA (aiRNA) can recruit the RNA-induced silencing complex (RISC) and lead to effective silencing of a variety of genes in mammalian cells by mediating sequence-specific cleavage of the target sequence between nucleotide 10 and 11 relative to the 5' end of the antisense strand (Sun et al., Nat. Biotech., 26:1379-1382 (2008)). Typically, an aiRNA molecule comprises a short RNA duplex having a sense strand and an antisense strand, wherein the duplex contains overhangs at the 3' and 5' ends of the antisense strand. The aiRNA is generally asymmetric because the sense strand is shorter on both ends when compared to the complementary antisense strand. In some aspects, aiRNA molecules may be designed, synthesized, and annealed under conditions similar to those used for siRNA molecules. As a non-limiting example, aiRNA sequences may be selected and generated using the methods described above for selecting siRNA sequences.

[0191] In another embodiment, aiRNA duplexes of various lengths (*e.g.*, about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 base pairs, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 base pairs) may be designed with overhangs at the 3' and 5' ends of the antisense strand to target an mRNA of interest. In certain instances, the sense strand of the aiRNA molecule is about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length. In certain other instances, the antisense strand of the aiRNA molecule is about 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 nucleotides in length.

[0192] In some embodiments, the 5' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*, "AA", "UU", "dTdT", *etc*.). In other embodiments, the 3' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*, "AA", "UU", "dTdT", *etc*.). In certain aspects, the aiRNA molecules described herein may comprise one or more modified nucleotides, *e.g.*, in the double-stranded (duplex) region and/or in the antisense overhangs. As a non-limiting example, aiRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0193] In certain embodiments, aiRNA molecules may comprise an antisense strand which corresponds to the antisense strand of an siRNA molecule, *e.g.*, one of the siRNA molecules described herein. In other embodiments, aiRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.*, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

3. miRNA

[0194] Generally, microRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. miRNAs are encoded by genes from whose DNA they are transcribed, but miRNAs are not translated into protein (non-coding RNA); instead, each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional mature miRNA. Mature miRNA molecules are either partially or completely complementary to one or more messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression. The identification of miRNA molecules is described, *e.g.,* in Lagos-Quintana et al., Science, 294:853-858; Lau et al., Science, 294:858-862; and Lee et al., Science, 294:862-864.

[0195] The genes encoding miRNA are much longer than the processed mature miRNA molecule. miRNA are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail and processed to short, ~70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha (Denli et al., Nature, 432:231-235 (2004)). These pre-miRNA are then processed to mature miRNA in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC) (Bernstein et al., Nature, 409:363-366 (2001). Either the sense strand or antisense strand of DNA can function as templates to give rise to miRNA.

[0196] When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC complex. This strand is known as the guide strand and is selected by the argonaute protein, the catalytically active RNase in the RISC complex, on the basis of the stability of the 5' end (Preall et al., Curr. Biol., 16:530-535 (2006)). The remaining strand, known as the anti-guide or passenger strand, is degraded as a RISC

complex substrate (Gregory et al., Cell, 123:631-640 (2005)). After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and induce target mRNA degradation and/or translational silencing.

[0197] Mammalian miRNA molecules are usually complementary to a site in the 3' UTR of the target mRNA sequence. In certain instances, the annealing of the miRNA to the target mRNA inhibits protein translation by blocking the protein translation machinery. In certain other instances, the annealing of the miRNA to the target mRNA facilitates the cleavage and degradation of the target mRNA through a process similar to RNA interference (RNAi). miRNA may also target methylation of genomic sites which correspond to targeted mRNA. Generally, miRNA function in association with a complement of proteins collectively termed the miRNP.

[0198] In certain aspects, the miRNA molecules described herein are about 15-100, 15-90, 15-80, 15-75, 15-70, 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In certain other aspects, miRNA molecules may comprise one or more modified nucleotides. As a non-limiting example, miRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0199] In some embodiments, miRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.,* genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0200] In other embodiments, one or more agents that block the activity of a miRNA targeting an mRNA of interest are administered using a lipid particle described herein (*e.g.,* a nucleic acid-lipid particle). Examples of blocking agents include, but are not limited to, steric blocking oligonucleotides, locked nucleic acid oligonucleotides, and Morpholino oligonucleotides. Such blocking agents may bind directly to the miRNA or to the miRNA binding site on the target mRNA.

4. Antisense Oligonucleotides

[0201] In one embodiment, the nucleic acid is an antisense oligonucleotide directed to a target gene or sequence of interest. The terms "antisense oligonucleotide" or "antisense" include oligonucleotides that are complementary to a targeted polynucleotide sequence. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence. Antisense RNA oligonucleotides prevent the translation of complementary RNA strands by binding to the RNA. Antisense DNA oligonucleotides can be used to target a specific, complementary (coding or non-coding) RNA. If binding occurs, this DNA/RNA hybrid can be degraded by the enzyme RNase H. In a particular embodiment, antisense oligonucleotides comprise from about 10 to about 60 nucleotides, more preferably from about 15 to about 30 nucleotides. The term also encompasses antisense oligonucleotides that may not be exactly complementary to the desired target gene. Thus, the invention can be utilized in instances where non-target specific-activities are found with antisense, or where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use.

[0202] Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein synthesis by a targeted gene. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (*see,* U.S. Patent Nos. 5,739,119 and 5,759,829). Furthermore, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDR1), ICAM-1, E-selectin, STK-1, striatal GABAA receptor, and human EGF (*see,* Jaskulski et al., Science, 240:1544-6 (1988); Vasanthakumar et al., Cancer Commun., 1:225-32 (1989); Peris et al., Brain Res Mol Brain Res., 15;57:310-20 (1998); and U.S. Patent Nos. 5,801,154; 5,789,573; 5,718,709 and 5,610,288). Moreover, antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.,* cancer (*see,* U.S. Patent Nos. 5,747,470; 5,591,317; and 5,783,683).

[0203] Methods of producing antisense oligonucleotides are known in the art and can be readily adapted to produce an antisense oligonucleotide that targets any polynucleotide sequence. Selection of antisense oligonucleotide sequences specific for a given target sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense oligonucleotides may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul et al., Nucleic Acids Res., 25:3389-402 (1997)).

5. Ribozymes

[0204] According to another embodiment of the present disclosure, nucleic acid-lipid particles are associated with ribozymes. Ribozymes are RNA-protein complexes having specific catalytic domains that possess endonuclease activity (*see,* Kim et al., Proc. Natl. Acad. Sci. USA., 84:8788-92 (1987); and Forster et al., Cell, 49:211-20 (1987)). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (*see,* Cech et al., Cell, 27:487-96 (1981); Michel et al., J. Mol. Biol., 216:585-610 (1990); Reinhold-Hurek et al., Nature, 357:173-6 (1992)). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

[0205] At least six basic varieties of naturally-occurring enzymatic RNA molecules are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

[0206] The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence), or Neurospora VS RNA motif, for example. Specific examples of hammerhead motifs are described in, *e.g.,* Rossi et al., Nucleic Acids Res., 20:4559-65 (1992). Examples of hairpin motifs are described in, *e.g.,* EP 0360257, Hampel et al., Biochemistry, 28:4929-33 (1989); Hampel et al., Nucleic Acids Res., 18:299-304 (1990); and U.S. Patent No. 5,631,359. An example of the hepatitis δ virus motif is described in, *e.g.,* Perrotta et al., Biochemistry, 31:11843-52 (1992). An example of the RNaseP motif is described in, *e.g.*, Guerrier-Takada et al., Cell, 35:849-57 (1983). Examples of the Neurospora VS RNA ribozyme motif is described in, *e.g.,* Saville et al., Cell, 61:685-96 (1990); Saville et al., Proc. Natl. Acad. Sci. USA, 88:8826-30 (1991); Collins et al., Biochemistry, 32:2795-9 (1993). An example of the Group I intron is described in, *e.g.,* U.S. Patent No. 4,987,071. Important characteristics of enzymatic nucleic acid molecules used according to the present disclosure are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus, the ribozyme constructs need not be limited to specific motifs mentioned herein.

[0207] Methods of producing a ribozyme targeted to any polynucleotide sequence are known in the art. Ribozymes may be designed as described in, *e.g.,* PCT Publication Nos. WO 93/23569 and WO 94/02595, and synthesized to be tested *in vitro* and/or *in vivo* as described therein.

[0208] Ribozyme activity can be optimized by altering the length of the ribozyme binding arms or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (*see, e.g.,* PCT Publication Nos. WO 92/07065, WO 93/15187, WO 91/03162, and WO 94/13688; EP 92110298.4; and U.S. Patent No. 5,334,711, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

6. Immunostimulatory Oligonucleotides

[0209] Nucleic acids associated with lipid particles of the present disclosure may be immunostimulatory, including immunostimulatory oligonucleotides (ISS; single-or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal such as a human. ISS include, *e.g.*, certain palindromes leading to hairpin secondary structures (*see,* Yamamoto et al., J. Immunol., 148:4072-6 (1992)), or CpG motifs, as well as other known ISS features (such as multi-G domains; *see;* PCT Publication No. WO 96/11266).

[0210] Immunostimulatory nucleic acids are considered to be non-sequence specific when it is not required that they specifically bind to and reduce the expression of a target sequence in order to provoke an immune response. Thus, certain immunostimulatory nucleic acids may comprise a sequence corresponding to a region of a naturally-occurring gene or mRNA, but they may still be considered non-sequence specific immunostimulatory nucleic acids.

[0211] In one embodiment, the immunostimulatory nucleic acid or oligonucleotide comprises at least one CpG dinucleotide. The oligonucleotide or CpG dinucleotide may be unmethylated or methylated. In another embodiment, the immunostimulatory nucleic acid comprises at least one CpG dinucleotide having a methylated cytosine. In one embodiment, the nucleic acid comprises a single CpG dinucleotide, wherein the cytosine in the CpG dinucleotide is methylated. In an alternative embodiment, the nucleic acid comprises at least two CpG dinucleotides, wherein at least one cytosine

in the CpG dinucleotides is methylated. In a further embodiment, each cytosine in the CpG dinucleotides present in the sequence is methylated. In another embodiment, the nucleic acid comprises a plurality of CpG dinucleotides, wherein at least one of the CpG dinucleotides comprises a methylated cytosine. Examples of immunostimulatory oligonucleotides suitable for use in the compositions and methods of the present disclosure are described in PCT Application No. PCT/US08/88676, filed December 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Patent No. 6,406,705, and Raney et al., J. Pharm. Exper. Ther., 298:1185-92 (2001). In certain embodiments, the oligonucleotides used in the compositions and methods of the disclosure have a phosphodiester ("PO") backbone or a phosphorothioate ("PS") backbone, and/or at least one methylated cytosine residue in a CpG motif.

B. Other Active Agents

[0212] In certain embodiments, the active agent associated with the lipid particles of the present disclosure may comprise one or more therapeutic proteins, polypeptides, or small organic molecules or compounds. Non-limiting examples of such therapeutically effective agents or drugs include oncology drugs (*e.g.,* chemotherapy drugs, hormonal therapaeutic agents, immunotherapeutic agents, radiotherapeutic agents, *etc*.), lipid-lowering agents, anti-viral drugs, anti-inflammatory compounds, antidepressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs such as antiarrhythmic agents, hormones, vasoconstrictors, and steroids. These active agents may be administered alone in the lipid particles of the present disclosure, or in combination (*e.g*., co-administered) with lipid particles of the present disclosure comprising nucleic acid such as interfering RNA.

[0213] Non-limiting examples of chemotherapy drugs include platinum-based drugs (*e.g*., oxaliplatin, cisplatin, carboplatin, spiroplatin, iproplatin, satraplatin, *etc*.), alkylating agents (*e.g*., cyclophosphamide, ifosfamide, chlorambucil, busulfan, melphalan, mechlorethamine, uramustine, thiotepa, nitrosoureas, *etc*.), anti-metabolites (*e.g*., 5-fluorouracil (5-FU), azathioprine, methotrexate, leucovorin, capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, pemetrexed, raltitrexed, *etc*.), plant alkaloids (*e.g*., vincristine, vinblastine, vinorelbine, vindesine, podophyllotoxin, paclitaxel (taxol), docetaxel, *etc*.), topoisomerase inhibitors (*e.g*., irinotecan (CPT-11; Camptosar), topotecan, amsacrine, etoposide (VP16), etoposide phosphate, teniposide, *etc*.), antitumor antibiotics (*e.g*., doxorubicin, adriamycin, daunorubicin, epirubicin, actinomycin, bleomycin, mitomycin, mitoxantrone, plicamycin, *etc*.), tyrosine kinase inhibitors (*e.g.,* gefitinib (Iressa®), sunitinib (Sutent®; SU11248), erlotinib (Tarceva®; OSI-1774), lapatinib (GW572016; GW2016), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), imatinib (Gleevec®; STI571), dasatinib (BMS-354825), leflunomide (SU101), vandetanib (Zactima™; ZD6474), *etc*.), pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and combinations thereof.

[0214] Examples of conventional hormonal therapaeutic agents include, without limitation, steroids (*e.g*., dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

[0215] Examples of conventional immunotherapeutic agents include, but are not limited to, immunostimulants (*e.g*., Bacillus Calmette-Guérin (BCG), levamisole, interleukin-2, alpha-interferon, *etc*.), monoclonal antibodies (*e.g*., anti-CD20, anti-HER2, anti-CD52, anti-HLA-DR, and anti-VEGF monoclonal antibodies), immunotoxins (*e.g*., anti-CD33 monoclonal antibody-calicheamicin conjugate, anti-CD22 monoclonal antibody-pseudomonas exotoxin conjugate, *etc*.), and radio immunotherapy (*e.g*., anti-CD20 monoclonal antibody conjugated to ^{111}In, ^{90}Y, or ^{131}I, *etc*.).

[0216] Examples of conventional radiotherapeutic agents include, but are not limited to, radionuclides such as ^{47}Sc, ^{64}Cu, ^{67}Cu, ^{89}Sr, ^{86}Y, ^{87}Y, ^{90}Y, ^{105}Rh, ^{111}Ag, ^{111}In, ^{117m}Sn, ^{149}Pm, ^{153}Sm ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi, optionally conjugated to antibodies directed against tumor antigens.

[0217] Additional oncology drugs that may be used according to the present disclosure include, but are not limited to, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, bexarotene, biCNU, carmustine, CCNU, celecoxib, cladribine, cyclosporin A, cytosine arabinoside, cytoxan, dexrazoxane, DTIC, estramustine, exemestane, FK506, gemtuzumab-ozogamicin, hydrea, hydroxyurea, idarubicin, interferon, letrozole, leustatin, leuprolide, litretinoin, megastrol, L-PAM, mesna, methoxsalen, mithramycin, nitrogen mustard, pamidronate, Pegademase, pentostatin, porfimer sodium, prednisone, rituxan, streptozocin, STI-571, taxotere, temozolamide, VM-26, toremifene, tretinoin, ATRA, valrubicin, and velban. Other examples of oncology drugs that may be used according to the present disclosure are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors, and camptothecins.

[0218] Non-limiting examples of lipid-lowering agents for treating a lipid disease or disorder associated with elevated triglycerides, cholesterol, and/or glucose include statins, fibrates, ezetimibe, thiazolidinediones, niacin, beta-blockers, nitroglycerin, calcium antagonists, fish oil, and mixtures thereof.

[0219] Examples of anti-viral drugs include, but are not limited to, abacavir, aciclovir, acyclovir, adefovir, amantadine, amprenavir, arbidol, atazanavir, atripla, cidofovir, combivir, darunavir, delavirdine, didanosine, docosanol, edoxudine, efavirenz, emtricitabine, enfuvirtide, entecavir, entry inhibitors, famciclovir, fixed dose combinations, fomivirsen, fosamprenavir, foscarnet, fosfonet, fusion inhibitors, ganciclovir, ibacitabine, imunovir, idoxuridine, imiquimod, indinavir, ino-

sine, integrase inhibitors, interferon type III (*e.g.*, IFN-λ molecules such as IFN-λ1, IFN-λ2, and IFN-λ3), interferon type II (*e.g.*, IFN-γ), interferon type I (*e.g.*, IFN-α such as PEGylated IFN-α, IFN-β, IFN-κ, IFN-δ, IFN-ε, IFN-τ, IFN-ω, and IFN-ζ), interferon, lamivudine, lopinavir, loviride, MK-0518, maraviroc, moroxydine, nelfinavir, nevirapine, nexavir, nucleoside analogues, oseltamivir, penciclovir, peramivir, pleconaril, podophyllotoxin, protease inhibitors, reverse transcriptase inhibitors, ribavirin, rimantadine, ritonavir, saquinavir, stavudine, synergistic enhancers, tenofovir, tenofovir disoproxil, tipranavir, trifluridine, trizivir, tromantadine, truvada, valaciclovir, valganciclovir, vicriviroc, vidarabine, viramidine, zalcitabine, zanamivir, zidovudine, pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and mixtures thereof.

V. Lipid Particles

[0220] The lipid particles of the present disclosure typically comprise an active agent or therapeutic agent, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans. The lipid particles of the present disclosure typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm.

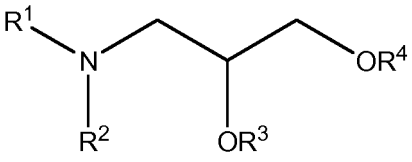
[0221] In preferred embodiments, the lipid particles of the present disclosure are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (*e.g.*, siRNA, aiRNA, and/or miRNA), a cationic lipid *(e.g.,* a cationic lipid of Formulas I, II, and/or III), a non-cationic lipid (*e.g.,* cholesterol alone or mixtures of one or more phospholipids and cholesterol), and a conjugated lipid that inhibits aggregation of the particles (*e.g.,* one or more PEG-lipid conjugates). The SNALP may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more unmodified and/or modified interfering RNA molecules. Nucleic acid-lipid particles and their method of preparation are described in, *e.g.,* U.S. Patent Nos. 5,753,613; 5,785,992; 5,705,385; 5,976,567; 5,981,501; 6,110,745; and 6,320,017; and PCT Publication No. WO 96/40964.

A. Cationic Lipids

[0222] Any of a variety of cationic lipids may be used in the lipid particles of the present disclosure (*e.g.,* SNALP), either alone or in combination with one or more other cationic lipid species or non-cationic lipid species.

[0223] Cationic lipids which are useful in the present invention can be any of a number of lipid species which carry a net positive charge at physiological pH. Such lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3.beta.-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-Dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), 1,2-Dilinoleoylcarbamyl-3-dimethylaminopropane (DLinCDAP), and mixtures thereof. A number of these lipids and related analogs have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390. Additionally, a number of commercial preparations of cationic lipids are available and can be used in the present invention. These include, *e.g.*, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic liposomes comprising DOGS from Promega Corp., Madison, Wisconsin, USA).

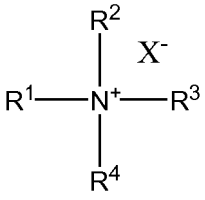
[0224] Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.



(I),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.,* R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.,* R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipid of Formula I is symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4 comprise at least three sites of unsaturation and are independently selected from, *e.g.*, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl. In particularly preferred embodiments, the cationic lipid of Formula I is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) or 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA).

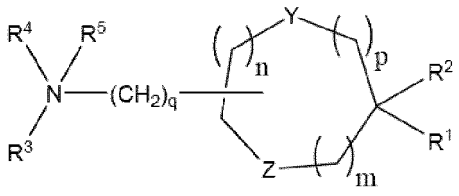
[0225] Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.



(II),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.,* R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.,* R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipids of the present invention are symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4 comprise at least three sites of unsaturation and are independently selected from, *e.g.,* dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl.

[0226] Moreover, cationic lipids of Formula III having the following structures (or salts thereof) are useful in the present invention.



(III),

[0227] Wherein R^1 and R^2 are either the same or different and independently optionally substituted C_{12}-C_{24} alkyl, optionally substituted C_{12}-C_{24} alkenyl, optionallysubstituted C_{12}-C_{24} alkynyl, or optionally substituted C_{12}-C_{24} acyl; R^3 and R^4 are either the same or different and independently optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 alkenyl, or optionally substituted C_1-C_6 alkynyl or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen; R^5 is either absent or hydrogen or C_1-C_6 alkyl to provide a quaternary amine; m, n, and p are either the same or different and independently either 0 or 1 with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; and Y and Z are either the same or different and independently O, S, or NH.

[0228] In some embodiments, the cationic lipid of Formula III is 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-

3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), or mixtures thereof. In preferred embodiments, the cationic lipid of Formula III is DLin-K-C2-DMA (XTC2).

[0229] The cationic lipid typically comprises from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0230] It will be readily apparent to one of skill in the art that depending on the intended use of the particles, the proportions of the components can be varied and the delivery efficiency of a particular formulation can be measured using, *e.g.*, an endosomal release parameter (ERP) assay.

B. Non-Cationic Lipids

[0231] The non-cationic lipids used in the lipid particles described herein (*e.g.,* SNALP) can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

[0232] Non-limiting examples of non-cationic lipids include phospholipids such as lecithin, phosphatidylethanolamine, lyso lecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C_{10}-C_{24} carbon chains, *e.g*., lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

[0233] Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof such as cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof.

[0234] In some embodiments, the non-cationic lipid present in the lipid particles (*e.g.,* SNALP) comprises or consists of cholesterol or a derivative thereof, *e.g.,* a phospholipid-free lipid particle formulation. In other embodiments, the non-cationic lipid present in the lipid particles (*e.g.,* SNALP) comprises or consists of one or more phospholipids, *e.g.,* a cholesterol-free lipid particle formulation. In further embodiments, the non-cationic lipid present in the lipid particles (*e.g.,* SNALP) comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof.

[0235] Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, *e.g.,* stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

[0236] In some embodiments, the non-cationic lipid comprises from about 13 mol % to about 49.5 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 35 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0237] In certain embodiments described herein, the cholesterol present in phospholipid-free lipid particles comprises from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, or from about 35 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle may comprise cholesterol at about 37 mol % of the total lipid present in the particle.

[0238] In certain other embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, or from about 35 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 34 mol % of the total lipid present in the particle.

[0239] In further embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 10 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising

a mixture of phospholipid and cholesterol may comprise cholesterol at about 20 mol % of the total lipid present in the particle.

[0240] In embodiments described herein where the lipid particles contain a mixture of phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 45, 50, 55, or 60 mol % of the total lipid present in the particle. In certain instances, the phospholipid component in the mixture may comprise from about 2 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, or from about 6 mol % to about 8 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (*e.g.,* in a mixture with about 34 mol % cholesterol) of the total lipid present in the particle. In certain other instances, the phospholipid component in the mixture may comprise from about 10 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As another non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 20 mol % (*e.g.,* in a mixture with about 20 mol % cholesterol) of the total lipid present in the particle.

C. Lipid Conjugate

[0241] In addition to cationic and non-cationic lipids, the lipid particles described herein(*e.g.*, SNALP) comprise a lipid conjugate. The conjugated lipid is useful in that it prevents the aggregation of particles. Suitable conjugated lipids include, but are not limited to, PEG-lipid conjugates, ATTA-lipid conjugates, cationic-polymer-lipid conjugates (CPLs), and mixtures thereof. In certain embodiments, the particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate together with a CPL.

[0242] In a preferred embodiment, the lipid conjugate is a PEG-lipid. Examples of PEG-lipids include, but are not limited to, PEG coupled to dialkyloxypropyls (PEG-DAA) as described in, *e.g.,* PCT Publication No. WO 05/026372, PEG coupled to diacylglycerol (PEG-DAG) as described in, *e.g.,* U.S. Patent Publication Nos. 20030077829 and 2005008689, PEG coupled to phospholipids such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides as described in, *e.g.,* U.S. Patent No. 5,885,613, PEG conjugated to cholesterol or a derivative thereof, and mixtures thereof. Additional PEG-lipids include, without limitation, PEG-C-DOMG, 2KPEG-DMG, and a mixture thereof.

[0243] PEG is a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG 2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, for example, the following: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH_2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM). Other PEGs such as those described in U.S. Patent Nos. 6,774,180 and 7,053,150 (*e.g.,* mPEG (20 KDa) amine) are also useful for preparing the PEG-lipid conjugates described herein. In addition, monomethoxypolyethyleneglycol-acetic acid (MePEG-CH_2COOH) is particularly useful for preparing PEG-lipid conjugates including, *e.g.*, PEG-DAA conjugates.

[0244] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc*.). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

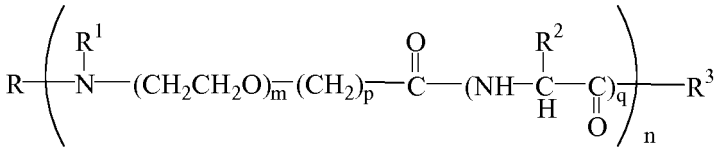
[0245] In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In a preferred embodiment, the linker moiety is a non-ester containing linker moiety. As used herein, the term "non-ester containing linker moiety" refers to a linker moiety that does not contain a carboxylic ester bond (-OC(O)-). Suitable non-ester containing linker moieties include, but are not limited to, amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulphide (-S-S-), ether (-O-), succinyl (-(O)CCH_2CH_2C(O)-), succinamidyl (-NHC(O)CH_2CH_2C(O)NH-), ether, disulphide, as well as combinations thereof (such as a linker containing both a carbamate linker moiety and an amido linker moiety). In a preferred embodiment, a carbamate linker is used to couple the PEG to the lipid.

[0246] In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, *e.g.*, carbonate (-OC(O)O-), succinoyl, phosphate esters (-O-(O)POH-O-), sulfonate esters, and combinations thereof.

[0247] Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of

saturation can be conjugated to PEG to form the lipid conjugate. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skilled in the art. Phosphatidylethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), and distearoyl-phosphatidylethanolamine (DSPE).

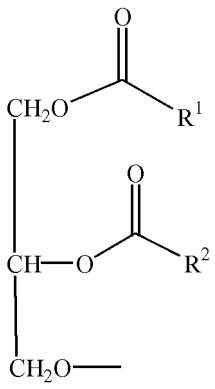
[0248] The term "ATTA" or "polyamide" refers to, without limitation, compounds described in U.S. Patent Nos. 6,320,017 and 6,586,559. These compounds include a compound having the formula:



(IV),

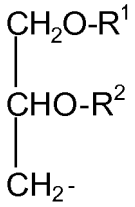
wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R^1 is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R^1 and the nitrogen to which they are bound form an azido moiety; R^2 is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; R^3 is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and NR^4R^5, wherein R^4 and R^5 are independently hydrogen or alkyl; n is 4 to 80; m is 2 to 6; p is 1 to 4; and q is 0 or 1. It will be apparent to those of skill in the art that other polyamides can be used in the compounds of the present disclosure.

[0249] The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.,* R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.* Diacylglycerols have the following general formula:



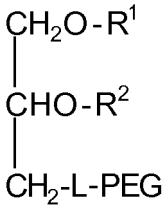
(V).

[0250] The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:



(VI).

[0251] In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:



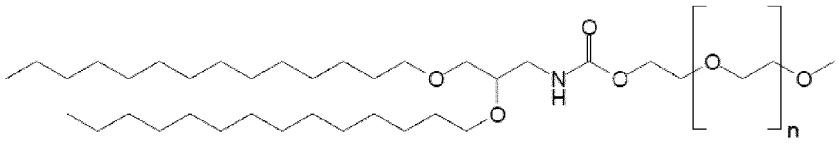
(VII),

wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.,* R^1 and R^2 are both myristyl (*i.e*., dimyristyl), R^1 and R^2 are both stearyl (*i.e*., distearyl), *etc.*

[0252] In Formula VII above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g*., from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG has an average molecular weight of about 2,000 daltons or about 750 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl, or aryl. In certain embodiments, the terminal hydroxyl group is substituted with a methoxy or methyl group.

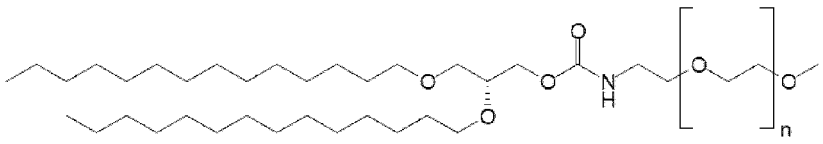
[0253] In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, a succinamidyl linker moiety, and combinations thereof. In a preferred embodiment, the non-ester containing linker moiety is a carbamate linker moiety (*i.e*., a PEG-C-DAA conjugate). In another preferred embodiment, the non-ester containing linker moiety is an amido linker moiety (*i.e*., a PEG-*A*-DAA conjugate). In yet another preferred embodiment, the non-ester containing linker moiety is a succinamidyl linker moiety (*i.e*., a PEG-S-DAA conjugate).

[0254] In particular embodiments, the PEG-lipid conjugate is selected from:



(PEG-C-DMA);

and



(PEG-C-DOMG).

[0255] The PEG-DAA conjugates are synthesized using standard techniques and reagents known to those of skill in the art. It will be recognized that the PEG-DAA conjugates will contain various amide, amine, ether, thio, carbamate, and urea linkages. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See, e.g.,* March, ADVANCED ORGANIC CHEMISTRY (Wiley 1992); Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY, 5th ed. (Longman 1989). It will also be appreciated that any functional groups present may require protection and deprotection at different points in the synthesis of the PEG-DAA conjugates. Those of skill in the art will recognize that such techniques are well known. *See, e.g.,* Green and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS (Wiley 1991).

[0256] Preferably, the PEG-DAA conjugate is a dilauryloxypropyl (C_{12})-PEG conjugate, dimyristyloxypropyl (C_{14})-PEG conjugate, a dipalmityloxypropyl (C_{16})-PEG conjugate, or a distearyloxypropyl (C_{18})-PEG conjugate. Those of skill in the art will readily appreciate that other dialkyloxypropyls can be used in the PEG-DAA conjugates of the present disclosure.

[0257] In addition to the foregoing, it will be readily apparent to those of skill in the art that other hydrophilic polymers can be used in place of PEG. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

[0258] In addition to the foregoing components, the particles (*e.g.,* SNALP or SPLP) of the present disclosure can further comprise cationic poly(ethylene glycol) (PEG) lipids or CPLs (*see, e.g.*, Chen et al., Bioconj. Chem., 11:433-437 (2000)). Suitable SPLPs and SPLP-CPLs for use in the present disclosure, and methods of making and using SPLPs and SPLP-CPLs, are disclosed, *e.g.*, in U.S. Patent No. 6,852,334 and PCT Publication No. WO 00/62813.

[0259] Suitable CPLs include compounds of Formula VIII:

$$A\text{-}W\text{-}Y \qquad \text{(VIII)},$$

wherein A, W, and Y are as described below.

[0260] With reference to Formula VIII, "A" is a lipid moiety such as an amphipathic lipid, a neutral lipid, or a hydrophobic lipid that acts as a lipid anchor. Suitable lipid examples include, but are not limited to, diacylglycerolyls, dialkylglycerolyls, N-N-dialkylaminos, 1,2-diacyloxy-3-aminopropanes, and 1,2-dialkyl-3-aminopropanes.

[0261] "W" is a polymer or an oligomer such as a hydrophilic polymer or oligomer. Preferably, the hydrophilic polymer is a biocompatable polymer that is nonimmunogenic or possesses low inherent immunogenicity. Alternatively, the hydrophilic polymer can be weakly antigenic if used with appropriate adjuvants. Suitable nonimmunogenic polymers include, but are not limited to, PEG, polyamides, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, and combinations thereof. In a preferred embodiment, the polymer has a molecular weight of from about 250 to about 7,000 daltons.

[0262] "Y" is a polycationic moiety. The term polycationic moiety refers to a compound, derivative, or functional group having a positive charge, preferably at least 2 positive charges at a selected pH, preferably physiological pH. Suitable polycationic moieties include basic amino acids and their derivatives such as arginine, asparagine, glutamine, lysine, and histidine; spermine; spermidine; cationic dendrimers; polyamines; polyamine sugars; and amino polysaccharides. The polycationic moieties can be linear, such as linear tetralysine, branched or dendrimeric in structure. Polycationic moieties have between about 2 to about 15 positive charges, preferably between about 2 to about 12 positive charges, and more preferably between about 2 to about 8 positive charges at selected pH values. The selection of which polycationic moiety to employ may be determined by the type of particle application which is desired.

[0263] The charges on the polycationic moieties can be either distributed around the entire particle moiety, or alternatively, they can be a discrete concentration of charge density in one particular area of the particle moiety *e.g.,* a charge spike. If the charge density is distributed on the particle, the charge density can be equally distributed or unequally distributed. All variations of charge distribution of the polycationic moiety are encompassed by the present disclosure.

[0264] The lipid "A" and the nonimmunogenic polymer "W" can be attached by various methods and preferably by covalent attachment. Methods known to those of skill in the art can be used for the covalent attachment of "A" and "W." Suitable linkages include, but are not limited to, amide, amine, carboxyl, carbonate, carbamate, ester, and hydrazone linkages. It will be apparent to those skilled in the art that "A" and "W" must have complementary functional groups to effectuate the linkage. The reaction of these two groups, one on the lipid and the other on the polymer, will provide the desired linkage. For example, when the lipid is a diacylglycerol and the terminal hydroxyl is activated, for instance with NHS and DCC, to form an active ester, and is then reacted with a polymer which contains an amino group, such as with a polyamide (*see, e.g.,* U.S. Patent Nos. 6,320,017 and 6,586,559), an amide bond will form between the two groups.

[0265] In certain instances, the polycationic moiety can have a ligand attached, such as a targeting ligand or a chelating moiety for complexing calcium. Preferably, after the ligand is attached, the cationic moiety maintains a positive charge. In certain instances, the ligand that is attached has a positive charge. Suitable ligands include, but are not limited to, a compound or device with a reactive functional group and include lipids, amphipathic lipids, carrier compounds, bioaffinity compounds, biomaterials, biopolymers, biomedical devices, analytically detectable compounds, therapeutically active compounds, enzymes, peptides, proteins, antibodies, immune stimulators, radiolabels, fluorogens, biotin, drugs, haptens, DNA, RNA, polysaccharides, liposomes, virosomes, micelles, immunoglobulins, functional groups, other targeting moieties, or toxins.

[0266] The lipid conjugate (*e.g.,* PEG-lipid) typically comprises from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 0.9 mol % to about 1.6 mol %, from about 0.9 mol % to about 1.8 mol %, from about 1 mol % to about 1.8 mol %, from about 1 mol % to about 1.7 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, or from about 1.4 mol % to about 1.5 mol % of the total lipid present in the particle.

[0267] One of ordinary skill in the art will appreciate that the concentration of the lipid conjugate can be varied depending

on the lipid conjugate employed and the rate at which the nucleic acid-lipid particle is to become fusogenic.

[0268] By controlling the composition and concentration of the lipid conjugate, one can control the rate at which the lipid conjugate exchanges out of the nucleic acid-lipid particle and, in turn, the rate at which the nucleic acid-lipid particle becomes fusogenic. For instance, when a PEG-phosphatidylethanolamine conjugate or a PEG-ceramide conjugate is used as the lipid conjugate, the rate at which the nucleic acid-lipid particle becomes fusogenic can be varied, for example, by varying the concentration of the lipid conjugate, by varying the molecular weight of the PEG, or by varying the chain length and degree of saturation of the acyl chain groups on the phosphatidylethanolamine or the ceramide. In addition, other variables including, for example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the nucleic acid-lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the nucleic acid-lipid particle becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure.

VI. Preparation of Lipid Particles

[0269] The lipid particles described herein, *e.g.*, SNALP, in which an active agent or therapeutic agent such as an interfering RNA is encapsulated in a lipid bilayer and is protected from degradation, can be formed by any method known in the art including, but not limited to, a continuous mixing method or a direct dilution process.

[0270] In preferred embodiments, the cationic lipids are lipids of Formula I, II, and III, or combinations thereof. In other preferred embodiments, the non-cationic lipids are egg sphingomyelin (ESM), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dipalmitoyl-phosphatidylcholine (DPPC), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, 14:0 PE (1,2-dimyristoyl-phosphatidylethanolamine (DMPE)), 16:0 PE (1,2-dipalmitoyl-phosphatidylethanolamine (DPPE)), 18:0 PE (1,2-distearoyl-phosphatidylethanolamine (DSPE)), 18:1 PE (1,2-dioleoyl-phosphatidylethanolamine (DOPE)), 18:1 trans PE (1,2-dielaidoyl-phosphatidylethanolamine (DEPE)), 18:0-18:1 PE (1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE)), 16:0-18:1 PE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)), polyethylene glycol-based polymers (*e.g.*, PEG 2000, PEG 5000, PEG-modified diacylglycerols, or PEG-modified dialkyloxypropyls), cholesterol, or combinations thereof.

[0271] In certain embodiments, the present disclosure provides for SNALP produced via a continuous mixing method, *e.g.*, a process that includes providing an aqueous solution comprising a nucleic acid such as an interfering RNA in a first reservoir, providing an organic lipid solution in a second reservoir, and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a liposome encapsulating the nucleic acid (*e.g.*, interfering RNA). This process and the apparatus for carrying this process are described in detail in U.S. Patent Publication No. 20040142025.

[0272] The action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a liposome substantially instantaneously upon mixing. As used herein, the phrase "continuously diluting a lipid solution with a buffer solution" (and variations) generally means that the lipid solution is diluted sufficiently rapidly in a hydration process with sufficient force to effectuate vesicle generation. By mixing the aqueous solution comprising a nucleic acid with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution in the presence of the buffer solution (*i.e.*, aqueous solution) to produce a nucleic acid-lipid particle.

[0273] The SNALP formed using the continuous mixing method typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0274] In another embodiment, the present disclosure provides for SNALP produced via a direct dilution process that includes forming a liposome solution and immediately and directly introducing the liposome solution into a collection vessel containing a controlled amount of dilution buffer. In preferred aspects, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In one aspect, the amount of dilution buffer present in the collection vessel is substantially equal to the volume of liposome solution introduced thereto. As a non-limiting example, a liposome solution in 45% ethanol when introduced into the collection vessel containing an equal volume of dilution buffer will advantageously yield smaller particles.

[0275] In yet another embodiment, the present disclosure provides for SNALP produced via a direct dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the liposome solution formed in a first mixing region is immediately and directly mixed with dilution buffer in the second mixing region. In preferred aspects, the second mixing region includes a T-connector arranged so that the liposome solution and the dilution buffer flows meet as opposing 180° flows; however, connectors providing shallower angles can be used, *e.g.*, from about 27° to about 180°. A pump mechanism delivers a controllable flow of buffer to the second mixing region. In one aspect, the flow rate of dilution buffer provided to the second mixing region is controlled to be

substantially equal to the flow rate of liposome solution introduced thereto from the first mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the liposome solution in the second mixing region, and therefore also the concentration of liposome solution in buffer throughout the second mixing process. Such control of the dilution buffer flow rate advantageously allows for small particle size formation at reduced concentrations.

[0276] These processes and the apparatuses for carrying out these direct dilution processes are described in detail in U.S. Patent Publication No. 20070042031.

[0277] The SNALP formed using the direct dilution process typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0278] If needed, the lipid particles described herein (*e.g.,* SNALP) can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

[0279] Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles, is described in U.S. Patent No. 4,737,323. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between about 60 and about 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

[0280] Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

[0281] In some embodiments, the nucleic acids in the SNALP are precondensed as described in, *e.g.*, U.S. Patent Application No. 09/744,103.

[0282] In other embodiments, the methods will further comprise adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions. Examples of suitable non-lipid polycations include, hexadimethrine bromide (sold under the brandname POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine, and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

[0283] In some embodiments, the nucleic acid to lipid ratios (mass/mass ratios) in a formed SNALP will range from about 0.01 to about 0.2, from about 0.02 to about 0.1, from about 0.03 to about 0.1, or from about 0.01 to about 0.08. The ratio of the starting materials also falls within this range. In other embodiments, the SNALP preparation uses about 400 μg nucleic acid per 10 mg total lipid or a nucleic acid to lipid mass ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 μg of nucleic acid. In other preferred embodiments, the particle has a nucleic acid:lipid mass ratio of about 0.08.

[0284] In other embodiments, the lipid to nucleic acid ratios (mass/mass ratios) in a formed SNALP will range from about 1 (1:1) to about 100 (100:1), from about 5 (5:1) to about 100 (100:1), from about 1 (1:1) to about 50 (50:1), from about 2 (2:1) to about 50 (50:1), from about 3 (3:1) to about 50 (50:1), from about 4 (4:1) to about 50 (50:1), from about 5 (5:1) to about 50 (50:1), from about 1 (1:1) to about 25 (25:1), from about 2 (2:1) to about 25 (25:1), from about 3 (3:1) to about 25 (25:1), from about 4 (4:1) to about 25 (25:1), from about 5 (5:1) to about 25 (25:1), from about 5 (5:1) to about 20 (20:1), from about 5 (5:1) to about 15 (15:1), from about 5 (5:1) to about 10 (10:1), about 5 (5:1), 6 (6:1), 7 (7:1), 8 (8:1), 9 (9:1), 10 (10:1), 11 (11:1), 12 (12:1), 13 (13:1), 14 (14:1), or 15 (15:1). The ratio of the starting materials also falls within this range.

[0285] As previously discussed, the conjugated lipid may further include a CPL. A variety of general methods for making SNALP-CPLs (CPL-containing SNALP) are discussed herein. Two general techniques include "post-insertion" technique, that is, insertion of a CPL into, for example, a pre-formed SNALP, and the "standard" technique, wherein the CPL is included in the lipid mixture during, for example, the SNALP formation steps. The post-insertion technique results in SNALP having CPLs mainly in the external face of the SNALP bilayer membrane, whereas standard techniques provide SNALP having CPLs on both internal and external faces. The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAAs and PEG-DAGs). Methods of making SNALP-CPL, are taught, for example, in U.S. Patent Nos. 5,705,385; 6,586,410; 5,981,501; 6,534,484; and 6,852,334; U.S. Patent Publication No. 20020072121; and PCT Publication No. WO 00/62813.

VII. Kits

[0286] The present disclosure also provides lipid particles (*e.g.,* SNALP) in kit form. The kit may comprise a container which is compartmentalized for holding the various elements of the lipid particles (*e.g.*, the active agents or therapeutic agents such as nucleic acids and the individual lipid components of the particles). In some embodiments, the kit may further comprise an endosomal membrane destabilizer (*e.g.*, calcium ions). The kit typically contains the lipid particle compositions of the present disclosure, preferably in dehydrated form, with instructions for their rehydration and administration.

[0287] As explained herein, the lipid particles described herein (*e.g.,* SNALP) can be tailored to preferentially target particular tissues, organs, or tumors of interest. In certain instances, preferential targeting of lipid particles such as SNALP may be carried out by controlling the composition of the particle itself. For instance, as set forth in Example 11, it has been found that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP formulation can be used to preferentially target the liver (including liver tumors).

[0288] In certain other instances, it may be desirable to have a targeting moiety attached to the surface of the lipid particle to further enhance the targeting of the particle. Methods of attaching targeting moieties (*e.g.*, antibodies, proteins, *etc*.) to lipids (such as those used in the present particles) are known to those of skill in the art.

VII. Administration of Lipid Particles

[0289] Once formed, the lipid particles described herein (*e.g.,* SNALP) are useful for the introduction of active agents or therapeutic agents (*e.g.*, nucleic acids such as interfering RNA) into cells. Accordingly, the present invention also provides methods for introducing an active agent or therapeutic agent such as a nucleic acid (*e.g.*, interfering RNA) into a cell. The methods are carried out *in vitro* or *in vivo* by first forming the particles as described above and then contacting the particles with the cells for a period of time sufficient for delivery of the active agent or therapeutic agent to the cells to occur.

[0290] The lipid particles described herein (*e.g.,* SNALP) can be adsorbed to almost any cell type with which they are mixed or contacted. Once adsorbed, the particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the active agent or therapeutic agent (*e.g.*, nucleic acid) portion of the particle can take place via any one of these pathways. In particular, when fusion takes place, the particle membrane is integrated into the cell membrane and the contents of the particle combine with the intracellular fluid.

[0291] The lipid particles described herein (*e.g.,* SNALP) can be administered either alone or in a mixture with a pharmaceutically-acceptable carrier (*e.g.,* physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice. Generally, normal buffered saline (*e.g.,* 135-150 mM NaCl) will be employed as the pharmaceutically-acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Additional suitable carriers are described in, *e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

[0292] The pharmaceutically-acceptable carrier is generally added following particle formation. Thus, after the particle is formed, the particle can be diluted into pharmaceutically-acceptable carriers such as normal buffered saline.

[0293] The concentration of particles in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2 to 5%, to as much as about 10 to 90% by weight, and will be selected primarily by fluid volumes, viscosities, *etc*., in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, particles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

[0294] The pharmaceutical compositions of the present invention may be sterilized by conventional, well-known sterilization techniques. Aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically-acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, and calcium chloride. Additionally, the particle suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

A. *In vivo* Administration

[0295] Systemic delivery for *in vivo* therapy, *e.g.,* delivery of a therapeutic nucleic acid to a distal target cell via body systems such as the circulation, has been achieved using nucleic acid-lipid particles such as those described in PCT Publication Nos. WO 05/007196, WO 05/121348, WO 05/120152, and WO 04/002453. The present disclosure also provides fully encapsulated lipid particles that protect the nucleic acid from nuclease degradation in serum, are nonimmunogenic, are small in size, and are suitable for repeat dosing.

[0296] For *in vivo* administration, administration can be in any manner known in the art, *e.g.*, by injection, oral administration, inhalation (*e.g.*, intransal or intratracheal), transdermal application, or rectal administration. Administration can be accomplished via single or divided doses. The pharmaceutical compositions can be administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In some embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection (*see, e.g.*, U.S. Patent No. 5,286,634). Intracellular nucleic acid delivery has also been discussed in Straubringer et al., Methods Enzymol., 101:512 (1983); Mannino et al., Biotechniques, 6:682 (1988); Nicolau et al., Crit. Rev. Ther. Drug Carrier Syst., 6:239 (1989); and Behr, Acc. Chem. Res., 26:274 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, U.S. Patent Nos. 3,993,754; 4,145,410; 4,235,871; 4,224,179; 4,522,803; and 4,588,578. The lipid particles can be administered by direct injection at the site of disease or by injection at a site distal from the site of disease (*see*, *e.g.*, Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71(1994)).

[0297] The compositions of the present disclosure, either alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation (*e.g.*, intranasally or intratracheally) (*see*, Brigham et al., Am. J. Sci., 298:278 (1989)). Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0298] In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering nucleic acid compositions directly to the lungs via nasal aerosol sprays have been described, *e.g.,* in U.S. Patent Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Similarly, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Patent No. 5,780,045.

[0299] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions are preferably administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically, or intrathecally.

[0300] Generally, when administered intravenously, the lipid particle formulations are formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present disclosure. Suitable formulations for use in the present invention are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc.* These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

[0301] In certain applications, the lipid particles disclosed herein may be delivered via oral administration to the individual. The particles may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, pills, lozenges, elixirs, mouthwash, suspensions, oral sprays, syrups, wafers, and the like (*see, e.g.,* U.S. Patent Nos. 5,641,515, 5,580,579, and 5,792,451,). These oral dosage forms may also contain the following: binders, gelatin; excipients, lubricants, and/or flavoring agents. When the unit dosage form is a capsule, it may contain, in addition to the materials described above, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. Of course, any material used in preparing any unit dosage form should be pharmaceutically pure and substantially non-toxic in the amounts employed.

[0302] Typically, these oral formulations may contain at least about 0.1% of the lipid particles or more, although the percentage of the particles may, of course, be varied and may conveniently be between about 1% or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of particles in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0303] Formulations suitable for oral administration can consist of: (a) liquid solutions, such as an effective amount of a packaged therapeutic agent such as nucleic acid (*e.g.*, interfering RNA) suspended in diluents such as water, saline, or PEG 400; (b) capsules, sachets, or tablets, each containing a predetermined amount of a therapeutic agent such as nucleic acid (*e.g.*, interfering RNA), as liquids, solids, granules, or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise a therapeutic agent such as nucleic acid (*e.g.*, interfering RNA) in a flavor, *e.g.*, sucrose, as well as pastilles comprising the therapeutic agent in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the therapeutic agent, carriers known in the art.

[0304] In another example of their use, lipid particles can be incorporated into a broad range of topical dosage forms. For instance, a suspension containing nucleic acid-lipid particles such as SNALP can be formulated and administered as gels, oils, emulsions, topical creams, pastes, ointments, lotions, foams, mousses, and the like.

[0305] When preparing pharmaceutical preparations of the lipid particles described herein , it is preferable to use quantities of the particles which have been purified to reduce or eliminate empty particles or particles with therapeutic agents such as nucleic acid associated with the external surface.

[0306] The methods of the present disclosure may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as primates (*e.g.*, humans and chimpanzees as well as other nonhuman primates), canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, rats and mice), lagomorphs, and swine.

[0307] The amount of particles administered will depend upon the ratio of therapeutic agent (*e.g.*, nucleic acid) to lipid, the particular therapeutic agent (*e.g.*, nucleic acid) used, the disease or disorder being treated, the age, weight, and condition of the patient, and the judgment of the clinician, but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight, or about 10^8-10^{10} particles per administration (*e.g.*, injection).

B. *In vitro* Administration

[0308] For *in vitro* applications, the delivery of therapeutic agents such as nucleic acids (*e.g.*, interfering RNA) can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells are animal cells, more preferably mammalian cells, and most preferably human cells.

[0309] Contact between the cells and the lipid particles, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of particles varies widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. Treatment of the cells with the lipid particles is generally carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

[0310] In one group of preferred embodiments, a lipid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/ml, more preferably about 2 x 10^4 cells/ml. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/ml, more preferably about 0.1 μg/ml.

[0311] Using an Endosomal Release Parameter (ERP) assay, the delivery efficiency of the SNALP or other lipid particle described herein can be optimized. An ERP assay is described in detail in U.S. Patent Publication No. 20030077829. More particularly, the purpose of an ERP assay is to distinguish the effect of various cationic lipids and helper lipid components of SNALP based on their relative effect on binding/uptake or fusion with/destabilization of the endosomal membrane. This assay allows one to determine quantitatively how each component of the SNALP or other lipid particle affects delivery efficiency, thereby optimizing the SNALP or other lipid particle. Usually, an ERP assay measures expression of a reporter protein (*e.g.*, luciferase, β-galactosidase, green fluorescent protein (GFP), *etc.*), and in some instances, a SNALP formulation optimized for an expression plasmid will also be appropriate for encapsulating an interfering RNA. In other instances, an ERP assay can be adapted to measure downregulation of transcription or translation of a target sequence in the presence or absence of an interfering RNA (*e.g.,* siRNA). By comparing the ERPs for each of the various SNALP or other lipid particles, one can readily determine the optimized system, *e.g.,* the SNALP or other lipid particle that has the greatest uptake in the cell.

C. Cells for Delivery of Lipid Particles

[0312] The compositions and methods of the present disclosure are used to treat a wide variety of cell types, *in vivo* and *in vitro.* Suitable cells include, *e.g*., hematopoietic precursor (stem) cells, fibroblasts, keratinocytes, hepatocytes, endothelial cells, skeletal and smooth muscle cells, osteoblasts, neurons, quiescent lymphocytes, terminally differentiated cells, slow or noncycling primary cells, parenchymal cells, lymphoid cells, epithelial cells, bone cells, and the like. In preferred embodiments, an active agent or therapeutic agent such as an interfering RNA (*e.g*., siRNA) is delivered to cancer cells such as, *e.g*., lung cancer cells, colon cancer cells, rectal cancer cells, anal cancer cells, bile duct cancer cells, small intestine cancer cells, stomach (gastric) cancer cells, esophageal cancer cells, gallbladder cancer cells, liver cancer cells, pancreatic cancer cells, appendix cancer cells, breast cancer cells, ovarian cancer cells, cervical cancer cells, prostate cancer cells, renal cancer cells, cancer cells of the central nervous system, glioblastoma tumor cells, skin cancer cells, lymphoma cells, choriocarcinoma tumor cells, head and neck cancer cells, osteogenic sarcoma tumor cells, and blood cancer cells.

[0313] *In vivo* delivery of lipid particles such as SNALP encapsulating an interfering RNA (*e.g.,* siRNA) is suited for targeting cells of any cell type. The methods and compositions can be employed with cells of a wide variety of vertebrates, including mammals, such as, *e.g,* canines, felines, equines, bovines, ovines, caprines, rodents (*e.g*., mice, rats, and guinea pigs), lagomorphs, swine, and primates (*e.g*. monkeys, chimpanzees, and humans).

[0314] To the extent that tissue culture of cells may be required, it is well-known in the art. For example, Freshney, Culture of Animal Cells, a Manual of Basic Technique, 3rd Ed., Wiley-Liss, New York (1994), Kuchler *et al*., Biochemical Methods in Cell Culture and Virology, Dowden, Hutchinson and Ross, Inc. (1977), and the references cited therein provide a general guide to the culture of cells. Cultured cell systems often will be in the form of monolayers of cells, although cell suspensions are also used.

D. Detection of Lipid Particles

[0315] In some embodiments, the lipid particles of the present disclosure (*e.g.,* SNALP) are detectable in the subject at about 1, 2, 3, 4, 5, 6, 7, 8 or more hours. In other embodiments, the lipid particles of the present disclosure (*e.g.,* SNALP) are detectable in the subject at about 8, 12, 24, 48, 60, 72, or 96 hours, or about 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 25, or 28 days after administration of the particles. The presence of the particles can be detected in the cells, tissues, or other biological samples from the subject. The particles may be detected, *e.g*., by direct detection of the particles, detection of a therapeutic nucleic acid such as an interfering RNA (*e.g*., siRNA) sequence, detection of the target sequence of interest (*i.e*., by detecting expression or reduced expression of the sequence of interest), or a combination thereof.

1. Detection of Particles

[0316] Lipid particles described herein such as SNALP can be detected using any method known in the art. For example, a label can be coupled directly or indirectly to a component of the lipid particle using methods well-known in the art. A wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the lipid particle component, stability requirements, and available instrumentation and disposal provisions. Suitable labels include, but are not limited to, spectral labels such as fluorescent dyes (*e.g*., fluorescein and derivatives, such as fluorescein isothiocyanate (FITC) and Oregon Green™; rhodamine and derivatives such Texas red, tetrarhodimine isothiocynate (TRITC), *etc*., digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like; radio labels such as ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, *etc*.; enzymes such as horse radish peroxidase, alkaline phosphatase, *etc*.; spectral colorimetric labels such as colloidal gold or colored glass or plastic beads such as polystyrene, polypropylene, latex, *etc.* The label can be detected using any means known in the art.

2. Detection of Nucleic Acids

[0317] Nucleic acids (*e.g*., interfering RNA) are detected and quantified herein by any of a number of means well-known to those of skill in the art. The detection of nucleic acids may proceed by well-known methods such as Southern analysis, Northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography. Additional analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography may also be employed.

[0318] The selection of a nucleic acid hybridization format is not critical. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in, *e.g*., "Nucleic Acid Hybridization, A Practical

Approach," Eds. Hames and Higgins, IRL Press (1985).

[0319] The sensitivity of the hybridization assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBA™) are found in Sambrook et al., In Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (2000); and Ausubel et al., SHORT PROTOCOLS IN MOLECULAR BIOLOGY, eds., Current Protocols, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (2002); as well as U.S. Patent No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis et al. eds.) Academic Press Inc. San Diego, CA (1990); Arnheim & Levinson (October 1, 1990), C&EN 36; The Journal Of NIH Research, 3:81 (1991); Kwoh et al., Proc. Natl. Acad. Sci. USA, 86:1173 (1989); Guatelli et al., Proc. Natl. Acad. Sci. USA, 87:1874 (1990); Lomell et al., J. Clin. Chem., 35:1826 (1989); Landegren et al., Science, 241:1077 (1988); Van Brunt, Biotechnology, 8:291 (1990); Wu and Wallace, Gene, 4:560 (1989); Barringer et al., Gene, 89:117 (1990); and Sooknanan and Malek, Biotechnology, 13:563 (1995). Improved methods of cloning *in vitro* amplified nucleic acids are described in U.S. Pat. No. 5,426,039. Other methods described in the art are the nucleic acid sequence based amplification (NASBA™, Cangene, Mississauga, Ontario) and Qβ-replicase systems. These systems can be used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using, for example, nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation.

[0320] Nucleic acids for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as inhibitor components are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage et al., Tetrahedron Letts., 22:1859 1862 (1981), *e.g.*, using an automated synthesizer, as described in Needham VanDevanter et al., Nucleic Acids Res., 12:6159 (1984). Purification of ploynucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion exchange HPLC as described in Pearson et al., J. Chrom., 255:137 149 (1983). The sequence of the synthetic poluyucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, Methods in Enzymology, 65:499.

[0321] An alternative means for determining the level of transcription is *in situ* hybridization. *In situ* hybridization assays are well-known and are generally described in Angerer et al., Methods Enzymol., 152:649 (1987). In an *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

VIII. Examples

[0322] The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

Example 1. Materials and Methods.

[0323] ***siRNA***: All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, CO). The siRNAs were desalted and annealed using standard procedures.

[0324] ***Lipid Encapsulation of siRNA*:** In some embodiments, siRNA molecules were encapsulated into nucleic acid-lipid particles composed of the following lipids: the lipid conjugate PEG-cDMA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropylamine); the cationic lipid DLinDMA (1,2-Dilinoleyloxy-3-(N,N-dimethyl)aminopropane); the phospholipid DPPC (1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids; Alabaster, AL); and synthetic cholesterol (Sigma-Aldrich Corp.; St. Louis, MO) in the molar ratio 1.4:57.1:7.1:34.3, respectively. In other words, siRNAs were encapsulated into SNALP of the following "1:57" formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol. In other embodiments, siRNA molecules were encapsulated into phospholipid-free SNALP composed of the following lipids: the lipid conjugate PEG-cDMA; the cationic lipid DLinDMA; and synthetic cholesterol in the molar ratio 1.5:61.5:36.9, respectively. In other words, siRNAs were encapsulated into phospholipid-free SNALP of the following "1:62" formulation: 1.5% PEG-cDMA; 61.5% DLinDMA; and 36.9% cholesterol. For vehicle controls, empty particles with identical lipid composition were formed in the absence of siRNA. It should be understood that the 1:57 formulation and 1:62 formulation are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the formulation may vary. Typically, in the 1:57

formulation, the amount of cationic lipid will be 57 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:57 formulation being made up of non-cationic lipid (*e.g.*, phospholipid, cholesterol, or a mixture of the two). Similarly, in the 1:62 formulation, the amount of cationic lipid will be 62 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:62 formulation being made up of the non-cationic lipid (*e.g.*, cholesterol).

Example 2. Eg5 siRNA Formulated as 1:57 SNALP Are Potent Inhibitors of Cell Growth *in vitro*.

[0325] SNALP formulations were prepared with an siRNA targeting Eg5 as the nucleic acid component. Eg5 is a member of kinesin-related proteins that are involved in functions related to movements of organelles, microtubules, or chromosomes along microtubules. These functions include axonal transport, microtubule sliding during nuclear fusion or division, and chromosome disjunction during meiosis and early mitosis. Eg5 plays a critical role in mitosis of mammalian cells. The Eg5 siRNA used in this study is provided in Table 1. The modifications involved introducing 2'OMe-uridine at selected positions in the sense and antisense strands of the Eg5 2263 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 1. siRNA duplex comprising sense and antisense Eg5 RNA polynucleotides.

Modification	Eg5 2263 siRNA sequence	% 2'OMe-Modified	% Modified in DS Region
U/U	5'-C**U**GAAGACC**U**GAAGACAA**U**dTdT-3' 3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUUA-5'	6/42 = 14.3 %	6/38 = 15.8%
Column 1: "U/U" = 2'OMe-uridine modified siRNA duplex; Column 2: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. "dT" = deoxythymidine. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.			

[0326] The lipid components and physical characteristics of the SNALP formulations are summarized in Table 2. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes et al., Journal of Controlled Release, 107:276-287 (2005).

Table 2. Characteristics of the SNALP formulations used in this study.

Sample No.	Formulation Composition, Mole % PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
1	2 | 40 | 10 | 48	12.4	57	0.07	90
2	1.8 | 36.4 | 18.2 | 43.6	14.0	72	0.12	89
3	1.4 | 27.0 | 6.8 | 64.9	16.5	70	0.12	92
4	1.3 | 25.3 | 12.7 | 60.8	18.1	76	0.07	93
5	3.9 | 39.2 | 9.8 | 47.1	13.5	53	0.27	86
6	3.6 | 35.7 | 17.9 | 42.9	15.1	58	0.18	87
7	2.7 | 26.7 | 6.7 | 64.0	17.6	56	0.17	92
8	2.5 | 25.0 | 12.5 | 60.0	19.2	61	0.13	92
9	1.4 | 57.1 | 7.1 | 34.3	17.8	84	0.10	88
10	1.3 | 53.3 | 13.3 | 32.0	19.5	83	0.10	89
11	1.1 | 42.6 | 5.3 | 51.1	22.0	80	0.10	93
12	1.0 | 40.4 | 10.1 | 48.5	23.6	78	0.11	88
13	2.8 | 56.3 | 7.0 | 33.8	19.0	62	0.14	80

(continued)

Sample No.	Formulation Composition, Mole % PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
14	2.6 | 52.6 | 13.2 | 31.6	20.6	66	0.14	82
15	2.1 | 42.1 | 5.3 | 50.5	23.1	71	0.16	91
16	2 | 40 | 10 | 48	24.7	67	0.14	92

[0327] Silencing of Eg5 by siRNA transfection causes mitotic arrest and apoptosis in mammalian cells. Cell viability following transfection with SNALP containing an siRNA targeting Eg5 therefore provides a simple biological readout of *in vitro* transfection efficiency. Cell viability of *in vitro* cell cultures was assessed using the commercial reagent CellTiter-Blue® (Promega Corp.; Madison, WI), a resazurin dye that is reduced by metabolically active cells to the flourogenic product resorufin. The human colon cancer cell line HT29 was cultured using standard tissue culture techniques. 72 hours after SNALP application, CellTiter-Blue® reagent was added to the culture to quantify the metabolic activity of the cells, which is a measure of cell viability. Data are presented as a percent of cell viability relative to ("untreated") control cells that received phosphate buffered saline (PBS) vehicle only.

[0328] Figure 1 shows that the 1:57 SNALP formulation containing Eg5 2263 U/U siRNA was among the most potent inhibitors of tumor cell growth at all siRNA concentrations tested (*see,* Figure 1B, Sample 9).

Example 3. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo.*

[0329] SNALP formulations were prepared with an siRNA targeting apolipoprotein B (ApoB) as the nucleic acid component. ApoB is the main apolipoprotein of chylomicrons and low density lipoproteins (LDL). Mutations in ApoB are associated with hypercholesterolemia. ApoB occurs in the plasma in 2 main forms, ApoB48 and ApoB100, which are synthesized in the intestine and liver, respectively, due to an organ-specific stop codon. The ApoB siRNA used in this study is provided in Table 3. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the ApoB siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 3. siRNA duplex comprising sense and antisense ApoB RNA polynucleotides.

Position	Modification	ApoB siRNA sequence	% 2'OMe-Modified	% Modified in DS Region
10048	U2/2 G1/2	5'-AGU**G**UCA**U**CACAC**U**GAAUACC-3' 3'-GU**U**CACAGUAGU**G**U**G**AC**U**UAU-5'	7/42 = 16.7%	7/38 = 18.4%
Column 1: The number refers to the nucleotide position of the 5' base of the sense strand relative to the mouse ApoB mRNA sequence XM_137955. Column 2: The numbers refer to the distribution of 2'OMe chemical modifications in each strand. Column 3: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. Column 4: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 5: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.				

[0330] The lipid components and physical characteristics of the formulations are summarized in Table 4. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes et al., Journal of Controlled Release, 107:276-287 (2005).

Table 4. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2 | 40 | 10 | 48	12.4	59	0.15	93
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 2.2 | 44.4 | 53.3	10.7	55	0.17	91
4	PEG(2000)-C-DMA | DLinDMA | DOPC | Cholesterol 2 | 40 | 10 | 48	12.5	59	0.16	92
5	PEG(2000)-C-DMA | DLinDMA | DMPC | Cholesterol 2 | 40 | 10 | 48	12.2	56	0.11	92
6	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.8 | 36.4 | 18.2 | 43.6	13.8	66	0.16	93
7	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 2 | 40 | 10 | 48	12.4	56	0.12	92
8	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 27.0 | 6.8 | 64.9	16.5	60	0.10	93
9	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.3 | 25.3 | 12.7 | 60.8	18.1	74	0.13	92
10	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2.5 | 25.0 | 12.5 | 60.0	19.2	60	0.13	93
11	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.4 | 34.3	17.8	79	0.09	94
12	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.0 | 40.4 | 10.1 | 48.5	23.6	72	0.11	93
13	PEG(2000)-C-DMA | DLinDMA | DPPC 2 | 70 | 28	8.7	73	0.09	87
14	PEG(2000)-C-DMA | DLinDMA | DPPC 1.6 | 54.7 | 43.8	11.3	65	0.11	87

[0331] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0332] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al*., *Molecular Therapy*, 13:494 (2006).

[0333] Figure 2 shows that the 1:57 SNALP formulation containing ApoB 10048 U2/2 G1/2 siRNA was the most potent at reducing ApoB expression *in vivo* (*see,* Group 11).

Example 4. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo.*

[0334] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 5. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes et al., Journal of Controlled Release, 107:276-287 (2005).

Table 5. Characteristics of the SNALP formulations used in this study.

SNALP (L:D ratio)	siRNA Payload	Particle Size (Polydispersity)	% Encapsulation
2:30 (13)	ApoB-10048 U2/2 G1/2	65 nm (0.16)	88
1:57 (9)	ApoB-10048 U2/2 G1/2	74 nm (0.10)	89

[0335] The 2:30 SNALP formulation used in this study is lipid composition 2:30:20:48 as described in molar percentages of PEG-C-DMA, DLinDMA, DSPC, and cholesterol (in that order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 13:1.

[0336] The 1:57 SNALP formulation used in this study is lipid composition 1.5:57.1:7:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 9:1.

[0337] BALB/c mice (female, 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Days 0, 1, 2, 3 & 4 for a total of 5 doses per animal. Daily dosage was either 1.0 (for 2:30 SNALP) or 0.1 (for 1:57 SNALP) mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given IV injections of phosphate buffered saline (PBS) vehicle. On Study Day 7, 72 h after the last treatment, animals were euthanized and liver tissue was collected in RNAlater.

[0338] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al*., *Molecular Therapy,* 13:494 (2006).

[0339] Figure 3 shows that the 1:57 SNALP containing ApoB 10048 U2/2 G1/2 siRNA was more than 10 times as efficacious as the 2:30 SNALP in mediating ApoB gene silencing in mouse liver at a 10-fold lower dose.

Example 5. ApoB siRNA Formulated as 1:57 or 1:62 SNALP Have Potent Silencing Activity *in vivo.*

[0340] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 6. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes et al., Journal of Controlled Release, 107:276-287 (2005).

Table 6. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	76	0.06	89
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 1.5 | 61.5 | 36.9	8.1	76	0.04	86
4	PEG(2000)-C-DMA | DODMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.0	72	0.05	95

(continued)

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
5	PEG(5000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.6	52	0.16	89
6	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 1.4 | 57.1 | 7.1 | 34.3	8.9	68	0.10	94
7	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	72	0.07	95
8	PEG(2000)-C-DMA | DLinDMA | DPPC 1.8 | 70.2 | 28.1	8.6	74	0.13	86

[0341] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.75 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0342] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al*., *Molecular Therapy*, 13:494 (2006).

[0343] Figure 4 shows that the 1:57 and 1:62 SNALP formulations had comparable ApoB silencing activity *in vivo* (*see, e.g.,* Groups 2 & 3).

Example 6. ApoB siRNA Formulated as 1:62 SNALP Have Potent Silencing Activity *in vivo.*

[0344] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 7. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes et al., Journal of Controlled Release, 107:276-287 (2005).

Table 7. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition, Mole % PEG(2000)-C-DMA | LinDMA | Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	1.5 | 61.5 | 36.9	6.1	80	0.07	92
3	1.4 | 54.8 | 43.8	6.6	74	0.05	89
4	2.0 | 61.2 | 36.7	6.2	71	0.11	91
5	1.8 | 54.5 | 43.6	6.7	67	0.09	91
6	1.3 | 68.1 | 30.6	7.4	91	0.06	89
7	1.2 | 61.8 | 37.1	8.0	87	0.10	90
8	1.7 | 67.8 | 30.5	7.6	81	0.07	91
9	1.4 | 56.3 | 42.3	8.6	75	0.11	92
10	1.9 | 61.3 | 36.8	8.2	72	0.10	91
11	1.8 | 56.1 | 42.1	8.8	70	0.10	90

(continued)

Group	Formulation Composition, Mole % PEG(2000)-C-DMA | LinDMA | Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
12	1.3 | 66.7 | 32.0	9.5	89	0.09	89
13	1.2 | 61.7 | 37.0	10.0	87	0.10	91
14	1.7 | 66.4 | 31.9	9.6	82	0.11	90
15	1.5 | 61.5 | 36.9	10.1	79	0.10	91

[0345] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0346] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al*., *Molecular Therapy*, 13:494 (2006).

[0347] Figure 5 shows that the 1:62 SNALP formulation was one of the most potent inhibitors of ApoB expression at two different lipid:drug ratios *(i.e.,* 6.1 & 10.1) among the phospholipid-free SNALP formulations tested (*see,* Groups 2 & 15).

Example 7. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Syringe Press or Gear Pump Process.

[0348] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by various manufacturing processes. In particular, 1:57 SNALP was prepared by a syringe press or gear pump process using either PBS or citrate buffer (post-blend dilution) and administered intravenously in mice.

Experimental Design

[0349]

Animal Model: Female BALB/c mice, 5 wks old, n=4 per group/cage.
siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

Tolerability:

[0350]

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
1	PBS vehicle	Standard 10 mL/kg volume	
2	1|57 Citrate Direct Dil, Syringe Press	7	77
3	1|57 PBS Direct Dil, Syringe Press	7	96
4	1|57 PBS Direct Dil, Gear Pump	7	79
5	1|57 Citrate Direct Dil, Syringe Press	9	99
6	1|57 PBS Direct Dil, Syringe Press	9	123
7	1|57 PBS Direct Dil, Gear Pump	9	102

Efficacy:

[0351]

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
8	PBS vehicle	Standard 10 mL/kg volume	
9	1|57 PBS Direct Dil, Syringe Press	0.05	0.68
10	1|57 PBS Direct Dil, Gear Pump	0.05	0.57
11	1|57 PBS Direct Dil, Syringe Press	0.1	1.36
12	1|57 PBS Direct Dil, Gear Pump	0.1	1.13

Formulation:

[0352] Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

[0353] Formulation Details:

1. Lipid composition "1|57 Citrate blend" used in this study is 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation has an input lipid to drug ratio of 8.9.
2. Gear pump set up included 0.8 mm T-connector and 400 mL/min speed.
3. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0354] Formulation Summary:

		Particle Size			Final L:D
	1:57 (9:1) + DOW siRNA	Zavg (nm)	Poly	% Encap	(mg:mg)
322-050807-1	Syringe PBS Blend	79	0.12	92	13.6
322-050807-2	Syringe Citrate Blend	86	0.11	91	11.0
322-050807-3	Gear PBS Blend	80	0.09	93	11.3

Procedures

[0355] **Treatment:** Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 μl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0356] **Group 1-7 Endpoint:** Animals are sacrificed on Day 1, 24 h after test article administration. Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein.

[0357] **Group 8-12 Endpoint:** Animals are sacrificed on Day 2, 48 h after test article administration. Blood is collected by cardiac puncture and processed for plasma. Immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed and weighed separately: liver and spleen. The bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at -20°C or -80°C for archival purposes. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0358] **Termination:** Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0359] **Data Analysis:** Tolerability of treatment regime is monitored by animal appearance and behavior as well as body weight. Blood clinical chemistry is measured by automated analyzer. ApoB and GAPDH mRNA levels in liver are measured via QG assay. ApoB protein in plasma is measured via ELISA. Total cholesterol in plasma is measured via standard enzymatic/colorimetric assay.

Results

[0360] There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. Figure 6 shows that the tolerability of SNALP prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters. There was a tolerability difference between syringe citrate and syringe PBS at constant siRNA dosage, but that was likely an artifact dependent on the different final lipid:drug (L:D) ratios of these two preparations.

[0361] Figure 7 shows that the efficacy of the 1:57 SNALP prepared by gear pump was similar to the same SNALP prepared by syringe press. The tolerability profile was improved with the gear pump process, which could be attributed to increased initial encapsulation rate and decreased final L:D ratio.

Example 8. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Direct Dilution or In-Line Dilution Process.

[0362] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by a direct dilution or in-line dilution process at an input lipid to drug ratio of 6:1 or 9:1.

Experimental Design

[0363]

Animal Model: Female BALB/c mice, 7 wks old.
siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

CBC/Diff:

[0364]

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
1	3	PBS	-	-
2	3	1|57 SNALP (9:1)	7 mg/kg	71 mg/kg
3	3	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Clinical Chemistry:

[0365]

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
4	4	PBS	-	-
5	4	1|57 SNALP (9:1)	9 mg/kg	92 mg/kg
6	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg
7	4	(6:1) New 1|57 SNALP	11 mg/kg	78 mg/kg
8	4	(6:1) New 1|57 SNALP	13 mg/kg	93 mg/kg
9	4	(6:1) New 1|57 SNALP	15 mg/kg	107 mg/kg

(continued)

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
10	4	(6:1) New 1|57 SNALP	17 mg/kg	121 mg/kg
11	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Activity:

[0366]

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
12	4	PBS	-	-
13	4	1|57 SNALP (9:1)	0.05 mg/kg	0.51 mg/kg
14	4	1|57 SNALP (9:1)	0.1 mg/kg	1.02 mg/kg
15	4	1|57 SNALP (9:1)	0.2 mg/kg	2.04 mg/kg
16	4	(6:1) New 1|57 SNALP	0.05 mg/kg	0.36 mg/kg
17	4	(6:1) New 1|57 SNALP	0.1 mg/kg	0.71 mg/kg
18	4	(6:1) New 1|57 SNALP	0.2 mg/kg	1.42 mg/kg
19	4	(6:1) New 1|57 SNALP	0.4 mg/kg	2.85 mg/kg

Formulation:

[0367] Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.
[0368] Formulation Details:

1. "1|57 SNALP" used in this study is lipid composition 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by gear pump at an input lipid to drug ratio of 9:1 (28 mM lipids) or 6:1 (14 mM lipids).
2. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0369] Formulation Summary:

	1|57 SNALP Gear PBS In-Line	Particle Size			Final L:D
		Zavg (nm)	Poly	% Encap	(mg:mg)
322-051407-1	Input 9:1	78	0.07	93	10.2
322-051407-2	Input 6:1	81	0.05	92	7.1

Procedures

[0370] **Treatment:** Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 μl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.
[0371] **Endpoint:** Animals are sacrificed on Day 1, 24 h after test article administration (Grps 1-10) or on Day 2, 48 h after test article administration (Grps 11-19).
[0372] **Groups 1-3:** Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into an EDTA

microtainer, mixed immediately to prevent coagulation, and sent for analysis of CBC/Diff profile. Perform brief necropsy.

[0373] **Groups 4-11:** Blood is collected by cardiac puncture into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein. Perform brief necropsy.

[0374] **Groups 12-19:** Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed: liver. The liver is not weighed; the bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at -80°C. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0375] **Termination:** Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0376] **Data Analysis:** Tolerability of treatment regime is monitored by animal appearance and behavior, and body weight. Blood clinical chemistry and CBC/Diff profile is measured by automated analyzer. Liver ApoB mRNA is measured using the QuantiGene Assay. Plasma ApoB-100 is measured using ELISA. Plasma total cholesterol is measured using a standard enzymatic assay.

Results

Tolerability:

[0377] Figure 8 shows that there was very little effect on body weight 24 hours after 1:57 SNALP administration. The maximum weight loss of 3.6 ± 0.7% was observed at the highest drug dose of 17 mg/kg. There was also no obvious change in animal appearance/behavior at any of the dosages tested.

[0378] Figure 9 shows that there were no obvious changes in platelet count. Reduction of platelets can cause the mean platelet volume to increase as the body produces new platelets in compensation for the treatment-related decrease. Under the conditions of this study, the mean platelet volume did not change in SNALP-treated groups.

[0379] Figure 10 shows that clinically significant liver enzyme elevations (3xULN) occurred at drug dosages of 11 mg/kg for 1:57 SNALP at a lipid:drug (L:D) ratio of 10, and at 13 mg/kg at a L:D of 7. A slight dose response trend upwards in plasma total protein and globulin was also observed.

Efficacy:

[0380] Figure 11 shows that based on the liver mRNA QuantiGene analysis, the potency of the lower L:D SNALP was as good as that of the higher L:D SNALP at the tested drug dosages. In fact, the ApoB silencing activity was identical at the 0.05 and 0.1 mg/kg dosages. As such, the potency of the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) was similar to the potency of the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) at reducing ApoB expression.

[0381] Figure 12 shows that ApoB protein and total cholesterol levels were reduced to a similar extent by the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

Therapeutic Index:

[0382] This study demonstrates that both the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) caused about 60% ApoB liver mRNA silencing with a drug dose of 0.1 mg/kg. Interpolating from the available data points in Figure 10, a 10:1 final L:D ratio at 10 mg/kg may cause a similar degree of enzyme elevation as a 7:1 final L:D ratio at 13 mg/kg. Using these activity and toxicity points, the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio is (10 mg/kg) / (0.1 mg/kg) = 100 and the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is (13 mg/kg) / (0.1 mg/kg) = 130. Using this dataset, the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is 30% greater than the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio.

Example 9. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Increases Survival of Hep3B Tumor-Bearing Mice.

[0383] SNALP containing polo-like kinase 1 (PLK-1) siRNA (1:57 SNALP formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) were tested for their effects on the survival of CD1 nu/nu mice bearing Hep3B

liver tumors. PLK-1 is a serine/threonine kinase containing two functional domains: (1) a kinase domain; and (2) a polo-box domain (*see, e.g.,* Barr et al., Nat. Rev. Mol. Cell Biol., 5:429-440 (2004)). The activity and cellular concentration of PLK-1 are crucial for the precise regulation of cell division. PLK-1 is overexpressed in many cancer types including hepatoma and colon cancer, and PLK-1 expression often correlates with poor patient prognosis. Overexpression of PLK-1 (wild-type or kinase inactive) results in multinucleation (genetic instability). Hyperactive PLK-1 overrides the DNA damage checkpoint. Constitutive PLK-1 expression causes transformation of NIH 3T3 cells. PLK-1 phosphorylates the p53 tumor suppressor, thereby inhibiting the pro-apoptotic effects of p53. The PLK-1 siRNA used in this study are provided in Table 8. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the PLK-1 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 8. siRNA duplexes comprising sense and antisense PLK-1 RNA polynucleotides.

siRNA	PLK-1 siRNA Sequence		% Modified in DS Region
PLK1424 U4/GU	5'-AGA**U**CACCC**U**CCU**U**CAAA**U**ANN-3' 3'-NNUC**U**AGUGGGAG**G**AAUUUAU-5'	(SEQ ID NO.57) (SEQ ID NO.54)	6/38 = 15.8%
PLK1424 U4/G	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' 3'-NNUCUA**G**UG**G**GA**G**GAAUUUAU-5'	(SEQ ID NO.57) (SEQ ID NO.56)	7/38 = 18.4%
Column 1: The number after "PLK" refers to the nucleotide position of the 5' base of the sense strand relative to the start codon (ATG) of the human PLK-1 mRNA sequence NM_005030. Column 2: 2'-O-methyl (2'OMe) nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. N = deoxythymidine (dT) nucleotide, uridine (U) ribonucleotide, or ribonucleotide having complementarity to the target sequence or the complementary strand thereof. Column 3: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.			

Experimental Groups

[0384] 20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay
A	20 to seed	I.H. $1.5x10^6$ Hep3B	Luc 1:57	9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10 x 2 mg/kg	When moribund	Survival Body Weights
B			PLK 1424 1:57	9				

Test Articles

[0385] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 20 ml of each SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	Luc U/U SNALP 1:57 (28mM lipid)
B	PLK1424 U4/GU SNALP 1:57 (28mM lipid) PLK1424 U4/G SNALP 1:57 (28mM lipid)

Procedures

[0386]

Day 0	Mice will receive Anafen by SC injection (100 μg in 20 μl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25 μl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 μl) and 30G (3/8") needle. Cells will be injected slowly (~ 30 s) and a swab applied to the puncture wound immediately after needle withdrawal. After any bleeding has stopped (~1 min), the incision is closed with 5-6 sutures in the muscle wall and 3-4 skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.
Day 1	All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 μg in 20 μl saline).
Day 10	Mice will be randomized into the appropriate treatment groups.
Day 11	**Groups A, B - Day 11:** All Animals will be administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg). Dosing will be repeated for 5 consecutive days based on initial weight.
Day 14-35	**Groups A, B - Days 14, 17, 21, 25, 28, 32, 35:** All Animals will be re-administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg). **Body weights Groups:** Mice will be weighed on the day of dosing for 5 weeks, then twice weekly until close of the study. **Endpoint:** Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the treatment or tumor burden are terminated at the discretion of the vivarium staff.
Termination:	Mice are anesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.
Data Analysis:	Survival and body weights are assayed.

Results

[0387] Figure 13 shows the mean body weights of mice during therapeutic dosing of PLK1424 SNALP in the Hep3B intrahepatic (I.H.) tumor model. The treatment regimen was well tolerated with no apparent signs of treatment-related toxicity.

[0388] Figure 14 shows that treatment with 1:57 SNALP-formulated PLK1424 caused a significant increase in the survival of Hep3B tumor-bearing mice. This *in vivo* anti-tumor effect was observed in the absence of any apparent toxicity or immune stimulation.

Example 10. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Induces Tumor Cell Apoptosis in Hep3B Tumor-Bearing Mice.

[0389] The objectives of this study were as follows:

1. To determine the level of mRNA silencing in established Hep3B liver tumors following a single IV administration of PLK1424 SNALP.
2. To confirm the mechanism of mRNA silencing by detecting specific RNA cleavage products using RACE-PCR.
3. To confirm induction of tumor cell apoptosis by histopathology.

[0390] The 1:57 SNALP formulation (1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) was used for this study.

Experimental Groups

[0391] 20 SCID/beige mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	Sacrifice	Assay
A	20 to seed	I.H. $1x10^6$ Hep3B	PBS	6	1 x 2 mg/kg Day 20	24 h after treatment	Tumor QG Tumor RACE-PCR Histopathology
			Luc 1:57	7			
B			PLK 1424 1:57	7			
C							

Test Articles

[0392] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 2 ml of SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	PBS
B	Luc U/U 1:57 SNALP
C	PLK1424 U4/GU 1:57 SNALP

Procedures

[0393]

Day 0 Mice will receive Anafen by SC injection (100 μg in 20 μl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25μl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 μl) and 30G (3/8") needle. Cells will be injected slowly (~30 s) and a swab applied to the puncture wound immediately after needle withdrawal. After any bleeding has stopped (~1 min), the muscle wall incision is closed with 5-6 sutures. The skin incision is then closed with 3-4 metal skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.

Day 1 All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 μg in 20 μl saline).

Day 7 Mice will be randomized into the appropriate treatment groups.

Day 20 **Groups A-C:** Mice will be weighed and then administered either PBS, Luc, or PLK1424 SNALP by IV injection via the lateral tail vein. SNALP will be dosed at 2 mg/kg or equivalent volume (10 ml/kg) according to body weight.

Day 21 **Groups A-C:** All mice will be weighed and then euthanized by lethal anesthesia. Tumor bearing liver lobes from all mice in each group will be weighed and collected into RNALater for RNA analysis.

Endpoint: Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the treatment or tumor burden are terminated at the discretion of the vivarium staff.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.

Data Analysis: mRNA analysis of liver tumors by bDNA (QG) assay and RACE-PCR.

(continued)

Tumor cell apoptosis by histopathology.

Results

[0394] Body weights were monitored from Day 14 onwards to assess tumor progression. On Day 20, 6 mice showing greatest weight loss were randomized into each of the 3 groups and treated. All six mice had substantial-large I.H. tumors at sacrifice (Day 21). Treatment of the remaining 14 mice was therefore initiated on the Day 21 (sacrifice Day 22). 10/14 mice had substantial tumors; 2/14 mice had small/probable tumors; and 2/14 mice had no visible tumor burden.

[0395] Figure 15 shows data from Quantigene assays used to measure human (tumor)-specific PLK-1 mRNA levels. A single 2 mg/kg dose of 1:57 SNALP reduced PLK-1 mRNA levels by about 50% in intrahepatic Hep3B tumors growing in mice.

[0396] Figure 16 shows that a specific cleavage product of PLK-1 mRNA was detectable in mice treated with PLK1424 SNALP by 5' RACE-PCR. No specific PCR product was detectable in mice treated with either PBS or control (Luc) SNALP. Nucleotide sequencing of the PCR product confirmed the predicted cleavage site by PLK1424 siRNA-mediated RNA interference in the PLK-1 mRNA.

[0397] Figure 17 shows Hep3B tumor histology in mice treated with either Luc SNALP (top) or PLK1424 SNALP (bottom). Luc SNALP-treated mice displayed normal mitoses in Hep3B tumors, whereas PLK1424 SNALP-treated mice exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors.

Conclusion

[0398] This example illustrates that a single administration of PLK1424 1:57 SNALP to Hep3B tumor-bearing mice induced significant *in vivo* silencing of PLK-1 mRNA. This reduction in PLK-1 mRNA was confirmed to be mediated by RNA interference using 5' RACE-PCR analysis. Importantly, PLK-1 mRNA silencing by the 1:57 SNALP formulation profoundly disrupted tumor cell proliferation (mitosis), causing subsequent apoptosis of tumor cells. As demonstrated in the previous example, this anti-tumor effect translated into extended survival times in the tumor-bearing mice.

Example 11. Comparison of 1:57 PLK-1 SNALP Containing Either PEG-cDMA or PEG-cDSA in a Subcutaneous Hep3B Tumor Model.

[0399] This example demonstrates the utility of the PEG-lipid PEG-cDSA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-distearyloxypropylamine) in the 1:57 formulation for systemically targeting distal (*e.g.*, subcutaneous) tumors. In particular, this example compares the tumor targeting ability of 1:57 PLK-1 SNALPs containing either PEG-cDMA (C_{14}) or PEG-cDSA (C_{18}). Readouts are tumor growth inhibition and PLK1 mRNA silencing. The PLK-1 siRNA used was PLK1424 U4/GU, the sequence of which is provided in Table 8.

[0400] Subcutaneous (S.C.) Hep3B tumors were established in scid/beige mice. Multidose anti-tumor efficacy of 1:57 PLK-1 SNALP was evaluated for the following groups (n=5 for each group): (1) "Luc-cDMA" - PEG-cDMA Luc SNALP; (2) "PLK-cDMA" - PEG-cDMA PLK-1 SNALP; and (3) "PLK-cDSA" - PEG-cDSA PLK-1 SNALP. Administration of 6 x 2mg/kg siRNA was initiated once tumors reached about 5 mm in diameter (Day 10). Dosing was performed on Days 10, 12, 14, 17, 19, and 21. Tumors were measured by caliper twice weekly.

[0401] Figure 18 shows that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B S.C. tumors. In particular, 5/5 tumors in the PLK1-cDSA treated mice appeared flat, measurable only by discoloration at the tumor site.

[0402] Figure 19 shows the mRNA silencing of 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration. The extent of silencing observed with the PLK1-cDSA SNALP correlated with the anti-tumor activity in the multi-dose study shown in Figure 18.

[0403] The Luc-cDMA SNALP-treated group, which had developed large S.C. tumors at Day 24, were then administered PLK-cDSA SNALP on Days 24, 26, 28, 31, 33, and 35. There was no additional dosing of the original PLK-1 SNALP-treated groups. The results from this crossover dosing study with large established tumors is provided in Figure 20, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0404] A comparison of the effect of PEG-cDMA and PEG-cDSA 1:57 SNALPs on PLK-1 mRNA silencing was performed using established intrahepatic Hep3B tumors in scid/beige mice. A single 2 mg/kg dose of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA was administered intravenously. Liver/tumor samples were collected at 24 and 96 hours after SNALP treatment. Control = 2 mg/kg Luc-cDMA SNALP at 24 hours.

[0405] Figure 21 shows that PLK-cDMA SNALP and PLK-cDSA SNALP had similar silencing activities after 24 hours, but that the PLK-cDSA SNALP may increase the duration of mRNA silencing in intrahepatic tumors.

[0406] Figure 22 shows the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA. The extended blood circulation times observed for the PLK-cDSA SNALP may enable the increased accumulation and activity at distal (*e.g.*, subcutaneous) tumor sites.

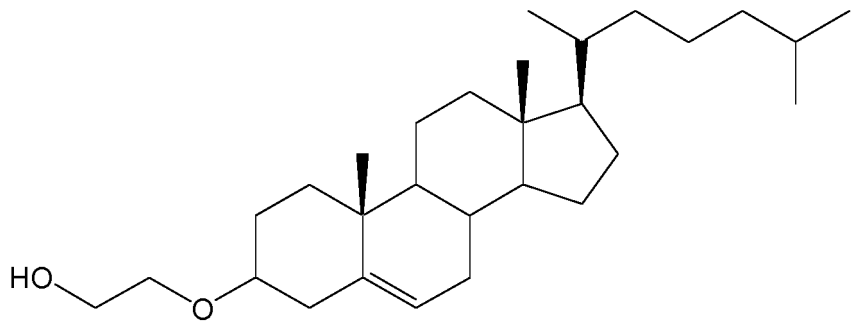
[0407] Thus, this study shows that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP can be used to preferentially target the liver.

Example 12. Synthesis of Cholesteryl-2'-Hydroxyethyl Ether.

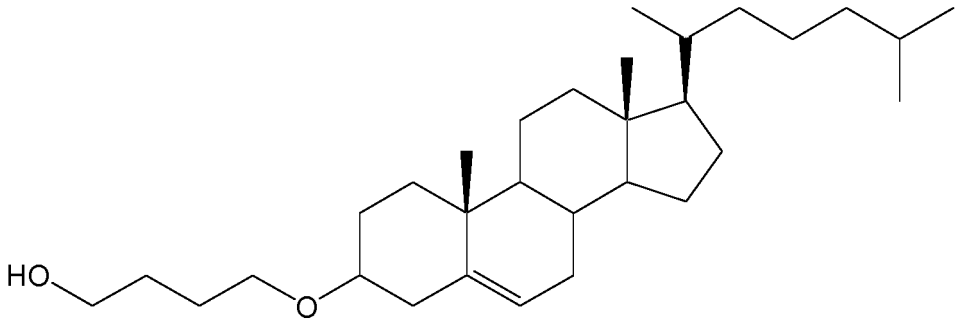
[0408] Step 1: A 250 ml round bottom flask containing cholesterol (5.0 g, 12.9 mmol) and a stir bar was sealed and flushed with nitrogen. Toluenesulphonyl chloride (5.0 g, 26.2 mmol) was weighed into a separate 100-mL round bottom flask, also sealed and flushed with nitrogen. Anhydrous pyridine (2 x 50 ml) was delivered to each flask. The toluenesulphonyl chloride solution was then transferred, via cannula, into the 250 ml flask, and the reaction stirred overnight. The pyridine was removed by rotovap, and methanol (80 ml) added to the residue. This was then stirred for 1 hour until a homogeneous suspension was obtained. The suspension was filtered, washed with acetonitrile (50 ml), and dried under vacuum to yield cholesteryl tosylate as a fluffy white solid (6.0 g, 86%).

[0409] Step 2: Cholesteryl tosylate (2.0 g, 3.7 mmol), 1,4-dioxane (50 mL), and ethylene glycol (4.6 g, 74 mmol) were added to a 100 ml flask containing a stir bar. The flask was fitted with a condenser, and refluxed overnight. The dioxane was then removed by rotovap, and the reaction mixture suspended in water (100 ml). The solution was transferred to a separating funnel and extracted with chloroform (3 x 100 ml). The organic phases were combined, washed with water (2 x 150 ml), dried over magnesium sulphate, and the solvent removed. The crude product was purified by column chromatography (5% acetone/hexane) to yield the product as a white solid (1.1 g, 69%).

[0410] The structures of the cholesterol derivatives cholesteryl-2'-hydroxyethyl ether and cholesteryl-4'-hydroxybutyl ether are as follows:



Cholesteryl-2'-hydroxyethyl ether



Cholesteryl-4'-hydroxybutyl ether

[0411] It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims.

Claims

1. A nucleic acid-lipid particle comprising:

(a) a nucleic acid;
(b) a cationic lipid comprising from 50 mol % to 65 mol % of the total lipid present in the particle;
(c) a non-cationic lipid comprising up to 49.5 mol % of the total lipid present in the particle and comprising a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle; and
(d) a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle.

2. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid comprises a small interfering RNA (siRNA).

3. The nucleic acid-lipid particle of claim 2, wherein:

(a) the siRNA comprises from about 15 to about 60 nucleotides; or
(b) the siRNA comprises at least one modified nucleotide; or
(c) the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide; or
(d) the siRNA is about 19 to about 25 base pairs in length; or
(e) the siRNA comprises 3' overhangs.

4. The nucleic acid-lipid particle of claim 1, wherein:

(a) the cationic lipid comprises 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), or a mixture thereof; or
(b) the cationic lipid comprises 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA); or
(c) the cationic lipid comprises from 50 mol % to 60 mol % of the total lipid present in the particle; or
(d) the cationic lipid comprises from 52 mol % to 62 mol % of the total lipid present in the particle.

5. The nucleic acid-lipid particle of claim 1, wherein:

(a) the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate; or
(b) the conjugated lipid that inhibits aggregation of particles comprises from 1 mol % to 2 mol % of the total lipid present in the particle.

6. The nucleic acid-lipid particle of claim 1, wherein:

(a) the nucleic acid in the nucleic acid-lipid particle is not substantially degraded after incubation of the particle in serum at 37°C for 30 minutes; or
(b) the nucleic acid is fully encapsulated in the nucleic acid-lipid particle; or
(c) the nucleic acid-lipid particle has a lipid:nucleic acid mass ratio of from about 5 to about 15; or
(d) the nucleic acid-lipid particle has a median diameter of from about 40 nm to about 150 nm.

7. The nucleic acid-lipid particle of claim 1, wherein:

(a) the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof; or
(b) the phospholipid comprises from 4 mol % to 10 mol % of the total lipid present in the particle..

8. The nucleic acid-lipid particle of claim 1, wherein:

(a) the cholesterol or derivative thereof comprises from 30 mol % to 35 mol % of the total lipid present in the particle; or
(b) the cholesterol or derivative thereof comprises from 32 mol % to 36 mol % of the total lipid present in the particle and the phospholipid comprises from 3 mol % to 15 mol % of the total lipid present in the particle.

9. The nucleic acid-lipid particle of claim 5(a), wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof; optionally wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof; preferably wherein the PEG in the PEG-DAA conjugate has an average molecular weight of about 2,000 daltons.

10. The nucleic acid-lipid particle of claim 5(a), wherein the nucleic acid is an siRNA and the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 7.1 mol % phospholipid, about 34.3 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

11. A pharmaceutical composition comprising a nucleic acid-lipid particle of any one of the preceding claims and a pharmaceutically acceptable carrier.

12. A method for introducing a nucleic acid into a cell, the method comprising:

contacting the cell *in vitro* with a nucleic acid-lipid particle of any one of claims 1 to 10, optionally wherein the cell is a mammalian cell.

13. A nucleic acid-lipid particle of any one of claims 1 to 10 for use in a method for the *in vivo* delivery of a nucleic acid, the method comprising administering said nucleic acid-lipid particle to a mammalian subject.

14. The nucleic acid-lipid particle for use according to claim 13, wherein the administration is selected from the group consisting of oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal.

15. A nucleic acid-lipid particle of any one of claims 1 to 10 for use in a method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising administering a therapeutically effective amount of said nucleic acid-lipid particle to the mammalian subject, wherein the disease or disorder is optionally selected from the group consisting of a viral infection, a liver disease or disorder, and cancer.

Patentansprüche

1. Nukleinsäure-Lipidteilchen, umfassend:

(a) eine Nukleinsäure;
(b) ein kationisches Lipid, umfassend 50 mol-% bis 65 mol-% des gesamten im Teilchen vorhandenen Lipids;
(c) ein nicht-kationisches Lipid, umfassend bis zu 49,5 mol-% des gesamten im Teilchen vorhandenen Lipids und umfassend ein Gemisch aus einem Phospholipid und Cholesterin oder einem Derivat davon; wobei das Cholesterin oder das Derivat davon 30 mol-% bis 40 mol-% des gesamten im Teilchen vorhandenen Lipids umfasst; und
(d) ein konjugiertes Lipid, das die Aggregation von Teilchen hemmt, die 0,5 mol-% bis 2 mol-% des gesamten im Teilchen vorhandenen Lipids umfassen.

2. Nukleinsäure-Lipidteilchen nach Anspruch 1, wobei die Nukleinsäure eine kleine Interferenz-RNA (siRNA) umfasst.

3. Nukleinsäure-Lipidteilchen nach Anspruch 2, wobei:

(a) die siRNA ca. 15 bis ca. 60 Nukleotide umfasst; oder
(b) die siRNA mindestens ein modifiziertes Nukleotid umfasst; oder
(c) die siRNA mindestens ein 2'-O-Methyl (2'OMe)-Nukleotid umfasst; oder
(d) die siRNA ca. 19 bis 25 Basenpaare lang ist; oder
(e) die siRNA 3'Überhänge umfasst.

4. Nukleinsäure-Lipidteilchen nach Anspruch 1, wobei:

(a) das kationische Lipid 1,2-Dilinoleyloxy-N,N-dimethylaminopropan (DLinDMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropan (DLenDMA) oder ein Gemisch davon umfasst; oder

(b) das kationische Lipid 2,2-Dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolan (DLin-K-C2-DMA) umfasst; oder
(c) das kationische Lipid 50 mol-% bis 60 mol-% des gesamten im Teilchen vorhandenen Lipids umfasst; oder
(d) das kationische Lipid 52 mol-% bis 62 mol-% des gesamten im Teilchen vorhandenen Lipids umfasst.

5. Nukleinsäure-Lipidteilchen nach Anspruch 1, wobei:

(a) das konjugierte Lipid, das eine Aggregation von Teilchen hemmt, ein Polyethylenglycol (PEG)-Lipidkonjugat umfasst; oder
(b) das konjugierte Lipid, das eine Aggregation von Teilchen hemmt, 1 mol-% bis 2 mol-% des gesamten im Teilchen vorhandenen Lipids umfasst.

6. Nukleinsäure-Lipidteilchen nach Anspruch 1, wobei:

(a) die Nukleinsäure im Nukleinsäure-Lipidteilchen nach einer Inkubation des Teilchens in Serum bei 37° C für 30 Minuten nicht wesentlich abgebaut wird; oder
(b) die Nukleinsäure vollständig im Nukleinsäure-Lipidteilchen eingekapselt ist; oder
(c) das Nukleinsäure-Lipidteilchen ein Lipid:Nukleinsäure-Massenverhältnis von ca. 5 zu ca. 15 hat; oder
(d) das Nukleinsäure-Lipidteilchen einen mittleren Durchmesser von ca. 40 nm bis ca. 150 nm hat.

7. Nukleinsäure-Lipidteilchen nach Anspruch 1, wobei:

(a) das Phospholipid Dipalmitoylphosphatidylcholin (DPPC), Distearoylphosphatidylcholin (DSPC) oder ein Gemisch davon umfasst; oder
(b) das Phospholipid 4 mol-% bis 10 mol-% des gesamten im Teilchen vorhandenen Lipids umfasst.

8. Nukleinsäure-Lipidteilchen nach Anspruch 1, wobei:

(a) das Cholesterin oder das Derivat davon 30 mol-% bis 35 mol-% des gesamten im Teilchen vorhandenen Lipids umfasst; oder
(b) das Cholesterin oder das Derivat davon 32 mol-% bis 36 mol-% des gesamten im Teilchen vorhandenen Lipids umfasst und das Phospholipid 3 mol-% bis 15 mol-% des gesamten im Teilchen vorhandenen Lipids umfasst.

9. Nukleinsäure-Lipidteilchen nach Anspruch 5(a), wobei das PEG-Lipidkonjugat ein PEG-Diacylglycerin (PEG-DAG)-Konjugat, ein PEG-Dialkyloxypropyl (PEG-DAA)-Konjugat oder ein Gemisch davon umfasst; wobei optional das PEG-DAA-Konjugat ein PEG-Dimyristyloxypropyl (PEG-DMA)-Konjugat, ein PEG-Distearyloxypropyl (PEG-DSA)-Konjugat oder ein Gemisch davon umfasst; wobei vorzugsweise das PEG im PEG-DAA-Konjugat ein durchschnittliches Molekulargewicht von ca. 2.000 Dalton hat.

10. Nukleinsäure-Lipidteilchen nach Anspruch 5(a), wobei die Nukleinsäure eine siRNA ist und das Nukleinsäure-Lipidteilchen ca. 57,1 mol-% kationisches Lipid, ca. 7,1 mol-% Phospholipid, ca. 34,3 mol-% Cholesterin oder ein Derivat davon und ca. 1,4 mol-% PEG-Lipidkonjugat umfasst.

11. Pharmazeutische Zusammensetzung, umfassend ein Nukleinsäure-Lipidteilchen nach einem der vorstehenden Ansprüche und eine pharmazeutische verträgliche Trägersubstanz.

12. Verfahren zur Einführung einer Nukleinsäure in eine Zelle, das Verfahren umfassend:

Kontaktieren der Zelle *in vitro* mit einem Nukleinsäure-Lipidteilchen nach einem der Ansprüche 1 bis 10, wobei die Zelle optional eine Säugetierzelle ist.

13. Nukleinsäure-Lipidteilchen nach einem der Ansprüche 1 bis 10 zur Verwendung in einem Verfahren für die *in vivo*-Verabreichung einer Nukleinsäure, das Verfahren umfassend das Verabreichen des Nukleinsäure-Lipidteilchens an ein Säugetiersubjekt.

14. Nukleinsäure-Lipidteilchen zur Verwendung nach Anspruch 13, wobei die Verabreichung ausgewählt ist aus der Gruppe bestehend aus oral, intranasal, intravenös, intraperitoneal, intramuskulär, intraartikulär, intraläsional, intratracheal, subkutan und intradermal.

15. Nukleinsäure-Lipidteilchen nach einem der Ansprüche 1 bis 10 zur Verwendung in einem Verfahren zur Behandlung einer Krankheit oder Störung in einem Säugetiersubjekt, das dieser bedarf, das Verfahren umfassend das Verabreichen einer therapeutisch wirkungsvollen Menge des Nukleinsäure-Lipidteilchens an das Säugetiersubjekt, wobei die Krankheit oder Störung optional ausgewählt ist aus der Gruppe bestehend aus einer viralen Infektion, einer Lebererkrankung oder -störung und einer Krebserkrankung.

Revendications

1. Particule d'acide nucléique lipide comprenant :

(a) un acide nucléique ;
(b) un lipide cationique comprenant de 50 % molaire à 65 % molaire du lipide total présent dans la particule ;
(c) un lipide non cationique comprenant jusqu'à 49,5 % molaire du lipide total présent dans la particule et comprenant un mélange d'un phospholipide et d'un cholestérol ou d'un dérivé de celui-ci, le cholestérol ou le dérivé de celui-ci comprenant de 30 % molaire à 40 % molaire du lipide total présent dans la particule ; et
(d) un lipide conjugué qui inhibe l'agrégation de particules comprenant de 0,5 % molaire à 2 % molaire du lipide total présent dans la particule.

2. Particule d'acide nucléique lipide selon la revendication 1, dans laquelle l'acide nucléique comprend un petit ARN interférent (ARNsi).

3. Particule d'acide nucléique lipide selon la revendication 2, dans laquelle :

(a) l'ARNsi comprend d'environ 15 à environ 60 nucléotides ; ou
(b) l'ARNsi comprend au moins un nucléotide modifié ; ou
(c) l'ARNsi comprend au moins un nucléotide 2'-O-méthyle (2'OMe) ; ou
(d) l'ARNsi représente environ 19 à environ 25 paires de base en longueur ; ou
(e) l'ARNsi comprend 3' saillies.

4. Particule d'acide nucléique lipide selon la revendication 1, dans laquelle :

(a) le lipide cationique comprend 1,2-dilinoleyloxy-N,N-diméthylaminopropane (DLinDMA), 1,2-dilinolényloxy-N,N-diméthylaminopropane (DLenDMA) ou un mélange de celui-ci ; ou
(b) le lipide cationique comprend 2,2-dilinoleyl-4-(2-diméthylaminoéthyl)-[1,3]-dioxolane (DLin-K-C2-DMA) ; ou
(c) le lipide cationique comprend de 50 % molaire à 60 % molaire du lipide total présent dans la particule ; ou
(d) le lipide cationique comprend de 52 % molaire à 62 % molaire du lipide total présent dans la particule.

5. Particule d'acide nucléique lipide selon la revendication 1, dans laquelle :

(a) le lipide conjugué qui inhibe l'agrégation de particules comprend un conjugué PEG (polyéthylèneglycol)-lipides ; ou
(b) le lipide conjugué qui inhibe l'agrégation de particules comprend de 1 % molaire à 2 % molaire du lipide total présent dans la particule.

6. Particule d'acide nucléique lipide selon la revendication 1, dans laquelle :

(a) l'acide nucléique dans la particule d'acide nucléique lipide n'est pas sensiblement dégradé après incubation de la particule dans du sérum à 37 °C pendant 30 minutes ; ou
(b) l'acide nucléique est complètement encapsulé dans la particule d'acide nucléique lipide ; ou
(c) la particule d'acide nucléique lipide a un rapport massique lipide/acide nucléique d'environ 5 à environ 15 ; ou
(d) la particule d'acide nucléique lipide présente un diamètre moyen d'environ 40 nm à environ 150 nm.

7. Particule d'acide nucléique lipide selon la revendication 1, dans laquelle :

(a) le phospholipide comprend la dipalmitoylphosphatidylcholine (DPPC), la distéaroylphosphatidylcholine (DSPC) ou un mélange de celle-ci ; ou
(b) le phospholipide comprend de 4 % molaire à 10 % molaire du lipide total présent dans la particule.

8. Particule d'acide nucléique lipide selon la revendication 1, dans laquelle :

(a) le cholestérol ou le dérivé de celui-ci comprend de 30 % molaire à 35 % molaire du lipide total présent dans la particule ; ou
(b) le cholestérol ou le dérivé de celui-ci comprend de 32 % molaire à 36 % molaire du lipide total présent dans la particule et le phospholipide comprend de 3 % molaire à 15 % molaire du lipide total présent dans la particule.

9. Particule d'acide nucléique lipide selon la revendication 5(a), dans laquelle le conjugué PEG-lipides comprend un conjugué PEG-diacylglycérol (PEG-DAG), un conjugué PEG-dialkyloxypropyle (PEG-DAA), ou un mélange de celui-ci ; le conjugué PEG-DAA comprenant éventuellement un PEG-dimyristyloxypropyle (PEG-DMA), un conjugué PEG-distéaryloxypropyle (PEG-DSA), ou un mélange de celui-ci ; le PEG dans le conjugué PEG-DAA ayant de préférence un poids moléculaire moyen d'environ 2000 dalton.

10. Particule d'acide nucléique lipide selon la revendication 5(a), dans laquelle l'acide nucléique est un ARNsi et la particule d'acide nucléique lipide comprend environ 57,1 % molaire de lipide cationique, environ 7,1 % molaire de phospholipide, environ 34,3 % molaire de cholestérol ou d'un dérivé de celui-ci et environ 1,4 % molaire de conjugué PEG-lipides.

11. Composition pharmaceutique comprenant une particule d'acide nucléique lipide selon l'une quelconque des revendications précédentes et un support pharmaceutiquement acceptable.

12. Méthode destinée à l'introduction d'un acide nucléique dans une cellule, la méthode comprenant : la mise en contact de la cellule *in vitro* avec une particule d'acide nucléique lipide selon l'une quelconque des revendications 1 à 10, la cellule étant éventuellement une cellule de mammifère.

13. Particule d'acide nucléique lipide selon l'une quelconque des revendications 1 à 10 en vue d'une utilisation dans une méthode destinée à l'administration *in vivo* d'un acide nucléique, la méthode comprenant l'administration de ladite particule d'acide nucléique lipide à un sujet mammifère.

14. Particule d'acide nucléique lipide en vue d'une utilisation selon la revendication 13, dans laquelle l'administration est choisie dans le groupe constitué par une administration orale, intranasale, intraveineuse, intrapéritonéale, intramusculaire, intra-articulaire, intralésionnelle, intratrachéale, sous-cutanée et intradermique.

15. Particule d'acide nucléique lipide selon l'une quelconque des revendications 1 à 10 en vue d'une utilisation dans une méthode de traitement d'une maladie ou d'un trouble chez un sujet mammifère ayant besoin de ce traitement, la méthode comprenant l'administration d'une quantité thérapeutiquement efficace de ladite particule d'acide nucléique lipide au sujet mammifère, la maladie ou le trouble étant éventuellement choisi dans le groupe constitué par une infection virale, une maladie ou un trouble hépatique et le cancer.

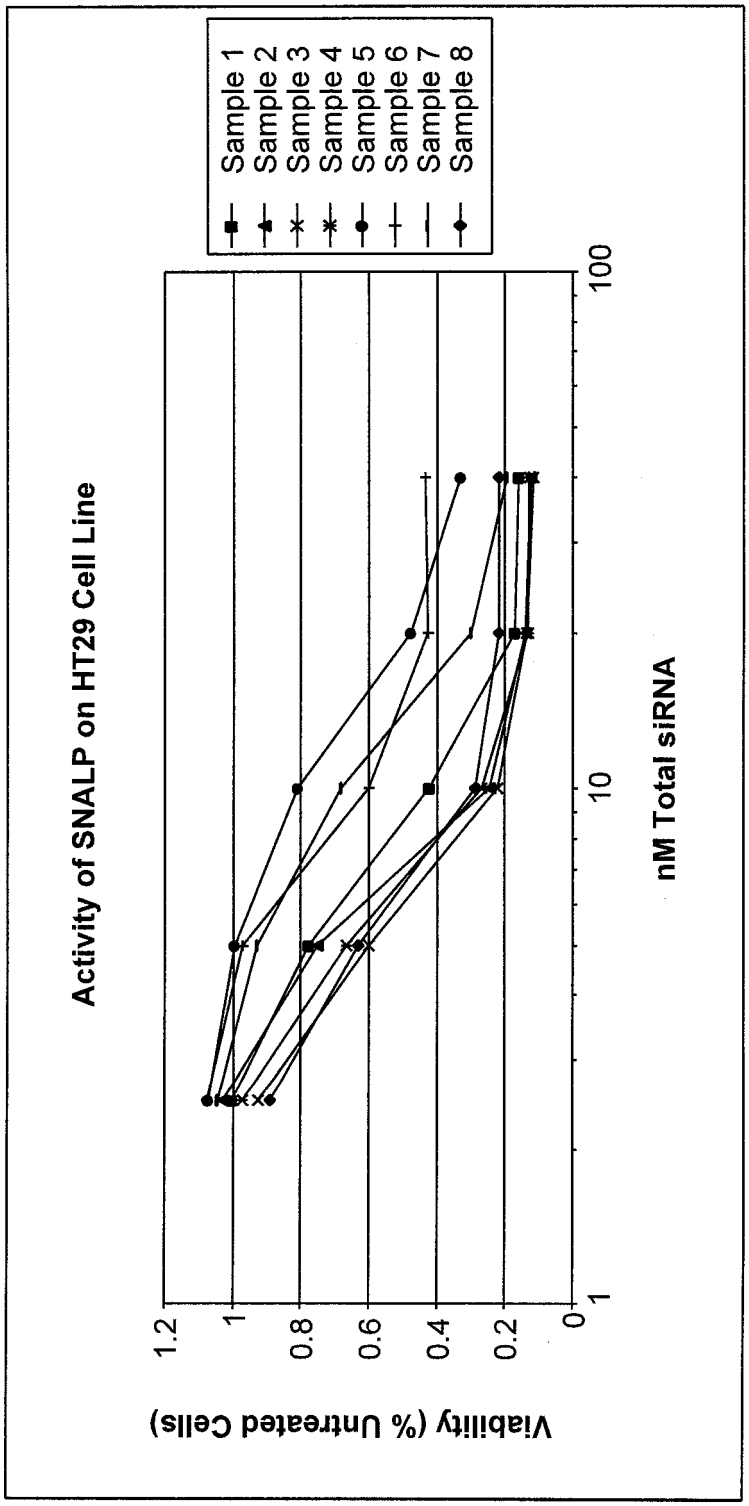

A
Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8

FIG. 1A

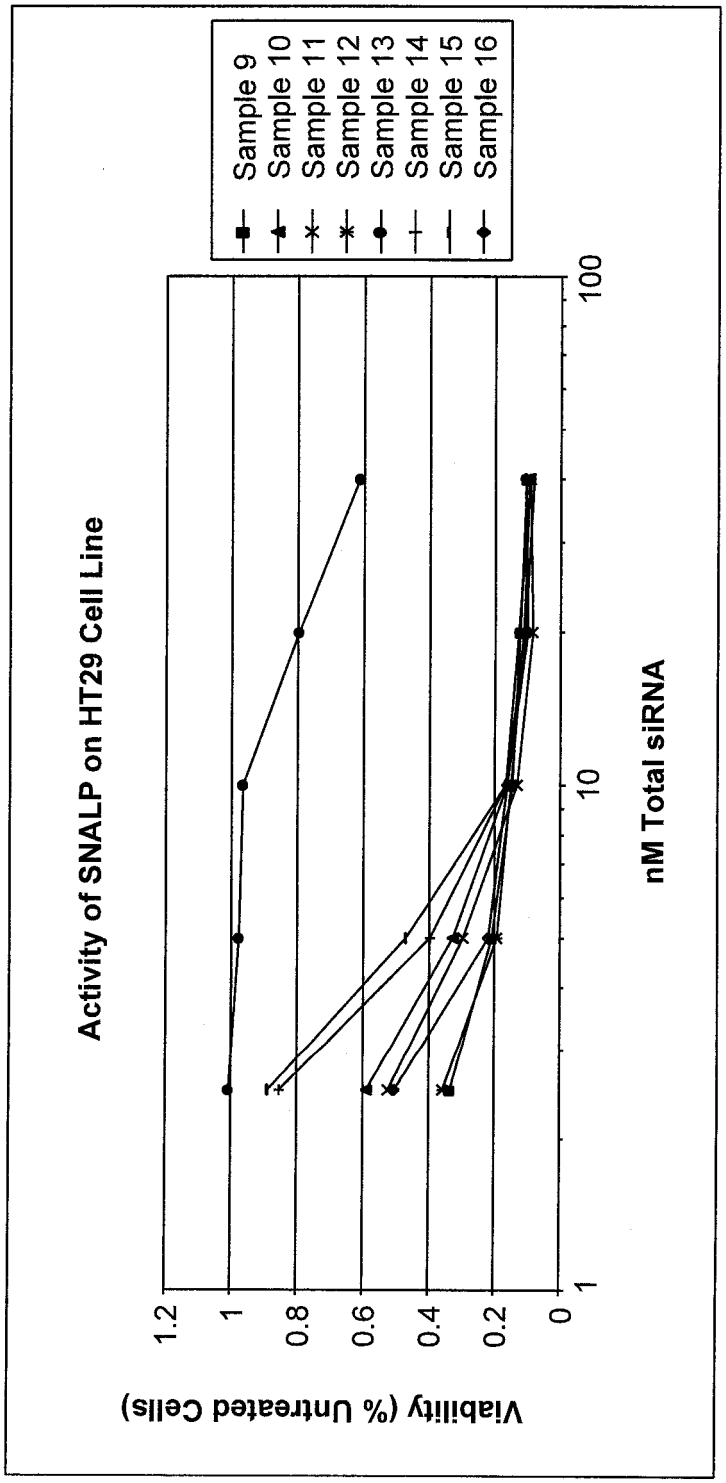

B
Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 9
Sample 10
Sample 11
Sample 12
Sample 13
Sample 14
Sample 15
Sample 16

FIG. 1B

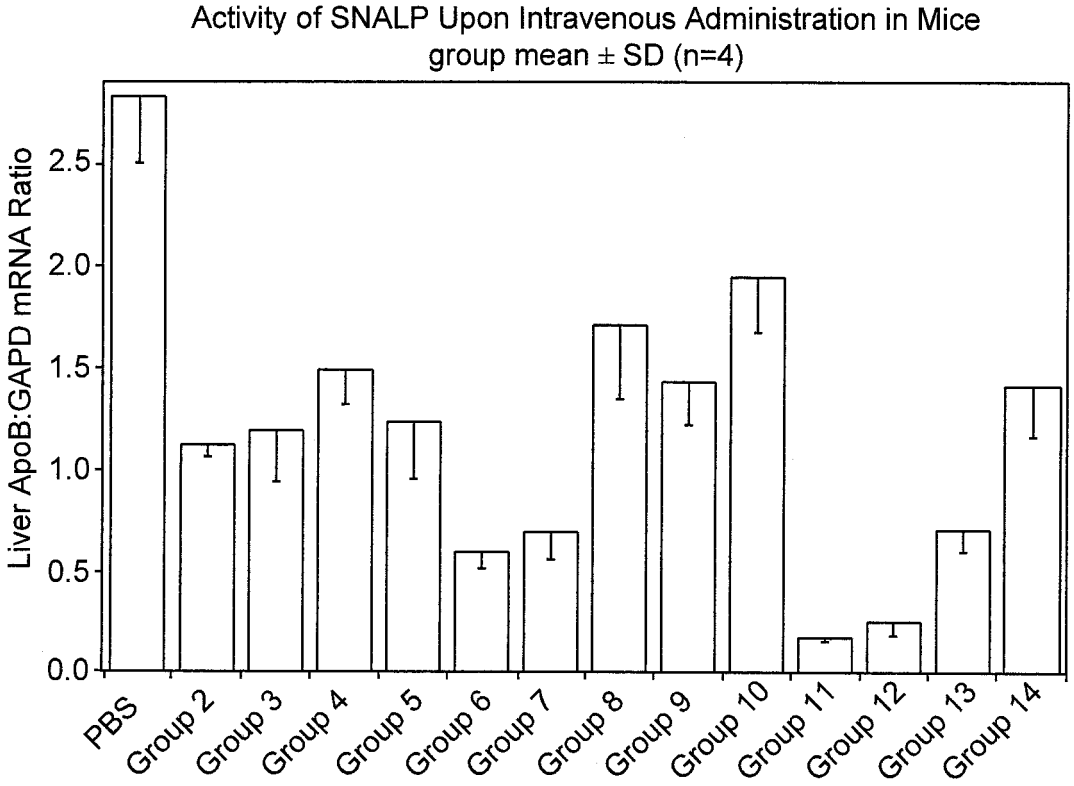

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
2.5
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

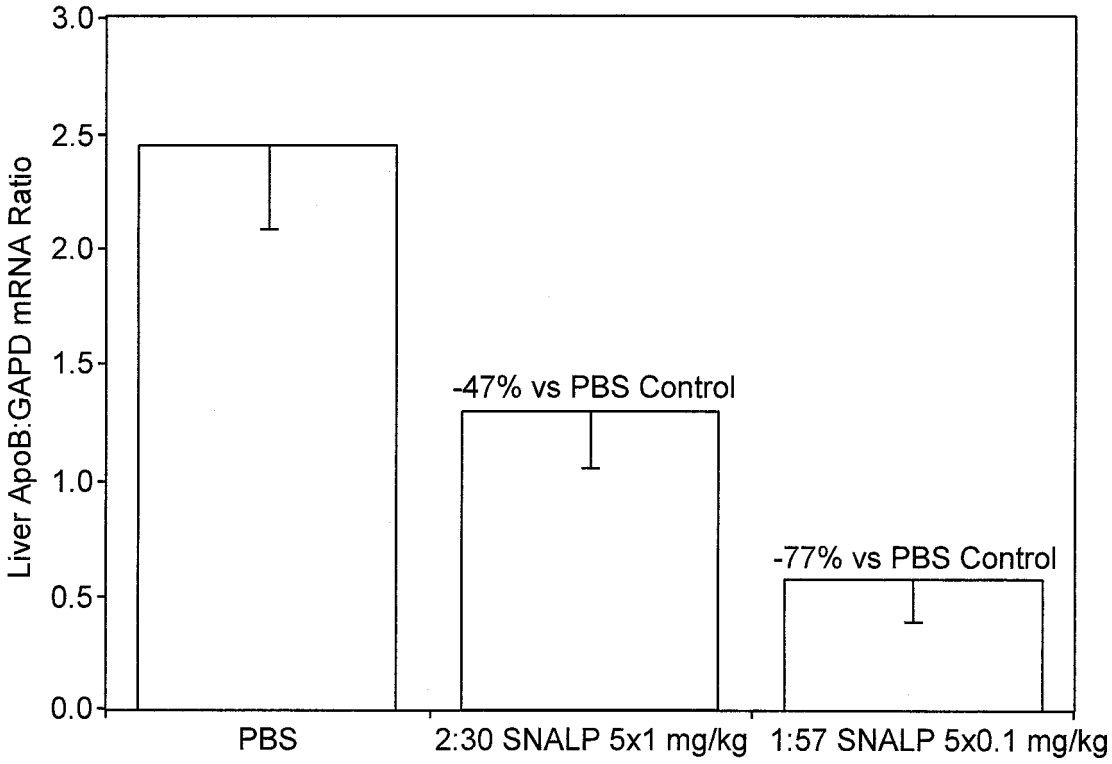

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Liver ApoB:GAPD mRNA Ratio
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5x1 mg/kg
1:57 SNALP 5x0.1 mg/kg

FIG. 3

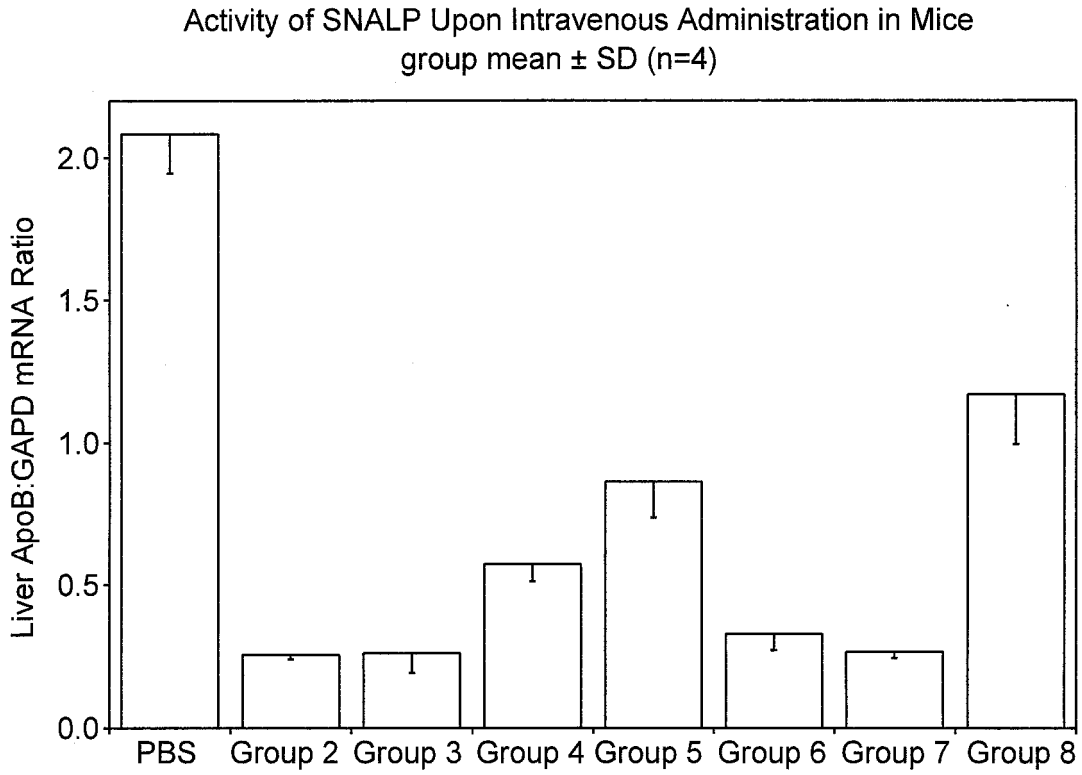

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

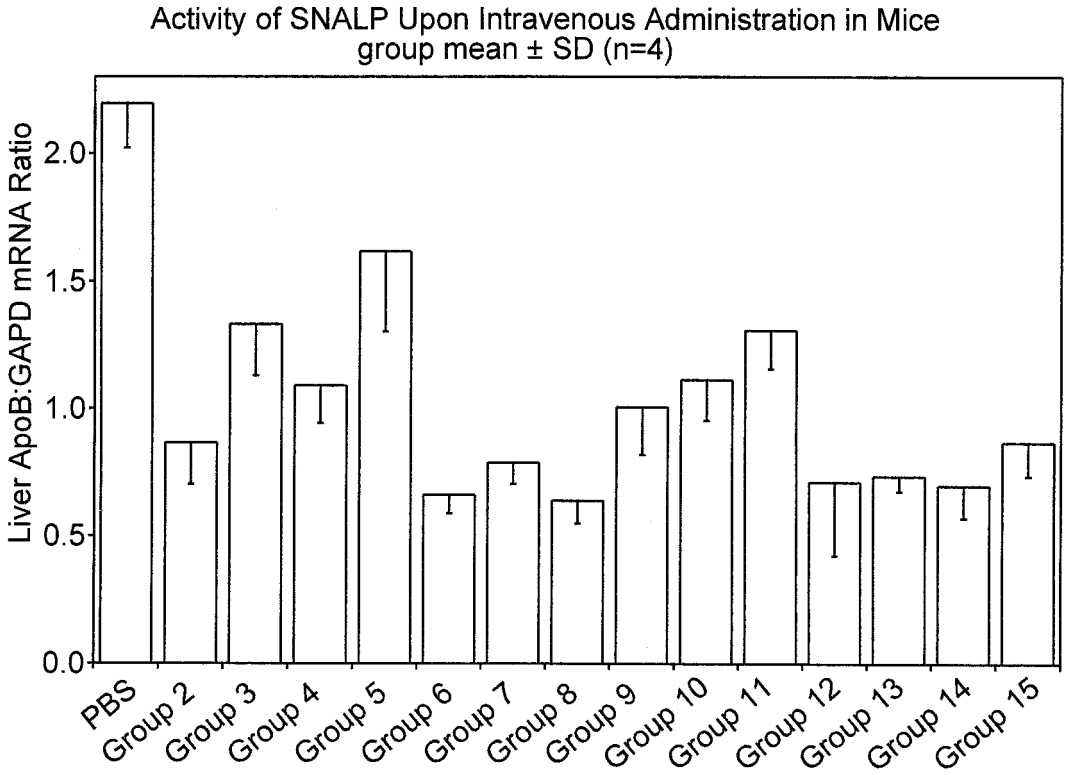

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

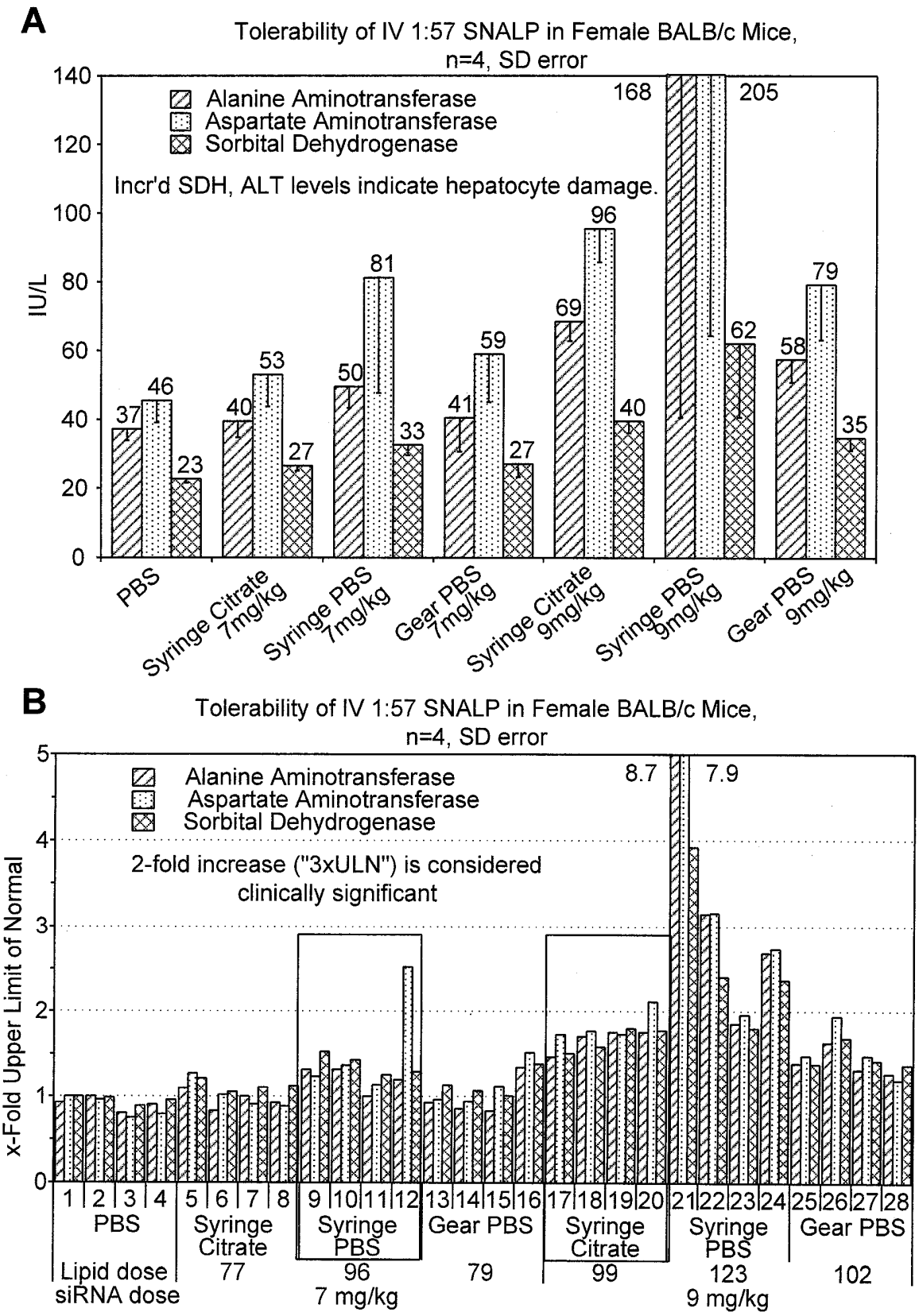
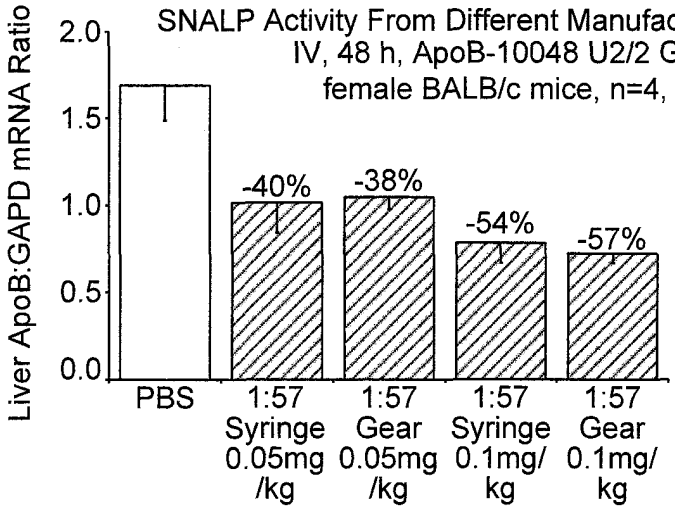
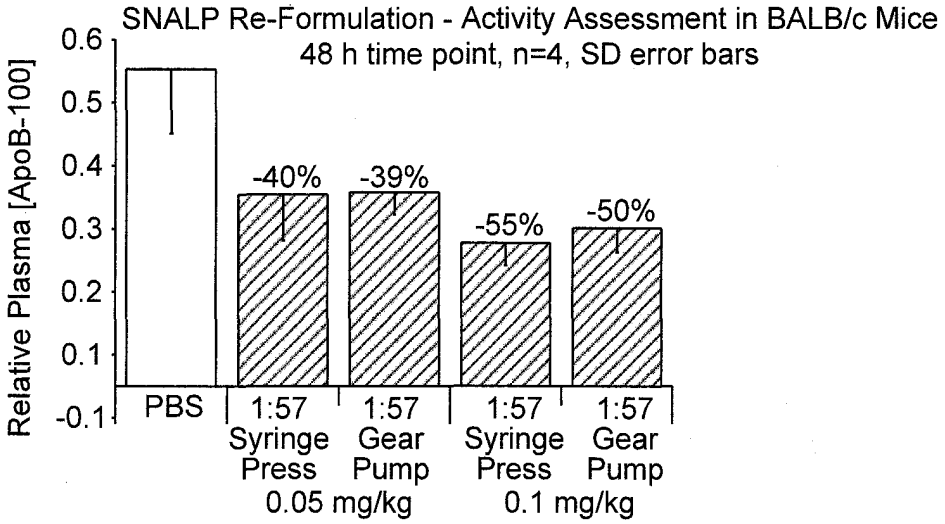

A
Tolerability of IV 1:57 SNALP in Female BALB/c Mice,
n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
140
120
100
80
60
40
20
0
168
205
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
62
58
79
35
PBS
Syringe Citrate 7mg/kg
Syringe PBS 7mg/kg
Gear PBS 7mg/kg
Syringe Citrate 9mg/kg
Syringe PBS 9mg/kg
Gear PBS 9mg/kg
B
Tolerability of IV 1:57 SNALP in Female BALB/c Mice,
n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
5
4
3
2
1
0
8.7
7.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
PBS
Syringe Citrate
Syringe PBS
Gear PBS
Syringe Citrate
Syringe PBS
Gear PBS
Lipid dose
siRNA dose
77
96
7 mg/kg
79
99
123
9 mg/kg
102

FIG. 6

A


SNALP Activity From Different Manufacturing Processes
IV, 48 h, ApoB-10048 U2/2 G1/2 (Dow),
female BALB/c mice, n=4, SD error
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
-40%
-38%
-54%
-57%
PBS
1:57 Syringe 0.05mg /kg
1:57 Gear 0.05mg /kg
1:57 Syringe 0.1mg/ kg
1:57 Gear 0.1mg/ kg

B


SNALP Re-Formulation - Activity Assessment in BALB/c Mice
48 h time point, n=4, SD error bars
Relative Plasma [ApoB-100]
0.6
0.5
0.4
0.3
0.2
0.1
-0.1
-40%
-39%
-55%
-50%
PBS
1:57 Syringe Press 0.05 mg/kg
1:57 Gear Pump 0.05 mg/kg
1:57 Syringe Press 0.1 mg/kg
1:57 Gear Pump 0.1 mg/kg

C

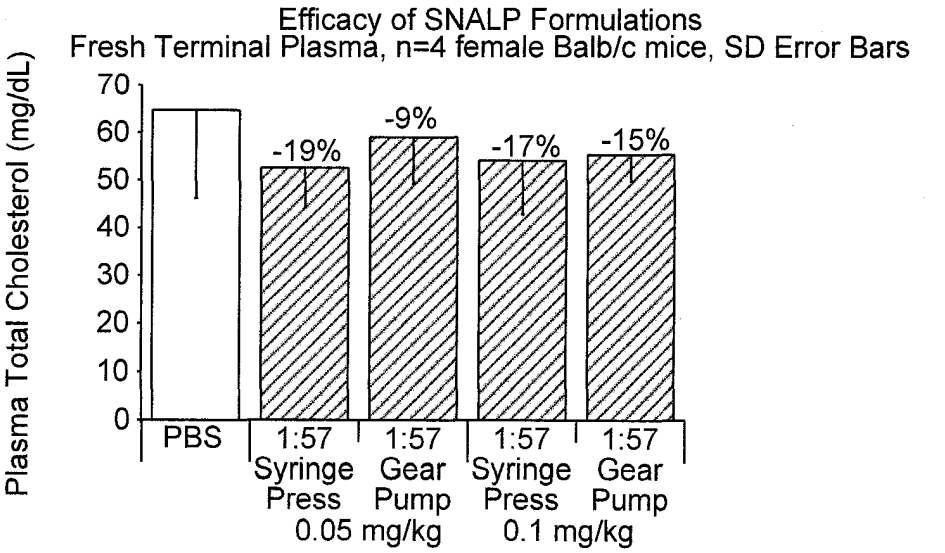

Efficacy of SNALP Formulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
70
60
50
40
30
20
10
0
-19%
-9%
-17%
-15%
PBS
1:57 Syringe Press 0.05 mg/kg
1:57 Gear Pump 0.05 mg/kg
1:57 Syringe Press 0.1 mg/kg
1:57 Gear Pump 0.1 mg/kg

FIG. 7

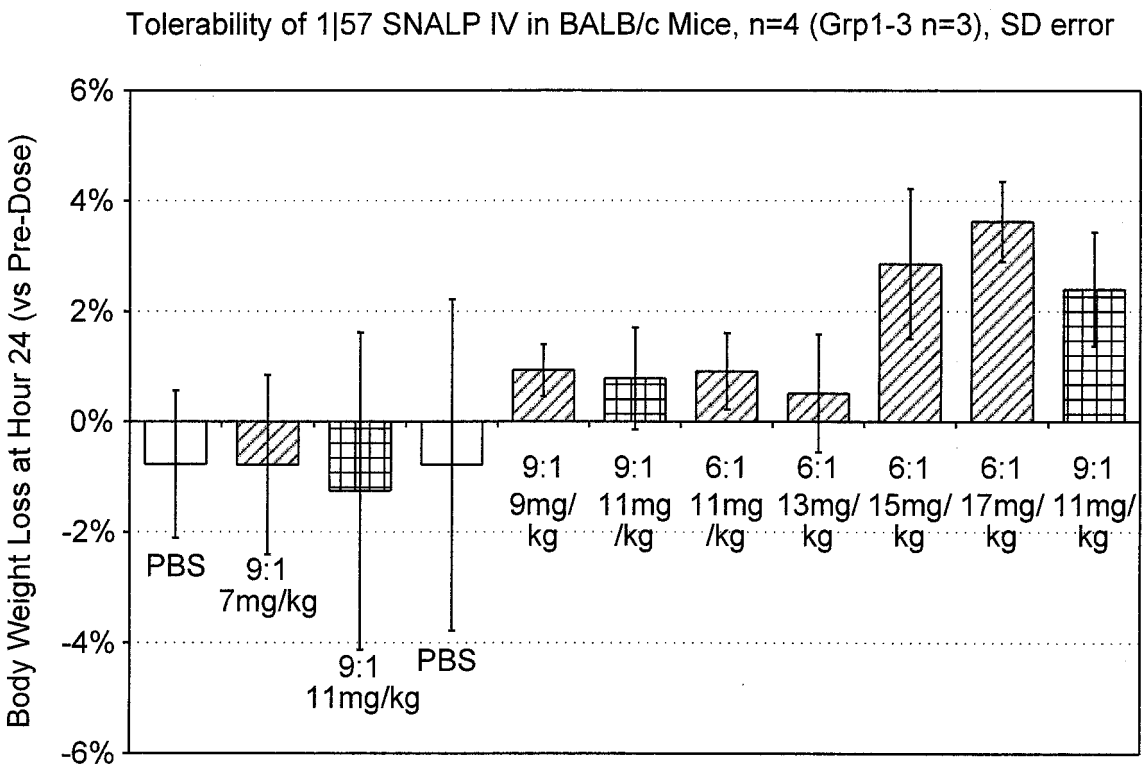

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

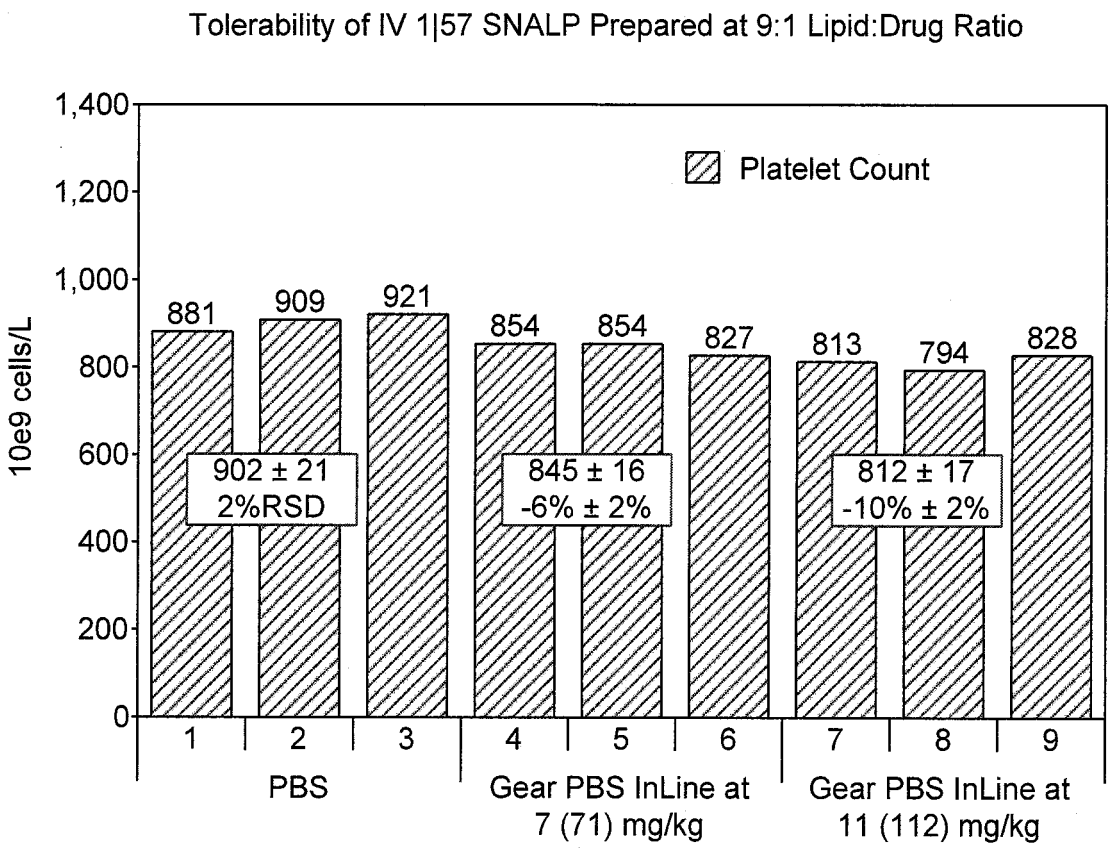

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
Platelet Count
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at
7 (71) mg/kg
Gear PBS InLine at
11 (112) mg/kg

FIG. 9

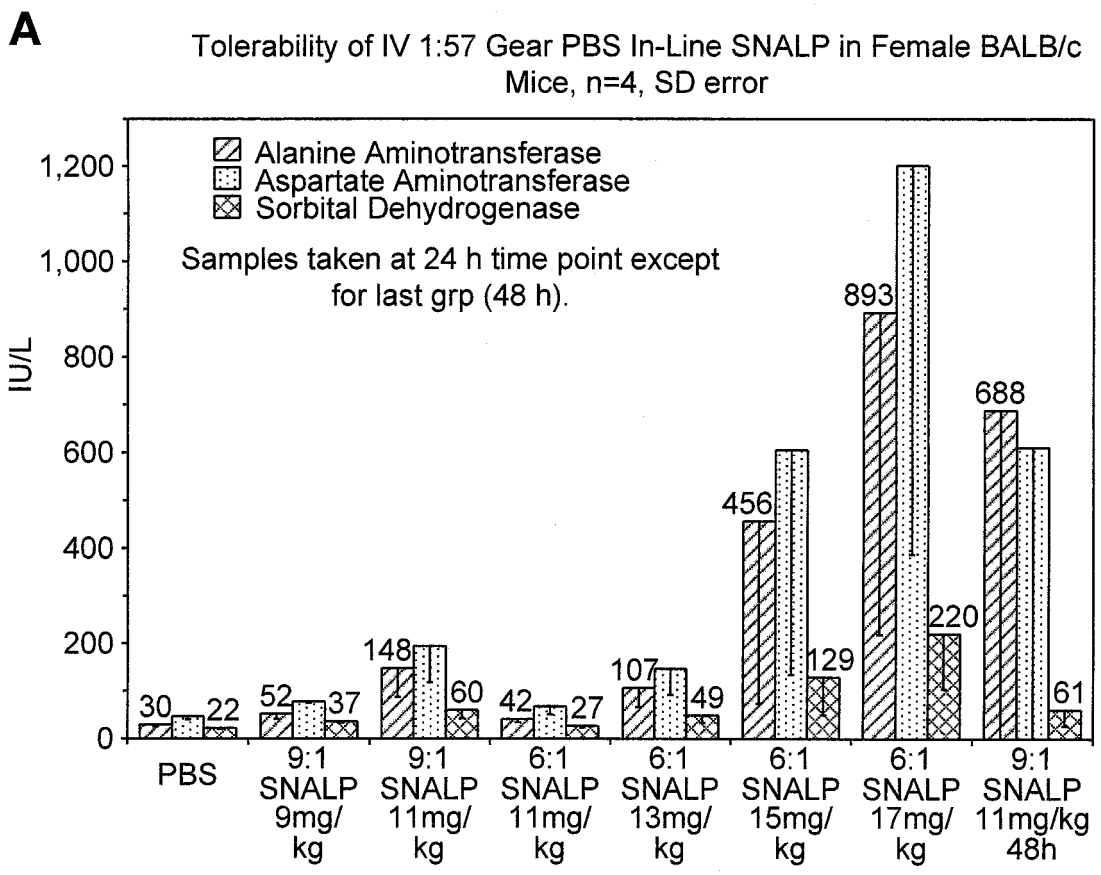

A
Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
688
61
PBS
9:1 SNALP 9mg/kg
9:1 SNALP 11mg/kg
6:1 SNALP 11mg/kg
6:1 SNALP 13mg/kg
6:1 SNALP 15mg/kg
6:1 SNALP 17mg/kg
9:1 SNALP 11mg/kg 48h

FIG. 10A

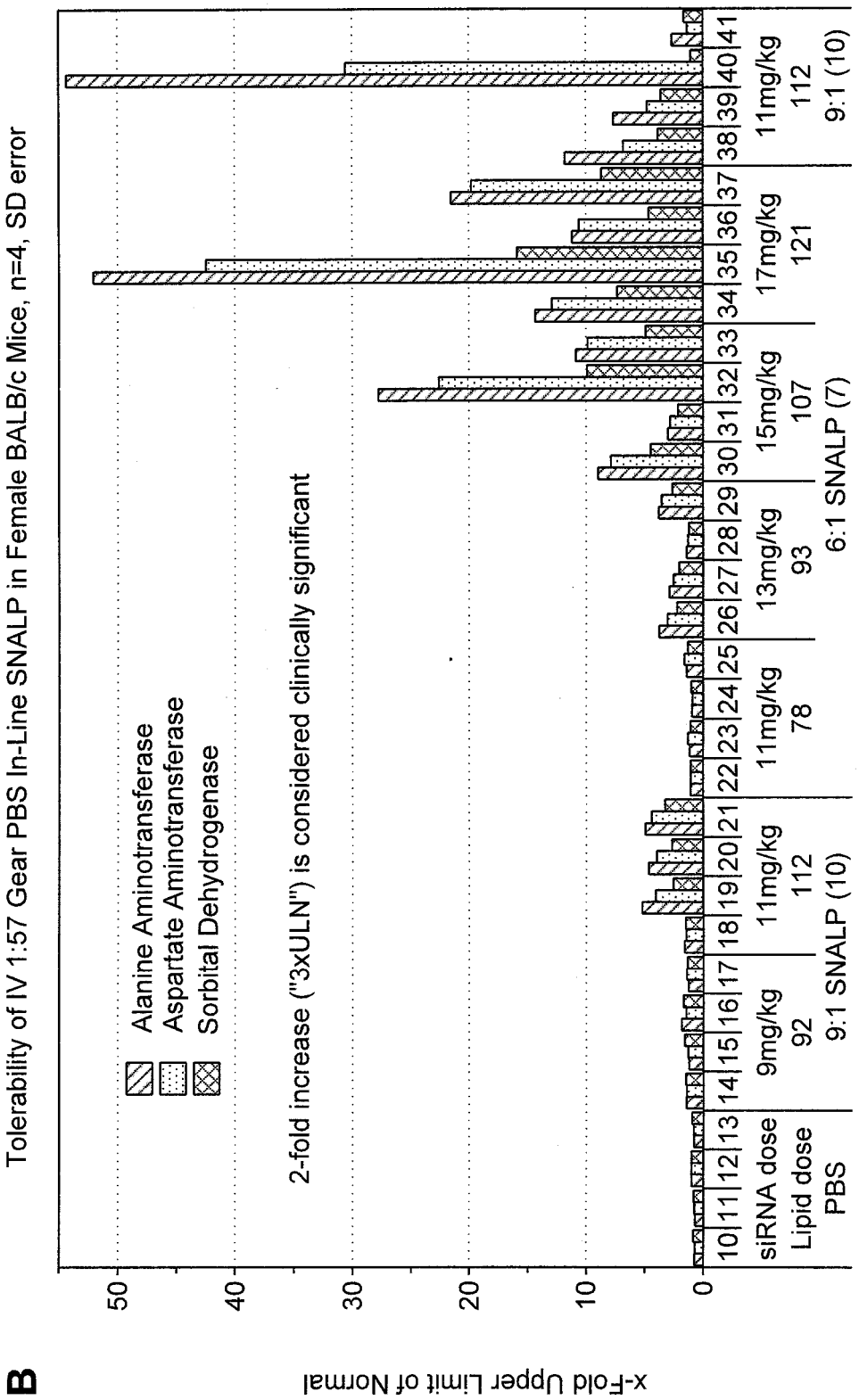

B
Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
10
20
30
40
50
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
siRNA dose
Lipid dose
PBS
9mg/kg
11mg/kg
11mg/kg
13mg/kg
15mg/kg
17mg/kg
11mg/kg
92
112
78
93
107
121
112
9:1 SNALP (10)
6:1 SNALP (7)
9:1 (10)

FIG. 10B

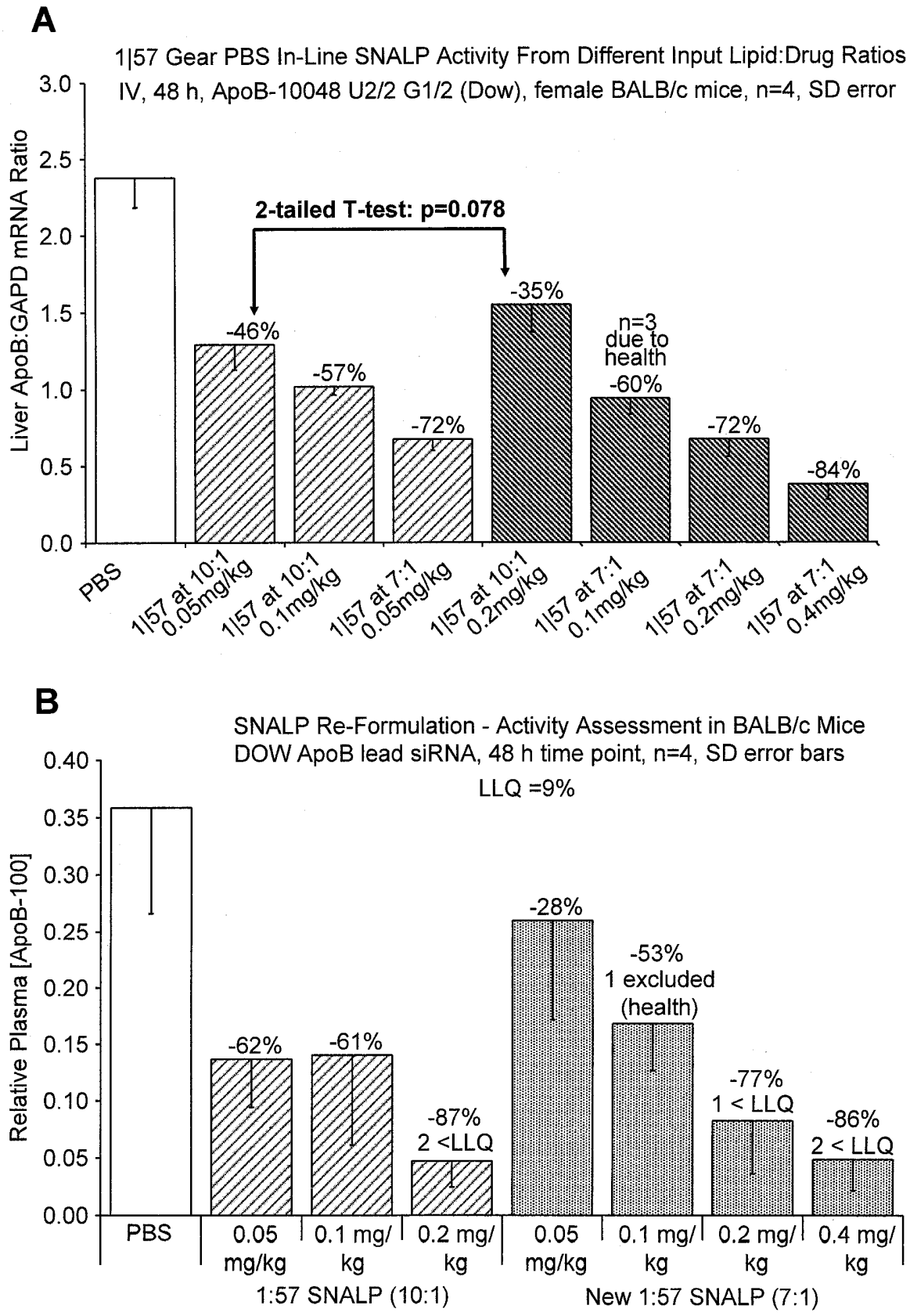

A
1|57 Gear PBS In-Line SNALP Activity From Different Input Lipid:Drug Ratios
IV, 48 h, ApoB-10048 U2/2 G1/2 (Dow), female BALB/c mice, n=4, SD error
Liver ApoB:GAPD mRNA Ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2-tailed T-test: p=0.078
-46%
-57%
-72%
-35%
n=3 due to health
-60%
-72%
-84%
PBS
1|57 at 10:1 0.05mg/kg
1|57 at 10:1 0.1mg/kg
1|57 at 7:1 0.05mg/kg
1|57 at 10:1 0.2mg/kg
1|57 at 7:1 0.1mg/kg
1|57 at 7:1 0.2mg/kg
1|57 at 7:1 0.4mg/kg
B
SNALP Re-Formulation - Activity Assessment in BALB/c Mice
DOW ApoB lead siRNA, 48 h time point, n=4, SD error bars
LLQ =9%
Relative Plasma [ApoB-100]
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-62%
-61%
-87%
2 <LLQ
-28%
-53%
1 excluded (health)
-77%
1 < LLQ
-86%
2 < LLQ
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 11

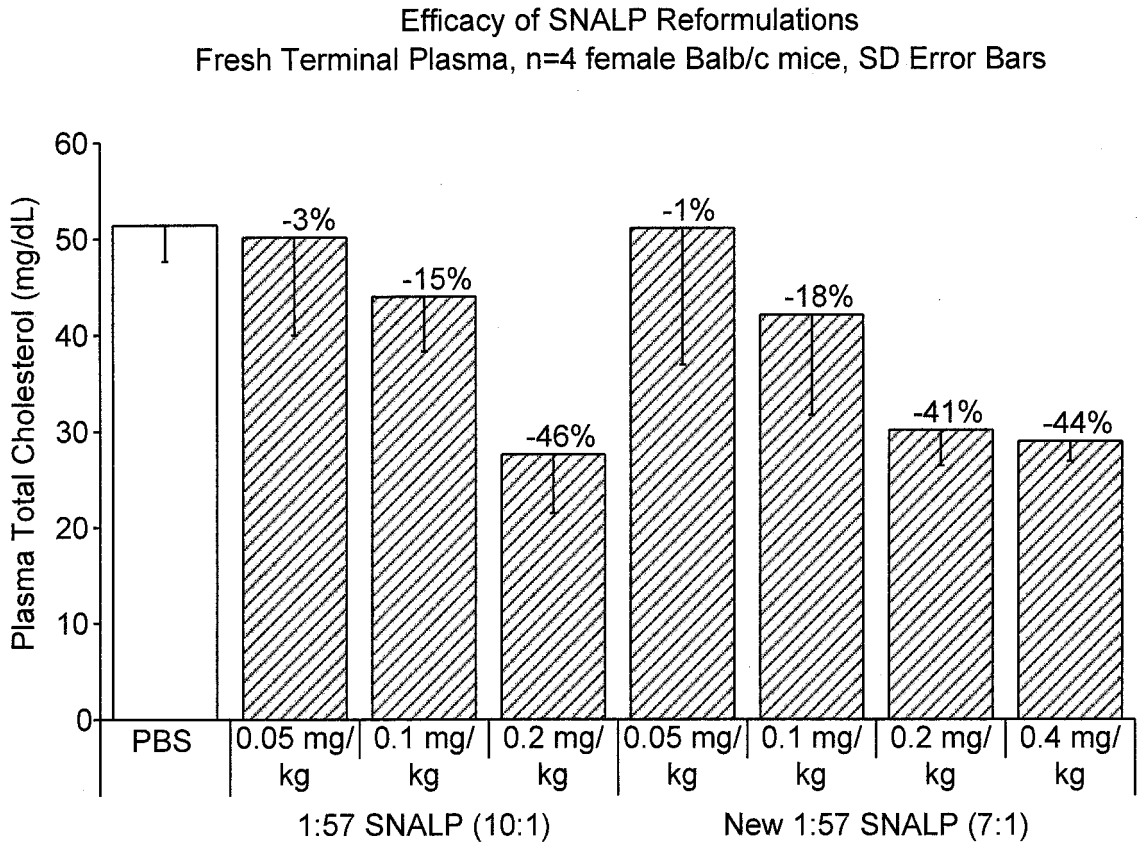

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 12

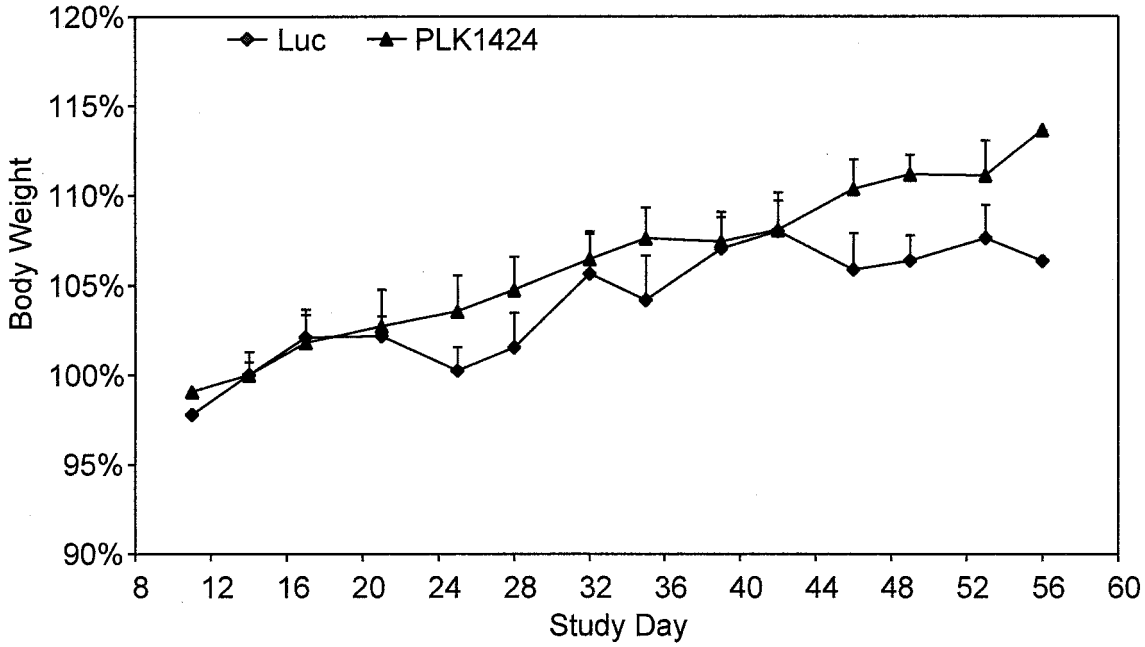

120%
115%
110%
105%
100%
95%
90%
Body Weight
Luc
PLK1424
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

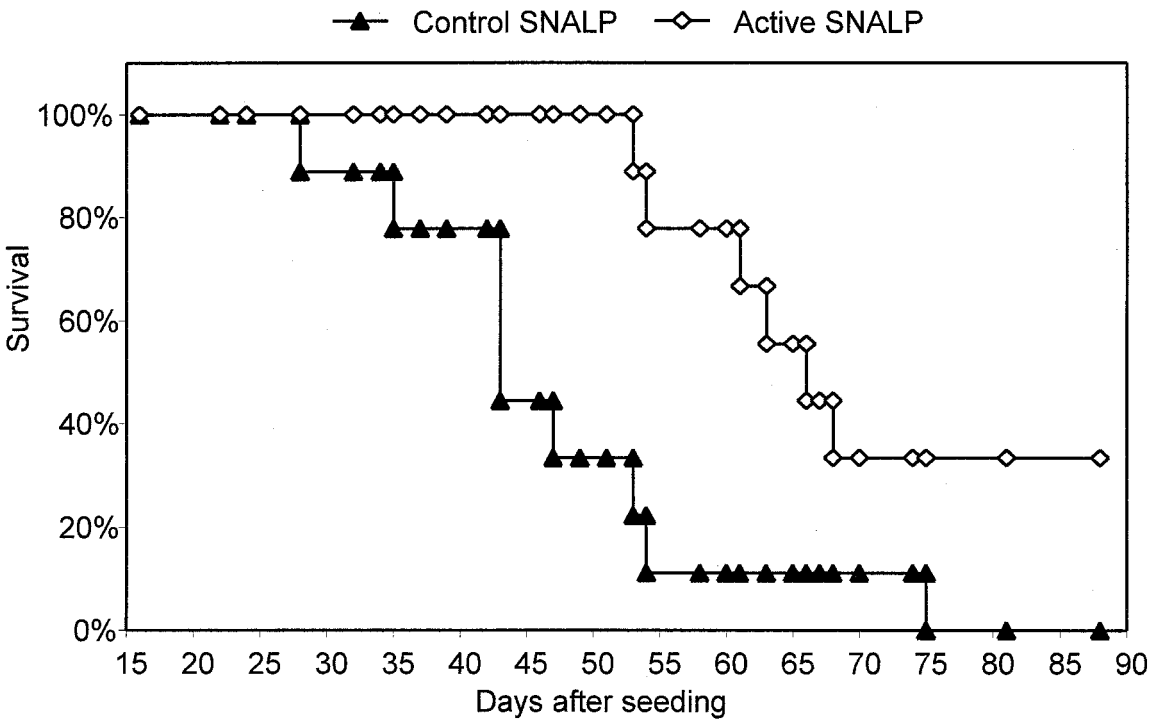

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
Days after seeding

FIG. 14

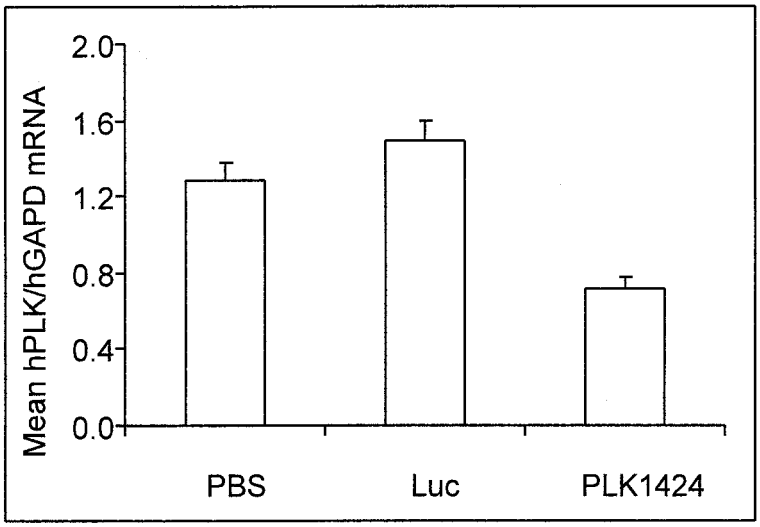


FIG. 15

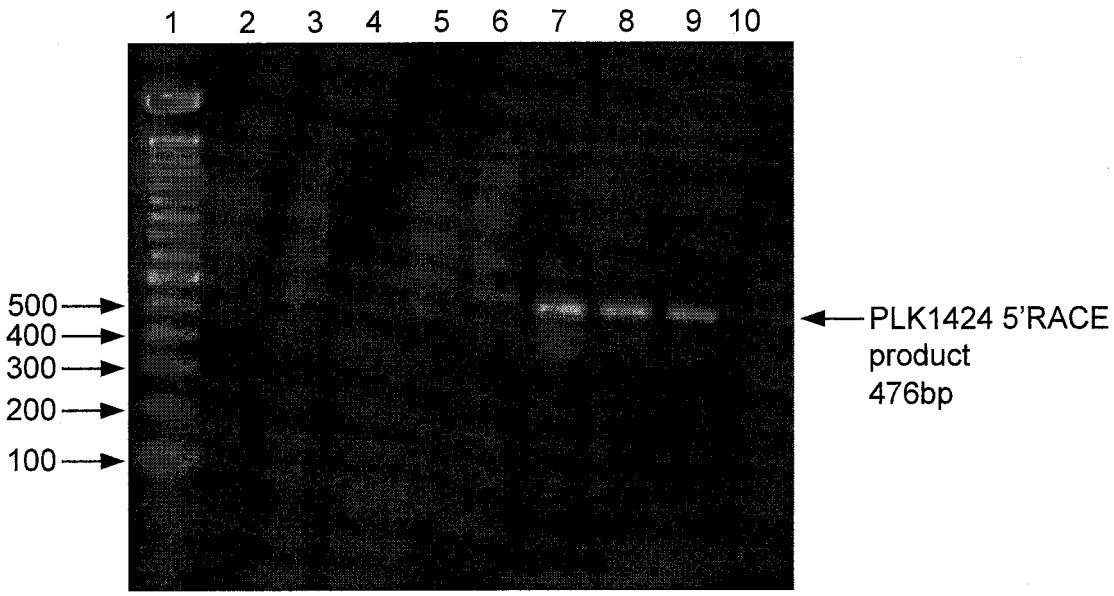
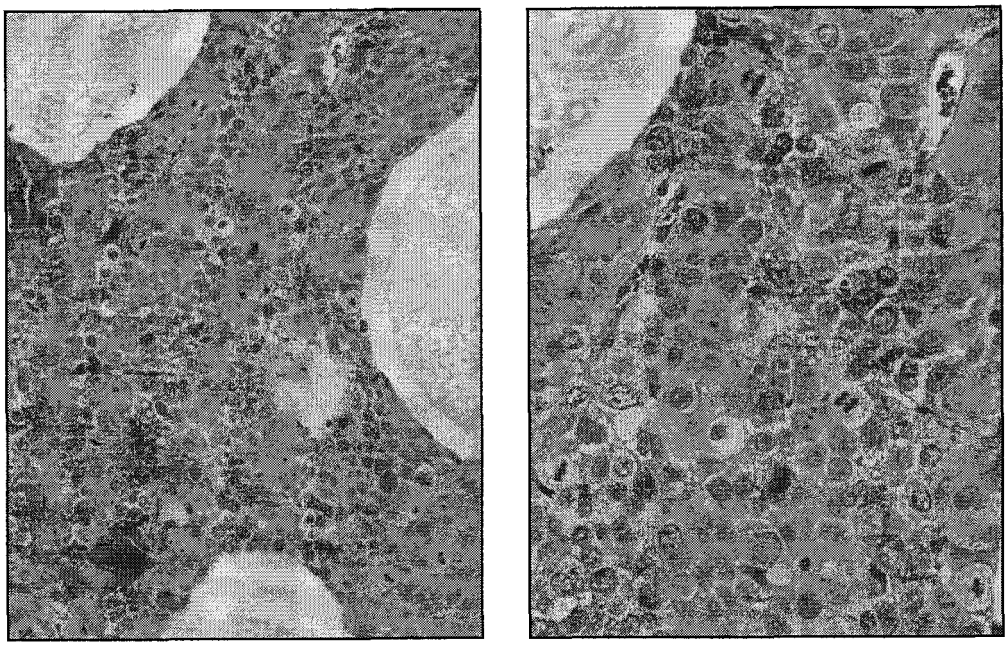
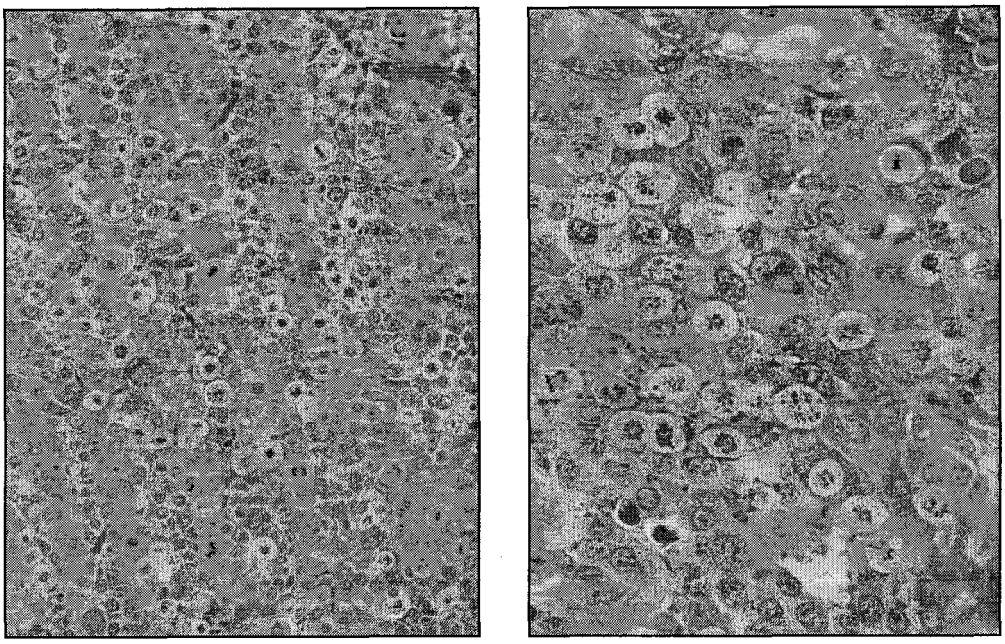

1
2
3
4
5
6
7
8
9
10
500
400
300
200
100
PLK1424 5'RACE
product
476bp

FIG. 16



x200 mag x400 mag



x200 mag x400 mag

FIG. 17

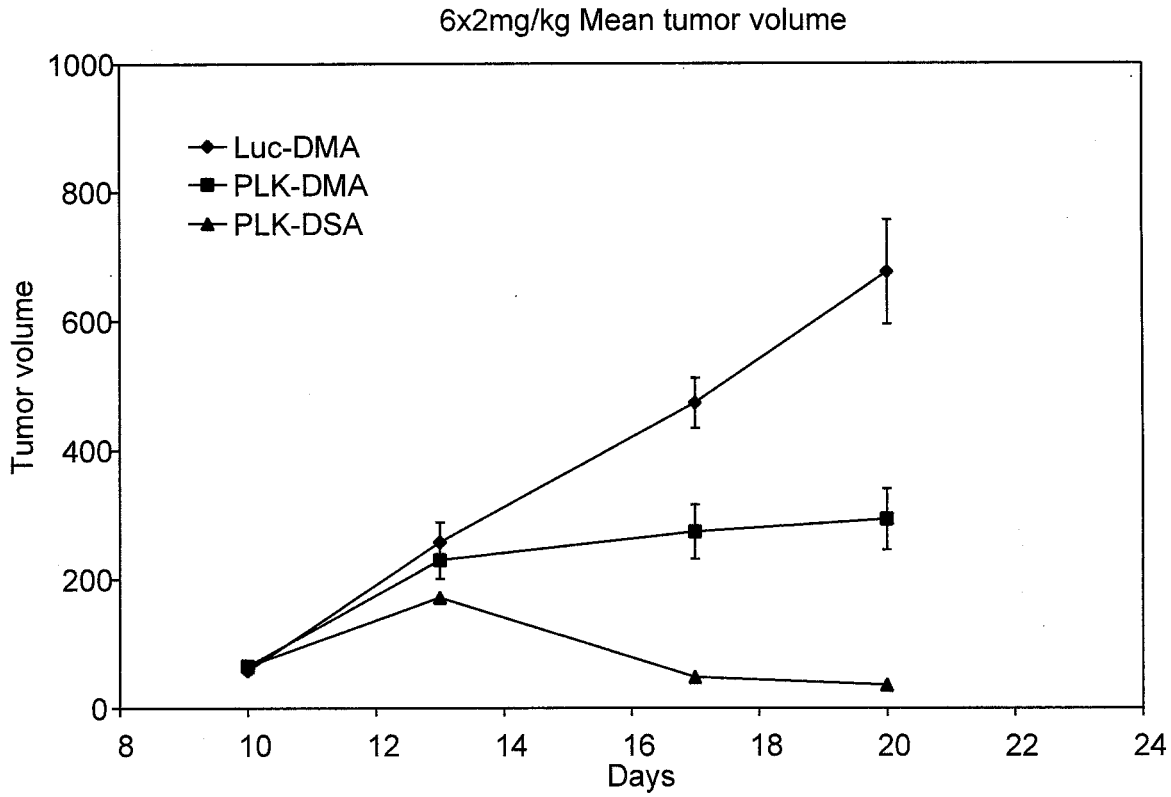

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

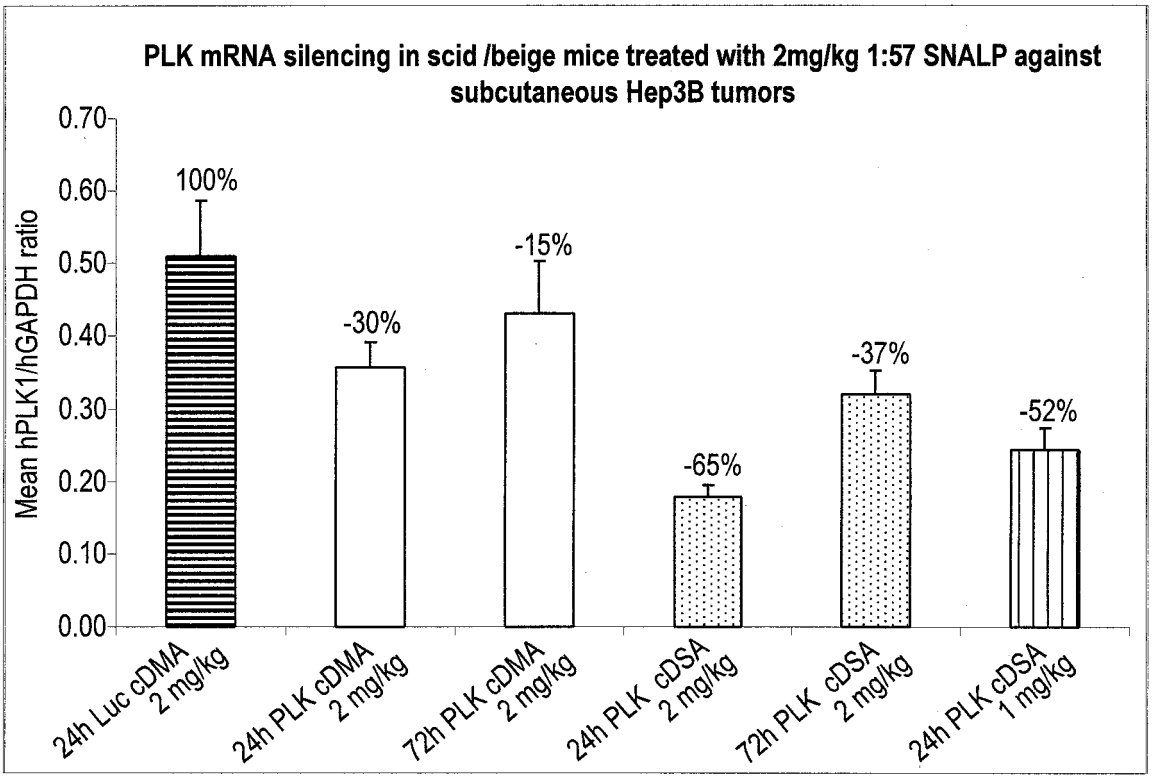

PLK mRNA silencing in scid /beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

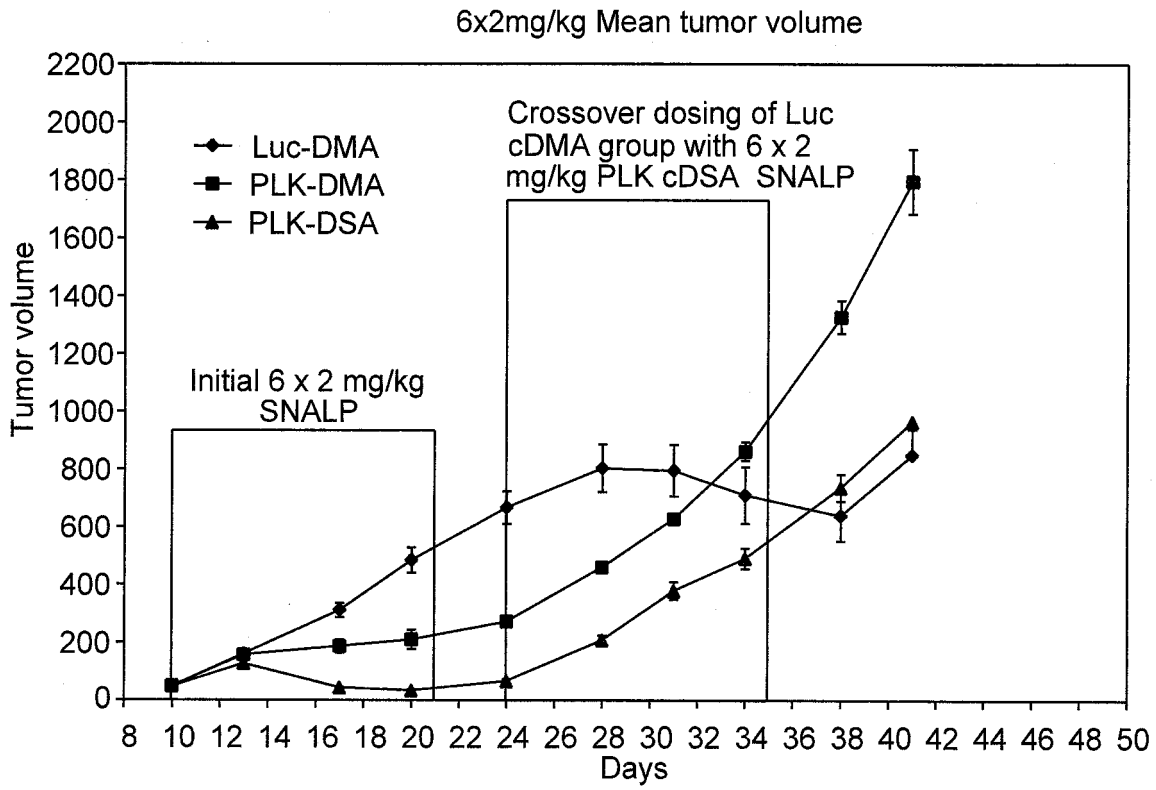

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Initial 6 x 2 mg/kg SNALP
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

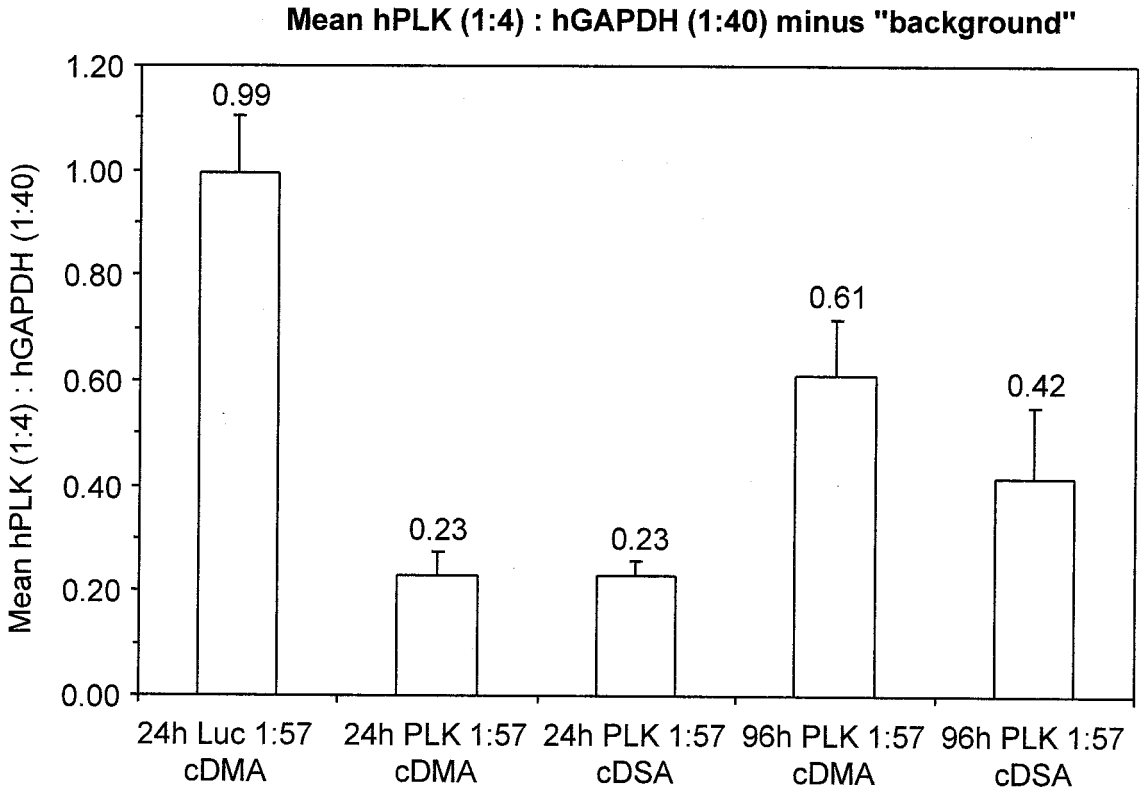

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

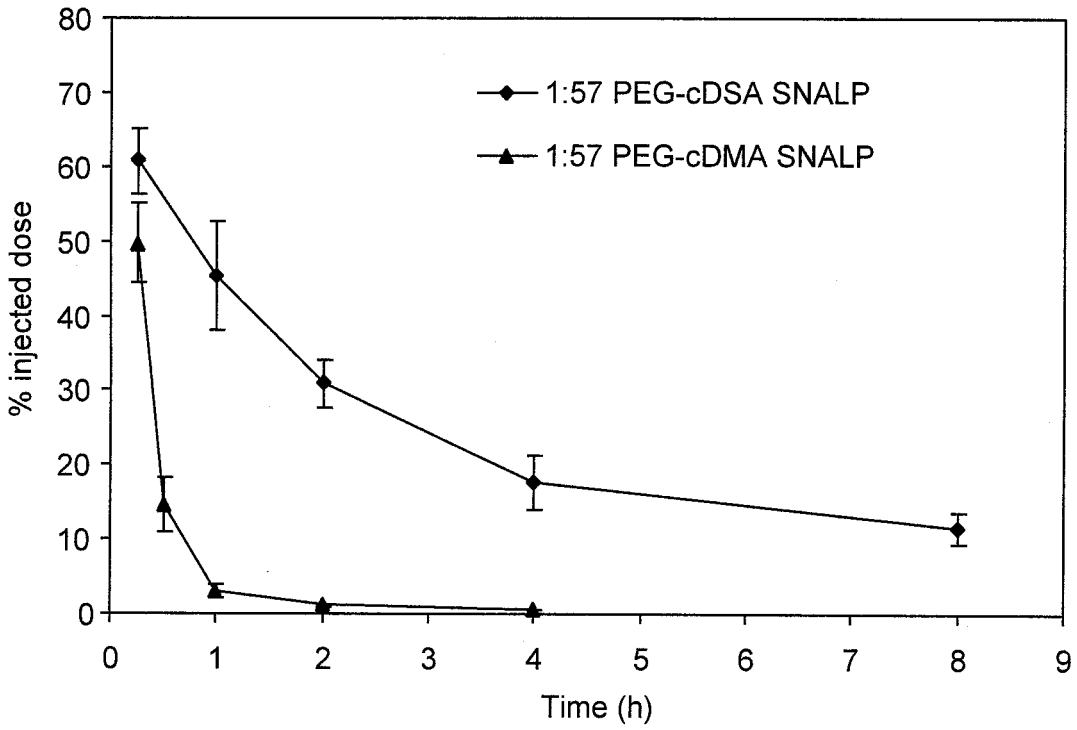

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- US 6458382 B **[0004]**
- US 20030073640 A **[0004]**
- US 6429200 B **[0006]**
- US 20030026831 A **[0006]**
- US 20020081736 A **[0006]**
- US 20030082103 A **[0006]**
- US 2005064595 A **[0010]**
- US 2006008910 A **[0011]**
- WO 2006053430 A **[0012]**
- WO 2007056861 A **[0012]**
- WO 2005035764 A **[0013]**
- WO 2006002538 A **[0014]**
- WO 0003683 A **[0055]**
- US 20040142025 A **[0056] [0271]**
- US 20070042031 A **[0056] [0276]**
- US 5885613 A **[0058] [0242]**
- US 20060083780 A **[0064] [0223]**
- US 20060240554 A **[0064] [0096] [0223]**
- US 5208036 A **[0064] [0223]**
- US 5264618 A **[0064] [0223]**
- US 5279833 A **[0064] [0223]**
- US 5283185 A **[0064] [0223]**
- US 5753613 A **[0064] [0221] [0223]**
- US 5785992 A **[0064] [0221] [0223]**
- WO 9610390 A **[0064] [0223]**
- US 61104212 A **[0096]**
- US 0888676 W **[0096] [0117] [0211]**
- US 7404969 B **[0117]**
- US 4376110 A, David **[0161]**
- US 4452901 A, Gordon **[0161]**
- US 3817827 A **[0161]**
- US 3850752 A **[0161]**
- US 3901654 A **[0161]**
- US 3935074 A **[0161]**
- US 3984533 A **[0161]**
- US 3996345 A **[0161]**
- US 4034074 A **[0161]**
- US 4098876 A **[0161]**
- US 4683195 A **[0167]**
- US 4683202 A **[0167] [0319]**
- US 5998203 A **[0172]**
- GB 2397818 B **[0173]**
- US 20040192626 A **[0173]**
- US 20050282188 A **[0173]**
- US 20070135372 A **[0173]**
- US 20050074771 A **[0175]**
- US 20050043219 A **[0175]**
- US 20050158727 A **[0175]**
- US 20030130186 A **[0175]**
- US 20040110296 A **[0175]**
- US 20040249178 A **[0175]**
- US 6753423 B **[0175]**
- US 20050119470 A **[0175]**
- US 20050107325 A **[0175]**
- US 20050153337 A **[0175]**
- US 20040167090 A **[0175]**
- US 20050239739 A **[0175]**
- US 6713069 B **[0178]**
- US 20070135370 A **[0178]**
- US 20070218122 A **[0179]**
- US 20060281175 A **[0180]**
- US 20050058982 A **[0180]**
- US 20070149470 A **[0180]**
- US 7348314 B **[0180]**
- US 61162127 A **[0180]**
- US 20060134189 A **[0181]**
- US 61147235 A **[0181]**
- US 80787207 A **[0182] [0183]**
- US 20050107316 A **[0182]**
- US 20070265438 A **[0182]**
- US 34334208 A **[0182]**
- US 61045251 A **[0182]**
- GB 2396864 A **[0185]**
- US 20040142895 A **[0185]**
- CA 2456444 **[0185]**
- US 6174861 B **[0186]**
- US 5639725 A **[0186]**
- US 5739119 A **[0202]**
- US 5759829 A **[0202]**
- US 5801154 A **[0202]**
- US 5789573 A **[0202]**
- US 5718709 A **[0202]**
- US 5610288 A **[0202]**
- US 5747470 A **[0202]**
- US 5591317 A **[0202]**
- US 5783683 A **[0202]**
- EP 0360257 A **[0206]**
- US 5631359 A **[0206]**
- US 4987071 A **[0206]**
- WO 9323569 A **[0207]**
- WO 9402595 A **[0207]**
- WO 9207065 A **[0208]**
- WO 9315187 A **[0208]**
- WO 9103162 A **[0208]**
- WO 9413688 A **[0208]**
- EP 92110298 A **[0208]**
- US 5334711 A **[0208]**
- WO 9611266 A **[0209]**

- WO 02069369 A **[0211]**
- WO 0115726 A **[0211]**
- US 6406705 B **[0211]**
- US 5705385 A **[0221] [0285]**
- US 5976567 A **[0221]**
- US 5981501 A **[0221] [0285]**
- US 6110745 A **[0221]**
- US 6320017 B **[0221] [0248] [0264]**
- WO 9640964 A **[0221]**
- WO 05026372 A **[0242]**
- US 20030077829 A **[0242] [0311]**
- US 2005008689 A **[0242]**
- US 6774180 B **[0243]**
- US 7053150 B **[0243]**
- US 6586559 B **[0248] [0264]**
- US 6852334 B **[0258] [0285]**
- WO 0062813 A **[0258] [0285]**
- US 4737323 A **[0279]**
- US 744103 A **[0281]**
- US 6586410 B **[0285]**
- US 6534484 B **[0285]**
- US 20020072121 A **[0285]**
- WO 05007196 A **[0295]**
- WO 05121348 A **[0295]**
- WO 05120152 A **[0295]**
- WO 04002453 A **[0295]**
- US 5286634 A **[0296]**
- US 3993754 A **[0296]**
- US 4145410 A **[0296]**
- US 4235871 A **[0296]**
- US 4224179 A **[0296]**
- US 4522803 A **[0296]**
- US 4588578 A **[0296]**
- US 5756353 A **[0298]**
- US 5804212 A **[0298]**
- US 5725871 A **[0298]**
- US 5780045 A **[0298]**
- US 5641515 A **[0301]**
- US 5580579 A **[0301]**
- US 5792451 A **[0301]**
- US 5426039 A **[0319]**

Non-patent literature cited in the description

- **ELBASHIR et al.** *Genes Dev.,* 2001, vol. 15, 188-200 **[0001]**
- **HAMMOND et al.** *Nat. Rev. Genet.,* 2001, vol. 2, 110-119 **[0001]**
- **WORGALL et al.** *Human Gene Therapy,* 1997, vol. 8, 37 **[0003]**
- **PEETERS et al.** *Human Gene Therapy,* 1996, vol. 7, 1693 **[0003]**
- **YEI et al.** *Gene Therapy,* 1994, vol. 1, 192 **[0003]**
- **HOPE et al.** *Molecular Membrane Biology,* 1998, vol. 15, 1 **[0003]**
- **FEIGNER.** *Scientific American,* 1997, vol. 276, 102 **[0004]**
- **CHONN et al.** *Current Opinion in Biotechnology,* 1995, vol. 6, 698 **[0004]**
- **HARRISON et al.** *Biotechniques,* 1995, vol. 19, 816 **[0005]**
- **LI et al.** *The Gene,* 1997, vol. 4, 891 **[0005]**
- **TAM et al.** *Gene Ther.,* 2000, vol. 7, 1867 **[0005]**
- **HUANG et al.** *Nature Biotechnology,* 1997, vol. 15, 620 **[0005]**
- **TEMPLETON et al.** *Nature Biotechnology,* 1997, vol. 15, 647 **[0005]**
- **HOFLAND et al.** *Pharmaceutical Research,* 1997, vol. 14, 742 **[0005]**
- **WHEELER et al.** *Gene Therapy,* 1999, vol. 6, 271 **[0008]**
- **YANG et al.** *Proc. Natl. Acad. Sci. USA,* 2002, vol. 99, 9942-9947 **[0036]**
- **CALEGARI et al.** *Proc. Natl. Acad. Sci. USA,* 2002, vol. 99, 14236 **[0036]**
- **BYROM et al.** *Ambion TechNotes,* 2003, vol. 10 (1), 4-6 **[0036]**
- **KAWASAKI et al.** *Nucleic Acids Res.,* 2003, vol. 31, 981-987 **[0036]**
- **KNIGHT et al.** *Science,* 2001, vol. 293, 2269-2271 **[0036]**
- **ROBERTSON et al.** *J. Biol. Chem.,* 1968, vol. 243, 82 **[0036]**
- **TIJSSEN.** Overview of principles of hybridization and the strategy of nucleic acid assays. *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes,* 1993 **[0042]**
- **INNIS et al.** PCR Protocols, A Guide to Methods and Applications. Academic Press, Inc, 1990 **[0043]**
- **SMITH ; WATERMAN.** *Adv. Appl. Math.,* 1981, vol. 2, 482 **[0047]**
- **NEEDLEMAN ; WUNSCH.** *J. Mol. Biol.,* 1970, vol. 48, 443 **[0047]**
- **PEARSON ; LIPMAN.** *Proc. Natl. Acad. Sci. USA,* 1988, vol. 85, 2444 **[0047]**
- Current Protocols in Molecular Biology. 1995 **[0047]**
- **ALTSCHUL et al.** *Nuc. Acids Res.,* 1977, vol. 25, 3389-3402 **[0048]**
- **ALTSCHUL et al.** *J. Mol. Biol,* 1990, vol. 215, 403-410 **[0048]**
- **KARLIN ; ALTSCHUL.** *Proc. Natl. Acad. Sci. USA,* 1993, vol. 90, 5873-5787 **[0049]**
- **BATZER et al.** *Nucleic Acid Res.,* 1991, vol. 19, 5081 **[0050]**
- **OHTSUKA et al.** *J. Biol. Chem.,* 1985, vol. 260, 2605-2608 **[0050]**
- **ROSSOLINI et al.** *Mol. Cell. Probes,* 1994, vol. 8, 91-98 **[0050]**
- **ELBASHIR et al.** *Genes Dev.,* 2001, vol. 15, 188 **[0150] [0164]**

- **NYKÄNEN et al.** *Cell,* 2001, vol. 107, 309 **[0150] [0164]**
- **ELBASHIR et al.** *Nature,* 2001, vol. 411, 494-498 **[0155]**
- **ELBASHIR et al.** *EMBO J.,* 2001, vol. 20, 6877-6888 **[0155] [0156]**
- **REYNOLDS et al.** *Nature Biotech.,* 2004, vol. 22 (3), 326-330 **[0155]**
- **KHVOROVA et al.** *Cell,* 2003, vol. 115, 209-216 **[0159]**
- **SCHWARZ et al.** *Cell,* 2003, vol. 115, 199-208 **[0159]**
- **LUO et al.** *Biophys. Res. Commun.,* 2004, vol. 318, 303-310 **[0159]**
- Radioimmunoassay Methods. 1970 **[0161]**
- **BROWN et al.** *J. Biol. Chem.,* 1980, vol. 255, 4980-4983 **[0161]**
- **RAINES et al.** *J. Biol. Chem.,* 1982, vol. 257, 5154-5160 **[0161]**
- **BROOKS et al.** *Clin. Exp. Immunol.,* 1980, vol. 39, 477 **[0161]**
- **BOWEN-POPE et al.** *Proc. Natl. Acad. Sci. USA,* 1984, vol. 81, 2396-2400 **[0161]**
- **JUDGE et al.** *Mol. Ther.,* 2006, vol. 13, 494-505 **[0162]**
- **KOHLER et al.** *Nature,* 1975, vol. 256, 495-497 **[0163]**
- **HARLOW ; LANE.** ANTIBODIES, A LABORATORY MANUAL. Cold Spring Harbor Publication, 1999 **[0163]**
- **BUHRING et al.** *Hybridoma,* 1991, vol. 10 (1), 77-78 **[0163]**
- **GUBLER ; HOFFMAN.** *Gene,* 1983, vol. 25, 263-269 **[0167]**
- PCR Protocols: A Guide to Methods and Applications. 1990 **[0167]**
- **SAMBROOK et al.** Molecular Cloning, A Laboratory Manual. 1989 **[0167]**
- **KRIEGLER.** Gene Transfer and Expression: A Laboratory Manual. 1990 **[0167]**
- Current Protocols in Molecular Biology. 1994 **[0167]**
- **USMAN et al.** *J. Am. Chem. Soc.,* 1987, vol. 109, 7845 **[0168]**
- **SCARINGE et al.** *Nucl. Acids Res.,* 1990, vol. 18, 5433 **[0168]**
- **WINCOTT et al.** *Nucl. Acids Res.,* 1995, vol. 23, 2677-2684 **[0168]**
- **WINCOTT et al.** *Methods Mol. Bio.,* 1997, vol. 74, 59 **[0168]**
- **SAENGER.** Principles of Nucleic Acid Structure. Springer-Verlag, 1984 **[0171]**
- **LIN et al.** *J. Am. Chem. Soc.,* 1998, vol. 120, 8531-8532 **[0171]**
- **LOAKES.** *Nucl. Acids Res.,* 2001, vol. 29, 2437-2447 **[0171]**
- **BEAUCAGE et al.** *Tetrahedron,* 1993, vol. 49, 1925 **[0172]**
- Nucleic Acid Analogues: Synthesis and Properties. **HUNZIKER et al.** Modern Synthetic Methods. VCH, 1995, 331-417 **[0172]**
- Novel Backbone Replacements for Oligonucleotides. **MESMAEKER et al.** Carbohydrate Modifications in Antisense Research. ACS, 1994, 24-39 **[0172]**
- **GEISBERT et al.** *J. Infect. Dis.,* 2006, vol. 193, 1650-1657 **[0177]**
- Arenaviridae: the viruses and their replication. **BUCHMEIER et al.** FIELDS VIROLOGY. Lippincott-Raven, 2001 **[0177]**
- **STEINHAUER et al.** *Annu Rev Genet.,* 2002, vol. 36, 305-332 **[0177]**
- **NEUMANN et al.** *J Gen Virol.,* 2002, vol. 83, 2635-2662 **[0177]**
- **HAMASAKI et al.** *FEBS Lett.,* 2003, vol. 543, 51 **[0177]**
- **YOKOTA et al.** *EMBO Rep.,* 2003, vol. 4, 602 **[0177]**
- **SCHLOMAI et al.** *Hepatology,* 2003, vol. 37, 764 **[0177]**
- **WILSON et al.** *Proc. Natl. Acad. Sci. USA,* 2003, vol. 100, 2783 **[0177]**
- **KAPADIA et al.** *Proc. Natl. Acad. Sci. USA,* 2003, vol. 100, 2014 **[0177]**
- FIELDS VIROLOGY. Lippincott-Raven, 2001 **[0177]**
- **BANERJEA et al.** *Mol. Ther.,* 2003, vol. 8, 62 **[0177]**
- **SONG et al.** *J. Virol.,* 2003, vol. 77, 7174 **[0177]**
- **STEPHENSON.** *JAMA,* 2003, vol. 289, 1494 **[0177]**
- **QIN et al.** *Proc. Natl. Acad. Sci. USA,* 2003, vol. 100, 183 **[0177]**
- **JIA et al.** *J. Virol.,* 2003, vol. 77, 3301 **[0177]**
- **HALL et al.** *J. Virol.,* 2003, vol. 77, 6066 **[0177]**
- **JIANG et al.** *Oncogene,* 2002, vol. 21, 6041 **[0177]**
- **SANCHEZ et al.** *Virus Res.,* 1993, vol. 29, 215-240 **[0178]**
- **WILL et al.** *J. Virol.,* 1993, vol. 67, 1203-1210 **[0178]**
- **VOLCHKOV et al.** *FEBS Lett.,* 1992, vol. 305, 181-184 **[0178]**
- **FORMAN et al.** *Cell,* 1995, vol. 81, 687 **[0181]**
- **SEOL et al.** *Mol. Endocrinol.,* 1995, vol. 9, 72 **[0181]**
- **ZAVACKI et al.** *Proc. Natl. Acad. Sci. USA,* 1997, vol. 94, 7909 **[0181]**
- **SAKAI et al.** *Cell,* 1996, vol. 85, 1037-1046 **[0181]**
- **DUNCAN et al.** *J. Biol. Chem.,* 1997, vol. 272, 12778-12785 **[0181]**
- **WILLY et al.** *Genes Dev.,* 1995, vol. 9, 1033-1045 **[0181]**
- **LEHMANN et al.** *J. Biol. Chem.,* 1997, vol. 272, 3137-3140 **[0181]**
- **JANOWSKI et al.** *Nature,* 1996, vol. 383, 728-731 **[0181]**
- **PEET et al.** *Cell,* 1998, vol. 93, 693-704 **[0181]**
- **BARR et al.** *Nat. Rev. Mol. Cell Biol.,* 2004, vol. 5, 429-440 **[0182] [0383]**
- **WILDA et al.** *Oncogene,* 2002, vol. 21, 5716 **[0183]**
- **SCHERR et al.** *Blood,* 2003, vol. 101, 1566 **[0183]**
- **HEIDENREICH et al.** *Blood,* 2003, vol. 101, 3157 **[0183]**

- **NIETH et al.** *FEBS Lett.,* 2003, vol. 545, 144 **[0183]**
- **WU et al.** *Cancer Res.,* 2003, vol. 63, 1515 **[0183]**
- **LI et al.** *Cancer Res.,* 2003, vol. 63, 3593 **[0183]**
- **ZOU et al.** *Genes Dev.,* 2002, vol. 16, 2923 **[0183]**
- **VERMA et al.** *Clin Cancer Res.,* 2003, vol. 9, 1291 **[0183]**
- **KOSCIOLEK et al.** *Mol Cancer Ther.,* 2003, vol. 2, 209 **[0183]**
- **NAGY et al.** *Exp. Cell Res.,* 2003, vol. 285, 39-49 **[0183]**
- **TUSCHL ; BORKHARDT.** *Mol. Interventions,* 2002, vol. 2, 158 **[0183]**
- **COLLIS et al.** *Cancer Res.,* 2003, vol. 63, 1550 **[0184]**
- **REICH et al.** *Mol. Vis.,* 2003, vol. 9, 210 **[0185]**
- **DECAUSSIN et al.** *J. Pathol.,* 1999, vol. 188, 369-377 **[0186]**
- **HILL et al.** *J. Immunol,* 2003, vol. 171, 691 **[0187]**
- **SONG et al.** *Nat. Med.,* 2003, vol. 9, 347 **[0187]**
- **HEINONEN et al.** *FEBS Lett.,* 2002, vol. 527, 274 **[0187]**
- **CAPLEN et al.** *Hum. Mol. Genet.,* 2002, vol. 11, 175 **[0188]**
- **SUN et al.** *Nat. Biotech.,* 2008, vol. 26, 1379-1382 **[0190]**
- **LAGOS-QUINTANA et al.** *Science,* vol. 294, 853-858 **[0194]**
- **LAU et al.** *Science,* vol. 294, 858-862 **[0194]**
- **LEE et al.** *Science,* vol. 294, 862-864 **[0194]**
- **DENLI et al.** *Nature,* 2004, vol. 432, 231-235 **[0195]**
- **BERNSTEIN et al.** *Nature,* 2001, vol. 409, 363-366 **[0195]**
- **PREALL et al.** *Curr. Biol.,* 2006, vol. 16, 530-535 **[0196]**
- **GREGORY et al.** *Cell,* 2005, vol. 123, 631-640 **[0196]**
- **JASKULSKI et al.** *Science,* 1988, vol. 240, 1544-6 **[0202]**
- **VASANTHAKUMAR et al.** *Cancer Commun.,* 1989, vol. 1, 225-32 **[0202]**
- **PERIS et al.** *Brain Res Mol Brain Res.,* 1998, vol. 15 (57), 310-20 **[0202]**
- **ALTSCHUL et al.** *Nucleic Acids Res,* 1997, vol. 25, 3389-402 **[0203]**
- **KIM et al.** *Proc. Natl. Acad. Sci. USA,* 1987, vol. 84, 8788-92 **[0204]**
- **FORSTER et al.** *Cell,* 1987, vol. 49, 211-20 **[0204]**
- **CECH et al.** *Cell,* 1981, vol. 27, 487-96 **[0204]**
- **MICHEL et al.** *J. Mol. Biol,* 1990, vol. 216, 585-610 **[0204]**
- **REINHOLD-HUREK et al.** *Nature,* 1992, vol. 357, 173-6 **[0204]**
- **ROSSI et al.** *Nucleic Acids Res,* 1992, vol. 20, 4559-65 **[0206]**
- **HAMPEL et al.** *Biochemistry,* 1989, vol. 28, 4929-33 **[0206]**
- **HAMPEL et al.** *Nucleic Acids Res,* 1990, vol. 18, 299-304 **[0206]**
- **PERROTTA et al.** *Biochemistry,* 1992, vol. 31, 11843-52 **[0206]**
- **GUERRIER-TAKADA et al.** *Cell,* 1983, vol. 35, 849-57 **[0206]**
- **SAVILLE et al.** *Cell,* 1990, vol. 61, 685-96 **[0206]**
- **SAVILLE et al.** *Proc. Natl. Acad. Sci. USA,* 1991, vol. 88, 8826-30 **[0206]**
- **COLLINS et al.** *Biochemistry,* 1993, vol. 32, 2795-9 **[0206]**
- **YAMAMOTO et al.** *J. Immunol,* 1992, vol. 148, 4072-6 **[0209]**
- **RANEY et al.** *J. Pharm. Exper. Ther.,* 2001, vol. 298, 1185-92 **[0211]**
- **MARCH.** ADVANCED ORGANIC CHEMISTRY. Wiley, 1992 **[0255]**
- **LAROCK.** COMPREHENSIVE ORGANIC TRANSFORMATIONS. VCH, 1989 **[0255]**
- **FURNISS.** VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY. Longman, 1989 **[0255]**
- **GREEN ; WUTS.** PROTECTIVE GROUPS IN ORGANIC SYNTHESIS. Wiley, 1991 **[0255]**
- **CHEN et al.** *Bioconj. Chem.,* 2000, vol. 11, 433-437 **[0258]**
- REMINGTON'S PHARMACEUTICAL SCIENCES. Mack Publishing Company, 1985 **[0291] [0300]**
- **STRAUBRINGER et al.** *Methods Enzymol,* 1983, vol. 101, 512 **[0296]**
- **MANNINO et al.** *Biotechniques,* 1988, vol. 6, 682 **[0296]**
- **NICOLAU et al.** *Crit. Rev. Ther. Drug Carrier Syst.,* 1989, vol. 6, 239 **[0296]**
- **BEHR.** *Acc. Chem. Res.,* 1993, vol. 26, 274 **[0296]**
- **CULVER.** HUMAN GENE THERAPY. MaryAnn Liebert, Inc, 1994, 70-71 **[0296]**
- **BRIGHAM et al.** *Am. J. Sci.,* 1989, vol. 298, 278 **[0297]**
- Nucleic Acid Hybridization, A Practical Approach. IRL Press, 1985 **[0318]**
- **SAMBROOK et al.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, 2000 **[0319]**
- **AUSUBEL et al.** SHORT PROTOCOLS IN MOLECULAR BIOLOGY. Current Protocols, 2002 **[0319]**
- PCR Protocols, A Guide to Methods and Applications. Academic Press Inc, 1990 **[0319]**
- **ARNHEIM ; LEVINSON.** *C&EN,* 01 October 1990, 36 **[0319]**
- *The Journal Of NIH Research,* 1991, vol. 3, 81 **[0319]**
- **KWOH et al.** *Proc. Natl. Acad. Sci. USA,* 1989, vol. 86, 1173 **[0319]**
- **GUATELLI et al.** *Proc. Natl. Acad. Sci. USA,* 1990, vol. 87, 1874 **[0319]**
- **LOMELL et al.** *J. Clin. Chem,* 1989, vol. 35, 1826 **[0319]**
- **LANDEGREN et al.** *Science,* 1988, vol. 241, 1077 **[0319]**
- **VAN BRUNT.** *Biotechnology,* 1990, vol. 8, 291 **[0319]**

- **WU ; WALLACE.** *Gene,* 1989, vol. 4, 560 **[0319]**
- **BARRINGER et al.** *Gene,* 1990, vol. 89, 117 **[0319]**
- **SOOKNANAN ; MALEK.** *Biotechnology,* 1995, vol. 13, 563 **[0319]**
- **BEAUCAGE et al.** *Tetrahedron Letts.,* 1981, vol. 22, 1859-1862 **[0320]**
- **NEEDHAM VANDEVANTER et al.** *Nucleic Acids Res,* 1984, vol. 12, 6159 **[0320]**
- **PEARSON et al.** *J. Chrom.,* 1983, vol. 255, 137-149 **[0320]**
- **MAXAM ; GILBERT.** Methods in Enzymology. Academic Press, 1980, vol. 65, 499 **[0320]**
- **ANGERER et al.** *Methods Enzymol.,* 1987, vol. 152, 649 **[0321]**
- **HEYES et al.** *Journal of Controlled Release,* 2005, vol. 107, 276-287 **[0326] [0330] [0334] [0340] [0344]**

JOINT APPENDIX 55

PTO/SB/05 (08-08)

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	020801-007710US
First Inventor	MacLachlan, Ian
Title	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
EFS Web Filing Date:	April 15, 2009

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☐ **Fee Transmittal Form** (e.g., PTO/SB/17)
2. ☐ **Applicant claims small entity status.** See 37 CFR 1.27.
3. ☒ **Specification** [*Total Pages* 119]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. ☒ **Drawing(s)** *(35 U.S.C.113)* [*Total Sheets* 22]
5. **Oath or Declaration (unsigned)** [*Total Sheets* 2]
   a. ☐ Newly executed (original or copy)
   b. ☐ A copy from a prior application (37 CFR 1.63 (d))
   (for a continuation/divisional with Box 18 completed)
   i. ☐ DELETION OF INVENTOR(S)
   Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☒ **Application Data Sheet.** See 37 CFR 1.76
7. ☐ **CD-ROM** or **CD-R** in duplicate, large table or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission**
   (if applicable, items a. - c. are required)
   a. ☐ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. ☐ CD-ROM or CD-R (2 copies); or
      ii. ☐ Paper
   c. ☐ Statements verifying identity of above copies

ADDRESS TO: **Commissioner for Patents**
P.O. Box 1450
Alexandria, VA 22313-1450

ACCOMPANYING APPLICATION PARTS

9. ☐ **Assignment Papers** (cover sheet & document(s))
   Name of Assignee _____
10. ☐ **37 CFR 3.73(b) Statement** *(when there is an assignee)* ☐ **Power of Attorney**
11. ☐ **English Translation Document** *(if applicable)*
12. ☐ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☐ Copies of citations attached
13. ☐ **Preliminary Amendment**
14. ☐ **Return Receipt Postcard** (MPEP 503)
    (Should be specifically itemized)
15. ☐ **Certified Copy of Priority Document(s)**
    (if foreign priority is claimed)
16. ☐ **Nonpublication Request** under 35 U.S.C. 122 (b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
17. ☒ Other: Cover page (1)

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: _____

Prior application information: *Examiner* _____ *Art Unit:* _____

19. CORRESPONDENCE ADDRESS

☒ The address associated with Customer Number: **20350** ***OR*** ☐ Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	Joe C. Hao	Date	April 15, 2009
Name (Print/Type)	Joe C. Hao	Registration No. (Attorney/Agent)	55,246

61905732 v1

Attorney Docket No.: 020801-007710US
Client Ref. No.:

PTO/SB/01A (07-07)

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☒ The attached application, or

☐ Application No. _____, filed on April 15, 2009,

☐ as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Ian MacLachlan Date: _____

Signature: _____ Citizen of: Canada/United Kingdom

Inventor two: Edward Yaworski Date: _____

Signature: _____ Citizen of: Canada

☒ Additional inventors or a legal representative are being named on 1 additional form(s) attached hereto.

61905727 v1

Attorney Docket No.: 020801-007710US
Client Ref. No.:

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76) – ADDITIONAL INVENTOR(S)
Supplemental Sheet 1 of 1

FULL NAME OF INVENTOR(S)	
Inventor three: Kieu Lam	Date:
Signature:	Citizen of: Canada
Inventor four:	Date:
Signature:	Citizen of:
Inventor five:	Date:
Signature:	Citizen of:
Inventor six:	Date:
Signature:	Citizen of:
Inventor seven:	Date:
Signature:	Citizen of:
Inventor eight:	Date:
Signature:	Citizen of:
Inventor nine:	Date:
Signature:	Citizen of:
Inventor ten:	Date:
Signature:	Citizen of:
Inventor eleven:	Date:
Signature:	Citizen of:
Inventor twelve:	Date:
Signature:	Citizen of:

61905727 v1

Application Data Sheet

Application Information

Application number::	
Filing Date::	04/15/09
Application Type::	Regular
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R??::	
Number of CD disks::	
Number of copies of CDs::	
Sequence Submission::	
Computer Readable Form (CRF)?::	
Number of copies of CRF::	
Title::	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
Attorney Docket Number::	020801-007710US
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	1
Total Drawing Sheets::	22
Small Entity?::	No
Latin name::	
Variety denomination name::	
Petition included?::	No
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers One::	
Secrecy Order in Parent Appl.::	No

Initial 4/15/09

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	Canada
Status::	Full Capacity
Given Name::	Ian
Middle Name::	
Family Name::	MacLachlan
Name Suffix::	
City of Residence::	Mission
State or Province of Residence::	BC
Country of Residence::	Canada
Street of Mailing Address::	8040 Aves Terrace
City of Mailing Address::	Mission
State or Province of mailing address::	BC
Country of mailing address::	Canada
Postal or Zip Code of mailing address::	V4S1E5

Applicant Authority Type::	Inventor
Primary Citizenship Country::	Canada
Status::	Full Capacity
Given Name::	Edward
Middle Name::	
Family Name::	Yaworski
Name Suffix::	
City of Residence::	Maple Ridge
State or Province of Residence::	BC
Country of Residence::	Canada
Street of Mailing Address::	23106-123B Avenue
City of Mailing Address::	Maple Ridge
State or Province of mailing address::	BC
Country of mailing address::	Canada

Postal or Zip Code of mailing address::	V2X9Z7

Applicant Authority Type::	Inventor
Primary Citizenship Country::	Canada
Status::	Full Capacity
Given Name::	Kieu
Middle Name::	
Family Name::	Lam
Name Suffix::	
City of Residence::	Surrey
State or Province of Residence::	BC
Country of Residence::	Canada
Street of Mailing Address::	18871 71 Avenue
City of Mailing Address::	Surrey
State or Province of mailing address::	BC
Country of mailing address::	Canada
Postal or Zip Code of mailing address::	V4N5M7

Correspondence Information

Correspondence Customer Number::	20350

Representative Information

Representative Customer Number::	20350

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	An Appn claiming benefit under 35 USC 119(e) of	61/045,228	04/15/08

Initial 4/15/09

Foreign Priority Information

Country::	Application number::	Filing Date::

Assignee Information

Assignee Name::	Protiva Biotherapeutics, Inc.
Street of mailing address::	100-8900 Glenlyon Parkway
City of mailing address::	Burnaby
State or Province of mailing address::	B.C.
Country of mailing address::	Canada
Postal or Zip Code of mailing address::	V5J5J8

Attorney Docket No.: 020801-007710US

PATENT APPLICATION

NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Inventors: Ian MacLachlan, a citizen of Canada and the United Kingdom, residing at
8040 Aves Terrace
Mission, B.C., Canada V4S 1E5

Edward Yaworski, a citizen of Canada, residing at
23106 - 123B Avenue
Maple Ridge, B.C., Canada V2X 9Z7

Kieu Lam, a citizen of Canada, residing at
18871 71 Avenue
Surrey, B.C., Canada V4N 5M7

Assignee: Protiva Biotherapeutics, Inc.
100-8900 Glenlyon Parkway
Burnaby, B.C., Canada V5J 5J8

Entity: Large

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 925-472-5000

NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Provisional Application No. 61/045,228, filed April 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not applicable.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

[0003] Not applicable.

REFERENCE TO A "SEQUENCE LISTING"

[0004] Not applicable.

BACKGROUND OF THE INVENTION

[0005] RNA interference (RNAi) is an evolutionarily conserved process in which recognition of double-stranded RNA (dsRNA) ultimately leads to posttranscriptional suppression of gene expression. This suppression is mediated by short dsRNA, also called small interfering RNA (siRNA), which induces specific degradation of mRNA through complementary base pairing. In several model systems, this natural response has been developed into a powerful tool for the investigation of gene function (*see, e.g.,* Elbashir *et al.*, *Genes Dev.*, 15:188-200 (2001); Hammond *et al.*, *Nat. Rev. Genet.*, 2:110-119 (2001)). More recently, it was discovered that introducing synthetic 21-nucleotide dsRNA duplexes into mammalian cells could efficiently silence gene expression.

[0006] Although the precise mechanism is still unclear, RNAi provides a potential new approach to downregulate or silence the transcription and translation of a gene of interest. For example, it is desirable to modulate (*e.g.*, reduce) the expression of certain genes for the treatment of neoplastic disorders such as cancer. It is also desirable to silence the expression of genes associated with liver diseases and disorders such as hepatitis. It is further desirable to reduce the expression of certain genes for the treatment of atherosclerosis and its manifestations, *e.g.,* hypercholesterolemia, myocardial infarction, and thrombosis.

[0007] A safe and effective nucleic acid delivery system is required for RNAi to be therapeutically useful. Viral vectors are relatively efficient gene delivery systems, but suffer from a variety of limitations, such as the potential for reversion to the wild-type as well as immune response concerns. As a result, nonviral gene delivery systems are receiving increasing attention (Worgall *et al.*, *Human Gene Therapy*, 8:37 (1997); Peeters *et al.*, *Human Gene Therapy*, 7:1693 (1996); Yei *et al.*, *Gene Therapy*, 1:192 (1994); Hope *et al.*, *Molecular Membrane Biology*, 15:1 (1998)). Furthermore, viral systems are rapidly cleared from the circulation, limiting transfection to "first-pass" organs such as the lungs, liver, and spleen. In addition, these systems induce immune responses that compromise delivery with subsequent injections.

[0008] Plasmid DNA-cationic liposome complexes are currently the most commonly employed nonviral gene delivery vehicles (Felgner, *Scientific American*, 276:102 (1997); Chonn *et al.*, *Current Opinion in Biotechnology*, 6:698 (1995)). For instance, cationic liposome complexes made of an amphipathic compound, a neutral lipid, and a detergent for transfecting insect cells are disclosed in U.S. Patent No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Publication No. 20030073640.

[0009] Cationic liposome complexes are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects (Harrison *et al.*, *Biotechniques*, 19:816 (1995); Li *et al.*, *The Gene*, 4:891 (1997); Tam *et al*, *Gene Ther.*, 7:1867 (2000)). As large, positively charged aggregates, lipoplexes are rapidly cleared when administered *in vivo*, with highest expression levels observed in first-pass organs, particularly the lungs (Huang *et al.*, *Nature Biotechnology*, 15:620 (1997); Templeton *et al.*, *Nature Biotechnology*, 15:647 (1997); Hofland *et al.*, *Pharmaceutical Research*, 14:742 (1997)).

[0010] Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Patent No. 6,429,200. Anionic liposomes are disclosed in U.S. Patent Publication No. 20030026831. Polymer liposomes that incorporate dextrin or glycerol-phosphocholine polymers are disclosed in U.S. Patent Publication Nos. 20020081736 and 20030082103, respectively.

[0011] A gene delivery system containing an encapsulated nucleic acid for systemic delivery should be small (*i.e.*, less than about 100 nm diameter) and should remain intact in the circulation for an extended period of time in order to achieve delivery to affected tissues. This requires a highly stable, serum-resistant nucleic acid-containing particle that does not interact with cells and other components of the vascular compartment. The particle should

also readily interact with target cells at a disease site in order to facilitate intracellular delivery of a desired nucleic acid.

[0012] Recent work has shown that nucleic acids can be encapsulated in small (*e.g.,* about 70 nm diameter) "stabilized plasmid-lipid particles" (SPLP) that consist of a single plasmid encapsulated within a bilayer lipid vesicle (Wheeler *et al.*, *Gene Therapy*, 6:271 (1999)). These SPLPs typically contain the "fusogenic" lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid, and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. SPLPs have systemic application as they exhibit extended circulation lifetimes following intravenous (i.v.) injection, accumulate preferentially at distal tumor sites due to the enhanced vascular permeability in such regions, and can mediate transgene expression at these tumor sites. The levels of transgene expression observed at the tumor site following i.v. injection of SPLPs containing the luciferase marker gene are superior to the levels that can be achieved employing plasmid DNA-cationic liposome complexes (lipoplexes) or naked DNA.

[0013] Thus, there remains a strong need in the art for novel and more efficient methods and compositions for introducing nucleic acids such as siRNA into cells. In addition, there is a need in the art for methods of downregulating the expression of genes of interest to treat or prevent diseases and disorders such as cancer and atherosclerosis. The present invention addresses these and other needs.

BRIEF SUMMARY OF THE INVENTION

[0014] The present invention provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.,* for the treatment of a disease or disorder).

[0015] In preferred embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.,* by a nuclease or protease. In other preferred embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0016] In one aspect, the present invention provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid

present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0017] More particularly, the present invention provides serum-stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (*e.g.,* one or more interfering RNA molecules such as siRNA, aiRNA, and/or miRNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP (*e.g.,* for the treatment of a disease or disorder).

[0018] In certain embodiments, the nucleic acid-lipid particle (*e.g.,* SNALP) comprises: (a) a nucleic acid (*e.g.,* an interfering RNA); (b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0019] In one preferred embodiment, the nucleic acid-lipid particle (*e.g.,* SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:62" formulation.

[0020] In another preferred embodiment, the nucleic acid-lipid particle (*e.g.,* SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:57" formulation.

[0021] The present invention also provides pharmaceutical compositions comprising a lipid particle described herein (*e.g.,* SNALP) and a pharmaceutically acceptable carrier.

[0022] In another aspect, the present invention provides methods for introducing an active agent or therapeutic agent (*e.g.,* nucleic acid) into a cell, the method comprising contacting the cell with a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.,* SNALP).

[0023] In yet another aspect, the present invention provides methods for the *in vivo* delivery of an active agent or therapeutic agent (*e.g.,* nucleic acid), the method comprising administering to a mammalian subject a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.,* SNALP).

[0024] In a further aspect, the present invention provides methods for treating a disease or disorder in a mammalian subject in need thereof, the method comprising administering to the mammalian subject a therapeutically effective amount of a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.,* SNALP).

[0025] Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] Figure 1 illustrates data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

[0027] Figure 2 illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

[0028] Figure 3 illustrates additional data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice. Each bar represents the group mean of five animals. Error bars indicate the standard deviation.

[0029] Figure 4 illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

[0030] Figure 5 illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

[0031] Figure 6 illustrates data demonstrating that the tolerability of 1:57 SNALP containing ApoB siRNA prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters.

[0032] Figure 7 illustrates data demonstrating that the efficacy of 1:57 SNALP containing ApoB siRNA prepared by gear pump was similar to the same SNALP prepared by syringe press.

[0033] Figure 8 illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

[0034] Figure 9 illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

[0035] Figure 10 illustrates data demonstrating that clinically significant liver enzyme elevations (3xULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

[0036] Figure 11 illustrates data demonstrating that the potency of the lower lipid:drug (L:D) 1:57 SNALP containing ApoB siRNA was as good as that of the higher L:D SNALP at the tested drug dosages.

[0037] Figure 12 illustrates data demonstrating that ApoB protein and total cholesterol levels were reduced to a similar extent by 1:57 SNALP containing ApoB siRNA at a 6:1 input L:D ratio (final ratio of 7:1) and 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

[0038] Figure 13 illustrates data demonstrating that a treatment regimen of 1:57 SNALP with siRNA targeting PLK-1 is well tolerated with no apparent signs of treatment related toxicity in mice bearing Hep3B liver tumors.

[0039] Figure 14 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA caused a significant increase in the survival of Hep3B tumor-bearing mice.

[0040] Figure 15 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA reduced PLK-1 mRNA levels by 50% in intrahepatic Hep3B tumors growing in mice 24 hours after SNALP administration.

[0041] Figure 16 illustrates data demonstrating that a specific cleavage product of PLK-1 mRNA was detectable by 5' RACE-PCR in mice treated with 1:57 SNALP containing PLK-1 siRNA. 10 µl PCR product/well were loaded onto a 1.5% agarose gel. Lane Nos.: (1) molecular weight (MW) marker; (2) PBS mouse 1; (3) PBS mouse 2; (4) PBS mouse 3; (5) Luc SNALP mouse 1; (6) Luc SNALP mouse 2; (7) PLK SNALP mouse 1; (8) PLK SNALP mouse 2; (9) PLK SNALP mouse 3; and (10) no template control.

[0042] Figure 17 illustrates data demonstrating that control (Luc) 1:57 SNALP-treated mice displayed normal mitoses in Hep3B tumors (top panels), whereas mice treated with 1:57 SNALP containing PLK-1 siRNA exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors (bottom panels).

[0043] Figure 18 illustrates data demonstrating that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B subcutaneous (S.C.) tumors.

[0044] Figure 19 illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

[0045] Figure 20 illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0046] Figure 21 illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

[0047] Figure 22 illustrates data demonstrating the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0048] The present invention is based, in part, upon the surprising discovery that lipid particles comprising from about 50 mol % to about 85 mol % of a cationic lipid, from about 13 mol % to about 49.5 mol % of a non-cationic lipid, and from about 0.5 mol % to about 2 mol % of a lipid conjugate provide advantages when used for the *in vitro* or *in vivo* delivery of an active agent, such as a therapeutic nucleic acid (*e.g.,* an interfering RNA). In particular, as illustrated by the Examples herein, the present invention provides stable nucleic acid-lipid particles (SNALP) that advantageously impart increased activity of the encapsulated nucleic acid (*e.g.,* an interfering RNA such as siRNA) and improved tolerability of the formulations *in vivo*, resulting in a significant increase in the therapeutic index as compared to nucleic acid-lipid particle compositions previously described. Additionally, the SNALP of the invention are stable in circulation, *e.g.,* resistant to degradation by nucleases in serum, and are substantially non-toxic to mammals such as humans. As a non-limiting example, Figure 3 of Example 4 shows that one SNALP embodiment of the invention ("1:57 SNALP") was more than 10 times as efficacious as compared to a nucleic acid-lipid particle previously described ("2:30 SNALP") in mediating target gene silencing at a 10-fold lower dose. Similarly, Figure 2 of Example 3 shows that the "1:57 SNALP" formulation was substantially more effective at silencing the expression of a target gene as compared to nucleic acid-lipid particles previously described ("2:40 SNALP").

[0049] In certain embodiments, the present invention provides improved compositions for the delivery of interfering RNA such as siRNA molecules. In particular, the Examples herein illustrate that the improved lipid particle formulations of the invention are highly effective in downregulating the mRNA and/or protein levels of target genes. Furthermore, the Examples herein illustrate that the presence of certain molar ratios of lipid components results in improved or enhanced activity of these lipid particle formulations of the present invention. For instance, the "1:57 SNALP" and "1:62 SNALP" formulations described herein are

exemplary formulations of the present invention that are particularly advantageous because they provide improved efficacy and tolerability *in vivo*, are serum-stable, are substantially non-toxic, are capable of accessing extravascular sites, and are capable of reaching target cell populations.

[0050] The lipid particles and compositions of the present invention may be used for a variety of purposes, including the delivery of associated or encapsulated therapeutic agents to cells, both *in vitro* and *in vivo*. Accordingly, the present invention provides methods for treating diseases or disorders in a subject in need thereof, by contacting the subject with a lipid particle described herein comprising one or more suitable therapeutic agents.

[0051] Various exemplary embodiments of the lipid particles of the invention, as well as compositions and formulations comprising the same, and their use to deliver therapeutic agents and modulate target gene and protein expression, are described in further detail below.

II. Definitions

[0052] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0053] The term "interfering RNA" or "RNAi" or "interfering RNA sequence" refers to single-stranded RNA (*e.g.,* mature miRNA) or double-stranded RNA (*i.e.*, duplex RNA such as siRNA, aiRNA, or pre-miRNA) that is capable of reducing or inhibiting the expression of a target gene or sequence (*e.g.,* by mediating the degradation or inhibiting the translation of mRNAs which are complementary to the interfering RNA sequence) when the interfering RNA is in the same cell as the target gene or sequence. Interfering RNA thus refers to the single-stranded RNA that is complementary to a target mRNA sequence or to the double-stranded RNA formed by two complementary strands or by a single, self-complementary strand. Interfering RNA may have substantial or complete identity to the target gene or sequence, or may comprise a region of mismatch (*i.e.,* a mismatch motif). The sequence of the interfering RNA can correspond to the full-length target gene, or a subsequence thereof.

[0054] Interfering RNA includes "small-interfering RNA" or "siRNA," *e.g.,* interfering RNA of about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, more typically about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.,* each complementary sequence of the double-stranded siRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, preferably about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded siRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, preferably

about 18-22, 19-20, or 19-21 base pairs in length). siRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides and 5' phosphate termini. Examples of siRNA include, without limitation, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand; a double-stranded polynucleotide molecule assembled from a single stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions; and a circular single-stranded polynucleotide molecule with two or more loop structures and a stem having self-complementary sense and antisense regions, where the circular polynucleotide can be processed *in vivo* or *in vitro* to generate an active double-stranded siRNA molecule.

[0055] Preferably, siRNA are chemically synthesized. siRNA can also be generated by cleavage of longer dsRNA (*e.g.*, dsRNA greater than about 25 nucleotides in length) with the *E. coli* RNase III or Dicer. These enzymes process the dsRNA into biologically active siRNA (*see*, *e.g.*, Yang *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:9942-9947 (2002); Calegari *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:14236 (2002); Byrom *et al.*, *Ambion TechNotes,* 10(1):4-6 (2003); Kawasaki *et al.*, *Nucleic Acids Res.,* 31:981-987 (2003); Knight *et al.*, *Science,* 293:2269-2271 (2001); and Robertson *et al.*, *J. Biol. Chem.,* 243:82 (1968)). Preferably, dsRNA are at least 50 nucleotides to about 100, 200, 300, 400, or 500 nucleotides in length. A dsRNA may be as long as 1000, 1500, 2000, 5000 nucleotides in length, or longer. The dsRNA can encode for an entire gene transcript or a partial gene transcript. In certain instances, siRNA may be encoded by a plasmid (*e.g.*, transcribed as sequences that automatically fold into duplexes with hairpin loops).

[0056] As used herein, the term "mismatch motif" or "mismatch region" refers to a portion of an interfering RNA (*e.g.,* siRNA, aiRNA, miRNA) sequence that does not have 100 % complementarity to its target sequence. An interfering RNA may have at least one, two, three, four, five, six, or more mismatch regions. The mismatch regions may be contiguous or may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides. The mismatch motifs or regions may comprise a single nucleotide or may comprise two, three, four, five, or more nucleotides.

[0057] An "effective amount" or "therapeutically effective amount" of an active agent or therapeutic agent such as an interfering RNA is an amount sufficient to produce the desired effect, *e.g.*, an inhibition of expression of a target sequence in comparison to the normal

expression level detected in the absence of an interfering RNA. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with an interfering RNA relative to the control is about 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, *e.g.,* examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0058] By "decrease," "decreasing," "reduce," or "reducing" of an immune response by an interfering RNA is intended to mean a detectable decrease of an immune response to a given interfering RNA (*e.g.*, a modified interfering RNA). The amount of decrease of an immune response by a modified interfering RNA may be determined relative to the level of an immune response in the presence of an unmodified interfering RNA. A detectable decrease can be about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more lower than the immune response detected in the presence of the unmodified interfering RNA. A decrease in the immune response to interfering RNA is typically measured by a decrease in cytokine production (*e.g.*, IFNγ, IFNα, TNFα, IL-6, or IL-12) by a responder cell *in vitro* or a decrease in cytokine production in the sera of a mammalian subject after administration of the interfering RNA.

[0059] As used herein, the term "responder cell" refers to a cell, preferably a mammalian cell, that produces a detectable immune response when contacted with an immunostimulatory interfering RNA such as an unmodified siRNA. Exemplary responder cells include, *e.g.*, dendritic cells, macrophages, peripheral blood mononuclear cells (PBMCs), splenocytes, and the like. Detectable immune responses include, *e.g.*, production of cytokines or growth factors such as TNF-α, IFN-α, IFN-β, IFN-γ, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TGF, and combinations thereof.

[0060] "Substantial identity" refers to a sequence that hybridizes to a reference sequence under stringent conditions, or to a sequence that has a specified percent identity over a specified region of a reference sequence.

[0061] The phrase "stringent hybridization conditions" refers to conditions under which a nucleic acid will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen,

Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0062] Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C-95°C for 30 sec.-2 min., an annealing phase lasting 30 sec.-2 min., and an extension phase of about 72°C for 1-2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, *e.g.,* in Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y. (1990).

[0063] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional

guidelines for determining hybridization parameters are provided in numerous references, *e.g.*, Current Protocols in Molecular Biology, Ausubel *et al.*, eds.

[0064] The terms "substantially identical" or "substantial identity," in the context of two or more nucleic acids, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (*i.e.,* at least about 60%, preferably at least about 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition, when the context indicates, also refers analogously to the complement of a sequence. Preferably, the substantial identity exists over a region that is at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length.

[0065] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0066] A "comparison window," as used herein, includes reference to a segment of any one of a number of contiguous positions selected from the group consisting of from about 5 to about 60, usually about 10 to about 45, more usually about 15 to about 30, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.,* by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.,* 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.,* 48:443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA,* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology*, Ausubel *et al.*, eds. (1995 supplement)).

[0067] A preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al., Nuc. Acids Res.,* 25:3389-3402 (1977) and Altschul *et al., J. Mol. Biol.*, 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

[0068] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul, *Proc. Natl. Acad. Sci. USA,* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0069] The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA and RNA. DNA may be in the form of, *e.g.*, antisense molecules, plasmid DNA, pre-condensed DNA, a PCR product, vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives and combinations of these groups. RNA may be in the form of siRNA, asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, tRNA, viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.,* degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third

position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.,* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.,* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes,* 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

[0070] The term "gene" refers to a nucleic acid (*e.g.*, DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

[0071] "Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

[0072] The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are characterized by being insoluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

[0073] A "lipid particle" is used herein to refer to a lipid formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), to a target site of interest. In the lipid particle of the invention, which is typically formed from a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of the particle, the active agent or therapeutic agent may be encapsulated in the lipid, thereby protecting the agent from enzymatic degradation.

[0074] As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a particle made from lipids (*e.g.*, a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of the particle), wherein the nucleic acid (*e.g.*, siRNA, aiRNA, miRNA, ssDNA, dsDNA, ssRNA, short hairpin RNA (shRNA), dsRNA, or a plasmid, including plasmids from which an interfering RNA is transcribed) is fully encapsulated within the lipid. As used herein, the term "SNALP" includes an SPLP, which is the term used to refer to a nucleic acid-lipid particle comprising a nucleic acid (*e.g.,* a plasmid) encapsulated within the lipid. SNALP and SPLP typically contain a cationic lipid, a

non-cationic lipid, and a lipid conjugate (*e.g.,* a PEG-lipid conjugate). SNALP and SPLP are extremely useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (i.v.) injection, they can accumulate at distal sites (*e.g.,* sites physically separated from the administration site), and they can mediate expression of the transfected gene or silencing of target gene expression at these distal sites. SPLP include "pSPLP," which comprise an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0075] The lipid particles of the invention (*e.g.,* SNALP) typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm, and are substantially non-toxic. In addition, nucleic acids, when present in the lipid particles of the invention, are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.,* U.S. Patent Publication Nos. 20040142025 and 20070042031, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0076] As used herein, "lipid encapsulated" can refer to a lipid particle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid is fully encapsulated in the lipid particle (*e.g.*, to form an SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle).

[0077] The term "lipid conjugate" refers to a conjugated lipid that inhibits aggregation of lipid particles. Such lipid conjugates include, but are not limited to, polyamide oligomers (*e.g.,* ATTA-lipid conjugates), PEG-lipid conjugates, such as PEG coupled to dialkyloxypropyls, PEG coupled to diacylglycerols, PEG coupled to cholesterol, PEG coupled to phosphatidylethanolamines, PEG conjugated to ceramides (*see, e.g.,* U.S. Patent No. 5,885,613, the disclosure of which is herein incorporated by reference in its entirety for all purposes), cationic PEG lipids, and mixtures thereof. PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.,* non-ester containing linker moieties and ester-containing linker moieties. In preferred embodiments, non-ester containing linker moieties are used.

[0078] The term "amphipathic lipid" refers, in part, to any suitable material wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the

hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxyl, and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids.

[0079] Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, are also within the group designated as amphipathic lipids. Additionally, the amphipathic lipids described above can be mixed with other lipids including triglycerides and sterols.

[0080] The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides, and diacylglycerols.

[0081] The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

[0082] The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerols, cardiolipins, diacylphosphatidylserines, diacylphosphatidic acids, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

[0083] The term "cationic lipid" refers to any of a number of lipid species that carry a net positive charge at a selected pH, such as physiological pH (*e.g.*, pH of about 7.0). It has been surprisingly found that cationic lipids comprising alkyl chains with multiple sites of unsaturation, *e.g.*, at least two or three sites of unsaturation, are particularly useful for

forming lipid particles with increased membrane fluidity. A number of cationic lipids and related analogs, which are also useful in the present invention, have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of cationic lipids are described in detail herein. In some cases, the cationic lipids comprise a protonatable tertiary amine (*e.g.,* pH titratable) head group, C18 alkyl chains, ether linkages between the head group and alkyl chains, and 0 to 3 double bonds. Such lipids include, *e.g.*, DSDMA, DLinDMA, DLenDMA, and DODMA.

[0084] The term "hydrophobic lipid" refers to compounds having apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups optionally substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Suitable examples include, but are not limited to, diacylglycerol, dialkylglycerol, N-N-dialkylamino, 1,2-diacyloxy-3-aminopropane, and 1,2-dialkyl-3-aminopropane.

[0085] The term "fusogenic" refers to the ability of a lipid particle, such as a SNALP, to fuse with the membranes of a cell. The membranes can be either the plasma membrane or membranes surrounding organelles, *e.g.*, endosome, nucleus, *etc.*

[0086] As used herein, the term "aqueous solution" refers to a composition comprising in whole, or in part, water.

[0087] As used herein, the term "organic lipid solution" refers to a composition comprising in whole, or in part, an organic solvent having a lipid.

[0088] "Distal site," as used herein, refers to a physically separated site, which is not limited to an adjacent capillary bed, but includes sites broadly distributed throughout an organism.

[0089] "Serum-stable" in relation to nucleic acid-lipid particles such as SNALP means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNAse assay, or an RNAse assay.

[0090] "Systemic delivery," as used herein, refers to delivery of lipid particles that leads to a broad biodistribution of an active agent or therapeutic agent such as an interfering RNA within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. To obtain broad biodistribution

generally requires a blood lifetime such that the agent is not rapidly degraded or cleared (such as by first pass organs (liver, lung, *etc.*) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration. Systemic delivery of lipid particles can be by any means known in the art including, for example, intravenous, subcutaneous, and intraperitoneal. In a preferred embodiment, systemic delivery of lipid particles is by intravenous delivery.

[0091] "Local delivery," as used herein, refers to delivery of an active agent or therapeutic agent such as an interfering RNA directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site such as a tumor or other target site such as a site of inflammation or a target organ such as the liver, heart, pancreas, kidney, and the like.

[0092] The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

[0093] The term "cancer" refers to any member of a class of diseases characterized by the uncontrolled growth of aberrant cells. The term includes all known cancers and neoplastic conditions, whether characterized as malignant, benign, soft tissue, or solid, and cancers of all stages and grades including pre- and post-metastatic cancers. Examples of different types of cancer include, but are not limited to, lung cancer, colon cancer, rectal cancer, anal cancer, bile duct cancer, small intestine cancer, stomach (gastric) cancer, esophageal cancer; gallbladder cancer, liver cancer, pancreatic cancer, appendix cancer, breast cancer, ovarian cancer; cervical cancer, prostate cancer, renal cancer (*e.g.,* renal cell carcinoma), cancer of the central nervous system, glioblastoma, skin cancer, lymphomas, choriocarcinomas, head and neck cancers, osteogenic sarcomas, and blood cancers. Non-limiting examples of specific types of liver cancer include hepatocellular carcinoma (HCC), secondary liver cancer (*e.g.,* caused by metastasis of some other non-liver cancer cell type), and hepatoblastoma. As used herein, a "tumor" comprises one or more cancerous cells.

III. Description of the Embodiments

[0094] The present invention provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.,* for the treatment of a disease or disorder).

[0095] In one aspect, the present invention provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from

about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0096] In certain embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.,* by a nuclease or protease. In certain other embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0097] In some embodiments, the active agent or therapeutic agent comprises a nucleic acid. In certain instances, the nucleic acid comprises an interfering RNA molecule such as, *e.g.,* an siRNA, aiRNA, miRNA, or mixtures thereof. In certain other instances, the nucleic acid comprises single-stranded or double-stranded DNA, RNA, or a DNA/RNA hybrid such as, *e.g.,* an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

[0098] In other embodiments, the active agent or therapeutic agent comprises a peptide or polypeptide. In certain instances, the peptide or polypeptide comprises an antibody such as, *e.g.,* a polyclonal antibody, a monoclonal antibody, an antibody fragment; a humanized antibody, a recombinant antibody, a recombinant human antibody, a Primatized™ antibody, or mixtures thereof. In certain other instances, the peptide or polypeptide comprises a cytokine, a growth factor, an apoptotic factor, a differentiation-inducing factor, a cell-surface receptor, a ligand, a hormone, a small molecule (*e.g.,* small organic molecule or compound), or mixtures thereof.

[0099] In preferred embodiments, the active agent or therapeutic agent comprises an siRNA. In one embodiment, the siRNA molecule comprises a double-stranded region of about 15 to about 60 nucleotides in length (*e.g.,* about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, or 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length). The siRNA molecules of the invention are capable of silencing the expression of a target sequence *in vitro* and/or *in vivo*.

[0100] In some embodiments, the siRNA molecule comprises at least one modified nucleotide. In certain preferred embodiments, the siRNA molecule comprises one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides in the double-stranded region. In certain instances, the siRNA comprises from about 1% to about 100%

(*e.g.,* about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region. In preferred embodiments, less than about 25% (*e.g.,* less than about 25%, 20%, 15%, 10%, or 5%) or from about 1% to about 25% (*e.g.,* from about 1%-25%, 5%-25%, 10%-25%, 15%-25%, 20%-25%, or 10%-20%) of the nucleotides in the double-stranded region comprise modified nucleotides.

[0101] In other embodiments, the siRNA molecule comprises modified nucleotides including, but not limited to, 2'-O-methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, locked nucleic acid (LNA) nucleotides, and mixtures thereof. In preferred embodiments, the siRNA comprises 2'OMe nucleotides (*e.g.,* 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof. In certain instances, the siRNA does not comprise 2'OMe-cytosine nucleotides. In other embodiments, the siRNA comprises a hairpin loop structure.

[0102] The siRNA may comprise modified nucleotides in one strand (*i.e.,* sense or antisense) or both strands of the double-stranded region of the siRNA molecule. Preferably, uridine and/or guanosine nucleotides are modified at selective positions in the double-stranded region of the siRNA duplex. With regard to uridine nucleotide modifications, at least one, two, three, four, five, six, or more of the uridine nucleotides in the sense and/or antisense strand can be a modified uridine nucleotide such as a 2'OMe-uridine nucleotide. In some embodiments, every uridine nucleotide in the sense and/or antisense strand is a 2'OMe-uridine nucleotide. With regard to guanosine nucleotide modifications, at least one, two, three, four, five, six, or more of the guanosine nucleotides in the sense and/or antisense strand can be a modified guanosine nucleotide such as a 2'OMe-guanosine nucleotide. In some embodiments, every guanosine nucleotide in the sense and/or antisense strand is a 2'OMe-guanosine nucleotide.

[0103] In certain embodiments, at least one, two, three, four, five, six, seven, or more 5'-GU-3' motifs in an siRNA sequence may be modified, *e.g.,* by introducing mismatches to eliminate the 5'-GU-3' motifs and/or by introducing modified nucleotides such as 2'OMe nucleotides. The 5'-GU-3' motif can be in the sense strand, the antisense strand, or both strands of the siRNA sequence. The 5'-GU-3' motifs may be adjacent to each other or, alternatively, they may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides.

[0104] In some preferred embodiments, a modified siRNA molecule is less immunostimulatory than a corresponding unmodified siRNA sequence. In such embodiments, the modified siRNA molecule with reduced immunostimulatory properties advantageously retains RNAi activity against the target sequence. In another embodiment, the immunostimulatory properties of the modified siRNA molecule and its ability to silence target gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the siRNA sequence such as, *e.g.,* within the double-stranded region of the siRNA duplex. In certain instances, the modified siRNA is at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% less immunostimulatory than the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that the immunostimulatory properties of the modified siRNA molecule and the corresponding unmodified siRNA molecule can be determined by, for example, measuring INF-α and/or IL-6 levels from about two to about twelve hours after systemic administration in a mammal or transfection of a mammalian responder cell using an appropriate lipid-based delivery system (such as the SNALP delivery system disclosed herein).

[0105] In certain embodiments, a modified siRNA molecule has an IC_{50} (*i.e.,* half-maximal inhibitory concentration) less than or equal to ten-fold that of the corresponding unmodified siRNA (*i.e.*, the modified siRNA has an IC_{50} that is less than or equal to ten-times the IC_{50} of the corresponding unmodified siRNA). In other embodiments, the modified siRNA has an IC_{50} less than or equal to three-fold that of the corresponding unmodified siRNA sequence. In yet other embodiments, the modified siRNA has an IC_{50} less than or equal to two-fold that of the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that a dose-response curve can be generated and the IC_{50} values for the modified siRNA and the corresponding unmodified siRNA can be readily determined using methods known to those of skill in the art.

[0106] In yet another embodiment, a modified siRNA molecule is capable of silencing at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the expression of the target sequence relative to the corresponding unmodified siRNA sequence.

[0107] In some embodiments, the siRNA molecule does not comprise phosphate backbone modifications, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In other embodiments, the siRNA comprises one, two, three, four, or more phosphate backbone

modifications, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise phosphate backbone modifications.

[0108] In further embodiments, the siRNA does not comprise 2'-deoxy nucleotides, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In yet further embodiments, the siRNA comprises one, two, three, four, or more 2'-deoxy nucleotides, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise 2'-deoxy nucleotides.

[0109] In certain instances, the nucleotide at the 3'-end of the double-stranded region in the sense and/or antisense strand is not a modified nucleotide. In certain other instances, the nucleotides near the 3'-end (*e.g.,* within one, two, three, or four nucleotides of the 3'-end) of the double-stranded region in the sense and/or antisense strand are not modified nucleotides.

[0110] The siRNA molecules described herein may have 3' overhangs of one, two, three, four, or more nucleotides on one or both sides of the double-stranded region, or may lack overhangs (*i.e.*, have blunt ends) on one or both sides of the double-stranded region. Preferably, the siRNA has 3' overhangs of two nucleotides on each side of the double-stranded region. In certain instances, the 3' overhang on the antisense strand has complementarity to the target sequence and the 3' overhang on the sense strand has complementarity to a complementary strand of the target sequence. Alternatively, the 3' overhangs do not have complementarity to the target sequence or the complementary strand thereof. In some embodiments, the 3' overhangs comprise one, two, three, four, or more nucleotides such as 2'-deoxy (2'H) nucleotides. In certain preferred embodiments, the 3' overhangs comprise deoxythymidine (dT) and/or uridine nucleotides. In other embodiments, one or more of the nucleotides in the 3' overhangs on one or both sides of the double-stranded region comprise modified nucleotides. Non-limiting examples of modified nucleotides are described above and include 2'OMe nucleotides, 2'-deoxy-2'F nucleotides, 2'-deoxy nucleotides, 2'-O-2-MOE nucleotides, LNA nucleotides, and mixtures thereof. In preferred embodiments, one, two, three, four, or more nucleotides in the 3' overhangs present on the sense and/or antisense strand of the siRNA comprise 2'OMe nucleotides (*e.g.,* 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof.

[0111] The siRNA may comprise at least one or a cocktail (*e.g.,* at least two, three, four, five, six, seven, eight, nine, ten, or more) of unmodified and/or modified siRNA sequences that silence target gene expression. The cocktail of siRNA may comprise sequences which

are directed to the same region or domain (*e.g.,* a "hot spot") and/or to different regions or domains of one or more target genes. In certain instances, one or more (*e.g.,* at least two, three, four, five, six, seven, eight, nine, ten, or more) modified siRNA that silence target gene expression are present in a cocktail. In certain other instances, one or more (*e.g.,* at least two, three, four, five, six, seven, eight, nine, ten, or more) unmodified siRNA sequences that silence target gene expression are present in a cocktail.

[0112] In some embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to the target sequence or a portion thereof. In other embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is 100% complementary to the target sequence or a portion thereof. In further embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that specifically hybridizes to the target sequence or a portion thereof.

[0113] In further embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the target sequence or a portion thereof. In additional embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is 100% identical to the target sequence or a portion thereof.

[0114] In the lipid particles of the invention (*e.g.,* SNALP comprising an interfering RNA such as siRNA), the cationic lipid may comprise, *e.g.,* one or more of the following: 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP),

3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), or mixtures thereof. In certain preferred embodiments, the cationic lipid is DLinDMA, DLin-K-C2-DMA ("XTC2"), or mixtures thereof.

[0115] The synthesis of cationic lipids such as DLin-K-C2-DMA ("XTC2"), DLin-K-C3-DMA, DLin-K-C4-DMA, DLin-K6-DMA, and DLin-K-MPZ, as well as additional cationic lipids, is described in U.S. Provisional Application No. 61/104,212, filed October 9, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as DLin-K-DMA, DLin-C-DAP, DLin-DAC, DLin-MA, DLinDAP, DLin-S-DMA, DLin-2-DMAP, DLin-TMA.Cl, DLin-TAP.Cl, DLin-MPZ, DLinAP, DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Application No. PCT/US08/88676, filed December 31, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as CLinDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060240554, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0116] In some embodiments, the cationic lipid may comprise from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 50 mol % to about 60 mol % of the total lipid present in the particle.

[0117] In other embodiments, the cationic lipid may comprise from about 55 mol % to about 90 mol %, from about 55 mol % to about 85 mol %, from about 55 mol % to about 80 mol %, from about 55 mol % to about 75 mol %, from about 55 mol % to about 70 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0118] In yet other embodiments, the cationic lipid may comprise from about 60 mol % to about 90 mol %, from about 60 mol % to about 85 mol %, from about 60 mol % to about 80 mol %, from about 60 mol % to about 75 mol %, or from about 60 mol % to about 70 mol % of the total lipid present in the particle.

[0119] In still yet other embodiments, the cationic lipid may comprise from about 65 mol % to about 90 mol %, from about 65 mol % to about 85 mol %, from about 65 mol % to about 80 mol %, or from about 65 mol % to about 75 mol % of the total lipid present in the particle.

[0120] In further embodiments, the cationic lipid may comprise from about 70 mol % to about 90 mol %, from about 70 mol % to about 85 mol %, from about 70 mol % to about 80 mol %, from about 75 mol % to about 90 mol %, from about 75 mol % to about 85 mol %, or from about 80 mol % to about 90 mol % of the total lipid present in the particle.

[0121] In additional embodiments, the cationic lipid may comprise (at least) about 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0122] In the lipid particles of the invention (*e.g.,* SNALP comprising an interfering RNA such as siRNA), the non-cationic lipid may comprise, *e.g.,* one or more anionic lipids and/or neutral lipids. In preferred embodiments, the non-cationic lipid comprises one of the following neutral lipid components: (1) cholesterol or a derivative thereof; (2) a phospholipid; or (3) a mixture of a phospholipid and cholesterol or a derivative thereof.

[0123] Examples of cholesterol derivatives include, but are not limited to, cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof. The synthesis of cholesteryl-2'-hydroxyethyl ether is described herein.

[0124] The phospholipid may be a neutral lipid including, but not limited to, dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-

phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), egg phosphatidylcholine (EPC), and mixtures thereof. In certain preferred embodiments, the phospholipid is DPPC, DSPC, or mixtures thereof.

[0125] In some embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 60 mol %, from about 15 mol % to about 60 mol %, from about 20 mol % to about 60 mol %, from about 25 mol % to about 60 mol %, from about 30 mol % to about 60 mol %, from about 10 mol % to about 55 mol %, from about 15 mol % to about 55 mol %, from about 20 mol % to about 55 mol %, from about 25 mol % to about 55 mol %, from about 30 mol % to about 55 mol %, from about 13 mol % to about 50 mol %, from about 15 mol % to about 50 mol % or from about 20 mol % to about 50 mol % of the total lipid present in the particle. When the non-cationic lipid is a mixture of a phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 50, or 60 mol % of the total lipid present in the particle.

[0126] In other embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 49.5 mol %, from about 13 mol % to about 49.5 mol %, from about 15 mol % to about 49.5 mol %, from about 20 mol % to about 49.5 mol %, from about 25 mol % to about 49.5 mol %, from about 30 mol % to about 49.5 mol %, from about 35 mol % to about 49.5 mol %, or from about 40 mol % to about 49.5 mol % of the total lipid present in the particle.

[0127] In yet other embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 45 mol %, from about 13 mol % to about 45 mol %, from about 15 mol % to about 45 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, or from about 35 mol % to about 45 mol % of the total lipid present in the particle.

[0128] In still yet other embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 40 mol %, from about 13 mol % to about 40 mol %, from about 15 mol % to about 40 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0129] In further embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 35 mol %, from about 13 mol % to about 35 mol %, from about 15 mol % to about 35 mol %, from about 20 mol % to

about 35 mol %, or from about 25 mol % to about 35 mol % of the total lipid present in the particle.

[0130] In yet further embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 30 mol %, from about 13 mol % to about 30 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 13 mol % to about 25 mol %, or from about 15 mol % to about 25 mol % of the total lipid present in the particle.

[0131] In additional embodiments, the non-cationic lipid (*e.g.,* one or more phospholipids and/or cholesterol) may comprise (at least) about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0132] In certain preferred embodiments, the non-cationic lipid comprises cholesterol or a derivative thereof of from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle of the invention may comprise cholesterol or a derivative thereof at about 37 mol % of the total lipid present in the particle. In other preferred embodiments, a phospholipid-free lipid particle of the invention may comprise cholesterol or a derivative thereof of from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 32 mol % to about 45 mol %, from about 32 mol % to about 42 mol %, from about 32 mol % to about 40 mol %, from about 34 mol % to about 45 mol %, from about 34 mol % to about 42 mol %, from about 34 mol % to about 40 mol %, or about 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0133] In certain other preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 4 mol % to about 10 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 30 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 7 mol % and cholesterol at about 34 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 3 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 4 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 4 mol % to about 8 mol

%, from about 5 mol % to about 12 mol %, from about 5 mol % to about 9 mol %, from about 6 mol % to about 12 mol %, from about 6 mol % to about 10 mol %, or about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 40 mol %, from about 25 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 28 mol % to about 40 mol %, from about 28 mol % to about 38 mol %, from about 30 mol % to about 38 mol %, from about 32 mol % to about 36 mol %, or about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0134] In further preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 20 mol % and cholesterol at about 20 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0135] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the conjugated lipid that inhibits aggregation of particles may comprise, *e.g.*, one or more of the following: a polyethyleneglycol (PEG)-lipid conjugate, a polyamide

(ATTA)-lipid conjugate, a cationic-polymer-lipid conjugates (CPLs), or mixtures thereof. In one preferred embodiment, the nucleic acid-lipid particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate. In certain embodiments, the PEG-lipid conjugate or ATTA-lipid conjugate is used together with a CPL. The conjugated lipid that inhibits aggregation of particles may comprise a PEG-lipid including, *e.g.*, a PEG-diacylglycerol (DAG), a PEG dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or mixtures thereof. The PEG-DAA conjugate may be PEG-dilauryloxypropyl (C12), a PEG-dimyristyloxypropyl (C14), a PEG-dipalmityloxypropyl (C16), a PEG-distearyloxypropyl (C18), or mixtures thereof.

[0136] Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-*sn*3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed December 31, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Yet additional PEG-lipid conjugates suitable for use in the invention include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol) (2KPEG-DMG). The synthesis of 2KPEG-DMG is described in U.S. Patent No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0137] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.,* from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

[0138] In some embodiments, the conjugated lipid that inhibits aggregation of particles is a CPL that has the formula: A-W-Y, wherein A is a lipid moiety, W is a hydrophilic polymer, and Y is a polycationic moiety. W may be a polymer selected from the group consisting of polyethyleneglycol (PEG), polyamide, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, or combinations thereof, the polymer having a molecular weight of from about 250 to about 7000 daltons. In some embodiments, Y has at least 4 positive charges at a selected pH. In some embodiments, Y may be lysine, arginine, asparagine, glutamine, derivatives thereof, or combinations thereof.

[0139] In certain instances, the conjugated lipid that inhibits aggregation of particles (*e.g.*, PEG-lipid conjugate) may comprise from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 1 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, from about 1.4 mol % to about 1.5 mol %, or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0140] In the lipid particles of the invention, the active agent or therapeutic agent may be fully encapsulated within the lipid portion of the particle, thereby protecting the active agent or therapeutic agent from enzymatic degradation. In preferred embodiments, a SNALP comprising a nucleic acid such as an interfering RNA (*e.g.*, siRNA) is fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the nucleic acid in the SNALP is not substantially degraded after exposure of the particle to a nuclease at 37°C for at least about 20, 30, 45, or 60 minutes. In certain other instances, the nucleic acid in the SNALP is not substantially degraded after incubation of the particle in serum at 37°C for at least about 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In other embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid such as siRNA) is complexed with the lipid portion of the particle. One of the benefits of the formulations of the present invention is that the lipid particle compositions are substantially non-toxic to mammals such as humans.

[0141] The term "fully encapsulated" indicates that the active agent or therapeutic agent in the lipid particle is not significantly degraded after exposure to serum or a nuclease or protease assay that would significantly degrade free DNA, RNA, or protein. In a fully encapsulated system, preferably less than about 25% of the active agent or therapeutic agent in the particle is degraded in a treatment that would normally degrade 100% of free active agent or therapeutic agent, more preferably less than about 10%, and most preferably less than about 5% of the active agent or therapeutic agent in the particle is degraded. In the context of nucleic acid therapeutic agents, full encapsulation may be determined by an Oligreen® assay. Oligreen® is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA or RNA in solution (available from Invitrogen Corporation; Carlsbad, CA). "Fully encapsulated" also indicates that the lipid particles are

serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

[0142] In another aspect, the present invention provides a lipid particle (*e.g.*, SNALP) composition comprising a plurality of lipid particles. In preferred embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid) is fully encapsulated within the lipid portion of the lipid particles (*e.g.*, SNALP), such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, %, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the lipid particles (*e.g.*, SNALP) have the active agent or therapeutic agent encapsulated therein.

[0143] Typically, the lipid particles (*e.g.,* SNALP) of the invention have a lipid:active agent (*e.g.,* lipid:nucleic acid) ratio (mass/mass ratio) of from about 1 to about 100. In some instances, the lipid:active agent (*e.g.,* lipid:nucleic acid) ratio (mass/mass ratio) ranges from about 1 to about 50, from about 2 to about 25, from about 3 to about 20, from about 4 to about 15, or from about 5 to about 10. In preferred embodiments, the lipid particles of the invention have a lipid:active agent (*e.g.,* lipid:nucleic acid) ratio (mass/mass ratio) of from about 5 to about 15, *e.g.,* about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 (or any fraction thereof or range therein).

[0144] Typically, the lipid particles (*e.g.,* SNALP) of the invention have a mean diameter of from about 40 nm to about 150 nm. In preferred embodiments, the lipid particles (*e.g.,* SNALP) of the invention have a mean diameter of from about 40 nm to about 130 nm, from about 40 nm to about 120 nm, from about 40 nm to about 100 nm, from about 50 nm to about 120 nm, from about 50 nm to about 100 nm, from about 60 nm to about 120 nm, from about 60 nm to about 110 nm, from about 60 nm to about 100 nm, from about 60 nm to about 90 nm, from about 60 nm to about 80 nm, from about 70 nm to about 120 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 70 nm to about 90 nm, from about 70 nm to about 80 nm, or less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm (or any fraction thereof or range therein).

[0145] In one specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.,* siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:62" formulation. In a preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is cholesterol, and the conjugated lipid is a PEG-DAA conjugate. Although these are preferred embodiments of the 1:62 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic lipids (including other cholesterol derivatives), and conjugated lipids can be used in the 1:62 formulation as described herein.

[0146] In another specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.,* siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:57" formulation. In one preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 5 mol % to about 9 mol % of the total lipid present in the particle (*e.g.,* about 7.1 mol %) and the cholesterol (or cholesterol derivative) comprises from about 32 mol % to about 37 mol % of the total lipid present in the particle (*e.g.,* about 34.3 mol %), and the PEG-lipid is a PEG-DAA (*e.g.,* PEG-cDMA). In another preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.,* about 20 mol %) and the cholesterol (or cholesterol derivative) comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.,* about 20 mol %), and the PEG-lipid is a PEG-DAA (*e.g.,* PEG-cDMA). Although these are preferred embodiments of the 1:57 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic

lipids (including other phospholipids and other cholesterol derivatives), and conjugated lipids can be used in the 1:57 formulation as described herein.

[0147] In preferred embodiments, the 1:62 SNALP formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-cDMA (or PEG-cDSA), about 61.5 mol % DLinDMA (or XTC2), and about 36.9 mol % cholesterol (or derivative thereof). In other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 7.1 mol % DPPC, and about 34.3 mol % cholesterol (or derivative thereof). In yet other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 20 mol % DPPC, and about 20 mol % cholesterol (or derivative thereof). It should be understood that these SNALP formulations are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the SNALP formulations may vary.

[0148] The present invention also provides a pharmaceutical composition comprising a lipid particle (*e.g.,* SNALP) described herein and a pharmaceutically acceptable carrier.

[0149] In a further aspect, the present invention provides a method for introducing one or more active agents or therapeutic agents (*e.g.,* nucleic acid) into a cell, comprising contacting the cell with a lipid particle (*e.g.,* SNALP) described herein. In one embodiment, the cell is in a mammal and the mammal is a human. In another embodiment, the present invention provides a method for the *in vivo* delivery of one or more active agents or therapeutic agents (*e.g.,* nucleic acid), comprising administering to a mammalian subject a lipid particle (*e.g.,* SNALP) described herein. In a preferred embodiment, the mode of administration includes, but is not limited to, oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. Preferably, the mammalian subject is a human.

[0150] In one embodiment, at least about 5%, 10%, 15%, 20%, or 25% of the total injected dose of the lipid particles (*e.g.,* SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In other embodiments, more than about 20%, 30%, 40% and as much as about 60%, 70% or 80% of the total injected dose of the lipid particles (*e.g.,* SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In certain instances, more than about 10% of a plurality of the particles is present in the plasma of a mammal about 1 hour after administration. In certain other instances, the presence of the lipid particles (*e.g.,*

SNALP) is detectable at least about 1 hour after administration of the particle. In certain embodiments, the presence of an active agent or therapeutic agent such as an interfering RNA (*e.g.,* siRNA) is detectable in cells of the lung, liver, tumor, or at a site of inflammation at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.,* siRNA) is detectable at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In yet other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.,* siRNA) occurs preferentially in tumor cells or in cells at a site of inflammation. In further embodiments, the presence or effect of an active agent or therapeutic agent such as an interfering RNA (*e.g.,* siRNA) in cells at a site proximal or distal to the site of administration or in cells of the lung, liver, or a tumor is detectable at about 12, 24, 48, 72, or 96 hours, or at about 6, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24, 26, or 28 days after administration. In additional embodiments, the lipid particles (*e.g.,* SNALP) of the invention are administered parenterally or intraperitoneally.

[0151] In some embodiments, the lipid particles (*e.g.,* SNALP) of the invention are particularly useful in methods for the therapeutic delivery of one or more nucleic acids comprising an interfering RNA sequence (*e.g.,* siRNA). In particular, it is an object of this invention to provide *in vitro* and *in vivo* methods for treatment of a disease or disorder in a mammal (*e.g.,* a rodent such as a mouse or a primate such as a human, chimpanzee, or monkey) by downregulating or silencing the transcription and/or translation of one or more target nucleic acid sequences or genes of interest. As a non-limiting example, the methods of the invention are useful for *in vivo* delivery of interfering RNA (*e.g.,* siRNA) to the liver and/or tumor of a mammalian subject. In certain embodiments, the disease or disorder is associated with expression and/or overexpression of a gene and expression or overexpression of the gene is reduced by the interfering RNA (*e.g.,* siRNA). In certain other embodiments, a therapeutically effective amount of the lipid particle (*e.g.,* SNALP) may be administered to the mammal. In some instances, an interfering RNA (*e.g.,* siRNA) is formulated into a SNALP, and the particles are administered to patients requiring such treatment. In other instances, cells are removed from a patient, the interfering RNA (*e.g.,* siRNA) is delivered *in vitro* (*e.g.,* using a SNALP described herein), and the cells are reinjected into the patient.

[0152] In an additional aspect, the present invention provides lipid particles (*e.g.,* SNALP) comprising asymmetrical interfering RNA (aiRNA) molecules that silence the expression of a target gene and methods of using such particles to silence target gene expression.

[0153] In one embodiment, the aiRNA molecule comprises a double-stranded (duplex) region of about 10 to about 25 (base paired) nucleotides in length, wherein the aiRNA molecule comprises an antisense strand comprising 5' and 3' overhangs, and wherein the aiRNA molecule is capable of silencing target gene expression.

[0154] In certain instances, the aiRNA molecule comprises a double-stranded (duplex) region of about 12-20, 12-19, 12-18, 13-17, or 14-17 (base paired) nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 (base paired) nucleotides in length. In certain other instances, the 5' and 3' overhangs on the antisense strand comprise sequences that are complementary to the target RNA sequence, and may optionally further comprise nontargeting sequences. In some embodiments, each of the 5' and 3' overhangs on the antisense strand comprises or consists of one, two, three, four, five, six, seven, or more nucleotides.

[0155] In other embodiments, the aiRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0156] In a related aspect, the present invention provides lipid particles (*e.g.,* SNALP) comprising microRNA (miRNA) molecules that silence the expression of a target gene and methods of using such compositions to silence target gene expression.

[0157] In one embodiment, the miRNA molecule comprises about 15 to about 60 nucleotides in length, wherein the miRNA molecule is capable of silencing target gene expression.

[0158] In certain instances, the miRNA molecule comprises about 15-50, 15-40, or 15-30 nucleotides in length, more typically about 15-25 or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In a preferred embodiment, the miRNA molecule is a mature miRNA molecule targeting an RNA sequence of interest.

[0159] In some embodiments, the miRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0160] As such, the lipid particles of the invention (*e.g.,* SNALP) are advantageous and suitable for use in the administration of active agents or therapeutic agents such as nucleic acid (*e.g.,* interfering RNA such as siRNA, aiRNA, and/or miRNA) to a subject (*e.g.*, a mammal such as a human) because they are stable in circulation, of a size required for pharmacodynamic behavior resulting in access to extravascular sites, and are capable of reaching target cell populations.

IV. Active Agents

[0161] Active agents (*e.g.,* therapeutic agents) include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be, *e.g.,* biological, physiological, and/or cosmetic. Active agents may be any type of molecule or compound including, but not limited to, nucleic acids, peptides, polypeptides, small molecules, and mixtures thereof. Non-limiting examples of nucleic acids include interfering RNA molecules (*e.g.,* siRNA, aiRNA, miRNA), antisense oligonucleotides, plasmids, ribozymes, immunostimulatory oligonucleotides, and mixtures thereof. Examples of peptides or polypeptides include, without limitation, antibodies (*e.g.,* polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, Primatized™ antibodies), cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell-surface receptors and their ligands, hormones, and mixtures thereof. Examples of small molecules include, but are not limited to, small organic molecules or compounds such as any conventional agent or drug known to those of skill in the art.

[0162] In some embodiments, the active agent is a therapeutic agent, or a salt or derivative thereof. Therapeutic agent derivatives may be therapeutically active themselves or they may be prodrugs, which become active upon further modification. Thus, in one embodiment, a therapeutic agent derivative retains some or all of the therapeutic activity as compared to the unmodified agent, while in another embodiment, a therapeutic agent derivative is a prodrug that lacks therapeutic activity, but becomes active upon further modification.

A. Nucleic Acids

[0163] In certain embodiments, lipid particles of the present invention are associated with a nucleic acid, resulting in a nucleic acid-lipid particle (*e.g.,* SNALP). In some embodiments, the nucleic acid is fully encapsulated in the lipid particle. As used herein, the term "nucleic acid" includes any oligonucleotide or polynucleotide, with fragments containing up to 60

nucleotides generally termed oligonucleotides, and longer fragments termed polynucleotides. In particular embodiments, oligonucletoides of the invention are from about 15 to about 60 nucleotides in length. Nucleic acid may be administered alone in the lipid particles of the invention, or in combination (*e.g.,* co-administered) with lipid particles of the invention comprising peptides, polypeptides, or small molecules such as conventional drugs.

[0164] In the context of this invention, the terms "polynucleotide" and "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally-occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also include polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake, reduced immunogenicity, and increased stability in the presence of nucleases.

[0165] Oligonucleotides are generally classified as deoxyribooligonucleotides or ribooligonucleotides. A deoxyribooligonucleotide consists of a 5-carbon sugar called deoxyribose joined covalently to phosphate at the 5′ and 3′ carbons of this sugar to form an alternating, unbranched polymer. A ribooligonucleotide consists of a similar repeating structure where the 5-carbon sugar is ribose.

[0166] The nucleic acid that is present in a lipid-nucleic acid particle according to this invention includes any form of nucleic acid that is known. The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Examples of double-stranded DNA are described herein and include, *e.g.,* structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA are described herein and include, *e.g.,* siRNA and other RNAi agents such as aiRNA and pre-miRNA. Single-stranded nucleic acids include, *e.g.,* antisense oligonucleotides, ribozymes, mature miRNA, and triplex-forming oligonucleotides.

[0167] Nucleic acids of the invention may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to about 100,000 nucleotide residues in length. In particular embodiments, oligonucleotides may range from about 10 to about 100 nucleotides in length. In various related embodiments, oligonucleotides, both single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 60 nucleotides, from about 15 to

about 60 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, or from about 20 to about 30 nucleotides in length.

[0168] In particular embodiments, an oligonucleotide (or a strand thereof) of the invention specifically hybridizes to or is complementary to a target polynucleotide sequence. The terms "specifically hybridizable" and "complementary" as used herein indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. In preferred embodiments, an oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target sequence interferes with the normal function of the target sequence to cause a loss of utility or expression therefrom, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.,* under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or, in the case of *in vitro* assays, under conditions in which the assays are conducted. Thus, the oligonucleotide may include 1, 2, 3, or more base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

1. siRNA

[0169] The siRNA component of the nucleic acid-lipid particles of the present invention is capable of silencing the expression of a target gene of interest. Each strand of the siRNA duplex is typically about 15 to about 60 nucleotides in length, preferably about 15 to about 30 nucleotides in length. In certain embodiments, the siRNA comprises at least one modified nucleotide. The modified siRNA is generally less immunostimulatory than a corresponding unmodified siRNA sequence and retains RNAi activity against the target gene of interest. In some embodiments, the modified siRNA contains at least one 2'OMe purine or pyrimidine nucleotide such as a 2'OMe-guanosine, 2'OMe-uridine, 2'OMe-adenosine, and/or 2'OMe-cytosine nucleotide. In preferred embodiments, one or more of the uridine and/or guanosine nucleotides are modified. The modified nucleotides can be present in one strand (*i.e.,* sense or antisense) or both strands of the siRNA. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell,* 107:309 (2001)), or may lack overhangs (*i.e.*, have blunt ends).

[0170] The modified siRNA generally comprises from about 1% to about 100% (*e.g.,* about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%,

19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region of the siRNA duplex. In certain embodiments, one, two, three, four, five, six, seven, eight, nine, ten, or more of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0171] In some embodiments, less than about 25% (*e.g.,* less than about 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0172] In other embodiments, from about 1% to about 25% (*e.g.,* from about 1%-25%, 2%-25%, 3%-25%, 4%-25%, 5%-25%, 6%-25%, 7%-25%, 8%-25%, 9%-25%, 10%-25%, 11%-25%, 12%-25%, 13%-25%, 14%-25%, 15%-25%, 16%-25%, 17%-25%, 18%-25%, 19%-25%, 20%-25%, 21%-25%, 22%-25%, 23%-25%, 24%-25%, *etc.*) or from about 1% to about 20% (*e.g.,* from about 1%-20%, 2%-20%, 3%-20%, 4%-20%, 5%-20%, 6%-20%, 7%-20%, 8%-20%, 9%-20%, 10%-20%, 11%-20%, 12%-20%, 13%-20%, 14%-20%, 15%-20%, 16%-20%, 17%-20%, 18%-20%, 19%-20%, 1%-19%, 2%-19%, 3%-19%, 4%-19%, 5%-19%, 6%-19%, 7%-19%, 8%-19%, 9%-19%, 10%-19%, 11%-19%, 12%-19%, 13%-19%, 14%-19%, 15%-19%, 16%-19%, 17%-19%, 18%-19%, 1%-18%, 2%-18%, 3%-18%, 4%-18%, 5%-18%, 6%-18%, 7%-18%, 8%-18%, 9%-18%, 10%-18%, 11%-18%, 12%-18%, 13%-18%, 14%-18%, 15%-18%, 16%-18%, 17%-18%, 1%-17%, 2%-17%, 3%-17%, 4%-17%, 5%-17%, 6%-17%, 7%-17%, 8%-17%, 9%-17%, 10%-17%, 11%-17%, 12%-17%, 13%-17%, 14%-17%, 15%-17%, 16%-17%, 1%-16%, 2%-16%, 3%-16%, 4%-16%, 5%-16%, 6%-16%, 7%-16%, 8%-16%, 9%-16%, 10%-16%, 11%-16%, 12%-16%, 13%-16%, 14%-16%, 15%-16%, 1%-15%, 2%-15%, 3%-15%, 4%-15%, 5%-15%, 6%-15%, 7%-15%, 8%-15%, 9%-15%, 10%-15%, 11%-15%, 12%-15%, 13%-15%, 14%-15%, *etc.*) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0173] In further embodiments, *e.g.,* when one or both strands of the siRNA are selectively modified at uridine and/or guanosine nucleotides, the resulting modified siRNA can comprise less than about 30% modified nucleotides (*e.g.,* less than about 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% modified nucleotides) or from about 1% to about 30% modified nucleotides (*e.g.,* from about 1%-30%, 2%-30%, 3%-30%, 4%-30%, 5%-30%, 6%-30%, 7%-30%, 8%-30%, 9%-30%, 10%-30%, 11%-30%, 12%-30%, 13%-30%, 14%-30%, 15%-30%, 16%-30%, 17%-30%, 18%-30%, 19%-30%, 20%-30%, 21%-30%, 22%-

30%, 23%-30%, 24%-30%, 25%-30%, 26%-30%, 27%-30%, 28%-30%, or 29%-30% modified nucleotides).

a. Selection of siRNA Sequences

[0174] Suitable siRNA sequences can be identified using any means known in the art. Typically, the methods described in Elbashir *et al.*, *Nature,* 411:494-498 (2001) and Elbashir *et al.*, *EMBO J.,* 20:6877-6888 (2001) are combined with rational design rules set forth in Reynolds *et al.*, *Nature Biotech.,* 22(3):326-330 (2004).

[0175] Generally, the nucleotide sequence 3' of the AUG start codon of a transcript from the target gene of interest is scanned for dinucleotide sequences (*e.g.*, AA, NA, CC, GG, or UU, wherein N = C, G, or U) (*see, e.g.*, Elbashir *et al.*, *EMBO J.,* 20:6877-6888 (2001)). The nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences (*i.e.*, a target sequence or a sense strand sequence). Typically, the 19, 21, 23, 25, 27, 29, 31, 33, 35, or more nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences. In some embodiments, the dinucleotide sequence is an AA or NA sequence and the 19 nucleotides immediately 3' to the AA or NA dinucleotide are identified as potential siRNA sequences. siRNA sequences are usually spaced at different positions along the length of the target gene. To further enhance silencing efficiency of the siRNA sequences, potential siRNA sequences may be analyzed to identify sites that do not contain regions of homology to other coding sequences, *e.g.,* in the target cell or organism. For example, a suitable siRNA sequence of about 21 base pairs typically will not have more than 16-17 contiguous base pairs of homology to coding sequences in the target cell or organism. If the siRNA sequences are to be expressed from an RNA Pol III promoter, siRNA sequences lacking more than 4 contiguous A's or T's are selected.

[0176] Once a potential siRNA sequence has been identified, a complementary sequence (*i.e.*, an antisense strand sequence) can be designed. A potential siRNA sequence can also be analyzed using a variety of criteria known in the art. For example, to enhance their silencing efficiency, the siRNA sequences may be analyzed by a rational design algorithm to identify sequences that have one or more of the following features: (1) G/C content of about 25% to about 60% G/C; (2) at least 3 A/Us at positions 15-19 of the sense strand; (3) no internal repeats; (4) an A at position 19 of the sense strand; (5) an A at position 3 of the sense strand; (6) a U at position 10 of the sense strand; (7) no G/C at position 19 of the sense strand; and (8) no G at position 13 of the sense strand. siRNA design tools that incorporate algorithms that assign suitable values of each of these features and are useful for selection of siRNA can

be found at, *e.g.*, http://boz094.ust.hk/RNAi/siRNA. One of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may be selected for further analysis and testing as potential siRNA sequences.

[0177] Additionally, potential siRNA sequences with one or more of the following criteria can often be eliminated as siRNA: (1) sequences comprising a stretch of 4 or more of the same base in a row; (2) sequences comprising homopolymers of Gs (*i.e.*, to reduce possible non-specific effects due to structural characteristics of these polymers; (3) sequences comprising triple base motifs (*e.g.*, GGG, CCC, AAA, or TTT); (4) sequences comprising stretches of 7 or more G/Cs in a row; and (5) sequences comprising direct repeats of 4 or more bases within the candidates resulting in internal fold-back structures. However, one of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may still be selected for further analysis and testing as potential siRNA sequences.

[0178] In some embodiments, potential siRNA sequences may be further analyzed based on siRNA duplex asymmetry as described in, *e.g.,* Khvorova *et al., Cell*, 115:209-216 (2003); and Schwarz *et al.*, *Cell*, 115:199-208 (2003). In other embodiments, potential siRNA sequences may be further analyzed based on secondary structure at the target site as described in, *e.g.,* Luo *et al.*, *Biophys. Res. Commun.*, 318:303-310 (2004). For example, secondary structure at the target site can be modeled using the Mfold algorithm (available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) to select siRNA sequences which favor accessibility at the target site where less secondary structure in the form of base-pairing and stem-loops is present.

[0179] Once a potential siRNA sequence has been identified, the sequence can be analyzed for the presence of any immunostimulatory properties, *e.g.,* using an *in vitro* cytokine assay or an *in vivo* animal model. Motifs in the sense and/or antisense strand of the siRNA sequence such as GU-rich motifs (*e.g.,* 5'-GU-3', 5'-UGU-3', 5'-GUGU-3', 5'-UGUGU-3', *etc.*) can also provide an indication of whether the sequence may be immunostimulatory. Once an siRNA molecule is found to be immunostimulatory, it can then be modified to decrease its immunostimulatory properties as described herein. As a non-limiting example, an siRNA sequence can be contacted with a mammalian responder cell under conditions such that the cell produces a detectable immune response to determine whether the siRNA is an immunostimulatory or a non-immunostimulatory siRNA. The mammalian responder cell may be from a naïve mammal (*i.e.*, a mammal that has not previously been in contact with the gene product of the siRNA sequence). The mammalian responder cell may be, *e.g.*, a peripheral blood mononuclear cell (PBMC), a macrophage, and the like. The detectable

immune response may comprise production of a cytokine or growth factor such as, *e.g.*, TNF-α, IFN-α, IFN-β, IFN-γ, IL-6, IL-12, or a combination thereof. An siRNA molecule identified as being immunostimulatory can then be modified to decrease its immunostimulatory properties by replacing at least one of the nucleotides on the sense and/or antisense strand with modified nucleotides. For example, less than about 30% (*e.g.,* less than about 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA duplex can be replaced with modified nucleotides such as 2'OMe nucleotides. The modified siRNA can then be contacted with a mammalian responder cell as described above to confirm that its immunostimulatory properties have been reduced or abrogated.

[0180] Suitable *in vitro* assays for detecting an immune response include, but are not limited to, the double monoclonal antibody sandwich immunoassay technique of David *et al.* (U.S. Patent No. 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide *et al.*, in Kirkham and Hunter, eds., *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh (1970)); the "Western blot" method of Gordon *et al.* (U.S. Patent No. 4,452,901); immunoprecipitation of labeled ligand (Brown *et al.*, *J. Biol. Chem.,* 255:4980-4983 (1980)); enzyme-linked immunosorbent assays (ELISA) as described, for example, by Raines *et al.*, *J. Biol. Chem.,* 257:5154-5160 (1982); immunocytochemical techniques, including the use of fluorochromes (Brooks *et al.*, *Clin. Exp. Immunol.,* 39:477 (1980)); and neutralization of activity (Bowen-Pope *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:2396-2400 (1984)). In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S. Patent Nos. 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0181] A non-limiting example of an *in vivo* model for detecting an immune response includes an *in vivo* mouse cytokine induction assay as described in, *e.g.,* Judge *et al.*, *Mol. Ther.*, 13:494-505 (2006). In certain embodiments, the assay that can be performed as follows: (1) siRNA can be administered by standard intravenous injection in the lateral tail vein; (2) blood can be collected by cardiac puncture about 6 hours after administration and processed as plasma for cytokine analysis; and (3) cytokines can be quantified using sandwich ELISA kits according to the manufacturer's instructions (*e.g.,* mouse and human IFN-α (PBL Biomedical; Piscataway, NJ); human IL-6 and TNF-α (eBioscience; San Diego, CA); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences; San Diego, CA)).

[0182] Monoclonal antibodies that specifically bind cytokines and growth factors are commercially available from multiple sources and can be generated using methods known in

the art (*see*, *e.g.*, Kohler *et al.*, *Nature*, 256: 495-497 (1975) and Harlow and Lane, ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publication, New York (1999)). Generation of monoclonal antibodies has been previously described and can be accomplished by any means known in the art (Buhring *et al.*, in Hybridoma, Vol. 10, No. 1, pp. 77-78 (1991)). In some methods, the monoclonal antibody is labeled (*e.g.*, with any composition detectable by spectroscopic, photochemical, biochemical, electrical, optical, or chemical means) to facilitate detection.

b. Generating siRNA Molecules

[0183] siRNA can be provided in several forms including, *e.g.*, as one or more isolated small-interfering RNA (siRNA) duplexes, as longer double-stranded RNA (dsRNA), or as siRNA or dsRNA transcribed from a transcriptional cassette in a DNA plasmid. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell*, 107:309 (2001), or may lack overhangs (*i.e.*, to have blunt ends).

[0184] An RNA population can be used to provide long precursor RNAs, or long precursor RNAs that have substantial or complete identity to a selected target sequence can be used to make the siRNA. The RNAs can be isolated from cells or tissue, synthesized, and/or cloned according to methods well known to those of skill in the art. The RNA can be a mixed population (obtained from cells or tissue, transcribed from cDNA, subtracted, selected, *etc.*), or can represent a single target sequence. RNA can be naturally occurring (*e.g.*, isolated from tissue or cell samples), synthesized *in vitro (e.g.,* using T7 or SP6 polymerase and PCR products or a cloned cDNA), or chemically synthesized.

[0185] To form a long dsRNA, for synthetic RNAs, the complement is also transcribed *in vitro* and hybridized to form a dsRNA. If a naturally occuring RNA population is used, the RNA complements are also provided (*e.g.*, to form dsRNA for digestion by *E. coli* RNAse III or Dicer), *e.g.*, by transcribing cDNAs corresponding to the RNA population, or by using RNA polymerases. The precursor RNAs are then hybridized to form double stranded RNAs for digestion. The dsRNAs can be directly administered to a subject or can be digested *in vitro* prior to administration.

[0186] Methods for isolating RNA, synthesizing RNA, hybridizing nucleic acids, making and screening cDNA libraries, and performing PCR are well known in the art (*see, e.g.,* Gubler and Hoffman, *Gene*, 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*), as are PCR methods (*see,* U.S. Patent Nos. 4,683,195 and 4,683,202; *PCR Protocols:*

A Guide to Methods and Applications (Innis *et al.*, eds, 1990)). Expression libraries are also well known to those of skill in the art. Additional basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0187] Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules of the invention can be synthesized using any of a variety of techniques known in the art, such as those described in Usman *et al.*, *J. Am. Chem. Soc.*, 109:7845 (1987); Scaringe *et al.*, *Nucl. Acids Res.*, 18:5433 (1990); Wincott *et al.*, *Nucl. Acids Res.*, 23:2677-2684 (1995); and Wincott *et al., Methods Mol. Bio.*, 74:59 (1997). The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example, small scale syntheses can be conducted on an Applied Biosystems synthesizer using a 0.2 µmol scale protocol. Alternatively, syntheses at the 0.2 µmol scale can be performed on a 96-well plate synthesizer from Protogene (Palo Alto, CA). However, a larger or smaller scale of synthesis is also within the scope of this invention. Suitable reagents for oligonucleotide synthesis, methods for RNA deprotection, and methods for RNA purification are known to those of skill in the art.

[0188] siRNA molecules can also be synthesized via a tandem synthesis technique, wherein both strands are synthesized as a single continuous oligonucleotide fragment or strand separated by a cleavable linker that is subsequently cleaved to provide separate fragments or strands that hybridize to form the siRNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siRNA can be readily adapted to both multiwell/multiplate synthesis platforms as well as large scale synthesis platforms employing batch reactors, synthesis columns, and the like. Alternatively, siRNA molecules can be assembled from two distinct oligonucleotides, wherein one oligonucleotide comprises the sense strand and the other comprises the antisense strand of the siRNA. For example, each strand can be synthesized separately and joined together by hybridization or ligation following synthesis and/or deprotection. In certain other instances, siRNA molecules can be synthesized as a single continuous oligonucleotide fragment, where the self-complementary sense and antisense regions hybridize to form an siRNA duplex having hairpin secondary structure.

c. Modifying siRNA Sequences

[0189] In certain aspects, siRNA molecules comprise a duplex having two strands and at least one modified nucleotide in the double-stranded region, wherein each strand is about 15 to about 60 nucleotides in length. Advantageously, the modified siRNA is less immunostimulatory than a corresponding unmodified siRNA sequence, but retains the capability of silencing the expression of a target sequence. In preferred embodiments, the degree of chemical modifications introduced into the siRNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the siRNA and retention of RNAi activity. As a non-limiting example, an siRNA molecule that targets a gene of interest can be minimally modified (*e.g.,* less than about 30%, 25%, 20%, 15%, 10%, or 5% modified) at selective uridine and/or guanosine nucleotides within the siRNA duplex to eliminate the immune response generated by the siRNA while retaining its capability to silence target gene expression.

[0190] Examples of modified nucleotides suitable for use in the invention include, but are not limited to, ribonucleotides having a 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl group. Modified nucleotides having a Northern conformation such as those described in, *e.g.,* Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag Ed. (1984), are also suitable for use in siRNA molecules. Such modified nucleotides include, without limitation, locked nucleic acid (LNA) nucleotides (*e.g.,* 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides), 2'-O-(2-methoxyethyl) (MOE) nucleotides, 2'-methyl-thio-ethyl nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy-2'-chloro (2'Cl) nucleotides, and 2'-azido nucleotides. In certain instances, the siRNA molecules described herein include one or more G-clamp nucleotides. A G-clamp nucleotide refers to a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine nucleotide within a duplex (*see, e.g.,* Lin *et al., J. Am. Chem. Soc.,* 120:8531-8532 (1998)). In addition, nucleotides having a nucleotide base analog such as, for example, C-phenyl, C-naphthyl, other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole (*see, e.g.,* Loakes, *Nucl. Acids Res.,* 29:2437-2447 (2001)) can be incorporated into siRNA molecules.

[0191] In certain embodiments, siRNA molecules may further comprise one or more chemical modifications such as terminal cap moieties, phosphate backbone modifications, and the like. Examples of terminal cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4',5'-methylene nucleotides, 1-(β-D-erythrofuranosyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α-nucleotides, modified base nucleotides, *threo*-pentofuranosyl nucleotides, acyclic 3',4'-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3'-3'-inverted nucleotide moieties, 3'-3'-inverted abasic moieties, 3'-2'-inverted nucleotide moieties, 3'-2'-inverted abasic moieties, 5'-5'-inverted nucleotide moieties, 5'-5'-inverted abasic moieties, 3'-5'-inverted deoxy abasic moieties, 5'-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, 6-aminohexyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3'-phosphoramidate, 5'-phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 5'-amino, 3'-phosphorothioate, 5'-phosphorothioate, phosphorodithioate, and bridging or non-bridging methylphosphonate or 5'-mercapto moieties (*see, e.g.,* U.S. Patent No. 5,998,203; Beaucage *et al.*, *Tetrahedron* 49:1925 (1993)). Non-limiting examples of phosphate backbone modifications (*i.e.,* resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and alkylsilyl substitutions (*see, e.g.,* Hunziker *et al.*, *Nucleic Acid Analogues: Synthesis and Properties*, in *Modern Synthetic Methods*, VCH, 331-417 (1995); Mesmaeker *et al.*, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39 (1994)). Such chemical modifications can occur at the 5'-end and/or 3'-end of the sense strand, antisense strand, or both strands of the siRNA. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0192] In some embodiments, the sense and/or antisense strand of the siRNA molecule can further comprise a 3'-terminal overhang having about 1 to about 4 (*e.g.,* 1, 2, 3, or 4) 2'-deoxy ribonucleotides and/or any combination of modified and unmodified nucleotides. Additional examples of modified nucleotides and types of chemical modifications that can be introduced into siRNA molecules are described, *e.g.,* in UK Patent No. GB 2,397,818 B and

U.S. Patent Publication Nos. 20040192626, 20050282188, and 20070135372, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0193] The siRNA molecules described herein can optionally comprise one or more non-nucleotides in one or both strands of the siRNA. As used herein, the term "non-nucleotide" refers to any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base such as adenosine, guanine, cytosine, uracil, or thymine and therefore lacks a base at the 1'-position.

[0194] In other embodiments, chemical modification of the siRNA comprises attaching a conjugate to the siRNA molecule. The conjugate can be attached at the 5' and/or 3'-end of the sense and/or antisense strand of the siRNA via a covalent attachment such as, *e.g.,* a biodegradable linker. The conjugate can also be attached to the siRNA, *e.g.,* through a carbamate group or other linking group (*see, e.g.,* U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). In certain instances, the conjugate is a molecule that facilitates the delivery of the siRNA into a cell. Examples of conjugate molecules suitable for attachment to siRNA include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (*e.g.,* folic acid, folate analogs and derivatives thereof), sugars (*e.g.,* galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, *etc.*), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof (*see, e.g.,* U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Patent No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule, oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Yet other examples include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidininium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples include the hydrophobic group, membrane active compound, cell penetrating compound, cell targeting signal, interaction modifier, and steric stabilizer conjugate molecules described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described in U.S. Patent Publication No. 20050239739. The type of conjugate used and the extent of

conjugation to the siRNA molecule can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the siRNA while retaining RNAi activity. As such, one skilled in the art can screen siRNA molecules having various conjugates attached thereto to identify ones having improved properties and full RNAi activity using any of a variety of well-known *in vitro* cell culture or *in vivo* animal models. The disclosures of the above-described patent documents are herein incorporated by reference in their entirety for all purposes.

d. Target Genes

[0195] The siRNA component of the nucleic acid-lipid particles described herein can be used to downregulate or silence the translation (*i.e.*, expression) of a gene of interest. Genes of interest include, but are not limited to, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders (*e.g.*, liver diseases and disorders), genes associated with tumorigenesis and cell transformation (*e.g.*, cancer), angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0196] Genes associated with viral infection and survival include those expressed by a virus in order to bind, enter, and replicate in a cell. Of particular interest are viral sequences associated with chronic viral diseases. Viral sequences of particular interest include sequences of Filoviruses such as Ebola virus and Marburg virus (*see, e.g.,* Geisbert *et al.*, *J. Infect. Dis.*, 193:1650-1657 (2006)); Arenaviruses such as Lassa virus, Junin virus, Machupo virus, Guanarito virus, and Sabia virus (Buchmeier *et al.*, *Arenaviridae*: the viruses and their replication, In: FIELDS VIROLOGY, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia, (2001)); Influenza viruses such as Influenza A, B, and C viruses, (*see, e.g.,* Steinhauer *et al.*, *Annu Rev Genet.,* 36:305-332 (2002); and Neumann *et al.*, *J Gen Virol.*, 83:2635-2662 (2002)); Hepatitis viruses (*see, e.g.,* Hamasaki *et al.*, *FEBS Lett.*, 543:51 (2003); Yokota *et al.*, *EMBO Rep.*, 4:602 (2003); Schlomai *et al.*, *Hepatology,* 37:764 (2003); Wilson *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2783 (2003); Kapadia *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2014 (2003); and FIELDS VIROLOGY, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia (2001)); Human Immunodeficiency Virus (HIV) (Banerjea *et al.*, *Mol. Ther.*, 8:62 (2003); Song *et al.*, *J. Virol.*, 77:7174 (2003); Stephenson, *JAMA,* 289:1494 (2003); Qin *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:183 (2003)); Herpes viruses (Jia *et al.*, *J. Virol.*, 77:3301 (2003)); and Human Papilloma Viruses (HPV) (Hall *et al.*, *J. Virol.*, 77:6066 (2003); Jiang *et al.*, *Oncogene,* 21:6041 (2002)).

[0197] Exemplary Filovirus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding structural proteins (*e.g.*, VP30, VP35, nucleoprotein (NP), polymerase protein (L-pol)) and membrane-associated proteins (*e.g.*, VP40, glycoprotein (GP), VP24). Complete genome sequences for Ebola virus are set forth in, *e.g.*, Genbank Accession Nos. NC_002549; AY769362; NC_006432; NC_004161; AY729654; AY354458; AY142960; AB050936; AF522874; AF499101; AF272001; and AF086833. Ebola virus VP24 sequences are set forth in, *e.g.*, Genbank Accession Nos. U77385 and AY058897. Ebola virus L-pol sequences are set forth in, *e.g.*, Genbank Accession No. X67110. Ebola virus VP40 sequences are set forth in, *e.g.*, Genbank Accession No. AY058896. Ebola virus NP sequences are set forth in, *e.g.*, Genbank Accession No. AY058895. Ebola virus GP sequences are set forth in, *e.g.*, Genbank Accession No. AY058898; Sanchez *et al.*, *Virus Res.*, 29:215-240 (1993); Will *et al.*, *J. Virol.*, 67:1203-1210 (1993); Volchkov *et al.*, *FEBS Lett.*, 305:181-184 (1992); and U.S. Patent No. 6,713,069. Additional Ebola virus sequences are set forth in, *e.g.*, Genbank Accession Nos. L11365 and X61274. Complete genome sequences for Marburg virus are set forth in, *e.g.*, Genbank Accession Nos. NC_001608; AY430365; AY430366; and AY358025. Marburg virus GP sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005734; AF005733; and AF005732. Marburg virus VP35 sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005731 and AF005730. Additional Marburg virus sequences are set forth in, *e.g.*, Genbank Accession Nos. X64406; Z29337; AF005735; and Z12132. Non-limiting examples of siRNA molecules targeting Ebola virus and Marburg virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070135370, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0198] Exemplary Influenza virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding nucleoprotein (NP), matrix proteins (M1 and M2), nonstructural proteins (NS1 and NS2), RNA polymerase (PA, PB1, PB2), neuraminidase (NA), and haemagglutinin (HA). Influenza A NP sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004522; AY818138; AB166863; AB188817; AB189046; AB189054; AB189062; AY646169; AY646177; AY651486; AY651493; AY651494; AY651495; AY651496; AY651497; AY651498; AY651499; AY651500; AY651501; AY651502; AY651503; AY651504; AY651505; AY651506; AY651507; AY651509; AY651528; AY770996; AY790308; AY818138; and AY818140. Influenza A PA sequences are set forth in, *e.g.*, Genbank Accession Nos. AY818132; AY790280; AY646171; AY818132;AY818133; AY646179; AY818134; AY551934; AY651613; AY651610;

AY651620; AY651617; AY651600; AY651611; AY651606; AY651618; AY651608; AY651607; AY651605; AY651609; AY651615; AY651616; AY651640; AY651614; AY651612; AY651621; AY651619; AY770995; and AY724786. Non-limiting examples of siRNA molecules targeting Influenza virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070218122, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0199] Exemplary hepatitis virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences involved in transcription and translation (*e.g.,* En1, En2, X, P) and nucleic acid sequences encoding structural proteins (*e.g.*, core proteins including C and C-related proteins, capsid and envelope proteins including S, M, and/or L proteins, or fragments thereof) (*see*, *e.g.*, FIELDS VIROLOGY, *supra*). Exemplary Hepatits C virus (HCV) nucleic acid sequences that can be silenced include, but are not limited to, the 5'-untranslated region (5'-UTR), the 3'-untranslated region (3'-UTR), the polyprotein translation initiation codon region, the internal ribosome entry site (IRES) sequence, and/or nucleic acid sequences encoding the core protein, the E1 protein, the E2 protein, the p7 protein, the NS2 protein, the NS3 protease/helicase, the NS4A protein, the NS4B protein, the NS5A protein, and/or the NS5B RNA-dependent RNA polymerase. HCV genome sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004102 (HCV genotype 1a), AJ238799 (HCV genotype 1b), NC_009823 (HCV genotype 2), NC_009824 (HCV genotype 3), NC_009825 (HCV genotype 4), NC_009826 (HCV genotype 5), and NC_009827 (HCV genotype 6). Hepatitis A virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001489; Hepatitis B virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_003977; Hepatitis D virus nucleic acid sequence are set forth in, *e.g.*, Genbank Accession No. NC_001653; Hepatitis E virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001434; and Hepatitis G virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001710. Silencing of sequences that encode genes associated with viral infection and survival can conveniently be used in combination with the administration of conventional agents used to treat the viral condition. Non-limiting examples of siRNA molecules targeting hepatitis virus nucleic acid sequences include those described in U.S. Patent Publication Nos. 20060281175, 20050058982, and 20070149470; U.S. Patent No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed March 20, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0200] Genes associated with metabolic diseases and disorders (*e.g.,* disorders in which the liver is the target and liver diseases and disorders) include, for example, genes expressed in dyslipidemia (*e.g.*, liver X receptors such as LXRα and LXRβ (Genback Accession No. NM_007121), farnesoid X receptors (FXR) (Genbank Accession No. NM_005123), sterol-regulatory element binding protein (SREBP), site-1 protease (S1P), 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG coenzyme-A reductase), apolipoprotein B (ApoB) (Genbank Accession No. NM_000384), apolipoprotein CIII (ApoC3) (Genbank Accession Nos. NM_000040 and NG_008949 REGION: 5001..8164), and apolipoprotein E (ApoE) (Genbank Accession Nos. NM_000041 and NG_007084 REGION: 5001..8612)); and diabetes (*e.g.*, glucose 6-phosphatase) (*see, e.g.*, Forman *et al., Cell,* 81:687 (1995); Seol *et al., Mol. Endocrinol.,* 9:72 (1995), Zavacki *et al., Proc. Natl. Acad. Sci. USA*, 94:7909 (1997); Sakai *et al., Cell,* 85:1037-1046 (1996); Duncan *et al., J. Biol. Chem.*, 272:12778-12785 (1997); Willy *et al., Genes Dev.,* 9:1033-1045 (1995); Lehmann *et al., J. Biol. Chem.*, 272:3137-3140 (1997); Janowski *et al., Nature,* 383:728-731 (1996); and Peet *et al., Cell,* 93:693-704 (1998)). One of skill in the art will appreciate that genes associated with metabolic diseases and disorders (*e.g.*, diseases and disorders in which the liver is a target and liver diseases and disorders) include genes that are expressed in the liver itself as well as and genes expressed in other organs and tissues. Silencing of sequences that encode genes associated with metabolic diseases and disorders can conveniently be used in combination with the administration of conventional agents used to treat the disease or disorder. Non-limiting examples of siRNA molecules targeting the ApoB gene include those described in U.S. Patent Publication No. 20060134189, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the ApoC3 gene include those described in U.S. Provisional Application No. 61/147,235, filed January 26, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0201] Examples of gene sequences associated with tumorigenesis and cell transformation (*e.g.*, cancer or other neoplasia) include mitotic kinesins such as Eg5 (KSP, KIF11; Genbank Accession No. NM_004523); serine/threonine kinases such as polo-like kinase 1 (PLK-1) (Genbank Accession No. NM_005030; Barr *et al., Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)); tyrosine kinases such as WEE1 (Genbank Accession Nos. NM_003390 and NM_001143976); inhibitors of apoptosis such as XIAP (Genbank Accession No. NM_001167); COP9 signalosome subunits such as CSN1, CSN2, CSN3, CSN4, CSN5

(JAB1; Genbank Accession No. NM_006837); CSN6, CSN7A, CSN7B, and CSN8; ubiquitin ligases such as COP1 (RFWD2; Genbank Accession Nos. NM_022457 and NM_001001740); and histone deacetylases such as HDAC1, HDAC2 (Genbank Accession No. NM_001527), HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, *etc.* Non-limiting examples of siRNA molecules targeting the Eg5 and XIAP genes include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the PLK-1 gene include those described in U.S. Patent Publication Nos. 20050107316 and 20070265438; and U.S. Patent Application No. 12/343,342, filed December 23, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of siRNA molecules targeting the CSN5 gene include those described in U.S. Provisional Application No. 61/045,251, filed April 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0202] Additional examples of gene sequences associated with tumorigenesis and cell transformation include translocation sequences such as MLL fusion genes, BCR-ABL (Wilda *et al.*, *Oncogene*, 21:5716 (2002); Scherr *et al.*, *Blood,* 101:1566 (2003)), TEL-AML1, EWS-FLI1, TLS-FUS, PAX3-FKHR, BCL-2, AML1-ETO, and AML1-MTG8 (Heidenreich *et al.*, *Blood,* 101:3157 (2003)); overexpressed sequences such as multidrug resistance genes (Nieth *et al.*, *FEBS Lett.*, 545:144 (2003); Wu *et al*, *Cancer Res.* 63:1515 (2003)), cyclins (Li *et al.*, *Cancer Res.*, 63:3593 (2003); Zou *et al.*, *Genes Dev.,* 16:2923 (2002)), beta-catenin (Verma *et al.*, *Clin Cancer Res.*, 9:1291 (2003)), telomerase genes (Kosciolek *et al.*, *Mol Cancer Ther.*, 2:209 (2003)), c-MYC, N-MYC, BCL-2, growth factor receptors (*e.g.,* EGFR/ErbB1 (Genbank Accession Nos. NM_005228, NM_201282, NM_201283, and NM_201284; *see also,* Nagy *et al. Exp. Cell Res.,* 285:39-49 (2003), ErbB2/HER-2 (Genbank Accession Nos. NM_004448 and NM_001005862), ErbB3 (Genbank Accession Nos. NM_001982 and NM_001005915), and ErbB4 (Genbank Accession Nos. NM_005235 and NM_001042599); and mutated sequences such as RAS (reviewed in Tuschl and Borkhardt, *Mol. Interventions*, 2:158 (2002)). Non-limiting examples of siRNA molecules targeting the EGFR gene include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0203] Silencing of sequences that encode DNA repair enzymes find use in combination with the administration of chemotherapeutic agents (Collis *et al.*, *Cancer Res.*, 63:1550 (2003)). Genes encoding proteins associated with tumor migration are also target sequences

of interest, for example, integrins, selectins, and metalloproteinases. The foregoing examples are not exclusive. Those of skill in the art will understand that any whole or partial gene sequence that facilitates or promotes tumorigenesis or cell transformation, tumor growth, or tumor migration can be included as a template sequence.

[0204] Angiogenic genes are able to promote the formation of new vessels. Of particular interest is vascular endothelial growth factor (VEGF) (Reich *et al.*, *Mol. Vis.*, 9:210 (2003)) or VEGFR. siRNA sequences that target VEGFR are set forth in, *e.g.*, GB 2396864; U.S. Patent Publication No. 20040142895; and CA 2456444, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0205] Anti-angiogenic genes are able to inhibit neovascularization. These genes are particularly useful for treating those cancers in which angiogenesis plays a role in the pathological development of the disease. Examples of anti-angiogenic genes include, but are not limited to, endostatin (*see, e.g.,* U.S. Patent No. 6,174,861), angiostatin (*see, e.g.*, U.S. Patent No. 5,639,725), and VEGFR2 (*see, e.g.,* Decaussin *et al.*, *J. Pathol.*, 188: 369-377 (1999)), the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0206] Immunomodulator genes are genes that modulate one or more immune responses. Examples of immunomodulator genes include, without limitation, cytokines such as growth factors (*e.g.*, TGF-α, TGF-β, EGF, FGF, IGF, NGF, PDGF, CGF, GM-CSF, SCF, *etc.*), interleukins (*e.g.*, IL-2, IL-4, IL-12 (Hill *et al.*, *J. Immunol.*, 171:691 (2003)), IL-15, IL-18, IL-20, *etc.*), interferons (*e.g.*, IFN-α, IFN-β, IFN-γ, *etc.*) and TNF. Fas and Fas ligand genes are also immunomodulator target sequences of interest (Song *et al.*, *Nat. Med.,* 9:347 (2003)). Genes encoding secondary signaling molecules in hematopoietic and lymphoid cells are also included in the present invention, for example, Tec family kinases such as Bruton's tyrosine kinase (Btk) (Heinonen *et al.*, *FEBS Lett.,* 527:274 (2002)).

[0207] Cell receptor ligands include ligands that are able to bind to cell surface receptors (*e.g.*, insulin receptor, EPO receptor, G-protein coupled receptors, receptors with tyrosine kinase activity, cytokine receptors, growth factor receptors, *etc.*), to modulate (*e.g.,* inhibit, activate, *etc.*) the physiological pathway that the receptor is involved in (*e.g.*, glucose level modulation, blood cell development, mitogenesis, *etc.*). Examples of cell receptor ligands include, but are not limited to, cytokines, growth factors, interleukins, interferons, erythropoietin (EPO), insulin, glucagon, G-protein coupled receptor ligands, *etc.* Templates coding for an expansion of trinucleotide repeats (*e.g.*, CAG repeats) find use in silencing pathogenic sequences in neurodegenerative disorders caused by the expansion of

trinucleotide repeats, such as spinobulbular muscular atrophy and Huntington's Disease (Caplen *et al.*, *Hum. Mol. Genet.*, 11:175 (2002)).

[0208] In addition to its utility in silencing the expression of any of the above-described genes for therapeutic purposes, the siRNA described herein are also useful in research and development applications as well as diagnostic, prophylactic, prognostic, clinical, and other healthcare applications. As a non-limiting example, the siRNA can be used in target validation studies directed at testing whether a gene of interest has the potential to be a therapeutic target. The siRNA can also be used in target identification studies aimed at discovering genes as potential therapeutic targets.

2. aiRNA

[0209] Like siRNA, asymmetrical interfering RNA (aiRNA) can recruit the RNA-induced silencing complex (RISC) and lead to effective silencing of a variety of genes in mammalian cells by mediating sequence-specific cleavage of the target sequence between nucleotide 10 and 11 relative to the 5' end of the antisense strand (Sun *et al.*, *Nat. Biotech.*, 26:1379-1382 (2008)). Typically, an aiRNA molecule comprises a short RNA duplex having a sense strand and an antisense strand, wherein the duplex contains overhangs at the 3' and 5' ends of the antisense strand. The aiRNA is generally asymmetric because the sense strand is shorter on both ends when compared to the complementary antisense strand. In some aspects, aiRNA molecules may be designed, synthesized, and annealed under conditions similar to those used for siRNA molecules. As a non-limiting example, aiRNA sequences may be selected and generated using the methods described above for selecting siRNA sequences.

[0210] In another embodiment, aiRNA duplexes of various lengths (*e.g.,* about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 base pairs, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 base pairs) may be designed with overhangs at the 3' and 5' ends of the antisense strand to target an mRNA of interest. In certain instances, the sense strand of the aiRNA molecule is about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length. In certain other instances, the antisense strand of the aiRNA molecule is about 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 nucleotides in length.

[0211] In some embodiments, the 5' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.,* "AA", "UU", "dTdT", *etc.*). In other embodiments, the 3' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.,*

"AA", "UU", "dTdT", *etc.*). In certain aspects, the aiRNA molecules described herein may comprise one or more modified nucleotides, *e.g.,* in the double-stranded (duplex) region and/or in the antisense overhangs. As a non-limiting example, aiRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0212] In certain embodiments, aiRNA molecules may comprise an antisense strand which corresponds to the antisense strand of an siRNA molecule, *e.g.,* one of the siRNA molecules described herein. In other embodiments, aiRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.,* genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

3. miRNA

[0213] Generally, microRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. miRNAs are encoded by genes from whose DNA they are transcribed, but miRNAs are not translated into protein (non-coding RNA); instead, each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional mature miRNA. Mature miRNA molecules are either partially or completely complementary to one or more messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression. The identification of miRNA molecules is described, *e.g.,* in Lagos-Quintana *et al.*, *Science*, 294:853-858; Lau *et al.*, *Science*, 294:858-862; and Lee *et al.*, *Science*, 294:862-864.

[0214] The genes encoding miRNA are much longer than the processed mature miRNA molecule. miRNA are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail and processed to short, ~70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha (Denli *et al.*, *Nature*, 432:231-235 (2004)). These pre-miRNA are then processed to mature miRNA in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC) (Bernstein

et al., *Nature*, 409:363-366 (2001). Either the sense strand or antisense strand of DNA can function as templates to give rise to miRNA.

[0215] When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC complex. This strand is known as the guide strand and is selected by the argonaute protein, the catalytically active RNase in the RISC complex, on the basis of the stability of the 5' end (Preall *et al.*, *Curr. Biol.*, 16:530-535 (2006)). The remaining strand, known as the anti-guide or passenger strand, is degraded as a RISC complex substrate (Gregory *et al.*, *Cell*, 123:631-640 (2005)). After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and induce target mRNA degradation and/or translational silencing.

[0216] Mammalian miRNA molecules are usually complementary to a site in the 3' UTR of the target mRNA sequence. In certain instances, the annealing of the miRNA to the target mRNA inhibits protein translation by blocking the protein translation machinery. In certain other instances, the annealing of the miRNA to the target mRNA facilitates the cleavage and degradation of the target mRNA through a process similar to RNA interference (RNAi). miRNA may also target methylation of genomic sites which correspond to targeted mRNA. Generally, miRNA function in association with a complement of proteins collectively termed the miRNP.

[0217] In certain aspects, the miRNA molecules described herein are about 15-100, 15-90, 15-80, 15-75, 15-70, 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In certain other aspects, miRNA molecules may comprise one or more modified nucleotides. As a non-limiting example, miRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0218] In some embodiments, miRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.,* genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0219] In other embodiments, one or more agents that block the activity of a miRNA targeting an mRNA of interest are administered using a lipid particle of the invention (*e.g.,* a

nucleic acid-lipid particle). Examples of blocking agents include, but are not limited to, steric blocking oligonucleotides, locked nucleic acid oligonucleotides, and Morpholino oligonucleotides. Such blocking agents may bind directly to the miRNA or to the miRNA binding site on the target mRNA.

4. Antisense Oligonucleotides

[0220] In one embodiment, the nucleic acid is an antisense oligonucleotide directed to a target gene or sequence of interest. The terms "antisense oligonucleotide" or "antisense" include oligonucleotides that are complementary to a targeted polynucleotide sequence. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence. Antisense RNA oligonucleotides prevent the translation of complementary RNA strands by binding to the RNA. Antisense DNA oligonucleotides can be used to target a specific, complementary (coding or non-coding) RNA. If binding occurs, this DNA/RNA hybrid can be degraded by the enzyme RNase H. In a particular embodiment, antisense oligonucleotides comprise from about 10 to about 60 nucleotides, more preferably from about 15 to about 30 nucleotides. The term also encompasses antisense oligonucleotides that may not be exactly complementary to the desired target gene. Thus, the invention can be utilized in instances where non-target specific-activities are found with antisense, or where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use.

[0221] Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein synthesis by a targeted gene. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (*see,* U.S. Patent Nos. 5,739,119 and 5,759,829). Furthermore, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDR1), ICAM-1, E-selectin, STK-1, striatal GABAA receptor, and human EGF (*see,* Jaskulski *et al.*, *Science*, 240:1544-6 (1988); Vasanthakumar *et al.*, *Cancer Commun.*, 1:225-32 (1989); Peris *et al.*, *Brain Res Mol Brain Res.*, 15;57:310-20 (1998); and U.S. Patent Nos. 5,801,154; 5,789,573; 5,718,709 and 5,610,288). Moreover, antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.,* cancer (*see,* U.S. Patent Nos.

5,747,470; 5,591,317; and 5,783,683). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0222] Methods of producing antisense oligonucleotides are known in the art and can be readily adapted to produce an antisense oligonucleotide that targets any polynucleotide sequence. Selection of antisense oligonucleotide sequences specific for a given target sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense oligonucleotides may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-402 (1997)).

5. Ribozymes

[0223] According to another embodiment of the invention, nucleic acid-lipid particles are associated with ribozymes. Ribozymes are RNA-protein complexes having specific catalytic domains that possess endonuclease activity (*see,* Kim *et al.*, *Proc. Natl. Acad. Sci. USA.*, 84:8788-92 (1987); and Forster *et al.*, *Cell*, 49:211-20 (1987)). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (*see,* Cech *et al.*, *Cell*, 27:487-96 (1981); Michel *et al.*, *J. Mol. Biol.*, 216:585-610 (1990); Reinhold-Hurek *et al.*, *Nature*, 357:173-6 (1992)). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

[0224] At least six basic varieties of naturally-occurring enzymatic RNA molecules are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-

pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

[0225] The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence), or Neurospora VS RNA motif, for example. Specific examples of hammerhead motifs are described in, *e.g.,* Rossi *et al.*, *Nucleic Acids Res.*, 20:4559-65 (1992). Examples of hairpin motifs are described in, *e.g.,* EP 0360257, Hampel *et al.*, *Biochemistry*, 28:4929-33 (1989); Hampel *et al.*, *Nucleic Acids Res.*, 18:299-304 (1990); and U.S. Patent No. 5,631,359. An example of the hepatitis δ virus motif is described in, *e.g.,* Perrotta *et al.*, *Biochemistry*, 31:11843-52 (1992). An example of the RNaseP motif is described in, *e.g.,* Guerrier-Takada *et al.*, *Cell*, 35:849-57 (1983). Examples of the Neurospora VS RNA ribozyme motif is described in, *e.g.,* Saville *et al.*, *Cell*, 61:685-96 (1990); Saville *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:8826-30 (1991); Collins *et al.*, *Biochemistry*, 32:2795-9 (1993). An example of the Group I intron is described in, *e.g.,* U.S. Patent No. 4,987,071. Important characteristics of enzymatic nucleic acid molecules used according to the invention are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus, the ribozyme constructs need not be limited to specific motifs mentioned herein. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0226] Methods of producing a ribozyme targeted to any polynucleotide sequence are known in the art. Ribozymes may be designed as described in, *e.g.,* PCT Publication Nos. WO 93/23569 and WO 94/02595, and synthesized to be tested *in vitro* and/or *in vivo* as described therein. The disclosures of these PCT publications are herein incorporated by reference in their entirety for all purposes.

[0227] Ribozyme activity can be optimized by altering the length of the ribozyme binding arms or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (*see, e.g.,* PCT Publication Nos. WO 92/07065, WO 93/15187, WO 91/03162, and WO 94/13688; EP 92110298.4; and U.S. Patent No. 5,334,711, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules, the disclosures of which are each herein incorporated by reference in their entirety

for all purposes), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

6. Immunostimulatory Oligonucleotides

[0228] Nucleic acids associated with lipid paticles of the present invention may be immunostimulatory, including immunostimulatory oligonucleotides (ISS; single-or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal such as a human. ISS include, *e.g.,* certain palindromes leading to hairpin secondary structures (*see,* Yamamoto *et al.*, *J. Immunol.*, 148:4072-6 (1992)), or CpG motifs, as well as other known ISS features (such as multi-G domains; *see;* PCT Publication No. WO 96/11266, the disclosure of which is herein incorporated by reference in its entirety for all purposes).

[0229] Immunostimulatory nucleic acids are considered to be non-sequence specific when it is not required that they specifically bind to and reduce the expression of a target sequence in order to provoke an immune response. Thus, certain immunostimulatory nucleic acids may comprise a sequence corresponding to a region of a naturally-occurring gene or mRNA, but they may still be considered non-sequence specific immunostimulatory nucleic acids.

[0230] In one embodiment, the immunostimulatory nucleic acid or oligonucleotide comprises at least one CpG dinucleotide. The oligonucleotide or CpG dinucleotide may be unmethylated or methylated. In another embodiment, the immunostimulatory nucleic acid comprises at least one CpG dinucleotide having a methylated cytosine. In one embodiment, the nucleic acid comprises a single CpG dinucleotide, wherein the cytosine in the CpG dinucleotide is methylated. In an alternative embodiment, the nucleic acid comprises at least two CpG dinucleotides, wherein at least one cytosine in the CpG dinucleotides is methylated. In a further embodiment, each cytosine in the CpG dinucleotides present in the sequence is methylated. In another embodiment, the nucleic acid comprises a plurality of CpG dinucleotides, wherein at least one of the CpG dinucleotides comprises a methylated cytosine. Examples of immunostimulatory oligonucleotides suitable for use in the compositions and methods of the present invention are described in PCT Application No. PCT/US08/88676, filed December 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Patent No. 6,406,705, and Raney *et al.*, *J. Pharm. Exper. Ther.*, 298:1185-92 (2001), the disclosures of which are each herein incorporated by reference in their entirety for all purposes. In certain embodiments, the oligonucleotides used in the compositions and

methods of the invention have a phosphodiester ("PO") backbone or a phosphorothioate ("PS") backbone, and/or at least one methylated cytosine residue in a CpG motif.

B. Other Active Agents

[0231] In certain embodiments, the active agent associated with the lipid paticles of the invention may comprise one or more therapeutic proteins, polypeptides, or small organic molecules or compounds. Non-limiting examples of such therapeutically effective agents or drugs include oncology drugs (*e.g.,* chemotherapy drugs, hormonal therapaeutic agents, immunotherapeutic agents, radiotherapeutic agents, *etc.*), lipid-lowering agents, anti-viral drugs, anti-inflammatory compounds, antidepressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs such as anti-arrhythmic agents, hormones, vasoconstrictors, and steroids. These active agents may be administered alone in the lipid particles of the invention, or in combination (*e.g.,* co-administered) with lipid particles of the invention comprising nucleic acid such as interfering RNA.

[0232] Non-limiting examples of chemotherapy drugs include platinum-based drugs (*e.g.,* oxaliplatin, cisplatin, carboplatin, spiroplatin, iproplatin, satraplatin, *etc.*), alkylating agents (*e.g.,* cyclophosphamide, ifosfamide, chlorambucil, busulfan, melphalan, mechlorethamine, uramustine, thiotepa, nitrosoureas, *etc.*), anti-metabolites (*e.g.,* 5-fluorouracil (5-FU), azathioprine, methotrexate, leucovorin, capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, pemetrexed, raltitrexed, *etc.*), plant alkaloids (*e.g.,* vincristine, vinblastine, vinorelbine, vindesine, podophyllotoxin, paclitaxel (taxol), docetaxel, *etc.*), topoisomerase inhibitors (*e.g.,* irinotecan (CPT-11; Camptosar), topotecan, amsacrine, etoposide (VP16), etoposide phosphate, teniposide, *etc.*), antitumor antibiotics (*e.g.,* doxorubicin, adriamycin, daunorubicin, epirubicin, actinomycin, bleomycin, mitomycin, mitoxantrone, plicamycin, *etc.*), tyrosine kinase inhibitors (*e.g.,* gefitinib (Iressa®), sunitinib (Sutent®; SU11248), erlotinib (Tarceva®; OSI-1774), lapatinib (GW572016; GW2016), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), imatinib (Gleevec®; STI571), dasatinib (BMS-354825), leflunomide (SU101), vandetanib (Zactima™; ZD6474), *etc.*), pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and combinations thereof.

[0233] Examples of conventional hormonal therapaeutic agents include, without limitation, steroids (*e.g.,* dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

[0234] Examples of conventional immunotherapeutic agents include, but are not limited to, immunostimulants (*e.g.,* Bacillus Calmette-Guérin (BCG), levamisole, interleukin-2, alpha-interferon, *etc.*), monoclonal antibodies (*e.g.,* anti-CD20, anti-HER2, anti-CD52, anti-HLA-DR, and anti-VEGF monoclonal antibodies), immunotoxins (*e.g.,* anti-CD33 monoclonal antibody-calicheamicin conjugate, anti-CD22 monoclonal antibody-pseudomonas exotoxin conjugate, *etc.*), and radioimmunotherapy (*e.g.,* anti-CD20 monoclonal antibody conjugated to ^{111}In, ^{90}Y, or ^{131}I, *etc.*).

[0235] Examples of conventional radiotherapeutic agents include, but are not limited to, radionuclides such as ^{47}Sc, ^{64}Cu, ^{67}Cu, ^{89}Sr, ^{86}Y, ^{87}Y, ^{90}Y, ^{105}Rh, ^{111}Ag, ^{111}In, ^{117m}Sn, ^{149}Pm, ^{153}Sm, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi, optionally conjugated to antibodies directed against tumor antigens.

[0236] Additional oncology drugs that may be used according to the invention include, but are not limited to, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, bexarotene, biCNU, carmustine, CCNU, celecoxib, cladribine, cyclosporin A, cytosine arabinoside, cytoxan, dexrazoxane, DTIC, estramustine, exemestane, FK506, gemtuzumab-ozogamicin, hydrea, hydroxyurea, idarubicin, interferon, letrozole, leustatin, leuprolide, litretinoin, megastrol, L-PAM, mesna, methoxsalen, mithramycin, nitrogen mustard, pamidronate, Pegademase, pentostatin, porfimer sodium, prednisone, rituxan, streptozocin, STI-571, taxotere, temozolamide, VM-26, toremifene, tretinoin, ATRA, valrubicin, and velban. Other examples of oncology drugs that may be used according to the invention are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors, and camptothecins.

[0237] Non-limiting examples of lipid-lowering agents for treating a lipid disease or disorder associated with elevated triglycerides, cholesterol, and/or glucose include statins, fibrates, ezetimibe, thiazolidinediones, niacin, beta-blockers, nitroglycerin, calcium antagonists, fish oil, and mixtures thereof.

[0238] Examples of anti-viral drugs include, but are not limited to, abacavir, aciclovir, acyclovir, adefovir, amantadine, amprenavir, arbidol, atazanavir, atripla, cidofovir, combivir, darunavir, delavirdine, didanosine, docosanol, edoxudine, efavirenz, emtricitabine, enfuvirtide, entecavir, entry inhibitors, famciclovir, fixed dose combinations, fomivirsen, fosamprenavir, foscarnet, fosfonet, fusion inhibitors, ganciclovir, ibacitabine, imunovir, idoxuridine, imiquimod, indinavir, inosine, integrase inhibitors, interferon type III (*e.g.,* IFN-λ molecules such as IFN-λ1, IFN-λ2, and IFN-λ3), interferon type II (*e.g.,* IFN-γ), interferon type I (*e.g.,* IFN-α such as PEGylated IFN-α, IFN-β, IFN-κ, IFN-δ, IFN-ε, IFN-τ, IFN-ω, and

IFN-ζ), interferon, lamivudine, lopinavir, loviride, MK-0518, maraviroc, moroxydine, nelfinavir, nevirapine, nexavir, nucleoside analogues, oseltamivir, penciclovir, peramivir, pleconaril, podophyllotoxin, protease inhibitors, reverse transcriptase inhibitors, ribavirin, rimantadine, ritonavir, saquinavir, stavudine, synergistic enhancers, tenofovir, tenofovir disoproxil, tipranavir, trifluridine, trizivir, tromantadine, truvada, valaciclovir, valganciclovir, vicriviroc, vidarabine, viramidine, zalcitabine, zanamivir, zidovudine, pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and mixtures thereof.

V. Lipid Particles

[0239] The lipid particles of the invention typically comprise an active agent or therapeutic agent, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.,* by a nuclease or protease. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans. The lipid particles of the invention typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm.

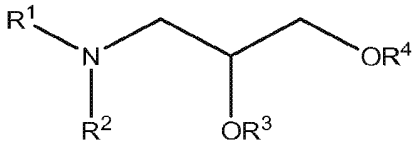
[0240] In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (*e.g.,* siRNA, aiRNA, and/or miRNA), a cationic lipid (*e.g.*, a cationic lipid of Formulas I, II, and/or III), a non-cationic lipid (*e.g.,* cholesterol alone or mixtures of one or more phospholipids and cholesterol), and a conjugated lipid that inhibits aggregation of the particles (*e.g.,* one or more PEG-lipid conjugates). The SNALP may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more unmodified and/or modified interfering RNA molecules. Nucleic acid-lipid particles and their method of preparation are described in, *e.g.,* U.S. Patent Nos. 5,753,613; 5,785,992; 5,705,385; 5,976,567; 5,981,501; 6,110,745; and 6,320,017; and PCT Publication No. WO 96/40964, the disclosures of which are each herein incorporated by reference in their entirety for all purposes.

A. Cationic Lipids

[0241] Any of a variety of cationic lipids may be used in the lipid particles of the invention (*e.g.,* SNALP), either alone or in combination with one or more other cationic lipid species or non-cationic lipid species.

[0242] Cationic lipids which are useful in the present invention can be any of a number of lipid species which carry a net positive charge at physiological pH. Such lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3.beta.-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-Dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), 1,2-Dilinoleoylcarbamyl-3-dimethylaminopropane (DLinCDAP), and mixtures thereof. A number of these lipids and related analogs have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are each herein incorporated by reference in their entirety for all purposes. Additionally, a number of commercial preparations of cationic lipids are available and can be used in the present invention. These include, *e.g.,* LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic liposomes comprising DOGS from Promega Corp., Madison, Wisconsin, USA).

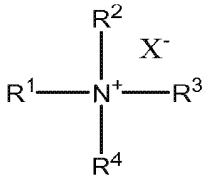
[0243] Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.



(I),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.,* R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.,* R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipid of Formula I is symmetrical, *i.e.,* R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4comprise at least three sites of unsaturation and are independently selected from, *e.g.,* dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl. In particularly preferred embodiments, the cationic lipid of Formula I is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) or 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA).

[0244] Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.

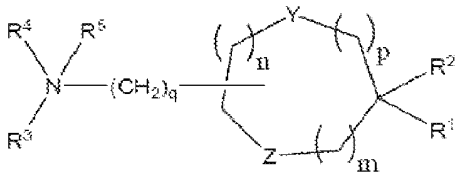


(II),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.,* R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.,* R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipids of the present invention are symmetrical, *i.e.,* R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments,

R^3 and R^4comprise at least three sites of unsaturation and are independently selected from, *e.g.,* dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl.

[0245] Moreover, cationic lipids of Formula III having the following structures (or salts thereof) are useful in the present invention.



(III),

Wherein R^1 and R^2 are either the same or different and independently optionally substituted C_{12}-C_{24} alkyl, optionally substituted C_{12}-C_{24} alkenyl, optionallysubstituted C_{12}-C_{24} alkynyl, or optionally substituted C_{12}-C_{24} acyl; R^3 and R^4 are either the same or different and independently optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 alkenyl, or optionally substituted C_1-C_6 alkynyl or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen; R^5 is either absent or hydrogen or C_1-C_6 alkyl to provide a quaternary amine; m, n, and p are either the same or different and independently either 0 or 1 with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; and Y and Z are either the same or different and independently O, S, or NH.

[0246] In some embodiments, the cationic lipid of Formula III is 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinolcoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), or mixtures

thereof. In preferred embodiments, the cationic lipid of Formula III is DLin-K-C2-DMA (XTC2).

[0247] The cationic lipid typically comprises from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0248] It will be readily apparent to one of skill in the art that depending on the intended use of the particles, the proportions of the components can be varied and the delivery efficiency of a particular formulation can be measured using, *e.g.,* an endosomal release parameter (ERP) assay.

B. Non-Cationic Lipids

[0249] The non-cationic lipids used in the lipid particles of the invention (*e.g.,* SNALP) can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

[0250] Non-limiting examples of non-cationic lipids include phospholipids such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C_{10}-C_{24} carbon chains, *e.g.,* lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

[0251] Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof such as cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof.

[0252] In some embodiments, the non-cationic lipid present in the lipid particles (*e.g.,* SNALP) comprises or consists of cholesterol or a derivative thereof, *e.g.,* a phospholipid-free lipid particle formulation. In other embodiments, the non-cationic lipid present in the lipid particles (*e.g.,* SNALP) comprises or consists of one or more phospholipids, *e.g.,* a cholesterol-free lipid particle formulation. In further embodiments, the non-cationic lipid present in the lipid particles (*e.g.,* SNALP) comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof.

[0253] Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, *e.g.,* stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

[0254] In some embodiments, the non-cationic lipid comprises from about 13 mol % to about 49.5 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 35 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0255] In certain embodiments, the cholesterol present in phospholipid-free lipid particles comprises from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, or from about 35 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle may comprise cholesterol at about 37 mol % of the total lipid present in the particle.

[0256] In certain other embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, or from about 35 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 34 mol % of the total lipid present in the particle.

[0257] In further embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 10 mol % to about 30 mol %,

from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 20 mol % of the total lipid present in the particle.

[0258] In embodiments where the lipid particles contain a mixture of phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 45, 50, 55, or 60 mol % of the total lipid present in the particle. In certain instances, the phospholipid component in the mixture may comprise from about 2 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, or from about 6 mol % to about 8 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (*e.g.,* in a mixture with about 34 mol % cholesterol) of the total lipid present in the particle. In certain other instances, the phospholipid component in the mixture may comprise from about 10 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As another non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 20 mol % (*e.g.,* in a mixture with about 20 mol % cholesterol) of the total lipid present in the particle.

C. Lipid Conjugate

[0259] In addition to cationic and non-cationic lipids, the lipid particles of the invention (*e.g.,* SNALP) comprise a lipid conjugate. The conjugated lipid is useful in that it prevents the aggregation of particles. Suitable conjugated lipids include, but are not limited to, PEG-lipid conjugates, ATTA-lipid conjugates, cationic-polymer-lipid conjugates (CPLs), and mixtures thereof. In certain embodiments, the particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate together with a CPL.

[0260] In a preferred embodiment, the lipid conjugate is a PEG-lipid. Examples of PEG-lipids include, but are not limited to, PEG coupled to dialkyloxypropyls (PEG-DAA) as described in, *e.g.,* PCT Publication No. WO 05/026372, PEG coupled to diacylglycerol (PEG-DAG) as described in, *e.g.,* U.S. Patent Publication Nos. 20030077829 and 2005008689, PEG coupled to phospholipids such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides as described in, *e.g.,* U.S. Patent No. 5,885,613, PEG conjugated to cholesterol or a derivative thereof, and mixtures thereof. The disclosures of

these patent documents are herein incorporated by reference in their entirety for all purposes. Additional PEG-lipids include, without limitation, PEG-C-DOMG, 2KPEG-DMG, and a mixture thereof.

[0261] PEG is a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG 2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, for example, the following: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH_2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM). Other PEGs such as those described in U.S. Patent Nos. 6,774,180 and 7,053,150 (*e.g.,* mPEG (20 KDa) amine) are also useful for preparing the PEG-lipid conjugates of the present invention. The disclosures of these patents are herein incorporated by reference in their entirety for all purposes. In addition, monomethoxypolyethyleneglycol-acetic acid (MePEG-CH_2COOH) is particularly useful for preparing PEG-lipid conjugates including, *e.g.*, PEG-DAA conjugates.

[0262] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.,* from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

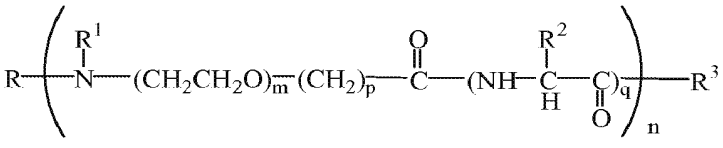
[0263] In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.,* non-ester containing linker moieties and ester-containing linker moieties. In a preferred embodiment, the linker moiety is a non-ester containing linker moiety. As used herein, the term "non-ester containing linker moiety" refers to a linker moiety that does not contain a carboxylic ester bond (-OC(O)-). Suitable non-ester containing linker moieties include, but are not limited to, amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulphide (-S-S-), ether (-O-), succinyl (-

(O)CCH$_2$CH$_2$C(O)-), succinamidyl (-NHC(O)CH$_2$CH$_2$C(O)NH-), ether, disulphide, as well as combinations thereof (such as a linker containing both a carbamate linker moiety and an amido linker moiety). In a preferred embodiment, a carbamate linker is used to couple the PEG to the lipid.

[0264] In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, *e.g.,* carbonate (-OC(O)O-), succinoyl, phosphate esters (-O-(O)POH-O-), sulfonate esters, and combinations thereof.

[0265] Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to PEG to form the lipid conjugate. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skilled in the art. Phosphatidyl-ethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), and distearoyl-phosphatidylethanolamine (DSPE).

[0266] The term "ATTA" or "polyamide" refers to, without limitation, compounds described in U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes. These compounds include a compound having the formula:

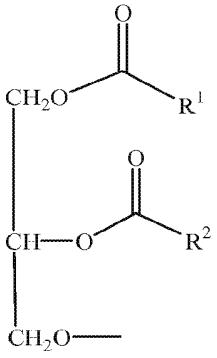


(IV),

wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R^1 is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R^1 and the nitrogen to which they are bound form an azido moiety; R^2 is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; R^3 is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and NR^4R^5, wherein R^4 and R^5 are independently hydrogen or alkyl; n is 4 to 80; m is 2 to 6; p is 1 to 4; and q is 0 or 1. It will

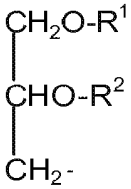
be apparent to those of skill in the art that other polyamides can be used in the compounds of the present invention.

[0267] The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.* Diacylglycerols have the following general formula:



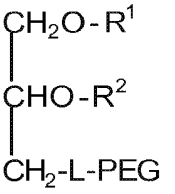
(V).

[0268] The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:



(VI).

[0269] In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:



(VII),

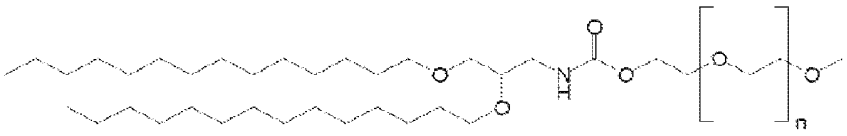
wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester

containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.*

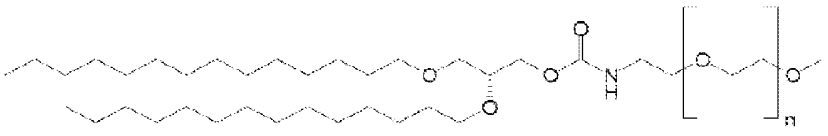
[0270] In Formula VII above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG has an average molecular weight of about 2,000 daltons or about 750 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl, or aryl. In certain embodiments, the terminal hydroxyl group is substituted with a methoxy or methyl group.

[0271] In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, a succinamidyl linker moiety, and combinations thereof. In a preferred embodiment, the non-ester containing linker moiety is a carbamate linker moiety (*i.e.*, a PEG-*C*-DAA conjugate). In another preferred embodiment, the non-ester containing linker moiety is an amido linker moiety (*i.e.*, a PEG-*A*-DAA conjugate). In yet another preferred embodiment, the non-ester containing linker moiety is a succinamidyl linker moiety (*i.e.*, a PEG-*S*-DAA conjugate).

[0272] In particular embodiments, the PEG-lipid conjugate is selected from:



(PEG-C-DMA); and



(PEG-C-DOMG).

[0273] The PEG-DAA conjugates are synthesized using standard techniques and reagents known to those of skill in the art. It will be recognized that the PEG-DAA conjugates will

contain various amide, amine, ether, thio, carbamate, and urea linkages. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See, e.g.,* March, ADVANCED ORGANIC CHEMISTRY (Wiley 1992); Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY, 5th ed. (Longman 1989). It will also be appreciated that any functional groups present may require protection and deprotection at different points in the synthesis of the PEG-DAA conjugates. Those of skill in the art will recognize that such techniques are well known. *See, e.g.,* Green and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS (Wiley 1991).

[0274] Preferably, the PEG-DAA conjugate is a dilauryloxypropyl (C_{12})-PEG conjugate, dimyristyloxypropyl (C_{14})-PEG conjugate, a dipalmityloxypropyl (C_{16})-PEG conjugate, or a distearyloxypropyl (C_{18})-PEG conjugate. Those of skill in the art will readily appreciate that other dialkyloxypropyls can be used in the PEG-DAA conjugates of the present invention.

[0275] In addition to the foregoing, it will be readily apparent to those of skill in the art that other hydrophilic polymers can be used in place of PEG. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

[0276] In addition to the foregoing components, the particles (*e.g.,* SNALP or SPLP) of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids or CPLs (*see, e.g.,* Chen *et al*., *Bioconj. Chem.,* 11:433-437 (2000)). Suitable SPLPs and SPLP-CPLs for use in the present invention, and methods of making and using SPLPs and SPLP-CPLs, are disclosed, *e.g.*, in U.S. Patent No. 6,852,334 and PCT Publication No. WO 00/62813, , the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0277] Suitable CPLs include compounds of Formula VIII:

A-W-Y (VIII),

wherein A, W, and Y are as described below.

[0278] With reference to Formula VIII, "A" is a lipid moiety such as an amphipathic lipid, a neutral lipid, or a hydrophobic lipid that acts as a lipid anchor. Suitable lipid examples include, but are not limited to, diacylglycerolyls, dialkylglycerolyls, N-N-dialkylaminos, 1,2-diacyloxy-3-aminopropanes, and 1,2-dialkyl-3-aminopropanes.

[0279] "W" is a polymer or an oligomer such as a hydrophilic polymer or oligomer. Preferably, the hydrophilic polymer is a biocompatable polymer that is nonimmunogenic or

possesses low inherent immunogenicity. Alternatively, the hydrophilic polymer can be weakly antigenic if used with appropriate adjuvants. Suitable nonimmunogenic polymers include, but are not limited to, PEG, polyamides, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, and combinations thereof. In a preferred embodiment, the polymer has a molecular weight of from about 250 to about 7,000 daltons.

[0280] "Y" is a polycationic moiety. The term polycationic moiety refers to a compound, derivative, or functional group having a positive charge, preferably at least 2 positive charges at a selected pH, preferably physiological pH. Suitable polycationic moieties include basic amino acids and their derivatives such as arginine, asparagine, glutamine, lysine, and histidine; spermine; spermidine; cationic dendrimers; polyamines; polyamine sugars; and amino polysaccharides. The polycationic moieties can be linear, such as linear tetralysine, branched or dendrimeric in structure. Polycationic moieties have between about 2 to about 15 positive charges, preferably between about 2 to about 12 positive charges, and more preferably between about 2 to about 8 positive charges at selected pH values. The selection of which polycationic moiety to employ may be determined by the type of particle application which is desired.

[0281] The charges on the polycationic moieties can be either distributed around the entire particle moiety, or alternatively, they can be a discrete concentration of charge density in one particular area of the particle moiety *e.g.,* a charge spike. If the charge density is distributed on the particle, the charge density can be equally distributed or unequally distributed. All variations of charge distribution of the polycationic moiety are encompassed by the present invention.

[0282] The lipid "A" and the nonimmunogenic polymer "W" can be attached by various methods and preferably by covalent attachment. Methods known to those of skill in the art can be used for the covalent attachment of "A" and "W." Suitable linkages include, but are not limited to, amide, amine, carboxyl, carbonate, carbamate, ester, and hydrazone linkages. It will be apparent to those skilled in the art that "A" and "W" must have complementary functional groups to effectuate the linkage. The reaction of these two groups, one on the lipid and the other on the polymer, will provide the desired linkage. For example, when the lipid is a diacylglycerol and the terminal hydroxyl is activated, for instance with NHS and DCC, to form an active ester, and is then reacted with a polymer which contains an amino group, such as with a polyamide (*see, e.g.,* U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes), an amide bond will form between the two groups.

[0283] In certain instances, the polycationic moiety can have a ligand attached, such as a targeting ligand or a chelating moiety for complexing calcium. Preferably, after the ligand is attached, the cationic moiety maintains a positive charge. In certain instances, the ligand that is attached has a positive charge. Suitable ligands include, but are not limited to, a compound or device with a reactive functional group and include lipids, amphipathic lipids, carrier compounds, bioaffinity compounds, biomaterials, biopolymers, biomedical devices, analytically detectable compounds, therapeutically active compounds, enzymes, peptides, proteins, antibodies, immune stimulators, radiolabels, fluorogens, biotin, drugs, haptens, DNA, RNA, polysaccharides, liposomes, virosomes, micelles, immunoglobulins, functional groups, other targeting moieties, or toxins.

[0284] The lipid conjugate (*e.g.,* PEG-lipid) typically comprises from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 0.9 mol % to about 1.6 mol %, from about 0.9 mol % to about 1.8 mol %, from about 1 mol % to about 1.8 mol %, from about 1 mol % to about 1.7 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, or from about 1.4 mol % to about 1.5 mol % of the total lipid present in the particle.

[0285] One of ordinary skill in the art will appreciate that the concentration of the lipid conjugate can be varied depending on the lipid conjugate employed and the rate at which the nucleic acid-lipid particle is to become fusogenic.

[0286] By controlling the composition and concentration of the lipid conjugate, one can control the rate at which the lipid conjugate exchanges out of the nucleic acid-lipid particle and, in turn, the rate at which the nucleic acid-lipid particle becomes fusogenic. For instance, when a PEG-phosphatidylethanolamine conjugate or a PEG-ceramide conjugate is used as the lipid conjugate, the rate at which the nucleic acid-lipid particle becomes fusogenic can be varied, for example, by varying the concentration of the lipid conjugate, by varying the molecular weight of the PEG, or by varying the chain length and degree of saturation of the acyl chain groups on the phosphatidylethanolamine or the ceramide. In addition, other variables including, for example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the nucleic acid-lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the nucleic acid-lipid particle becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure.

VI. Preparation of Lipid Particles

[0287] The lipid particles of the present invention, *e.g.,* SNALP, in which an active agent or therapeutic agent such as an interfering RNA is encapsulated in a lipid bilayer and is protected from degradation, can be formed by any method known in the art including, but not limited to, a continuous mixing method or a direct dilution process.

[0288] In preferred embodiments, the cationic lipids are lipids of Formula I, II, and III, or combinations thereof. In other preferred embodiments, the non-cationic lipids are egg sphingomyelin (ESM), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dipalmitoyl-phosphatidylcholine (DPPC), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, 14:0 PE (1,2-dimyristoyl-phosphatidylethanolamine (DMPE)), 16:0 PE (1,2-dipalmitoyl-phosphatidylethanolamine (DPPE)), 18:0 PE (1,2-distearoyl-phosphatidylethanolamine (DSPE)), 18:1 PE (1,2-dioleoyl-phosphatidylethanolamine (DOPE)), 18:1 trans PE (1,2-dielaidoyl-phosphatidylethanolamine (DEPE)), 18:0-18:1 PE (1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE)), 16:0-18:1 PE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)), polyethylene glycol-based polymers (*e.g.,* PEG 2000, PEG 5000, PEG-modified diacylglycerols, or PEG-modified dialkyloxypropyls), cholesterol, or combinations thereof.

[0289] In certain embodiments, the present invention provides for SNALP produced via a continuous mixing method, *e.g.*, a process that includes providing an aqueous solution comprising a nucleic acid such as an interfering RNA in a first reservoir, providing an organic lipid solution in a second reservoir, and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a liposome encapsulating the nucleic acid (*e.g.*, interfering RNA). This process and the apparatus for carrying this process are described in detail in U.S. Patent Publication No. 20040142025, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0290] The action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a liposome substantially instantaneously upon mixing. As used herein, the phrase "continuously diluting a lipid solution with a buffer solution" (and variations) generally means that the lipid solution is diluted sufficiently rapidly in a hydration process with sufficient force to effectuate vesicle generation. By mixing the

aqueous solution comprising a nucleic acid with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution in the presence of the buffer solution (*i.e.*, aqueous solution) to produce a nucleic acid-lipid particle.

[0291] The SNALP formed using the continuous mixing method typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0292] In another embodiment, the present invention provides for SNALP produced via a direct dilution process that includes forming a liposome solution and immediately and directly introducing the liposome solution into a collection vessel containing a controlled amount of dilution buffer. In preferred aspects, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In one aspect, the amount of dilution buffer present in the collection vessel is substantially equal to the volume of liposome solution introduced thereto. As a non-limiting example, a liposome solution in 45% ethanol when introduced into the collection vessel containing an equal volume of dilution buffer will advantageously yield smaller particles.

[0293] In yet another embodiment, the present invention provides for SNALP produced via a direct dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the liposome solution formed in a first mixing region is immediately and directly mixed with dilution buffer in the second mixing region. In preferred aspects, the second mixing region includes a T-connector arranged so that the liposome solution and the dilution buffer flows meet as opposing 180° flows; however, connectors providing shallower angles can be used, *e.g.,* from about 27° to about 180°. A pump mechanism delivers a controllable flow of buffer to the second mixing region. In one aspect, the flow rate of dilution buffer provided to the second mixing region is controlled to be substantially equal to the flow rate of liposome solution introduced thereto from the first mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the liposome solution in the second mixing region, and therefore also the concentration of liposome solution in buffer throughout the second mixing process. Such control of the dilution buffer flow rate advantageously allows for small particle size formation at reduced concentrations.

[0294] These processes and the apparatuses for carrying out these direct dilution processes are described in detail in U.S. Patent Publication No. 20070042031, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0295] The SNALP formed using the direct dilution process typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0296] If needed, the lipid particles of the invention (*e.g.,* SNALP) can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

[0297] Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles, is described in U.S. Patent No. 4,737,323, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between about 60 and about 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

[0298] Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

[0299] In some embodiments, the nucleic acids in the SNALP are precondensed as described in, *e.g.*, U.S. Patent Application No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0300] In other embodiments, the methods will further comprise adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions. Examples of suitable non-lipid polycations include, hexadimethrine bromide (sold under the brandname POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or

other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine, and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

[0301] In some embodiments, the nucleic acid to lipid ratios (mass/mass ratios) in a formed SNALP will range from about 0.01 to about 0.2, from about 0.02 to about 0.1, from about 0.03 to about 0.1, or from about 0.01 to about 0.08. The ratio of the starting materials also falls within this range. In other embodiments, the SNALP preparation uses about 400 μg nucleic acid per 10 mg total lipid or a nucleic acid to lipid mass ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 μg of nucleic acid. In other preferred embodiments, the particle has a nucleic acid:lipid mass ratio of about 0.08.

[0302] In other embodiments, the lipid to nucleic acid ratios (mass/mass ratios) in a formed SNALP will range from about 1 (1:1) to about 100 (100:1), from about 5 (5:1) to about 100 (100:1), from about 1 (1:1) to about 50 (50:1), from about 2 (2:1) to about 50 (50:1), from about 3 (3:1) to about 50 (50:1), from about 4 (4:1) to about 50 (50:1), from about 5 (5:1) to about 50 (50:1), from about 1 (1:1) to about 25 (25:1), from about 2 (2:1) to about 25 (25:1), from about 3 (3:1) to about 25 (25:1), from about 4 (4:1) to about 25 (25:1), from about 5 (5:1) to about 25 (25:1), from about 5 (5:1) to about 20 (20:1), from about 5 (5:1) to about 15 (15:1), from about 5 (5:1) to about 10 (10:1), about 5 (5:1), 6 (6:1), 7 (7:1), 8 (8:1), 9 (9:1), 10 (10:1), 11 (11:1), 12 (12:1), 13 (13:1), 14 (14:1), or 15 (15:1). The ratio of the starting materials also falls within this range.

[0303] As previously discussed, the conjugated lipid may further include a CPL. A variety of general methods for making SNALP-CPLs (CPL-containing SNALP) are discussed herein. Two general techniques include "post-insertion" technique, that is, insertion of a CPL into, for example, a pre-formed SNALP, and the "standard" technique, wherein the CPL is included in the lipid mixture during, for example, the SNALP formation steps. The post-insertion technique results in SNALP having CPLs mainly in the external face of the SNALP bilayer membrane, whereas standard techniques provide SNALP having CPLs on both internal and external faces. The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAAs and PEG-DAGs). Methods of making SNALP-CPL, are taught, for example, in U.S. Patent Nos. 5,705,385; 6,586,410; 5,981,501; 6,534,484; and 6,852,334; U.S. Patent Publication No. 20020072121; and PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

VII. Kits

[0304] The present invention also provides lipid particles (*e.g.,* SNALP) in kit form. The kit may comprise a container which is compartmentalized for holding the various elements of the lipid particles (*e.g.*, the active agents or therapeutic agents such as nucleic acids and the individual lipid components of the particles). In some embodiments, the kit may further comprise an endosomal membrane destabilizer (*e.g.*, calcium ions). The kit typically contains the lipid particle compositions of the present invention, preferably in dehydrated form, with instructions for their rehydration and administration.

[0305] As explained herein, the lipid particles of the invention (*e.g.,* SNALP) can be tailored to preferentially target particular tissues, organs, or tumors of interest. In certain instances, preferential targeting of lipid particles such as SNALP may be carried out by controlling the composition of the particle itself. For instance, as set forth in Example 11, it has been found that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP formulation can be used to preferentially target the liver (including liver tumors).

[0306] In certain other instances, it may be desirable to have a targeting moiety attached to the surface of the lipid particle to further enhance the targeting of the particle. Methods of attaching targeting moieties (*e.g.*, antibodies, proteins, *etc.*) to lipids (such as those used in the present particles) are known to those of skill in the art.

VII. Administration of Lipid Particles

[0307] Once formed, the lipid particles of the invention (*e.g.,* SNALP) are useful for the introduction of active agents or therapeutic agents (*e.g.*, nucleic acids such as interfering RNA) into cells. Accordingly, the present invention also provides methods for introducing an active agent or therapeutic agent such as a nucleic acid (*e.g.*, interfering RNA) into a cell. The methods are carried out *in vitro* or *in vivo* by first forming the particles as described above and then contacting the particles with the cells for a period of time sufficient for delivery of the active agent or therapeutic agent to the cells to occur.

[0308] The lipid particles of the invention (*e.g.,* SNALP) can be adsorbed to almost any cell type with which they are mixed or contacted. Once adsorbed, the particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the active agent or therapeutic agent (*e.g.*, nucleic acid) portion of the particle can take place via any one of these pathways. In particular, when

fusion takes place, the particle membrane is integrated into the cell membrane and the contents of the particle combine with the intracellular fluid.

[0309] The lipid particles of the invention (*e.g.*, SNALP) can be administered either alone or in a mixture with a pharmaceutically-acceptable carrier (*e.g.*, physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice. Generally, normal buffered saline (*e.g.*, 135-150 mM NaCl) will be employed as the pharmaceutically-acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Additional suitable carriers are described in, *e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

[0310] The pharmaceutically-acceptable carrier is generally added following particle formation. Thus, after the particle is formed, the particle can be diluted into pharmaceutically-acceptable carriers such as normal buffered saline.

[0311] The concentration of particles in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2 to 5%, to as much as about 10 to 90% by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, particles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

[0312] The pharmaceutical compositions of the present invention may be sterilized by conventional, well-known sterilization techniques. Aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically-acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium

chloride, and calcium chloride. Additionally, the particle suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

A. *In vivo* Administration

[0313] Systemic delivery for *in vivo* therapy, *e.g.*, delivery of a therapeutic nucleic acid to a distal target cell via body systems such as the circulation, has been achieved using nucleic acid-lipid particles such as those described in PCT Publication Nos. WO 05/007196, WO 05/121348, WO 05/120152, and WO 04/002453, the disclosures of which are herein incorporated by reference in their entirety for all purposes. The present invention also provides fully encapsulated lipid particles that protect the nucleic acid from nuclease degradation in serum, are nonimmunogenic, are small in size, and are suitable for repeat dosing.

[0314] For *in vivo* administration, administration can be in any manner known in the art, *e.g.*, by injection, oral administration, inhalation (*e.g.*, intransal or intratracheal), transdermal application, or rectal administration. Administration can be accomplished via single or divided doses. The pharmaceutical compositions can be administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In some embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection (*see*, *e.g.*, U.S. Patent No. 5,286,634). Intracellular nucleic acid delivery has also been discussed in Straubringer *et al.*, *Methods Enzymol.*, 101:512 (1983); Mannino *et al.*, *Biotechniques,* 6:682 (1988); Nicolau *et al.*, *Crit. Rev. Ther. Drug Carrier Syst.*, 6:239 (1989); and Behr, *Acc. Chem. Res.*, 26:274 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, U.S. Patent Nos. 3,993,754; 4,145,410; 4,235,871; 4,224,179; 4,522,803; and 4,588,578. The lipid particles can be administered by direct injection at the site of disease or by injection at a site distal from the site of disease (*see*, *e.g.*, Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71(1994)). The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0315] The compositions of the present invention, either alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation (*e.g.*, intranasally or intratracheally) (*see*, Brigham *et al.*, *Am.*

J. Sci., 298:278 (1989)). Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0316] In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering nucleic acid compositions directly to the lungs via nasal aerosol sprays have been described, *e.g.,* in U.S. Patent Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Similarly, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Patent No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

[0317] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions are preferably administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically, or intrathecally.

[0318] Generally, when administered intravenously, the lipid particle formulations are formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium

chloride, sorbitan monolaurate, triethanolamine oleate, *etc*. These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

[0319] In certain applications, the lipid particles disclosed herein may be delivered via oral administration to the individual. The particles may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, pills, lozenges, elixirs, mouthwash, suspensions, oral sprays, syrups, wafers, and the like (*see, e.g.,* U.S. Patent Nos. 5,641,515, 5,580,579, and 5,792,451, the disclosures of which are herein incorporated by reference in their entirety for all purposes). These oral dosage forms may also contain the following: binders, gelatin; excipients, lubricants, and/or flavoring agents. When the unit dosage form is a capsule, it may contain, in addition to the materials described above, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. Of course, any material used in preparing any unit dosage form should be pharmaceutically pure and substantially non-toxic in the amounts employed.

[0320] Typically, these oral formulations may contain at least about 0.1% of the lipid particles or more, although the percentage of the particles may, of course, be varied and may conveniently be between about 1% or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of particles in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0321] Formulations suitable for oral administration can consist of: (a) liquid solutions, such as an effective amount of a packaged therapeutic agent such as nucleic acid (*e.g.,* interfering RNA) suspended in diluents such as water, saline, or PEG 400; (b) capsules, sachets, or tablets, each containing a predetermined amount of a therapeutic agent such as nucleic acid (*e.g.,* interfering RNA), as liquids, solids, granules, or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic

acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise a therapeutic agent such as nucleic acid (*e.g.,* interfering RNA) in a flavor, *e.g.*, sucrose, as well as pastilles comprising the therapeutic agent in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the therapeutic agent, carriers known in the art.

[0322] In another example of their use, lipid particles can be incorporated into a broad range of topical dosage forms. For instance, a suspension containing nucleic acid-lipid particles such as SNALP can be formulated and administered as gels, oils, emulsions, topical creams, pastes, ointments, lotions, foams, mousses, and the like.

[0323] When preparing pharmaceutical preparations of the lipid particles of the invention, it is preferable to use quantities of the particles which have been purified to reduce or eliminate empty particles or particles with therapeutic agents such as nucleic acid associated with the external surface.

[0324] The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as primates (*e.g.*, humans and chimpanzees as well as other nonhuman primates), canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, rats and mice), lagomorphs, and swine.

[0325] The amount of particles administered will depend upon the ratio of therapeutic agent (*e.g.,* nucleic acid) to lipid, the particular therapeutic agent (*e.g.,* nucleic acid) used, the disease or disorder being treated, the age, weight, and condition of the patient, and the judgment of the clinician, but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight, or about 10^8-10^{10} particles per administration (*e.g.,* injection).

B. *In vitro* Administration

[0326] For *in vitro* applications, the delivery of therapeutic agents such as nucleic acids (*e.g.,* interfering RNA) can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells are animal cells, more preferably mammalian cells, and most preferably human cells.

[0327] Contact between the cells and the lipid particles, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of particles varies widely depending on the particular application, but is generally between about 1 μmol and about 10

mmol. Treatment of the cells with the lipid particles is generally carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

[0328] In one group of preferred embodiments, a lipid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/ml, more preferably about 2 x 10^4 cells/ml. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/ml, more preferably about 0.1 μg/ml.

[0329] Using an Endosomal Release Parameter (ERP) assay, the delivery efficiency of the SNALP or other lipid particle of the invention can be optimized. An ERP assay is described in detail in U.S. Patent Publication No. 20030077829, the disclosure of which is herein incorporated by reference in its entirety for all purposes. More particularly, the purpose of an ERP assay is to distinguish the effect of various cationic lipids and helper lipid components of SNALP based on their relative effect on binding/uptake or fusion with/destabilization of the endosomal membrane. This assay allows one to determine quantitatively how each component of the SNALP or other lipid particle affects delivery efficiency, thereby optimizing the SNALP or other lipid particle. Usually, an ERP assay measures expression of a reporter protein (*e.g.*, luciferase, β-galactosidase, green fluorescent protein (GFP), *etc.*), and in some instances, a SNALP formulation optimized for an expression plasmid will also be appropriate for encapsulating an interfering RNA. In other instances, an ERP assay can be adapted to measure downregulation of transcription or translation of a target sequence in the presence or absence of an interfering RNA (*e.g.,* siRNA). By comparing the ERPs for each of the various SNALP or other lipid particles, one can readily determine the optimized system, *e.g.*, the SNALP or other lipid particle that has the greatest uptake in the cell.

C. Cells for Delivery of Lipid Particles

[0330] The compositions and methods of the present invention are used to treat a wide variety of cell types, *in vivo* and *in vitro*. Suitable cells include, *e.g.*, hematopoietic precursor (stem) cells, fibroblasts, keratinocytes, hepatocytes, endothelial cells, skeletal and smooth muscle cells, osteoblasts, neurons, quiescent lymphocytes, terminally differentiated cells, slow or noncycling primary cells, parenchymal cells, lymphoid cells, epithelial cells, bone cells, and the like. In preferred embodiments, an active agent or therapeutic agent such as an interfering RNA (*e.g.,* siRNA) is delivered to cancer cells such as, *e.g.*, lung cancer cells, colon cancer cells, rectal cancer cells, anal cancer cells, bile duct cancer cells, small intestine cancer cells, stomach (gastric) cancer cells, esophageal cancer cells, gallbladder cancer cells,

liver cancer cells, pancreatic cancer cells, appendix cancer cells, breast cancer cells, ovarian cancer cells, cervical cancer cells, prostate cancer cells, renal cancer cells, cancer cells of the central nervous system, glioblastoma tumor cells, skin cancer cells, lymphoma cells, choriocarcinoma tumor cells, head and neck cancer cells, osteogenic sarcoma tumor cells, and blood cancer cells.

[0331] *In vivo* delivery of lipid particles such as SNALP encapsulating an interfering RNA (*e.g.,* siRNA) is suited for targeting cells of any cell type. The methods and compositions can be employed with cells of a wide variety of vertebrates, including mammals, such as, *e.g,* canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, mice, rats, and guinea pigs), lagomorphs, swine, and primates (*e.g.* monkeys, chimpanzees, and humans).

[0332] To the extent that tissue culture of cells may be required, it is well-known in the art. For example, Freshney, Culture of Animal Cells, a Manual of Basic Technique, 3rd Ed., Wiley-Liss, New York (1994), Kuchler *et al.*, Biochemical Methods in Cell Culture and Virology, Dowden, Hutchinson and Ross, Inc. (1977), and the references cited therein provide a general guide to the culture of cells. Cultured cell systems often will be in the form of monolayers of cells, although cell suspensions are also used.

D. Detection of Lipid Particles

[0333] In some embodiments, the lipid particles of the present invention (*e.g.,* SNALP) are detectable in the subject at about 1, 2, 3, 4, 5, 6, 7, 8 or more hours. In other embodiments, the lipid particles of the present invention (*e.g.,* SNALP) are detectable in the subject at about 8, 12, 24, 48, 60, 72, or 96 hours, or about 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 25, or 28 days after administration of the particles. The presence of the particles can be detected in the cells, tissues, or other biological samples from the subject. The particles may be detected, *e.g.*, by direct detection of the particles, detection of a therapeutic nucleic acid such as an interfering RNA (*e.g.,* siRNA) sequence, detection of the target sequence of interest (*i.e.*, by detecting expression or reduced expression of the sequence of interest), or a combination thereof.

1. Detection of Particles

[0334] Lipid particles of the invention such as SNALP can be detected using any method known in the art. For example, a label can be coupled directly or indirectly to a component of the lipid particle using methods well-known in the art. A wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the lipid particle component, stability requirements, and available instrumentation and disposal

provisions. Suitable labels include, but are not limited to, spectral labels such as fluorescent dyes (*e.g.*, fluorescein and derivatives, such as fluorescein isothiocyanate (FITC) and Oregon Green™; rhodamine and derivatives such Texas red, tetrarhodimine isothiocynate (TRITC), *etc.*, digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like; radiolabels such as ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, *etc.*; enzymes such as horse radish peroxidase, alkaline phosphatase, *etc.*; spectral colorimetric labels such as colloidal gold or colored glass or plastic beads such as polystyrene, polypropylene, latex, *etc.* The label can be detected using any means known in the art.

2. Detection of Nucleic Acids

[0335] Nucleic acids (*e.g.*, interfering RNA) are detected and quantified herein by any of a number of means well-known to those of skill in the art. The detection of nucleic acids may proceed by well-known methods such as Southern analysis, Northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography. Additional analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography may also be employed.

[0336] The selection of a nucleic acid hybridization format is not critical. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in, *e.g.*, "Nucleic Acid Hybridization, A Practical Approach," Eds. Hames and Higgins, IRL Press (1985).

[0337] The sensitivity of the hybridization assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBA™) are found in Sambrook *et al.*, In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (2000); and Ausubel *et al.*, SHORT PROTOCOLS IN MOLECULAR BIOLOGY, eds., Current Protocols, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (2002); as well as U.S. Patent No. 4,683,202; PCR Protocols, A Guide to Methods and

Applications (Innis *et al.* eds.) Academic Press Inc. San Diego, CA (1990); Arnheim & Levinson (October 1, 1990), *C&EN* 36; The *Journal Of NIH Research*, 3:81 (1991); Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173 (1989); Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874 (1990); Lomell *et al.*, *J. Clin. Chem.*, 35:1826 (1989); Landegren *et al.*, *Science*, 241:1077 (1988); Van Brunt, *Biotechnology*, 8:291 (1990); Wu and Wallace, *Gene*, 4:560 (1989); Barringer *et al.*, *Gene*, 89:117 (1990); and Sooknanan and Malek, *Biotechnology*, 13:563 (1995). Improved methods of cloning *in vitro* amplified nucleic acids are described in U.S. Pat. No. 5,426,039. Other methods described in the art are the nucleic acid sequence based amplification (NASBA™, Cangene, Mississauga, Ontario) and Qβ-replicase systems. These systems can be used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using, for example, nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation. The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0338] Nucleic acids for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as inhibitor components are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage *et al.*, *Tetrahedron Letts.*, 22:1859 1862 (1981), *e.g.*, using an automated synthesizer, as described in Needham VanDevanter *et al.*, *Nucleic Acids Res.*, 12:6159 (1984). Purification of ploynucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion exchange HPLC as described in Pearson *et al.*, *J. Chrom.*, 255:137 149 (1983). The sequence of the synthetic poluyucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology*, 65:499.

[0339] An alternative means for determining the level of transcription is *in situ* hybridization. *In situ* hybridization assays are well-known and are generally described in Angerer *et al.*, *Methods Enzymol.*, 152:649 (1987). In an *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

VIII. Examples

[0340] The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

Example 1. Materials and Methods.

[0341] ***siRNA***: All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, CO). The siRNAs were desalted and annealed using standard procedures.

[0342] ***Lipid Encapsulation of siRNA***: In some embodiments, siRNA molecules were encapsulated into nucleic acid-lipid particles composed of the following lipids: the lipid conjugate PEG-cDMA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropylamine); the cationic lipid DLinDMA (1,2-Dilinoleyloxy-3-(N,N-dimethyl)aminopropane); the phospholipid DPPC (1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids; Alabaster, AL); and synthetic cholesterol (Sigma-Aldrich Corp.; St. Louis, MO) in the molar ratio 1.4:57.1:7.1:34.3, respectively. In other words, siRNAs were encapsulated into SNALP of the following "1:57" formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol. In other embodiments, siRNA molecules were encapsulated into phospholipid-free SNALP composed of the following lipids: the lipid conjugate PEG-cDMA; the cationic lipid DLinDMA; and synthetic cholesterol in the molar ratio 1.5:61.5:36.9, respectively. In other words, siRNAs were encapsulated into phospholipid-free SNALP of the following "1:62" formulation: 1.5% PEG-cDMA; 61.5% DLinDMA; and 36.9% cholesterol. For vehicle controls, empty particles with identical lipid composition were formed in the absence of siRNA. It should be understood that the 1:57 formulation and 1:62 formulation are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the formulation may vary. Typically, in the 1:57 formulation, the amount of cationic lipid will be 57 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:57 formulation being made up of non-cationic lipid (*e.g.*, phospholipid, cholesterol, or a mixture of the two). Similarly, in the 1:62 formulation, the amount of cationic lipid will be 62 mol % ± 5 mol %, and the amount of lipid conjugate

will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:62 formulation being made up of the non-cationic lipid (*e.g.*, cholesterol).

Example 2. Eg5 siRNA Formulated as 1:57 SNALP Are Potent Inhibitors of Cell Growth *in vitro*.

[0343] SNALP formulations were prepared with an siRNA targeting Eg5 as the nucleic acid component. Eg5 is a member of kinesin-related proteins that are involved in functions related to movements of organelles, microtubules, or chromosomes along microtubules. These functions include axonal transport, microtubule sliding during nuclear fusion or division, and chromosome disjunction during meiosis and early mitosis. Eg5 plays a critical role in mitosis of mammalian cells. The Eg5 siRNA used in this study is provided in Table 1. The modifications involved introducing 2'OMe-uridine at selected positions in the sense and antisense strands of the Eg5 2263 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 1. siRNA duplex comprising sense and antisense Eg5 RNA polynucleotides.

Modification	Eg5 2263 siRNA sequence	% 2'OMe-Modified	% Modified in DS Region
U/U	5'-C**U**GAAGACC**U**GAAGACAA**U**dTdT-3' 3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUUA-5'	6/42 = 14.3%	6/38 = 15.8%

Column 1: "U/U" = 2'OMe-uridine modified siRNA duplex; Column 2: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. "dT" = deoxythymidine. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

[0344] The lipid components and physical characteristics of the SNALP formulations are summarized in Table 2. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 2. Characteristics of the SNALP formulations used in this study.

Sample No.	Formulation Composition, Mole % PEG(2000)-C-DMA I DLinDMA I DPPC I Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
1	2 I 40 I 10 I 48	12.4	57	0.07	90
2	1.8 I 36.4 I 18.2 I 43.6	14.0	72	0.12	89
3	1.4 I 27.0 I 6.8 I 64.9	16.5	70	0.12	92
4	1.3 I 25.3 I 12.7 I 60.8	18.1	76	0.07	93
5	3.9 I 39.2 I 9.8 I 47.1	13.5	53	0.27	86
6	3.6 I 35.7 I 17.9 I 42.9	15.1	58	0.18	87
7	2.7 I 26.7 I 6.7 I 64.0	17.6	56	0.17	92
8	2.5 I 25.0 I 12.5 I 60.0	19.2	61	0.13	92
9	1.4 I 57.1 I 7.1 I 34.3	17.8	84	0.10	88
10	1.3 I 53.3 I 13.3 I 32.0	19.5	83	0.10	89
11	1.1 I 42.6 I 5.3 I 51.1	22.0	80	0.10	93
12	1.0 I 40.4 I 10.1 I 48.5	23.6	78	0.11	88
13	2.8 I 56.3 I 7.0 I 33.8	19.0	62	0.14	80
14	2.6 I 52.6 I 13.2 I 31.6	20.6	66	0.14	82
15	2.1 I 42.1 I 5.3 I 50.5	23.1	71	0.16	91
16	2 I 40 I 10 I 48	24.7	67	0.14	92

[0345] Silencing of Eg5 by siRNA transfection causes mitotic arrest and apoptosis in mammalian cells. Cell viability following transfection with SNALP containing an siRNA targeting Eg5 therefore provides a simple biological readout of *in vitro* transfection efficiency. Cell viability of *in vitro* cell cultures was assessed using the commercial reagent CellTiter-Blue® (Promega Corp.; Madison, WI), a resazurin dye that is reduced by metabolically active cells to the flourogenic product resorufin. The human colon cancer cell line HT29 was cultured using standard tissue culture techniques. 72 hours after SNALP application, CellTiter-Blue® reagent was added to the culture to quantify the metabolic activity of the cells, which is a measure of cell viability. Data are presented as a percent of cell viability relative to ("untreated") control cells that received phosphate buffered saline (PBS) vehicle only.

[0346] Figure 1 shows that the 1:57 SNALP formulation containing Eg5 2263 U/U siRNA was among the most potent inhibitors of tumor cell growth at all siRNA concentrations tested (*see,* Figure 1B, Sample 9).

Example 3. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo*.

[0347] SNALP formulations were prepared with an siRNA targeting apolipoprotein B (ApoB) as the nucleic acid component. ApoB is the main apolipoprotein of chylomicrons and low density lipoproteins (LDL). Mutations in ApoB are associated with

hypercholesterolemia. ApoB occurs in the plasma in 2 main forms, ApoB48 and ApoB100, which are synthesized in the intestine and liver, respectively, due to an organ-specific stop codon. The ApoB siRNA used in this study is provided in Table 3. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the ApoB siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 3. siRNA duplex comprising sense and antisense ApoB RNA polynucleotides.

Position	Modification	ApoB siRNA sequence	% 2'OMe-Modified	% Modified in DS Region
10048	U2/2 G1/2	5'-AGU**G**UCA**U**CACAC**U**GAAUACC-3' 3'-GU**U**CACAGUAGU**G**U**G**AC**U**UAU-5'	7/42 = 16.7%	7/38 = 18.4%

Column 1: The number refers to the nucleotide position of the 5' base of the sense strand relative to the mouse ApoB mRNA sequence XM_137955. Column 2: The numbers refer to the distribution of 2'OMe chemical modifications in each strand. Column 3: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. Column 4: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 5: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

[0348] The lipid components and physical characteristics of the formulations are summarized in Table 4. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 4. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2 | 40 | 10 | 48	12.4	59	0.15	93
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 2.2 | 44.4 | 53.3	10.7	55	0.17	91
4	PEG(2000)-C-DMA | DLinDMA | DOPC | Cholesterol 2 | 40 | 10 | 48	12.5	59	0.16	92
5	PEG(2000)-C-DMA | DLinDMA | DMPC | Cholesterol 2 | 40 | 10 | 48	12.2	56	0.11	92
6	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.8 | 36.4 | 18.2 | 43.6	13.8	66	0.16	93
7	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 2 | 40 | 10 | 48	12.4	56	0.12	92
8	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 27.0 | 6.8 | 64.9	16.5	60	0.10	93
9	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.3 | 25.3 | 12.7 | 60.8	18.1	74	0.13	92
10	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2.5 | 25.0 | 12.5 | 60.0	19.2	60	0.13	93
11	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.4 | 34.3	17.8	79	0.09	94

12	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.0 | 40.4 | 10.1 | 48.5	23.6	72	0.11	93
13	PEG(2000)-C-DMA | DLinDMA | DPPC 2 | 70 | 28	8.7	73	0.09	87
14	PEG(2000)-C-DMA | DLinDMA | DPPC 1.6 | 54.7 | 43.8	11.3	65	0.11	87

[0349] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 µl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0350] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0351] Figure 2 shows that the 1:57 SNALP formulation containing ApoB 10048 U2/2 G1/2 siRNA was the most potent at reducing ApoB expression *in vivo* (*see,* Group 11).

Example 4. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo*.

[0352] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 5. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 5. Characteristics of the SNALP formulations used in this study.

SNALP (L:D ratio)	siRNA Payload	Particle Size (Polydispersity)	% Encapsulation
2:30 (13)	ApoB-10048 U2/2 G1/2	65 nm (0.16)	88
1:57 (9)	ApoB-10048 U2/2 G1/2	74 nm (0.10)	89

[0353] The 2:30 SNALP formulation used in this study is lipid composition 2:30:20:48 as described in molar percentages of PEG-C-DMA, DLinDMA, DSPC, and cholesterol (in that

order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 13:1.

[0354] The 1:57 SNALP formulation used in this study is lipid composition 1.5:57.1:7:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 9:1.

[0355] BALB/c mice (female, 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Days 0, 1, 2, 3 & 4 for a total of 5 doses per animal. Daily dosage was either 1.0 (for 2:30 SNALP) or 0.1 (for 1:57 SNALP) mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 µl). As a negative control, one group of animals was given IV injections of phosphate buffered saline (PBS) vehicle. On Study Day 7, 72 h after the last treatment, animals were euthanized and liver tissue was collected in RNAlater.

[0356] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0357] Figure 3 shows that the 1:57 SNALP containing ApoB 10048 U2/2 G1/2 siRNA was more than 10 times as efficacious as the 2:30 SNALP in mediating ApoB gene silencing in mouse liver at a 10-fold lower dose.

Example 5. ApoB siRNA Formulated as 1:57 or 1:62 SNALP Have Potent Silencing Activity *in vivo*.

[0358] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 6. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 6. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	76	0.06	89
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 1.5 | 61.5 | 36.9	8.1	76	0.04	86
4	PEG(2000)-C-DMA | DODMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.0	72	0.05	95
5	PEG(5000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.6	52	0.16	89
6	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 1.4 | 57.1 | 7.1 | 34.3	8.9	68	0.10	94
7	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	72	0.07	95
8	PEG(2000)-C-DMA | DLinDMA | DPPC 1.8 | 70.2 | 28.1	8.6	74	0.13	86

[0359] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.75 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 µl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0360] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0361] Figure 4 shows that the 1:57 and 1:62 SNALP formulations had comparable ApoB silencing activity *in vivo* (*see, e.g.,* Groups 2 & 3).

Example 6. ApoB siRNA Formulated as 1:62 SNALP Have Potent Silencing Activity *in vivo*.

[0362] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 7. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 7. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition, Mole % PEG(2000)-C-DMA I DLinDMA I Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	1.5 I 61.5 I 36.9	6.1	80	0.07	92
3	1.4 I 54.8 I 43.8	6.6	74	0.05	89
4	2.0 I 61.2 I 36.7	6.2	71	0.11	91
5	1.8 I 54.5 I 43.6	6.7	67	0.09	91
6	1.3 I 68.1 I 30.6	7.4	91	0.06	89
7	1.2 I 61.8 I 37.1	8.0	87	0.10	90
8	1.7 I 67.8 I 30.5	7.6	81	0.07	91
9	1.4 I 56.3 I 42.3	8.6	75	0.11	92
10	1.9 I 61.3 I 36.8	8.2	72	0.10	91
11	1.8 I 56.1 I 42.1	8.8	70	0.10	90
12	1.3 I 66.7 I 32.0	9.5	89	0.09	89
13	1.2 I 61.7 I 37.0	10.0	87	0.10	91
14	1.7 I 66.4 I 31.9	9.6	82	0.11	90
15	1.5 I 61.5 I 36.9	10.1	79	0.10	91

[0363] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 µl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0364] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0365] Figure 5 shows that the 1:62 SNALP formulation was one of the most potent inhibitors of ApoB expression at two different lipid:drug ratios (*i.e.,* 6.1 & 10.1) among the phospholipid-free SNALP formulations tested (*see,* Groups 2 & 15).

Example 7. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Syringe Press or Gear Pump Process.

[0366] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by various manufacturing processes. In particular, 1:57 SNALP was prepared by a syringe press or gear pump process

using either PBS or citrate buffer (post-blend dilution) and administered intravenously in mice.

Experimental Design

[0367] Animal Model: Female BALB/c mice, 5 wks old, n=4 per group/cage.

[0368] siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

Tolerability:

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
1	PBS vehicle	Standard 10 mL/kg volume	
2	1|57 Citrate Direct Dil, Syringe Press	7	77
3	1|57 PBS Direct Dil, Syringe Press	7	96
4	1|57 PBS Direct Dil, Gear Pump	7	79
5	1|57 Citrate Direct Dil, Syringe Press	9	99
6	1|57 PBS Direct Dil, Syringe Press	9	123
7	1|57 PBS Direct Dil, Gear Pump	9	102

Efficacy:

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
8	PBS vehicle	Standard 10 mL/kg volume	
9	1|57 PBS Direct Dil, Syringe Press	0.05	0.68
10	1|57 PBS Direct Dil, Gear Pump	0.05	0.57
11	1|57 PBS Direct Dil, Syringe Press	0.1	1.36
12	1|57 PBS Direct Dil, Gear Pump	0.1	1.13

Formulation:

[0369] Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 µm filter sterilized in crimp top vials.

[0370] Formulation Details:

1. Lipid composition "1|57 Citrate blend" used in this study is 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation has an input lipid to drug ratio of 8.9.
2. Gear pump set up included 0.8 mm T-connector and 400 mL/min speed.
3. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0371] Formulation Summary:

		Particle Size			Final L:D
	1:57 (9:1) + DOW siRNA	Zavg (nm)	Poly	% Encap	(mg:mg)
322-050807-1	Syringe PBS Blend	79	0.12	92	13.6
322-050807-2	Syringe Citrate Blend	86	0.11	91	11.0
322-050807-3	Gear PBS Blend	80	0.09	93	11.3

Procedures

[0372] **Treatment:** Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 µl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0373] **Group 1-7 Endpoint:** Animals are sacrificed on Day 1, 24 h after test article administration. Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein.

[0374] **Group 8-12 Endpoint:** Animals are sacrificed on Day 2, 48 h after test article administration. Blood is collected by cardiac puncture and processed for plasma. Immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed and weighed separately: liver and spleen. The bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at -20°C or -80°C for archival purposes. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0375] **Termination:** Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0376] **Data Analysis:** Tolerability of treatment regime is monitored by animal appearance and behavior as well as body weight. Blood clinical chemistry is measured by automated analyzer. ApoB and GAPDH mRNA levels in liver are measured via QG assay. ApoB

protein in plasma is measured via ELISA. Total cholesterol in plasma is measured via standard enzymatic/colorimetric assay.

Results

[0377] There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. Figure 6 shows that the tolerability of SNALP prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters. There was a tolerability difference between syringe citrate and syringe PBS at constant siRNA dosage, but that was likely an artifact dependent on the different final lipid:drug (L:D) ratios of these two preparations.

[0378] Figure 7 shows that the efficacy of the 1:57 SNALP prepared by gear pump was similar to the same SNALP prepared by syringe press. The tolerability profile was improved with the gear pump process, which could be attributed to increased initial encapsulation rate and decreased final L:D ratio.

Example 8. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Direct Dilution or In-Line Dilution Process.

[0379] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by a direct dilution or in-line dilution process at an input lipid to drug ratio of 6:1 or 9:1.

Experimental Design

[0380] Animal Model: Female BALB/c mice, 7 wks old.

[0381] siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

CBC/Diff:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
1	3	PBS	-	-
2	3	1|57 SNALP (9:1)	7 mg/kg	71 mg/kg
3	3	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Clinical Chemistry:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
4	4	PBS	-	-
5	4	1|57 SNALP (9:1)	9 mg/kg	92 mg/kg
6	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg
7	4	(6:1) New 1|57 SNALP	11 mg/kg	78 mg/kg

8	4	(6:1) New 1|57 SNALP	13 mg/kg	93 mg/kg
9	4	(6:1) New 1|57 SNALP	15 mg/kg	107 mg/kg
10	4	(6:1) New 1|57 SNALP	17 mg/kg	121 mg/kg
11	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Activity:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
12	4	PBS	-	-
13	4	1|57 SNALP (9:1)	0.05 mg/kg	0.51 mg/kg
14	4	1|57 SNALP (9:1)	0.1 mg/kg	1.02 mg/kg
15	4	1|57 SNALP (9:1)	0.2 mg/kg	2.04 mg/kg
16	4	(6:1) New 1|57 SNALP	0.05 mg/kg	0.36 mg/kg
17	4	(6:1) New 1|57 SNALP	0.1 mg/kg	0.71 mg/kg
18	4	(6:1) New 1|57 SNALP	0.2 mg/kg	1.42 mg/kg
19	4	(6:1) New 1|57 SNALP	0.4 mg/kg	2.85 mg/kg

Formulation:

[0382] Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 µm filter sterilized in crimp top vials.

[0383] Formulation Details:

1. "1|57 SNALP" used in this study is lipid composition 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by gear pump at an input lipid to drug ratio of 9:1 (28 mM lipids) or 6:1 (14 mM lipids).
2. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0384] Formulation Summary:

	1|57 SNALP Gear PBS In-Line	Particle Size			Final L:D
		Zavg (nm)	Poly	% Encap	(mg:mg)
322-051407-1	Input 9:1	78	0.07	93	10.2
322-051407-2	Input 6:1	81	0.05	92	7.1

Procedures

[0385] **Treatment:** Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 µl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0386] **Endpoint:** Animals are sacrificed on Day 1, 24 h after test article administration (Grps 1-10) or on Day 2, 48 h after test article administration (Grps 11-19).

[0387] **Groups 1-3:** Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into an EDTA microtainer, mixed immediately to prevent coagulation, and sent for analysis of CBC/Diff profile. Perform brief necropsy.

[0388] **Groups 4-11:** Blood is collected by cardiac puncture into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein. Perform brief necropsy.

[0389] **Groups 12-19:** Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed: liver. The liver is not weighed; the bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at –80°C. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0390] **Termination:** Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0391] **Data Analysis:** Tolerability of treatment regime is monitored by animal appearance and behavior, and body weight. Blood clinical chemistry and CBC/Diff profile is measured by automated analyzer. Liver ApoB mRNA is measured using the QuantiGene Assay. Plasma ApoB-100 is measured using ELISA. Plasma total cholesterol is measured using a standard enzymatic assay.

Results

Tolerability:

[0392] Figure 8 shows that there was very little effect on body weight 24 hours after 1:57 SNALP administration. The maximum weight loss of 3.6 ± 0.7% was observed at the highest drug dose of 17 mg/kg. There was also no obvious change in animal appearance/behavior at any of the dosages tested.

[0393] Figure 9 shows that there were no obvious changes in platelet count. Reduction of platelets can cause the mean platelet volume to increase as the body produces new platelets in compensation for the treatment-related decrease. Under the conditions of this study, the mean platelet volume did not change in SNALP-treated groups.

[0394] Figure 10 shows that clinically significant liver enzyme elevations (3xULN) occurred at drug dosages of 11 mg/kg for 1:57 SNALP at a lipid:drug (L:D) ratio of 10, and at 13 mg/kg at a L:D of 7. A slight dose response trend upwards in plasma total protein and globulin was also observed.

Efficacy:

[0395] Figure 11 shows that based on the liver mRNA QuantiGene analysis, the potency of the lower L:D SNALP was as good as that of the higher L:D SNALP at the tested drug dosages. In fact, the ApoB silencing activity was identical at the 0.05 and 0.1 mg/kg dosages. As such, the potency of the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) was similar to the potency of the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) at reducing ApoB expression.

[0396] Figure 12 shows that ApoB protein and total cholesterol levels were reduced to a similar extent by the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

Therapeutic Index:

[0397] This study demonstrates that both the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) caused about 60% ApoB liver mRNA silencing with a drug dose of 0.1 mg/kg. Interpolating from the available data points in Figure 10, a 10:1 final L:D ratio at 10 mg/kg may cause a similar degree of enzyme elevation as a 7:1 final L:D ratio at 13 mg/kg. Using these activity and toxicity points, the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio is (10 mg/kg) / (0.1 mg/kg) = 100 and the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is (13 mg/kg) / (0.1 mg/kg) = 130. Using this dataset, the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is 30% greater than the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio.

Example 9. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Increases Survival of Hep3B Tumor-Bearing Mice.

[0398] SNALP containing polo-like kinase 1 (PLK-1) siRNA (1:57 SNALP formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) were tested for their effects on the survival of CD1 nu/nu mice bearing Hep3B liver tumors. PLK-1 is a serine/threonine kinase containing two functional domains: (1) a kinase domain; and (2) a polo-box domain (*see, e.g.,* Barr *et al.*, *Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)). The activity and cellular concentration of PLK-1 are crucial for the precise regulation of cell division. PLK-1 is overexpressed in many cancer types including hepatoma and colon cancer, and PLK-1 expression often correlates with poor patient prognosis. Overexpression of PLK-1 (wild-type or kinase inactive) results in multinucleation (genetic instability). Hyperactive PLK-1 overrides the DNA damage checkpoint. Constitutive PLK-1 expression causes transformation of NIH 3T3 cells. PLK-1 phosphorylates the p53 tumor suppressor, thereby inhibiting the pro-apoptotic effects of p53. The PLK-1 siRNA used in this study are provided in Table 8. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the PLK-1 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 8. siRNA duplexes comprising sense and antisense PLK-1 RNA polynucleotides.

siRNA	PLK-1 siRNA Sequence	% Modified in DS Region
PLK1424 U4/GU	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' (SEQ ID NO.57) 3'-NNUC**U**AGUGGGAG**G**AAUUUAU-5' (SEQ ID NO.54)	6/38 = 15.8%
PLK1424 U4/G	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' (SEQ ID NO.57) 3'-NNUCUA**G**UG**G**GA**G**GAAUUUAU-5' (SEQ ID NO.56)	7/38 = 18.4%

Column 1: The number after "PLK" refers to the nucleotide position of the 5' base of the sense strand relative to the start codon (ATG) of the human PLK-1 mRNA sequence NM_005030. Column 2: 2'-O-methyl (2'OMe) nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. N = deoxythymidine (dT) nucleotide, uridine (U) ribonucleotide, or ribonucleotide having complementarity to the target sequence or the complementary strand thereof. Column 3: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

Experimental Groups

[0399] 20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay

A	20 to seed	I.H. $1.5x10^6$ Hep3B	Luc 1:57	9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10 x 2 mg/kg	When moribund	Survival Body Weights
B			PLK 1424 1:57	9				

Test Articles

[0400] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 20 ml of each SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	Luc U/U SNALP 1:57 (28mM lipid)
B	PLK1424 U4/GU SNALP 1:57 (28mM lipid) PLK1424 U4/G SNALP 1:57 (28mM lipid)

Procedures

Day 0 Mice will receive Anafen by SC injection (100 µg in 20 µl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25 µl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 µl) and 30G (3/8") needle. Cells will be injected slowly (~ 30 s) and a swab applied to the puncture wound immediately after needle withdrawal. After any bleeding has stopped (~1 min), the incision is closed with 5-6 sutures in the muscle wall and 3-4 skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.

Day 1	All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 µg in 20 µl saline).
Day 10	Mice will be randomized into the appropriate treatment groups.
Day 11	**Groups A, B – Day 11:** All Animals will be administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg). Dosing will be repeated for 5 consecutive days based on initial weight.
Day 14-35	**Groups A, B – Days 14, 17, 21, 25, 28, 32, 35:** All Animals will be re-administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg). **Body weights Groups:** Mice will be weighed on the day of dosing for 5 weeks, then twice weekly until close of the study. **Endpoint:** Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the treatment or tumor burden are terminated at the discretion of the vivarium staff.
Termination:	Mice are anesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.
Data Analysis:	Survival and body weights are assayed.

Results

[0401] Figure 13 shows the mean body weights of mice during therapeutic dosing of PLK1424 SNALP in the Hep3B intrahepatic (I.H.) tumor model. The treatment regimen was well tolerated with no apparent signs of treatment-related toxicity.

[0402] Figure 14 shows that treatment with 1:57 SNALP-formulated PLK1424 caused a significant increase in the survival of Hep3B tumor-bearing mice. This *in vivo* anti-tumor effect was observed in the absence of any apparent toxicity or immune stimulation.

Example 10. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Induces Tumor Cell Apoptosis in Hep3B Tumor-Bearing Mice.

[0403] The objectives of this study were as follows:

1. To determine the level of mRNA silencing in established Hep3B liver tumors following a single IV administration of PLK1424 SNALP.
2. To confirm the mechanism of mRNA silencing by detecting specific RNA cleavage products using RACE-PCR.
3. To confirm induction of tumor cell apoptosis by histopathology.

[0404] The 1:57 SNALP formulation (1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) was used for this study.

Experimental Groups

[0405] 20 SCID/beige mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	Sacrifice	Assay
A	20 to seed	I.H. $1x10^6$ Hep3B	PBS	6	1 x 2 mg/kg Day 20	24 h after treatment	Tumor QG Tumor RACE-PCR Histopathology
			Luc 1:57	7			
B			PLK 1424 1:57	7			
C							

Test Articles

[0406] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 2 ml of SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	PBS
B	Luc U/U 1:57 SNALP
C	PLK1424 U4/GU 1:57 SNALP

Procedures

Day 0 Mice will receive Anafen by SC injection (100 µg in 20 µl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the

	sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25µl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 µl) and 30G (3/8") needle. Cells will be injected slowly (~30 s) and a swab applied to the puncture wound immediately after needle withdrawal. After any bleeding has stopped (~1 min), the muscle wall incision is closed with 5-6 sutures. The skin incision is then closed with 3-4 metal skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.
Day 1	All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 µg in 20 µl saline**).**
Day 7	Mice will be randomized into the appropriate treatment groups.
Day 20	**Groups A-C:** Mice will be weighed and then administered either PBS, Luc, or PLK1424 SNALP by IV injection via the lateral tail vein. SNALP will be dosed at 2 mg/kg or equivalent volume (10 ml/kg) according to body weight.
Day 21	**Groups A-C**: All mice will be weighed and then euthanized by lethal anesthesia. Tumor bearing liver lobes from all mice in each group will be weighed and collected into RNALater for RNA analysis.
	Endpoint: Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the treatment or tumor burden are terminated at the discretion of the vivarium staff.
Termination:	Mice are anaesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.

Data Analysis: mRNA analysis of liver tumors by bDNA (QG) assay and RACE-PCR.

Tumor cell apoptosis by histopathology.

Results

[0407] Body weights were monitored from Day 14 onwards to assess tumor progression. On Day 20, 6 mice showing greatest weight loss were randomized into each of the 3 groups and treated. All six mice had substantial-large I.H. tumors at sacrifice (Day 21). Treatment of the remaining 14 mice was therefore initiated on the Day 21 (sacrifice Day 22). 10/14 mice had substantial tumors; 2/14 mice had small/probable tumors; and 2/14 mice had no visible tumor burden.

[0408] Figure 15 shows data from Quantigene assays used to measure human (tumor)-specific PLK-1 mRNA levels. A single 2 mg/kg dose of 1:57 SNALP reduced PLK-1 mRNA levels by about 50% in intrahepatic Hep3B tumors growing in mice.

[0409] Figure 16 shows that a specific cleavage product of PLK-1 mRNA was detectable in mice treated with PLK1424 SNALP by 5' RACE-PCR. No specific PCR product was detectable in mice treated with either PBS or control (Luc) SNALP. Nucleotide sequencing of the PCR product confirmed the predicted cleavage site by PLK1424 siRNA-mediated RNA interference in the PLK-1 mRNA.

[0410] Figure 17 shows Hep3B tumor histology in mice treated with either Luc SNALP (top) or PLK1424 SNALP (bottom). Luc SNALP-treated mice displayed normal mitoses in Hep3B tumors, whereas PLK1424 SNALP-treated mice exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors.

Conclusion

[0411] This example illustrates that a single administration of PLK1424 1:57 SNALP to Hep3B tumor-bearing mice induced significant *in vivo* silencing of PLK-1 mRNA. This reduction in PLK-1 mRNA was confirmed to be mediated by RNA interference using 5' RACE-PCR analysis. Importantly, PLK-1 mRNA silencing by the 1:57 SNALP formulation profoundly disrupted tumor cell proliferation (mitosis), causing subsequent apoptosis of tumor cells. As demonstrated in the previous example, this anti-tumor effect translated into extended survival times in the tumor-bearing mice.

Example 11. Comparison of 1:57 PLK-1 SNALP Containing Either PEG-cDMA or PEG-cDSA in a Subcutaneous Hep3B Tumor Model.

[0412] This example demonstrates the utility of the PEG-lipid PEG-cDSA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-distearyloxypropylamine) in the 1:57 formulation for systemically targeting distal (*e.g.,* subcutaneous) tumors. In particular, this example compares the tumor targeting ability of 1:57 PLK-1 SNALPs containing either PEG-cDMA (C_{14}) or PEG-cDSA (C_{18}). Readouts are tumor growth inhibition and PLK1 mRNA silencing. The PLK-1 siRNA used was PLK1424 U4/GU, the sequence of which is provided in Table 8.

[0413] Subcutaneous (S.C.) Hep3B tumors were established in scid/beige mice. Multidose anti-tumor efficacy of 1:57 PLK-1 SNALP was evaluated for the following groups (n=5 for each group): (1) "Luc-cDMA" - PEG-cDMA Luc SNALP; (2) "PLK-cDMA" - PEG-cDMA PLK-1 SNALP; and (3) "PLK-cDSA" - PEG-cDSA PLK-1 SNALP. Administration of 6 x 2mg/kg siRNA was initiated once tumors reached about 5 mm in diameter (Day 10). Dosing was performed on Days 10, 12, 14, 17, 19, and 21. Tumors were measured by caliper twice weekly.

[0414] Figure 18 shows that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B S.C. tumors. In particular, 5/5 tumors in the PLK1-cDSA treated mice appeared flat, measurable only by discoloration at the tumor site.

[0415] Figure 19 shows the mRNA silencing of 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration. The extent of silencing observed with the PLK1-cDSA SNALP correlated with the anti-tumor activity in the multi-dose study shown in Figure 18.

[0416] The Luc-cDMA SNALP-treated group, which had developed large S.C. tumors at Day 24, were then administered PLK-cDSA SNALP on Days 24, 26, 28, 31, 33, and 35. There was no additional dosing of the original PLK-1 SNALP-treated groups. The results from this crossover dosing study with large established tumors is provided in Figure 20, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0417] A comparison of the effect of PEG-cDMA and PEG-cDSA 1:57 SNALPs on PLK-1 mRNA silencing was performed using established intrahepatic Hep3B tumors in scid/beige mice. A single 2 mg/kg dose of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA was administered intravenously. Liver/tumor samples were collected at 24 and 96 hours after SNALP treatment. Control = 2 mg/kg Luc-cDMA SNALP at 24 hours.

[0418] Figure 21 shows that PLK-cDMA SNALP and PLK-cDSA SNALP had similar silencing activities after 24 hours, but that the PLK-cDSA SNALP may increase the duration of mRNA silencing in intrahepatic tumors.

[0419] Figure 22 shows the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA. The extended blood circulation times observed for the PLK-cDSA SNALP may enable the increased accumulation and activity at distal (*e.g.*, subcutaneous) tumor sites.

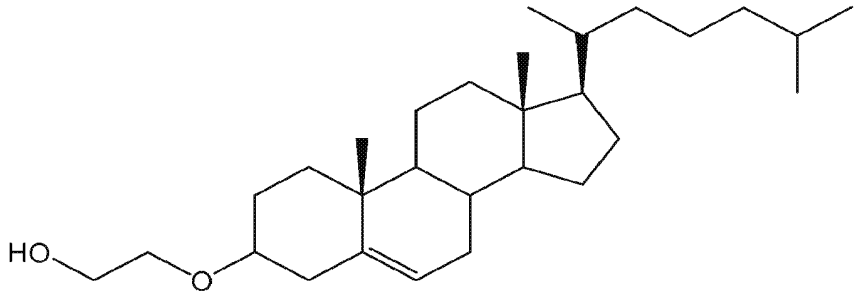
[0420] Thus, this study shows that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP can be used to preferentially target the liver.

Example 12. Synthesis of Cholesteryl-2'-Hydroxyethyl Ether.

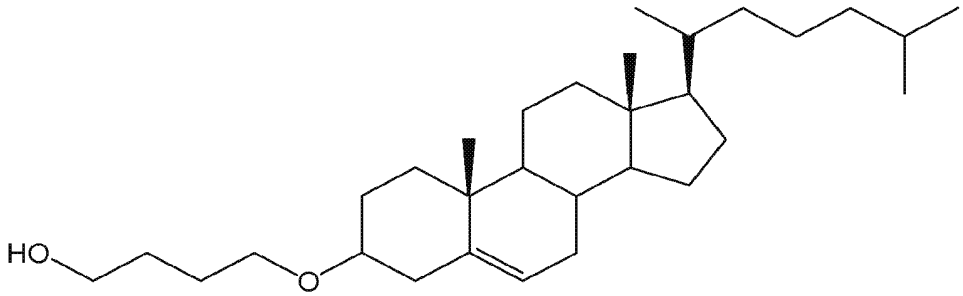
[0421] Step 1: A 250 ml round bottom flask containing cholesterol (5.0 g, 12.9 mmol) and a stir bar was sealed and flushed with nitrogen. Toluenesulphonyl chloride (5.0 g, 26.2 mmol) was weighed into a separate 100-mL round bottom flask, also sealed and flushed with nitrogen. Anhydrous pyridine (2 x 50 ml) was delivered to each flask. The toluenesulphonyl chloride solution was then transferred, via cannula, into the 250 ml flask, and the reaction stirred overnight. The pyridine was removed by rotovap, and methanol (80 ml) added to the residue. This was then stirred for 1 hour until a homogeneous suspension was obtained. The suspension was filtered, washed with acetonitrile (50 ml), and dried under vacuum to yield cholesteryl tosylate as a fluffy white solid (6.0 g, 86%).

[0422] Step 2: Cholesteryl tosylate (2.0 g, 3.7 mmol), 1,4-dioxane (50 mL), and ethylene glycol (4.6 g, 74 mmol) were added to a 100 ml flask containing a stir bar. The flask was fitted with a condenser, and refluxed overnight. The dioxane was then removed by rotovap, and the reaction mixture suspended in water (100 ml). The solution was transferred to a separating funnel and extracted with chloroform (3 x 100 ml). The organic phases were combined, washed with water (2 x 150 ml), dried over magnesium sulphate, and the solvent removed. The crude product was purified by column chromatography (5% acetone/hexane) to yield the product as a white solid (1.1 g, 69%).

[0423] The structures of the cholesterol derivatives cholesteryl-2'-hydroxyethyl ether and cholesteryl-4'-hydroxybutyl ether are as follows:



Cholesteryl-2'-hydroxyethyl ether



Cholesteryl-4'-hydroxybutyl ether

[0424] It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent applications, patents, PCT publications, and Genbank Accession Nos., are incorporated herein by reference for all purposes.

WHAT IS CLAIMED IS:

1. A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

2. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid comprises a small interfering RNA (siRNA).

3. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises from about 15 to about 60 nucleotides.

4. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one modified nucleotide.

5. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

6. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), or a mixture thereof.

7. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA).

8. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle.

9. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 52 mol % to about 62 mol % of the total lipid present in the particle.

10. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises cholesterol or a derivative thereof.

11. The nucleic acid-lipid particle of claim 10, wherein the cholesterol or derivative thereof comprises from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle.

12. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a phospholipid.

13. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.

14. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

15. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises from about 4 mol % to about 10 mol % of the total lipid present in the particle and the cholesterol comprises from about 30 mol % to about 40 mol % of the total lipid present in the particle.

16. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises from about 10 mol % to about 30 mol % of the total lipid present in the particle and the cholesterol comprises from about 10 mol % to about 30 mol % of the total lipid present in the particle.

17. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

18. The nucleic acid-lipid particle of claim 17, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

19. The nucleic acid-lipid particle of claim 18, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

20. The nucleic acid-lipid particle of claim 19, wherein the PEG has an average molecular weight of about 2,000 daltons.

21. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from about 1 mol % to about 2 mol % of the total lipid present in the particle.

22. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid in the nucleic acid-lipid particle is not substantially degraded after incubation of the particle in serum at 37°C for 30 minutes.

23. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

24. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a lipid:nucleic acid mass ratio of from about 5 to about 15.

25. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a median diameter of from about 40 nm to about 150 nm.

26. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.

27. A nucleic acid-lipid particle comprising:

(a) an siRNA;

(b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle;

(c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and

(d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

28. The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises DLinDMA.

29. The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises DLin-K-C2-DMA.

30. The nucleic acid-lipid particle of claim 27, wherein the PEG-lipid conjugate comprises a PEG-DAA conjugate.

31. The nucleic acid-lipid particle of claim 27, wherein the nucleic acid-lipid particle comprises about 61.5 mol % cationic lipid, about 36.9% cholesterol or a derivative thereof, and about 1.5 mol % PEG-lipid conjugate.

32. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 27 and a pharmaceutically acceptable carrier.

33. A nucleic acid-lipid particle, comprising:

(a) an siRNA;

(b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle;

(c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and

(d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

34. The nucleic acid-lipid particle of claim 33, wherein the cationic lipid comprises DLinDMA.

35. The nucleic acid-lipid particle of claim 33, wherein the cationic lipid comprises DLin-K-C2-DMA.

36. The nucleic acid-lipid particle of claim 33, wherein the phospholipid comprises DPPC.

37. The nucleic acid-lipid particle of claim 33, wherein the PEG-lipid conjugate comprises a PEG-DAA conjugate.

38. The nucleic acid-lipid particle of claim 33, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 7.1 mol % phospholipid, about 34.3 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

39. The nucleic acid-lipid particle of claim 33, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 20 mol % phospholipid, about 20 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

40. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 33 and a pharmaceutically acceptable carrier.

41. A method for introducing a nucleic acid into a cell, the method comprising:

contacting the cell with a nucleic acid-lipid particle of claim 1, 27, or 33.

42. The method of claim 41, wherein the cell is in a mammal.

43. A method for the *in vivo* delivery of a nucleic acid, the method comprising:

administering to a mammalian subject a nucleic acid-lipid particle of claim 1, 27, or 33.

44. The method of claim 43, wherein the administration is selected from the group consisting of oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal.

45. A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising:

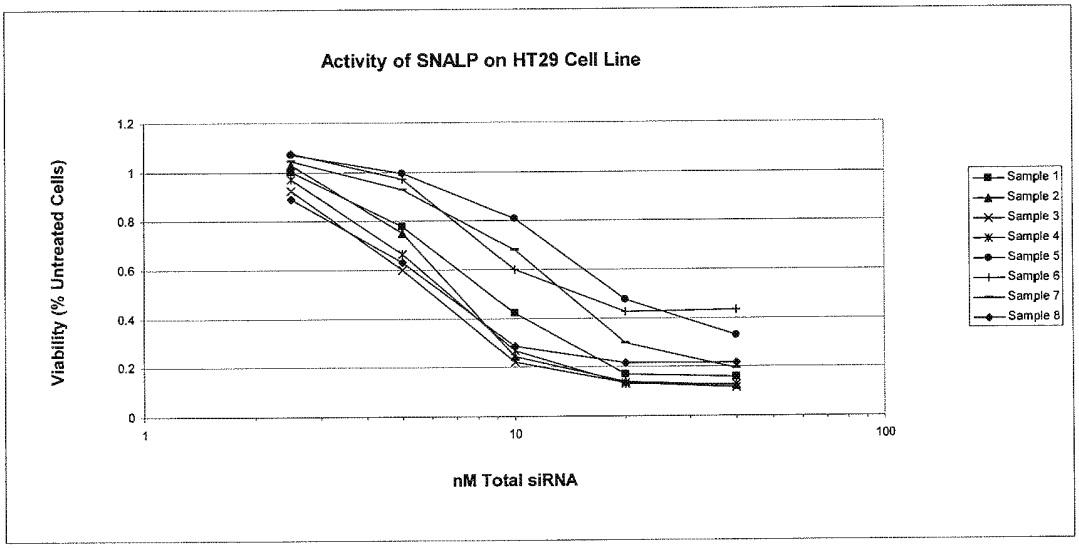
administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 1, 27, or 33.

46. The method of claim 45, wherein the disease or disorder is selected from the group consisting of a viral infection, a liver disease or disorder, and cancer.

ABSTRACT OF THE DISCLOSURE

The present invention provides novel, stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles. More particularly, the present invention provides stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (such as one or more interfering RNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP.

A


Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8

B

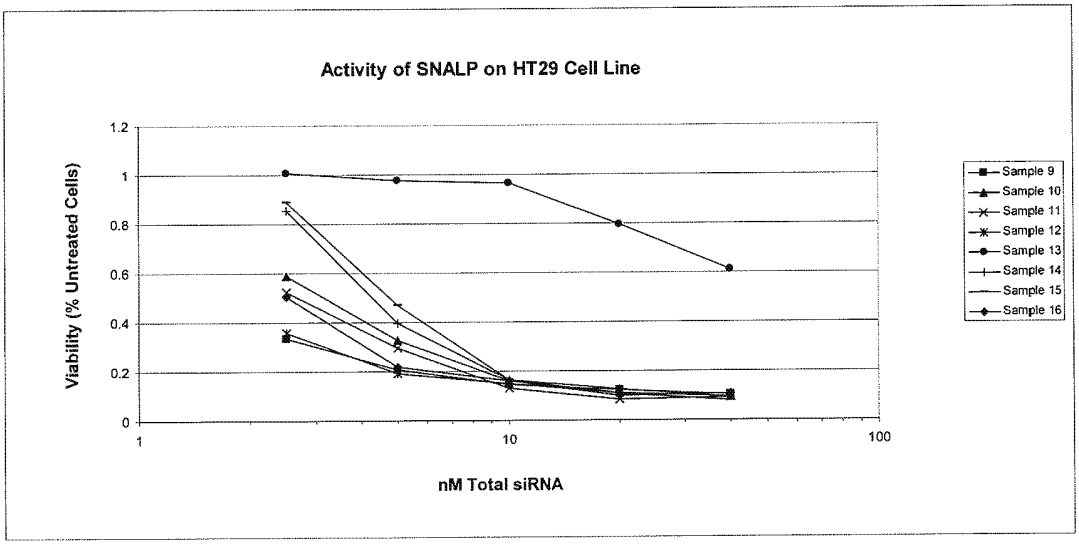

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 9
Sample 10
Sample 11
Sample 12
Sample 13
Sample 14
Sample 15
Sample 16

FIG. 1

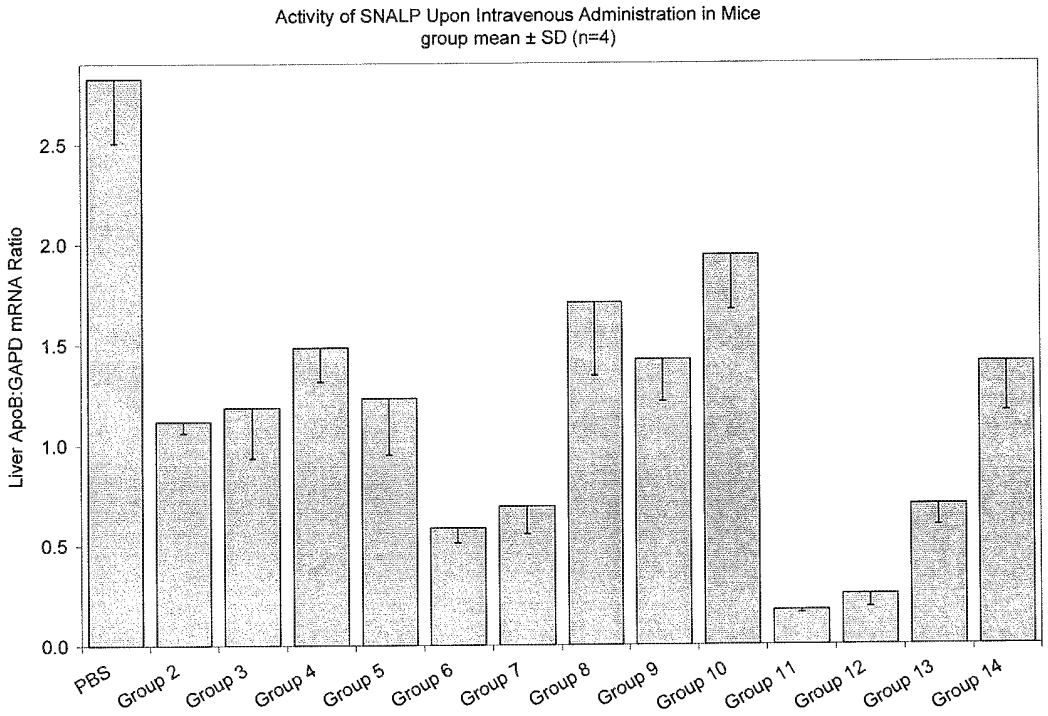

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.5
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

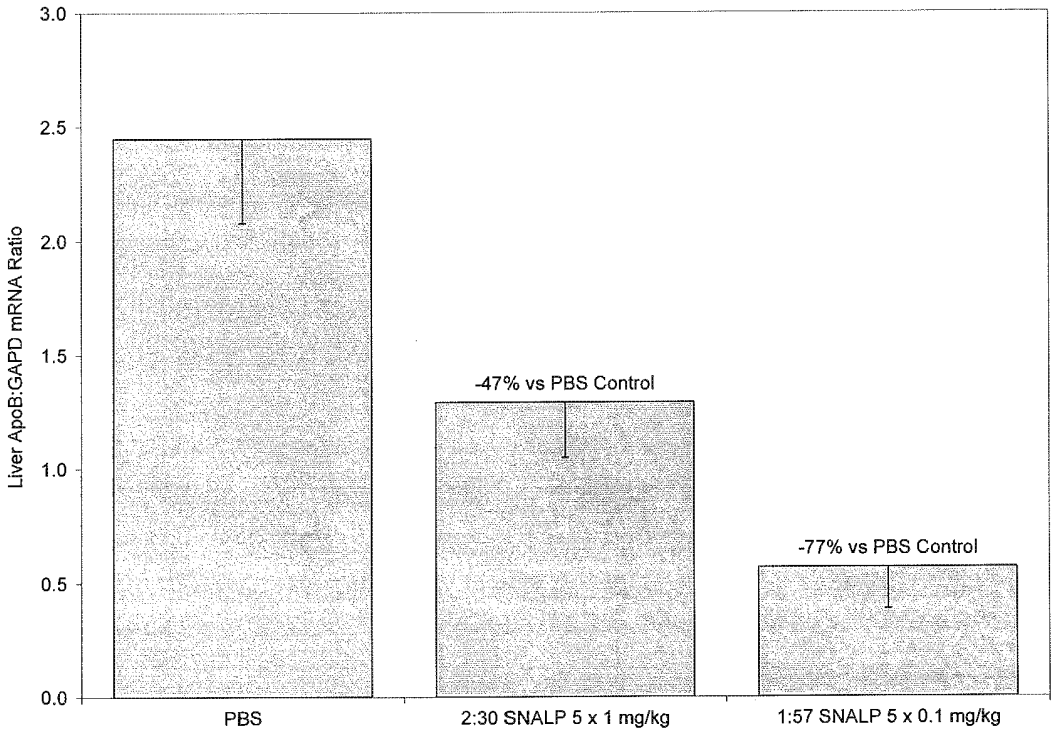

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Liver ApoB:GAPD mRNA Ratio
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5 x 1 mg/kg
1:57 SNALP 5 x 0.1 mg/kg

FIG. 2

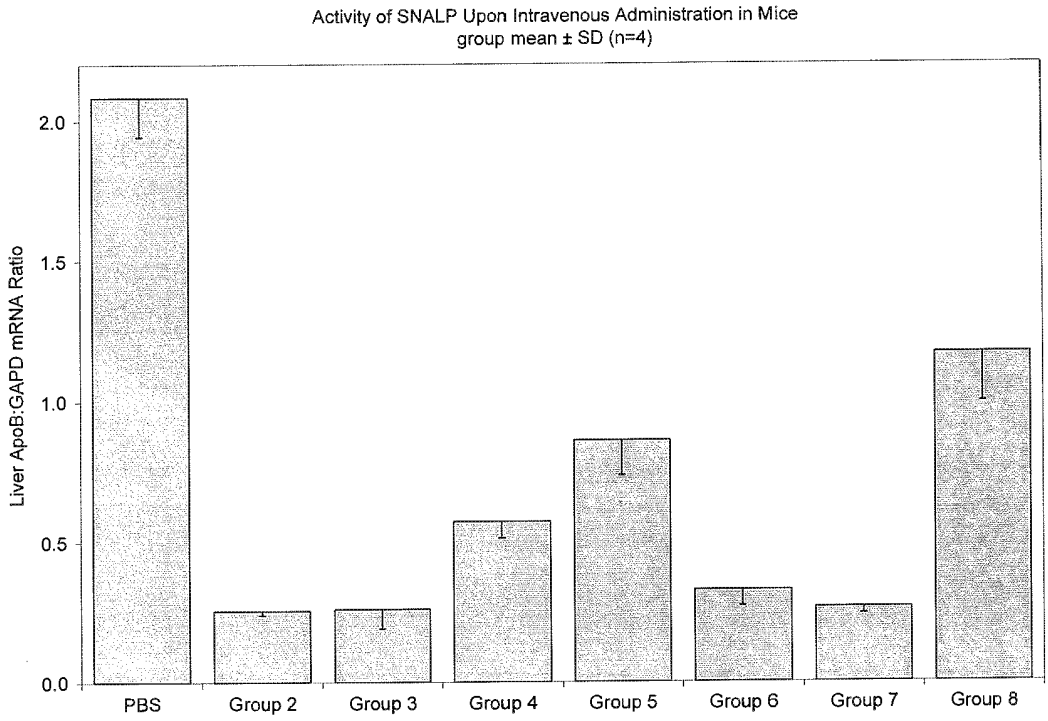

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

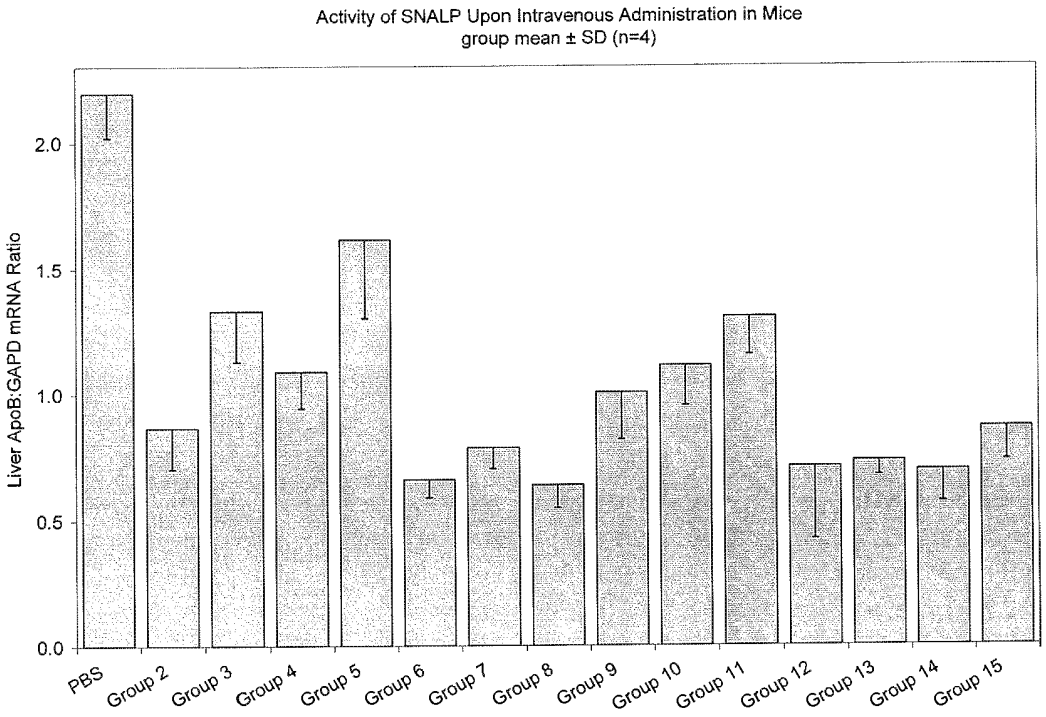
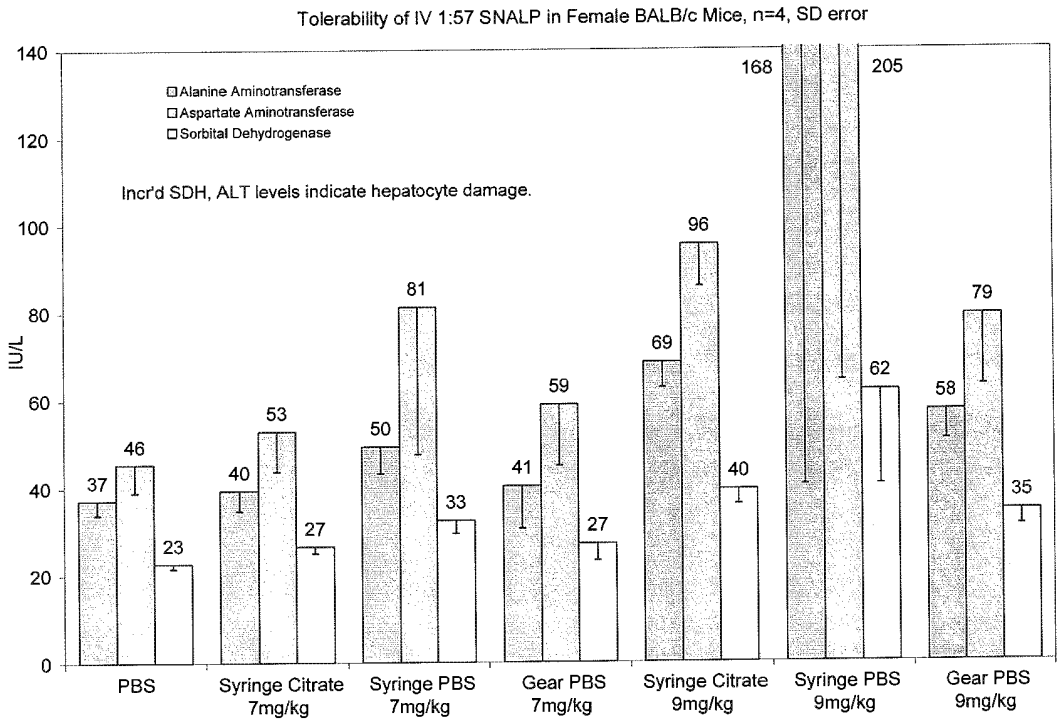

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

A


Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
0
20
40
60
80
100
120
140
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
168
205
62
58
79
35
PBS
Syringe Citrate 7mg/kg
Syringe PBS 7mg/kg
Gear PBS 7mg/kg
Syringe Citrate 9mg/kg
Syringe PBS 9mg/kg
Gear PBS 9mg/kg

B

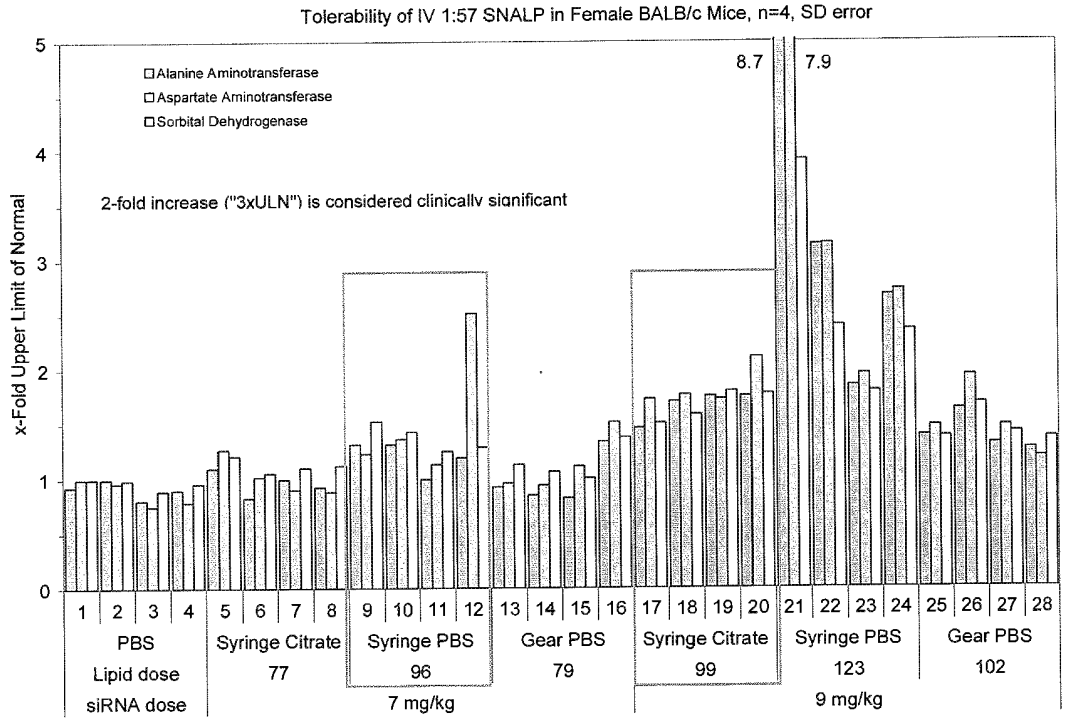
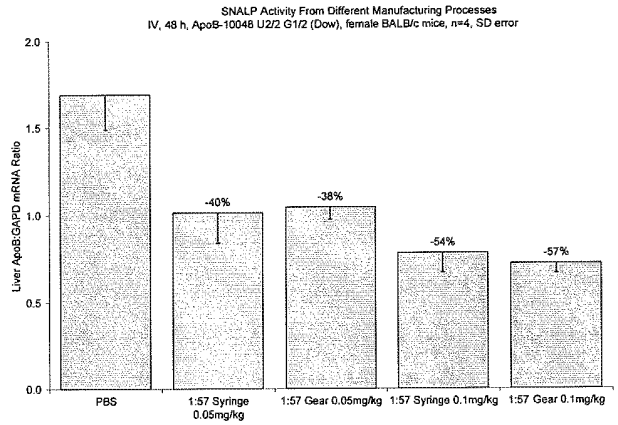
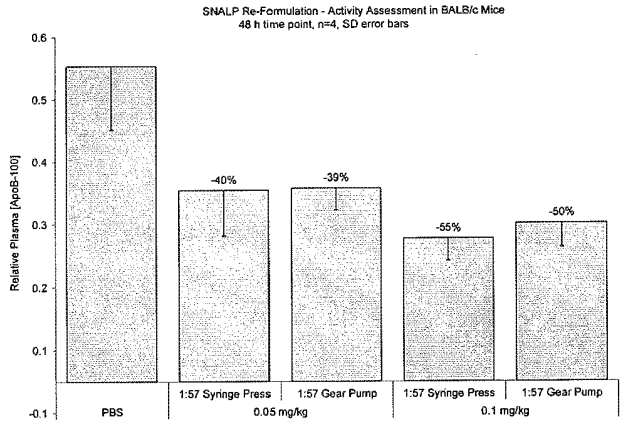

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
1
2
3
4
5
8.7
7.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
PBS
Lipid dose
siRNA dose
Syringe Citrate
77
Syringe PBS
96
7 mg/kg
Gear PBS
79
Syringe Citrate
99
Syringe PBS
123
9 mg/kg
Gear PBS
102

FIG. 6

A


SNALP Activity From Different Manufacturing Processes
IV, 48 h, ApoB-10048 U2/2 G1/2 (Dow), female BALB/c mice, n=4, SD error
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
-40%
-38%
-54%
-57%
PBS
1:57 Syringe 0.05mg/kg
1:57 Gear 0.05mg/kg
1:57 Syringe 0.1mg/kg
1:57 Gear 0.1mg/kg

B


SNALP Re-Formulation - Activity Assessment in BALB/c Mice
48 h time point, n=4, SD error bars
Relative Plasma [ApoB-100]
0.6
0.5
0.4
0.3
0.2
0.1
-0.1
-40%
-39%
-55%
-50%
PBS
1:57 Syringe Press
1:57 Gear Pump
0.05 mg/kg
1:57 Syringe Press
1:57 Gear Pump
0.1 mg/kg

C

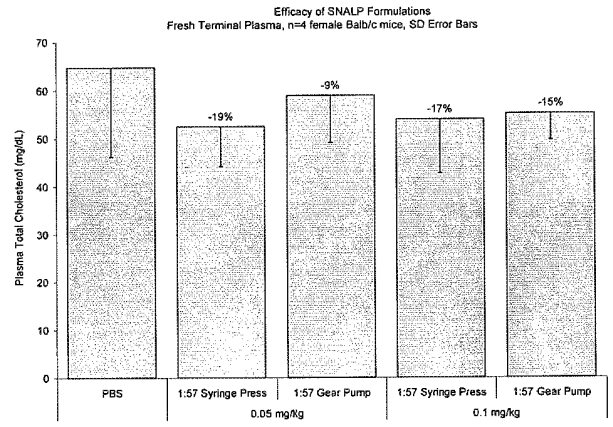

Efficacy of SNALP Formulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
70
60
50
40
30
20
10
0
-19%
-9%
-17%
-15%
PBS
1:57 Syringe Press
1:57 Gear Pump
0.05 mg/kg
1:57 Syringe Press
1:57 Gear Pump
0.1 mg/kg

FIG. 7

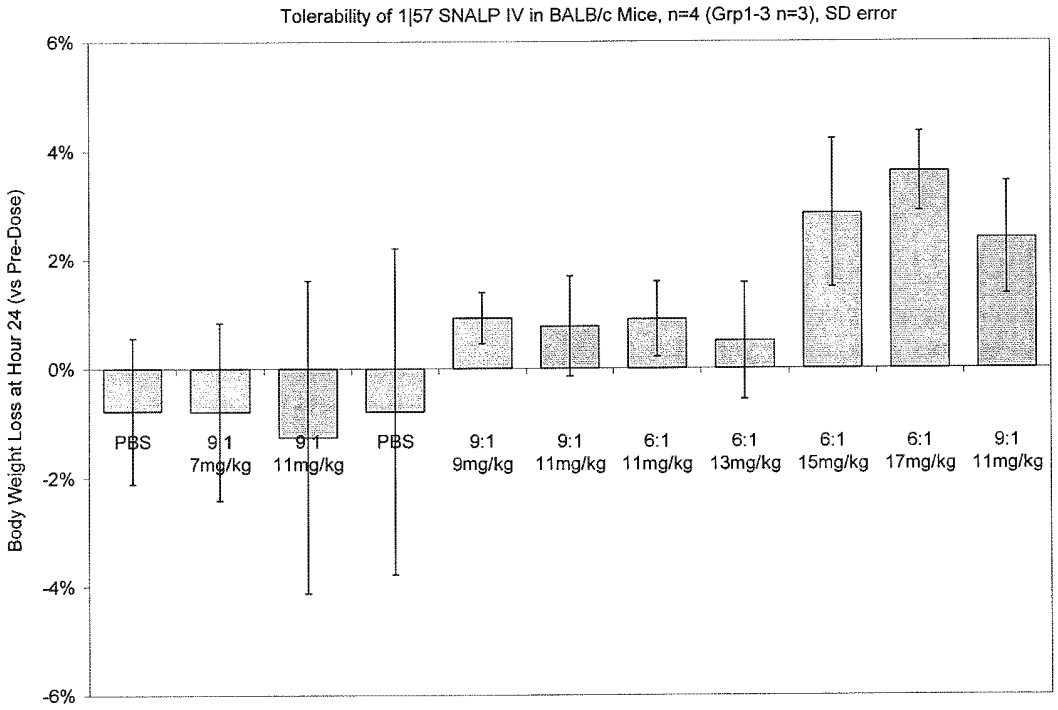

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

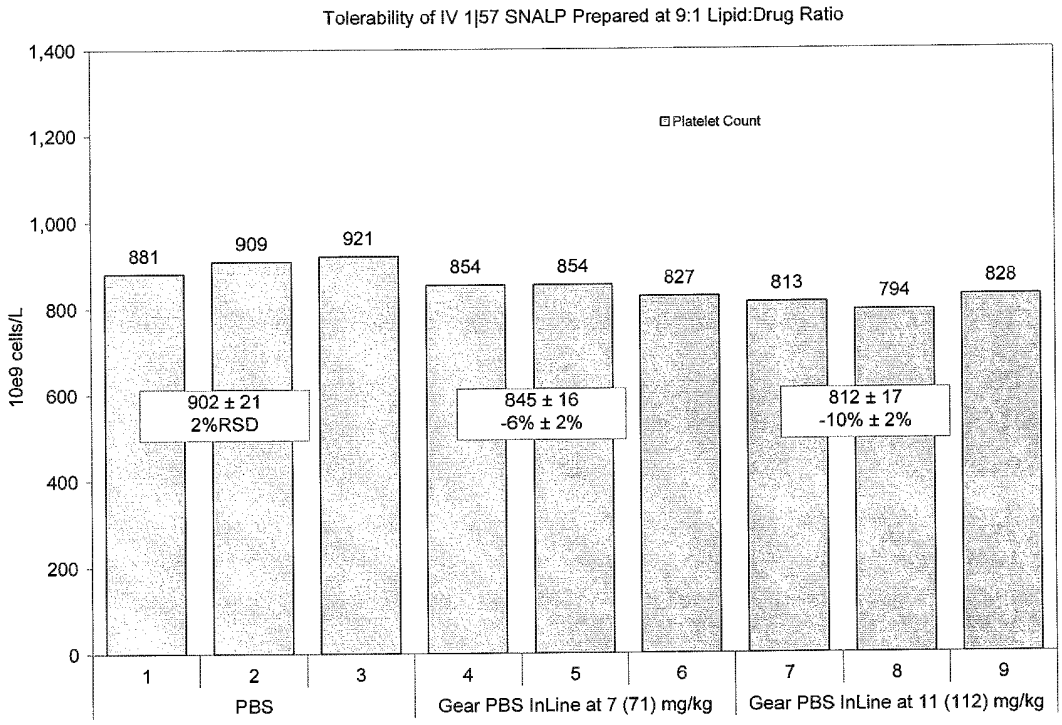
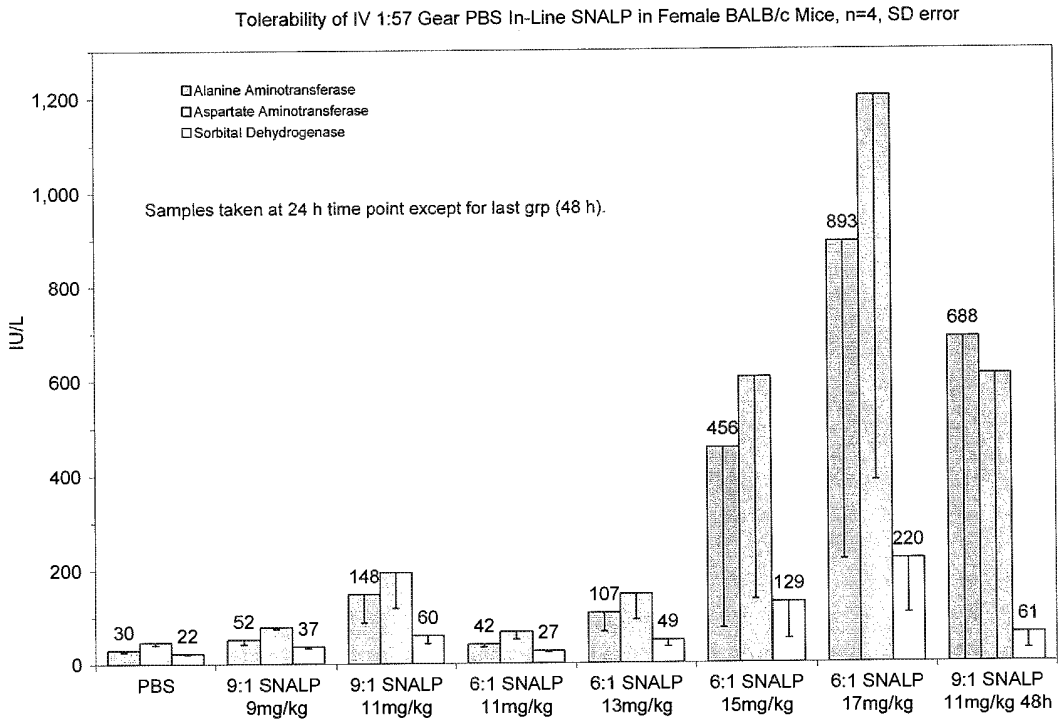

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
Platelet Count
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at 7 (71) mg/kg
Gear PBS InLine at 11 (112) mg/kg

FIG. 9

A


Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
688
61
PBS
9:1 SNALP 9mg/kg
9:1 SNALP 11mg/kg
6:1 SNALP 11mg/kg
6:1 SNALP 13mg/kg
6:1 SNALP 15mg/kg
6:1 SNALP 17mg/kg
9:1 SNALP 11mg/kg 48h

B

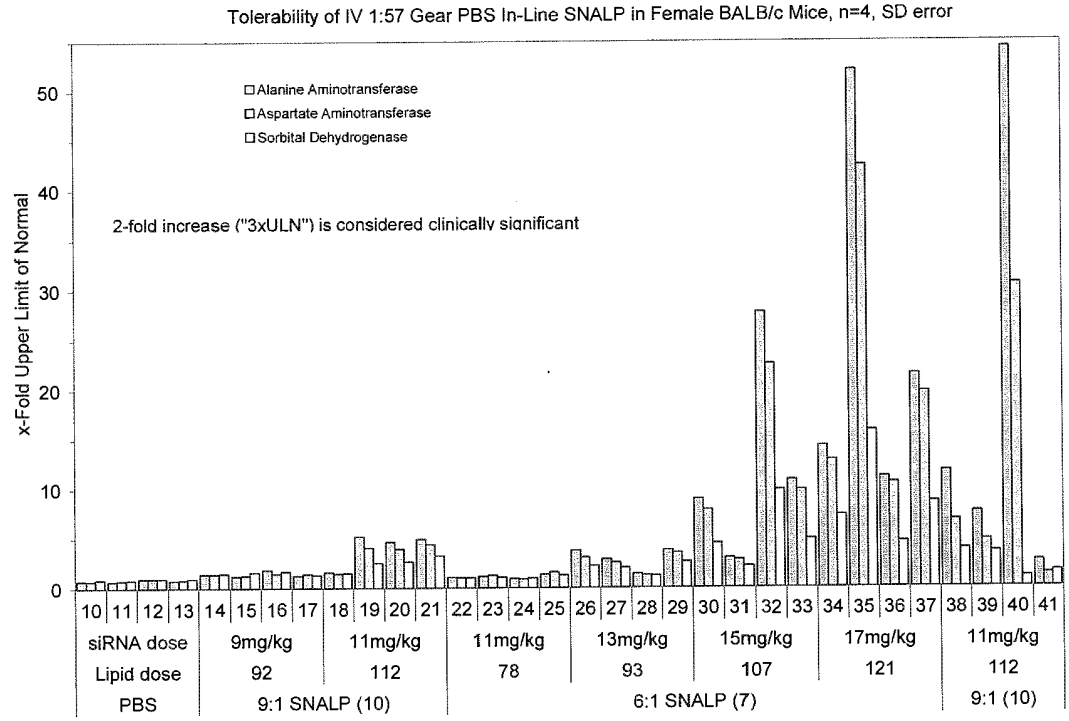
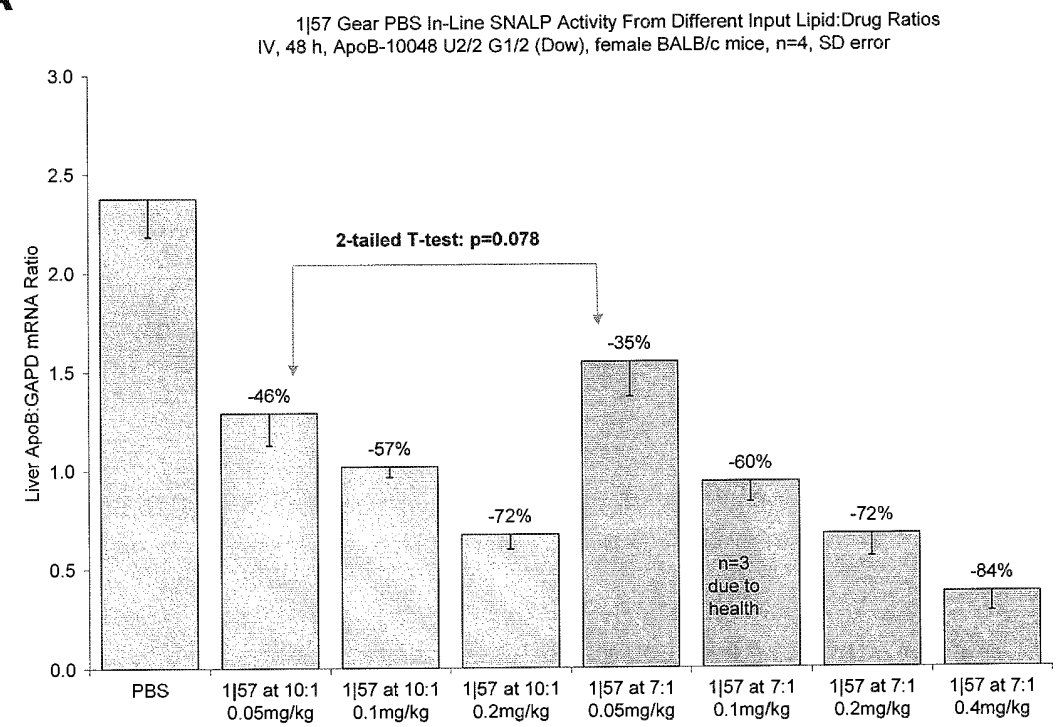

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
50
40
30
20
10
0
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
siRNA dose
9mg/kg
11mg/kg
11mg/kg
13mg/kg
15mg/kg
17mg/kg
11mg/kg
Lipid dose
92
112
78
93
107
121
112
PBS
9:1 SNALP (10)
6:1 SNALP (7)
9:1 (10)

FIG. 10

A


1|57 Gear PBS In-Line SNALP Activity From Different Input Lipid:Drug Ratios
IV, 48 h, ApoB-10048 U2/2 G1/2 (Dow), female BALB/c mice, n=4, SD error
Liver ApoB:GAPD mRNA Ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2-tailed T-test: p=0.078
-46%
-57%
-72%
-35%
-60%
n=3 due to health
-72%
-84%
PBS
1|57 at 10:1 0.05mg/kg
1|57 at 10:1 0.1mg/kg
1|57 at 10:1 0.2mg/kg
1|57 at 7:1 0.05mg/kg
1|57 at 7:1 0.1mg/kg
1|57 at 7:1 0.2mg/kg
1|57 at 7:1 0.4mg/kg

B

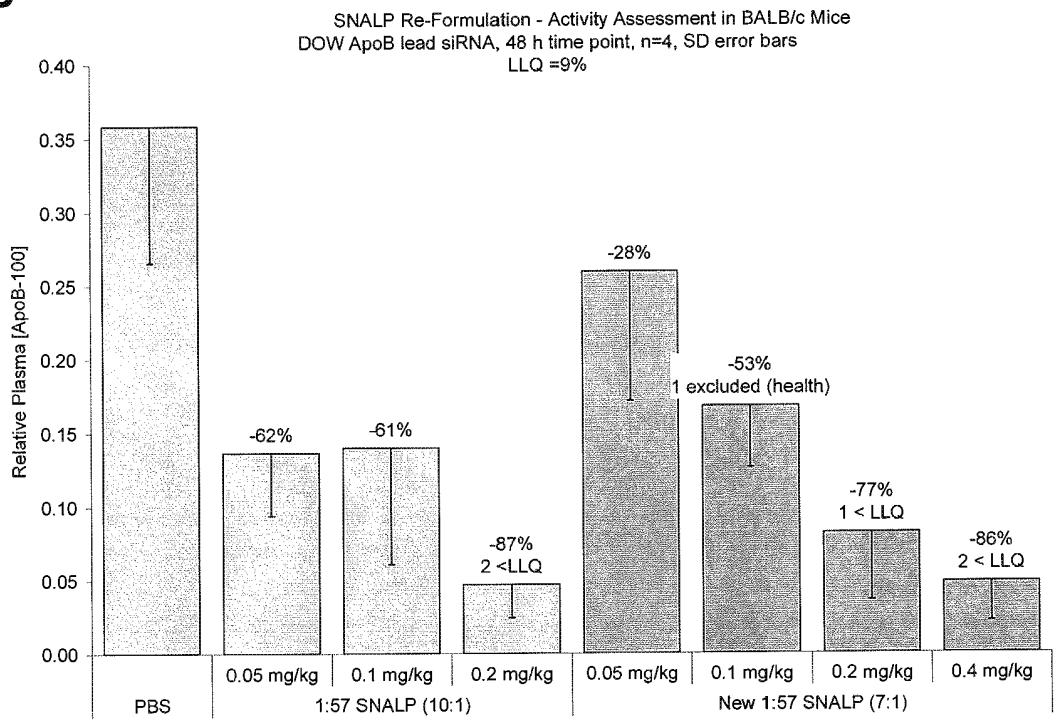

SNALP Re-Formulation - Activity Assessment in BALB/c Mice
DOW ApoB lead siRNA, 48 h time point, n=4, SD error bars
LLQ =9%
Relative Plasma [ApoB-100]
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-62%
-61%
-87%
2 <LLQ
-28%
-53%
1 excluded (health)
-77%
1 < LLQ
-86%
2 < LLQ
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
1:57 SNALP (10:1)
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
New 1:57 SNALP (7:1)

FIG. 11

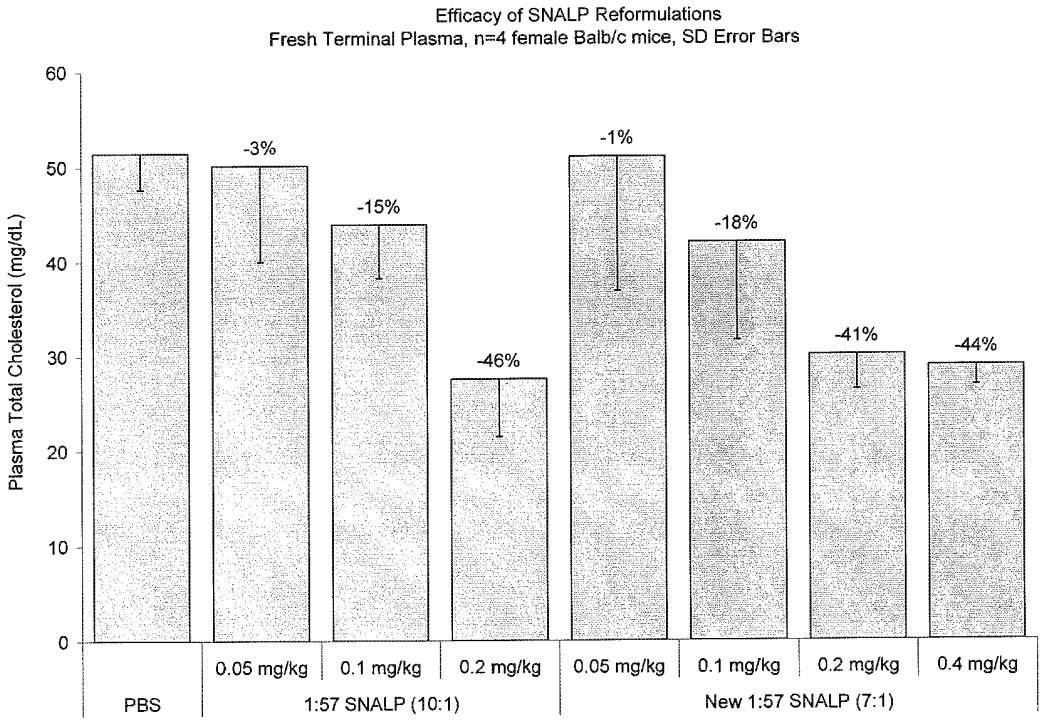

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
1:57 SNALP (10:1)
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
New 1:57 SNALP (7:1)

FIG. 12

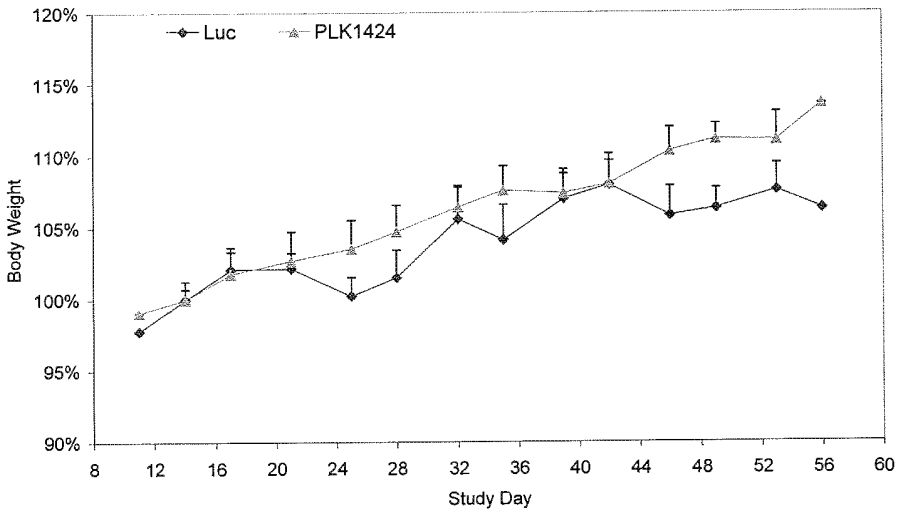

Luc
PLK1424
Body Weight
120%
115%
110%
105%
100%
95%
90%
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

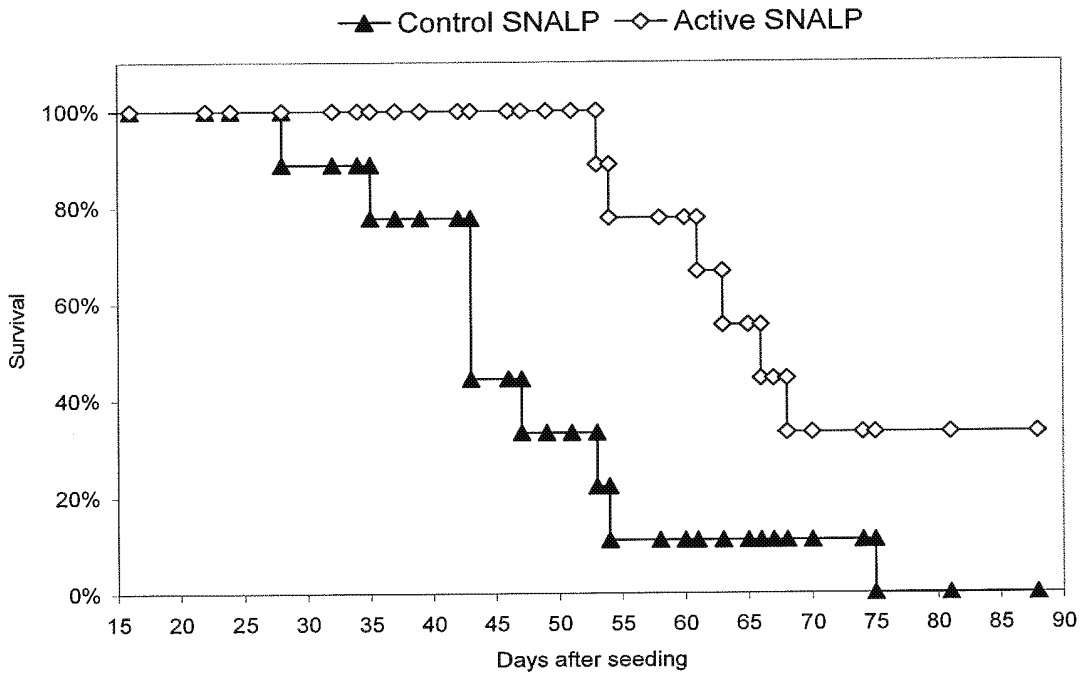

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
Days after seeding

FIG. 14

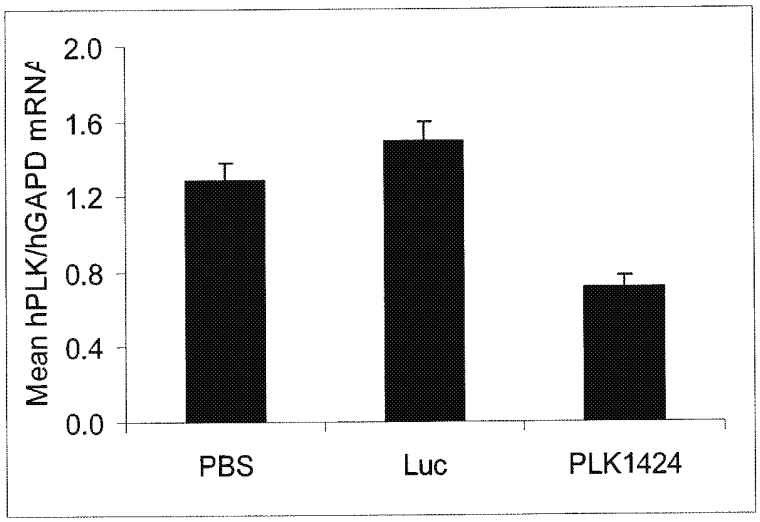


FIG. 15

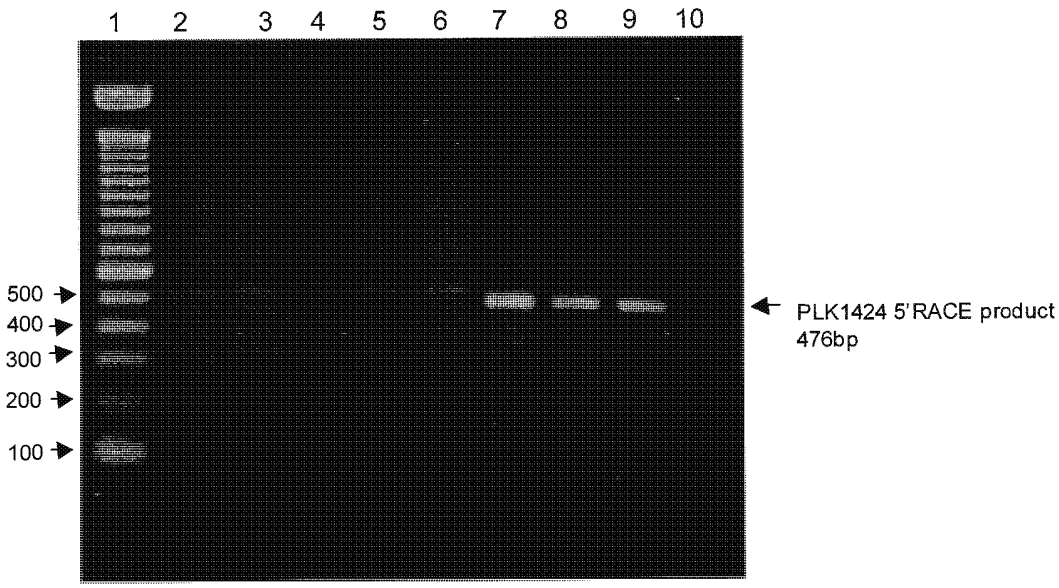
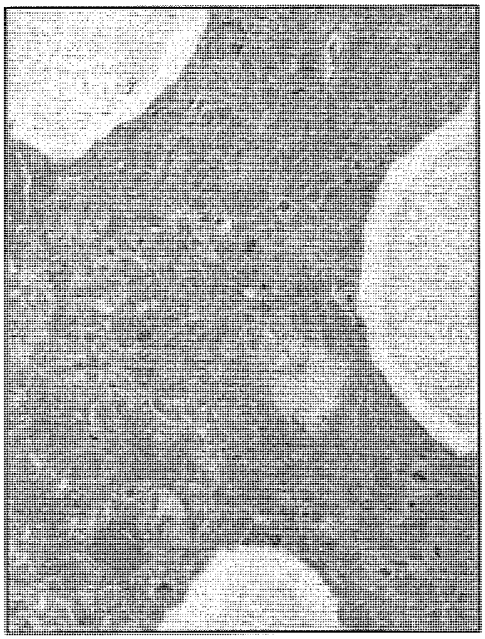
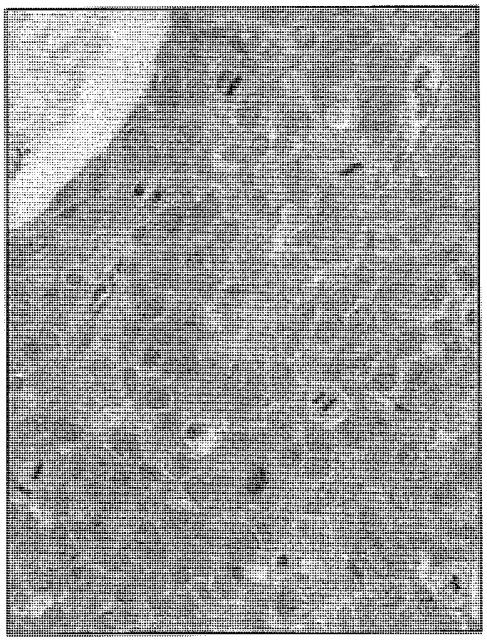

1
2
3
4
5
6
7
8
9
10
500
400
300
200
100
PLK1424 5'RACE product
476bp

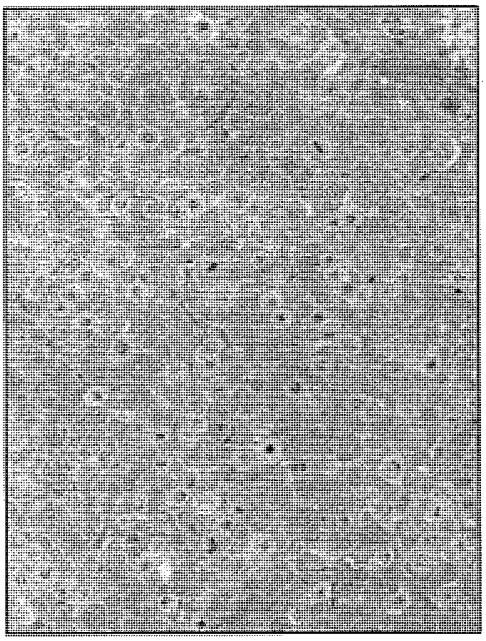
FIG. 16



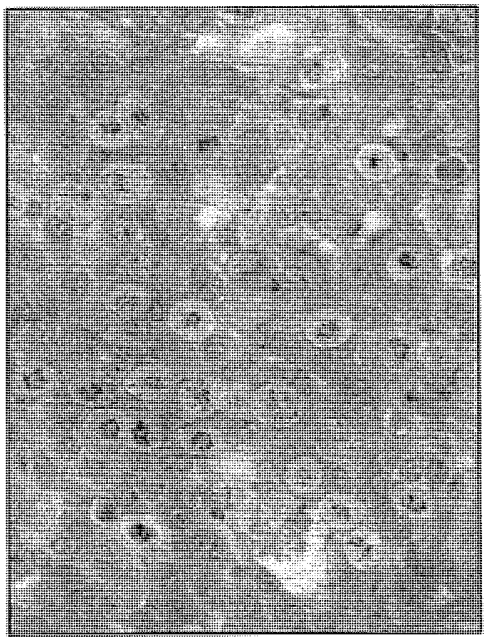
x200 mag



x400 mag



x200 mag



x400 mag

FIG. 17

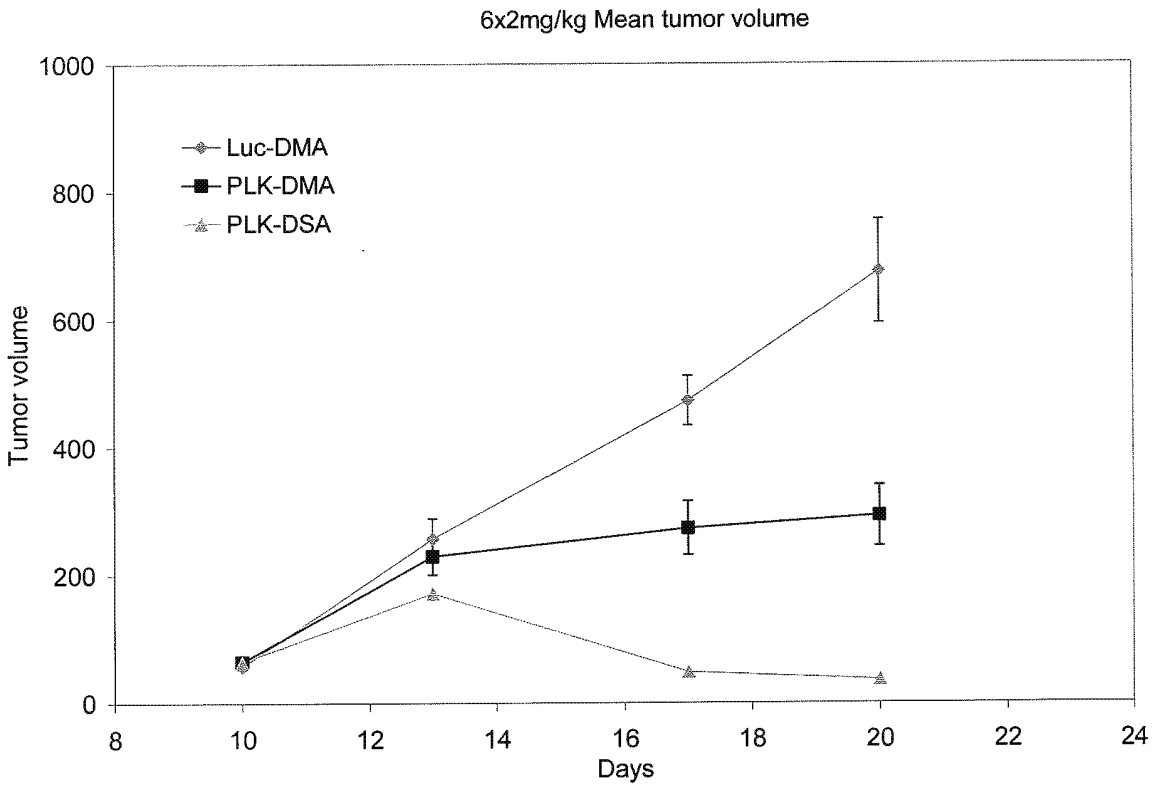

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

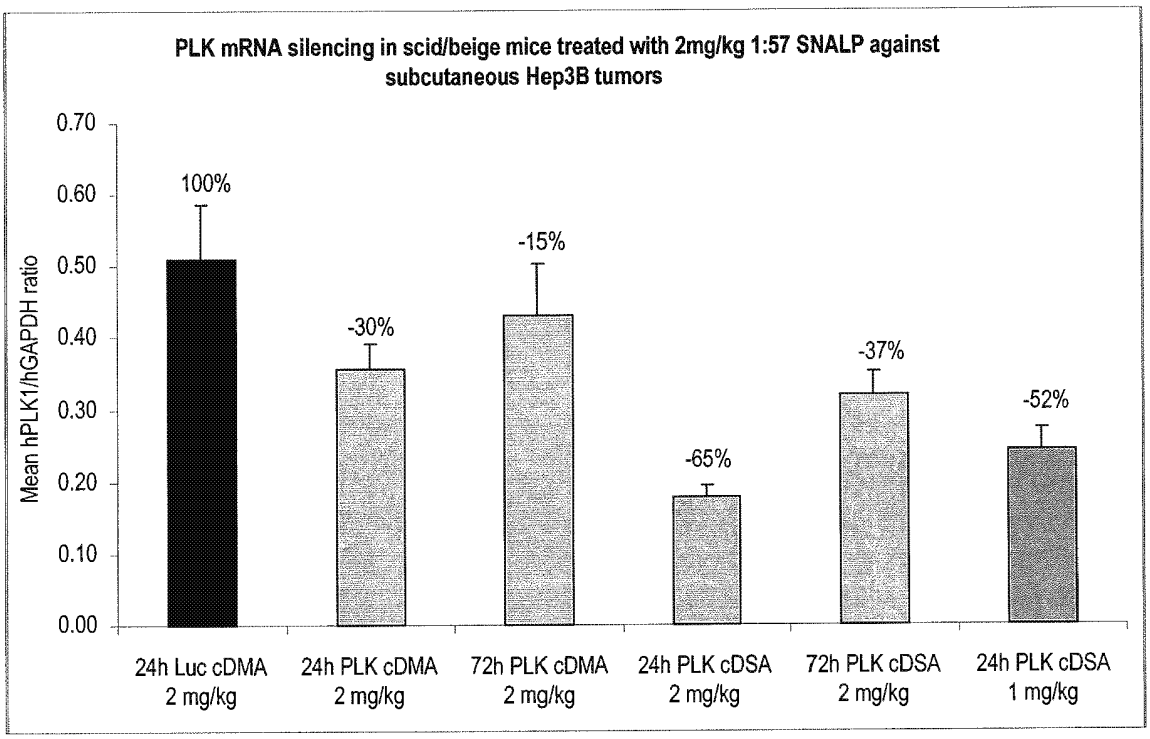

PLK mRNA silencing in scid/beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

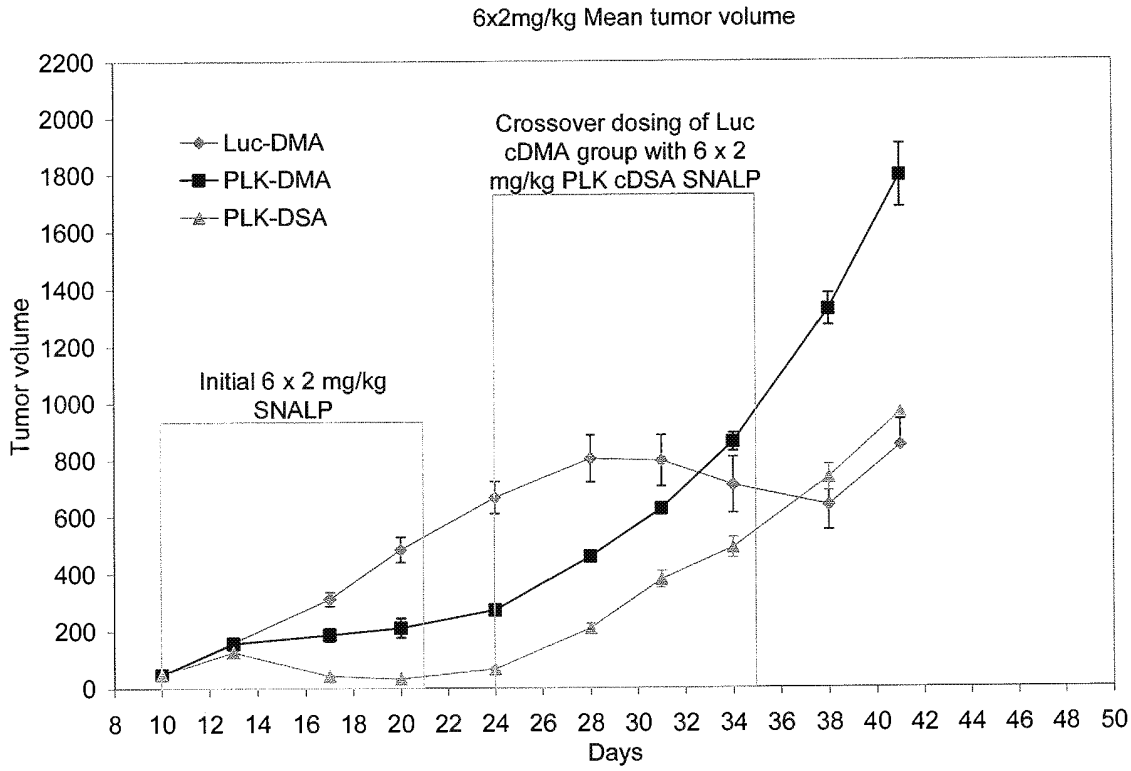

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
Initial 6 x 2 mg/kg SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

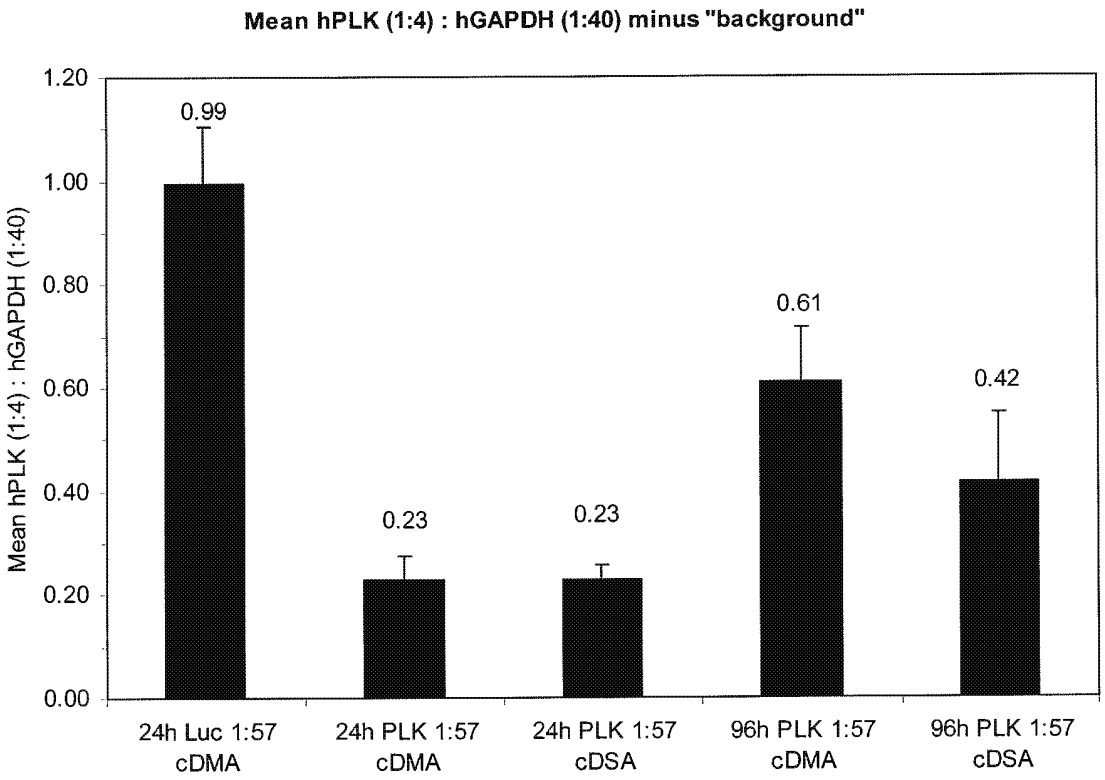

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

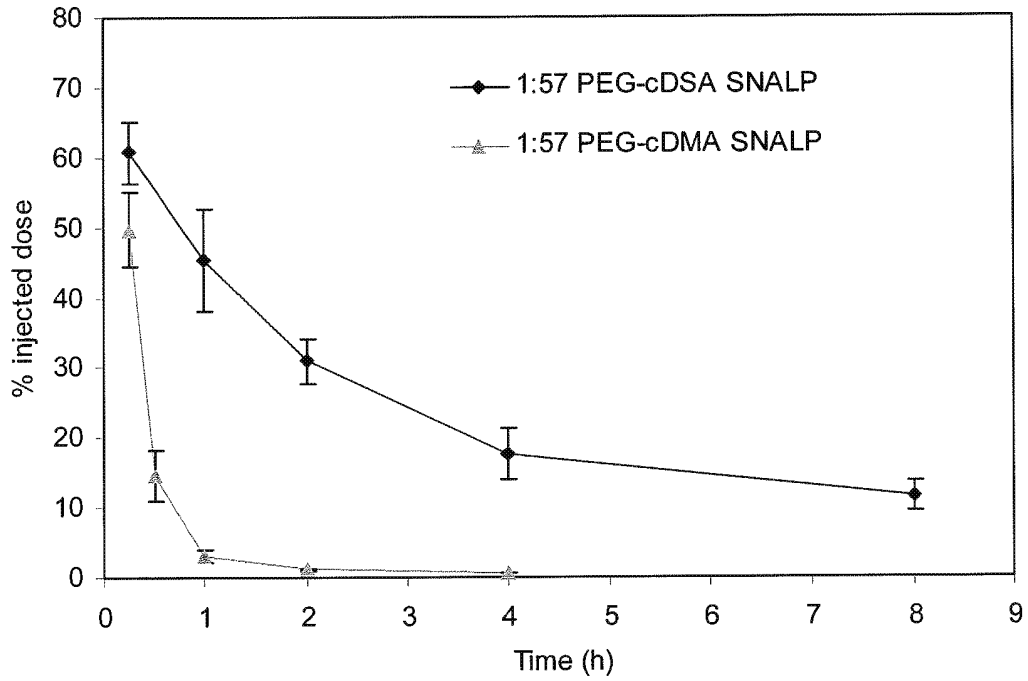

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

Electronic Acknowledgement Receipt

EFS ID:	5161378
Application Number:	12424367
International Application Number:	
Confirmation Number:	1406
Title of Invention:	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
First Named Inventor/Applicant Name:	Ian MacLachlan
Customer Number:	20350
Filer:	Joe Chao-Peng/Judith Cotham
Filer Authorized By:	Joe Chao-Peng
Attorney Docket Number:	020801-007710US
Receipt Date:	15-APR-2009
Filing Date:	
Time Stamp:	17:48:04
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		020801_007710US_trans_app_041509.PDF	290238 9ad6f59cbac1d1ef276a5d6ba83c223225fe33db	yes	7

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Transmittal of New Application	1	1
	Oath or Declaration filed	2	3
	Application Data Sheet	4	7

Warnings:					
Information:					
2		020801_007710US_nonprov_app_041509.pdf	547090 ba736335356eaf8797aa887c2e26e7083f4e9bfd	yes	120

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Specification	1	114
	Claims	115	119
	Abstract	120	120

Warnings:					
Information:					
3	Drawings-other than black and white line drawings	020801_007710US_figs_041509.PDF	1905563 c93a611c02bdb90b1a8c5fc86180a52367920acb	no	22
Warnings:					
Information:					
		Total Files Size (in bytes):	2742891		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

DocCode - SCORE

SCORE Placeholder Sheet for IFW Content

Application Number: 12424367 Document Date: 4/15/2009 5:48:04 PM

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

- Drawings – Other than Black and White Line Drawings

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

To access the documents in the SCORE database, refer to instructions developed by SIRA.

At the time of document entry (noted above):

- Examiners may access SCORE content via the eDAN interface.
- Other USPTO employees can bookmark the current SCORE URL (http://es/ScoreAccessWeb/).
- External customers may access SCORE content via the Public and Private PAIR interfaces.

Form Revision Date: February 8, 2006

JOINT APPENDIX 56

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on November, 2009

TOWNSEND and TOWNSEND and CREW LLP

By: Judith Cotham

PATENT
Attorney Docket No.: 020801-007710US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

MacLACHLAN *et al.*

Application No.: 12/424,367

Filed: April 15, 2009

For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Customer No.: 20350

Confirmation No. 1406

Examiner: Not yet assigned

Art Unit: 1636

COMMUNICATION UNDER

37 C.F.R. §§ 1.821-1.825

AND

PRELIMINARY AMENDMENT

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

Prior to examination of the above-referenced application, please enter the following amendments and remarks:

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims which begins on page 4 of this paper.

Amendments to the Drawings begin on page 9 of this paper and include both an attached replacement sheet and an annotated sheet showing changes.

Remarks begin on page 10 of this paper.

Amendments to the Specification:

Please replace Table 1 beginning at page 92, line 14, with the following:

--Table 1. siRNA duplex comprising sense and antisense Eg5 RNA polynucleotides.

Modification	Eg5 2263 siRNA sequence	SEQ ID NO:	% 2'OMe-Modified	% Modified in DS Region
U/U	5'-C**U**GAAGACC**U**GAAGACAA**U**dTdT-3' 3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUUA-5'	1 2	6/42 = 14.3%	6/38 = 15.8%

Column 1: "U/U" = 2'OMe-uridine modified siRNA duplex; Column 2: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. "dT" = deoxythymidine. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.--

Please replace Table 3 beginning at page 94, line 7, with the following:

--Table 3. siRNA duplex comprising sense and antisense ApoB RNA polynucleotides.

Position	Modification	ApoB siRNA sequence	SEQ ID NO:	% 2'OMe-Modified	% Modified in DS Region
10048	U2/2 G1/2	5'-AGU**G**UCA**U**CACAC**U**GAAUACC-3' 3'-GU**U**CACAGUAGU**G**U**G**AC**U**UAU-5'	3 4	7/42 = 16.7%	7/38 = 18.4%

Column 1: The number refers to the nucleotide position of the 5' base of the sense strand relative to the mouse ApoB mRNA sequence XM_137955. Column 2: The numbers refer to the distribution of 2'OMe chemical modifications in each strand. Column 3: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. Column 4: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 5: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.--

Please replace Table 8 beginning at page 105, line 19, with the following:

--Table 8. siRNA duplexes comprising sense and antisense PLK-1 RNA polynucleotides.

siRNA	PLK-1 siRNA Sequence	SEQ ID NO:	% Modified in DS Region
PLK1424 U4/GU	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' ~~(SEQ ID NO.57)~~ 3'-NNUC**U**AGUGGGAG**G**AAUUUAU-5' ~~(SEQ ID NO.54)~~	5 6	6/38 = 15.8%
PLK1424 U4/G	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' ~~(SEQ ID NO.57)~~ 3'-NNUCUA**G**UG**G**GA**G**GAAUUUAU-5' ~~(SEQ ID NO.56)~~	5 7	7/38 = 18.4%

Column 1: The number after "PLK" refers to the nucleotide position of the 5' base of the sense strand relative to the start codon (ATG) of the human PLK-1 mRNA sequence NM_005030. Column 2: 2'-O-methyl (2'OMe) nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. N = deoxythymidine (dT) nucleotide, uridine (U) ribonucleotide, or ribonucleotide having complementarity to the target sequence (antisense strand) or the complementary strand thereof (sense strand). Column 3: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.--

Please insert the accompanying paper copy of the Sequence Listing, page numbers 1 to 4, at the end of the application.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1. (Currently amended) A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from about 50 mol % to about **[[85]]** 65 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising ~~**from about 13 mol % to about 49.5 mol %**~~ a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the phospholipid comprises from about 4 mol % to about 10 mol % of the total lipid present in the particle and the cholesterol or derivative thereof comprises from about 30 mol % to about 40 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

2. (Original) The nucleic acid-lipid particle of claim 1, wherein the nucleic acid comprises a small interfering RNA (siRNA).

3. (Original) The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises from about 15 to about 60 nucleotides.

4. (Original) The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one modified nucleotide.

5. (Original) The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

6. (Original) The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), or a mixture thereof.

7-8. (Canceled)

9. (Original) The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 52 mol % to about 62 mol % of the total lipid present in the particle.

10-13. (Canceled)

14. (Currently amended) The nucleic acid-lipid particle of claim **[[13]]** 1, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

15-16. (Canceled)

17. (Original) The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

18. (Original) The nucleic acid-lipid particle of claim 17, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

19. (Original) The nucleic acid-lipid particle of claim 18, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

20. (Original) The nucleic acid-lipid particle of claim 19, wherein the PEG has an average molecular weight of about 2,000 daltons.

21. (Original) The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from about 1 mol % to about 2 mol % of the total lipid present in the particle.

22. (Original) The nucleic acid-lipid particle of claim 1, wherein the nucleic acid in the nucleic acid-lipid particle is not substantially degraded after incubation of the particle in serum at 37°C for 30 minutes.

23. (Original) The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

24. (Original) The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a lipid:nucleic acid mass ratio of from about 5 to about 15.

25. (Original) The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a median diameter of from about 40 nm to about 150 nm.

26. (Original) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.

27. (Currently amended) A nucleic acid-lipid particle comprising:

(a) ~~an siRNA~~ a nucleic acid;

(b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle;

(c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and

(d) a ~~PEG-lipid conjugate~~ conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

28-30. (Canceled)

31. (Currently amended) The nucleic acid-lipid particle of claim **[[27]]** 51, wherein the nucleic acid-lipid particle comprises about 61.5 mol % cationic lipid, about 36.9% cholesterol or a derivative thereof, and about 1.5 mol % PEG-lipid conjugate.

32. (Original) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 27 and a pharmaceutically acceptable carrier.

33-37. (Canceled)

38. (Currently amended) The nucleic acid-lipid particle of claim **[[33]]** 17, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 7.1 mol % phospholipid, about 34.3 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

39-46. (Canceled)

47. (New) The nucleic acid-lipid particle of claim 1, wherein the phospholipid comprises from about 5 mol % to about 9 mol % of the total lipid present in the particle.

48. (New) The nucleic acid-lipid particle of claim 1, wherein the cholesterol or derivative thereof comprises from about 32 mol % to about 36 mol % of the total lipid present in the particle.

49. (New) The nucleic acid-lipid particle of claim 27, wherein the nucleic acid comprises a small interfering RNA (siRNA).

50. (New) The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), or a mixture thereof.

51. (New) The nucleic acid-lipid particle of claim 27, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

52. (New) The nucleic acid-lipid particle of claim 51, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

53. (New) The nucleic acid-lipid particle of claim 52, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

54. (New) The nucleic acid-lipid particle of claim 27, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

Amendments to the Drawings:

The attached replacement sheets of drawings includes changes to the margins and formatting only, as required by the Notice to File Missing Parts. No new matter has been added.

Attachment: Replacement Sheets (22 sheets)

REMARKS

I. STATUS OF THE CLAIMS

After entry of this amendment, claims 1-6, 9, 14, 17-27, 31, 32, 38, and 47-54 are pending in this application and are presented for examination. Claims 7, 8, 10-13, 15, 16, 28-30, 33-37, and 39-46 have been canceled without prejudice to future prosecution. Claims 1, 14, 27, 31, and 38 have been amended. Claims 47-54 are newly added.

Support for the amendments to claim 1 is found, for example, in original claims 13 and 15, and in paragraph [0116] on page 24 of the instant specification. Support for the amendments to claim 27 is found, for example, in original claim 1 and in paragraph [0145] on page 32 of the instant specification.

Claim 14 has been amended to establish dependency from claim 1. Claim 31 has been amended to establish dependency from claim 51. Claim 38 has been amended to establish dependency from claim 17.

Newly added claims 47 and 48 find support, for example, in paragraph [0133] on pages 27-28 of the instant specification.

Newly added claims 49-54 find support, for example, in original claims 2, 6, 17-19, and 23, respectively.

As such, no new matter has been introduced with the foregoing amendments.

II. SEQUENCE LISTING

In response to the request to comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, 37 C.F.R. §§ 1.821-1.825, that accompanied the Notice to File Missing Parts of Nonprovisional Application mailed May 13, 2009, Applicants submit herewith the required paper copy and computer readable copy of the Sequence Listing.

Applicants request entry of this amendment in adherence with 37 C.F.R. §§1.821 to 1.825. The computer readable form contains the above named sequences, SEQ ID NOS:1-7, and the paper copy of the sequence information was printed from the computer readable form.

The information contained in the computer readable form was prepared through the use of the software program "FastSEQ" and is identical to that of the paper copy. This amendment contains no new matter.

CONCLUSION

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

Joe C. Hao
Reg. No. 55,246

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 415-576-0200
Fax: 415-576-0300
Attachments
JCH:dmw
62298904 v1

JOINT APPENDIX 57

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on June 1, 2010.

TOWNSEND and TOWNSEND and CREW LLP

By: /Judith Cotham/

PATENT
Attorney Docket No.: 020801-007710US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Edward Yaworski et al.

Application No.: 12/424,367

Filed: April 15, 2009

For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Customer No.: 20350

Confirmation No. 1406

Examiner: Pitrak, Jennifer S.

Technology Center/Art Unit: 1635

RESPONSE TO RESTRICTION REQUIREMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Restriction Requirement mailed March 31, 2010, please enter the following amendments and remarks. A Petition for a one-month extension of time accompanies this response.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks begin on page 6 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1. (Previously presented) A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from about 50 mol % to about 65 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the phospholipid comprises from about 4 mol % to about 10 mol % of the total lipid present in the particle and the cholesterol or derivative thereof comprises from about 30 mol % to about 40 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

2. (Original) The nucleic acid-lipid particle of claim 1, wherein the nucleic acid comprises a small interfering RNA (siRNA).

3. (Original) The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises from about 15 to about 60 nucleotides.

4. (Original) The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one modified nucleotide.

5. (Original) The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

6-8. (Canceled)

9. (Original) The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 52 mol % to about 62 mol % of the total lipid present in the particle.

10-13. (Canceled)

14. (Previously presented) The nucleic acid-lipid particle of claim 1, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

15-16. (Canceled)

17. (Original) The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

18. (Original) The nucleic acid-lipid particle of claim 17, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

19. (Original) The nucleic acid-lipid particle of claim 18, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

20. (Original) The nucleic acid-lipid particle of claim 19, wherein the PEG has an average molecular weight of about 2,000 daltons.

21. (Original) The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from about 1 mol % to about 2 mol % of the total lipid present in the particle.

22. (Original) The nucleic acid-lipid particle of claim 1, wherein the nucleic acid in the nucleic acid-lipid particle is not substantially degraded after incubation of the particle in serum at 37°C for 30 minutes.

23. (Original) The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

24. (Original) The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a lipid:nucleic acid mass ratio of from about 5 to about 15.

25. (Original) The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a median diameter of from about 40 nm to about 150 nm.

26. (Original) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.

27-37. (Canceled)

38. (Previously presented) The nucleic acid-lipid particle of claim 17, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 7.1 mol % phospholipid, about 34.3 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

39-46. (Canceled)

47. (Previously presented) The nucleic acid-lipid particle of claim 1, wherein the phospholipid comprises from about 5 mol % to about 9 mol % of the total lipid present in the particle.

48. (Previously presented) The nucleic acid-lipid particle of claim 1, wherein the cholesterol or derivative thereof comprises from about 32 mol % to about 36 mol % of the total lipid present in the particle.

49-54. (Canceled)

55. (New) The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA).

REMARKS

I. STATUS OF THE CLAIMS

After entry of this amendment, claims 1-5, 9, 14, 17-26, 38, 47-48, and 55 are pending in this application and are presented for examination. Claims 6-8, 10-13, 15-16, 27-37, 39-46, and 49-54 have been canceled without prejudice to future prosecution. Claim 55 is newly added and finds support in original claim 7.

As such, no new matter has been introduced with the foregoing amendments. Reconsideration is respectfully requested.

II. ELECTION OF SPECIES

The Examiner has indicated that an election of species is required under 35 U.S.C. § 121. In response, Applicants elect herewith the following species and provide a listing of the claims readable thereon:

A. Species 1

Applicants elect herewith a nucleic acid-lipid particle comprising a non-cationic lipid comprising a mixture of a phospholipid and cholesterol or a derivative thereof. Claims 1-5, 9, 14, 17-26, 38, 47-48, and 55 read on the elected species. Applicants note that the Examiner acknowledges that the species in this group are patentably distinct because the phospholipid component imparts specific qualities or functions to the particles (*see,* page 2 of the Restriction Requirement).

Applicants request that upon allowance of a generic claim, the Examiner consider rejoinder of withdrawn species if they are embraced by the allowed generic claim pursuant to 37 C.F.R. § 1.141.

B. Species 2

Applicants have canceled claim 6 without prejudice to future prosecution, thereby rendering the species election moot with respect to this claim. Applicants note that the Examiner acknowledges that the species in this group are patentably distinct because each cationic lipid imparts distinct qualities to the claimed particles (*see,* page 4 of the Restriction Requirement).

C. Species 3

Applicants elect herewith dipalmitoylphosphatidylcholine (DPPC). Claims 1-5, 9, 14, 17-26, 38, 47-48, and 55 read on the elected species. Applicants note that the Examiner acknowledges that the species in this group are patentably distinct because each phospholipid imparts distinct qualities to the claimed particles (*see,* page 6 of the Restriction Requirement).

Applicants request that upon allowance of a generic claim, the Examiner consider rejoinder of withdrawn species if they are embraced by the allowed generic claim pursuant to 37 C.F.R. § 1.141.

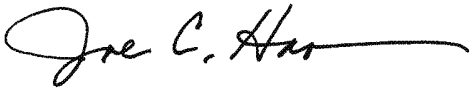
D. Species 4

Applicants elect herewith a PEG-dialkyloxypropyl (PEG-DAA) conjugate. Claims 1-5, 9, 14, 17-26, 38, 47-48, and 55 read on the elected species. Applicants note that the Examiner acknowledges that the species in this group are patentably distinct because each PEG-lipid conjugate imparts distinct qualities to the claimed particles (*see,* page 7 of the Restriction Requirement).

Applicants request that upon allowance of a generic claim, the Examiner consider rejoinder of withdrawn species if they are embraced by the allowed generic claim pursuant to 37 C.F.R. § 1.141.

In view of the foregoing, Applicants respectfully request early action on the merits. If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,



Joe C. Hao
Reg. No. 55,246

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 925-472-5000
Fax: 415-576-0300
62652628 v1

JOINT APPENDIX 58

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/424,367	04/15/2009	Edward Yaworski	020801-007710US	1406

20350 7590 07/30/2010

TOWNSEND AND TOWNSEND AND CREW, LLP
TWO EMBARCADERO CENTER
EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

EXAMINER	
PITRAK, JENNIFER S	
ART UNIT	**PAPER NUMBER**
1635	

MAIL DATE	DELIVERY MODE
07/30/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	12/424,367	YAWORSKI ET AL.	
	Examiner	Art Unit	
	JENNIFER PITRAK	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *01 June 2010*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-5,9,14,17-26,38,47,48 and 55* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-5,9,14,17-26,38,47,48 and 55* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *6/8/10*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

The requirement for election of species presented in the Office Action mailed 3/31/2010 has been withdrawn. Upon further consideration, the species are deemed to be patentably indistinct.

Claims 1-5, 9, 14, 17-26, 38, 47, 48, and 55 are pending and are under examination.

Priority

Applicant's claim for the benefit of a prior-filed application under 35 U.S.C. 119(e) or under 35 U.S.C. 120, 121, or 365(c) is acknowledged. Applicant has not complied with one or more conditions for receiving the benefit of an earlier filing date under 35 U.S.C. 120 as follows: The later-filed application must be an application for a patent for an invention which is also disclosed in the prior application (the parent or original nonprovisional application or provisional application). The disclosure of the invention in the parent application and in the later-filed application must be sufficient to comply with the requirements of the first paragraph of 35 U.S.C. 112. See *Transco Products, Inc. v. Performance Contracting, Inc.*, 38 F.3d 551, 32 USPQ2d 1077 (Fed. Cir. 1994).

The disclosure of the prior-filed application, Application No. 61/045228, fails to provide adequate support or enablement in the manner provided by the first paragraph of 35 U.S.C. 112

for one or more claims of this application. Application No. 61/046228 does not provide adequate support for claim 55. **Therefore, claim 55 is afforded the benefit of the instant filing date, 04/15/2009.**

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

MacLachlan, *et al.* (US 2006/0008910, copending application 11/148152)

Claims 1-4, 9, 14, 17-26, 38, 47, and 48 are rejected under 35 U.S.C. 103(a) as being unpatentable over MacLachlan, *et al.* (US 2006/0008910, of record, item 10 on 06/08/2010 IDS) ("MacLachlan").

The claims are to a nucleic acid lipid particle comprising a nucleic acid, a cationic lipid, a noncationic lipid mixture of phospholipid and cholesterol, and a conjugated lipid. The claims are further directed to the particle wherein the nucleic acid is an siRNA, the relative amounts of components are specified, and the lipids are specified.

MacLachlan teaches lipid encapsulated interfering RNA in the form of stable nucleic acid-lipid particles ("SNALP") comprising an siRNA, a cationic lipid, phospholipid, cholesterol,

and a conjugated lipid (page 4, paragraph 56; pages 7-11, paragraphs 84-119; claim 5) wherein the siRNA is from about 15-60 nucleotides (claim 6), the conjugated lipid is PEG-DMA and has an average molecular weight of about 2000 daltons (claim 13; paragraphs 91, 95, and 96), and the phospholipid is DSPC (paragraphs 62 and 91). MacLachlan also teaches the SNALP wherein the cationic lipid is from about 2 mol % to about 60 mol % of the total lipid present in the particle (paragraph 85), the phospholipid is from about 5% to about 90% or from about 10% to about 85% of the total lipid present in the particle (paragraph 85), the cholesterol is from about 20% to about 55% of the total lipid present in the particle (paragraph 85, top of page 8), and the conjugated lipid is from about 1% to about 20% of the total lipid present in the particle (paragraph 85). MacLachlan teaches that it will be readily apparent to one of skill in the art that the proportions of the components of the nucleic acid lipid particles may be varied (p.8, paragraph 85). MacLachlan teaches that the particles can be formulated in pharmaceutically acceptable carriers (page 18, paragraphs 205-7). MacLachlan teaches the particles having a lipid:nucleic acid mass ratio of from 12.5-100 (nucleic acid:lipid ratio from 0.01-0.08, page 15, paragraph 162) and having a median diameter of less than about 150 nm (claims 4 and 20). MacLachlan also teaches that the nucleic acids of the particles can comprise modified nucleotides (page 6, paragraph 73).

It would have been obvious to one of skill in the art at the time the instant invention was made to make a nucleic acid lipid particle comprising an siRNA, a cationic lipid, a phospholipid, cholesterol, and a PEG-conjugate because MacLachlan teaches such a particle. It would have been obvious to make the particle comprising the instantly claimed components and having the instantly claimed physical properties of claims 23-25 because MacLachlan teaches the particles

comprising such components. It further would have been obvious to formulate the particles with the instantly claimed amounts of the individual components because MacLachlan teaches particles formulated with ranges of amounts that overlap with the instantly claimed ranges and teaches that the proportions of the components can be varied by those of skill in the art. Thus, by routine experimentation towards optimization, one of skill in the art could arrive at the instantly claimed proportions. Absent evidence to the contrary, the nucleic acid lipid particles of MacLachlan would not be substantially degraded after incubation of the particle in serum at 37°C for 30 minutes (claim 22). Therefore, the claims would have been *prima facie* obvious at the time the instant invention was made.

MacLachlan, *et al.* (US 2006/0008910) and Fosnaugh, *et al.* (US 2003/0143732)

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 are rejected under 35 U.S.C. 103(a) as being unpatentable over MacLachlan, *et al.* (US 2006/0008910, of record, item 10 on 06/08/2010 IDS) as applied to claims 1-4, 9, 14, 17-26, 38, 47, and 48 above, and further in view of Fosnaugh, *et al.* (US 2003/0143732, of record, item 3 on 06/08/2010 IDS).

Claims 1-4, 9, 14, 17-26, 38, 47, and 48 are described above and are obvious as described in the preceding rejection.

Claim 5 is to the nucleic acid lipid particle wherein the siRNA of the particle comprises a 2'-O-methyl modification.

MacLachlan teaches SNALPs as described in the preceding rejection. MacLachlan does not teach the SNALPs wherein the siRNA is 2'-O-methyl-modified.

Fosnaugh teaches siRNAs and that siRNAs can be modified with 2'-O-methyl modifications to increase siRNA nuclease resistance (pages 4-5, paragraph 34).

It would have been obvious to make the SNALP of MacLachlan with an siRNA comprising a 2'-O-methyl modification because Fosnaugh teaches that such modification enhances siRNA resistance to nuclease degradation. One of skill would recognize that increasing nuclease resistance of the siRNA would improve the serum stability of the siRNA, which would add to the serum stability afforded to the siRNA by its inclusion in the SNALP. Therefore, the claims would have been *prima facie* obvious at the time the instant invention was made.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claim 55 is rejected under 35 U.S.C. 102(e) as being anticipated by MacLachlan, *et al.* (US 2009/0291131) ("MacLachlan 2").

The applied reference has a common inventor with the instant application. Based upon the earlier effective U.S. filing date of the reference, it constitutes prior art under 35 U.S.C.

102(e). This rejection under 35 U.S.C. 102(e) might be overcome either by a showing under 37 CFR 1.132 that any invention disclosed but not claimed in the reference was derived from the inventor of this application and is thus not the invention "by another," or by an appropriate showing under 37 CFR 1.131.

Claim 55 is to a nucleic acid lipid particle as described previously wherein the cationic lipid is DLin-K-C2-DMA.

MacLachlan 2 teaches SNALPs comprising siRNAs, cationic lipid, phospholipid, cholesterol, and a conjugated lipid. MacLachlan 2 teaches that the cationic lipid can be 2,2 -dilinoleyl-4 -dimethylaminoethyl-[1,3] -dioxolane (DLin-K-XTC2-DMA), which is the same as the instantly claimed DLin-K-C2-DMA (page 3, paragraph 22). MacLachlan 2 teaches that the SNALPs may comprise the cationic lipid from about 50 mol % to about 65 mol %, the phospholipid from about 4 mol % to about 10 mol %, cholesterol from about 30 mol % to about 40 mol %, and the conjugated lipid from about 0.5 mol % to about 2 mol % of the total lipid in the particle. Therefore, MacLachlan 2 anticipates the instant claim.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined

application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

12/343342

Claims 1-5, 14, 17-19, 23-26, 38, and 55 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 24, 25, 26, 30-32, 43, 44, , 120, 123, 124, and 125 of copending Application No. 12/343342. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '342 application are directed to siRNA-lipid particles having the same proportions

of components and the same components as instantly claimed, except for the conjugated lipid component, which is limited to about 0.5 mol % to about 2 mol % in the instant case and is limited to about 1 mol % to about 15 mol % in the '342 claims. The lower limit of the mol % of the conjugated lipid component in the '342 claims overlaps with the instantly claimed mol % range. The instant claims would have been obvious by routine optimization of the '342 claims. The instant claim 55 is obvious over the '342 claims because the '342 application teaches that DLin-K-XTC2-DMA is one cationic lipid useful in the claimed nucleic acid-lipid particles (see rejection under 35 USC 102(e) above).

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

11/148152

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-15 and 38-46 of copending Application No. 11/148152 in view of Fosnaugh, et al. (US 2003/0143732, of record, item 3 on 06/08/2010 IDS). Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '152 application are directed to siRNA-lipid particles comprising the instantly claimed components and the disclosure describes that the components can comprise the particles in proportions that overlap with the instantly claimed proportions as indicated above in the rejection under 35 USC § 103. Fosnaugh, et al. teaches that siRNAs can be modified with 2'-O-methyl groups, rendering the instant claim 5

obvious also as indicated above. See rejection under 35 USC 103(a) for the obviousness analysis of the instant claims.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

11/174453

Claims 1-5, 9, 14, 17-19, 21, 22, 23, 25, 26, 38, 47, and 48 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 10, 11, 13, 14, 15, 16, 17, 18, 19, 22, 23, 24, 25, 26, 27, 43, 44, 45, 47, 48, 49, 51, and 53 of copending Application No. 11/174453 in view of Fosnaugh, *et al.* (US 2003/0143732, of record, item 3 on 06/08/2010 IDS).

Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '453 application are directed to siRNA-lipid particles comprising the instantly claimed components in ranges of proportions that encompass or overlap with the instantly claimed proportions. Thus, by routine experimentation towards optimization, one of skill in the art could arrive at the instantly claimed proportions. Fosnaugh, et al. teaches that siRNAs can be modified with 2'-O-methyl groups to enhance siRNA stability. Therefore, it would have been obvious to one of skill in the art to modify the siRNA of the '453 claims with a 2'-O-methyl modification to improve the stability of the siRNA.

This is a provisional obviousness-type double patenting rejection.

11/283550

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4, 5, 7, 9, 10, 11, 13, 14, 15, 16, 17, 19, 22, 24, 26, 27, 29, 30, 31, 32, 33, 34, 35, 37, 38, 39, 57, 58, 59, 60, 61, 62, 64, 65, 66, 67, 68, 69, and 71 of copending Application No. 11/283550. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '550 application are directed to siRNA-lipid particles comprising the instantly claimed components in ranges of proportions that encompass or overlap with the instantly claimed proportions. Thus, by routine experimentation towards optimization, one of skill in the art could arrive at the instantly claimed proportions of components.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

11/426907

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-4, 6-11, 52, 55-60, 63-66, and 68-76 of copending Application No. 11/426907. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '907 application are directed to siRNA-lipid particles comprising the instantly claimed components in ranges of proportions that encompass or overlap with the instantly claimed proportions. Thus, by routine experimentation towards optimization, one of skill in the art could arrive at the instantly claimed proportions of components.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

11/511855

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 70, 71, 72, 73, 74, and 75 of copending Application No. 11/511855. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '855 application are directed to methods of using siRNA-lipid particles and the disclosure teaches that the particles may comprising the instantly claimed components in ranges of proportions that encompass or overlap with the instantly claimed proportions. Thus, by routine experimentation towards optimization, one of skill in the art could arrive at the instantly claimed proportions of components.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

11/584341

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 16, 17, 18, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 31, 33, 34, 35, 36, 38-42, 44-46, 73, 74, 76, 77, 78, 80, and 91-95 of copending Application No. 11/584341. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '341 application are

directed to siRNA-lipid particles comprising the instantly claimed components in ranges of proportions that encompass or overlap with the instantly claimed proportions. Thus, by routine experimentation towards optimization, one of skill in the art could arrive at the instantly claimed proportions of components.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

11/592756

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 59-61, 63-72, 74, 76-79, 81-89, 182, , 183, 184, and 187 of copending Application No. 11/92756. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '756 application are directed to siRNA-lipid particles comprising the instantly claimed components in ranges of proportions that encompass or overlap with the instantly claimed proportions. Thus, by routine experimentation towards optimization, one of skill in the art could arrive at the instantly claimed proportions of components.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

11/807872

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 44-55 of

copending Application No. 11/807872. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '872 application are directed to siRNA-lipid particles comprising the instantly claimed components in ranges of proportions that encompass or overlap with the instantly claimed proportions and the disclosure describes that the components can comprise the particles in proportions that encompass or overlap with the instantly claimed proportions. Thus, by routine experimentation towards optimization, one of skill in the art could arrive at the instantly claimed proportions of components.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

12/359119

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 29-39 of copending Application No. 12/359119. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '119 application are directed to siRNA-lipid particles comprising the instantly claimed components in ranges of proportions that encompass or overlap with the instantly claimed proportions and the disclosure describes that the components can comprise the particles in proportions that encompass or overlap with the instantly claimed proportions. Thus, by routine experimentation towards optimization, one of skill in the art could arrive at the instantly claimed proportions of components.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to JENNIFER PITRAK whose telephone number is (571)270-3061. The examiner can normally be reached on Monday-Friday, 8:30AM-5:00PM, EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christopher Low can be reached on 571-272-0951. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Jennifer Pitrak/
Examiner, Art Unit 1635

JOINT APPENDIX 59

PTO/SB/05 (08-08)

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	86399-802191(007720US)
First Inventor	Edward Yaworski
Title	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
EFS Web Filing Date	October 5, 2011

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☐ **Fee Transmittal Form** (e.g., PTO/SB/17)
2. ☐ **Applicant claims small entity status.** See 37 CFR 1.27.
3. ☒ **Specification** [*Total Pages* 119]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. ☒ **Drawing(s)** *(35 U.S.C.113)* [*Total Sheets* 24]
5. **Oath or Declaration** [*Total Sheets* 2]
   a. ☐ Newly executed (original or copy)
   b. ☒ A copy from a prior application (37 CFR 1.63 (d))
   (for a continuation/divisional with Box 18 completed)
   i. ☐ DELETION OF INVENTOR(S)
   Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☒ **Application Data Sheet.** See 37 CFR 1.76
7. ☐ **CD-ROM** or **CD-R** in duplicate, large table or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission**
   (if applicable, items a. - c. are required)
   a. ☐ Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
   i. ☐ CD-ROM or CD-R (2 copies); or
   ii. ☒ Paper
   c. ☐ Statements verifying identity of above copies

ADDRESS TO: **Commissioner for Patents**
P.O. Box 1450
Alexandria, VA 22313-1450

ACCOMPANYING APPLICATION PARTS

9. ☐ **Assignment Papers** (cover sheet & document(s))
   Name of Assignee _____
10. ☐ **37 CFR 3.73(b) Statement** *(when there is an assignee)* ☐ **Power of Attorney**
11. ☐ **English Translation Document** *(if applicable)*
12. ☐ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☐ Copies of citations attached
13. ☐ **Preliminary Amendment**
14. ☐ **Return Receipt Postcard** (MPEP 503)
    (Should be specifically itemized)
15. ☐ **Certified Copy of Priority Document(s)**
    (if foreign priority is claimed)
16. ☐ **Nonpublication Request** under 35 U.S.C. 122 (b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
17. ☐ Other: _____

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*

☒ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No: 12/424,367

Prior application information: *Examiner* Brian Whiteman *Art Unit:* 1635

19. CORRESPONDENCE ADDRESS

☒ The address associated with Customer Number: **20350** *OR* ☐ Correspondence address below

Name					
Address					
City		State		Zip Code	
Country		Telephone		Email	

Signature	Joe C. Hao	Date	October 5, 2011
Name (Print/Type)	Joe C. Hao	Registration No. (Attorney/Agent)	55,246

63760646 v1

Attorney Docket No.: 020801-007710US
Client Ref. No.:

PTO/SB/01A (07-07)

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or

☒ Application No. 12/424,367, filed on April 15, 2009,

☐ as amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: Edward Yaworski — Date: Nov 06/09

Signature: [signature] — Citizen of: Canada

Inventor two: Kieu Lam — Date: NOV 09/09

Signature: [signature] — Citizen of: Canada

☒ Additional inventors or a legal representative are being named on 1 additional form(s) attached hereto.

62247018 v1

Attorney Docket No.: 020801-007710US
Client Ref. No.:

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76) – ADDITIONAL INVENTOR(S)
Supplemental Sheet 1 of 1

FULL NAME OF INVENTOR(S)	
Inventor three: Lloyd Jeffs Signature:	Date: 06 NOV 2009 Citizen of: Canada/United Kingdom
Inventor four: Lorne Palmer Signature:	Date: Nov 6, 2009 Citizen of: Canada
Inventor five: Ian MacLachlan Signature:	Date: Nov. 9, 2009. Citizen of: Canada/United Kingdom
Inventor six: Signature:	Date: Citizen of:
Inventor seven: Signature:	Date: Citizen of:
Inventor eight: Signature:	Date: Citizen of:
Inventor nine: Signature:	Date: Citizen of:
Inventor ten: Signature:	Date: Citizen of:
Inventor eleven: Signature:	Date: Citizen of:
Inventor twelve: Signature:	Date: Citizen of:

62247018 v1

Application Data Sheet

Application Information

Application number::	
Filing Date::	10/05/11
Application Type::	Regular
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R??::	
Number of CD disks::	
Number of copies of CDs::	
Sequence Submission::	
Computer Readable Form (CRF)?::	
Number of copies of CRF::	
Title::	NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
Attorney Docket Number::	86399-802191(007720US)
Request for Early Publication::	No
Request for Non-Publication::	No
Suggested Drawing Figure::	1
Total Drawing Sheets::	24
Small Entity?::	No
Latin name::	
Variety denomination name::	
Petition included?::	No
Petition Type::	
Licensed US Govt. Agency::	
Contract or Grant Numbers One::	
Secrecy Order in Parent Appl.::	No

Initial 10/05/11

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	Canada
Status::	Full Capacity
Given Name::	Edward
Middle Name::	
Family Name::	Yaworski
Name Suffix::	
City of Residence::	Maple Ridge
State or Province of Residence::	BC
Country of Residence::	Canada
Street of Mailing Address::	23106-123B Avenue
City of Mailing Address::	Maple Ridge
State or Province of mailing address::	BC
Country of mailing address::	Canada
Postal or Zip Code of mailing address::	V2X9Z7

Applicant Authority Type::	Inventor
Primary Citizenship Country::	Canada
Status::	Full Capacity
Given Name::	Kieu
Middle Name::	
Family Name::	Lam
Name Suffix::	
City of Residence::	Surrey
State or Province of Residence::	BC
Country of Residence::	Canada
Street of Mailing Address::	18871 71 Avenue
City of Mailing Address::	Surrey
State or Province of mailing address::	BC
Country of mailing address::	Canada

Initial 10/05/11

Postal or Zip Code of mailing address::	V4N5M7

Applicant Authority Type::	Inventor
Primary Citizenship Country::	United Kingdom
Status::	Full Capacity
Given Name::	Lloyd
Middle Name::	
Family Name::	Jeffs
Name Suffix::	
City of Residence::	Delta
State or Province of Residence::	BC
Country of Residence::	Canada
Street of Mailing Address::	5218 Walnut Place
City of Mailing Address::	Delta
State or Province of mailing address::	BC
Country of mailing address::	Canada
Postal or Zip Code of mailing address::	V4K3B4

Applicant Authority Type::	Inventor
Primary Citizenship Country::	Canada
Status::	Full Capacity
Given Name::	Lorne
Middle Name::	
Family Name::	Palmer
Name Suffix::	
City of Residence::	Vancouver
State or Province of Residence::	BC
Country of Residence::	Canada
Street of Mailing Address::	8076 Elliott Street
City of Mailing Address::	Vancouver
State or Province of mailing address::	BC

Initial 10/05/11

Country of mailing address::	Canada
Postal or Zip Code of mailing address::	V5S2P2

Applicant Authority Type::	Inventor
Primary Citizenship Country::	Canada/United Kingdom
Status::	Full Capacity
Given Name::	Ian
Middle Name::	
Family Name::	MacLachlan
Name Suffix::	
City of Residence::	Mission
State or Province of Residence::	BC
Country of Residence::	Canada
Street of Mailing Address::	8040 Aves Terrace
City of Mailing Address::	Mission
State or Province of mailing address::	BC
Country of mailing address::	Canada
Postal or Zip Code of mailing address::	V4S1E5

Correspondence Information

Correspondence Customer Number::	20350

Representative Information

Representative Customer Number::	20350

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application 12/424,367	Continuation of An Appn claiming benefit under 35 USC 119(e) of	12/424,367 61/045,228	04/15/09 04/15/08

Initial 10/05/11

Foreign Priority Information

Country::	Application number::	Filing Date::

Assignee Information

Assignee Name::	Protiva Biotherapeutics, Inc.
Street of mailing address::	100-8900 Glenlyon Parkway
City of mailing address::	Burnaby
State or Province of mailing address::	BC
Country of mailing address::	Canada
Postal or Zip Code of mailing address::	V5J5J8

NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] The present application is a continuation of U.S. Application No. 12/424,367 filed April 15, 2009 which application claims priority to U.S. Provisional Application No. 61/045,228, filed April 15, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not applicable.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

[0003] Not applicable.

REFERENCE TO A "SEQUENCE LISTING"

[0004] Not applicable.

BACKGROUND OF THE INVENTION

[0005] RNA interference (RNAi) is an evolutionarily conserved process in which recognition of double-stranded RNA (dsRNA) ultimately leads to posttranscriptional suppression of gene expression. This suppression is mediated by short dsRNA, also called small interfering RNA (siRNA), which induces specific degradation of mRNA through complementary base pairing. In several model systems, this natural response has been developed into a powerful tool for the investigation of gene function (*see, e.g.,* Elbashir *et al.*, *Genes Dev.*, 15:188-200 (2001); Hammond *et al.*, *Nat. Rev. Genet.*, 2:110-119 (2001)). More recently, it was discovered that introducing synthetic 21-nucleotide dsRNA duplexes into mammalian cells could efficiently silence gene expression.

[0006] Although the precise mechanism is still unclear, RNAi provides a potential new approach to downregulate or silence the transcription and translation of a gene of interest. For example, it is desirable to modulate (*e.g.*, reduce) the expression of certain genes for the treatment of neoplastic disorders such as cancer. It is also desirable to silence the expression of genes associated with liver diseases and disorders such as hepatitis. It is further desirable to reduce the expression of certain genes for the treatment of atherosclerosis and its manifestations, *e.g.,* hypercholesterolemia, myocardial infarction, and thrombosis.

[0007] A safe and effective nucleic acid delivery system is required for RNAi to be therapeutically useful. Viral vectors are relatively efficient gene delivery systems, but suffer from a variety of limitations, such as the potential for reversion to the wild-type as well as immune response concerns. As a result, nonviral gene delivery systems are receiving increasing attention (Worgall *et al.*, *Human Gene Therapy*, 8:37 (1997); Peeters *et al.*, *Human Gene Therapy*, 7:1693 (1996); Yei *et al.*, *Gene Therapy*, 1:192 (1994); Hope *et al.*, *Molecular Membrane Biology*, 15:1 (1998)). Furthermore, viral systems are rapidly cleared from the circulation, limiting transfection to "first-pass" organs such as the lungs, liver, and spleen. In addition, these systems induce immune responses that compromise delivery with subsequent injections.

[0008] Plasmid DNA-cationic liposome complexes are currently the most commonly employed nonviral gene delivery vehicles (Felgner, *Scientific American*, 276:102 (1997); Chonn *et al.*, *Current Opinion in Biotechnology*, 6:698 (1995)). For instance, cationic liposome complexes made of an amphipathic compound, a neutral lipid, and a detergent for transfecting insect cells are disclosed in U.S. Patent No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Publication No. 20030073640.

[0009] Cationic liposome complexes are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects (Harrison *et al.*, *Biotechniques*, 19:816 (1995); Li *et al.*, *The Gene*, 4:891 (1997); Tam *et al*, *Gene Ther.*, 7:1867 (2000)). As large, positively charged aggregates, lipoplexes are rapidly cleared when administered *in vivo*, with highest expression levels observed in first-pass organs, particularly the lungs (Huang *et al.*, *Nature Biotechnology*, 15:620 (1997); Templeton *et al.*, *Nature Biotechnology*, 15:647 (1997); Hofland *et al.*, *Pharmaceutical Research*, 14:742 (1997)).

[0010] Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Patent No. 6,429,200. Anionic liposomes are disclosed in U.S. Patent Publication No. 20030026831. Polymer liposomes that incorporate dextrin or glycerol-phosphocholine polymers are disclosed in U.S. Patent Publication Nos. 20020081736 and 20030082103, respectively.

[0011] A gene delivery system containing an encapsulated nucleic acid for systemic delivery should be small (*i.e.*, less than about 100 nm diameter) and should remain intact in the circulation for an extended period of time in order to achieve delivery to affected tissues. This requires a highly stable, serum-resistant nucleic acid-containing particle that does not interact with cells and other components of the vascular compartment. The particle should

also readily interact with target cells at a disease site in order to facilitate intracellular delivery of a desired nucleic acid.

[0012] Recent work has shown that nucleic acids can be encapsulated in small (*e.g.*, about 70 nm diameter) "stabilized plasmid-lipid particles" (SPLP) that consist of a single plasmid encapsulated within a bilayer lipid vesicle (Wheeler *et al.*, *Gene Therapy*, 6:271 (1999)). These SPLPs typically contain the "fusogenic" lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid, and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. SPLPs have systemic application as they exhibit extended circulation lifetimes following intravenous (i.v.) injection, accumulate preferentially at distal tumor sites due to the enhanced vascular permeability in such regions, and can mediate transgene expression at these tumor sites. The levels of transgene expression observed at the tumor site following i.v. injection of SPLPs containing the luciferase marker gene are superior to the levels that can be achieved employing plasmid DNA-cationic liposome complexes (lipoplexes) or naked DNA.

[0013] Thus, there remains a strong need in the art for novel and more efficient methods and compositions for introducing nucleic acids such as siRNA into cells. In addition, there is a need in the art for methods of downregulating the expression of genes of interest to treat or prevent diseases and disorders such as cancer and atherosclerosis. The present invention addresses these and other needs.

BRIEF SUMMARY OF THE INVENTION

[0014] The present invention provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.*, for the treatment of a disease or disorder).

[0015] In preferred embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In other preferred embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0016] In one aspect, the present invention provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid

present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0017] More particularly, the present invention provides serum-stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (*e.g.*, one or more interfering RNA molecules such as siRNA, aiRNA, and/or miRNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP (*e.g.*, for the treatment of a disease or disorder).

[0018] In certain embodiments, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) a nucleic acid (*e.g.*, an interfering RNA); (b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0019] In one preferred embodiment, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:62" formulation.

[0020] In another preferred embodiment, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:57" formulation.

[0021] The present invention also provides pharmaceutical compositions comprising a lipid particle described herein (*e.g.*, SNALP) and a pharmaceutically acceptable carrier.

[0022] In another aspect, the present invention provides methods for introducing an active agent or therapeutic agent (*e.g.*, nucleic acid) into a cell, the method comprising contacting the cell with a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0023] In yet another aspect, the present invention provides methods for the *in vivo* delivery of an active agent or therapeutic agent (*e.g.*, nucleic acid), the method comprising administering to a mammalian subject a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0024] In a further aspect, the present invention provides methods for treating a disease or disorder in a mammalian subject in need thereof, the method comprising administering to the mammalian subject a therapeutically effective amount of a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0025] Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] Figure 1 illustrates data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

[0027] Figure 2 illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

[0028] Figure 3 illustrates additional data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice. Each bar represents the group mean of five animals. Error bars indicate the standard deviation.

[0029] Figure 4 illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

[0030] Figure 5 illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

[0031] Figure 6 illustrates data demonstrating that the tolerability of 1:57 SNALP containing ApoB siRNA prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters.

[0032] Figure 7 illustrates data demonstrating that the efficacy of 1:57 SNALP containing ApoB siRNA prepared by gear pump was similar to the same SNALP prepared by syringe press.

[0033] Figure 8 illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

[0034] Figure 9 illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

[0035] Figure 10 illustrates data demonstrating that clinically significant liver enzyme elevations (3xULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

[0036] Figure 11 illustrates data demonstrating that the potency of the lower lipid:drug (L:D) 1:57 SNALP containing ApoB siRNA was as good as that of the higher L:D SNALP at the tested drug dosages.

[0037] Figure 12 illustrates data demonstrating that ApoB protein and total cholesterol levels were reduced to a similar extent by 1:57 SNALP containing ApoB siRNA at a 6:1 input L:D ratio (final ratio of 7:1) and 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

[0038] Figure 13 illustrates data demonstrating that a treatment regimen of 1:57 SNALP with siRNA targeting PLK-1 is well tolerated with no apparent signs of treatment related toxicity in mice bearing Hep3B liver tumors.

[0039] Figure 14 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA caused a significant increase in the survival of Hep3B tumor-bearing mice.

[0040] Figure 15 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA reduced PLK-1 mRNA levels by 50% in intrahepatic Hep3B tumors growing in mice 24 hours after SNALP administration.

[0041] Figure 16 illustrates data demonstrating that a specific cleavage product of PLK-1 mRNA was detectable by 5' RACE-PCR in mice treated with 1:57 SNALP containing PLK-1 siRNA. 10 μl PCR product/well were loaded onto a 1.5% agarose gel. Lane Nos.: (1) molecular weight (MW) marker; (2) PBS mouse 1; (3) PBS mouse 2; (4) PBS mouse 3; (5) Luc SNALP mouse 1; (6) Luc SNALP mouse 2; (7) PLK SNALP mouse 1; (8) PLK SNALP mouse 2; (9) PLK SNALP mouse 3; and (10) no template control.

[0042] Figure 17 illustrates data demonstrating that control (Luc) 1:57 SNALP-treated mice displayed normal mitoses in Hep3B tumors (top panels), whereas mice treated with 1:57 SNALP containing PLK-1 siRNA exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors (bottom panels).

[0043] Figure 18 illustrates data demonstrating that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B subcutaneous (S.C.) tumors.

[0044] Figure 19 illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

[0045] Figure 20 illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0046] Figure 21 illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

[0047] Figure 22 illustrates data demonstrating the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0048] The present invention is based, in part, upon the surprising discovery that lipid particles comprising from about 50 mol % to about 85 mol % of a cationic lipid, from about 13 mol % to about 49.5 mol % of a non-cationic lipid, and from about 0.5 mol % to about 2 mol % of a lipid conjugate provide advantages when used for the *in vitro* or *in vivo* delivery of an active agent, such as a therapeutic nucleic acid (*e.g.*, an interfering RNA). In particular, as illustrated by the Examples herein, the present invention provides stable nucleic acid-lipid particles (SNALP) that advantageously impart increased activity of the encapsulated nucleic acid (*e.g.*, an interfering RNA such as siRNA) and improved tolerability of the formulations *in vivo*, resulting in a significant increase in the therapeutic index as compared to nucleic acid-lipid particle compositions previously described. Additionally, the SNALP of the invention are stable in circulation, *e.g.*, resistant to degradation by nucleases in serum, and are substantially non-toxic to mammals such as humans. As a non-limiting example, Figure 3 of Example 4 shows that one SNALP embodiment of the invention ("1:57 SNALP") was more than 10 times as efficacious as compared to a nucleic acid-lipid particle previously described ("2:30 SNALP") in mediating target gene silencing at a 10-fold lower dose. Similarly, Figure 2 of Example 3 shows that the "1:57 SNALP" formulation was substantially more effective at silencing the expression of a target gene as compared to nucleic acid-lipid particles previously described ("2:40 SNALP").

[0049] In certain embodiments, the present invention provides improved compositions for the delivery of interfering RNA such as siRNA molecules. In particular, the Examples herein illustrate that the improved lipid particle formulations of the invention are highly effective in downregulating the mRNA and/or protein levels of target genes. Furthermore, the Examples herein illustrate that the presence of certain molar ratios of lipid components results in improved or enhanced activity of these lipid particle formulations of the present invention. For instance, the "1:57 SNALP" and "1:62 SNALP" formulations described herein are

exemplary formulations of the present invention that are particularly advantageous because they provide improved efficacy and tolerability *in vivo*, are serum-stable, are substantially non-toxic, are capable of accessing extravascular sites, and are capable of reaching target cell populations.

[0050] The lipid particles and compositions of the present invention may be used for a variety of purposes, including the delivery of associated or encapsulated therapeutic agents to cells, both *in vitro* and *in vivo*. Accordingly, the present invention provides methods for treating diseases or disorders in a subject in need thereof, by contacting the subject with a lipid particle described herein comprising one or more suitable therapeutic agents.

[0051] Various exemplary embodiments of the lipid particles of the invention, as well as compositions and formulations comprising the same, and their use to deliver therapeutic agents and modulate target gene and protein expression, are described in further detail below.

II. Definitions

[0052] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0053] The term "interfering RNA" or "RNAi" or "interfering RNA sequence" refers to single-stranded RNA (*e.g.*, mature miRNA) or double-stranded RNA (*i.e.*, duplex RNA such as siRNA, aiRNA, or pre-miRNA) that is capable of reducing or inhibiting the expression of a target gene or sequence (*e.g.*, by mediating the degradation or inhibiting the translation of mRNAs which are complementary to the interfering RNA sequence) when the interfering RNA is in the same cell as the target gene or sequence. Interfering RNA thus refers to the single-stranded RNA that is complementary to a target mRNA sequence or to the double-stranded RNA formed by two complementary strands or by a single, self-complementary strand. Interfering RNA may have substantial or complete identity to the target gene or sequence, or may comprise a region of mismatch (*i.e.*, a mismatch motif). The sequence of the interfering RNA can correspond to the full-length target gene, or a subsequence thereof.

[0054] Interfering RNA includes "small-interfering RNA" or "siRNA," *e.g.*, interfering RNA of about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, more typically about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.*, each complementary sequence of the double-stranded siRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, preferably about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded siRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, preferably

about 18-22, 19-20, or 19-21 base pairs in length). siRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides and 5' phosphate termini. Examples of siRNA include, without limitation, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand; a double-stranded polynucleotide molecule assembled from a single stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions; and a circular single-stranded polynucleotide molecule with two or more loop structures and a stem having self-complementary sense and antisense regions, where the circular polynucleotide can be processed *in vivo* or *in vitro* to generate an active double-stranded siRNA molecule.

[0055] Preferably, siRNA are chemically synthesized. siRNA can also be generated by cleavage of longer dsRNA (*e.g.*, dsRNA greater than about 25 nucleotides in length) with the *E. coli* RNase III or Dicer. These enzymes process the dsRNA into biologically active siRNA (*see*, *e.g.*, Yang *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:9942-9947 (2002); Calegari *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:14236 (2002); Byrom *et al.*, *Ambion TechNotes,* 10(1):4-6 (2003); Kawasaki *et al.*, *Nucleic Acids Res.,* 31:981-987 (2003); Knight *et al.*, *Science,* 293:2269-2271 (2001); and Robertson *et al.*, *J. Biol. Chem.,* 243:82 (1968)). Preferably, dsRNA are at least 50 nucleotides to about 100, 200, 300, 400, or 500 nucleotides in length. A dsRNA may be as long as 1000, 1500, 2000, 5000 nucleotides in length, or longer. The dsRNA can encode for an entire gene transcript or a partial gene transcript. In certain instances, siRNA may be encoded by a plasmid (*e.g.*, transcribed as sequences that automatically fold into duplexes with hairpin loops).

[0056] As used herein, the term "mismatch motif" or "mismatch region" refers to a portion of an interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) sequence that does not have 100 % complementarity to its target sequence. An interfering RNA may have at least one, two, three, four, five, six, or more mismatch regions. The mismatch regions may be contiguous or may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides. The mismatch motifs or regions may comprise a single nucleotide or may comprise two, three, four, five, or more nucleotides.

[0057] An "effective amount" or "therapeutically effective amount" of an active agent or therapeutic agent such as an interfering RNA is an amount sufficient to produce the desired effect, *e.g.*, an inhibition of expression of a target sequence in comparison to the normal

expression level detected in the absence of an interfering RNA. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with an interfering RNA relative to the control is about 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, *e.g.*, examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0058] By "decrease," "decreasing," "reduce," or "reducing" of an immune response by an interfering RNA is intended to mean a detectable decrease of an immune response to a given interfering RNA (*e.g.*, a modified interfering RNA). The amount of decrease of an immune response by a modified interfering RNA may be determined relative to the level of an immune response in the presence of an unmodified interfering RNA. A detectable decrease can be about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more lower than the immune response detected in the presence of the unmodified interfering RNA. A decrease in the immune response to interfering RNA is typically measured by a decrease in cytokine production (*e.g.*, IFNγ, IFNα, TNFα, IL-6, or IL-12) by a responder cell *in vitro* or a decrease in cytokine production in the sera of a mammalian subject after administration of the interfering RNA.

[0059] As used herein, the term "responder cell" refers to a cell, preferably a mammalian cell, that produces a detectable immune response when contacted with an immunostimulatory interfering RNA such as an unmodified siRNA. Exemplary responder cells include, *e.g.*, dendritic cells, macrophages, peripheral blood mononuclear cells (PBMCs), splenocytes, and the like. Detectable immune responses include, *e.g.*, production of cytokines or growth factors such as TNF-α, IFN-α, IFN-β, IFN-γ, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TGF, and combinations thereof.

[0060] "Substantial identity" refers to a sequence that hybridizes to a reference sequence under stringent conditions, or to a sequence that has a specified percent identity over a specified region of a reference sequence.

[0061] The phrase "stringent hybridization conditions" refers to conditions under which a nucleic acid will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen,

Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0062] Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C-95°C for 30 sec.-2 min., an annealing phase lasting 30 sec.-2 min., and an extension phase of about 72°C for 1-2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, *e.g.*, in Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y. (1990).

[0063] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional

guidelines for determining hybridization parameters are provided in numerous references, *e.g.*, Current Protocols in Molecular Biology, Ausubel *et al.*, eds.

[0064] The terms "substantially identical" or "substantial identity," in the context of two or more nucleic acids, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (*i.e.*, at least about 60%, preferably at least about 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition, when the context indicates, also refers analogously to the complement of a sequence. Preferably, the substantial identity exists over a region that is at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length.

[0065] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0066] A "comparison window," as used herein, includes reference to a segment of any one of a number of contiguous positions selected from the group consisting of from about 5 to about 60, usually about 10 to about 45, more usually about 15 to about 30, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.*, 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.*, 48:443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*, 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology*, Ausubel *et al.*, eds. (1995 supplement)).

[0067] A preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.*, 25:3389-3402 (1977) and Altschul *et al.*, *J. Mol. Biol.*, 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

[0068] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul, *Proc. Natl. Acad. Sci. USA,* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0069] The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA and RNA. DNA may be in the form of, *e.g.*, antisense molecules, plasmid DNA, pre-condensed DNA, a PCR product, vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives and combinations of these groups. RNA may be in the form of siRNA, asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, tRNA, viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third

position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.*, 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.*, 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes*, 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

[0070] The term "gene" refers to a nucleic acid (*e.g.*, DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

[0071] "Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

[0072] The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are characterized by being insoluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

[0073] A "lipid particle" is used herein to refer to a lipid formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), to a target site of interest. In the lipid particle of the invention, which is typically formed from a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of the particle, the active agent or therapeutic agent may be encapsulated in the lipid, thereby protecting the agent from enzymatic degradation.

[0074] As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a particle made from lipids (*e.g.*, a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of the particle), wherein the nucleic acid (*e.g.*, siRNA, aiRNA, miRNA, ssDNA, dsDNA, ssRNA, short hairpin RNA (shRNA), dsRNA, or a plasmid, including plasmids from which an interfering RNA is transcribed) is fully encapsulated within the lipid. As used herein, the term "SNALP" includes an SPLP, which is the term used to refer to a nucleic acid-lipid particle comprising a nucleic acid (*e.g.*, a plasmid) encapsulated within the lipid. SNALP and SPLP typically contain a cationic lipid, a

non-cationic lipid, and a lipid conjugate (*e.g.*, a PEG-lipid conjugate). SNALP and SPLP are extremely useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (i.v.) injection, they can accumulate at distal sites (*e.g.*, sites physically separated from the administration site), and they can mediate expression of the transfected gene or silencing of target gene expression at these distal sites. SPLP include "pSPLP," which comprise an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0075] The lipid particles of the invention (*e.g.*, SNALP) typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm, and are substantially non-toxic. In addition, nucleic acids, when present in the lipid particles of the invention, are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Publication Nos. 20040142025 and 20070042031, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0076] As used herein, "lipid encapsulated" can refer to a lipid particle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid is fully encapsulated in the lipid particle (*e.g.*, to form an SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle).

[0077] The term "lipid conjugate" refers to a conjugated lipid that inhibits aggregation of lipid particles. Such lipid conjugates include, but are not limited to, polyamide oligomers (*e.g.*, ATTA-lipid conjugates), PEG-lipid conjugates, such as PEG coupled to dialkyloxypropyls, PEG coupled to diacylglycerols, PEG coupled to cholesterol, PEG coupled to phosphatidylethanolamines, PEG conjugated to ceramides (*see, e.g.*, U.S. Patent No. 5,885,613, the disclosure of which is herein incorporated by reference in its entirety for all purposes), cationic PEG lipids, and mixtures thereof. PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In preferred embodiments, non-ester containing linker moieties are used.

[0078] The term "amphipathic lipid" refers, in part, to any suitable material wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the

hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxyl, and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids.

[0079] Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, are also within the group designated as amphipathic lipids. Additionally, the amphipathic lipids described above can be mixed with other lipids including triglycerides and sterols.

[0080] The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides, and diacylglycerols.

[0081] The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

[0082] The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerols, cardiolipins, diacylphosphatidylserines, diacylphosphatidic acids, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

[0083] The term "cationic lipid" refers to any of a number of lipid species that carry a net positive charge at a selected pH, such as physiological pH (*e.g.*, pH of about 7.0). It has been surprisingly found that cationic lipids comprising alkyl chains with multiple sites of unsaturation, *e.g.*, at least two or three sites of unsaturation, are particularly useful for

forming lipid particles with increased membrane fluidity. A number of cationic lipids and related analogs, which are also useful in the present invention, have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of cationic lipids are described in detail herein. In some cases, the cationic lipids comprise a protonatable tertiary amine (*e.g.*, pH titratable) head group, C18 alkyl chains, ether linkages between the head group and alkyl chains, and 0 to 3 double bonds. Such lipids include, *e.g.*, DSDMA, DLinDMA, DLenDMA, and DODMA.

[0084] The term "hydrophobic lipid" refers to compounds having apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups optionally substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Suitable examples include, but are not limited to, diacylglycerol, dialkylglycerol, N-N-dialkylamino, 1,2-diacyloxy-3-aminopropane, and 1,2-dialkyl-3-aminopropane.

[0085] The term "fusogenic" refers to the ability of a lipid particle, such as a SNALP, to fuse with the membranes of a cell. The membranes can be either the plasma membrane or membranes surrounding organelles, *e.g.*, endosome, nucleus, *etc.*

[0086] As used herein, the term "aqueous solution" refers to a composition comprising in whole, or in part, water.

[0087] As used herein, the term "organic lipid solution" refers to a composition comprising in whole, or in part, an organic solvent having a lipid.

[0088] "Distal site," as used herein, refers to a physically separated site, which is not limited to an adjacent capillary bed, but includes sites broadly distributed throughout an organism.

[0089] "Serum-stable" in relation to nucleic acid-lipid particles such as SNALP means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNAse assay, or an RNAse assay.

[0090] "Systemic delivery," as used herein, refers to delivery of lipid particles that leads to a broad biodistribution of an active agent or therapeutic agent such as an interfering RNA within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. To obtain broad biodistribution

generally requires a blood lifetime such that the agent is not rapidly degraded or cleared (such as by first pass organs (liver, lung, *etc.*) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration. Systemic delivery of lipid particles can be by any means known in the art including, for example, intravenous, subcutaneous, and intraperitoneal. In a preferred embodiment, systemic delivery of lipid particles is by intravenous delivery.

[0091] "Local delivery," as used herein, refers to delivery of an active agent or therapeutic agent such as an interfering RNA directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site such as a tumor or other target site such as a site of inflammation or a target organ such as the liver, heart, pancreas, kidney, and the like.

[0092] The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

[0093] The term "cancer" refers to any member of a class of diseases characterized by the uncontrolled growth of aberrant cells. The term includes all known cancers and neoplastic conditions, whether characterized as malignant, benign, soft tissue, or solid, and cancers of all stages and grades including pre- and post-metastatic cancers. Examples of different types of cancer include, but are not limited to, lung cancer, colon cancer, rectal cancer, anal cancer, bile duct cancer, small intestine cancer, stomach (gastric) cancer, esophageal cancer; gallbladder cancer, liver cancer, pancreatic cancer, appendix cancer, breast cancer, ovarian cancer; cervical cancer, prostate cancer, renal cancer (*e.g.*, renal cell carcinoma), cancer of the central nervous system, glioblastoma, skin cancer, lymphomas, choriocarcinomas, head and neck cancers, osteogenic sarcomas, and blood cancers. Non-limiting examples of specific types of liver cancer include hepatocellular carcinoma (HCC), secondary liver cancer (*e.g.*, caused by metastasis of some other non-liver cancer cell type), and hepatoblastoma. As used herein, a "tumor" comprises one or more cancerous cells.

III. Description of the Embodiments

[0094] The present invention provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.*, for the treatment of a disease or disorder).

[0095] In one aspect, the present invention provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from

about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0096] In certain embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In certain other embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0097] In some embodiments, the active agent or therapeutic agent comprises a nucleic acid. In certain instances, the nucleic acid comprises an interfering RNA molecule such as, *e.g.*, an siRNA, aiRNA, miRNA, or mixtures thereof. In certain other instances, the nucleic acid comprises single-stranded or double-stranded DNA, RNA, or a DNA/RNA hybrid such as, *e.g.*, an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

[0098] In other embodiments, the active agent or therapeutic agent comprises a peptide or polypeptide. In certain instances, the peptide or polypeptide comprises an antibody such as, *e.g.*, a polyclonal antibody, a monoclonal antibody, an antibody fragment; a humanized antibody, a recombinant antibody, a recombinant human antibody, a Primatized™ antibody, or mixtures thereof. In certain other instances, the peptide or polypeptide comprises a cytokine, a growth factor, an apoptotic factor, a differentiation-inducing factor, a cell-surface receptor, a ligand, a hormone, a small molecule (*e.g.*, small organic molecule or compound), or mixtures thereof.

[0099] In preferred embodiments, the active agent or therapeutic agent comprises an siRNA. In one embodiment, the siRNA molecule comprises a double-stranded region of about 15 to about 60 nucleotides in length (*e.g.*, about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, or 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length). The siRNA molecules of the invention are capable of silencing the expression of a target sequence *in vitro* and/or *in vivo*.

[0100] In some embodiments, the siRNA molecule comprises at least one modified nucleotide. In certain preferred embodiments, the siRNA molecule comprises one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides in the double-stranded region. In certain instances, the siRNA comprises from about 1% to about 100%

(*e.g.*, about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region. In preferred embodiments, less than about 25% (*e.g.*, less than about 25%, 20%, 15%, 10%, or 5%) or from about 1% to about 25% (*e.g.*, from about 1%-25%, 5%-25%, 10%-25%, 15%-25%, 20%-25%, or 10%-20%) of the nucleotides in the double-stranded region comprise modified nucleotides.

[0101] In other embodiments, the siRNA molecule comprises modified nucleotides including, but not limited to, 2'-O-methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, locked nucleic acid (LNA) nucleotides, and mixtures thereof. In preferred embodiments, the siRNA comprises 2'OMe nucleotides (*e.g.*, 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof. In certain instances, the siRNA does not comprise 2'OMe-cytosine nucleotides. In other embodiments, the siRNA comprises a hairpin loop structure.

[0102] The siRNA may comprise modified nucleotides in one strand (*i.e.*, sense or antisense) or both strands of the double-stranded region of the siRNA molecule. Preferably, uridine and/or guanosine nucleotides are modified at selective positions in the double-stranded region of the siRNA duplex. With regard to uridine nucleotide modifications, at least one, two, three, four, five, six, or more of the uridine nucleotides in the sense and/or antisense strand can be a modified uridine nucleotide such as a 2'OMe-uridine nucleotide. In some embodiments, every uridine nucleotide in the sense and/or antisense strand is a 2'OMe-uridine nucleotide. With regard to guanosine nucleotide modifications, at least one, two, three, four, five, six, or more of the guanosine nucleotides in the sense and/or antisense strand can be a modified guanosine nucleotide such as a 2'OMe-guanosine nucleotide. In some embodiments, every guanosine nucleotide in the sense and/or antisense strand is a 2'OMe-guanosine nucleotide.

[0103] In certain embodiments, at least one, two, three, four, five, six, seven, or more 5'-GU-3' motifs in an siRNA sequence may be modified, *e.g.*, by introducing mismatches to eliminate the 5'-GU-3' motifs and/or by introducing modified nucleotides such as 2'OMe nucleotides. The 5'-GU-3' motif can be in the sense strand, the antisense strand, or both strands of the siRNA sequence. The 5'-GU-3' motifs may be adjacent to each other or, alternatively, they may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides.

[0104] In some preferred embodiments, a modified siRNA molecule is less immunostimulatory than a corresponding unmodified siRNA sequence. In such embodiments, the modified siRNA molecule with reduced immunostimulatory properties advantageously retains RNAi activity against the target sequence. In another embodiment, the immunostimulatory properties of the modified siRNA molecule and its ability to silence target gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the siRNA sequence such as, *e.g.*, within the double-stranded region of the siRNA duplex. In certain instances, the modified siRNA is at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% less immunostimulatory than the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that the immunostimulatory properties of the modified siRNA molecule and the corresponding unmodified siRNA molecule can be determined by, for example, measuring INF-α and/or IL-6 levels from about two to about twelve hours after systemic administration in a mammal or transfection of a mammalian responder cell using an appropriate lipid-based delivery system (such as the SNALP delivery system disclosed herein).

[0105] In certain embodiments, a modified siRNA molecule has an IC_{50} (*i.e.*, half-maximal inhibitory concentration) less than or equal to ten-fold that of the corresponding unmodified siRNA (*i.e.*, the modified siRNA has an IC_{50} that is less than or equal to ten-times the IC_{50} of the corresponding unmodified siRNA). In other embodiments, the modified siRNA has an IC_{50} less than or equal to three-fold that of the corresponding unmodified siRNA sequence. In yet other embodiments, the modified siRNA has an IC_{50} less than or equal to two-fold that of the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that a dose-response curve can be generated and the IC_{50} values for the modified siRNA and the corresponding unmodified siRNA can be readily determined using methods known to those of skill in the art.

[0106] In yet another embodiment, a modified siRNA molecule is capable of silencing at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the expression of the target sequence relative to the corresponding unmodified siRNA sequence.

[0107] In some embodiments, the siRNA molecule does not comprise phosphate backbone modifications, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In other embodiments, the siRNA comprises one, two, three, four, or more phosphate backbone

modifications, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise phosphate backbone modifications.

[0108] In further embodiments, the siRNA does not comprise 2'-deoxy nucleotides, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In yet further embodiments, the siRNA comprises one, two, three, four, or more 2'-deoxy nucleotides, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise 2'-deoxy nucleotides.

[0109] In certain instances, the nucleotide at the 3'-end of the double-stranded region in the sense and/or antisense strand is not a modified nucleotide. In certain other instances, the nucleotides near the 3'-end (*e.g.*, within one, two, three, or four nucleotides of the 3'-end) of the double-stranded region in the sense and/or antisense strand are not modified nucleotides.

[0110] The siRNA molecules described herein may have 3' overhangs of one, two, three, four, or more nucleotides on one or both sides of the double-stranded region, or may lack overhangs (*i.e.*, have blunt ends) on one or both sides of the double-stranded region. Preferably, the siRNA has 3' overhangs of two nucleotides on each side of the double-stranded region. In certain instances, the 3' overhang on the antisense strand has complementarity to the target sequence and the 3' overhang on the sense strand has complementarity to a complementary strand of the target sequence. Alternatively, the 3' overhangs do not have complementarity to the target sequence or the complementary strand thereof. In some embodiments, the 3' overhangs comprise one, two, three, four, or more nucleotides such as 2'-deoxy (2'H) nucleotides. In certain preferred embodiments, the 3' overhangs comprise deoxythymidine (dT) and/or uridine nucleotides. In other embodiments, one or more of the nucleotides in the 3' overhangs on one or both sides of the double-stranded region comprise modified nucleotides. Non-limiting examples of modified nucleotides are described above and include 2'OMe nucleotides, 2'-deoxy-2'F nucleotides, 2'-deoxy nucleotides, 2'-O-2-MOE nucleotides, LNA nucleotides, and mixtures thereof. In preferred embodiments, one, two, three, four, or more nucleotides in the 3' overhangs present on the sense and/or antisense strand of the siRNA comprise 2'OMe nucleotides (*e.g.*, 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof.

[0111] The siRNA may comprise at least one or a cocktail (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) of unmodified and/or modified siRNA sequences that silence target gene expression. The cocktail of siRNA may comprise sequences which

are directed to the same region or domain (*e.g.*, a "hot spot") and/or to different regions or domains of one or more target genes. In certain instances, one or more (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) modified siRNA that silence target gene expression are present in a cocktail. In certain other instances, one or more (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) unmodified siRNA sequences that silence target gene expression are present in a cocktail.

[0112] In some embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to the target sequence or a portion thereof. In other embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is 100% complementary to the target sequence or a portion thereof. In further embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that specifically hybridizes to the target sequence or a portion thereof.

[0113] In further embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the target sequence or a portion thereof. In additional embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is 100% identical to the target sequence or a portion thereof.

[0114] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the cationic lipid may comprise, *e.g.*, one or more of the following: 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinolcylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP),

3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), or mixtures thereof. In certain preferred embodiments, the cationic lipid is DLinDMA, DLin-K-C2-DMA ("XTC2"), or mixtures thereof.

[0115] The synthesis of cationic lipids such as DLin-K-C2-DMA ("XTC2"), DLin-K-C3-DMA, DLin-K-C4-DMA, DLin-K6-DMA, and DLin-K-MPZ, as well as additional cationic lipids, is described in U.S. Provisional Application No. 61/104,212, filed October 9, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as DLin-K-DMA, DLin-C-DAP, DLin-DAC, DLin-MA, DLinDAP, DLin-S-DMA, DLin-2-DMAP, DLin-TMA.Cl, DLin-TAP.Cl, DLin-MPZ, DLinAP, DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Application No. PCT/US08/88676, filed December 31, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as CLinDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060240554, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0116] In some embodiments, the cationic lipid may comprise from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 50 mol % to about 60 mol % of the total lipid present in the particle.

[0117] In other embodiments, the cationic lipid may comprise from about 55 mol % to about 90 mol %, from about 55 mol % to about 85 mol %, from about 55 mol % to about 80 mol %, from about 55 mol % to about 75 mol %, from about 55 mol % to about 70 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0118] In yet other embodiments, the cationic lipid may comprise from about 60 mol % to about 90 mol %, from about 60 mol % to about 85 mol %, from about 60 mol % to about 80 mol %, from about 60 mol % to about 75 mol %, or from about 60 mol % to about 70 mol % of the total lipid present in the particle.

[0119] In still yet other embodiments, the cationic lipid may comprise from about 65 mol % to about 90 mol %, from about 65 mol % to about 85 mol %, from about 65 mol % to about 80 mol %, or from about 65 mol % to about 75 mol % of the total lipid present in the particle.

[0120] In further embodiments, the cationic lipid may comprise from about 70 mol % to about 90 mol %, from about 70 mol % to about 85 mol %, from about 70 mol % to about 80 mol %, from about 75 mol % to about 90 mol %, from about 75 mol % to about 85 mol %, or from about 80 mol % to about 90 mol % of the total lipid present in the particle.

[0121] In additional embodiments, the cationic lipid may comprise (at least) about 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0122] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the non-cationic lipid may comprise, *e.g.*, one or more anionic lipids and/or neutral lipids. In preferred embodiments, the non-cationic lipid comprises one of the following neutral lipid components: (1) cholesterol or a derivative thereof; (2) a phospholipid; or (3) a mixture of a phospholipid and cholesterol or a derivative thereof.

[0123] Examples of cholesterol derivatives include, but are not limited to, cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof. The synthesis of cholesteryl-2'-hydroxyethyl ether is described herein.

[0124] The phospholipid may be a neutral lipid including, but not limited to, dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-

phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), egg phosphatidylcholine (EPC), and mixtures thereof. In certain preferred embodiments, the phospholipid is DPPC, DSPC, or mixtures thereof.

[0125] In some embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 60 mol %, from about 15 mol % to about 60 mol %, from about 20 mol % to about 60 mol %, from about 25 mol % to about 60 mol %, from about 30 mol % to about 60 mol %, from about 10 mol % to about 55 mol %, from about 15 mol % to about 55 mol %, from about 20 mol % to about 55 mol %, from about 25 mol % to about 55 mol %, from about 30 mol % to about 55 mol %, from about 13 mol % to about 50 mol %, from about 15 mol % to about 50 mol % or from about 20 mol % to about 50 mol % of the total lipid present in the particle. When the non-cationic lipid is a mixture of a phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 50, or 60 mol % of the total lipid present in the particle.

[0126] In other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 49.5 mol %, from about 13 mol % to about 49.5 mol %, from about 15 mol % to about 49.5 mol %, from about 20 mol % to about 49.5 mol %, from about 25 mol % to about 49.5 mol %, from about 30 mol % to about 49.5 mol %, from about 35 mol % to about 49.5 mol %, or from about 40 mol % to about 49.5 mol % of the total lipid present in the particle.

[0127] In yet other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 45 mol %, from about 13 mol % to about 45 mol %, from about 15 mol % to about 45 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, or from about 35 mol % to about 45 mol % of the total lipid present in the particle.

[0128] In still yet other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 40 mol %, from about 13 mol % to about 40 mol %, from about 15 mol % to about 40 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0129] In further embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 35 mol %, from about 13 mol % to about 35 mol %, from about 15 mol % to about 35 mol %, from about 20 mol % to

about 35 mol %, or from about 25 mol % to about 35 mol % of the total lipid present in the particle.

[0130] In yet further embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 30 mol %, from about 13 mol % to about 30 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 13 mol % to about 25 mol %, or from about 15 mol % to about 25 mol % of the total lipid present in the particle.

[0131] In additional embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise (at least) about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0132] In certain preferred embodiments, the non-cationic lipid comprises cholesterol or a derivative thereof of from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle of the invention may comprise cholesterol or a derivative thereof at about 37 mol % of the total lipid present in the particle. In other preferred embodiments, a phospholipid-free lipid particle of the invention may comprise cholesterol or a derivative thereof of from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 32 mol % to about 45 mol %, from about 32 mol % to about 42 mol %, from about 32 mol % to about 40 mol %, from about 34 mol % to about 45 mol %, from about 34 mol % to about 42 mol %, from about 34 mol % to about 40 mol %, or about 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0133] In certain other preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 4 mol % to about 10 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 30 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 7 mol % and cholesterol at about 34 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 3 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 4 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 4 mol % to about 8 mol

%, from about 5 mol % to about 12 mol %, from about 5 mol % to about 9 mol %, from about 6 mol % to about 12 mol %, from about 6 mol % to about 10 mol %, or about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 40 mol %, from about 25 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 28 mol % to about 40 mol %, from about 28 mol % to about 38 mol %, from about 30 mol % to about 38 mol %, from about 32 mol % to about 36 mol %, or about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0134] In further preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 20 mol % and cholesterol at about 20 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0135] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the conjugated lipid that inhibits aggregation of particles may comprise, *e.g.*, one or more of the following: a polyethyleneglycol (PEG)-lipid conjugate, a polyamide

(ATTA)-lipid conjugate, a cationic-polymer-lipid conjugates (CPLs), or mixtures thereof. In one preferred embodiment, the nucleic acid-lipid particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate. In certain embodiments, the PEG-lipid conjugate or ATTA-lipid conjugate is used together with a CPL. The conjugated lipid that inhibits aggregation of particles may comprise a PEG-lipid including, *e.g.*, a PEG-diacylglycerol (DAG), a PEG dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or mixtures thereof. The PEG-DAA conjugate may be PEG-dilauryloxypropyl (C12), a PEG-dimyristyloxypropyl (C14), a PEG-dipalmityloxypropyl (C16), a PEG-distearyloxypropyl (C18), or mixtures thereof.

[0136] Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-*sn*3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed December 31, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Yet additional PEG-lipid conjugates suitable for use in the invention include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol) (2KPEG-DMG). The synthesis of 2KPEG-DMG is described in U.S. Patent No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0137] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.,* from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

[0138] In some embodiments, the conjugated lipid that inhibits aggregation of particles is a CPL that has the formula: A-W-Y, wherein A is a lipid moiety, W is a hydrophilic polymer, and Y is a polycationic moiety. W may be a polymer selected from the group consisting of polyethyleneglycol (PEG), polyamide, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, or combinations thereof, the polymer having a molecular weight of from about 250 to about 7000 daltons. In some embodiments, Y has at least 4 positive charges at a selected pH. In some embodiments, Y may be lysine, arginine, asparagine, glutamine, derivatives thereof, or combinations thereof.

[0139] In certain instances, the conjugated lipid that inhibits aggregation of particles (*e.g.*, PEG-lipid conjugate) may comprise from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 1 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, from about 1.4 mol % to about 1.5 mol %, or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0140] In the lipid particles of the invention, the active agent or therapeutic agent may be fully encapsulated within the lipid portion of the particle, thereby protecting the active agent or therapeutic agent from enzymatic degradation. In preferred embodiments, a SNALP comprising a nucleic acid such as an interfering RNA (*e.g.*, siRNA) is fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the nucleic acid in the SNALP is not substantially degraded after exposure of the particle to a nuclease at 37°C for at least about 20, 30, 45, or 60 minutes. In certain other instances, the nucleic acid in the SNALP is not substantially degraded after incubation of the particle in serum at 37°C for at least about 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In other embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid such as siRNA) is complexed with the lipid portion of the particle. One of the benefits of the formulations of the present invention is that the lipid particle compositions are substantially non-toxic to mammals such as humans.

[0141] The term "fully encapsulated" indicates that the active agent or therapeutic agent in the lipid particle is not significantly degraded after exposure to serum or a nuclease or protease assay that would significantly degrade free DNA, RNA, or protein. In a fully encapsulated system, preferably less than about 25% of the active agent or therapeutic agent in the particle is degraded in a treatment that would normally degrade 100% of free active agent or therapeutic agent, more preferably less than about 10%, and most preferably less than about 5% of the active agent or therapeutic agent in the particle is degraded. In the context of nucleic acid therapeutic agents, full encapsulation may be determined by an Oligreen® assay. Oligreen® is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA or RNA in solution (available from Invitrogen Corporation; Carlsbad, CA). "Fully encapsulated" also indicates that the lipid particles are

serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

[0142] In another aspect, the present invention provides a lipid particle (*e.g.*, SNALP) composition comprising a plurality of lipid particles. In preferred embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid) is fully encapsulated within the lipid portion of the lipid particles (*e.g.*, SNALP), such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, %, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the lipid particles (*e.g.*, SNALP) have the active agent or therapeutic agent encapsulated therein.

[0143] Typically, the lipid particles (*e.g.*, SNALP) of the invention have a lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 1 to about 100. In some instances, the lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) ranges from about 1 to about 50, from about 2 to about 25, from about 3 to about 20, from about 4 to about 15, or from about 5 to about 10. In preferred embodiments, the lipid particles of the invention have a lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 5 to about 15, *e.g.*, about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 (or any fraction thereof or range therein).

[0144] Typically, the lipid particles (*e.g.*, SNALP) of the invention have a mean diameter of from about 40 nm to about 150 nm. In preferred embodiments, the lipid particles (*e.g.*, SNALP) of the invention have a mean diameter of from about 40 nm to about 130 nm, from about 40 nm to about 120 nm, from about 40 nm to about 100 nm, from about 50 nm to about 120 nm, from about 50 nm to about 100 nm, from about 60 nm to about 120 nm, from about 60 nm to about 110 nm, from about 60 nm to about 100 nm, from about 60 nm to about 90 nm, from about 60 nm to about 80 nm, from about 70 nm to about 120 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 70 nm to about 90 nm, from about 70 nm to about 80 nm, or less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm (or any fraction thereof or range therein).

[0145] In one specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:62" formulation. In a preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is cholesterol, and the conjugated lipid is a PEG-DAA conjugate. Although these are preferred embodiments of the 1:62 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic lipids (including other cholesterol derivatives), and conjugated lipids can be used in the 1:62 formulation as described herein.

[0146] In another specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:57" formulation. In one preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 5 mol % to about 9 mol % of the total lipid present in the particle (*e.g.*, about 7.1 rmol %) and the cholesterol (or cholesterol derivative) comprises from about 32 mol % to about 37 mol % of the total lipid present in the particle (*e.g.*, about 34.3 mol %), and the PEG-lipid is a PEG-DAA (*e.g.*, PEG-cDMA). In another preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.*, about 20 mol %) and the cholesterol (or cholesterol derivative) comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.*, about 20 mol %), and the PEG-lipid is a PEG-DAA (*e.g.*, PEG-cDMA). Although these are preferred embodiments of the 1:57 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic

lipids (including other phospholipids and other cholesterol derivatives), and conjugated lipids can be used in the 1:57 formulation as described herein.

[0147] In preferred embodiments, the 1:62 SNALP formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-cDMA (or PEG-cDSA), about 61.5 mol % DLinDMA (or XTC2), and about 36.9 mol % cholesterol (or derivative thereof). In other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 7.1 mol % DPPC, and about 34.3 mol % cholesterol (or derivative thereof). In yet other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 20 mol % DPPC, and about 20 mol % cholesterol (or derivative thereof). It should be understood that these SNALP formulations are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the SNALP formulations may vary.

[0148] The present invention also provides a pharmaceutical composition comprising a lipid particle (*e.g.*, SNALP) described herein and a pharmaceutically acceptable carrier.

[0149] In a further aspect, the present invention provides a method for introducing one or more active agents or therapeutic agents (*e.g.*, nucleic acid) into a cell, comprising contacting the cell with a lipid particle (*e.g.*, SNALP) described herein. In one embodiment, the cell is in a mammal and the mammal is a human. In another embodiment, the present invention provides a method for the *in vivo* delivery of one or more active agents or therapeutic agents (*e.g.*, nucleic acid), comprising administering to a mammalian subject a lipid particle (*e.g.*, SNALP) described herein. In a preferred embodiment, the mode of administration includes, but is not limited to, oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. Preferably, the mammalian subject is a human.

[0150] In one embodiment, at least about 5%, 10%, 15%, 20%, or 25% of the total injected dose of the lipid particles (*e.g.*, SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In other embodiments, more than about 20%, 30%, 40% and as much as about 60%, 70% or 80% of the total injected dose of the lipid particles (*e.g.*, SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In certain instances, more than about 10% of a plurality of the particles is present in the plasma of a mammal about 1 hour after administration. In certain other instances, the presence of the lipid particles (*e.g.*,

SNALP) is detectable at least about 1 hour after administration of the particle. In certain embodiments, the presence of an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is detectable in cells of the lung, liver, tumor, or at a site of inflammation at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is detectable at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In yet other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) occurs preferentially in tumor cells or in cells at a site of inflammation. In further embodiments, the presence or effect of an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) in cells at a site proximal or distal to the site of administration or in cells of the lung, liver, or a tumor is detectable at about 12, 24, 48, 72, or 96 hours, or at about 6, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24, 26, or 28 days after administration. In additional embodiments, the lipid particles (*e.g.*, SNALP) of the invention are administered parenterally or intraperitoneally.

[0151] In some embodiments, the lipid particles (*e.g.*, SNALP) of the invention are particularly useful in methods for the therapeutic delivery of one or more nucleic acids comprising an interfering RNA sequence (*e.g.*, siRNA). In particular, it is an object of this invention to provide *in vitro* and *in vivo* methods for treatment of a disease or disorder in a mammal (*e.g.*, a rodent such as a mouse or a primate such as a human, chimpanzee, or monkey) by downregulating or silencing the transcription and/or translation of one or more target nucleic acid sequences or genes of interest. As a non-limiting example, the methods of the invention are useful for *in vivo* delivery of interfering RNA (*e.g.*, siRNA) to the liver and/or tumor of a mammalian subject. In certain embodiments, the disease or disorder is associated with expression and/or overexpression of a gene and expression or overexpression of the gene is reduced by the interfering RNA (*e.g.*, siRNA). In certain other embodiments, a therapeutically effective amount of the lipid particle (*e.g.*, SNALP) may be administered to the mammal. In some instances, an interfering RNA (*e.g.*, siRNA) is formulated into a SNALP, and the particles are administered to patients requiring such treatment. In other instances, cells are removed from a patient, the interfering RNA (*e.g.*, siRNA) is delivered *in vitro* (*e.g.*, using a SNALP described herein), and the cells are reinjected into the patient.

[0152] In an additional aspect, the present invention provides lipid particles (*e.g.*, SNALP) comprising asymmetrical interfering RNA (aiRNA) molecules that silence the expression of a target gene and methods of using such particles to silence target gene expression.

[0153] In one embodiment, the aiRNA molecule comprises a double-stranded (duplex) region of about 10 to about 25 (base paired) nucleotides in length, wherein the aiRNA molecule comprises an antisense strand comprising 5' and 3' overhangs, and wherein the aiRNA molecule is capable of silencing target gene expression.

[0154] In certain instances, the aiRNA molecule comprises a double-stranded (duplex) region of about 12-20, 12-19, 12-18, 13-17, or 14-17 (base paired) nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 (base paired) nucleotides in length. In certain other instances, the 5' and 3' overhangs on the antisense strand comprise sequences that are complementary to the target RNA sequence, and may optionally further comprise nontargeting sequences. In some embodiments, each of the 5' and 3' overhangs on the antisense strand comprises or consists of one, two, three, four, five, six, seven, or more nucleotides.

[0155] In other embodiments, the aiRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0156] In a related aspect, the present invention provides lipid particles (*e.g.*, SNALP) comprising microRNA (miRNA) molecules that silence the expression of a target gene and methods of using such compositions to silence target gene expression.

[0157] In one embodiment, the miRNA molecule comprises about 15 to about 60 nucleotides in length, wherein the miRNA molecule is capable of silencing target gene expression.

[0158] In certain instances, the miRNA molecule comprises about 15-50, 15-40, or 15-30 nucleotides in length, more typically about 15-25 or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In a preferred embodiment, the miRNA molecule is a mature miRNA molecule targeting an RNA sequence of interest.

[0159] In some embodiments, the miRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0160] As such, the lipid particles of the invention (*e.g.*, SNALP) are advantageous and suitable for use in the administration of active agents or therapeutic agents such as nucleic acid (*e.g.*, interfering RNA such as siRNA, aiRNA, and/or miRNA) to a subject (*e.g.*, a mammal such as a human) because they are stable in circulation, of a size required for pharmacodynamic behavior resulting in access to extravascular sites, and are capable of reaching target cell populations.

IV. Active Agents

[0161] Active agents (*e.g.*, therapeutic agents) include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be, *e.g.*, biological, physiological, and/or cosmetic. Active agents may be any type of molecule or compound including, but not limited to, nucleic acids, peptides, polypeptides, small molecules, and mixtures thereof. Non-limiting examples of nucleic acids include interfering RNA molecules (*e.g.*, siRNA, aiRNA, miRNA), antisense oligonucleotides, plasmids, ribozymes, immunostimulatory oligonucleotides, and mixtures thereof. Examples of peptides or polypeptides include, without limitation, antibodies (*e.g.*, polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, Primatized™ antibodies), cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell-surface receptors and their ligands, hormones, and mixtures thereof. Examples of small molecules include, but are not limited to, small organic molecules or compounds such as any conventional agent or drug known to those of skill in the art.

[0162] In some embodiments, the active agent is a therapeutic agent, or a salt or derivative thereof. Therapeutic agent derivatives may be therapeutically active themselves or they may be prodrugs, which become active upon further modification. Thus, in one embodiment, a therapeutic agent derivative retains some or all of the therapeutic activity as compared to the unmodified agent, while in another embodiment, a therapeutic agent derivative is a prodrug that lacks therapeutic activity, but becomes active upon further modification.

A. Nucleic Acids

[0163] In certain embodiments, lipid particles of the present invention are associated with a nucleic acid, resulting in a nucleic acid-lipid particle (*e.g.*, SNALP). In some embodiments, the nucleic acid is fully encapsulated in the lipid particle. As used herein, the term "nucleic acid" includes any oligonucleotide or polynucleotide, with fragments containing up to 60

nucleotides generally termed oligonucleotides, and longer fragments termed polynucleotides. In particular embodiments, oligonucletoides of the invention are from about 15 to about 60 nucleotides in length. Nucleic acid may be administered alone in the lipid particles of the invention, or in combination (*e.g.*, co-administered) with lipid particles of the invention comprising peptides, polypeptides, or small molecules such as conventional drugs.

[0164] In the context of this invention, the terms "polynucleotide" and "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally-occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also include polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake, reduced immunogenicity, and increased stability in the presence of nucleases.

[0165] Oligonucleotides are generally classified as deoxyribooligonucleotides or ribooligonucleotides. A deoxyribooligonucleotide consists of a 5-carbon sugar called deoxyribose joined covalently to phosphate at the 5′ and 3′ carbons of this sugar to form an alternating, unbranched polymer. A ribooligonucleotide consists of a similar repeating structure where the 5-carbon sugar is ribose.

[0166] The nucleic acid that is present in a lipid-nucleic acid particle according to this invention includes any form of nucleic acid that is known. The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Examples of double-stranded DNA are described herein and include, *e.g.*, structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA are described herein and include, *e.g.*, siRNA and other RNAi agents such as aiRNA and pre-miRNA. Single-stranded nucleic acids include, *e.g.*, antisense oligonucleotides, ribozymes, mature miRNA, and triplex-forming oligonucleotides.

[0167] Nucleic acids of the invention may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to about 100,000 nucleotide residues in length. In particular embodiments, oligonucleotides may range from about 10 to about 100 nucleotides in length. In various related embodiments, oligonucleotides, both single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 60 nucleotides, from about 15 to

about 60 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, or from about 20 to about 30 nucleotides in length.

[0168] In particular embodiments, an oligonucleotide (or a strand thereof) of the invention specifically hybridizes to or is complementary to a target polynucleotide sequence. The terms "specifically hybridizable" and "complementary" as used herein indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. In preferred embodiments, an oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target sequence interferes with the normal function of the target sequence to cause a loss of utility or expression therefrom, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or, in the case of *in vitro* assays, under conditions in which the assays are conducted. Thus, the oligonucleotide may include 1, 2, 3, or more base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

1. siRNA

[0169] The siRNA component of the nucleic acid-lipid particles of the present invention is capable of silencing the expression of a target gene of interest. Each strand of the siRNA duplex is typically about 15 to about 60 nucleotides in length, preferably about 15 to about 30 nucleotides in length. In certain embodiments, the siRNA comprises at least one modified nucleotide. The modified siRNA is generally less immunostimulatory than a corresponding unmodified siRNA sequence and retains RNAi activity against the target gene of interest. In some embodiments, the modified siRNA contains at least one 2'OMe purine or pyrimidine nucleotide such as a 2'OMe-guanosine, 2'OMe-uridine, 2'OMe-adenosine, and/or 2'OMe-cytosine nucleotide. In preferred embodiments, one or more of the uridine and/or guanosine nucleotides are modified. The modified nucleotides can be present in one strand (*i.e.*, sense or antisense) or both strands of the siRNA. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell*, 107:309 (2001)), or may lack overhangs (*i.e.*, have blunt ends).

[0170] The modified siRNA generally comprises from about 1% to about 100% (*e.g.*, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%,

19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region of the siRNA duplex. In certain embodiments, one, two, three, four, five, six, seven, eight, nine, ten, or more of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0171] In some embodiments, less than about 25% (*e.g.*, less than about 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0172] In other embodiments, from about 1% to about 25% (*e.g.*, from about 1%-25%, 2%-25%, 3%-25%, 4%-25%, 5%-25%, 6%-25%, 7%-25%, 8%-25%, 9%-25%, 10%-25%, 11%-25%, 12%-25%, 13%-25%, 14%-25%, 15%-25%, 16%-25%, 17%-25%, 18%-25%, 19%-25%, 20%-25%, 21%-25%, 22%-25%, 23%-25%, 24%-25%, *etc.*) or from about 1% to about 20% (*e.g.*, from about 1%-20%, 2%-20%, 3%-20%, 4%-20%, 5%-20%, 6%-20%, 7%-20%, 8%-20%, 9%-20%, 10%-20%, 11%-20%, 12%-20%, 13%-20%, 14%-20%, 15%-20%, 16%-20%, 17%-20%, 18%-20%, 19%-20%, 1%-19%, 2%-19%, 3%-19%, 4%-19%, 5%-19%, 6%-19%, 7%-19%, 8%-19%, 9%-19%, 10%-19%, 11%-19%, 12%-19%, 13%-19%, 14%-19%, 15%-19%, 16%-19%, 17%-19%, 18%-19%, 1%-18%, 2%-18%, 3%-18%, 4%-18%, 5%-18%, 6%-18%, 7%-18%, 8%-18%, 9%-18%, 10%-18%, 11%-18%, 12%-18%, 13%-18%, 14%-18%, 15%-18%, 16%-18%, 17%-18%, 1%-17%, 2%-17%, 3%-17%, 4%-17%, 5%-17%, 6%-17%, 7%-17%, 8%-17%, 9%-17%, 10%-17%, 11%-17%, 12%-17%, 13%-17%, 14%-17%, 15%-17%, 16%-17%, 1%-16%, 2%-16%, 3%-16%, 4%-16%, 5%-16%, 6%-16%, 7%-16%, 8%-16%, 9%-16%, 10%-16%, 11%-16%, 12%-16%, 13%-16%, 14%-16%, 15%-16%, 1%-15%, 2%-15%, 3%-15%, 4%-15%, 5%-15%, 6%-15%, 7%-15%, 8%-15%, 9%-15%, 10%-15%, 11%-15%, 12%-15%, 13%-15%, 14%-15%, *etc.*) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0173] In further embodiments, *e.g.*, when one or both strands of the siRNA are selectively modified at uridine and/or guanosine nucleotides, the resulting modified siRNA can comprise less than about 30% modified nucleotides (*e.g.*, less than about 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% modified nucleotides) or from about 1% to about 30% modified nucleotides (*e.g.*, from about 1%-30%, 2%-30%, 3%-30%, 4%-30%, 5%-30%, 6%-30%, 7%-30%, 8%-30%, 9%-30%, 10%-30%, 11%-30%, 12%-30%, 13%-30%, 14%-30%, 15%-30%, 16%-30%, 17%-30%, 18%-30%, 19%-30%, 20%-30%, 21%-30%, 22%-

30%, 23%-30%, 24%-30%, 25%-30%, 26%-30%, 27%-30%, 28%-30%, or 29%-30% modified nucleotides).

a. Selection of siRNA Sequences

[0174] Suitable siRNA sequences can be identified using any means known in the art. Typically, the methods described in Elbashir *et al.*, *Nature,* 411:494-498 (2001) and Elbashir *et al.*, *EMBO J.,* 20:6877-6888 (2001) are combined with rational design rules set forth in Reynolds *et al.*, *Nature Biotech.*, 22(3):326-330 (2004).

[0175] Generally, the nucleotide sequence 3' of the AUG start codon of a transcript from the target gene of interest is scanned for dinucleotide sequences (*e.g.*, AA, NA, CC, GG, or UU, wherein N = C, G, or U) (*see*, *e.g.*, Elbashir *et al.*, *EMBO J.,* 20:6877-6888 (2001)). The nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences (*i.e.*, a target sequence or a sense strand sequence). Typically, the 19, 21, 23, 25, 27, 29, 31, 33, 35, or more nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences. In some embodiments, the dinucleotide sequence is an AA or NA sequence and the 19 nucleotides immediately 3' to the AA or NA dinucleotide are identified as potential siRNA sequences. siRNA sequences are usually spaced at different positions along the length of the target gene. To further enhance silencing efficiency of the siRNA sequences, potential siRNA sequences may be analyzed to identify sites that do not contain regions of homology to other coding sequences, *e.g.,* in the target cell or organism. For example, a suitable siRNA sequence of about 21 base pairs typically will not have more than 16-17 contiguous base pairs of homology to coding sequences in the target cell or organism. If the siRNA sequences are to be expressed from an RNA Pol III promoter, siRNA sequences lacking more than 4 contiguous A's or T's are selected.

[0176] Once a potential siRNA sequence has been identified, a complementary sequence (*i.e.*, an antisense strand sequence) can be designed. A potential siRNA sequence can also be analyzed using a variety of criteria known in the art. For example, to enhance their silencing efficiency, the siRNA sequences may be analyzed by a rational design algorithm to identify sequences that have one or more of the following features: (1) G/C content of about 25% to about 60% G/C; (2) at least 3 A/Us at positions 15-19 of the sense strand; (3) no internal repeats; (4) an A at position 19 of the sense strand; (5) an A at position 3 of the sense strand; (6) a U at position 10 of the sense strand; (7) no G/C at position 19 of the sense strand; and (8) no G at position 13 of the sense strand. siRNA design tools that incorporate algorithms that assign suitable values of each of these features and are useful for selection of siRNA can

be found at, *e.g.*, http://boz094.ust.hk/RNAi/siRNA. One of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may be selected for further analysis and testing as potential siRNA sequences.

[0177] Additionally, potential siRNA sequences with one or more of the following criteria can often be eliminated as siRNA: (1) sequences comprising a stretch of 4 or more of the same base in a row; (2) sequences comprising homopolymers of Gs (*i.e.*, to reduce possible non-specific effects due to structural characteristics of these polymers; (3) sequences comprising triple base motifs (*e.g.*, GGG, CCC, AAA, or TTT); (4) sequences comprising stretches of 7 or more G/Cs in a row; and (5) sequences comprising direct repeats of 4 or more bases within the candidates resulting in internal fold-back structures. However, one of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may still be selected for further analysis and testing as potential siRNA sequences.

[0178] In some embodiments, potential siRNA sequences may be further analyzed based on siRNA duplex asymmetry as described in, *e.g.*, Khvorova *et al.*, *Cell*, 115:209-216 (2003); and Schwarz *et al.*, *Cell*, 115:199-208 (2003). In other embodiments, potential siRNA sequences may be further analyzed based on secondary structure at the target site as described in, *e.g.*, Luo *et al.*, *Biophys. Res. Commun.*, 318:303-310 (2004). For example, secondary structure at the target site can be modeled using the Mfold algorithm (available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) to select siRNA sequences which favor accessibility at the target site where less secondary structure in the form of base-pairing and stem-loops is present.

[0179] Once a potential siRNA sequence has been identified, the sequence can be analyzed for the presence of any immunostimulatory properties, *e.g.*, using an *in vitro* cytokine assay or an *in vivo* animal model. Motifs in the sense and/or antisense strand of the siRNA sequence such as GU-rich motifs (*e.g.*, 5'-GU-3', 5'-UGU-3', 5'-GUGU-3', 5'-UGUGU-3', *etc.*) can also provide an indication of whether the sequence may be immunostimulatory. Once an siRNA molecule is found to be immunostimulatory, it can then be modified to decrease its immunostimulatory properties as described herein. As a non-limiting example, an siRNA sequence can be contacted with a mammalian responder cell under conditions such that the cell produces a detectable immune response to determine whether the siRNA is an immunostimulatory or a non-immunostimulatory siRNA. The mammalian responder cell may be from a naïve mammal (*i.e.*, a mammal that has not previously been in contact with the gene product of the siRNA sequence). The mammalian responder cell may be, *e.g.*, a peripheral blood mononuclear cell (PBMC), a macrophage, and the like. The detectable

immune response may comprise production of a cytokine or growth factor such as, *e.g.*, TNF-α, IFN-α, IFN-β, IFN-γ, IL-6, IL-12, or a combination thereof. An siRNA molecule identified as being immunostimulatory can then be modified to decrease its immunostimulatory properties by replacing at least one of the nucleotides on the sense and/or antisense strand with modified nucleotides. For example, less than about 30% (*e.g.*, less than about 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA duplex can be replaced with modified nucleotides such as 2'OMe nucleotides. The modified siRNA can then be contacted with a mammalian responder cell as described above to confirm that its immunostimulatory properties have been reduced or abrogated.

[0180] Suitable *in vitro* assays for detecting an immune response include, but are not limited to, the double monoclonal antibody sandwich immunoassay technique of David *et al.* (U.S. Patent No. 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide *et al.*, in Kirkham and Hunter, eds., *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh (1970)); the "Western blot" method of Gordon *et al.* (U.S. Patent No. 4,452,901); immunoprecipitation of labeled ligand (Brown *et al.*, *J. Biol. Chem.*, 255:4980-4983 (1980)); enzyme-linked immunosorbent assays (ELISA) as described, for example, by Raines *et al.*, *J. Biol. Chem.*, 257:5154-5160 (1982); immunocytochemical techniques, including the use of fluorochromes (Brooks *et al.*, *Clin. Exp. Immunol.*, 39:477 (1980)); and neutralization of activity (Bowen-Pope *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:2396-2400 (1984)). In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S. Patent Nos. 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0181] A non-limiting example of an *in vivo* model for detecting an immune response includes an *in vivo* mouse cytokine induction assay as described in, *e.g.*, Judge *et al.*, *Mol. Ther.*, 13:494-505 (2006). In certain embodiments, the assay that can be performed as follows: (1) siRNA can be administered by standard intravenous injection in the lateral tail vein; (2) blood can be collected by cardiac puncture about 6 hours after administration and processed as plasma for cytokine analysis; and (3) cytokines can be quantified using sandwich ELISA kits according to the manufacturer's instructions (*e.g.*, mouse and human IFN-α (PBL Biomedical; Piscataway, NJ); human IL-6 and TNF-α (eBioscience; San Diego, CA); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences; San Diego, CA)).

[0182] Monoclonal antibodies that specifically bind cytokines and growth factors are commercially available from multiple sources and can be generated using methods known in

the art (*see, e.g.*, Kohler *et al.*, *Nature*, 256: 495-497 (1975) and Harlow and Lane, ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publication, New York (1999)). Generation of monoclonal antibodies has been previously described and can be accomplished by any means known in the art (Buhring *et al.*, in Hybridoma, Vol. 10, No. 1, pp. 77-78 (1991)). In some methods, the monoclonal antibody is labeled (*e.g.*, with any composition detectable by spectroscopic, photochemical, biochemical, electrical, optical, or chemical means) to facilitate detection.

b. Generating siRNA Molecules

[0183] siRNA can be provided in several forms including, *e.g.*, as one or more isolated small-interfering RNA (siRNA) duplexes, as longer double-stranded RNA (dsRNA), or as siRNA or dsRNA transcribed from a transcriptional cassette in a DNA plasmid. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell*, 107:309 (2001), or may lack overhangs (*i.e.*, to have blunt ends).

[0184] An RNA population can be used to provide long precursor RNAs, or long precursor RNAs that have substantial or complete identity to a selected target sequence can be used to make the siRNA. The RNAs can be isolated from cells or tissue, synthesized, and/or cloned according to methods well known to those of skill in the art. The RNA can be a mixed population (obtained from cells or tissue, transcribed from cDNA, subtracted, selected, *etc.*), or can represent a single target sequence. RNA can be naturally occurring (*e.g.*, isolated from tissue or cell samples), synthesized *in vitro (e.g.,* using T7 or SP6 polymerase and PCR products or a cloned cDNA), or chemically synthesized.

[0185] To form a long dsRNA, for synthetic RNAs, the complement is also transcribed *in vitro* and hybridized to form a dsRNA. If a naturally occuring RNA population is used, the RNA complements are also provided (*e.g.*, to form dsRNA for digestion by *E. coli* RNAse III or Dicer), *e.g.*, by transcribing cDNAs corresponding to the RNA population, or by using RNA polymerases. The precursor RNAs are then hybridized to form double stranded RNAs for digestion. The dsRNAs can be directly administered to a subject or can be digested *in vitro* prior to administration.

[0186] Methods for isolating RNA, synthesizing RNA, hybridizing nucleic acids, making and screening cDNA libraries, and performing PCR are well known in the art (*see, e.g.,* Gubler and Hoffman, *Gene*, 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*), as are PCR methods (*see,* U.S. Patent Nos. 4,683,195 and 4,683,202; *PCR Protocols:*

A Guide to Methods and Applications (Innis *et al.*, eds, 1990)). Expression libraries are also well known to those of skill in the art. Additional basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0187] Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules of the invention can be synthesized using any of a variety of techniques known in the art, such as those described in Usman *et al.*, *J. Am. Chem. Soc.*, 109:7845 (1987); Scaringe *et al.*, *Nucl. Acids Res.*, 18:5433 (1990); Wincott *et al.*, *Nucl. Acids Res.*, 23:2677-2684 (1995); and Wincott *et al., Methods Mol. Bio.*, 74:59 (1997). The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example, small scale syntheses can be conducted on an Applied Biosystems synthesizer using a 0.2 μmol scale protocol. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer from Protogene (Palo Alto, CA). However, a larger or smaller scale of synthesis is also within the scope of this invention. Suitable reagents for oligonucleotide synthesis, methods for RNA deprotection, and methods for RNA purification are known to those of skill in the art.

[0188] siRNA molecules can also be synthesized via a tandem synthesis technique, wherein both strands are synthesized as a single continuous oligonucleotide fragment or strand separated by a cleavable linker that is subsequently cleaved to provide separate fragments or strands that hybridize to form the siRNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siRNA can be readily adapted to both multiwell/multiplate synthesis platforms as well as large scale synthesis platforms employing batch reactors, synthesis columns, and the like. Alternatively, siRNA molecules can be assembled from two distinct oligonucleotides, wherein one oligonucleotide comprises the sense strand and the other comprises the antisense strand of the siRNA. For example, each strand can be synthesized separately and joined together by hybridization or ligation following synthesis and/or deprotection. In certain other instances, siRNA molecules can be synthesized as a single continuous oligonucleotide fragment, where the self-complementary sense and antisense regions hybridize to form an siRNA duplex having hairpin secondary structure.

c. Modifying siRNA Sequences

[0189] In certain aspects, siRNA molecules comprise a duplex having two strands and at least one modified nucleotide in the double-stranded region, wherein each strand is about 15 to about 60 nucleotides in length. Advantageously, the modified siRNA is less immunostimulatory than a corresponding unmodified siRNA sequence, but retains the capability of silencing the expression of a target sequence. In preferred embodiments, the degree of chemical modifications introduced into the siRNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the siRNA and retention of RNAi activity. As a non-limiting example, an siRNA molecule that targets a gene of interest can be minimally modified (*e.g.*, less than about 30%, 25%, 20%, 15%, 10%, or 5% modified) at selective uridine and/or guanosine nucleotides within the siRNA duplex to eliminate the immune response generated by the siRNA while retaining its capability to silence target gene expression.

[0190] Examples of modified nucleotides suitable for use in the invention include, but are not limited to, ribonucleotides having a 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl group. Modified nucleotides having a Northern conformation such as those described in, *e.g.*, Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag Ed. (1984), are also suitable for use in siRNA molecules. Such modified nucleotides include, without limitation, locked nucleic acid (LNA) nucleotides (*e.g.*, 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides), 2'-O-(2-methoxyethyl) (MOE) nucleotides, 2'-methyl-thio-ethyl nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy-2'-chloro (2'Cl) nucleotides, and 2'-azido nucleotides. In certain instances, the siRNA molecules described herein include one or more G-clamp nucleotides. A G-clamp nucleotide refers to a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine nucleotide within a duplex (*see, e.g.*, Lin *et al.*, *J. Am. Chem. Soc.*, 120:8531-8532 (1998)). In addition, nucleotides having a nucleotide base analog such as, for example, C-phenyl, C-naphthyl, other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole (*see, e.g.*, Loakes, *Nucl. Acids Res.*, 29:2437-2447 (2001)) can be incorporated into siRNA molecules.

[0191] In certain embodiments, siRNA molecules may further comprise one or more chemical modifications such as terminal cap moieties, phosphate backbone modifications, and the like. Examples of terminal cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4',5'-methylene nucleotides, 1-(β-D-erythrofuranosyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α-nucleotides, modified base nucleotides, *threo*-pentofuranosyl nucleotides, acyclic 3',4'-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3'-3'-inverted nucleotide moieties, 3'-3'-inverted abasic moieties, 3'-2'-inverted nucleotide moieties, 3'-2'-inverted abasic moieties, 5'-5'-inverted nucleotide moieties, 5'-5'-inverted abasic moieties, 3'-5'-inverted deoxy abasic moieties, 5'-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, 6-aminohexyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3'-phosphoramidate, 5'-phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 5'-amino, 3'-phosphorothioate, 5'-phosphorothioate, phosphorodithioate, and bridging or non-bridging methylphosphonate or 5'-mercapto moieties (*see, e.g.,* U.S. Patent No. 5,998,203; Beaucage *et al.*, *Tetrahedron* 49:1925 (1993)). Non-limiting examples of phosphate backbone modifications (*i.e.,* resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and alkylsilyl substitutions (*see, e.g.,* Hunziker *et al.*, *Nucleic Acid Analogues: Synthesis and Properties*, in *Modern Synthetic Methods*, VCH, 331-417 (1995); Mesmaeker *et al.*, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39 (1994)). Such chemical modifications can occur at the 5'-end and/or 3'-end of the sense strand, antisense strand, or both strands of the siRNA. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0192] In some embodiments, the sense and/or antisense strand of the siRNA molecule can further comprise a 3'-terminal overhang having about 1 to about 4 (*e.g.,* 1, 2, 3, or 4) 2'-deoxy ribonucleotides and/or any combination of modified and unmodified nucleotides. Additional examples of modified nucleotides and types of chemical modifications that can be introduced into siRNA molecules are described, *e.g.,* in UK Patent No. GB 2,397,818 B and

U.S. Patent Publication Nos. 20040192626, 20050282188, and 20070135372, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0193] The siRNA molecules described herein can optionally comprise one or more non-nucleotides in one or both strands of the siRNA. As used herein, the term "non-nucleotide" refers to any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base such as adenosine, guanine, cytosine, uracil, or thymine and therefore lacks a base at the 1'-position.

[0194] In other embodiments, chemical modification of the siRNA comprises attaching a conjugate to the siRNA molecule. The conjugate can be attached at the 5' and/or 3'-end of the sense and/or antisense strand of the siRNA via a covalent attachment such as, *e.g.*, a biodegradable linker. The conjugate can also be attached to the siRNA, *e.g.*, through a carbamate group or other linking group (*see, e.g.*, U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). In certain instances, the conjugate is a molecule that facilitates the delivery of the siRNA into a cell. Examples of conjugate molecules suitable for attachment to siRNA include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (*e.g.*, folic acid, folate analogs and derivatives thereof), sugars (*e.g.*, galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, *etc.*), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof (*see, e.g.*, U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Patent No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule, oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Yet other examples include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidininium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples include the hydrophobic group, membrane active compound, cell penetrating compound, cell targeting signal, interaction modifier, and steric stabilizer conjugate molecules described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described in U.S. Patent Publication No. 20050239739. The type of conjugate used and the extent of

conjugation to the siRNA molecule can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the siRNA while retaining RNAi activity. As such, one skilled in the art can screen siRNA molecules having various conjugates attached thereto to identify ones having improved properties and full RNAi activity using any of a variety of well-known *in vitro* cell culture or *in vivo* animal models. The disclosures of the above-described patent documents are herein incorporated by reference in their entirety for all purposes.

d. Target Genes

[0195] The siRNA component of the nucleic acid-lipid particles described herein can be used to downregulate or silence the translation (*i.e.*, expression) of a gene of interest. Genes of interest include, but are not limited to, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders (*e.g.*, liver diseases and disorders), genes associated with tumorigenesis and cell transformation (*e.g.*, cancer), angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0196] Genes associated with viral infection and survival include those expressed by a virus in order to bind, enter, and replicate in a cell. Of particular interest are viral sequences associated with chronic viral diseases. Viral sequences of particular interest include sequences of Filoviruses such as Ebola virus and Marburg virus (*see, e.g.,* Geisbert *et al.*, *J. Infect. Dis.*, 193:1650-1657 (2006)); Arenaviruses such as Lassa virus, Junin virus, Machupo virus, Guanarito virus, and Sabia virus (Buchmeier *et al.*, *Arenaviridae*: the viruses and their replication, In: FIELDS VIROLOGY, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia, (2001)); Influenza viruses such as Influenza A, B, and C viruses, (*see, e.g.,* Steinhauer *et al.*, *Annu Rev Genet.,* 36:305-332 (2002); and Neumann *et al.*, *J Gen Virol.*, 83:2635-2662 (2002)); Hepatitis viruses (*see, e.g.,* Hamasaki *et al.*, *FEBS Lett.*, 543:51 (2003); Yokota *et al.*, *EMBO Rep.*, 4:602 (2003); Schlomai *et al.*, *Hepatology,* 37:764 (2003); Wilson *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2783 (2003); Kapadia *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2014 (2003); and FIELDS VIROLOGY, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia (2001)); Human Immunodeficiency Virus (HIV) (Banerjea *et al.*, *Mol. Ther.*, 8:62 (2003); Song *et al.*, *J. Virol.*, 77:7174 (2003); Stephenson, *JAMA,* 289:1494 (2003); Qin *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:183 (2003)); Herpes viruses (Jia *et al.*, *J. Virol.*, 77:3301 (2003)); and Human Papilloma Viruses (HPV) (Hall *et al.*, *J. Virol.*, 77:6066 (2003); Jiang *et al.*, *Oncogene,* 21:6041 (2002)).

[0197] Exemplary Filovirus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding structural proteins (*e.g.*, VP30, VP35, nucleoprotein (NP), polymerase protein (L-pol)) and membrane-associated proteins (*e.g.*, VP40, glycoprotein (GP), VP24). Complete genome sequences for Ebola virus are set forth in, *e.g.*, Genbank Accession Nos. NC_002549; AY769362; NC_006432; NC_004161; AY729654; AY354458; AY142960; AB050936; AF522874; AF499101; AF272001; and AF086833. Ebola virus VP24 sequences are set forth in, *e.g.*, Genbank Accession Nos. U77385 and AY058897. Ebola virus L-pol sequences are set forth in, *e.g.*, Genbank Accession No. X67110. Ebola virus VP40 sequences are set forth in, *e.g.*, Genbank Accession No. AY058896. Ebola virus NP sequences are set forth in, *e.g.*, Genbank Accession No. AY058895. Ebola virus GP sequences are set forth in, *e.g.*, Genbank Accession No. AY058898; Sanchez *et al.*, *Virus Res.*, 29:215-240 (1993); Will *et al.*, *J. Virol.*, 67:1203-1210 (1993); Volchkov *et al.*, *FEBS Lett.*, 305:181-184 (1992); and U.S. Patent No. 6,713,069. Additional Ebola virus sequences are set forth in, *e.g.*, Genbank Accession Nos. L11365 and X61274. Complete genome sequences for Marburg virus are set forth in, *e.g.*, Genbank Accession Nos. NC_001608; AY430365; AY430366; and AY358025. Marburg virus GP sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005734; AF005733; and AF005732. Marburg virus VP35 sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005731 and AF005730. Additional Marburg virus sequences are set forth in, *e.g.*, Genbank Accession Nos. X64406; Z29337; AF005735; and Z12132. Non-limiting examples of siRNA molecules targeting Ebola virus and Marburg virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070135370, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0198] Exemplary Influenza virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding nucleoprotein (NP), matrix proteins (M1 and M2), nonstructural proteins (NS1 and NS2), RNA polymerase (PA, PB1, PB2), neuraminidase (NA), and haemagglutinin (HA). Influenza A NP sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004522; AY818138; AB166863; AB188817; AB189046; AB189054; AB189062; AY646169; AY646177; AY651486; AY651493; AY651494; AY651495; AY651496; AY651497; AY651498; AY651499; AY651500; AY651501; AY651502; AY651503; AY651504; AY651505; AY651506; AY651507; AY651509; AY651528; AY770996; AY790308; AY818138; and AY818140. Influenza A PA sequences are set forth in, *e.g.*, Genbank Accession Nos. AY818132; AY790280; AY646171; AY818132;AY818133; AY646179; AY818134; AY551934; AY651613; AY651610;

AY651620; AY651617; AY651600; AY651611; AY651606; AY651618; AY651608; AY651607; AY651605; AY651609; AY651615; AY651616; AY651640; AY651614; AY651612; AY651621; AY651619; AY770995; and AY724786. Non-limiting examples of siRNA molecules targeting Influenza virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070218122, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0199] Exemplary hepatitis virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences involved in transcription and translation (*e.g.*, En1, En2, X, P) and nucleic acid sequences encoding structural proteins (*e.g.*, core proteins including C and C-related proteins, capsid and envelope proteins including S, M, and/or L proteins, or fragments thereof) (*see*, *e.g.*, FIELDS VIROLOGY, *supra*). Exemplary Hepatits C virus (HCV) nucleic acid sequences that can be silenced include, but are not limited to, the 5'-untranslated region (5'-UTR), the 3'-untranslated region (3'-UTR), the polyprotein translation initiation codon region, the internal ribosome entry site (IRES) sequence, and/or nucleic acid sequences encoding the core protein, the E1 protein, the E2 protein, the p7 protein, the NS2 protein, the NS3 protease/helicase, the NS4A protein, the NS4B protein, the NS5A protein, and/or the NS5B RNA-dependent RNA polymerase. HCV genome sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004102 (HCV genotype 1a), AJ238799 (HCV genotype 1b), NC_009823 (HCV genotype 2), NC_009824 (HCV genotype 3), NC_009825 (HCV genotype 4), NC_009826 (HCV genotype 5), and NC_009827 (HCV genotype 6). Hepatitis A virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001489; Hepatitis B virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_003977; Hepatitis D virus nucleic acid sequence are set forth in, *e.g.*, Genbank Accession No. NC_001653; Hepatitis E virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001434; and Hepatitis G virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001710. Silencing of sequences that encode genes associated with viral infection and survival can conveniently be used in combination with the administration of conventional agents used to treat the viral condition. Non-limiting examples of siRNA molecules targeting hepatitis virus nucleic acid sequences include those described in U.S. Patent Publication Nos. 20060281175, 20050058982, and 20070149470; U.S. Patent No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed March 20, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0200] Genes associated with metabolic diseases and disorders (*e.g.*, disorders in which the liver is the target and liver diseases and disorders) include, for example, genes expressed in dyslipidemia (*e.g.*, liver X receptors such as LXRα and LXRβ (Genback Accession No. NM_007121), farnesoid X receptors (FXR) (Genbank Accession No. NM_005123), sterol-regulatory element binding protein (SREBP), site-1 protease (S1P), 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG coenzyme-A reductase), apolipoprotein B (ApoB) (Genbank Accession No. NM_000384), apolipoprotein CIII (ApoC3) (Genbank Accession Nos. NM_000040 and NG_008949 REGION: 5001..8164), and apolipoprotein E (ApoE) (Genbank Accession Nos. NM_000041 and NG_007084 REGION: 5001..8612)); and diabetes (*e.g.*, glucose 6-phosphatase) (*see, e.g.*, Forman *et al., Cell,* 81:687 (1995); Seol *et al., Mol. Endocrinol.,* 9:72 (1995), Zavacki *et al., Proc. Natl. Acad. Sci. USA*, 94:7909 (1997); Sakai *et al., Cell,* 85:1037-1046 (1996); Duncan *et al., J. Biol. Chem.*, 272:12778-12785 (1997); Willy *et al., Genes Dev.*, 9:1033-1045 (1995); Lehmann *et al., J. Biol. Chem.*, 272:3137-3140 (1997); Janowski *et al., Nature,* 383:728-731 (1996); and Peet *et al., Cell,* 93:693-704 (1998)). One of skill in the art will appreciate that genes associated with metabolic diseases and disorders (*e.g.*, diseases and disorders in which the liver is a target and liver diseases and disorders) include genes that are expressed in the liver itself as well as and genes expressed in other organs and tissues. Silencing of sequences that encode genes associated with metabolic diseases and disorders can conveniently be used in combination with the administration of conventional agents used to treat the disease or disorder. Non-limiting examples of siRNA molecules targeting the ApoB gene include those described in U.S. Patent Publication No. 20060134189, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the ApoC3 gene include those described in U.S. Provisional Application No. 61/147,235, filed January 26, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0201] Examples of gene sequences associated with tumorigenesis and cell transformation (*e.g.*, cancer or other neoplasia) include mitotic kinesins such as Eg5 (KSP, KIF11; Genbank Accession No. NM_004523); serine/threonine kinases such as polo-like kinase 1 (PLK-1) (Genbank Accession No. NM_005030; Barr *et al.*, *Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)); tyrosine kinases such as WEE1 (Genbank Accession Nos. NM_003390 and NM_001143976); inhibitors of apoptosis such as XIAP (Genbank Accession No. NM_001167); COP9 signalosome subunits such as CSN1, CSN2, CSN3, CSN4, CSN5

(JAB1; Genbank Accession No. NM_006837); CSN6, CSN7A, CSN7B, and CSN8; ubiquitin ligases such as COP1 (RFWD2; Genbank Accession Nos. NM_022457 and NM_001001740); and histone deacetylases such as HDAC1, HDAC2 (Genbank Accession No. NM_001527), HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, *etc.*

Non-limiting examples of siRNA molecules targeting the Eg5 and XIAP genes include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the PLK-1 gene include those described in U.S. Patent Publication Nos. 20050107316 and 20070265438; and U.S. Patent Application No. 12/343,342, filed December 23, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of siRNA molecules targeting the CSN5 gene include those described in U.S. Provisional Application No. 61/045,251, filed April 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0202] Additional examples of gene sequences associated with tumorigenesis and cell transformation include translocation sequences such as MLL fusion genes, BCR-ABL (Wilda *et al.*, *Oncogene*, 21:5716 (2002); Scherr *et al.*, *Blood,* 101:1566 (2003)), TEL-AML1, EWS-FLI1, TLS-FUS, PAX3-FKHR, BCL-2, AML1-ETO, and AML1-MTG8 (Heidenreich *et al.*, *Blood,* 101:3157 (2003)); overexpressed sequences such as multidrug resistance genes (Nieth *et al.*, *FEBS Lett.*, 545:144 (2003); Wu *et al*, *Cancer Res.* 63:1515 (2003)), cyclins (Li *et al.*, *Cancer Res.*, 63:3593 (2003); Zou *et al.*, *Genes Dev.,* 16:2923 (2002)), beta-catenin (Verma *et al.*, *Clin Cancer Res.*, 9:1291 (2003)), telomerase genes (Kosciolek *et al.*, *Mol Cancer Ther.*, 2:209 (2003)), c-MYC, N-MYC, BCL-2, growth factor receptors (*e.g.,* EGFR/ErbB1 (Genbank Accession Nos. NM_005228, NM_201282, NM_201283, and NM_201284; *see also,* Nagy *et al. Exp. Cell Res.,* 285:39-49 (2003), ErbB2/HER-2 (Genbank Accession Nos. NM_004448 and NM_001005862), ErbB3 (Genbank Accession Nos. NM_001982 and NM_001005915), and ErbB4 (Genbank Accession Nos. NM_005235 and NM_001042599); and mutated sequences such as RAS (reviewed in Tuschl and Borkhardt, *Mol. Interventions*, 2:158 (2002)). Non-limiting examples of siRNA molecules targeting the EGFR gene include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0203] Silencing of sequences that encode DNA repair enzymes find use in combination with the administration of chemotherapeutic agents (Collis *et al.*, *Cancer Res.*, 63:1550 (2003)). Genes encoding proteins associated with tumor migration are also target sequences

of interest, for example, integrins, selectins, and metalloproteinases. The foregoing examples are not exclusive. Those of skill in the art will understand that any whole or partial gene sequence that facilitates or promotes tumorigenesis or cell transformation, tumor growth, or tumor migration can be included as a template sequence.

[0204] Angiogenic genes are able to promote the formation of new vessels. Of particular interest is vascular endothelial growth factor (VEGF) (Reich *et al.*, *Mol. Vis.*, 9:210 (2003)) or VEGFR. siRNA sequences that target VEGFR are set forth in, *e.g.*, GB 2396864; U.S. Patent Publication No. 20040142895; and CA 2456444, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0205] Anti-angiogenic genes are able to inhibit neovascularization. These genes are particularly useful for treating those cancers in which angiogenesis plays a role in the pathological development of the disease. Examples of anti-angiogenic genes include, but are not limited to, endostatin (*see, e.g.,* U.S. Patent No. 6,174,861), angiostatin (*see, e.g.*, U.S. Patent No. 5,639,725), and VEGFR2 (*see, e.g.,* Decaussin *et al.*, *J. Pathol.*, 188: 369-377 (1999)), the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0206] Immunomodulator genes are genes that modulate one or more immune responses. Examples of immunomodulator genes include, without limitation, cytokines such as growth factors (*e.g.*, TGF-α, TGF-β, EGF, FGF, IGF, NGF, PDGF, CGF, GM-CSF, SCF, *etc.*), interleukins (*e.g.*, IL-2, IL-4, IL-12 (Hill *et al.*, *J. Immunol.*, 171:691 (2003)), IL-15, IL-18, IL-20, *etc.*), interferons (*e.g.*, IFN-α, IFN-β, IFN-γ, *etc.*) and TNF. Fas and Fas ligand genes are also immunomodulator target sequences of interest (Song *et al.*, *Nat. Med.*, 9:347 (2003)). Genes encoding secondary signaling molecules in hematopoietic and lymphoid cells are also included in the present invention, for example, Tec family kinases such as Bruton's tyrosine kinase (Btk) (Heinonen *et al.*, *FEBS Lett.*, 527:274 (2002)).

[0207] Cell receptor ligands include ligands that are able to bind to cell surface receptors (*e.g.*, insulin receptor, EPO receptor, G-protein coupled receptors, receptors with tyrosine kinase activity, cytokine receptors, growth factor receptors, *etc.*), to modulate (*e.g.*, inhibit, activate, *etc.*) the physiological pathway that the receptor is involved in (*e.g.*, glucose level modulation, blood cell development, mitogenesis, *etc.*). Examples of cell receptor ligands include, but are not limited to, cytokines, growth factors, interleukins, interferons, erythropoietin (EPO), insulin, glucagon, G-protein coupled receptor ligands, *etc.* Templates coding for an expansion of trinucleotide repeats (*e.g.*, CAG repeats) find use in silencing pathogenic sequences in neurodegenerative disorders caused by the expansion of

trinucleotide repeats, such as spinobulbular muscular atrophy and Huntington's Disease (Caplen *et al.*, *Hum. Mol. Genet.*, 11:175 (2002)).

[0208] In addition to its utility in silencing the expression of any of the above-described genes for therapeutic purposes, the siRNA described herein are also useful in research and development applications as well as diagnostic, prophylactic, prognostic, clinical, and other healthcare applications. As a non-limiting example, the siRNA can be used in target validation studies directed at testing whether a gene of interest has the potential to be a therapeutic target. The siRNA can also be used in target identification studies aimed at discovering genes as potential therapeutic targets.

2. aiRNA

[0209] Like siRNA, asymmetrical interfering RNA (aiRNA) can recruit the RNA-induced silencing complex (RISC) and lead to effective silencing of a variety of genes in mammalian cells by mediating sequence-specific cleavage of the target sequence between nucleotide 10 and 11 relative to the 5' end of the antisense strand (Sun *et al.*, *Nat. Biotech.*, 26:1379-1382 (2008)). Typically, an aiRNA molecule comprises a short RNA duplex having a sense strand and an antisense strand, wherein the duplex contains overhangs at the 3' and 5' ends of the antisense strand. The aiRNA is generally asymmetric because the sense strand is shorter on both ends when compared to the complementary antisense strand. In some aspects, aiRNA molecules may be designed, synthesized, and annealed under conditions similar to those used for siRNA molecules. As a non-limiting example, aiRNA sequences may be selected and generated using the methods described above for selecting siRNA sequences.

[0210] In another embodiment, aiRNA duplexes of various lengths (*e.g.*, about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 base pairs, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 base pairs) may be designed with overhangs at the 3' and 5' ends of the antisense strand to target an mRNA of interest. In certain instances, the sense strand of the aiRNA molecule is about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length. In certain other instances, the antisense strand of the aiRNA molecule is about 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 nucleotides in length.

[0211] In some embodiments, the 5' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*, "AA", "UU", "dTdT", *etc.*). In other embodiments, the 3' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*,

"AA", "UU", "dTdT", *etc.*). In certain aspects, the aiRNA molecules described herein may comprise one or more modified nucleotides, *e.g.,* in the double-stranded (duplex) region and/or in the antisense overhangs. As a non-limiting example, aiRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0212] In certain embodiments, aiRNA molecules may comprise an antisense strand which corresponds to the antisense strand of an siRNA molecule, *e.g.,* one of the siRNA molecules described herein. In other embodiments, aiRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.,* genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

3. miRNA

[0213] Generally, microRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. miRNAs are encoded by genes from whose DNA they are transcribed, but miRNAs are not translated into protein (non-coding RNA); instead, each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional mature miRNA. Mature miRNA molecules are either partially or completely complementary to one or more messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression. The identification of miRNA molecules is described, *e.g.,* in Lagos-Quintana *et al.*, *Science*, 294:853-858; Lau *et al.*, *Science*, 294:858-862; and Lee *et al.*, *Science*, 294:862-864.

[0214] The genes encoding miRNA are much longer than the processed mature miRNA molecule. miRNA are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail and processed to short, ~70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha (Denli *et al.*, *Nature*, 432:231-235 (2004)). These pre-miRNA are then processed to mature miRNA in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC) (Bernstein

et al., *Nature*, 409:363-366 (2001). Either the sense strand or antisense strand of DNA can function as templates to give rise to miRNA.

[0215] When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC complex. This strand is known as the guide strand and is selected by the argonaute protein, the catalytically active RNase in the RISC complex, on the basis of the stability of the 5' end (Preall *et al.*, *Curr. Biol.*, 16:530-535 (2006)). The remaining strand, known as the anti-guide or passenger strand, is degraded as a RISC complex substrate (Gregory *et al.*, *Cell*, 123:631-640 (2005)). After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and induce target mRNA degradation and/or translational silencing.

[0216] Mammalian miRNA molecules are usually complementary to a site in the 3' UTR of the target mRNA sequence. In certain instances, the annealing of the miRNA to the target mRNA inhibits protein translation by blocking the protein translation machinery. In certain other instances, the annealing of the miRNA to the target mRNA facilitates the cleavage and degradation of the target mRNA through a process similar to RNA interference (RNAi). miRNA may also target methylation of genomic sites which correspond to targeted mRNA. Generally, miRNA function in association with a complement of proteins collectively termed the miRNP.

[0217] In certain aspects, the miRNA molecules described herein are about 15-100, 15-90, 15-80, 15-75, 15-70, 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In certain other aspects, miRNA molecules may comprise one or more modified nucleotides. As a non-limiting example, miRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0218] In some embodiments, miRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.*, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0219] In other embodiments, one or more agents that block the activity of a miRNA targeting an mRNA of interest are administered using a lipid particle of the invention (*e.g.*, a

nucleic acid-lipid particle). Examples of blocking agents include, but are not limited to, steric blocking oligonucleotides, locked nucleic acid oligonucleotides, and Morpholino oligonucleotides. Such blocking agents may bind directly to the miRNA or to the miRNA binding site on the target mRNA.

4. Antisense Oligonucleotides

[0220] In one embodiment, the nucleic acid is an antisense oligonucleotide directed to a target gene or sequence of interest. The terms "antisense oligonucleotide" or "antisense" include oligonucleotides that are complementary to a targeted polynucleotide sequence. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence. Antisense RNA oligonucleotides prevent the translation of complementary RNA strands by binding to the RNA. Antisense DNA oligonucleotides can be used to target a specific, complementary (coding or non-coding) RNA. If binding occurs, this DNA/RNA hybrid can be degraded by the enzyme RNase H. In a particular embodiment, antisense oligonucleotides comprise from about 10 to about 60 nucleotides, more preferably from about 15 to about 30 nucleotides. The term also encompasses antisense oligonucleotides that may not be exactly complementary to the desired target gene. Thus, the invention can be utilized in instances where non-target specific-activities are found with antisense, or where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use.

[0221] Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein synthesis by a targeted gene. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (*see,* U.S. Patent Nos. 5,739,119 and 5,759,829). Furthermore, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDR1), ICAM-1, E-selectin, STK-1, striatal GABAA receptor, and human EGF (*see,* Jaskulski *et al.*, *Science*, 240:1544-6 (1988); Vasanthakumar *et al.*, *Cancer Commun.*, 1:225-32 (1989); Peris *et al.*, *Brain Res Mol Brain Res.*, 15;57:310-20 (1998); and U.S. Patent Nos. 5,801,154; 5,789,573; 5,718,709 and 5,610,288). Moreover, antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.,* cancer (*see,* U.S. Patent Nos.

5,747,470; 5,591,317; and 5,783,683). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0222] Methods of producing antisense oligonucleotides are known in the art and can be readily adapted to produce an antisense oligonucleotide that targets any polynucleotide sequence. Selection of antisense oligonucleotide sequences specific for a given target sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense oligonucleotides may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-402 (1997)).

5. Ribozymes

[0223] According to another embodiment of the invention, nucleic acid-lipid particles are associated with ribozymes. Ribozymes are RNA-protein complexes having specific catalytic domains that possess endonuclease activity (*see,* Kim *et al.*, *Proc. Natl. Acad. Sci. USA.*, 84:8788-92 (1987); and Forster *et al.*, *Cell*, 49:211-20 (1987)). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (*see,* Cech *et al.*, *Cell*, 27:487-96 (1981); Michel *et al.*, *J. Mol. Biol.*, 216:585-610 (1990); Reinhold-Hurek *et al.*, *Nature*, 357:173-6 (1992)). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

[0224] At least six basic varieties of naturally-occurring enzymatic RNA molecules are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-

pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

[0225] The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence), or Neurospora VS RNA motif, for example. Specific examples of hammerhead motifs are described in, *e.g.,* Rossi *et al.*, *Nucleic Acids Res.*, 20:4559-65 (1992). Examples of hairpin motifs are described in, *e.g.,* EP 0360257, Hampel *et al.*, *Biochemistry*, 28:4929-33 (1989); Hampel *et al.*, *Nucleic Acids Res.*, 18:299-304 (1990); and U.S. Patent No. 5,631,359. An example of the hepatitis δ virus motif is described in, *e.g.,* Perrotta *et al.*, *Biochemistry*, 31:11843-52 (1992). An example of the RNaseP motif is described in, *e.g.,* Guerrier-Takada *et al.*, *Cell*, 35:849-57 (1983). Examples of the Neurospora VS RNA ribozyme motif is described in, *e.g.,* Saville *et al.*, *Cell*, 61:685-96 (1990); Saville *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:8826-30 (1991); Collins *et al.*, *Biochemistry*, 32:2795-9 (1993). An example of the Group I intron is described in, *e.g.,* U.S. Patent No. 4,987,071. Important characteristics of enzymatic nucleic acid molecules used according to the invention are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus, the ribozyme constructs need not be limited to specific motifs mentioned herein. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0226] Methods of producing a ribozyme targeted to any polynucleotide sequence are known in the art. Ribozymes may be designed as described in, *e.g.,* PCT Publication Nos. WO 93/23569 and WO 94/02595, and synthesized to be tested *in vitro* and/or *in vivo* as described therein. The disclosures of these PCT publications are herein incorporated by reference in their entirety for all purposes.

[0227] Ribozyme activity can be optimized by altering the length of the ribozyme binding arms or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (*see, e.g.,* PCT Publication Nos. WO 92/07065, WO 93/15187, WO 91/03162, and WO 94/13688; EP 92110298.4; and U.S. Patent No. 5,334,711, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules, the disclosures of which are each herein incorporated by reference in their entirety

for all purposes), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

6. Immunostimulatory Oligonucleotides

[0228] Nucleic acids associated with lipid paticles of the present invention may be immunostimulatory, including immunostimulatory oligonucleotides (ISS; single-or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal such as a human. ISS include, *e.g.,* certain palindromes leading to hairpin secondary structures (*see,* Yamamoto *et al.*, *J. Immunol.*, 148:4072-6 (1992)), or CpG motifs, as well as other known ISS features (such as multi-G domains; *see;* PCT Publication No. WO 96/11266, the disclosure of which is herein incorporated by reference in its entirety for all purposes).

[0229] Immunostimulatory nucleic acids are considered to be non-sequence specific when it is not required that they specifically bind to and reduce the expression of a target sequence in order to provoke an immune response. Thus, certain immunostimulatory nucleic acids may comprise a sequence corresponding to a region of a naturally-occurring gene or mRNA, but they may still be considered non-sequence specific immunostimulatory nucleic acids.

[0230] In one embodiment, the immunostimulatory nucleic acid or oligonucleotide comprises at least one CpG dinucleotide. The oligonucleotide or CpG dinucleotide may be unmethylated or methylated. In another embodiment, the immunostimulatory nucleic acid comprises at least one CpG dinucleotide having a methylated cytosine. In one embodiment, the nucleic acid comprises a single CpG dinucleotide, wherein the cytosine in the CpG dinucleotide is methylated. In an alternative embodiment, the nucleic acid comprises at least two CpG dinucleotides, wherein at least one cytosine in the CpG dinucleotides is methylated. In a further embodiment, each cytosine in the CpG dinucleotides present in the sequence is methylated. In another embodiment, the nucleic acid comprises a plurality of CpG dinucleotides, wherein at least one of the CpG dinucleotides comprises a methylated cytosine. Examples of immunostimulatory oligonucleotides suitable for use in the compositions and methods of the present invention are described in PCT Application No. PCT/US08/88676, filed December 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Patent No. 6,406,705, and Raney *et al.*, *J. Pharm. Exper. Ther.*, 298:1185-92 (2001), the disclosures of which are each herein incorporated by reference in their entirety for all purposes. In certain embodiments, the oligonucleotides used in the compositions and

methods of the invention have a phosphodiester ("PO") backbone or a phosphorothioate ("PS") backbone, and/or at least one methylated cytosine residue in a CpG motif.

B. Other Active Agents

[0231] In certain embodiments, the active agent associated with the lipid paticles of the invention may comprise one or more therapeutic proteins, polypeptides, or small organic molecules or compounds. Non-limiting examples of such therapeutically effective agents or drugs include oncology drugs (*e.g.*, chemotherapy drugs, hormonal therapaeutic agents, immunotherapeutic agents, radiotherapeutic agents, *etc.*), lipid-lowering agents, anti-viral drugs, anti-inflammatory compounds, antidepressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs such as anti-arrhythmic agents, hormones, vasoconstrictors, and steroids. These active agents may be administered alone in the lipid particles of the invention, or in combination (*e.g.*, co-administered) with lipid particles of the invention comprising nucleic acid such as interfering RNA.

[0232] Non-limiting examples of chemotherapy drugs include platinum-based drugs (*e.g.*, oxaliplatin, cisplatin, carboplatin, spiroplatin, iproplatin, satraplatin, *etc.*), alkylating agents (*e.g.*, cyclophosphamide, ifosfamide, chlorambucil, busulfan, melphalan, mechlorethamine, uramustine, thiotepa, nitrosoureas, *etc.*), anti-metabolites (*e.g.*, 5-fluorouracil (5-FU), azathioprine, methotrexate, leucovorin, capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, pemetrexed, raltitrexed, *etc.*), plant alkaloids (*e.g.*, vincristine, vinblastine, vinorelbine, vindesine, podophyllotoxin, paclitaxel (taxol), docetaxel, *etc.*), topoisomerase inhibitors (*e.g.*, irinotecan (CPT-11; Camptosar), topotecan, amsacrine, etoposide (VP16), etoposide phosphate, teniposide, *etc.*), antitumor antibiotics (*e.g.*, doxorubicin, adriamycin, daunorubicin, epirubicin, actinomycin, bleomycin, mitomycin, mitoxantrone, plicamycin, *etc.*), tyrosine kinase inhibitors (*e.g.*, gefitinib (Iressa®), sunitinib (Sutent®; SU11248), erlotinib (Tarceva®; OSI-1774), lapatinib (GW572016; GW2016), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), imatinib (Gleevec®; STI571), dasatinib (BMS-354825), leflunomide (SU101), vandetanib (Zactima™; ZD6474), *etc.*), pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and combinations thereof.

[0233] Examples of conventional hormonal therapaeutic agents include, without limitation, steroids (*e.g.*, dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

[0234] Examples of conventional immunotherapeutic agents include, but are not limited to, immunostimulants (*e.g.*, Bacillus Calmette-Guérin (BCG), levamisole, interleukin-2, alpha-interferon, *etc.*), monoclonal antibodies (*e.g.*, anti-CD20, anti-HER2, anti-CD52, anti-HLA-DR, and anti-VEGF monoclonal antibodies), immunotoxins (*e.g.*, anti-CD33 monoclonal antibody-calicheamicin conjugate, anti-CD22 monoclonal antibody-pseudomonas exotoxin conjugate, *etc.*), and radioimmunotherapy (*e.g.*, anti-CD20 monoclonal antibody conjugated to ^{111}In, ^{90}Y, or ^{131}I, *etc.*).

[0235] Examples of conventional radiotherapeutic agents include, but are not limited to, radionuclides such as ^{47}Sc, ^{64}Cu, ^{67}Cu, ^{89}Sr, ^{86}Y, ^{87}Y, ^{90}Y, ^{105}Rh, ^{111}Ag, ^{111}In, ^{117m}Sn, ^{149}Pm, ^{153}Sm, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi, optionally conjugated to antibodies directed against tumor antigens.

[0236] Additional oncology drugs that may be used according to the invention include, but are not limited to, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, bexarotene, biCNU, carmustine, CCNU, celecoxib, cladribine, cyclosporin A, cytosine arabinoside, cytoxan, dexrazoxane, DTIC, estramustine, exemestane, FK506, gemtuzumab-ozogamicin, hydrea, hydroxyurea, idarubicin, interferon, letrozole, leustatin, leuprolide, litretinoin, megastrol, L-PAM, mesna, methoxsalen, mithramycin, nitrogen mustard, pamidronate, Pegademase, pentostatin, porfimer sodium, prednisone, rituxan, streptozocin, STI-571, taxotere, temozolamide, VM-26, toremifene, tretinoin, ATRA, valrubicin, and velban. Other examples of oncology drugs that may be used according to the invention are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors, and camptothecins.

[0237] Non-limiting examples of lipid-lowering agents for treating a lipid disease or disorder associated with elevated triglycerides, cholesterol, and/or glucose include statins, fibrates, ezetimibe, thiazolidinediones, niacin, beta-blockers, nitroglycerin, calcium antagonists, fish oil, and mixtures thereof.

[0238] Examples of anti-viral drugs include, but are not limited to, abacavir, aciclovir, acyclovir, adefovir, amantadine, amprenavir, arbidol, atazanavir, atripla, cidofovir, combivir, darunavir, delavirdine, didanosine, docosanol, edoxudine, efavirenz, emtricitabine, enfuvirtide, entecavir, entry inhibitors, famciclovir, fixed dose combinations, fomivirsen, fosamprenavir, foscarnet, fosfonet, fusion inhibitors, ganciclovir, ibacitabine, imunovir, idoxuridine, imiquimod, indinavir, inosine, integrase inhibitors, interferon type III (*e.g.*, IFN-λ molecules such as IFN-λ1, IFN-λ2, and IFN-λ3), interferon type II (*e.g.*, IFN-γ), interferon type I (*e.g.*, IFN-α such as PEGylated IFN-α, IFN-β, IFN-κ, IFN-δ, IFN-ε, IFN-τ, IFN-ω, and

IFN-ζ), interferon, lamivudine, lopinavir, loviride, MK-0518, maraviroc, moroxydine, nelfinavir, nevirapine, nexavir, nucleoside analogues, oseltamivir, penciclovir, peramivir, pleconaril, podophyllotoxin, protease inhibitors, reverse transcriptase inhibitors, ribavirin, rimantadine, ritonavir, saquinavir, stavudine, synergistic enhancers, tenofovir, tenofovir disoproxil, tipranavir, trifluridine, trizivir, tromantadine, truvada, valaciclovir, valganciclovir, vicriviroc, vidarabine, viramidine, zalcitabine, zanamivir, zidovudine, pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and mixtures thereof.

V. Lipid Particles

[0239] The lipid particles of the invention typically comprise an active agent or therapeutic agent, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.,* by a nuclease or protease. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans. The lipid particles of the invention typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm.

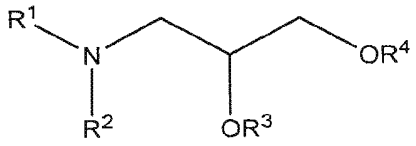
[0240] In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (*e.g.,* siRNA, aiRNA, and/or miRNA), a cationic lipid (*e.g.*, a cationic lipid of Formulas I, II, and/or III), a non-cationic lipid (*e.g.,* cholesterol alone or mixtures of one or more phospholipids and cholesterol), and a conjugated lipid that inhibits aggregation of the particles (*e.g.,* one or more PEG-lipid conjugates). The SNALP may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more unmodified and/or modified interfering RNA molecules. Nucleic acid-lipid particles and their method of preparation are described in, *e.g.,* U.S. Patent Nos. 5,753,613; 5,785,992; 5,705,385; 5,976,567; 5,981,501; 6,110,745; and 6,320,017; and PCT Publication No. WO 96/40964, the disclosures of which are each herein incorporated by reference in their entirety for all purposes.

A. Cationic Lipids

[0241] Any of a variety of cationic lipids may be used in the lipid particles of the invention (*e.g.,* SNALP), either alone or in combination with one or more other cationic lipid species or non-cationic lipid species.

[0242] Cationic lipids which are useful in the present invention can be any of a number of lipid species which carry a net positive charge at physiological pH. Such lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3.beta.-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-Dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), 1,2-Dilinoleoylcarbamyl-3-dimethylaminopropane (DLinCDAP), and mixtures thereof. A number of these lipids and related analogs have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are each herein incorporated by reference in their entirety for all purposes. Additionally, a number of commercial preparations of cationic lipids are available and can be used in the present invention. These include, *e.g.,* LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic liposomes comprising DOGS from Promega Corp., Madison, Wisconsin, USA).

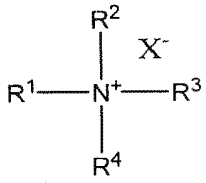
[0243] Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.



(I),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.*, R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.*, R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipid of Formula I is symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4comprise at least three sites of unsaturation and are independently selected from, *e.g.*, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl. In particularly preferred embodiments, the cationic lipid of Formula I is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) or 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA).

[0244] Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.

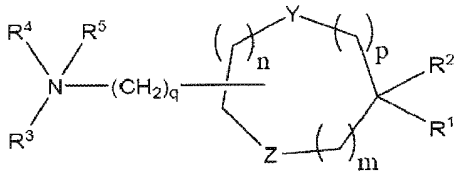


(II),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.*, R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.*, R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipids of the present invention are symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments,

R^3 and R^4comprise at least three sites of unsaturation and are independently selected from, *e.g.*, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl.

[0245] Moreover, cationic lipids of Formula III having the following structures (or salts thereof) are useful in the present invention.



(III),

Wherein R^1 and R^2 are either the same or different and independently optionally substituted C_{12}-C_{24} alkyl, optionally substituted C_{12}-C_{24} alkenyl, optionallysubstituted C_{12}-C_{24} alkynyl, or optionally substituted C_{12}-C_{24} acyl; R^3 and R^4 are either the same or different and independently optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 alkenyl, or optionally substituted C_1-C_6 alkynyl or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen; R^5 is either absent or hydrogen or C_1-C_6 alkyl to provide a quaternary amine; m, n, and p are either the same or different and independently either 0 or 1 with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; and Y and Z are either the same or different and independently O, S, or NH.

[0246] In some embodiments, the cationic lipid of Formula III is 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), or mixtures

thereof. In preferred embodiments, the cationic lipid of Formula III is DLin-K-C2-DMA (XTC2).

[0247] The cationic lipid typically comprises from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0248] It will be readily apparent to one of skill in the art that depending on the intended use of the particles, the proportions of the components can be varied and the delivery efficiency of a particular formulation can be measured using, *e.g.*, an endosomal release parameter (ERP) assay.

B. Non-Cationic Lipids

[0249] The non-cationic lipids used in the lipid particles of the invention (*e.g.*, SNALP) can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

[0250] Non-limiting examples of non-cationic lipids include phospholipids such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C_{10}-C_{24} carbon chains, *e.g.*, lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

[0251] Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof such as cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof.

[0252] In some embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of cholesterol or a derivative thereof, *e.g.*, a phospholipid-free lipid particle formulation. In other embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of one or more phospholipids, *e.g.*, a cholesterol-free lipid particle formulation. In further embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof.

[0253] Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, *e.g.*, stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

[0254] In some embodiments, the non-cationic lipid comprises from about 13 mol % to about 49.5 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 35 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0255] In certain embodiments, the cholesterol present in phospholipid-free lipid particles comprises from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, or from about 35 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle may comprise cholesterol at about 37 mol % of the total lipid present in the particle.

[0256] In certain other embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, or from about 35 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 34 mol % of the total lipid present in the particle.

[0257] In further embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 10 mol % to about 30 mol %,

from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 20 mol % of the total lipid present in the particle.

[0258] In embodiments where the lipid particles contain a mixture of phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 45, 50, 55, or 60 mol % of the total lipid present in the particle. In certain instances, the phospholipid component in the mixture may comprise from about 2 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, or from about 6 mol % to about 8 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (*e.g.*, in a mixture with about 34 mol % cholesterol) of the total lipid present in the particle. In certain other instances, the phospholipid component in the mixture may comprise from about 10 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As another non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 20 mol % (*e.g.*, in a mixture with about 20 mol % cholesterol) of the total lipid present in the particle.

C. Lipid Conjugate

[0259] In addition to cationic and non-cationic lipids, the lipid particles of the invention (*e.g.*, SNALP) comprise a lipid conjugate. The conjugated lipid is useful in that it prevents the aggregation of particles. Suitable conjugated lipids include, but are not limited to, PEG-lipid conjugates, ATTA-lipid conjugates, cationic-polymer-lipid conjugates (CPLs), and mixtures thereof. In certain embodiments, the particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate together with a CPL.

[0260] In a preferred embodiment, the lipid conjugate is a PEG-lipid. Examples of PEG-lipids include, but are not limited to, PEG coupled to dialkyloxypropyls (PEG-DAA) as described in, *e.g.*, PCT Publication No. WO 05/026372, PEG coupled to diacylglycerol (PEG-DAG) as described in, *e.g.*, U.S. Patent Publication Nos. 20030077829 and 2005008689, PEG coupled to phospholipids such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides as described in, *e.g.*, U.S. Patent No. 5,885,613, PEG conjugated to cholesterol or a derivative thereof, and mixtures thereof. The disclosures of

these patent documents are herein incorporated by reference in their entirety for all purposes. Additional PEG-lipids include, without limitation, PEG-C-DOMG, 2KPEG-DMG, and a mixture thereof.

[0261] PEG is a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG 2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, for example, the following: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH_2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM). Other PEGs such as those described in U.S. Patent Nos. 6,774,180 and 7,053,150 (*e.g.*, mPEG (20 KDa) amine) are also useful for preparing the PEG-lipid conjugates of the present invention. The disclosures of these patents are herein incorporated by reference in their entirety for all purposes. In addition, monomethoxypolyethyleneglycol-acetic acid (MePEG-CH_2COOH) is particularly useful for preparing PEG-lipid conjugates including, *e.g.*, PEG-DAA conjugates.

[0262] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

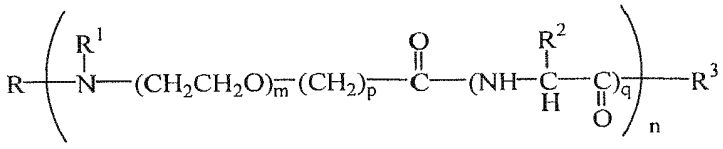
[0263] In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In a preferred embodiment, the linker moiety is a non-ester containing linker moiety. As used herein, the term "non-ester containing linker moiety" refers to a linker moiety that does not contain a carboxylic ester bond (-OC(O)-). Suitable non-ester containing linker moieties include, but are not limited to, amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulphide (-S-S-), ether (-O-), succinyl (-

(O)CCH_2CH_2C(O)-), succinamidyl (-NHC(O)CH_2CH_2C(O)NH-), ether, disulphide, as well as combinations thereof (such as a linker containing both a carbamate linker moiety and an amido linker moiety). In a preferred embodiment, a carbamate linker is used to couple the PEG to the lipid.

[0264] In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, *e.g.*, carbonate (-OC(O)O-), succinoyl, phosphate esters (-O-(O)POH-O-), sulfonate esters, and combinations thereof.

[0265] Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to PEG to form the lipid conjugate. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skilled in the art. Phosphatidyl-ethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), and distearoyl-phosphatidylethanolamine (DSPE).

[0266] The term "ATTA" or "polyamide" refers to, without limitation, compounds described in U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes. These compounds include a compound having the formula:

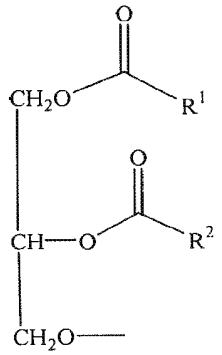


(IV),

wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R^1 is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R^1 and the nitrogen to which they are bound form an azido moiety; R^2 is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; R^3 is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and NR^4R^5, wherein R^4 and R^5 are independently hydrogen or alkyl; n is 4 to 80; m is 2 to 6; p is 1 to 4; and q is 0 or 1. It will

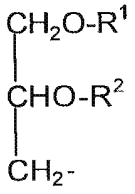
be apparent to those of skill in the art that other polyamides can be used in the compounds of the present invention.

[0267] The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.* Diacylglycerols have the following general formula:



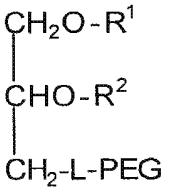
(V).

[0268] The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:



(VI).

[0269] In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:



(VII),

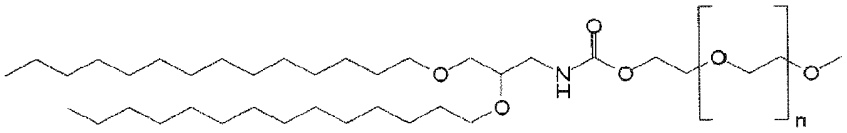
wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester

containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.*

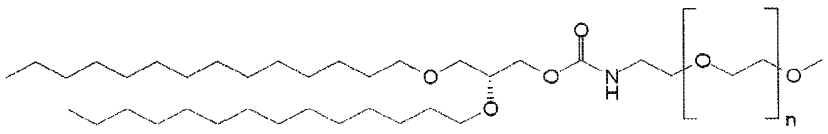
[0270] In Formula VII above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG has an average molecular weight of about 2,000 daltons or about 750 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl, or aryl. In certain embodiments, the terminal hydroxyl group is substituted with a methoxy or methyl group.

[0271] In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, a succinamidyl linker moiety, and combinations thereof. In a preferred embodiment, the non-ester containing linker moiety is a carbamate linker moiety (*i.e.*, a PEG-*C*-DAA conjugate). In another preferred embodiment, the non-ester containing linker moiety is an amido linker moiety (*i.e.*, a PEG-*A*-DAA conjugate). In yet another preferred embodiment, the non-ester containing linker moiety is a succinamidyl linker moiety (*i.e.*, a PEG-*S*-DAA conjugate).

[0272] In particular embodiments, the PEG-lipid conjugate is selected from:



(PEG-C-DMA); and



(PEG-C-DOMG).

[0273] The PEG-DAA conjugates are synthesized using standard techniques and reagents known to those of skill in the art. It will be recognized that the PEG-DAA conjugates will

contain various amide, amine, ether, thio, carbamate, and urea linkages. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See, e.g.,* March, ADVANCED ORGANIC CHEMISTRY (Wiley 1992); Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY, 5th ed. (Longman 1989). It will also be appreciated that any functional groups present may require protection and deprotection at different points in the synthesis of the PEG-DAA conjugates. Those of skill in the art will recognize that such techniques are well known. *See, e.g.,* Green and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS (Wiley 1991).

[0274] Preferably, the PEG-DAA conjugate is a dilauryloxypropyl (C_{12})-PEG conjugate, dimyristyloxypropyl (C_{14})-PEG conjugate, a dipalmityloxypropyl (C_{16})-PEG conjugate, or a distearyloxypropyl (C_{18})-PEG conjugate. Those of skill in the art will readily appreciate that other dialkyloxypropyls can be used in the PEG-DAA conjugates of the present invention.

[0275] In addition to the foregoing, it will be readily apparent to those of skill in the art that other hydrophilic polymers can be used in place of PEG. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

[0276] In addition to the foregoing components, the particles (*e.g.,* SNALP or SPLP) of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids or CPLs (*see, e.g.,* Chen *et al.*, *Bioconj. Chem.,* 11:433-437 (2000)). Suitable SPLPs and SPLP-CPLs for use in the present invention, and methods of making and using SPLPs and SPLP-CPLs, are disclosed, *e.g.*, in U.S. Patent No. 6,852,334 and PCT Publication No. WO 00/62813, , the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0277] Suitable CPLs include compounds of Formula VIII:

A-W-Y (VIII),

wherein A, W, and Y are as described below.

[0278] With reference to Formula VIII, "A" is a lipid moiety such as an amphipathic lipid, a neutral lipid, or a hydrophobic lipid that acts as a lipid anchor. Suitable lipid examples include, but are not limited to, diacylglycerolyls, dialkylglycerolyls, N-N-dialkylaminos, 1,2-diacyloxy-3-aminopropanes, and 1,2-dialkyl-3-aminopropanes.

[0279] "W" is a polymer or an oligomer such as a hydrophilic polymer or oligomer. Preferably, the hydrophilic polymer is a biocompatable polymer that is nonimmunogenic or

possesses low inherent immunogenicity. Alternatively, the hydrophilic polymer can be weakly antigenic if used with appropriate adjuvants. Suitable nonimmunogenic polymers include, but are not limited to, PEG, polyamides, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, and combinations thereof. In a preferred embodiment, the polymer has a molecular weight of from about 250 to about 7,000 daltons.

[0280] "Y" is a polycationic moiety. The term polycationic moiety refers to a compound, derivative, or functional group having a positive charge, preferably at least 2 positive charges at a selected pH, preferably physiological pH. Suitable polycationic moieties include basic amino acids and their derivatives such as arginine, asparagine, glutamine, lysine, and histidine; spermine; spermidine; cationic dendrimers; polyamines; polyamine sugars; and amino polysaccharides. The polycationic moieties can be linear, such as linear tetralysine, branched or dendrimeric in structure. Polycationic moieties have between about 2 to about 15 positive charges, preferably between about 2 to about 12 positive charges, and more preferably between about 2 to about 8 positive charges at selected pH values. The selection of which polycationic moiety to employ may be determined by the type of particle application which is desired.

[0281] The charges on the polycationic moieties can be either distributed around the entire particle moiety, or alternatively, they can be a discrete concentration of charge density in one particular area of the particle moiety *e.g.,* a charge spike. If the charge density is distributed on the particle, the charge density can be equally distributed or unequally distributed. All variations of charge distribution of the polycationic moiety are encompassed by the present invention.

[0282] The lipid "A" and the nonimmunogenic polymer "W" can be attached by various methods and preferably by covalent attachment. Methods known to those of skill in the art can be used for the covalent attachment of "A" and "W." Suitable linkages include, but are not limited to, amide, amine, carboxyl, carbonate, carbamate, ester, and hydrazone linkages. It will be apparent to those skilled in the art that "A" and "W" must have complementary functional groups to effectuate the linkage. The reaction of these two groups, one on the lipid and the other on the polymer, will provide the desired linkage. For example, when the lipid is a diacylglycerol and the terminal hydroxyl is activated, for instance with NHS and DCC, to form an active ester, and is then reacted with a polymer which contains an amino group, such as with a polyamide (*see, e.g.,* U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes), an amide bond will form between the two groups.

[0283] In certain instances, the polycationic moiety can have a ligand attached, such as a targeting ligand or a chelating moiety for complexing calcium. Preferably, after the ligand is attached, the cationic moiety maintains a positive charge. In certain instances, the ligand that is attached has a positive charge. Suitable ligands include, but are not limited to, a compound or device with a reactive functional group and include lipids, amphipathic lipids, carrier compounds, bioaffinity compounds, biomaterials, biopolymers, biomedical devices, analytically detectable compounds, therapeutically active compounds, enzymes, peptides, proteins, antibodies, immune stimulators, radiolabels, fluorogens, biotin, drugs, haptens, DNA, RNA, polysaccharides, liposomes, virosomes, micelles, immunoglobulins, functional groups, other targeting moieties, or toxins.

[0284] The lipid conjugate (*e.g.*, PEG-lipid) typically comprises from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 0.9 mol % to about 1.6 mol %, from about 0.9 mol % to about 1.8 mol %, from about 1 mol % to about 1.8 mol %, from about 1 mol % to about 1.7 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, or from about 1.4 mol % to about 1.5 mol % of the total lipid present in the particle.

[0285] One of ordinary skill in the art will appreciate that the concentration of the lipid conjugate can be varied depending on the lipid conjugate employed and the rate at which the nucleic acid-lipid particle is to become fusogenic.

[0286] By controlling the composition and concentration of the lipid conjugate, one can control the rate at which the lipid conjugate exchanges out of the nucleic acid-lipid particle and, in turn, the rate at which the nucleic acid-lipid particle becomes fusogenic. For instance, when a PEG-phosphatidylethanolamine conjugate or a PEG-ceramide conjugate is used as the lipid conjugate, the rate at which the nucleic acid-lipid particle becomes fusogenic can be varied, for example, by varying the concentration of the lipid conjugate, by varying the molecular weight of the PEG, or by varying the chain length and degree of saturation of the acyl chain groups on the phosphatidylethanolamine or the ceramide. In addition, other variables including, for example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the nucleic acid-lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the nucleic acid-lipid particle becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure.

VI. Preparation of Lipid Particles

[0287] The lipid particles of the present invention, *e.g.*, SNALP, in which an active agent or therapeutic agent such as an interfering RNA is encapsulated in a lipid bilayer and is protected from degradation, can be formed by any method known in the art including, but not limited to, a continuous mixing method or a direct dilution process.

[0288] In preferred embodiments, the cationic lipids are lipids of Formula I, II, and III, or combinations thereof. In other preferred embodiments, the non-cationic lipids are egg sphingomyelin (ESM), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dipalmitoyl-phosphatidylcholine (DPPC), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, 14:0 PE (1,2-dimyristoyl-phosphatidylethanolamine (DMPE)), 16:0 PE (1,2-dipalmitoyl-phosphatidylethanolamine (DPPE)), 18:0 PE (1,2-distearoyl-phosphatidylethanolamine (DSPE)), 18:1 PE (1,2-dioleoyl-phosphatidylethanolamine (DOPE)), 18:1 trans PE (1,2-dielaidoyl-phosphatidylethanolamine (DEPE)), 18:0-18:1 PE (1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE)), 16:0-18:1 PE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)), polyethylene glycol-based polymers (*e.g.*, PEG 2000, PEG 5000, PEG-modified diacylglycerols, or PEG-modified dialkyloxypropyls), cholesterol, or combinations thereof.

[0289] In certain embodiments, the present invention provides for SNALP produced via a continuous mixing method, *e.g.*, a process that includes providing an aqueous solution comprising a nucleic acid such as an interfering RNA in a first reservoir, providing an organic lipid solution in a second reservoir, and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a liposome encapsulating the nucleic acid (*e.g.*, interfering RNA). This process and the apparatus for carrying this process are described in detail in U.S. Patent Publication No. 20040142025, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0290] The action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a liposome substantially instantaneously upon mixing. As used herein, the phrase "continuously diluting a lipid solution with a buffer solution" (and variations) generally means that the lipid solution is diluted sufficiently rapidly in a hydration process with sufficient force to effectuate vesicle generation. By mixing the

aqueous solution comprising a nucleic acid with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution in the presence of the buffer solution (*i.e.*, aqueous solution) to produce a nucleic acid-lipid particle.

[0291] The SNALP formed using the continuous mixing method typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0292] In another embodiment, the present invention provides for SNALP produced via a direct dilution process that includes forming a liposome solution and immediately and directly introducing the liposome solution into a collection vessel containing a controlled amount of dilution buffer. In preferred aspects, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In one aspect, the amount of dilution buffer present in the collection vessel is substantially equal to the volume of liposome solution introduced thereto. As a non-limiting example, a liposome solution in 45% ethanol when introduced into the collection vessel containing an equal volume of dilution buffer will advantageously yield smaller particles.

[0293] In yet another embodiment, the present invention provides for SNALP produced via a direct dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the liposome solution formed in a first mixing region is immediately and directly mixed with dilution buffer in the second mixing region. In preferred aspects, the second mixing region includes a T-connector arranged so that the liposome solution and the dilution buffer flows meet as opposing 180° flows; however, connectors providing shallower angles can be used, *e.g.*, from about 27° to about 180°. A pump mechanism delivers a controllable flow of buffer to the second mixing region. In one aspect, the flow rate of dilution buffer provided to the second mixing region is controlled to be substantially equal to the flow rate of liposome solution introduced thereto from the first mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the liposome solution in the second mixing region, and therefore also the concentration of liposome solution in buffer throughout the second mixing process. Such control of the dilution buffer flow rate advantageously allows for small particle size formation at reduced concentrations.

[0294] These processes and the apparatuses for carrying out these direct dilution processes are described in detail in U.S. Patent Publication No. 20070042031, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0295] The SNALP formed using the direct dilution process typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0296] If needed, the lipid particles of the invention (*e.g.,* SNALP) can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

[0297] Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles, is described in U.S. Patent No. 4,737,323, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between about 60 and about 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

[0298] Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

[0299] In some embodiments, the nucleic acids in the SNALP are precondensed as described in, *e.g.*, U.S. Patent Application No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0300] In other embodiments, the methods will further comprise adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions. Examples of suitable non-lipid polycations include, hexadimethrine bromide (sold under the brandname POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or

other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine, and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

[0301] In some embodiments, the nucleic acid to lipid ratios (mass/mass ratios) in a formed SNALP will range from about 0.01 to about 0.2, from about 0.02 to about 0.1, from about 0.03 to about 0.1, or from about 0.01 to about 0.08. The ratio of the starting materials also falls within this range. In other embodiments, the SNALP preparation uses about 400 μg nucleic acid per 10 mg total lipid or a nucleic acid to lipid mass ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 μg of nucleic acid. In other preferred embodiments, the particle has a nucleic acid:lipid mass ratio of about 0.08.

[0302] In other embodiments, the lipid to nucleic acid ratios (mass/mass ratios) in a formed SNALP will range from about 1 (1:1) to about 100 (100:1), from about 5 (5:1) to about 100 (100:1), from about 1 (1:1) to about 50 (50:1), from about 2 (2:1) to about 50 (50:1), from about 3 (3:1) to about 50 (50:1), from about 4 (4:1) to about 50 (50:1), from about 5 (5:1) to about 50 (50:1), from about 1 (1:1) to about 25 (25:1), from about 2 (2:1) to about 25 (25:1), from about 3 (3:1) to about 25 (25:1), from about 4 (4:1) to about 25 (25:1), from about 5 (5:1) to about 25 (25:1), from about 5 (5:1) to about 20 (20:1), from about 5 (5:1) to about 15 (15:1), from about 5 (5:1) to about 10 (10:1), about 5 (5:1), 6 (6:1), 7 (7:1), 8 (8:1), 9 (9:1), 10 (10:1), 11 (11:1), 12 (12:1), 13 (13:1), 14 (14:1), or 15 (15:1). The ratio of the starting materials also falls within this range.

[0303] As previously discussed, the conjugated lipid may further include a CPL. A variety of general methods for making SNALP-CPLs (CPL-containing SNALP) are discussed herein. Two general techniques include "post-insertion" technique, that is, insertion of a CPL into, for example, a pre-formed SNALP, and the "standard" technique, wherein the CPL is included in the lipid mixture during, for example, the SNALP formation steps. The post-insertion technique results in SNALP having CPLs mainly in the external face of the SNALP bilayer membrane, whereas standard techniques provide SNALP having CPLs on both internal and external faces. The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAAs and PEG-DAGs). Methods of making SNALP-CPL, are taught, for example, in U.S. Patent Nos. 5,705,385; 6,586,410; 5,981,501; 6,534,484; and 6,852,334; U.S. Patent Publication No. 20020072121; and PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

VII. Kits

[0304] The present invention also provides lipid particles (*e.g.*, SNALP) in kit form. The kit may comprise a container which is compartmentalized for holding the various elements of the lipid particles (*e.g.*, the active agents or therapeutic agents such as nucleic acids and the individual lipid components of the particles). In some embodiments, the kit may further comprise an endosomal membrane destabilizer (*e.g.*, calcium ions). The kit typically contains the lipid particle compositions of the present invention, preferably in dehydrated form, with instructions for their rehydration and administration.

[0305] As explained herein, the lipid particles of the invention (*e.g.*, SNALP) can be tailored to preferentially target particular tissues, organs, or tumors of interest. In certain instances, preferential targeting of lipid particles such as SNALP may be carried out by controlling the composition of the particle itself. For instance, as set forth in Example 11, it has been found that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP formulation can be used to preferentially target the liver (including liver tumors).

[0306] In certain other instances, it may be desirable to have a targeting moiety attached to the surface of the lipid particle to further enhance the targeting of the particle. Methods of attaching targeting moieties (*e.g.*, antibodies, proteins, *etc.*) to lipids (such as those used in the present particles) are known to those of skill in the art.

VII. Administration of Lipid Particles

[0307] Once formed, the lipid particles of the invention (*e.g.*, SNALP) are useful for the introduction of active agents or therapeutic agents (*e.g.*, nucleic acids such as interfering RNA) into cells. Accordingly, the present invention also provides methods for introducing an active agent or therapeutic agent such as a nucleic acid (*e.g.*, interfering RNA) into a cell. The methods are carried out *in vitro* or *in vivo* by first forming the particles as described above and then contacting the particles with the cells for a period of time sufficient for delivery of the active agent or therapeutic agent to the cells to occur.

[0308] The lipid particles of the invention (*e.g.*, SNALP) can be adsorbed to almost any cell type with which they are mixed or contacted. Once adsorbed, the particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the active agent or therapeutic agent (*e.g.*, nucleic acid) portion of the particle can take place via any one of these pathways. In particular, when

fusion takes place, the particle membrane is integrated into the cell membrane and the contents of the particle combine with the intracellular fluid.

[0309] The lipid particles of the invention (*e.g.*, SNALP) can be administered either alone or in a mixture with a pharmaceutically-acceptable carrier (*e.g.*, physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice. Generally, normal buffered saline (*e.g.*, 135-150 mM NaCl) will be employed as the pharmaceutically-acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc*. Additional suitable carriers are described in, *e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

[0310] The pharmaceutically-acceptable carrier is generally added following particle formation. Thus, after the particle is formed, the particle can be diluted into pharmaceutically-acceptable carriers such as normal buffered saline.

[0311] The concentration of particles in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2 to 5%, to as much as about 10 to 90% by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, particles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

[0312] The pharmaceutical compositions of the present invention may be sterilized by conventional, well-known sterilization techniques. Aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically-acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium

chloride, and calcium chloride. Additionally, the particle suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

A. *In vivo* Administration

[0313] Systemic delivery for *in vivo* therapy, *e.g.*, delivery of a therapeutic nucleic acid to a distal target cell via body systems such as the circulation, has been achieved using nucleic acid-lipid particles such as those described in PCT Publication Nos. WO 05/007196, WO 05/121348, WO 05/120152, and WO 04/002453, the disclosures of which are herein incorporated by reference in their entirety for all purposes. The present invention also provides fully encapsulated lipid particles that protect the nucleic acid from nuclease degradation in serum, are nonimmunogenic, are small in size, and are suitable for repeat dosing.

[0314] For *in vivo* administration, administration can be in any manner known in the art, *e.g.*, by injection, oral administration, inhalation (*e.g.*, intransal or intratracheal), transdermal application, or rectal administration. Administration can be accomplished via single or divided doses. The pharmaceutical compositions can be administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In some embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection (*see*, *e.g.*, U.S. Patent No. 5,286,634). Intracellular nucleic acid delivery has also been discussed in Straubringer *et al.*, *Methods Enzymol.*, 101:512 (1983); Mannino *et al.*, *Biotechniques*, 6:682 (1988); Nicolau *et al.*, *Crit. Rev. Ther. Drug Carrier Syst.*, 6:239 (1989); and Behr, *Acc. Chem. Res.*, 26:274 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, U.S. Patent Nos. 3,993,754; 4,145,410; 4,235,871; 4,224,179; 4,522,803; and 4,588,578. The lipid particles can be administered by direct injection at the site of disease or by injection at a site distal from the site of disease (*see*, *e.g.*, Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71(1994)). The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0315] The compositions of the present invention, either alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation (*e.g.*, intranasally or intratracheally) (*see*, Brigham *et al.*, *Am.*

J. Sci., 298:278 (1989)). Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0316] In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering nucleic acid compositions directly to the lungs via nasal aerosol sprays have been described, *e.g.*, in U.S. Patent Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Similarly, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Patent No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

[0317] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions are preferably administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically, or intrathecally.

[0318] Generally, when administered intravenously, the lipid particle formulations are formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium

chloride, sorbitan monolaurate, triethanolamine oleate, *etc.* These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

[0319] In certain applications, the lipid particles disclosed herein may be delivered via oral administration to the individual. The particles may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, pills, lozenges, elixirs, mouthwash, suspensions, oral sprays, syrups, wafers, and the like (*see, e.g.,* U.S. Patent Nos. 5,641,515, 5,580,579, and 5,792,451, the disclosures of which are herein incorporated by reference in their entirety for all purposes). These oral dosage forms may also contain the following: binders, gelatin; excipients, lubricants, and/or flavoring agents. When the unit dosage form is a capsule, it may contain, in addition to the materials described above, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. Of course, any material used in preparing any unit dosage form should be pharmaceutically pure and substantially non-toxic in the amounts employed.

[0320] Typically, these oral formulations may contain at least about 0.1% of the lipid particles or more, although the percentage of the particles may, of course, be varied and may conveniently be between about 1% or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of particles in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0321] Formulations suitable for oral administration can consist of: (a) liquid solutions, such as an effective amount of a packaged therapeutic agent such as nucleic acid (*e.g.,* interfering RNA) suspended in diluents such as water, saline, or PEG 400; (b) capsules, sachets, or tablets, each containing a predetermined amount of a therapeutic agent such as nucleic acid (*e.g.,* interfering RNA), as liquids, solids, granules, or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic

acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise a therapeutic agent such as nucleic acid (*e.g.,* interfering RNA) in a flavor, *e.g.*, sucrose, as well as pastilles comprising the therapeutic agent in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the therapeutic agent, carriers known in the art.

[0322] In another example of their use, lipid particles can be incorporated into a broad range of topical dosage forms. For instance, a suspension containing nucleic acid-lipid particles such as SNALP can be formulated and administered as gels, oils, emulsions, topical creams, pastes, ointments, lotions, foams, mousses, and the like.

[0323] When preparing pharmaceutical preparations of the lipid particles of the invention, it is preferable to use quantities of the particles which have been purified to reduce or eliminate empty particles or particles with therapeutic agents such as nucleic acid associated with the external surface.

[0324] The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as primates (*e.g.*, humans and chimpanzees as well as other nonhuman primates), canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, rats and mice), lagomorphs, and swine.

[0325] The amount of particles administered will depend upon the ratio of therapeutic agent (*e.g.,* nucleic acid) to lipid, the particular therapeutic agent (*e.g.,* nucleic acid) used, the disease or disorder being treated, the age, weight, and condition of the patient, and the judgment of the clinician, but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight, or about 10^8-10^{10} particles per administration (*e.g.,* injection).

B. *In vitro* Administration

[0326] For *in vitro* applications, the delivery of therapeutic agents such as nucleic acids (*e.g.,* interfering RNA) can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells are animal cells, more preferably mammalian cells, and most preferably human cells.

[0327] Contact between the cells and the lipid particles, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of particles varies widely depending on the particular application, but is generally between about 1 μmol and about 10

mmol. Treatment of the cells with the lipid particles is generally carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

[0328] In one group of preferred embodiments, a lipid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/ml, more preferably about 2×10^4 cells/ml. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/ml, more preferably about 0.1 μg/ml.

[0329] Using an Endosomal Release Parameter (ERP) assay, the delivery efficiency of the SNALP or other lipid particle of the invention can be optimized. An ERP assay is described in detail in U.S. Patent Publication No. 20030077829, the disclosure of which is herein incorporated by reference in its entirety for all purposes. More particularly, the purpose of an ERP assay is to distinguish the effect of various cationic lipids and helper lipid components of SNALP based on their relative effect on binding/uptake or fusion with/destabilization of the endosomal membrane. This assay allows one to determine quantitatively how each component of the SNALP or other lipid particle affects delivery efficiency, thereby optimizing the SNALP or other lipid particle. Usually, an ERP assay measures expression of a reporter protein (*e.g.*, luciferase, β-galactosidase, green fluorescent protein (GFP), *etc.*), and in some instances, a SNALP formulation optimized for an expression plasmid will also be appropriate for encapsulating an interfering RNA. In other instances, an ERP assay can be adapted to measure downregulation of transcription or translation of a target sequence in the presence or absence of an interfering RNA (*e.g.*, siRNA). By comparing the ERPs for each of the various SNALP or other lipid particles, one can readily determine the optimized system, *e.g.*, the SNALP or other lipid particle that has the greatest uptake in the cell.

C. Cells for Delivery of Lipid Particles

[0330] The compositions and methods of the present invention are used to treat a wide variety of cell types, *in vivo* and *in vitro*. Suitable cells include, *e.g.*, hematopoietic precursor (stem) cells, fibroblasts, keratinocytes, hepatocytes, endothelial cells, skeletal and smooth muscle cells, osteoblasts, neurons, quiescent lymphocytes, terminally differentiated cells, slow or noncycling primary cells, parenchymal cells, lymphoid cells, epithelial cells, bone cells, and the like. In preferred embodiments, an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is delivered to cancer cells such as, *e.g.*, lung cancer cells, colon cancer cells, rectal cancer cells, anal cancer cells, bile duct cancer cells, small intestine cancer cells, stomach (gastric) cancer cells, esophageal cancer cells, gallbladder cancer cells,

liver cancer cells, pancreatic cancer cells, appendix cancer cells, breast cancer cells, ovarian cancer cells, cervical cancer cells, prostate cancer cells, renal cancer cells, cancer cells of the central nervous system, glioblastoma tumor cells, skin cancer cells, lymphoma cells, choriocarcinoma tumor cells, head and neck cancer cells, osteogenic sarcoma tumor cells, and blood cancer cells.

[0331] *In vivo* delivery of lipid particles such as SNALP encapsulating an interfering RNA (*e.g.*, siRNA) is suited for targeting cells of any cell type. The methods and compositions can be employed with cells of a wide variety of vertebrates, including mammals, such as, *e.g*, canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, mice, rats, and guinea pigs), lagomorphs, swine, and primates (*e.g.* monkeys, chimpanzees, and humans).

[0332] To the extent that tissue culture of cells may be required, it is well-known in the art. For example, Freshney, Culture of Animal Cells, a Manual of Basic Technique, 3rd Ed., Wiley-Liss, New York (1994), Kuchler *et al.*, Biochemical Methods in Cell Culture and Virology, Dowden, Hutchinson and Ross, Inc. (1977), and the references cited therein provide a general guide to the culture of cells. Cultured cell systems often will be in the form of monolayers of cells, although cell suspensions are also used.

D. Detection of Lipid Particles

[0333] In some embodiments, the lipid particles of the present invention (*e.g.*, SNALP) are detectable in the subject at about 1, 2, 3, 4, 5, 6, 7, 8 or more hours. In other embodiments, the lipid particles of the present invention (*e.g.*, SNALP) are detectable in the subject at about 8, 12, 24, 48, 60, 72, or 96 hours, or about 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 25, or 28 days after administration of the particles. The presence of the particles can be detected in the cells, tissues, or other biological samples from the subject. The particles may be detected, *e.g.*, by direct detection of the particles, detection of a therapeutic nucleic acid such as an interfering RNA (*e.g.*, siRNA) sequence, detection of the target sequence of interest (*i.e.*, by detecting expression or reduced expression of the sequence of interest), or a combination thereof.

1. Detection of Particles

[0334] Lipid particles of the invention such as SNALP can be detected using any method known in the art. For example, a label can be coupled directly or indirectly to a component of the lipid particle using methods well-known in the art. A wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the lipid particle component, stability requirements, and available instrumentation and disposal

provisions. Suitable labels include, but are not limited to, spectral labels such as fluorescent dyes (*e.g.*, fluorescein and derivatives, such as fluorescein isothiocyanate (FITC) and Oregon Green™; rhodamine and derivatives such Texas red, tetrarhodimine isothiocynate (TRITC), *etc.*, digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like; radiolabels such as ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, *etc.*; enzymes such as horse radish peroxidase, alkaline phosphatase, *etc.*; spectral colorimetric labels such as colloidal gold or colored glass or plastic beads such as polystyrene, polypropylene, latex, *etc.* The label can be detected using any means known in the art.

2. Detection of Nucleic Acids

[0335] Nucleic acids (*e.g.*, interfering RNA) are detected and quantified herein by any of a number of means well-known to those of skill in the art. The detection of nucleic acids may proceed by well-known methods such as Southern analysis, Northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography. Additional analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography may also be employed.

[0336] The selection of a nucleic acid hybridization format is not critical. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in, *e.g.*, "Nucleic Acid Hybridization, A Practical Approach," Eds. Hames and Higgins, IRL Press (1985).

[0337] The sensitivity of the hybridization assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBA™) are found in Sambrook *et al.*, In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (2000); and Ausubel *et al.*, SHORT PROTOCOLS IN MOLECULAR BIOLOGY, eds., Current Protocols, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (2002); as well as U.S. Patent No. 4,683,202; PCR Protocols, A Guide to Methods and

Applications (Innis *et al.* eds.) Academic Press Inc. San Diego, CA (1990); Arnheim & Levinson (October 1, 1990), *C&EN* 36; The *Journal Of NIH Research*, 3:81 (1991); Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173 (1989); Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874 (1990); Lomell *et al.*, *J. Clin. Chem.*, 35:1826 (1989); Landegren *et al.*, *Science*, 241:1077 (1988); Van Brunt, *Biotechnology*, 8:291 (1990); Wu and Wallace, *Gene*, 4:560 (1989); Barringer *et al.*, *Gene*, 89:117 (1990); and Sooknanan and Malek, *Biotechnology*, 13:563 (1995). Improved methods of cloning *in vitro* amplified nucleic acids are described in U.S. Pat. No. 5,426,039. Other methods described in the art are the nucleic acid sequence based amplification (NASBA™, Cangene, Mississauga, Ontario) and Qβ-replicase systems. These systems can be used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using, for example, nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation. The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0338] Nucleic acids for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as inhibitor components are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage *et al.*, *Tetrahedron Letts.*, 22:1859 1862 (1981), *e.g.*, using an automated synthesizer, as described in Needham VanDevanter *et al.*, *Nucleic Acids Res.*, 12:6159 (1984). Purification of ploynucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion exchange HPLC as described in Pearson *et al.*, *J. Chrom.*, 255:137 149 (1983). The sequence of the synthetic poluyucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology*, 65:499.

[0339] An alternative means for determining the level of transcription is *in situ* hybridization. *In situ* hybridization assays are well-known and are generally described in Angerer *et al.*, *Methods Enzymol.*, 152:649 (1987). In an *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

VIII. Examples

[0340] The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

Example 1. Materials and Methods.

[0341] ***siRNA***: All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, CO). The siRNAs were desalted and annealed using standard procedures.

[0342] ***Lipid Encapsulation of siRNA***: In some embodiments, siRNA molecules were encapsulated into nucleic acid-lipid particles composed of the following lipids: the lipid conjugate PEG-cDMA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropylamine); the cationic lipid DLinDMA (1,2-Dilinoleyloxy-3-(N,N-dimethyl)aminopropane); the phospholipid DPPC (1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids; Alabaster, AL); and synthetic cholesterol (Sigma-Aldrich Corp.; St. Louis, MO) in the molar ratio 1.4:57.1:7.1:34.3, respectively. In other words, siRNAs were encapsulated into SNALP of the following "1:57" formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol. In other embodiments, siRNA molecules were encapsulated into phospholipid-free SNALP composed of the following lipids: the lipid conjugate PEG-cDMA; the cationic lipid DLinDMA; and synthetic cholesterol in the molar ratio 1.5:61.5:36.9, respectively. In other words, siRNAs were encapsulated into phospholipid-free SNALP of the following "1:62" formulation: 1.5% PEG-cDMA; 61.5% DLinDMA; and 36.9% cholesterol. For vehicle controls, empty particles with identical lipid composition were formed in the absence of siRNA. It should be understood that the 1:57 formulation and 1:62 formulation are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the formulation may vary. Typically, in the 1:57 formulation, the amount of cationic lipid will be 57 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:57 formulation being made up of non-cationic lipid (*e.g.*, phospholipid, cholesterol, or a mixture of the two). Similarly, in the 1:62 formulation, the amount of cationic lipid will be 62 mol % ± 5 mol %, and the amount of lipid conjugate

will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:62 formulation being made up of the non-cationic lipid (*e.g.*, cholesterol).

Example 2. Eg5 siRNA Formulated as 1:57 SNALP Are Potent Inhibitors of Cell Growth *in vitro*.

[0343] SNALP formulations were prepared with an siRNA targeting Eg5 as the nucleic acid component. Eg5 is a member of kinesin-related proteins that are involved in functions related to movements of organelles, microtubules, or chromosomes along microtubules. These functions include axonal transport, microtubule sliding during nuclear fusion or division, and chromosome disjunction during meiosis and early mitosis. Eg5 plays a critical role in mitosis of mammalian cells. The Eg5 siRNA used in this study is provided in Table 1. The modifications involved introducing 2'OMe-uridine at selected positions in the sense and antisense strands of the Eg5 2263 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 1. siRNA duplex comprising sense and antisense Eg5 RNA polynucleotides.

Modification	Eg5 2263 siRNA sequence	% 2'OMe-Modified	% Modified in DS Region
U/U	5'-C**U**GAAGACC**U**GAAGACAA**U**dTdT-3' 3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUUA-5'	6/42 = 14.3%	6/38 = 15.8%

Column 1: "U/U" = 2'OMe-uridine modified siRNA duplex; Column 2: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. "dT" = deoxythymidine. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

[0344] The lipid components and physical characteristics of the SNALP formulations are summarized in Table 2. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 2. Characteristics of the SNALP formulations used in this study.

Sample No.	Formulation Composition, Mole % PEG(2000)-C-DMA I DLinDMA I DPPC I Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
1	2 I 40 I 10 I 48	12.4	57	0.07	90
2	1.8 I 36.4 I 18.2 I 43.6	14.0	72	0.12	89
3	1.4 I 27.0 I 6.8 I 64.9	16.5	70	0.12	92
4	1.3 I 25.3 I 12.7 I 60.8	18.1	76	0.07	93
5	3.9 I 39.2 I 9.8 I 47.1	13.5	53	0.27	86
6	3.6 I 35.7 I 17.9 I 42.9	15.1	58	0.18	87
7	2.7 I 26.7 I 6.7 I 64.0	17.6	56	0.17	92
8	2.5 I 25.0 I 12.5 I 60.0	19.2	61	0.13	92
9	1.4 I 57.1 I 7.1 I 34.3	17.8	84	0.10	88
10	1.3 I 53.3 I 13.3 I 32.0	19.5	83	0.10	89
11	1.1 I 42.6 I 5.3 I 51.1	22.0	80	0.10	93
12	1.0 I 40.4 I 10.1 I 48.5	23.6	78	0.11	88
13	2.8 I 56.3 I 7.0 I 33.8	19.0	62	0.14	80
14	2.6 I 52.6 I 13.2 I 31.6	20.6	66	0.14	82
15	2.1 I 42.1 I 5.3 I 50.5	23.1	71	0.16	91
16	2 I 40 I 10 I 48	24.7	67	0.14	92

[0345] Silencing of Eg5 by siRNA transfection causes mitotic arrest and apoptosis in mammalian cells. Cell viability following transfection with SNALP containing an siRNA targeting Eg5 therefore provides a simple biological readout of *in vitro* transfection efficiency. Cell viability of *in vitro* cell cultures was assessed using the commercial reagent CellTiter-Blue® (Promega Corp.; Madison, WI), a resazurin dye that is reduced by metabolically active cells to the flourogenic product resorufin. The human colon cancer cell line HT29 was cultured using standard tissue culture techniques. 72 hours after SNALP application, CellTiter-Blue® reagent was added to the culture to quantify the metabolic activity of the cells, which is a measure of cell viability. Data are presented as a percent of cell viability relative to ("untreated") control cells that received phosphate buffered saline (PBS) vehicle only.

[0346] Figure 1 shows that the 1:57 SNALP formulation containing Eg5 2263 U/U siRNA was among the most potent inhibitors of tumor cell growth at all siRNA concentrations tested (*see,* Figure 1B, Sample 9).

Example 3. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo*.

[0347] SNALP formulations were prepared with an siRNA targeting apolipoprotein B (ApoB) as the nucleic acid component. ApoB is the main apolipoprotein of chylomicrons and low density lipoproteins (LDL). Mutations in ApoB are associated with

hypercholesterolemia. ApoB occurs in the plasma in 2 main forms, ApoB48 and ApoB100, which are synthesized in the intestine and liver, respectively, due to an organ-specific stop codon. The ApoB siRNA used in this study is provided in Table 3. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the ApoB siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 3. siRNA duplex comprising sense and antisense ApoB RNA polynucleotides.

Position	Modification	ApoB siRNA sequence	% 2'OMe-Modified	% Modified in DS Region
10048	U2/2 G1/2	5'-AGU**G**UCA**U**CACAC**U**GAAUACC-3' 3'-GU**U**CACAGUAGU**G**U**G**AC**U**UAU-5'	7/42 = 16.7%	7/38 = 18.4%

Column 1: The number refers to the nucleotide position of the 5' base of the sense strand relative to the mouse ApoB mRNA sequence XM_137955. Column 2: The numbers refer to the distribution of 2'OMe chemical modifications in each strand. Column 3: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. Column 4: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 5: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

[0348] The lipid components and physical characteristics of the formulations are summarized in Table 4. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 4. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2 | 40 | 10 | 48	12.4	59	0.15	93
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 2.2 | 44.4 | 53.3	10.7	55	0.17	91
4	PEG(2000)-C-DMA | DLinDMA | DOPC | Cholesterol 2 | 40 | 10 | 48	12.5	59	0.16	92
5	PEG(2000)-C-DMA | DLinDMA | DMPC | Cholesterol 2 | 40 | 10 | 48	12.2	56	0.11	92
6	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.8 | 36.4 | 18.2 | 43.6	13.8	66	0.16	93
7	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 2 | 40 | 10 | 48	12.4	56	0.12	92
8	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 27.0 | 6.8 | 64.9	16.5	60	0.10	93
9	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.3 | 25.3 | 12.7 | 60.8	18.1	74	0.13	92
10	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2.5 | 25.0 | 12.5 | 60.0	19.2	60	0.13	93
11	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.4 | 34.3	17.8	79	0.09	94

12	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.0 | 40.4 | 10.1 | 48.5	23.6	72	0.11	93
13	PEG(2000)-C-DMA | DLinDMA | DPPC 2 | 70 | 28	8.7	73	0.09	87
14	PEG(2000)-C-DMA | DLinDMA | DPPC 1.6 | 54.7 | 43.8	11.3	65	0.11	87

[0349] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0350] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0351] Figure 2 shows that the 1:57 SNALP formulation containing ApoB 10048 U2/2 G1/2 siRNA was the most potent at reducing ApoB expression *in vivo* (*see,* Group 11).

Example 4. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo*.

[0352] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 5. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 5. Characteristics of the SNALP formulations used in this study.

SNALP (L:D ratio)	siRNA Payload	Particle Size (Polydispersity)	% Encapsulation
2:30 (13)	ApoB-10048 U2/2 G1/2	65 nm (0.16)	88
1:57 (9)	ApoB-10048 U2/2 G1/2	74 nm (0.10)	89

[0353] The 2:30 SNALP formulation used in this study is lipid composition 2:30:20:48 as described in molar percentages of PEG-C-DMA, DLinDMA, DSPC, and cholesterol (in that

order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 13:1.

[0354] The 1:57 SNALP formulation used in this study is lipid composition 1.5:57.1:7:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 9:1.

[0355] BALB/c mice (female, 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Days 0, 1, 2, 3 & 4 for a total of 5 doses per animal. Daily dosage was either 1.0 (for 2:30 SNALP) or 0.1 (for 1:57 SNALP) mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given IV injections of phosphate buffered saline (PBS) vehicle. On Study Day 7, 72 h after the last treatment, animals were euthanized and liver tissue was collected in RNAlater.

[0356] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0357] Figure 3 shows that the 1:57 SNALP containing ApoB 10048 U2/2 G1/2 siRNA was more than 10 times as efficacious as the 2:30 SNALP in mediating ApoB gene silencing in mouse liver at a 10-fold lower dose.

Example 5. ApoB siRNA Formulated as 1:57 or 1:62 SNALP Have Potent Silencing Activity *in vivo*.

[0358] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 6. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 6. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	76	0.06	89
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 1.5 | 61.5 | 36.9	8.1	76	0.04	86
4	PEG(2000)-C-DMA | DODMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.0	72	0.05	95
5	PEG(5000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.6	52	0.16	89
6	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 1.4 | 57.1 | 7.1 | 34.3	8.9	68	0.10	94
7	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	72	0.07	95
8	PEG(2000)-C-DMA | DLinDMA | DPPC 1.8 | 70.2 | 28.1	8.6	74	0.13	86

[0359] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.75 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0360] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0361] Figure 4 shows that the 1:57 and 1:62 SNALP formulations had comparable ApoB silencing activity *in vivo* (*see, e.g.*, Groups 2 & 3).

Example 6. ApoB siRNA Formulated as 1:62 SNALP Have Potent Silencing Activity *in vivo*.

[0362] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 7. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 7. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition, Mole % PEG(2000)-C-DMA I DLinDMA I Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	1.5 I 61.5 I 36.9	6.1	80	0.07	92
3	1.4 I 54.8 I 43.8	6.6	74	0.05	89
4	2.0 I 61.2 I 36.7	6.2	71	0.11	91
5	1.8 I 54.5 I 43.6	6.7	67	0.09	91
6	1.3 I 68.1 I 30.6	7.4	91	0.06	89
7	1.2 I 61.8 I 37.1	8.0	87	0.10	90
8	1.7 I 67.8 I 30.5	7.6	81	0.07	91
9	1.4 I 56.3 I 42.3	8.6	75	0.11	92
10	1.9 I 61.3 I 36.8	8.2	72	0.10	91
11	1.8 I 56.1 I 42.1	8.8	70	0.10	90
12	1.3 I 66.7 I 32.0	9.5	89	0.09	89
13	1.2 I 61.7 I 37.0	10.0	87	0.10	91
14	1.7 I 66.4 I 31.9	9.6	82	0.11	90
15	1.5 I 61.5 I 36.9	10.1	79	0.10	91

[0363] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0364] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0365] Figure 5 shows that the 1:62 SNALP formulation was one of the most potent inhibitors of ApoB expression at two different lipid:drug ratios (*i.e.*, 6.1 & 10.1) among the phospholipid-free SNALP formulations tested (*see*, Groups 2 & 15).

Example 7. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Syringe Press or Gear Pump Process.

[0366] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by various manufacturing processes. In particular, 1:57 SNALP was prepared by a syringe press or gear pump process

using either PBS or citrate buffer (post-blend dilution) and administered intravenously in mice.

Experimental Design

[0367] Animal Model: Female BALB/c mice, 5 wks old, n=4 per group/cage.

[0368] siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

Tolerability:

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
1	PBS vehicle	Standard 10 mL/kg volume	
2	1|57 Citrate Direct Dil, Syringe Press	7	77
3	1|57 PBS Direct Dil, Syringe Press	7	96
4	1|57 PBS Direct Dil, Gear Pump	7	79
5	1|57 Citrate Direct Dil, Syringe Press	9	99
6	1|57 PBS Direct Dil, Syringe Press	9	123
7	1|57 PBS Direct Dil, Gear Pump	9	102

Efficacy:

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
8	PBS vehicle	Standard 10 mL/kg volume	
9	1|57 PBS Direct Dil, Syringe Press	0.05	0.68
10	1|57 PBS Direct Dil, Gear Pump	0.05	0.57
11	1|57 PBS Direct Dil, Syringe Press	0.1	1.36
12	1|57 PBS Direct Dil, Gear Pump	0.1	1.13

Formulation:

[0369] Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

[0370] Formulation Details:

1. Lipid composition "1|57 Citrate blend" used in this study is 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation has an input lipid to drug ratio of 8.9.
2. Gear pump set up included 0.8 mm T-connector and 400 mL/min speed.
3. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0371] Formulation Summary:

		Particle Size			Final L:D
	1:57 (9:1) + DOW siRNA	Zavg (nm)	Poly	% Encap	(mg:mg)
322-050807-1	Syringe PBS Blend	79	0.12	92	13.6
322-050807-2	Syringe Citrate Blend	86	0.11	91	11.0
322-050807-3	Gear PBS Blend	80	0.09	93	11.3

Procedures

[0372] **Treatment:** Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 μl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0373] **Group 1-7 Endpoint:** Animals are sacrificed on Day 1, 24 h after test article administration. Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein.

[0374] **Group 8-12 Endpoint:** Animals are sacrificed on Day 2, 48 h after test article administration. Blood is collected by cardiac puncture and processed for plasma. Immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed and weighed separately: liver and spleen. The bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at -20°C or -80°C for archival purposes. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0375] **Termination:** Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0376] **Data Analysis:** Tolerability of treatment regime is monitored by animal appearance and behavior as well as body weight. Blood clinical chemistry is measured by automated analyzer. ApoB and GAPDH mRNA levels in liver are measured via QG assay. ApoB

protein in plasma is measured via ELISA. Total cholesterol in plasma is measured via standard enzymatic/colorimetric assay.

Results

[0377] There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. Figure 6 shows that the tolerability of SNALP prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters. There was a tolerability difference between syringe citrate and syringe PBS at constant siRNA dosage, but that was likely an artifact dependent on the different final lipid:drug (L:D) ratios of these two preparations.

[0378] Figure 7 shows that the efficacy of the 1:57 SNALP prepared by gear pump was similar to the same SNALP prepared by syringe press. The tolerability profile was improved with the gear pump process, which could be attributed to increased initial encapsulation rate and decreased final L:D ratio.

Example 8. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Direct Dilution or In-Line Dilution Process.

[0379] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by a direct dilution or in-line dilution process at an input lipid to drug ratio of 6:1 or 9:1.

Experimental Design

[0380] Animal Model: Female BALB/c mice, 7 wks old.

[0381] siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

CBC/Diff:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
1	3	PBS	-	-
2	3	1|57 SNALP (9:1)	7 mg/kg	71 mg/kg
3	3	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Clinical Chemistry:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
4	4	PBS	-	-
5	4	1|57 SNALP (9:1)	9 mg/kg	92 mg/kg
6	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg
7	4	(6:1) New 1|57 SNALP	11 mg/kg	78 mg/kg

8	4	(6:1) New 1|57 SNALP	13 mg/kg	93 mg/kg
9	4	(6:1) New 1|57 SNALP	15 mg/kg	107 mg/kg
10	4	(6:1) New 1|57 SNALP	17 mg/kg	121 mg/kg
11	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Activity:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
12	4	PBS	-	-
13	4	1|57 SNALP (9:1)	0.05 mg/kg	0.51 mg/kg
14	4	1|57 SNALP (9:1)	0.1 mg/kg	1.02 mg/kg
15	4	1|57 SNALP (9:1)	0.2 mg/kg	2.04 mg/kg
16	4	(6:1) New 1|57 SNALP	0.05 mg/kg	0.36 mg/kg
17	4	(6:1) New 1|57 SNALP	0.1 mg/kg	0.71 mg/kg
18	4	(6:1) New 1|57 SNALP	0.2 mg/kg	1.42 mg/kg
19	4	(6:1) New 1|57 SNALP	0.4 mg/kg	2.85 mg/kg

Formulation:

[0382] Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

[0383] Formulation Details:

1. "1|57 SNALP" used in this study is lipid composition 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by gear pump at an input lipid to drug ratio of 9:1 (28 mM lipids) or 6:1 (14 mM lipids).
2. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0384] Formulation Summary:

	1|57 SNALP Gear PBS In-Line	Particle Size			Final L:D
		Zavg (nm)	Poly	% Encap	(mg:mg)
322-051407-1	Input 9:1	78	0.07	93	10.2
322-051407-2	Input 6:1	81	0.05	92	7.1

Procedures

[0385] **Treatment:** Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 μl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0386] **Endpoint:** Animals are sacrificed on Day 1, 24 h after test article administration (Grps 1-10) or on Day 2, 48 h after test article administration (Grps 11-19).

[0387] **Groups 1-3:** Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into an EDTA microtainer, mixed immediately to prevent coagulation, and sent for analysis of CBC/Diff profile. Perform brief necropsy.

[0388] **Groups 4-11:** Blood is collected by cardiac puncture into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein. Perform brief necropsy.

[0389] **Groups 12-19:** Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed: liver. The liver is not weighed; the bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at –80°C. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0390] **Termination:** Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0391] **Data Analysis:** Tolerability of treatment regime is monitored by animal appearance and behavior, and body weight. Blood clinical chemistry and CBC/Diff profile is measured by automated analyzer. Liver ApoB mRNA is measured using the QuantiGene Assay. Plasma ApoB-100 is measured using ELISA. Plasma total cholesterol is measured using a standard enzymatic assay.

Results

Tolerability:

[0392] Figure 8 shows that there was very little effect on body weight 24 hours after 1:57 SNALP administration. The maximum weight loss of 3.6 ± 0.7% was observed at the highest drug dose of 17 mg/kg. There was also no obvious change in animal appearance/behavior at any of the dosages tested.

[0393] Figure 9 shows that there were no obvious changes in platelet count. Reduction of platelets can cause the mean platelet volume to increase as the body produces new platelets in compensation for the treatment-related decrease. Under the conditions of this study, the mean platelet volume did not change in SNALP-treated groups.

[0394] Figure 10 shows that clinically significant liver enzyme elevations (3xULN) occurred at drug dosages of 11 mg/kg for 1:57 SNALP at a lipid:drug (L:D) ratio of 10, and at 13 mg/kg at a L:D of 7. A slight dose response trend upwards in plasma total protein and globulin was also observed.

Efficacy:

[0395] Figure 11 shows that based on the liver mRNA QuantiGene analysis, the potency of the lower L:D SNALP was as good as that of the higher L:D SNALP at the tested drug dosages. In fact, the ApoB silencing activity was identical at the 0.05 and 0.1 mg/kg dosages. As such, the potency of the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) was similar to the potency of the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) at reducing ApoB expression.

[0396] Figure 12 shows that ApoB protein and total cholesterol levels were reduced to a similar extent by the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

Therapeutic Index:

[0397] This study demonstrates that both the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) caused about 60% ApoB liver mRNA silencing with a drug dose of 0.1 mg/kg. Interpolating from the available data points in Figure 10, a 10:1 final L:D ratio at 10 mg/kg may cause a similar degree of enzyme elevation as a 7:1 final L:D ratio at 13 mg/kg. Using these activity and toxicity points, the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio is (10 mg/kg) / (0.1 mg/kg) = 100 and the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is (13 mg/kg) / (0.1 mg/kg) = 130. Using this dataset, the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is 30% greater than the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio.

Example 9. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Increases Survival of Hep3B Tumor-Bearing Mice.

[0398] SNALP containing polo-like kinase 1 (PLK-1) siRNA (1:57 SNALP formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) were tested for their effects on the survival of CD1 nu/nu mice bearing Hep3B liver tumors. PLK-1 is a serine/threonine kinase containing two functional domains: (1) a kinase domain; and (2) a polo-box domain (*see, e.g.,* Barr *et al.*, *Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)). The activity and cellular concentration of PLK-1 are crucial for the precise regulation of cell division. PLK-1 is overexpressed in many cancer types including hepatoma and colon cancer, and PLK-1 expression often correlates with poor patient prognosis. Overexpression of PLK-1 (wild-type or kinase inactive) results in multinucleation (genetic instability). Hyperactive PLK-1 overrides the DNA damage checkpoint. Constitutive PLK-1 expression causes transformation of NIH 3T3 cells. PLK-1 phosphorylates the p53 tumor suppressor, thereby inhibiting the pro-apoptotic effects of p53. The PLK-1 siRNA used in this study are provided in Table 8. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the PLK-1 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 8. siRNA duplexes comprising sense and antisense PLK-1 RNA polynucleotides.

siRNA	PLK-1 siRNA Sequence	% Modified in DS Region
PLK1424 U4/GU	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' (SEQ ID NO.57) 3'-NNUC**U**AGUGGGAG**G**AAUUUAU-5' (SEQ ID NO.54)	6/38 = 15.8%
PLK1424 U4/G	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' (SEQ ID NO.57) 3'-NNUCUAG**U**GG**G**AG**G**AAUUUAU-5' (SEQ ID NO.56)	7/38 = 18.4%

Column 1: The number after "PLK" refers to the nucleotide position of the 5' base of the sense strand relative to the start codon (ATG) of the human PLK-1 mRNA sequence NM_005030. Column 2: 2'-O-methyl (2'OMe) nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. N = deoxythymidine (dT) nucleotide, uridine (U) ribonucleotide, or ribonucleotide having complementarity to the target sequence or the complementary strand thereof. Column 3: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

Experimental Groups

[0399] 20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay

A	20 to seed	I.H. $1.5x10^6$ Hep3B	Luc 1:57	9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10 x 2 mg/kg	When moribund	Survival Body Weights
B			PLK 1424 1:57	9				

Test Articles

[0400] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 20 ml of each SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	Luc U/U SNALP 1:57 (28mM lipid)
B	PLK1424 U4/GU SNALP 1:57 (28mM lipid) PLK1424 U4/G SNALP 1:57 (28mM lipid)

Procedures

Day 0 Mice will receive Anafen by SC injection (100 µg in 20 µl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25 µl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 µl) and 30G (3/8") needle. Cells will be injected slowly (~ 30 s) and a swab applied to the puncture wound immediately after needle withdrawal. After any bleeding has stopped (~1 min), the incision is closed with 5-6 sutures in the muscle wall and 3-4 skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.

Day 1	All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 μg in 20 μl saline).
Day 10	Mice will be randomized into the appropriate treatment groups.
Day 11	**Groups A, B – Day 11:** All Animals will be administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg). Dosing will be repeated for 5 consecutive days based on initial weight.
Day 14-35	**Groups A, B – Days 14, 17, 21, 25, 28, 32, 35:** All Animals will be re-administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg). **Body weights Groups:** Mice will be weighed on the day of dosing for 5 weeks, then twice weekly until close of the study. **Endpoint:** Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the treatment or tumor burden are terminated at the discretion of the vivarium staff.
Termination:	Mice are anesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.
Data Analysis:	Survival and body weights are assayed.

Results

[0401] Figure 13 shows the mean body weights of mice during therapeutic dosing of PLK1424 SNALP in the Hep3B intrahepatic (I.H.) tumor model. The treatment regimen was well tolerated with no apparent signs of treatment-related toxicity.

[0402] Figure 14 shows that treatment with 1:57 SNALP-formulated PLK1424 caused a significant increase in the survival of Hep3B tumor-bearing mice. This *in vivo* anti-tumor effect was observed in the absence of any apparent toxicity or immune stimulation.

Example 10. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Induces Tumor Cell Apoptosis in Hep3B Tumor-Bearing Mice.

[0403] The objectives of this study were as follows:

1. To determine the level of mRNA silencing in established Hep3B liver tumors following a single IV administration of PLK1424 SNALP.
2. To confirm the mechanism of mRNA silencing by detecting specific RNA cleavage products using RACE-PCR.
3. To confirm induction of tumor cell apoptosis by histopathology.

[0404] The 1:57 SNALP formulation (1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) was used for this study.

Experimental Groups

[0405] 20 SCID/beige mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	Sacrifice	Assay
A	20 to seed	I.H. $1x10^6$ Hep3B	PBS	6	1 x 2 mg/kg Day 20	24 h after treatment	Tumor QG Tumor RACE-PCR Histopathology
			Luc 1:57	7			
B			PLK 1424 1:57	7			
C							

Test Articles

[0406] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 2 ml of SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	PBS
B	Luc U/U 1:57 SNALP
C	PLK1424 U4/GU 1:57 SNALP

Procedures

Day 0 Mice will receive Anafen by SC injection (100 µg in 20 µl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the

sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25µl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 µl) and 30G (3/8") needle. Cells will be injected slowly (~30 s) and a swab applied to the puncture wound immediately after needle withdrawal. After any bleeding has stopped (~1 min), the muscle wall incision is closed with 5-6 sutures. The skin incision is then closed with 3-4 metal skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.

Day 1 All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 µg in 20 µl saline).

Day 7 Mice will be randomized into the appropriate treatment groups.

Day 20 **Groups A-C:** Mice will be weighed and then administered either PBS, Luc, or PLK1424 SNALP by IV injection via the lateral tail vein. SNALP will be dosed at 2 mg/kg or equivalent volume (10 ml/kg) according to body weight.

Day 21 **Groups A-C**: All mice will be weighed and then euthanized by lethal anesthesia.

Tumor bearing liver lobes from all mice in each group will be weighed and collected into RNALater for RNA analysis.

Endpoint: Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the treatment or tumor burden are terminated at the discretion of the vivarium staff.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.

Data Analysis: mRNA analysis of liver tumors by bDNA (QG) assay and RACE-PCR.

Tumor cell apoptosis by histopathology.

Results

[0407] Body weights were monitored from Day 14 onwards to assess tumor progression. On Day 20, 6 mice showing greatest weight loss were randomized into each of the 3 groups and treated. All six mice had substantial-large I.H. tumors at sacrifice (Day 21). Treatment of the remaining 14 mice was therefore initiated on the Day 21 (sacrifice Day 22). 10/14 mice had substantial tumors; 2/14 mice had small/probable tumors; and 2/14 mice had no visible tumor burden.

[0408] Figure 15 shows data from Quantigene assays used to measure human (tumor)-specific PLK-1 mRNA levels. A single 2 mg/kg dose of 1:57 SNALP reduced PLK-1 mRNA levels by about 50% in intrahepatic Hep3B tumors growing in mice.

[0409] Figure 16 shows that a specific cleavage product of PLK-1 mRNA was detectable in mice treated with PLK1424 SNALP by 5' RACE-PCR. No specific PCR product was detectable in mice treated with either PBS or control (Luc) SNALP. Nucleotide sequencing of the PCR product confirmed the predicted cleavage site by PLK1424 siRNA-mediated RNA interference in the PLK-1 mRNA.

[0410] Figure 17 shows Hep3B tumor histology in mice treated with either Luc SNALP (top) or PLK1424 SNALP (bottom). Luc SNALP-treated mice displayed normal mitoses in Hep3B tumors, whereas PLK1424 SNALP-treated mice exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors.

Conclusion

[0411] This example illustrates that a single administration of PLK1424 1:57 SNALP to Hep3B tumor-bearing mice induced significant *in vivo* silencing of PLK-1 mRNA. This reduction in PLK-1 mRNA was confirmed to be mediated by RNA interference using 5' RACE-PCR analysis. Importantly, PLK-1 mRNA silencing by the 1:57 SNALP formulation profoundly disrupted tumor cell proliferation (mitosis), causing subsequent apoptosis of tumor cells. As demonstrated in the previous example, this anti-tumor effect translated into extended survival times in the tumor-bearing mice.

Example 11. Comparison of 1:57 PLK-1 SNALP Containing Either PEG-cDMA or PEG-cDSA in a Subcutaneous Hep3B Tumor Model.

[0412] This example demonstrates the utility of the PEG-lipid PEG-cDSA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-distearyloxypropylamine) in the 1:57 formulation for systemically targeting distal (*e.g.*, subcutaneous) tumors. In particular, this example compares the tumor targeting ability of 1:57 PLK-1 SNALPs containing either PEG-cDMA (C_{14}) or PEG-cDSA (C_{18}). Readouts are tumor growth inhibition and PLK1 mRNA silencing. The PLK-1 siRNA used was PLK1424 U4/GU, the sequence of which is provided in Table 8.

[0413] Subcutaneous (S.C.) Hep3B tumors were established in scid/beige mice. Multidose anti-tumor efficacy of 1:57 PLK-1 SNALP was evaluated for the following groups (n=5 for each group): (1) "Luc-cDMA" - PEG-cDMA Luc SNALP; (2) "PLK-cDMA" - PEG-cDMA PLK-1 SNALP; and (3) "PLK-cDSA" - PEG-cDSA PLK-1 SNALP. Administration of 6 x 2mg/kg siRNA was initiated once tumors reached about 5 mm in diameter (Day 10). Dosing was performed on Days 10, 12, 14, 17, 19, and 21. Tumors were measured by caliper twice weekly.

[0414] Figure 18 shows that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B S.C. tumors. In particular, 5/5 tumors in the PLK1-cDSA treated mice appeared flat, measurable only by discoloration at the tumor site.

[0415] Figure 19 shows the mRNA silencing of 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration. The extent of silencing observed with the PLK1-cDSA SNALP correlated with the anti-tumor activity in the multi-dose study shown in Figure 18.

[0416] The Luc-cDMA SNALP-treated group, which had developed large S.C. tumors at Day 24, were then administered PLK-cDSA SNALP on Days 24, 26, 28, 31, 33, and 35. There was no additional dosing of the original PLK-1 SNALP-treated groups. The results from this crossover dosing study with large established tumors is provided in Figure 20, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0417] A comparison of the effect of PEG-cDMA and PEG-cDSA 1:57 SNALPs on PLK-1 mRNA silencing was performed using established intrahepatic Hep3B tumors in scid/beige mice. A single 2 mg/kg dose of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA was administered intravenously. Liver/tumor samples were collected at 24 and 96 hours after SNALP treatment. Control = 2 mg/kg Luc-cDMA SNALP at 24 hours.

[0418] Figure 21 shows that PLK-cDMA SNALP and PLK-cDSA SNALP had similar silencing activities after 24 hours, but that the PLK-cDSA SNALP may increase the duration of mRNA silencing in intrahepatic tumors.

[0419] Figure 22 shows the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA. The extended blood circulation times observed for the PLK-cDSA SNALP may enable the increased accumulation and activity at distal (*e.g.*, subcutaneous) tumor sites.

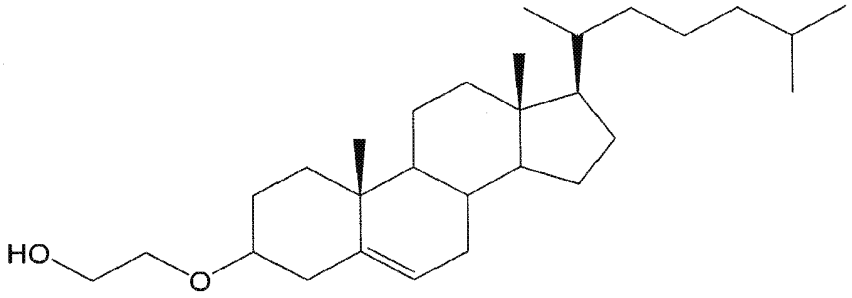
[0420] Thus, this study shows that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP can be used to preferentially target the liver.

Example 12. Synthesis of Cholesteryl-2'-Hydroxyethyl Ether.

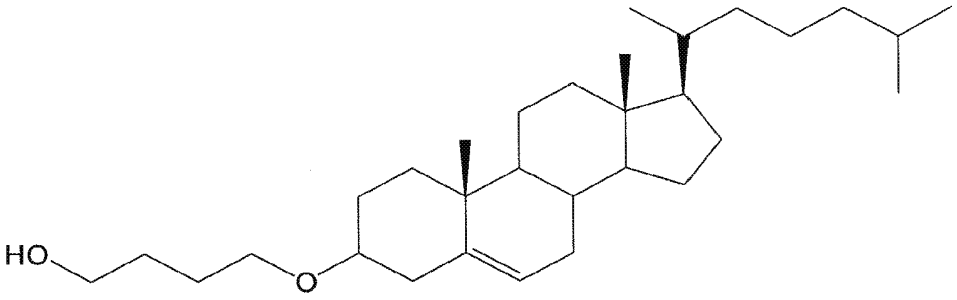
[0421] Step 1: A 250 ml round bottom flask containing cholesterol (5.0 g, 12.9 mmol) and a stir bar was sealed and flushed with nitrogen. Toluenesulphonyl chloride (5.0 g, 26.2 mmol) was weighed into a separate 100-mL round bottom flask, also sealed and flushed with nitrogen. Anhydrous pyridine (2 x 50 ml) was delivered to each flask. The toluenesulphonyl chloride solution was then transferred, via cannula, into the 250 ml flask, and the reaction stirred overnight. The pyridine was removed by rotovap, and methanol (80 ml) added to the residue. This was then stirred for 1 hour until a homogeneous suspension was obtained. The suspension was filtered, washed with acetonitrile (50 ml), and dried under vacuum to yield cholesteryl tosylate as a fluffy white solid (6.0 g, 86%).

[0422] Step 2: Cholesteryl tosylate (2.0 g, 3.7 mmol), 1,4-dioxane (50 mL), and ethylene glycol (4.6 g, 74 mmol) were added to a 100 ml flask containing a stir bar. The flask was fitted with a condenser, and refluxed overnight. The dioxane was then removed by rotovap, and the reaction mixture suspended in water (100 ml). The solution was transferred to a separating funnel and extracted with chloroform (3 x 100 ml). The organic phases were combined, washed with water (2 x 150 ml), dried over magnesium sulphate, and the solvent removed. The crude product was purified by column chromatography (5% acetone/hexane) to yield the product as a white solid (1.1 g, 69%).

[0423] The structures of the cholesterol derivatives cholesteryl-2'-hydroxyethyl ether and cholesteryl-4'-hydroxybutyl ether are as follows:



Cholesteryl-2'-hydroxyethyl ether



Cholesteryl-4'-hydroxybutyl ether

[0424] It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent applications, patents, PCT publications, and Genbank Accession Nos., are incorporated herein by reference for all purposes.

WHAT IS CLAIMED IS:

1. A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

2. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid comprises a small interfering RNA (siRNA).

3. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises from about 15 to about 60 nucleotides.

4. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one modified nucleotide.

5. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

6. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), or a mixture thereof.

7. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA).

8. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle.

9. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 52 mol % to about 62 mol % of the total lipid present in the particle.

10. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises cholesterol or a derivative thereof.

11. The nucleic acid-lipid particle of claim 10, wherein the cholesterol or derivative thereof comprises from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle.

12. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a phospholipid.

13. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.

14. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

15. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises from about 4 mol % to about 10 mol % of the total lipid present in the particle and the cholesterol comprises from about 30 mol % to about 40 mol % of the total lipid present in the particle.

16. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises from about 10 mol % to about 30 mol % of the total lipid present in the particle and the cholesterol comprises from about 10 mol % to about 30 mol % of the total lipid present in the particle.

17. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

18. The nucleic acid-lipid particle of claim 17, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

19. The nucleic acid-lipid particle of claim 18, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

20. The nucleic acid-lipid particle of claim 19, wherein the PEG has an average molecular weight of about 2,000 daltons.

21. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from about 1 mol % to about 2 mol % of the total lipid present in the particle.

22. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid in the nucleic acid-lipid particle is not substantially degraded after incubation of the particle in serum at 37°C for 30 minutes.

23. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

24. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a lipid:nucleic acid mass ratio of from about 5 to about 15.

25. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a median diameter of from about 40 nm to about 150 nm.

26. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.

27. A nucleic acid-lipid particle comprising:

(a) an siRNA;

(b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle;

(c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and

(d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

28. The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises DLinDMA.

29. The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises DLin-K-C2-DMA.

30. The nucleic acid-lipid particle of claim 27, wherein the PEG-lipid conjugate comprises a PEG-DAA conjugate.

31. The nucleic acid-lipid particle of claim 27, wherein the nucleic acid-lipid particle comprises about 61.5 mol % cationic lipid, about 36.9% cholesterol or a derivative thereof, and about 1.5 mol % PEG-lipid conjugate.

32. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 27 and a pharmaceutically acceptable carrier.

33. A nucleic acid-lipid particle, comprising:

(a) an siRNA;

(b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle;

(c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and

(d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

34. The nucleic acid-lipid particle of claim 33, wherein the cationic lipid comprises DLinDMA.

35. The nucleic acid-lipid particle of claim 33, wherein the cationic lipid comprises DLin-K-C2-DMA.

36. The nucleic acid-lipid particle of claim 33, wherein the phospholipid comprises DPPC.

37. The nucleic acid-lipid particle of claim 33, wherein the PEG-lipid conjugate comprises a PEG-DAA conjugate.

38. The nucleic acid-lipid particle of claim 33, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 7.1 mol % phospholipid, about 34.3 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

39. The nucleic acid-lipid particle of claim 33, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 20 mol % phospholipid, about 20 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

40. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 33 and a pharmaceutically acceptable carrier.

41. A method for introducing a nucleic acid into a cell, the method comprising:

contacting the cell with a nucleic acid-lipid particle of claim 1, 27, or 33.

42. The method of claim 41, wherein the cell is in a mammal.

43. A method for the *in vivo* delivery of a nucleic acid, the method comprising:

administering to a mammalian subject a nucleic acid-lipid particle of claim 1, 27, or 33.

44. The method of claim 43, wherein the administration is selected from the group consisting of oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal.

45. A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising:

administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 1, 27, or 33.

46. The method of claim 45, wherein the disease or disorder is selected from the group consisting of a viral infection, a liver disease or disorder, and cancer.

ABSTRACT OF THE DISCLOSURE

The present invention provides novel, stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles. More particularly, the present invention provides stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (such as one or more interfering RNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP.

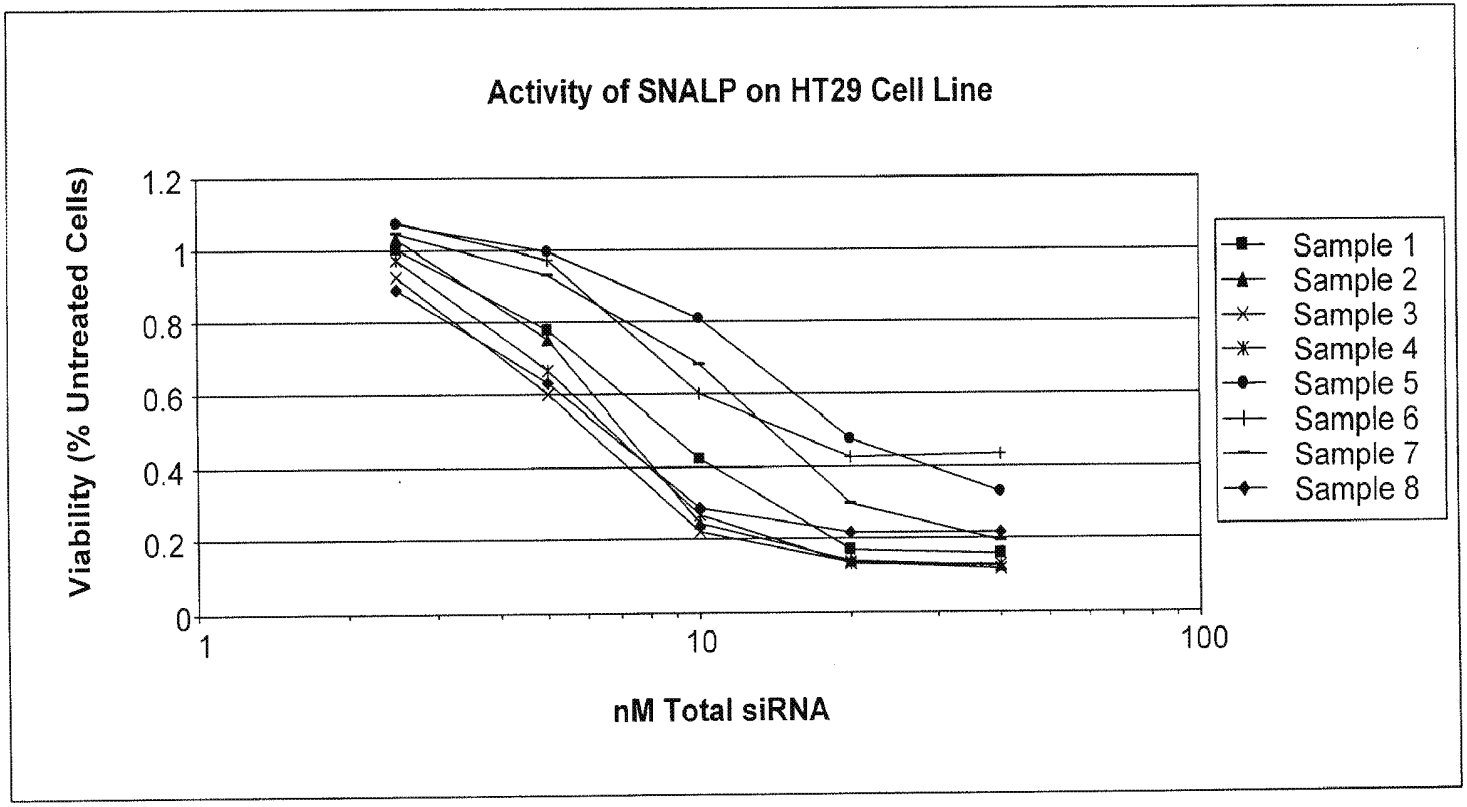


FIG. 1A

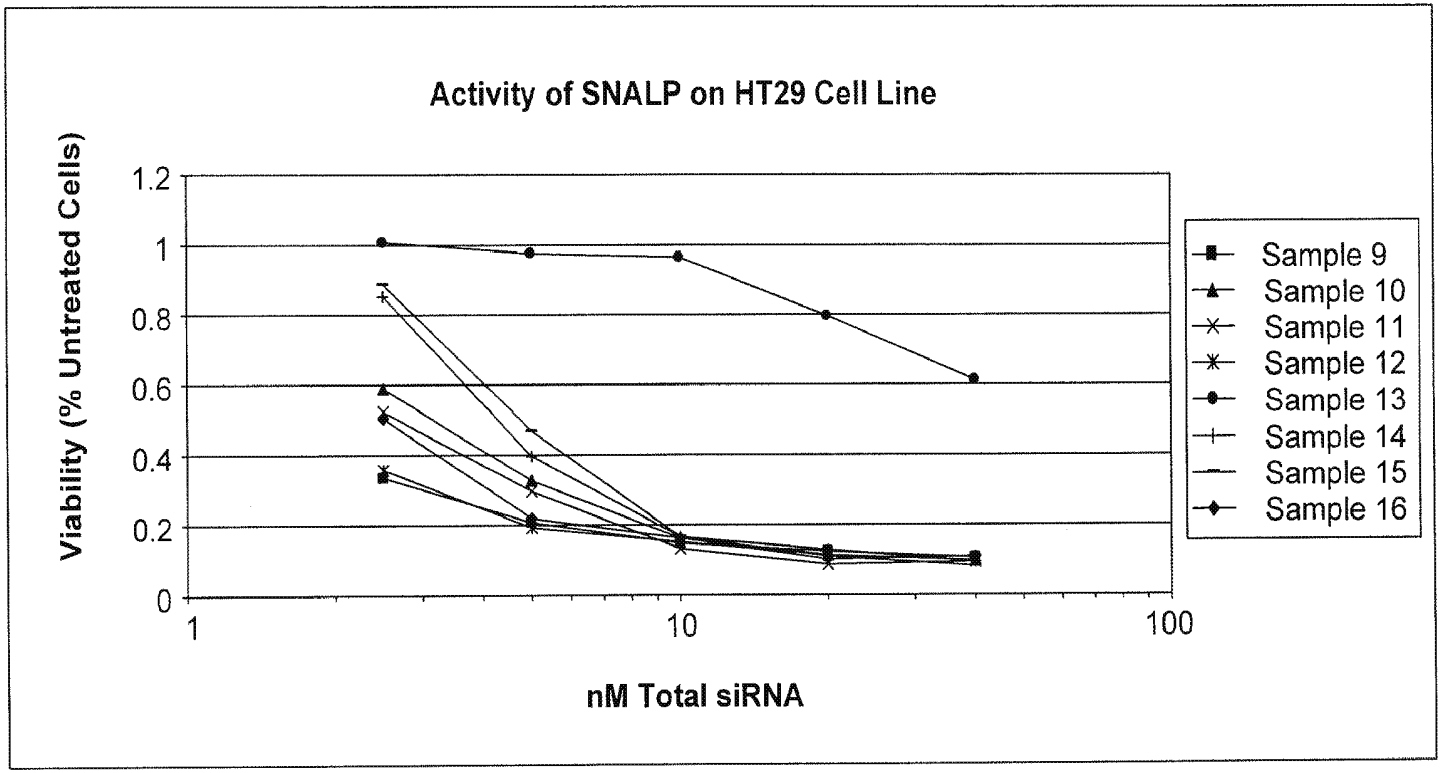


FIG. 1B

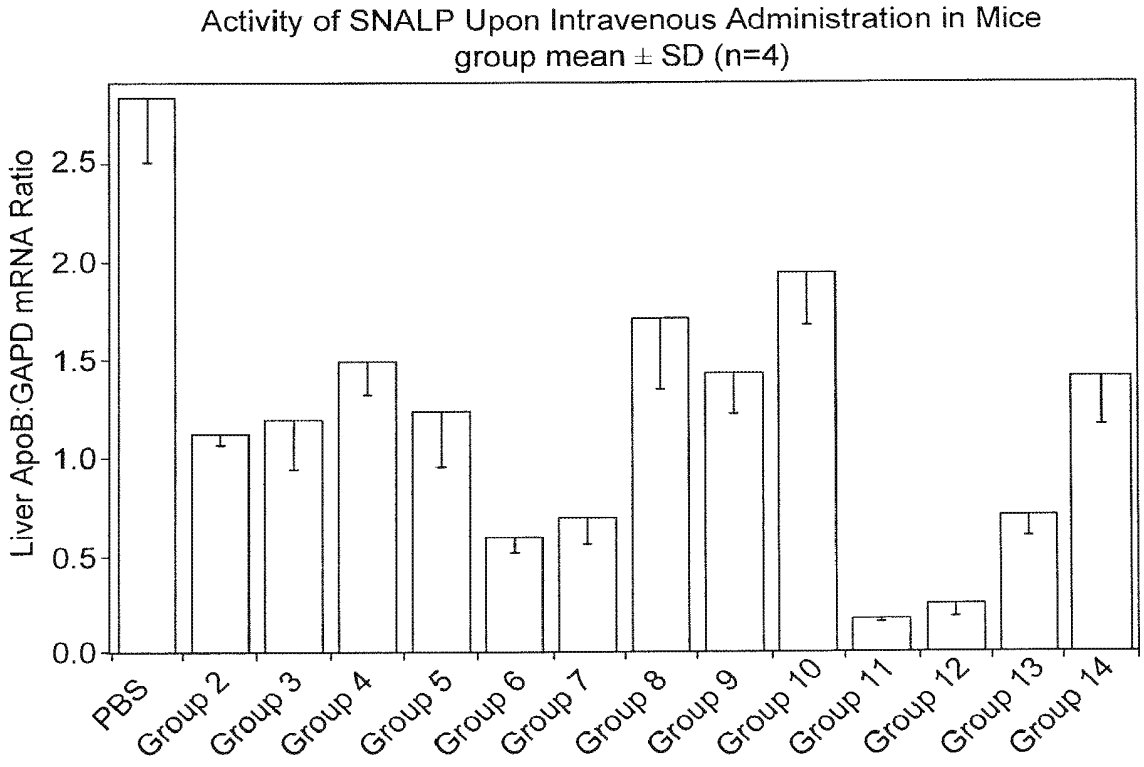

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
2.5
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

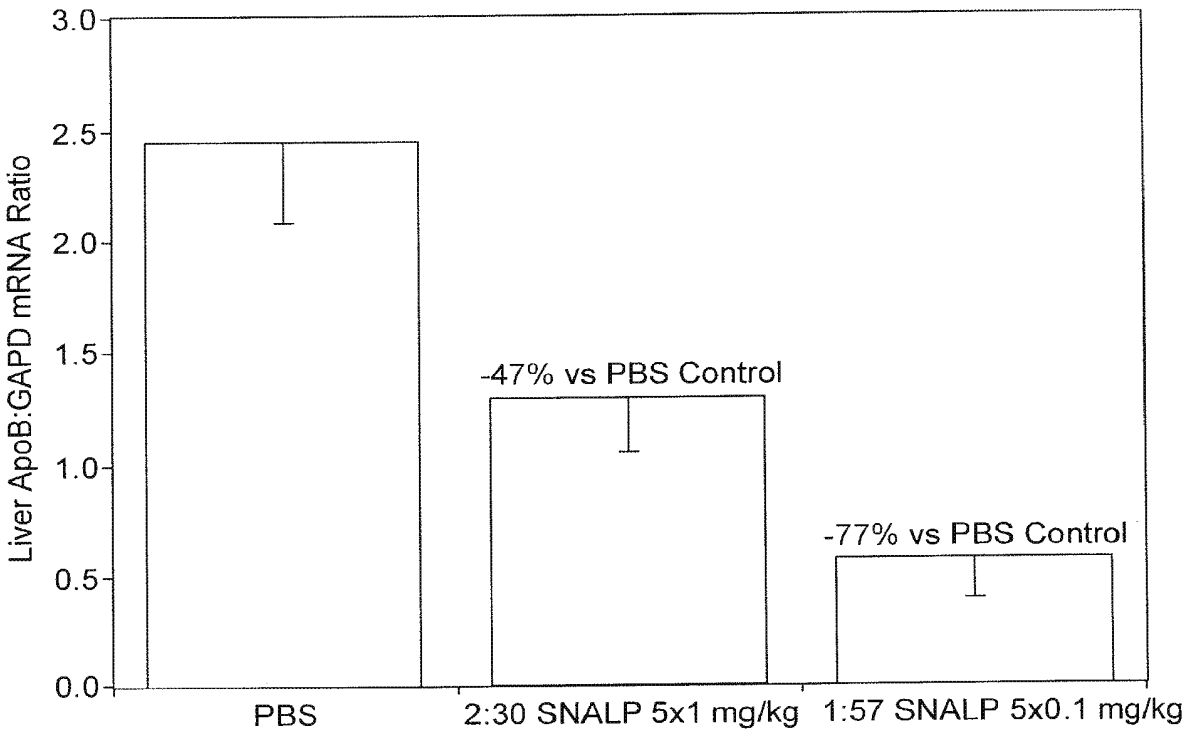

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Liver ApoB:GAPD mRNA Ratio
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5x1 mg/kg
1:57 SNALP 5x0.1 mg/kg

FIG. 3

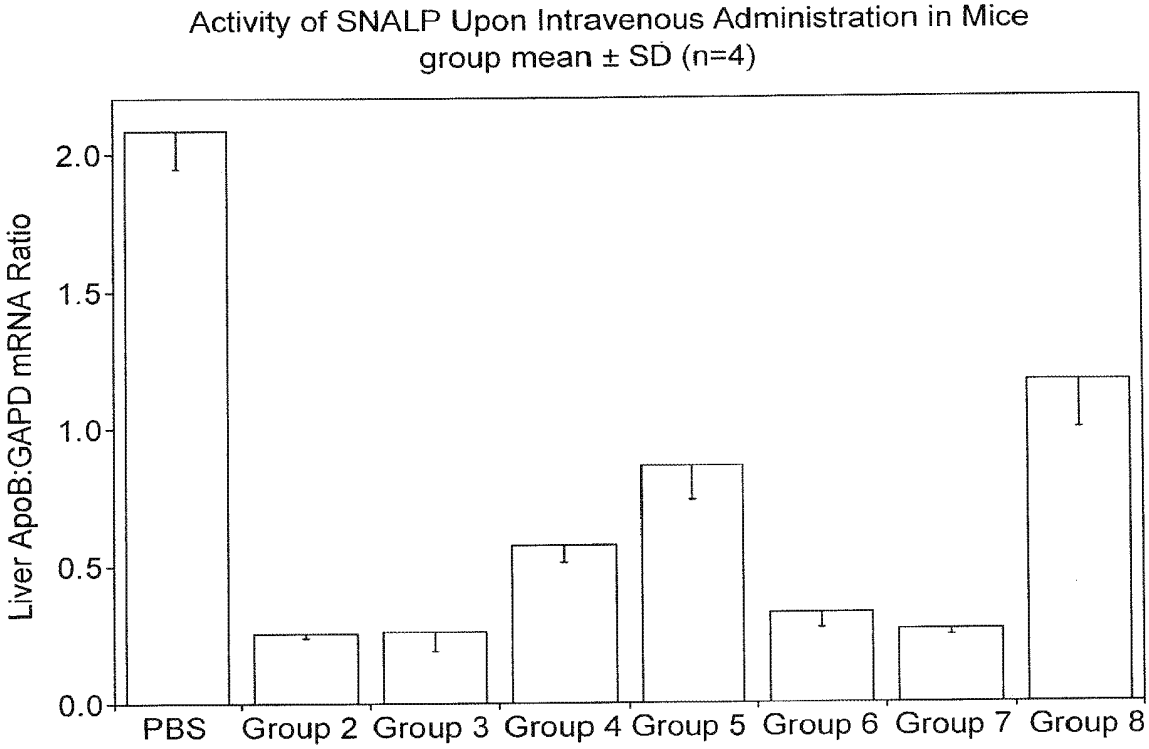

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

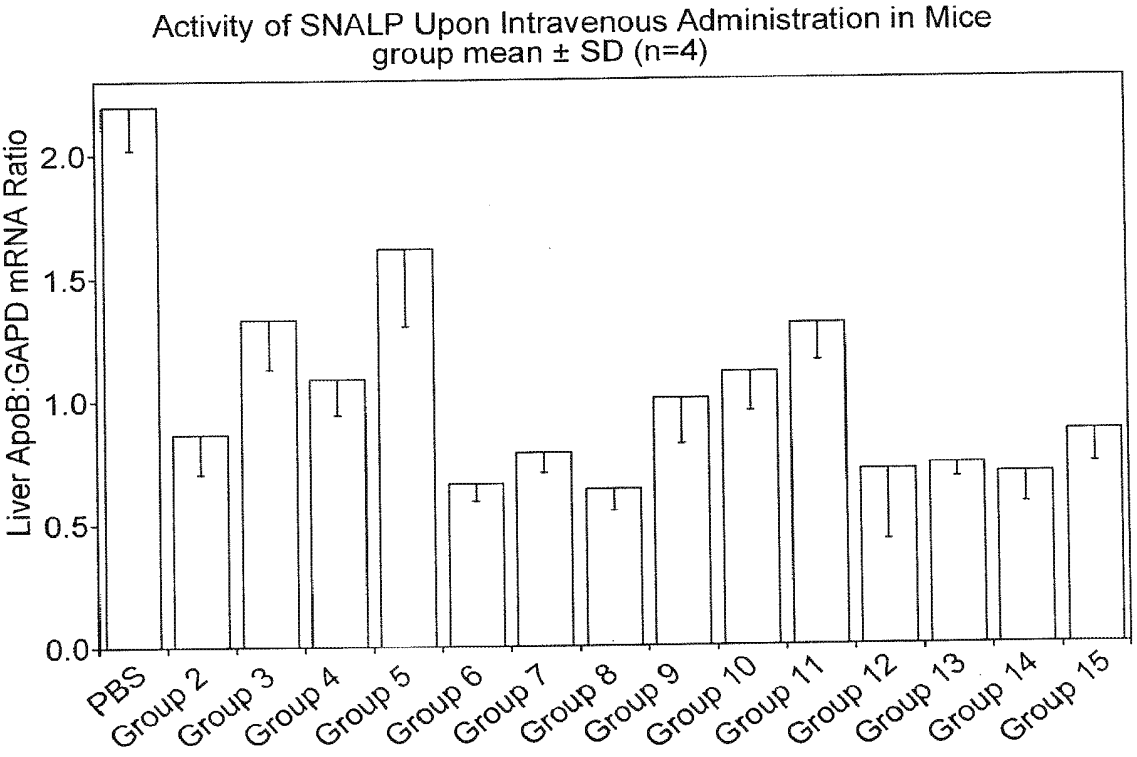

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

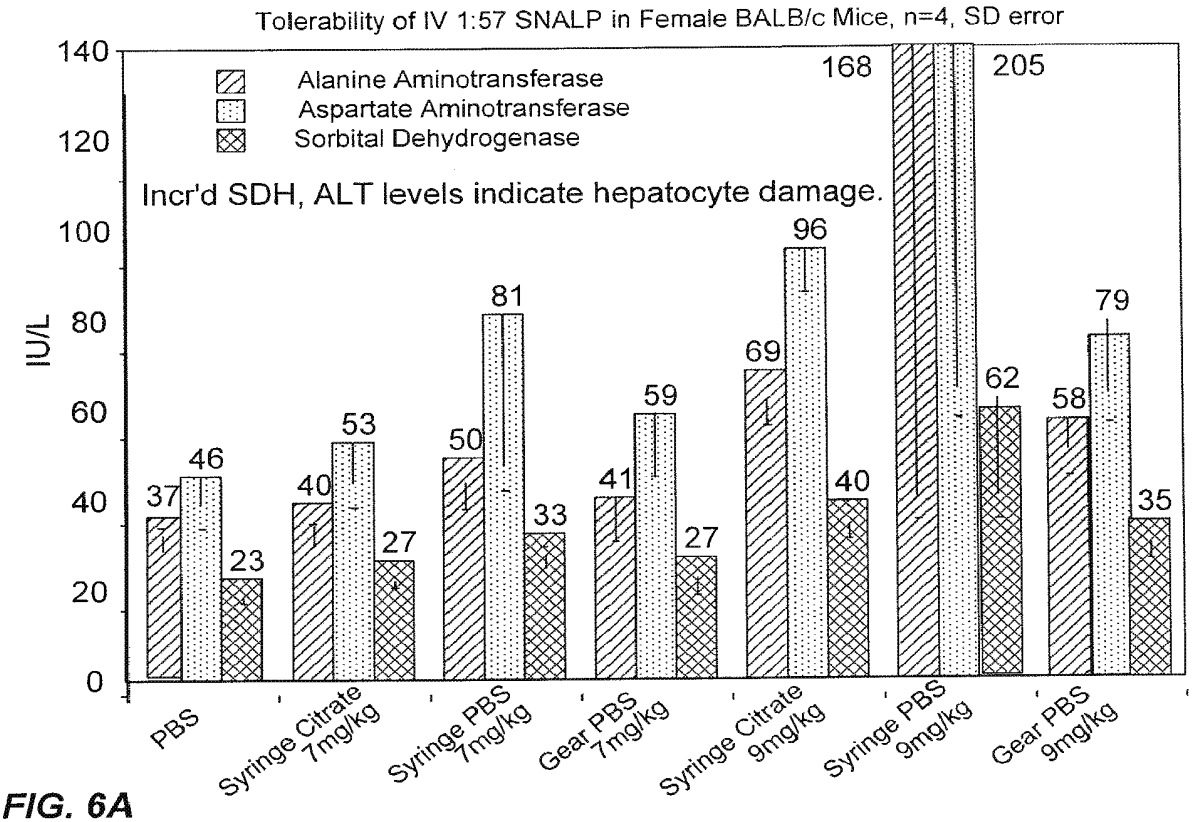

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
0
20
40
60
80
100
120
140
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
168
205
62
58
79
35
PBS
Syringe Citrate 7mg/kg
Syringe PBS 7mg/kg
Gear PBS 7mg/kg
Syringe Citrate 9mg/kg
Syringe PBS 9mg/kg
Gear PBS 9mg/kg

FIG. 6A

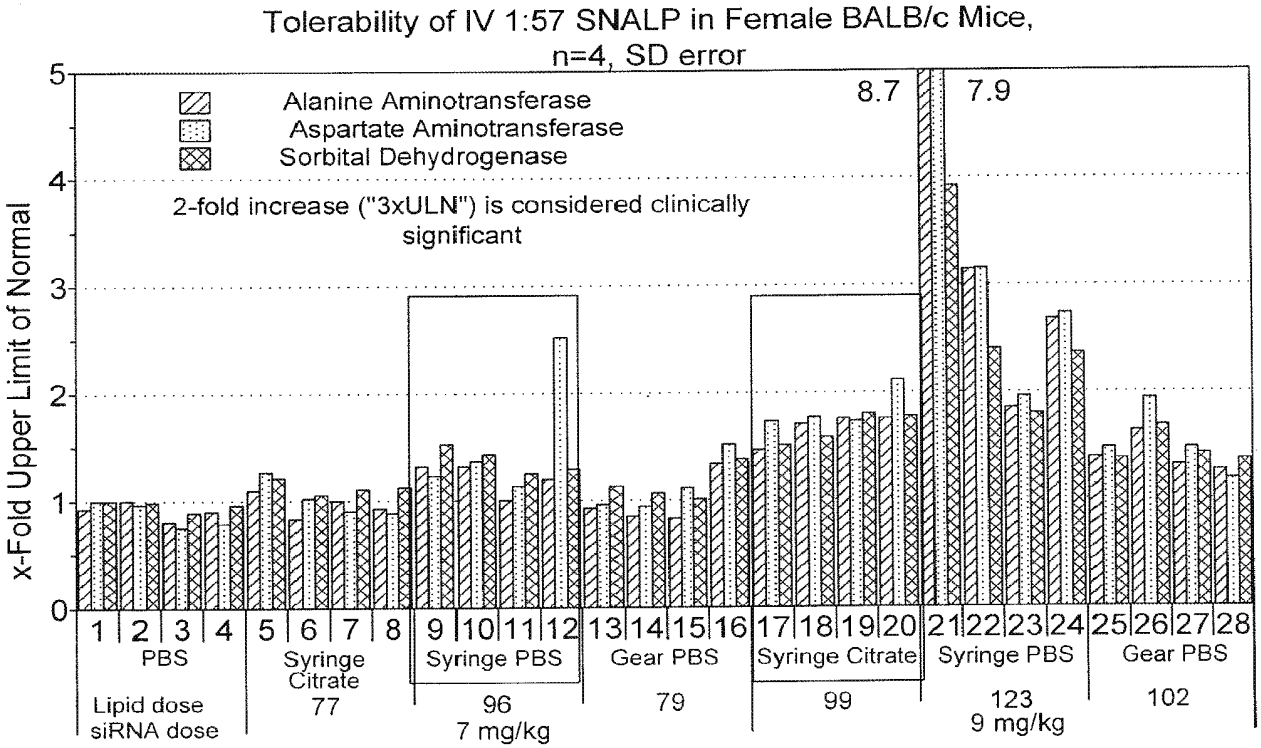

Tolerability of IV 1:57 SNALP in Female BALB/c Mice,
n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
1
2
3
4
5
8.7
7.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
PBS
Syringe Citrate
Syringe PBS
Gear PBS
Syringe Citrate
Syringe PBS
Gear PBS
Lipid dose
siRNA dose
77
96
7 mg/kg
79
99
123
9 mg/kg
102

FIG. 6B

FIG. 7A

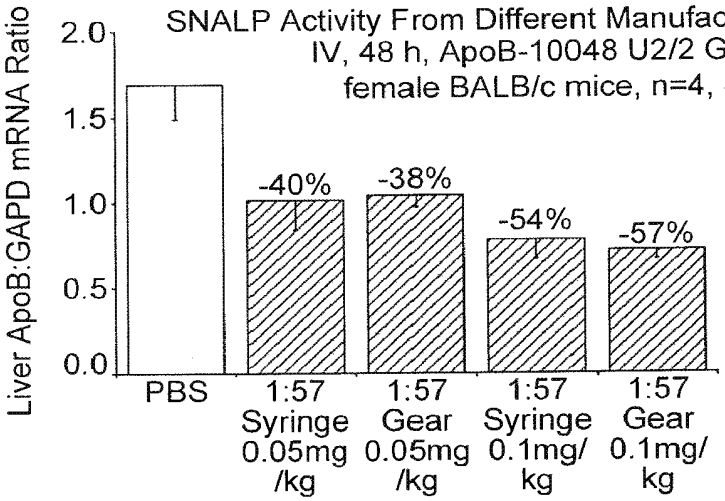


FIG. 7B

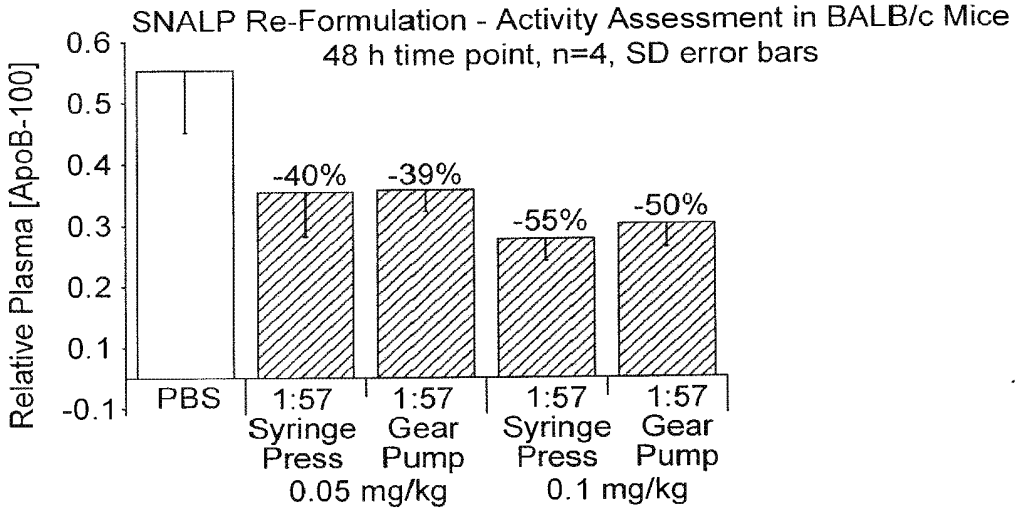
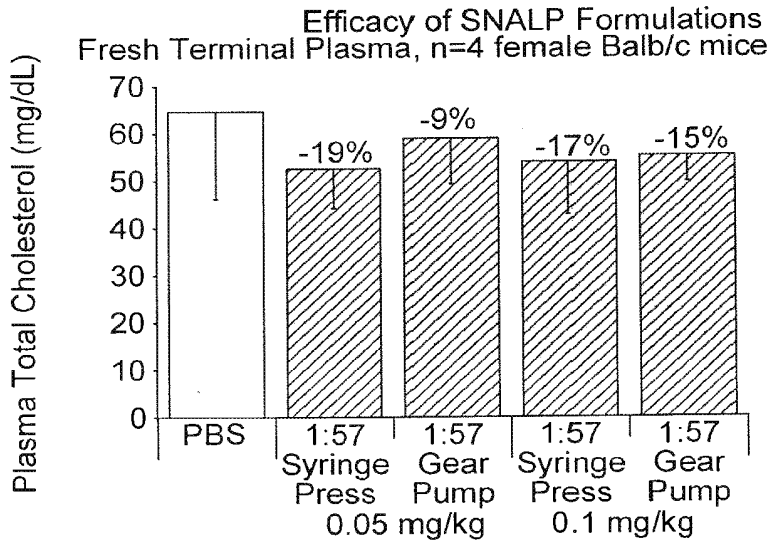


FIG. 7C



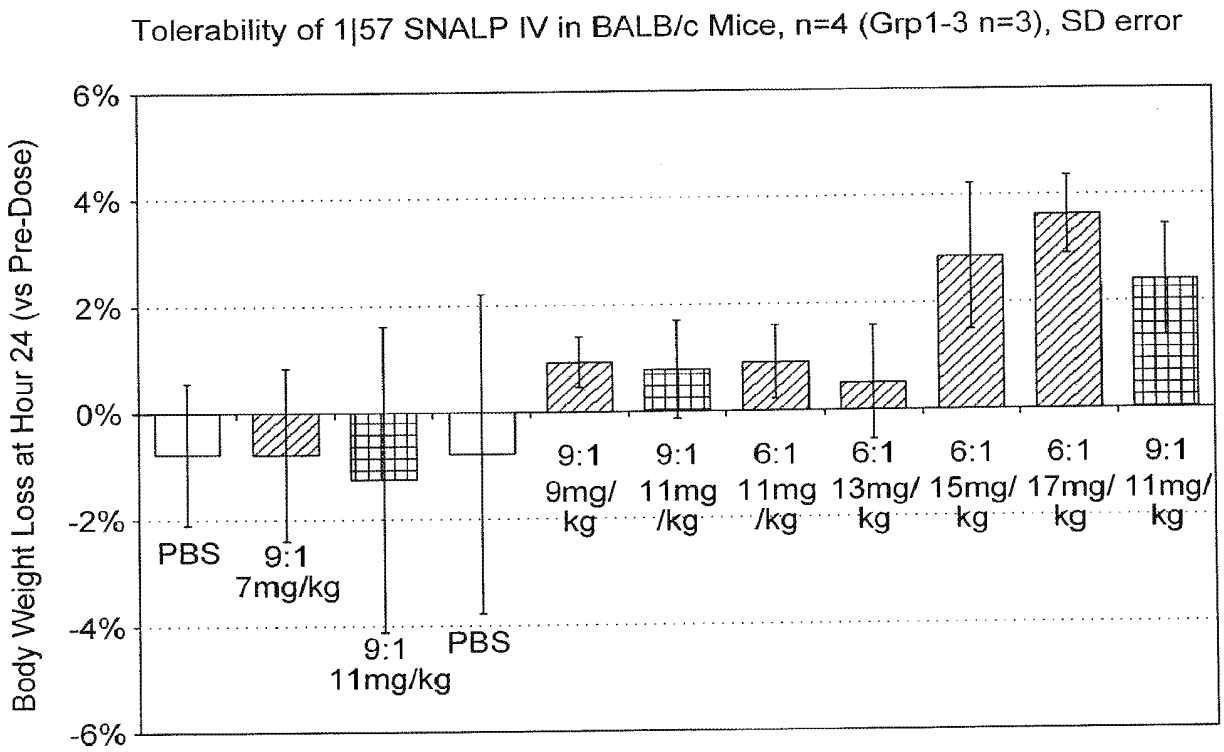

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

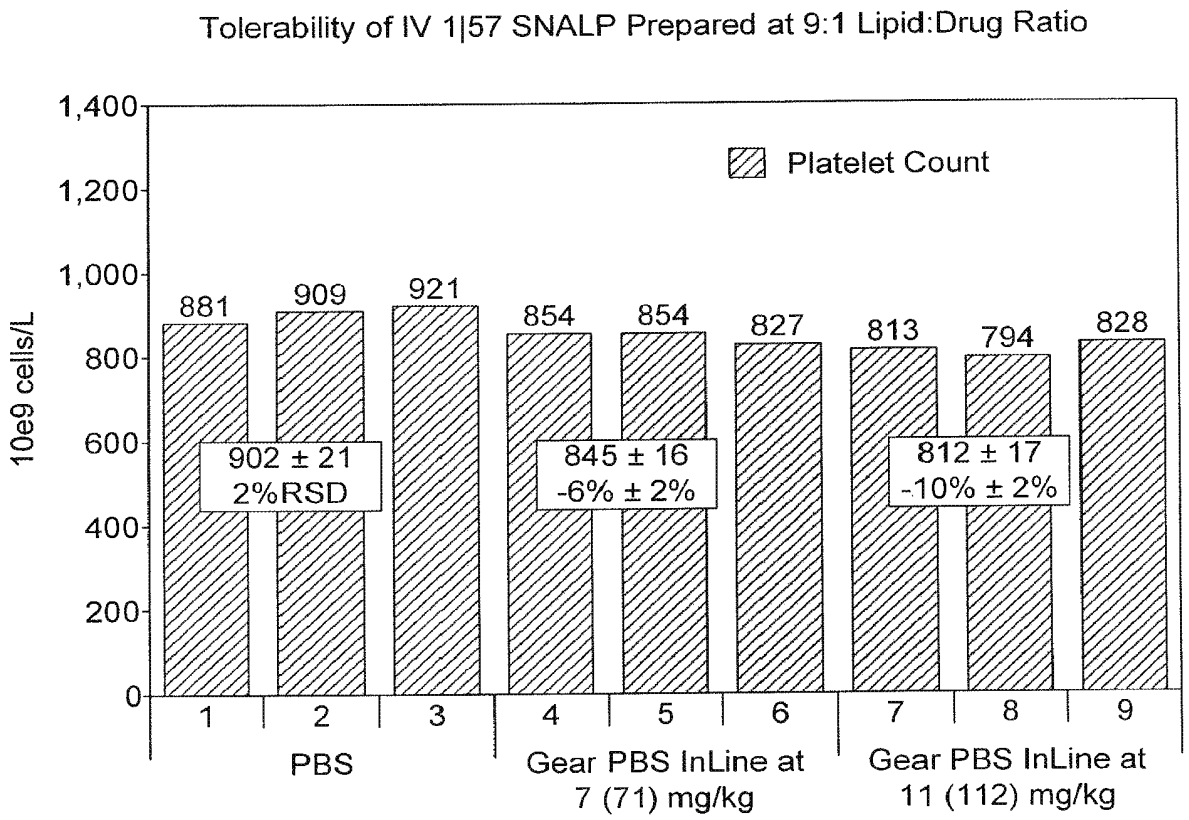

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
Platelet Count
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at
7 (71) mg/kg
Gear PBS InLine at
11 (112) mg/kg

FIG. 9

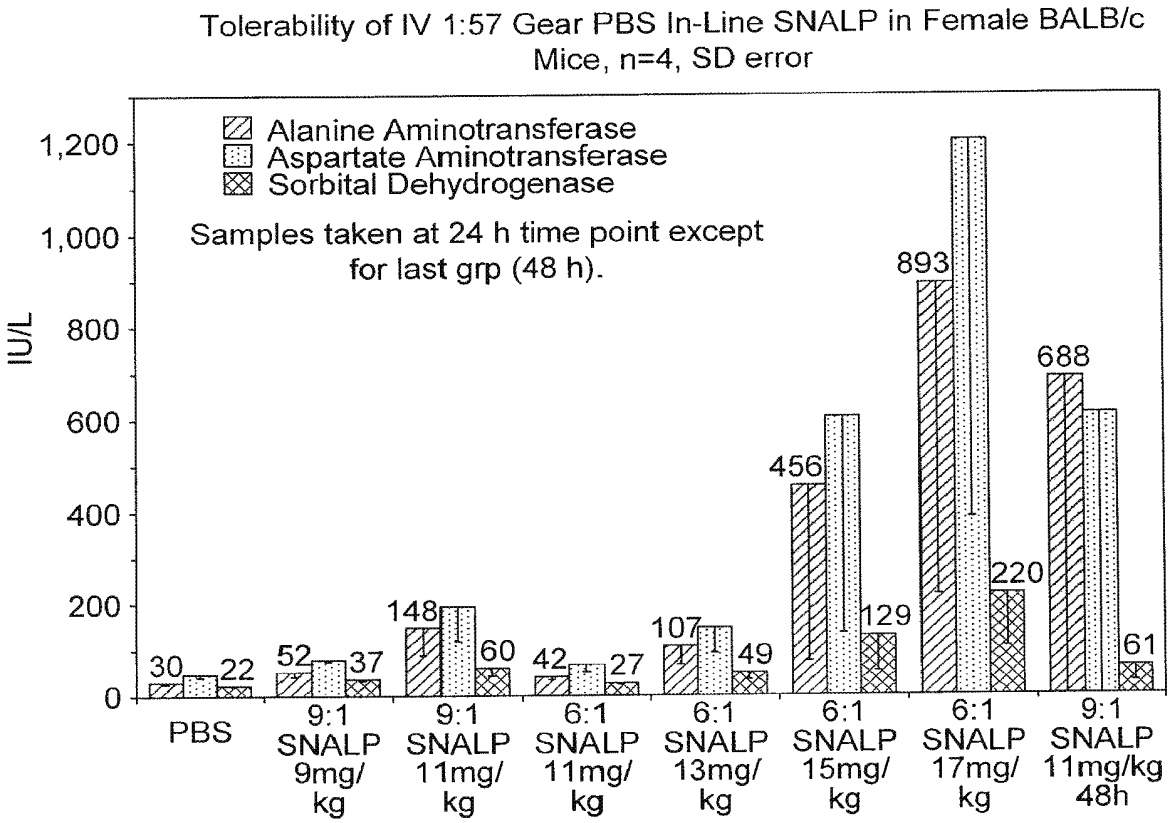

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
688
61
PBS
9:1 SNALP 9mg/kg
9:1 SNALP 11mg/kg
6:1 SNALP 11mg/kg
6:1 SNALP 13mg/kg
6:1 SNALP 15mg/kg
6:1 SNALP 17mg/kg
9:1 SNALP 11mg/kg 48h

FIG. 10A

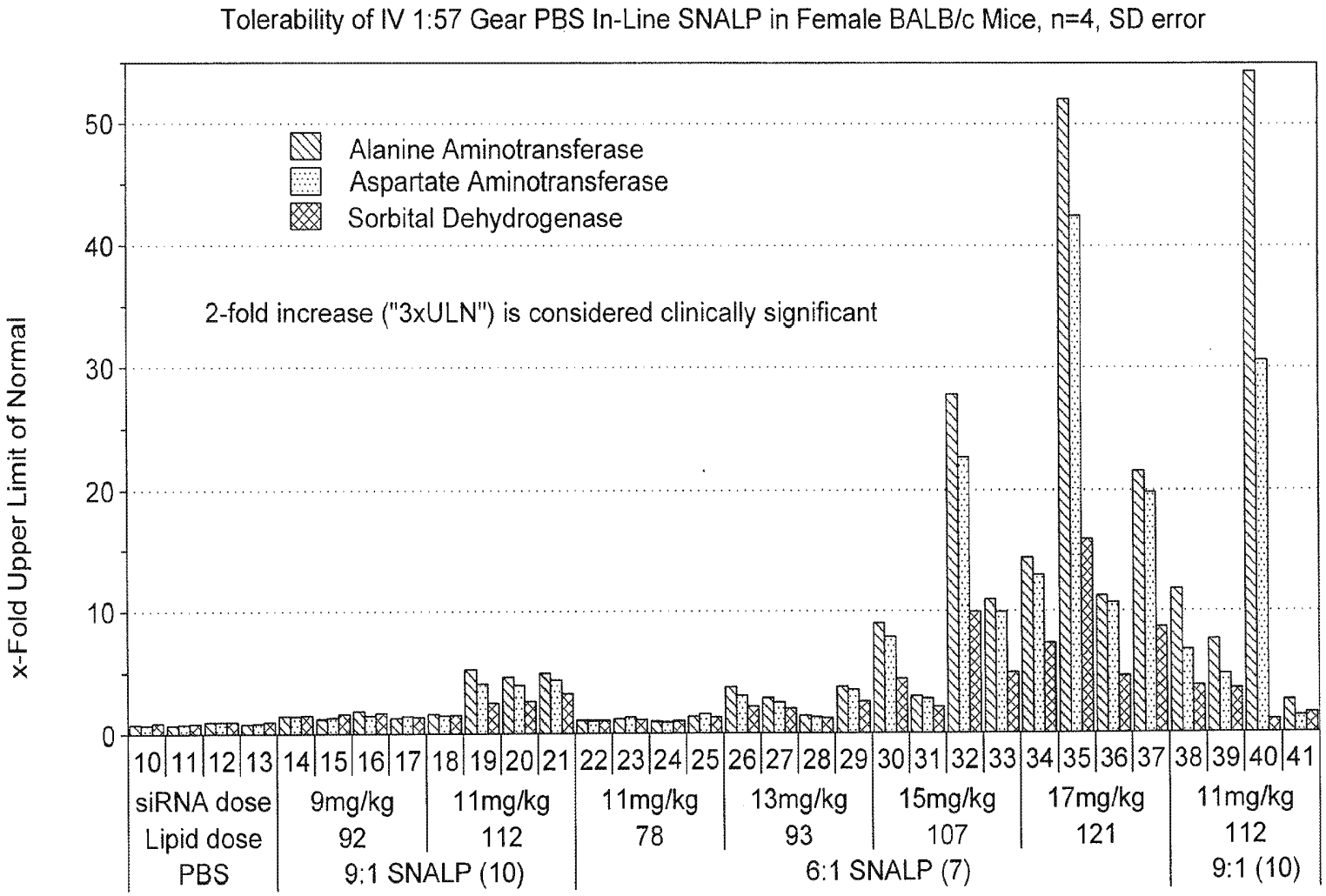

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
x-Fold Upper Limit of Normal
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
0
10
20
30
40
50
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
siRNA dose
Lipid dose
PBS
9mg/kg
92
9:1 SNALP (10)
11mg/kg
112
11mg/kg
78
13mg/kg
93
15mg/kg
107
6:1 SNALP (7)
17mg/kg
121
11mg/kg
112
9:1 (10)

FIG. 10B

FIG. 11A

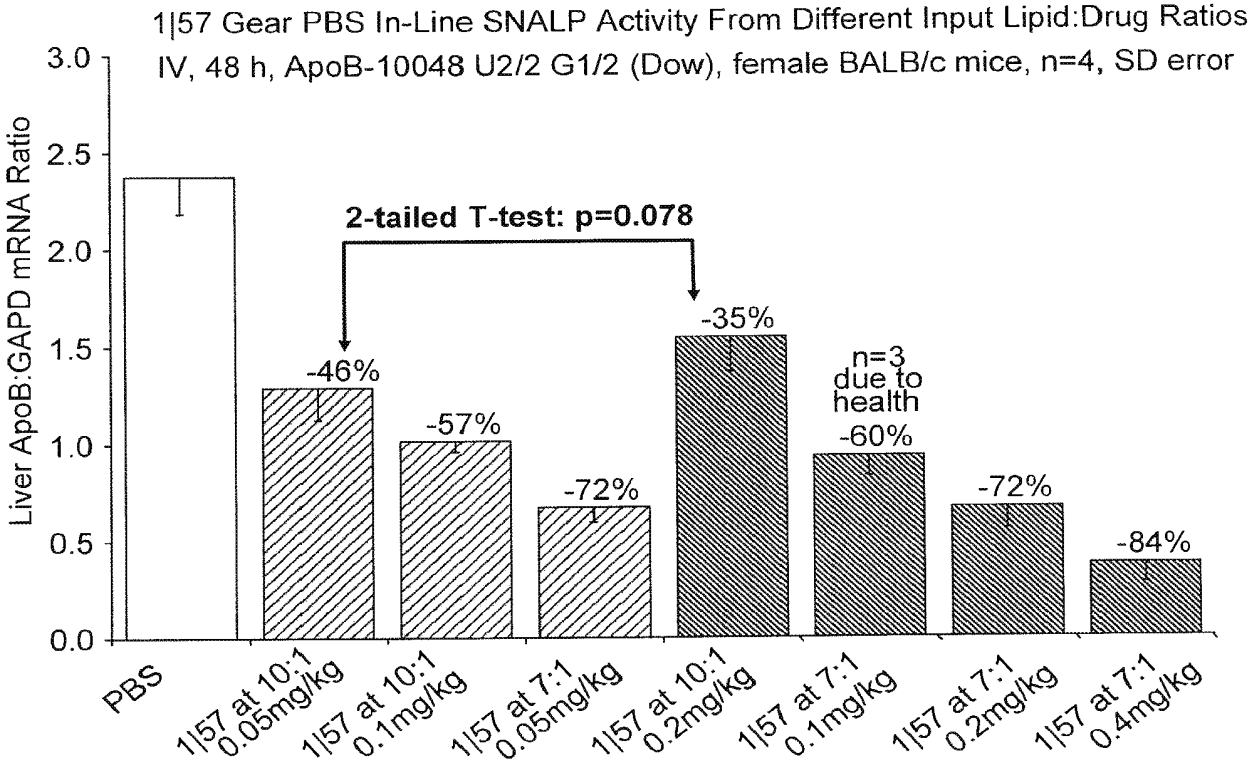
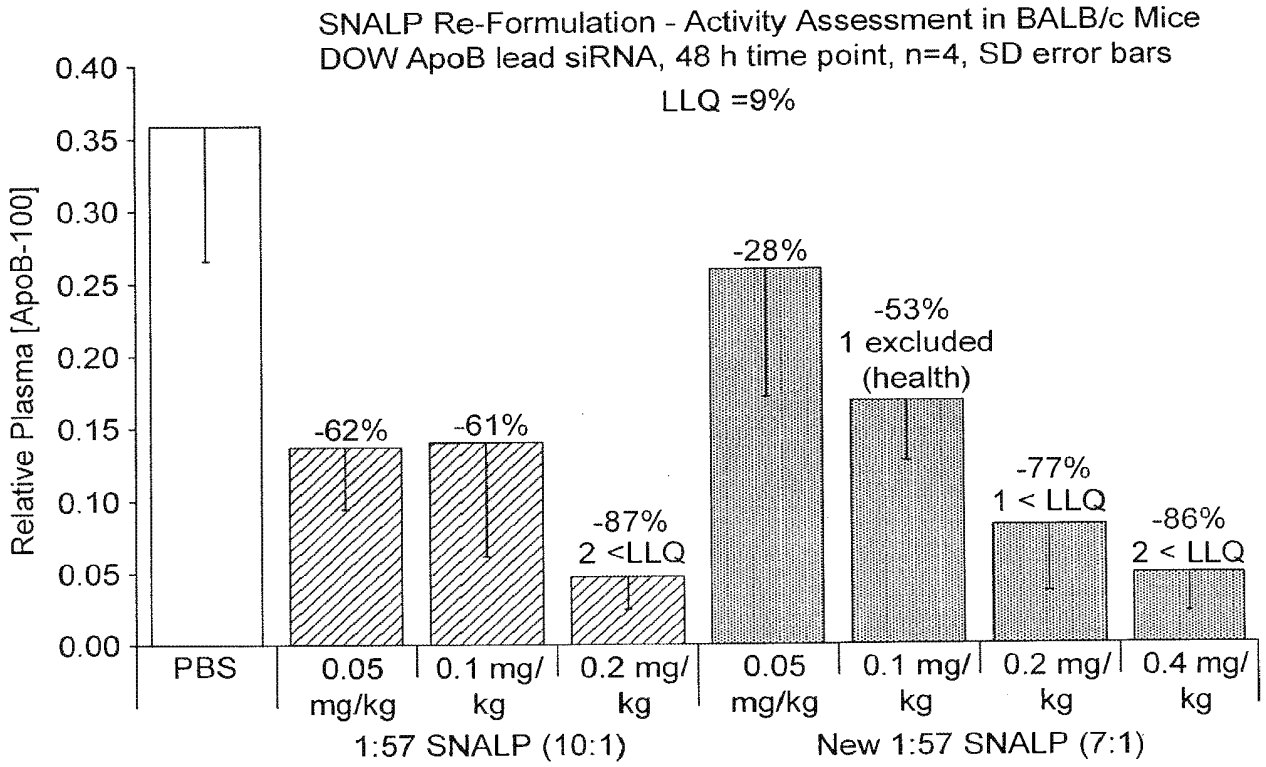


FIG. 11B



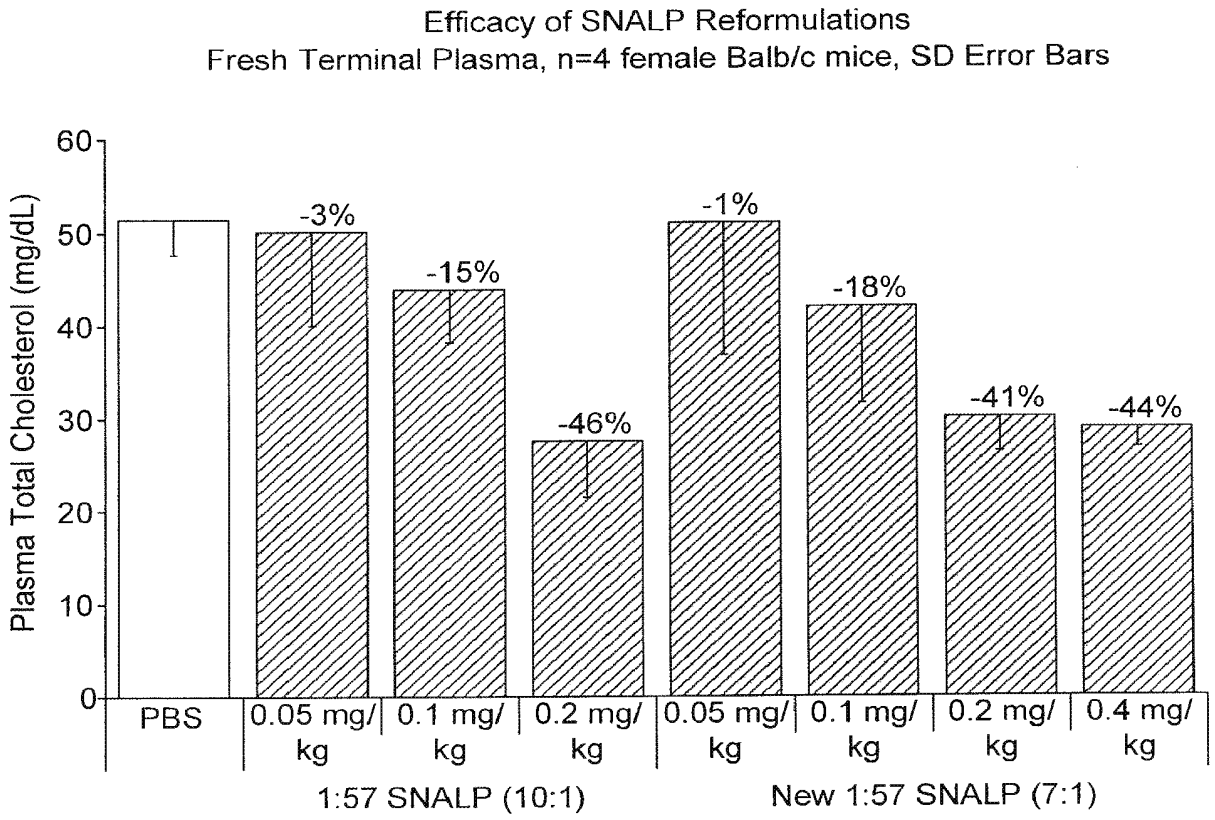

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 12

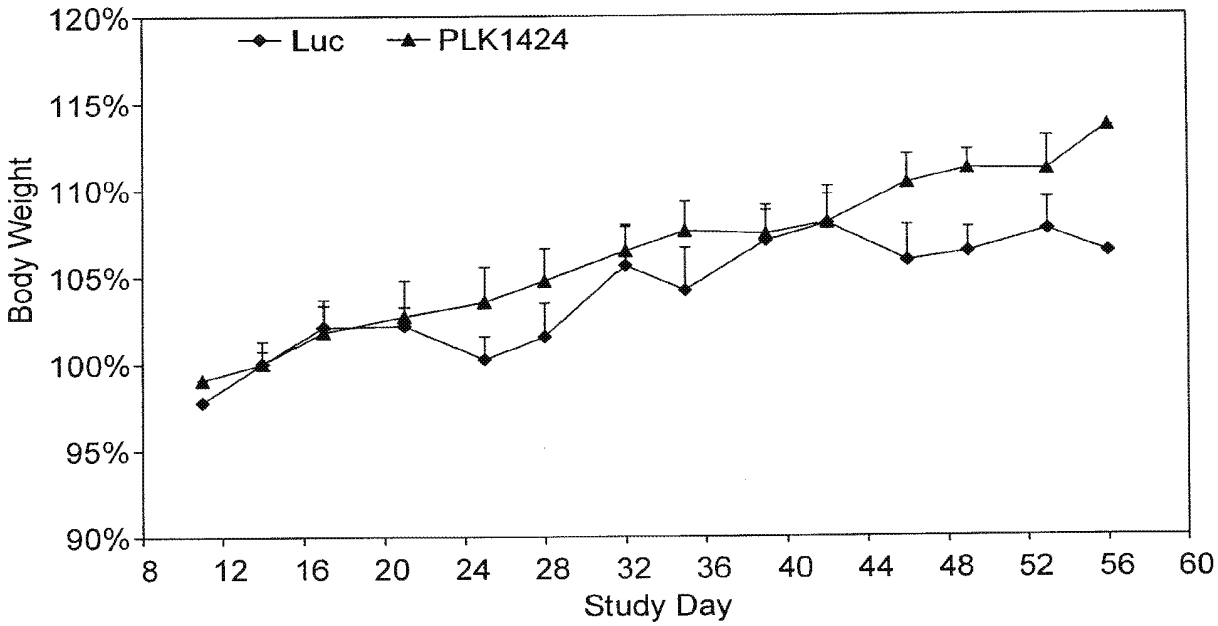

Luc
PLK1424
Body Weight
120%
115%
110%
105%
100%
95%
90%
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

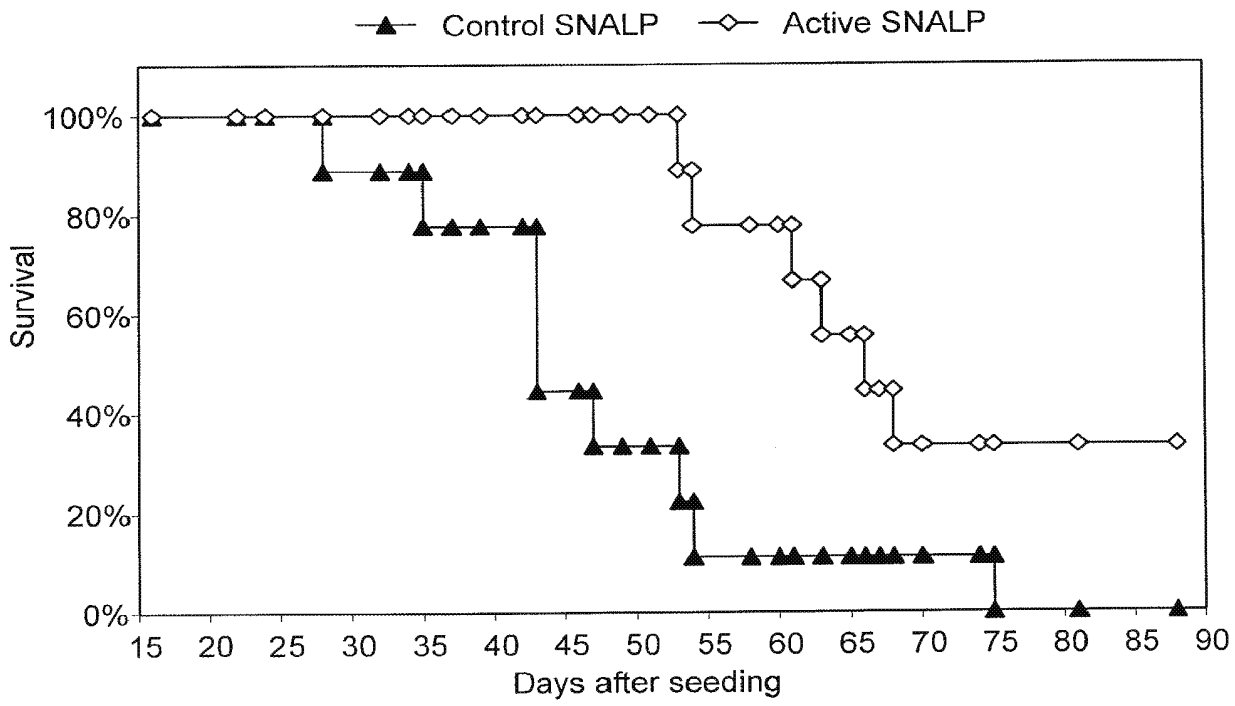

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
Days after seeding

FIG. 14

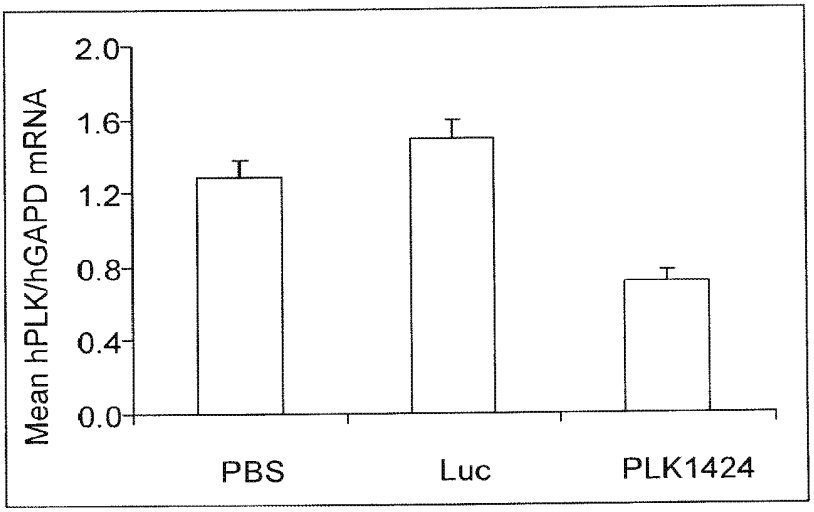


FIG. 15

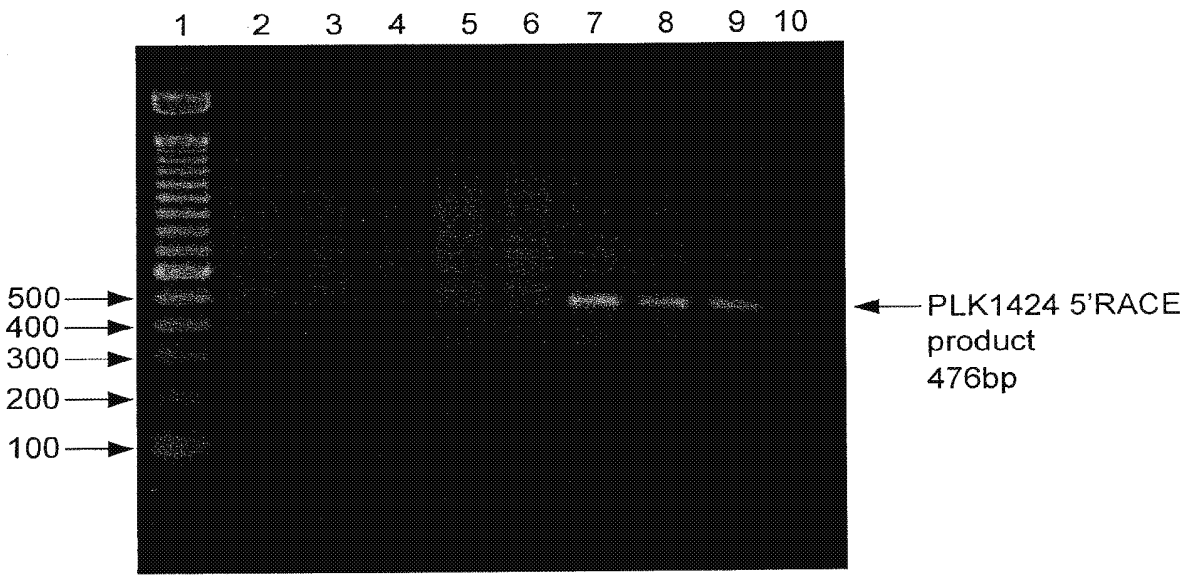
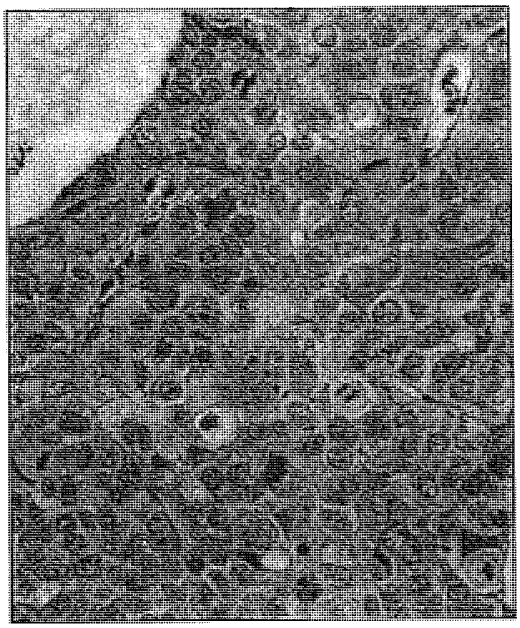
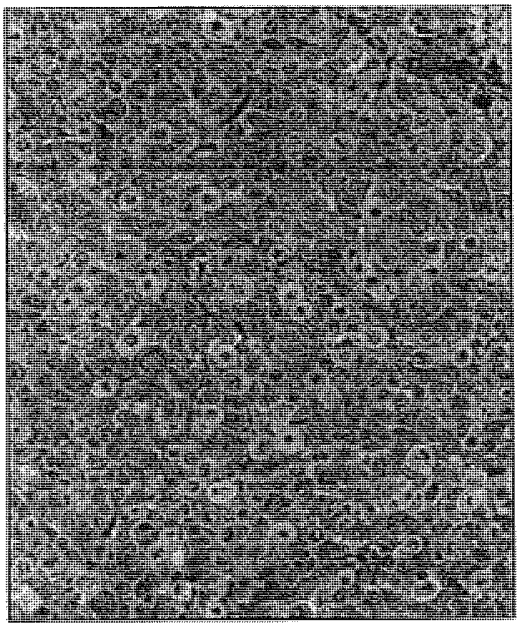

1 2 3 4 5 6 7 8 9 10
500
400
300
200
100
PLK1424 5'RACE
product
476bp

FIG. 16

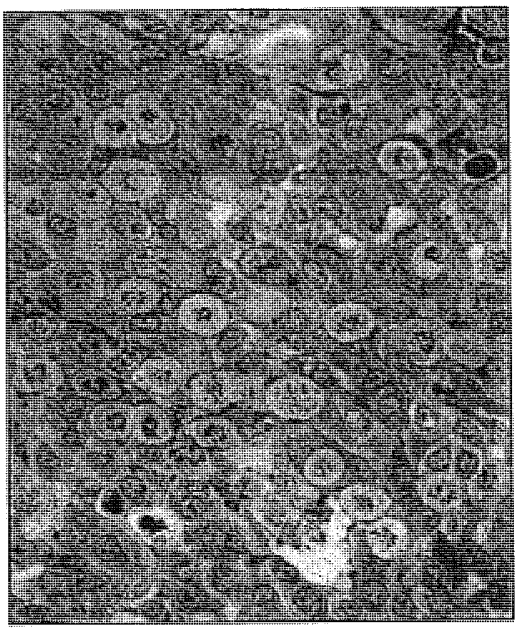
x200 mag



x400 mag



x200 mag



x400 mag

FIG. 17

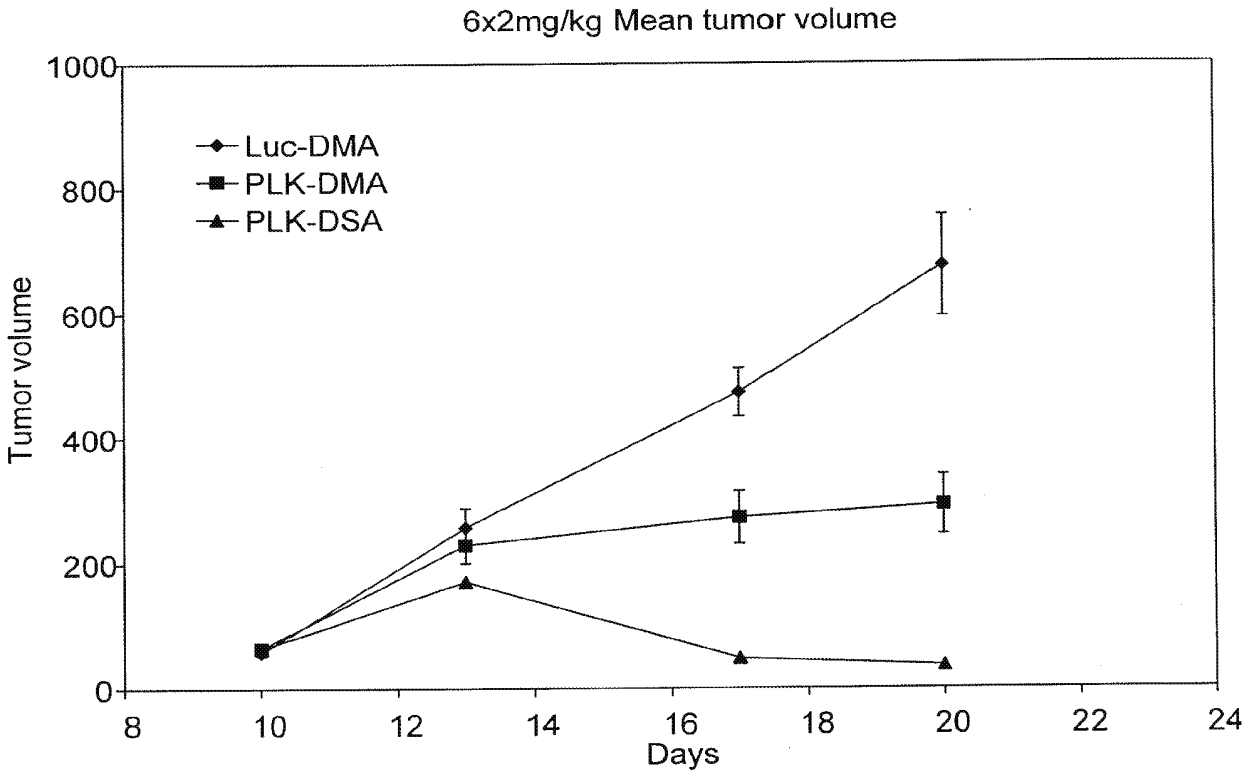

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

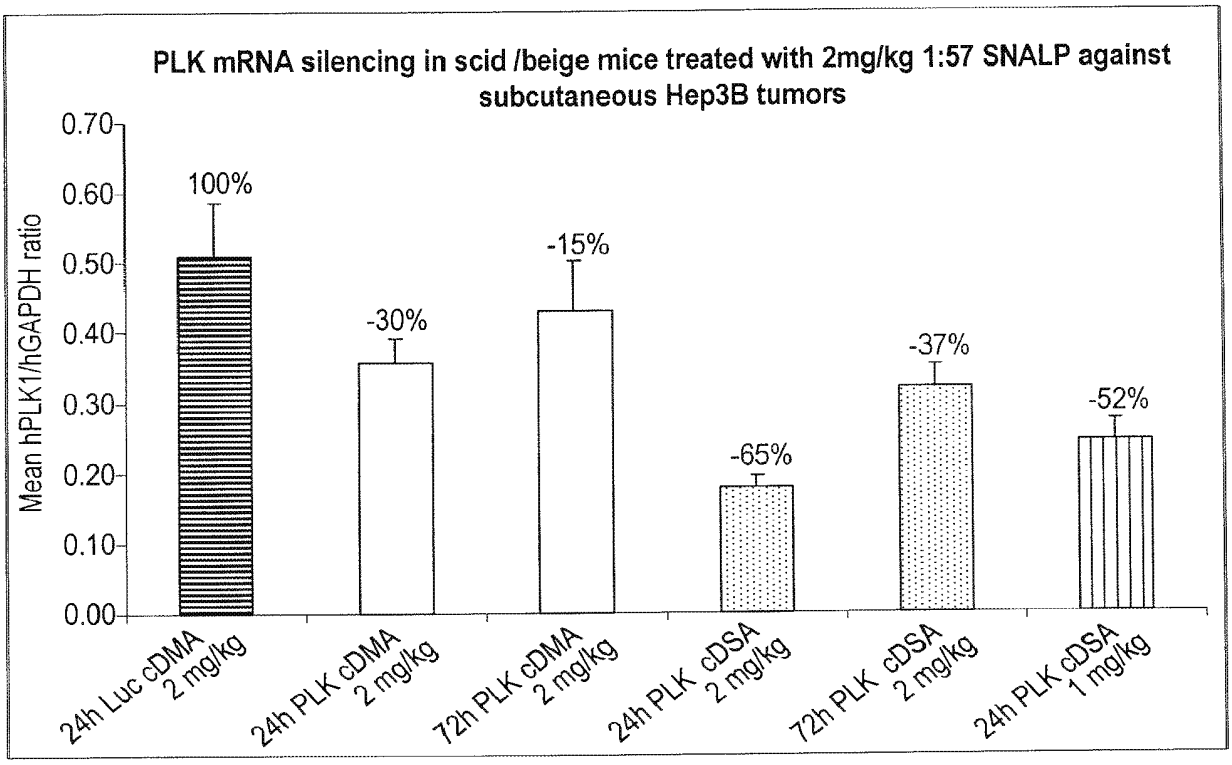

PLK mRNA silencing in scid /beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

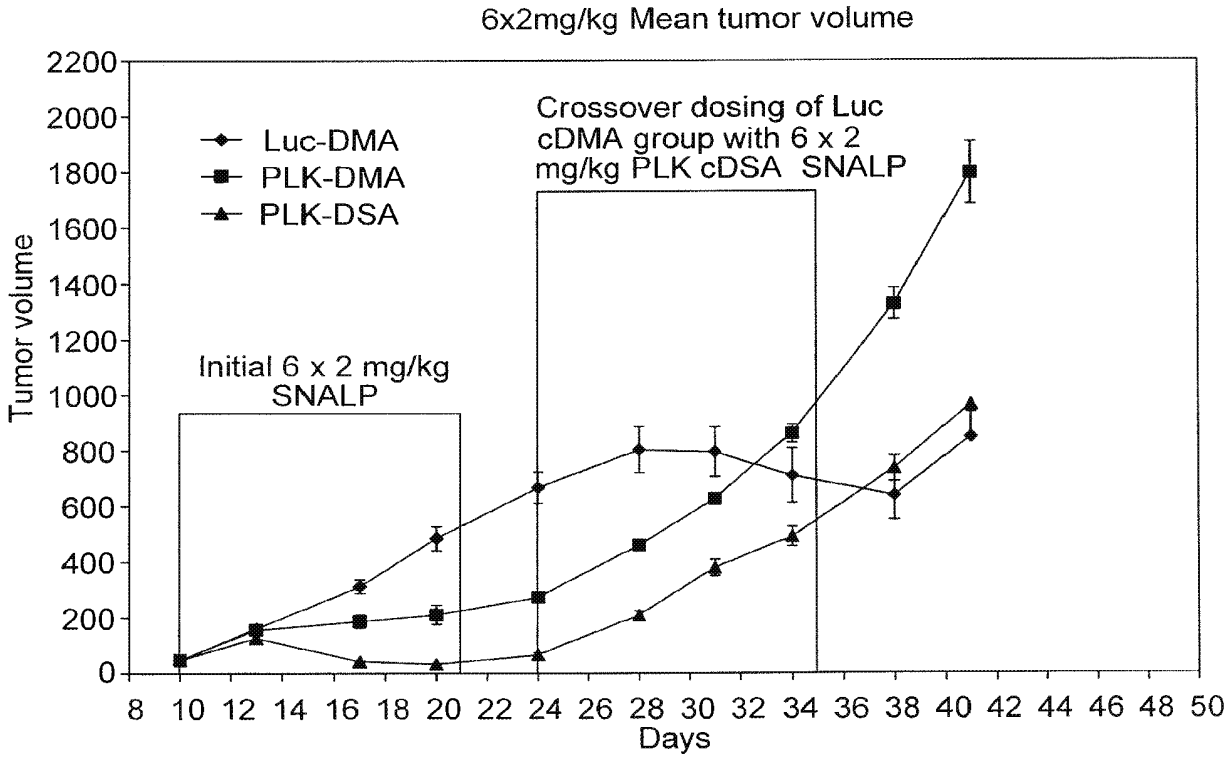

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Initial 6 x 2 mg/kg SNALP
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

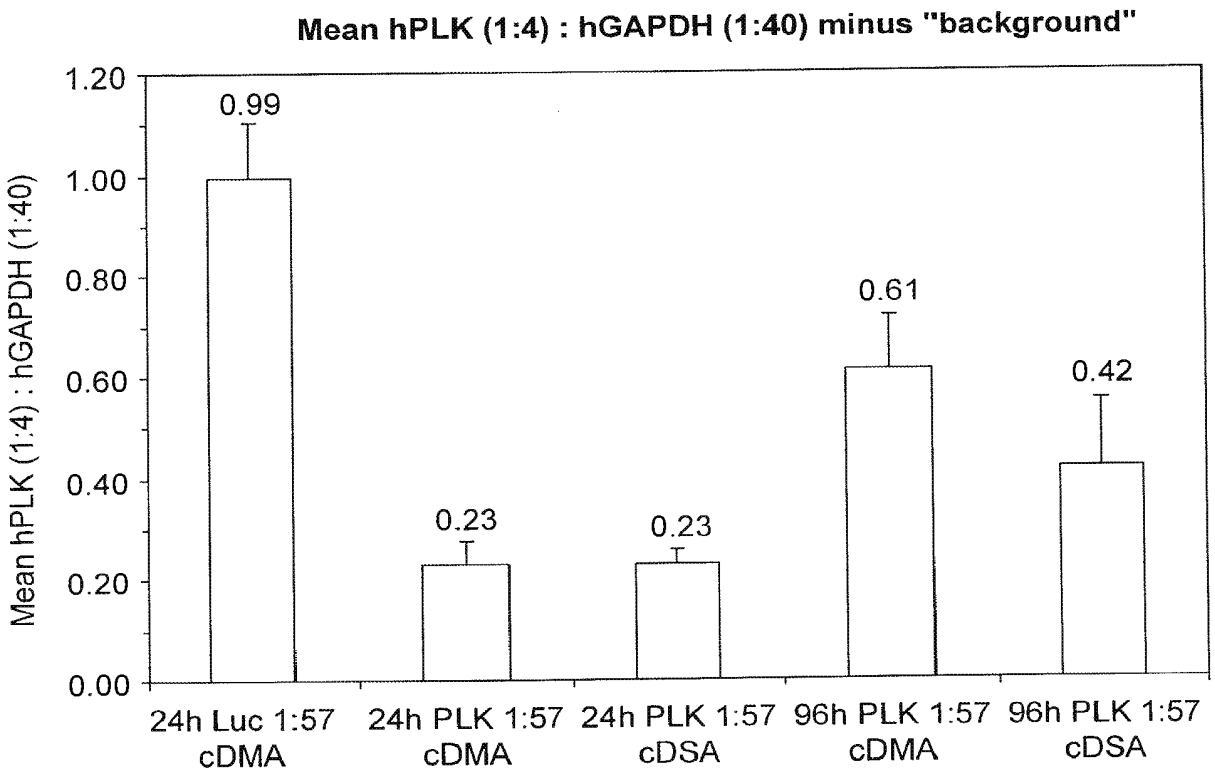

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

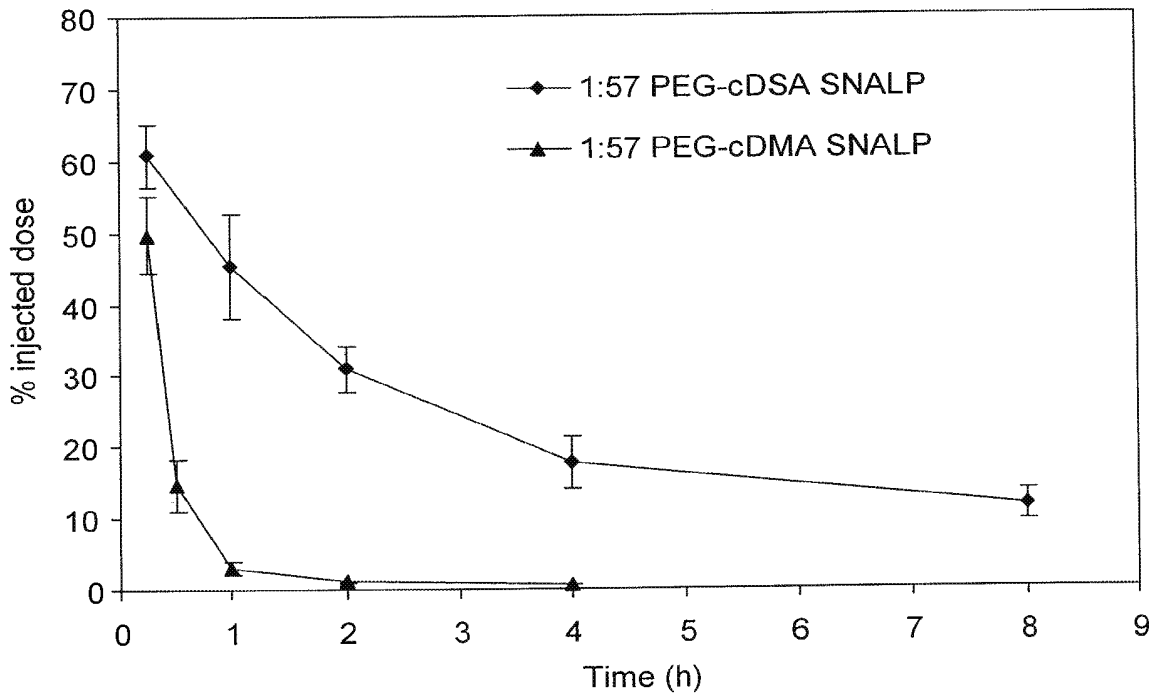

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

SEQUENCE LISTING

<110> Yaworski, Edward
Lam, Kieu
Jeffs, Lloyd
Palmer, Lorne
MacLachlan, Ian

<120> Novel Lipid Formulations for Nucleic Acid Delivery

<130> 020801-007710US

<140> US 12/424,367
<141> 2009-04-15

<150> US 61/045,228
<151> 2008-04-15

<160> 7

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic E5 kinesin-related protein 2263 U/U siRNA sense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic E5 kinesin-related protein 2263 U/U siRNA sense strand of siRNA duplex

<221> modified_base
<222> (2)...(19)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT)

<400> 1
cngaagaccn gaagacaann n 21

<210> 2
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic E5 kinesin-related protein 2263 U/U siRNA antisense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic E5 kinesin-related protein 2263 U/U siRNA antisense strand of siRNA duplex

<221> modified_base
<222> (8)...(16)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT)

<400> 2
auugucunca ggncuncagn n 21

<210> 3
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic apolipoprotein B (ApoB) 10048 U2/2 G1/2 siRNA sense strand of siRNA duplex

<221> modified_base
<222> (4)...(4)
<223> n = gm

<221> modified_base
<222> (8)...(14)
<223> n = um

<400> 3
agunucanca cacngaauac c 21

<210> 4
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic apolipoprotein B (ApoB) 10048 U2/2 G1/2 siRNA antisense strand of siRNA duplex

<221> modified_base
<222> (4)...(4)
<223> n = um

<221> modified_base
<222> (7)...(9)
<223> n = gm

<221> modified_base
<222> (19)...(19)
<223> n = um

<400> 4
uauncanunu gaugacacnu g 21

<210> 5
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/GU and PLK1424 U4/G siRNA sense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/GU and PLK1424 U4/G siRNA sense strand of siRNA duplex

<221> modified_base
<222> (4)...(18)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT), u or a ribonucleotide complementary to target complementary sequence

<400> 5
agancacccn ccunaaanan n 21

<210> 6
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/GU siRNA antisense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/GU siRNA antisense strand of siRNA duplex

<221> modified_base
<222> (8)...(8)
<223> n = gm

<221> modified_base
<222> (17)...(17)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT), u or a ribonucleotide complementary to target sequence

<400> 6
uauuuaanga gggugancun n 21

<210> 7
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/G siRNA antisense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/G siRNA antisense strand of siRNA duplex

<221> modified_base
<222> (9)...(15)
<223> n = gm

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT), u or a ribonucleotide complementary to target sequence

<400> 7
uauuuaagna gngunaucun n 21

JOINT APPENDIX 60

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on March 28, 2012.

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Judith Cotham/

PATENT

Attorney Docket No.: 86399-007720US-802191

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

MacLACHLAN *et al.*

Application No.: 13/253,917

Filed: October 5, 2011

For: NOVEL LIPID FORMULATIONS FOR NULEIC ACID DELIVERY

Customer No.: 20350

Confirmation No. 4479

Examiner: Not yet assigned

Art Unit: 1653

COMMUNICATION UNDER

37 C.F.R. §§ 1.821-1.825

AND

PRELIMINARY AMENDMENT

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the request to comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, 37 C.F.R. §§ 1.821-1.825, that accompanied the Notice to File Missing Parts of Nonprovisional Application mailed October 28, 2011, Applicants submit herewith the required computer readable copy of the Substitute Sequence Listing. Please amend the specification as follows.

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims which begins on page 4 of this paper.

Remarks begin on page 7 of this paper.

Amendments to the Specification:

Please replace paragraph **[0001]** beginning at page 1, line 3, with the following:

[0001] The present application is a continuation of U.S. Application No. 12/424,367, filed April 15, 2009, now U.S. Patent No. 8,058,069, which application claims priority to U.S. Provisional Application No. 61/045,228, filed April 15, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

Please replace paragraph **[0004]** beginning at page 1, line 12, with the following:

REFERENCE TO A "SEQUENCE LISTING[["]]," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

[0004] ~~Not applicable.~~ The Sequence Listing written in file -77-2.TXT, created on March 13, 2012, 8,192 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference in its entirety for all purposes.

Please replace Table 1 beginning at page 92, line 14, with the following:

Table 1. siRNA duplex comprising sense and antisense Eg5 RNA polynucleotides.

Modification	Eg5 2263 siRNA sequence	SEQ ID NO:	% 2'OMe-Modified	% Modified in DS Region
U/U	5'-C**U**GAAGACC**U**GAAGACAA**U**dTdT-3' 3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUU**A**-5'	1 2	6/42 = 14.3%	6/38 = 15.8%

Column 1: "U/U" = 2'OMe-uridine modified siRNA duplex; Column 2: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. "dT" = deoxythymidine. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

Please replace Table 3 beginning at page 94, line 7, with the following:

Table 3. siRNA duplex comprising sense and antisense ApoB RNA polynucleotides.

Position	Modification	ApoB siRNA sequence	SEQ ID NO:	% 2'OMe-Modified	% Modified in DS Region
10048	U2/2 G1/2	5'-AGU**G**UCA**U**CACAC**U**GAAUACC-3' 3'-GU**U**CACAGUAGU**G**U**G**AC**U**UAU-5'	3 4	7/42 = 16.7%	7/38 = 18.4%

Column 1: The number refers to the nucleotide position of the 5' base of the sense strand relative to the mouse ApoB mRNA sequence XM_137955. Column 2: The numbers refer to the distribution of 2'OMe chemical modifications in each strand. Column 3: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. Column 4: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 5: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

Please cancel the present "SEQUENCE LISTING", pages 1 through 4, submitted October 5, 2011, and insert therefor the accompanying paper copy of the Substitute Sequence Listing, page numbers 1 to 4, at the end of the application.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-46. Canceled

47. (New) A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from 50 mol % to 65 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the phospholipid comprises from 3 mol % to 15 mol % of the total lipid present in the particle and the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle.

48. (New) The nucleic acid-lipid particle of claim 47, wherein the nucleic acid comprises a small interfering RNA (siRNA).

49. (New) The nucleic acid-lipid particle of claim 48, wherein the siRNA comprises at least one modified nucleotide.

50. (New) The nucleic acid-lipid particle of claim 48, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

51. (New) The nucleic acid-lipid particle of claim 48, wherein the siRNA is about 19 to about 25 base pairs in length.

52. (New) The nucleic acid-lipid particle of claim 48, wherein the siRNA comprises 3' overhangs.

53. (New) The nucleic acid-lipid particle of claim 47, wherein the cationic lipid comprises from 50 mol % to 60 mol % of the total lipid present in the particle.

54. (New) The nucleic acid-lipid particle of claim 47, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

55. (New) The nucleic acid-lipid particle of claim 47, wherein the phospholipid comprises from 4 mol % to 15 mol % of the total lipid present in the particle.

56. (New) The nucleic acid-lipid particle of claim 47, wherein the phospholipid comprises from 4 mol % to 12 mol % of the total lipid present in the particle.

57. (New) The nucleic acid-lipid particle of claim 47, wherein the phospholipid comprises from 5 mol % to 12 mol % of the total lipid present in the particle.

58. (New) The nucleic acid-lipid particle of claim 47, wherein the phospholipid comprises from 6 mol % to 12 mol % of the total lipid present in the particle.

59. (New) The nucleic acid-lipid particle of claim 47, wherein the cholesterol or derivative thereof comprises from 30 mol % to 35 mol % of the total lipid present in the particle.

60. (New) The nucleic acid-lipid particle of claim 47, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

61. (New) The nucleic acid-lipid particle of claim 60, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

62. (New) The nucleic acid-lipid particle of claim 61, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

63. (New) The nucleic acid-lipid particle of claim 62, wherein the PEG has an average molecular weight of about 2,000 daltons.

64. (New) The nucleic acid-lipid particle of claim 47, wherein the conjugated lipid that inhibits aggregation of particles comprises from 1 mol % to 2 mol % of the total lipid present in the particle.

65. (New) The nucleic acid-lipid particle of claim 60, wherein the nucleic acid-lipid particle comprises about 55 mol % cationic lipid, about 11 mol % phospholipid, about 33 mol % cholesterol or a derivative thereof, and about 1.6 mol % PEG-lipid conjugate.

66. (New) The nucleic acid-lipid particle of claim 47, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

67. (New) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 47 and a pharmaceutically acceptable carrier.

REMARKS

I. STATUS OF THE CLAIMS

After entry of this amendment, claims 47-67 are pending in this application and are presented for examination. Claims 1-46 have been canceled without prejudice to future prosecution. Claims 47-67 are newly added.

Support for new claims 47-67 is found throughout the specification as filed. In particular, new claim 47 finds support, for example, in original claim 1, in paragraph [0116] on page 24, and in paragraph [0133] on pages 27-28. New claim 48 finds support, for example, in original claim 2. New claim 49 finds support, for example, in original claim 4. New claim 50 finds support, for example, in original claim 5. New claims 51 and 52 find support, for example, in paragraph [0054] on pages 8-9. New claim 53 finds support, for example, in paragraph [0116] on page 24. New claim 54 finds support, for example, in original claim 14. New claims 55-59 find support, for example, in paragraph [0133] on pages 27-28. New claims 60-64 find support, for example, in original claims 17-21, respectively. New claim 65 finds support, for example, in paragraph [0121] on page 25, in paragraph [0133] on pages 27-28, and in paragraph [0139] on page 30. New claim 66 finds support, for example, in original claim 23. New claim 67 finds support, for example, in original claim 26.

As such, no new matter has been introduced. Reconsideration is respectfully requested.

II. SUBSTITUTE SEQUENCE LISTING

This amendment is accompanied by a computer readable form containing the above named sequences, SEQ ID NOS:1-7. The Substitute Sequence Listing in computer readable form was prepared through the use of the software program "FastSEQ" in accordance with 37 C.F.R. §§1.821 to 1.825. The Substitute Sequence Listing does not include new matter or matter that goes beyond the disclosure of the application as filed.

According to the Legal Framework for EFS-Web (September 2008), if a sequence listing text file submitted via EFS-Web complies with the requirements of 37 CFR 1.824(a)(2)-(6) and (b), the text file will serve as both the paper copy required by 37 CFR 1.821(c) and the

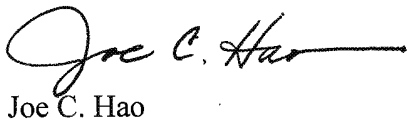
CRF required by 37 CFR 1.821(e). Therefore, a paper copy of the referenced Substitute Sequence Listing is not included with this amendment.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,



Joe C. Hao
Reg. No. 55,246

KILPATRICK TOWNSEND & STOCKTON LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 925-472-5000
Fax: 415-576-0300
Attachments
JCH:dmw
64033847 v1

JOINT APPENDIX 61

NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] The present application is a continuation of U.S. Application No. 13/928,309, filed June 26, 2013, which application is a continuation of 13/253,917, filed October 5, 2011, now U.S. Patent No. 8,492,359, which application is a continuation of 12/424,367 filed April 15, 2009, now U.S. Patent No. 8,058,069, which application claims priority to U.S. Provisional Application No. 61/045,228, filed April 15, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not applicable.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

[0003] Not applicable.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

[0004] The Sequence Listing written in file -77-3.TXT, created on August 22, 2013, 8,192 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

[0005] RNA interference (RNAi) is an evolutionarily conserved process in which recognition of double-stranded RNA (dsRNA) ultimately leads to posttranscriptional suppression of gene expression. This suppression is mediated by short dsRNA, also called small interfering RNA (siRNA), which induces specific degradation of mRNA through complementary base pairing. In several model systems, this natural response has been developed into a powerful tool for the investigation of gene function (*see, e.g.*, Elbashir *et al.*, *Genes Dev.*, 15:188-200 (2001); Hammond *et al.*, *Nat. Rev. Genet.*, 2:110-119 (2001)). More recently, it was discovered that introducing synthetic 21-nucleotide dsRNA duplexes into mammalian cells could efficiently silence gene expression.

[0006] Although the precise mechanism is still unclear, RNAi provides a potential new approach to downregulate or silence the transcription and translation of a gene of interest. For example, it is desirable to modulate (*e.g.*, reduce) the expression of certain genes for the treatment of neoplastic disorders such as cancer. It is also desirable to silence the expression of genes associated with liver diseases and disorders such as hepatitis. It is further desirable to reduce the expression of certain genes for the treatment of atherosclerosis and its manifestations, *e.g.,* hypercholesterolemia, myocardial infarction, and thrombosis.

[0007] A safe and effective nucleic acid delivery system is required for RNAi to be therapeutically useful. Viral vectors are relatively efficient gene delivery systems, but suffer from a variety of limitations, such as the potential for reversion to the wild-type as well as immune response concerns. As a result, nonviral gene delivery systems are receiving increasing attention (Worgall *et al.*, *Human Gene Therapy*, 8:37 (1997); Peeters *et al.*, *Human Gene Therapy*, 7:1693 (1996); Yei *et al.*, *Gene Therapy*, 1:192 (1994); Hope *et al.*, *Molecular Membrane Biology*, 15:1 (1998)). Furthermore, viral systems are rapidly cleared from the circulation, limiting transfection to "first-pass" organs such as the lungs, liver, and spleen. In addition, these systems induce immune responses that compromise delivery with subsequent injections.

[0008] Plasmid DNA-cationic liposome complexes are currently the most commonly employed nonviral gene delivery vehicles (Felgner, *Scientific American*, 276:102 (1997); Chonn *et al.*, *Current Opinion in Biotechnology*, 6:698 (1995)). For instance, cationic liposome complexes made of an amphipathic compound, a neutral lipid, and a detergent for transfecting insect cells are disclosed in U.S. Patent No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Publication No. 20030073640.

[0009] Cationic liposome complexes are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects (Harrison *et al.*, *Biotechniques*, 19:816 (1995); Li *et al.*, *The Gene*, 4:891 (1997); Tam *et al*, *Gene Ther.*, 7:1867 (2000)). As large, positively charged aggregates, lipoplexes are rapidly cleared when administered *in vivo*, with highest expression levels observed in first-pass organs, particularly the lungs (Huang *et al.*, *Nature Biotechnology*, 15:620 (1997); Templeton *et al.*, *Nature Biotechnology*, 15:647 (1997); Hofland *et al.*, *Pharmaceutical Research*, 14:742 (1997)).

[0010] Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Patent No. 6,429,200. Anionic liposomes are disclosed in U.S. Patent Publication No. 20030026831.

Polymer liposomes that incorporate dextrin or glycerol-phosphocholine polymers are disclosed in U.S. Patent Publication Nos. 20020081736 and 20030082103, respectively.

[0011] A gene delivery system containing an encapsulated nucleic acid for systemic delivery should be small (*i.e.*, less than about 100 nm diameter) and should remain intact in the circulation for an extended period of time in order to achieve delivery to affected tissues. This requires a highly stable, serum-resistant nucleic acid-containing particle that does not interact with cells and other components of the vascular compartment. The particle should also readily interact with target cells at a disease site in order to facilitate intracellular delivery of a desired nucleic acid.

[0012] Recent work has shown that nucleic acids can be encapsulated in small (*e.g.*, about 70 nm diameter) "stabilized plasmid-lipid particles" (SPLP) that consist of a single plasmid encapsulated within a bilayer lipid vesicle (Wheeler *et al.*, *Gene Therapy*, 6:271 (1999)). These SPLPs typically contain the "fusogenic" lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid, and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. SPLPs have systemic application as they exhibit extended circulation lifetimes following intravenous (i.v.) injection, accumulate preferentially at distal tumor sites due to the enhanced vascular permeability in such regions, and can mediate transgene expression at these tumor sites. The levels of transgene expression observed at the tumor site following i.v. injection of SPLPs containing the luciferase marker gene are superior to the levels that can be achieved employing plasmid DNA-cationic liposome complexes (lipoplexes) or naked DNA.

[0013] Thus, there remains a strong need in the art for novel and more efficient methods and compositions for introducing nucleic acids such as siRNA into cells. In addition, there is a need in the art for methods of downregulating the expression of genes of interest to treat or prevent diseases and disorders such as cancer and atherosclerosis. The present invention addresses these and other needs.

BRIEF SUMMARY OF THE INVENTION

[0014] The present invention provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.*, for the treatment of a disease or disorder).

[0015] In preferred embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in

the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In other preferred embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0016] In one aspect, the present invention provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0017] More particularly, the present invention provides serum-stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (*e.g.*, one or more interfering RNA molecules such as siRNA, aiRNA, and/or miRNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP (*e.g.*, for the treatment of a disease or disorder).

[0018] In certain embodiments, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) a nucleic acid (*e.g.*, an interfering RNA); (b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0019] In one preferred embodiment, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:62" formulation.

[0020] In another preferred embodiment, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:57" formulation.

[0021] The present invention also provides pharmaceutical compositions comprising a lipid particle described herein (*e.g.*, SNALP) and a pharmaceutically acceptable carrier.

[0022] In another aspect, the present invention provides methods for introducing an active agent or therapeutic agent (*e.g.*, nucleic acid) into a cell, the method comprising contacting the cell with a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0023] In yet another aspect, the present invention provides methods for the *in vivo* delivery of an active agent or therapeutic agent (*e.g.*, nucleic acid), the method comprising administering to a mammalian subject a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0024] In a further aspect, the present invention provides methods for treating a disease or disorder in a mammalian subject in need thereof, the method comprising administering to the mammalian subject a therapeutically effective amount of a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0025] Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] Figure 1A (Samples 1-8) and Figure 1B (Samples 9-16) illustrate data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

[0027] Figure 2 illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

[0028] Figure 3 illustrates additional data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice. Each bar represents the group mean of five animals. Error bars indicate the standard deviation.

[0029] Figure 4 illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

[0030] Figure 5 illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

[0031] Figure 6A (expressed as IU/L) and Figure 6B (expressed as x-Fold Upper Limit of Normal) illustrate data demonstrating that the tolerability of 1:57 SNALP containing ApoB siRNA prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters.

[0032] Figure 7A (expressed as liver ApoB:GAPD mRNA ratio), Figure 7B (expressed as relative plasma ApoB-100 concentration), and Figure 7C (expressed as plasma total cholesterol illustrate data demonstrating that the efficacy of 1:57 SNALP containing ApoB siRNA prepared by gear pump was similar to the same SNALP prepared by syringe press.

[0033] Figure 8 illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

[0034] Figure 9 illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

[0035] Figure 10A (expressed as IU/L) and Figure 10B (expressed as x-Fold Upper Limit of Normal) illustrate data demonstrating that clinically significant liver enzyme elevations (3xULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

[0036] Figure 11A (expressed as liver ApoB:GAPD mRNA ratio) and Figure 11B (expressed as relative plasma ApoB-100 concentration) illustrate data demonstrating that the potency of the lower lipid:drug (L:D) 1:57 SNALP containing ApoB siRNA was as good as that of the higher L:D SNALP at the tested drug dosages.

[0037] Figure 12 illustrates data demonstrating that ApoB protein and total cholesterol levels were reduced to a similar extent by 1:57 SNALP containing ApoB siRNA at a 6:1 input L:D ratio (final ratio of 7:1) and 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

[0038] Figure 13 illustrates data demonstrating that a treatment regimen of 1:57 SNALP with siRNA targeting PLK-1 is well tolerated with no apparent signs of treatment related toxicity in mice bearing Hep3B liver tumors.

[0039] Figure 14 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA caused a significant increase in the survival of Hep3B tumor-bearing mice.

[0040] Figure 15 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA reduced PLK-1 mRNA levels by 50% in intrahepatic Hep3B tumors growing in mice 24 hours after SNALP administration.

[0041] Figure 16 illustrates data demonstrating that a specific cleavage product of PLK-1 mRNA was detectable by 5' RACE-PCR in mice treated with 1:57 SNALP containing PLK-1 siRNA. 10 μl PCR product/well were loaded onto a 1.5% agarose gel. Lane Nos.: (1) molecular weight (MW) marker; (2) PBS mouse 1; (3) PBS mouse 2; (4) PBS mouse 3; (5) Luc SNALP mouse 1; (6) Luc SNALP mouse 2; (7) PLK SNALP mouse 1; (8) PLK SNALP mouse 2; (9) PLK SNALP mouse 3; and (10) no template control.

[0042] Figure 17 illustrates data demonstrating that control (Luc) 1:57 SNALP-treated mice displayed normal mitoses in Hep3B tumors (top panels), whereas mice treated with 1:57 SNALP containing PLK-1 siRNA exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors (bottom panels).

[0043] Figure 18 illustrates data demonstrating that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B subcutaneous (S.C.) tumors.

[0044] Figure 19 illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

[0045] Figure 20 illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0046] Figure 21 illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

[0047] Figure 22 illustrates data demonstrating the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0048] The present invention is based, in part, upon the surprising discovery that lipid particles comprising from about 50 mol % to about 85 mol % of a cationic lipid, from about 13 mol % to about 49.5 mol % of a non-cationic lipid, and from about 0.5 mol % to about 2 mol % of a lipid conjugate provide advantages when used for the *in vitro* or *in vivo* delivery of an active agent, such as a therapeutic nucleic acid (*e.g.*, an interfering RNA). In particular, as illustrated by the Examples herein, the present invention provides stable nucleic acid-lipid particles (SNALP) that advantageously impart increased activity of the encapsulated nucleic acid (*e.g.*, an interfering RNA such as siRNA) and improved tolerability of the formulations *in vivo*, resulting in a significant increase in the therapeutic index as compared to nucleic acid-lipid particle compositions previously described. Additionally, the SNALP of the invention are stable in circulation, *e.g.*, resistant to degradation by nucleases in serum, and are substantially non-toxic to mammals such as humans. As a non-limiting example, Figure 3 of Example 4 shows that one SNALP embodiment of the invention ("1:57 SNALP") was more than 10 times as efficacious as compared to a nucleic acid-lipid particle previously described ("2:30 SNALP") in mediating target gene silencing at a 10-fold lower dose. Similarly, Figure 2 of Example 3 shows that the "1:57 SNALP" formulation was substantially

more effective at silencing the expression of a target gene as compared to nucleic acid-lipid particles previously described ("2:40 SNALP").

[0049] In certain embodiments, the present invention provides improved compositions for the delivery of interfering RNA such as siRNA molecules. In particular, the Examples herein illustrate that the improved lipid particle formulations of the invention are highly effective in downregulating the mRNA and/or protein levels of target genes. Furthermore, the Examples herein illustrate that the presence of certain molar ratios of lipid components results in improved or enhanced activity of these lipid particle formulations of the present invention. For instance, the "1:57 SNALP" and "1:62 SNALP" formulations described herein are exemplary formulations of the present invention that are particularly advantageous because they provide improved efficacy and tolerability *in vivo*, are serum-stable, are substantially non-toxic, are capable of accessing extravascular sites, and are capable of reaching target cell populations.

[0050] The lipid particles and compositions of the present invention may be used for a variety of purposes, including the delivery of associated or encapsulated therapeutic agents to cells, both *in vitro* and *in vivo*. Accordingly, the present invention provides methods for treating diseases or disorders in a subject in need thereof, by contacting the subject with a lipid particle described herein comprising one or more suitable therapeutic agents.

[0051] Various exemplary embodiments of the lipid particles of the invention, as well as compositions and formulations comprising the same, and their use to deliver therapeutic agents and modulate target gene and protein expression, are described in further detail below.

II. Definitions

[0052] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0053] The term "interfering RNA" or "RNAi" or "interfering RNA sequence" refers to single-stranded RNA (*e.g.*, mature miRNA) or double-stranded RNA (*i.e.*, duplex RNA such as siRNA, aiRNA, or pre-miRNA) that is capable of reducing or inhibiting the expression of a target gene or sequence (*e.g.*, by mediating the degradation or inhibiting the translation of mRNAs which are complementary to the interfering RNA sequence) when the interfering RNA is in the same cell as the target gene or sequence. Interfering RNA thus refers to the single-stranded RNA that is complementary to a target mRNA sequence or to the double-stranded RNA formed by two complementary strands or by a single, self-complementary strand. Interfering RNA may have substantial or complete identity to the target gene or

sequence, or may comprise a region of mismatch (*i.e.*, a mismatch motif). The sequence of the interfering RNA can correspond to the full-length target gene, or a subsequence thereof.

[0054] Interfering RNA includes "small-interfering RNA" or "siRNA," *e.g.*, interfering RNA of about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, more typically about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.*, each complementary sequence of the double-stranded siRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, preferably about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded siRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, preferably about 18-22, 19-20, or 19-21 base pairs in length). siRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides and 5' phosphate termini. Examples of siRNA include, without limitation, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand; a double-stranded polynucleotide molecule assembled from a single stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions; and a circular single-stranded polynucleotide molecule with two or more loop structures and a stem having self-complementary sense and antisense regions, where the circular polynucleotide can be processed *in vivo* or *in vitro* to generate an active double-stranded siRNA molecule.

[0055] Preferably, siRNA are chemically synthesized. siRNA can also be generated by cleavage of longer dsRNA (*e.g.*, dsRNA greater than about 25 nucleotides in length) with the *E. coli* RNase III or Dicer. These enzymes process the dsRNA into biologically active siRNA (*see*, *e.g.*, Yang *et al.*, *Proc. Natl. Acad. Sci. USA*, 99:9942-9947 (2002); Calegari *et al.*, *Proc. Natl. Acad. Sci. USA*, 99:14236 (2002); Byrom *et al.*, *Ambion TechNotes*, 10(1):4-6 (2003); Kawasaki *et al.*, *Nucleic Acids Res.*, 31:981-987 (2003); Knight *et al.*, *Science*, 293:2269-2271 (2001); and Robertson *et al.*, *J. Biol. Chem.*, 243:82 (1968)). Preferably, dsRNA are at least 50 nucleotides to about 100, 200, 300, 400, or 500 nucleotides in length. A dsRNA may be as long as 1000, 1500, 2000, 5000 nucleotides in length, or longer. The dsRNA can encode for an entire gene transcript or a partial gene transcript. In certain instances, siRNA may be encoded by a plasmid (*e.g.*, transcribed as sequences that automatically fold into duplexes with hairpin loops).

[0056] As used herein, the term "mismatch motif" or "mismatch region" refers to a portion of an interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) sequence that does not have 100 % complementarity to its target sequence. An interfering RNA may have at least one, two, three, four, five, six, or more mismatch regions. The mismatch regions may be contiguous or may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides. The mismatch motifs or regions may comprise a single nucleotide or may comprise two, three, four, five, or more nucleotides.

[0057] An "effective amount" or "therapeutically effective amount" of an active agent or therapeutic agent such as an interfering RNA is an amount sufficient to produce the desired effect, *e.g.*, an inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of an interfering RNA. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with an interfering RNA relative to the control is about 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, *e.g.*, examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0058] By "decrease," "decreasing," "reduce," or "reducing" of an immune response by an interfering RNA is intended to mean a detectable decrease of an immune response to a given interfering RNA (*e.g.*, a modified interfering RNA). The amount of decrease of an immune response by a modified interfering RNA may be determined relative to the level of an immune response in the presence of an unmodified interfering RNA. A detectable decrease can be about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more lower than the immune response detected in the presence of the unmodified interfering RNA. A decrease in the immune response to interfering RNA is typically measured by a decrease in cytokine production (*e.g.*, IFNγ, IFNα, TNFα, IL-6, or IL-12) by a responder cell *in vitro* or a decrease in cytokine production in the sera of a mammalian subject after administration of the interfering RNA.

[0059] As used herein, the term "responder cell" refers to a cell, preferably a mammalian cell, that produces a detectable immune response when contacted with an immunostimulatory interfering RNA such as an unmodified siRNA. Exemplary responder cells include, *e.g.*, dendritic cells, macrophages, peripheral blood mononuclear cells (PBMCs), splenocytes, and the like. Detectable immune responses include, *e.g.*, production of cytokines or growth

factors such as TNF-α, IFN-α, IFN-β, IFN-γ, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TGF, and combinations thereof.

[0060] "Substantial identity" refers to a sequence that hybridizes to a reference sequence under stringent conditions, or to a sequence that has a specified percent identity over a specified region of a reference sequence.

[0061] The phrase "stringent hybridization conditions" refers to conditions under which a nucleic acid will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0062] Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C-95°C for 30 sec.-2 min., an annealing phase lasting 30 sec.-2 min., and an extension phase of about 72°C for 1-2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, *e.g.*, in Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y. (1990).

[0063] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous references, *e.g.*, Current Protocols in Molecular Biology, Ausubel *et al.*, eds.

[0064] The terms "substantially identical" or "substantial identity," in the context of two or more nucleic acids, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (*i.e.*, at least about 60%, preferably at least about 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition, when the context indicates, also refers analogously to the complement of a sequence. Preferably, the substantial identity exists over a region that is at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length.

[0065] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0066] A "comparison window," as used herein, includes reference to a segment of any one of a number of contiguous positions selected from the group consisting of from about 5 to about 60, usually about 10 to about 45, more usually about 15 to about 30, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences

for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.*, 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.*, 48:443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*, 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology*, Ausubel *et al.*, eds. (1995 supplement)).

[0067] A preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al., Nuc. Acids Res.*, 25:3389-3402 (1977) and Altschul *et al., J. Mol. Biol.*, 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

[0068] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul, *Proc. Natl. Acad. Sci. USA*, 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0069] The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA and RNA. DNA may be in the form of, *e.g.*, antisense molecules, plasmid DNA, pre-condensed DNA, a PCR product, vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives and combinations of these groups. RNA may be in the form of siRNA, asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, tRNA, viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the

reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.,* degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.,* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.,* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes,* 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

[0070] The term "gene" refers to a nucleic acid (*e.g.*, DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

[0071] "Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

[0072] The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are characterized by being insoluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

[0073] A "lipid particle" is used herein to refer to a lipid formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), to a target site of interest. In the lipid particle of the invention, which is typically formed from a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of

the particle, the active agent or therapeutic agent may be encapsulated in the lipid, thereby protecting the agent from enzymatic degradation.

[0074] As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a particle made from lipids (*e.g.*, a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of the particle), wherein the nucleic acid (*e.g.*, siRNA, aiRNA, miRNA, ssDNA, dsDNA, ssRNA, short hairpin RNA (shRNA), dsRNA, or a plasmid, including plasmids from which an interfering RNA is transcribed) is fully encapsulated within the lipid. As used herein, the term "SNALP" includes an SPLP, which is the term used to refer to a nucleic acid-lipid particle comprising a nucleic acid (*e.g.*, a plasmid) encapsulated within the lipid. SNALP and SPLP typically contain a cationic lipid, a non-cationic lipid, and a lipid conjugate (*e.g.*, a PEG-lipid conjugate). SNALP and SPLP are extremely useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (i.v.) injection, they can accumulate at distal sites (*e.g.*, sites physically separated from the administration site), and they can mediate expression of the transfected gene or silencing of target gene expression at these distal sites. SPLP include "pSPLP," which comprise an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0075] The lipid particles of the invention (*e.g.*, SNALP) typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm, and are substantially non-toxic. In addition, nucleic acids, when present in the lipid particles of the invention, are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Publication Nos. 20040142025 and 20070042031, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0076] As used herein, "lipid encapsulated" can refer to a lipid particle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid is fully encapsulated in the lipid particle (*e.g.*, to form an SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle).

[0077] The term "lipid conjugate" refers to a conjugated lipid that inhibits aggregation of lipid particles. Such lipid conjugates include, but are not limited to, polyamide oligomers (*e.g.*, ATTA-lipid conjugates), PEG-lipid conjugates, such as PEG coupled to

dialkyloxypropyls, PEG coupled to diacylglycerols, PEG coupled to cholesterol, PEG coupled to phosphatidylethanolamines, PEG conjugated to ceramides (*see, e.g.*, U.S. Patent No. 5,885,613, the disclosure of which is herein incorporated by reference in its entirety for all purposes), cationic PEG lipids, and mixtures thereof. PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In preferred embodiments, non-ester containing linker moieties are used.

[0078] The term "amphipathic lipid" refers, in part, to any suitable material wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxyl, and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids.

[0079] Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, are also within the group designated as amphipathic lipids. Additionally, the amphipathic lipids described above can be mixed with other lipids including triglycerides and sterols.

[0080] The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides, and diacylglycerols.

[0081] The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

[0082] The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerols, cardiolipins, diacylphosphatidylserines, diacylphosphatidic acids, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

[0083] The term "cationic lipid" refers to any of a number of lipid species that carry a net positive charge at a selected pH, such as physiological pH (*e.g.*, pH of about 7.0). It has been surprisingly found that cationic lipids comprising alkyl chains with multiple sites of unsaturation, *e.g.*, at least two or three sites of unsaturation, are particularly useful for forming lipid particles with increased membrane fluidity. A number of cationic lipids and related analogs, which are also useful in the present invention, have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of cationic lipids are described in detail herein. In some cases, the cationic lipids comprise a protonatable tertiary amine (*e.g.*, pH titratable) head group, C18 alkyl chains, ether linkages between the head group and alkyl chains, and 0 to 3 double bonds. Such lipids include, *e.g.*, DSDMA, DLinDMA, DLenDMA, and DODMA.

[0084] The term "hydrophobic lipid" refers to compounds having apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups optionally substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Suitable examples include, but are not limited to, diacylglycerol, dialkylglycerol, N-N-dialkylamino, 1,2-diacyloxy-3-aminopropane, and 1,2-dialkyl-3-aminopropane.

[0085] The term "fusogenic" refers to the ability of a lipid particle, such as a SNALP, to fuse with the membranes of a cell. The membranes can be either the plasma membrane or membranes surrounding organelles, *e.g.*, endosome, nucleus, *etc.*

[0086] As used herein, the term "aqueous solution" refers to a composition comprising in whole, or in part, water.

[0087] As used herein, the term "organic lipid solution" refers to a composition comprising in whole, or in part, an organic solvent having a lipid.

[0088] "Distal site," as used herein, refers to a physically separated site, which is not limited to an adjacent capillary bed, but includes sites broadly distributed throughout an organism.

[0089] "Serum-stable" in relation to nucleic acid-lipid particles such as SNALP means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNAse assay, or an RNAse assay.

[0090] "Systemic delivery," as used herein, refers to delivery of lipid particles that leads to a broad biodistribution of an active agent or therapeutic agent such as an interfering RNA within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. To obtain broad biodistribution generally requires a blood lifetime such that the agent is not rapidly degraded or cleared (such as by first pass organs (liver, lung, *etc.*) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration. Systemic delivery of lipid particles can be by any means known in the art including, for example, intravenous, subcutaneous, and intraperitoneal. In a preferred embodiment, systemic delivery of lipid particles is by intravenous delivery.

[0091] "Local delivery," as used herein, refers to delivery of an active agent or therapeutic agent such as an interfering RNA directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site such as a tumor or other target site such as a site of inflammation or a target organ such as the liver, heart, pancreas, kidney, and the like.

[0092] The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

[0093] The term "cancer" refers to any member of a class of diseases characterized by the uncontrolled growth of aberrant cells. The term includes all known cancers and neoplastic conditions, whether characterized as malignant, benign, soft tissue, or solid, and cancers of all stages and grades including pre- and post-metastatic cancers. Examples of different types of cancer include, but are not limited to, lung cancer, colon cancer, rectal cancer, anal cancer, bile duct cancer, small intestine cancer, stomach (gastric) cancer, esophageal cancer; gallbladder cancer, liver cancer, pancreatic cancer, appendix cancer, breast cancer, ovarian cancer; cervical cancer, prostate cancer, renal cancer (*e.g.*, renal cell carcinoma), cancer of the central nervous system, glioblastoma, skin cancer, lymphomas, choriocarcinomas, head

and neck cancers, osteogenic sarcomas, and blood cancers. Non-limiting examples of specific types of liver cancer include hepatocellular carcinoma (HCC), secondary liver cancer (*e.g.*, caused by metastasis of some other non-liver cancer cell type), and hepatoblastoma. As used herein, a "tumor" comprises one or more cancerous cells.

III. Description of the Embodiments

[0094] The present invention provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.*, for the treatment of a disease or disorder).

[0095] In one aspect, the present invention provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0096] In certain embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In certain other embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0097] In some embodiments, the active agent or therapeutic agent comprises a nucleic acid. In certain instances, the nucleic acid comprises an interfering RNA molecule such as, *e.g.*, an siRNA, aiRNA, miRNA, or mixtures thereof. In certain other instances, the nucleic acid comprises single-stranded or double-stranded DNA, RNA, or a DNA/RNA hybrid such as, *e.g.*, an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

[0098] In other embodiments, the active agent or therapeutic agent comprises a peptide or polypeptide. In certain instances, the peptide or polypeptide comprises an antibody such as, *e.g.*, a polyclonal antibody, a monoclonal antibody, an antibody fragment; a humanized antibody, a recombinant antibody, a recombinant human antibody, a Primatized™ antibody, or mixtures thereof. In certain other instances, the peptide or polypeptide comprises a cytokine, a growth factor, an apoptotic factor, a differentiation-inducing factor, a cell-surface

receptor, a ligand, a hormone, a small molecule (*e.g.*, small organic molecule or compound), or mixtures thereof.

[0099] In preferred embodiments, the active agent or therapeutic agent comprises an siRNA. In one embodiment, the siRNA molecule comprises a double-stranded region of about 15 to about 60 nucleotides in length (*e.g.*, about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, or 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length). The siRNA molecules of the invention are capable of silencing the expression of a target sequence *in vitro* and/or *in vivo*.

[0100] In some embodiments, the siRNA molecule comprises at least one modified nucleotide. In certain preferred embodiments, the siRNA molecule comprises one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides in the double-stranded region. In certain instances, the siRNA comprises from about 1% to about 100% (*e.g.*, about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region. In preferred embodiments, less than about 25% (*e.g.*, less than about 25%, 20%, 15%, 10%, or 5%) or from about 1% to about 25% (*e.g.*, from about 1%-25%, 5%-25%, 10%-25%, 15%-25%, 20%-25%, or 10%-20%) of the nucleotides in the double-stranded region comprise modified nucleotides.

[0101] In other embodiments, the siRNA molecule comprises modified nucleotides including, but not limited to, 2'-O-methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, locked nucleic acid (LNA) nucleotides, and mixtures thereof. In preferred embodiments, the siRNA comprises 2'OMe nucleotides (*e.g.*, 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof. In certain instances, the siRNA does not comprise 2'OMe-cytosine nucleotides. In other embodiments, the siRNA comprises a hairpin loop structure.

[0102] The siRNA may comprise modified nucleotides in one strand (*i.e.*, sense or antisense) or both strands of the double-stranded region of the siRNA molecule. Preferably, uridine and/or guanosine nucleotides are modified at selective positions in the double-stranded region of the siRNA duplex. With regard to uridine nucleotide modifications, at least one, two, three, four, five, six, or more of the uridine nucleotides in the sense and/or antisense strand can be a modified uridine nucleotide such as a 2'OMe-uridine nucleotide. In some embodiments, every uridine nucleotide in the sense and/or antisense strand is a 2'OMe-

uridine nucleotide. With regard to guanosine nucleotide modifications, at least one, two, three, four, five, six, or more of the guanosine nucleotides in the sense and/or antisense strand can be a modified guanosine nucleotide such as a 2'OMe-guanosine nucleotide. In some embodiments, every guanosine nucleotide in the sense and/or antisense strand is a 2'OMe-guanosine nucleotide.

[0103] In certain embodiments, at least one, two, three, four, five, six, seven, or more 5'-GU-3' motifs in an siRNA sequence may be modified, *e.g.*, by introducing mismatches to eliminate the 5'-GU-3' motifs and/or by introducing modified nucleotides such as 2'OMe nucleotides. The 5'-GU-3' motif can be in the sense strand, the antisense strand, or both strands of the siRNA sequence. The 5'-GU-3' motifs may be adjacent to each other or, alternatively, they may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides.

[0104] In some preferred embodiments, a modified siRNA molecule is less immunostimulatory than a corresponding unmodified siRNA sequence. In such embodiments, the modified siRNA molecule with reduced immunostimulatory properties advantageously retains RNAi activity against the target sequence. In another embodiment, the immunostimulatory properties of the modified siRNA molecule and its ability to silence target gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the siRNA sequence such as, *e.g.*, within the double-stranded region of the siRNA duplex. In certain instances, the modified siRNA is at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% less immunostimulatory than the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that the immunostimulatory properties of the modified siRNA molecule and the corresponding unmodified siRNA molecule can be determined by, for example, measuring INF-α and/or IL-6 levels from about two to about twelve hours after systemic administration in a mammal or transfection of a mammalian responder cell using an appropriate lipid-based delivery system (such as the SNALP delivery system disclosed herein).

[0105] In certain embodiments, a modified siRNA molecule has an IC_{50} (*i.e.*, half-maximal inhibitory concentration) less than or equal to ten-fold that of the corresponding unmodified siRNA (*i.e.*, the modified siRNA has an IC_{50} that is less than or equal to ten-times the IC_{50} of the corresponding unmodified siRNA). In other embodiments, the modified siRNA has an IC_{50} less than or equal to three-fold that of the corresponding unmodified siRNA sequence. In yet other embodiments, the modified siRNA has an IC_{50} less than or equal to two-fold that

of the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that a dose-response curve can be generated and the IC_{50} values for the modified siRNA and the corresponding unmodified siRNA can be readily determined using methods known to those of skill in the art.

[0106] In yet another embodiment, a modified siRNA molecule is capable of silencing at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the expression of the target sequence relative to the corresponding unmodified siRNA sequence.

[0107] In some embodiments, the siRNA molecule does not comprise phosphate backbone modifications, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In other embodiments, the siRNA comprises one, two, three, four, or more phosphate backbone modifications, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise phosphate backbone modifications.

[0108] In further embodiments, the siRNA does not comprise 2'-deoxy nucleotides, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In yet further embodiments, the siRNA comprises one, two, three, four, or more 2'-deoxy nucleotides, *e.g.*, in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise 2'-deoxy nucleotides.

[0109] In certain instances, the nucleotide at the 3'-end of the double-stranded region in the sense and/or antisense strand is not a modified nucleotide. In certain other instances, the nucleotides near the 3'-end (*e.g.*, within one, two, three, or four nucleotides of the 3'-end) of the double-stranded region in the sense and/or antisense strand are not modified nucleotides.

[0110] The siRNA molecules described herein may have 3' overhangs of one, two, three, four, or more nucleotides on one or both sides of the double-stranded region, or may lack overhangs (*i.e.*, have blunt ends) on one or both sides of the double-stranded region. Preferably, the siRNA has 3' overhangs of two nucleotides on each side of the double-stranded region. In certain instances, the 3' overhang on the antisense strand has complementarity to the target sequence and the 3' overhang on the sense strand has complementarity to a complementary strand of the target sequence. Alternatively, the 3' overhangs do not have complementarity to the target sequence or the complementary strand thereof. In some embodiments, the 3' overhangs comprise one, two, three, four, or more nucleotides such as 2'-deoxy (2'H) nucleotides. In certain preferred embodiments, the 3' overhangs comprise deoxythymidine (dT) and/or uridine nucleotides. In other embodiments, one or more of the nucleotides in the 3' overhangs on one or both sides of the double-

stranded region comprise modified nucleotides. Non-limiting examples of modified nucleotides are described above and include 2'OMe nucleotides, 2'-deoxy-2'F nucleotides, 2'-deoxy nucleotides, 2'-O-2-MOE nucleotides, LNA nucleotides, and mixtures thereof. In preferred embodiments, one, two, three, four, or more nucleotides in the 3' overhangs present on the sense and/or antisense strand of the siRNA comprise 2'OMe nucleotides (*e.g.*, 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof.

[0111] The siRNA may comprise at least one or a cocktail (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) of unmodified and/or modified siRNA sequences that silence target gene expression. The cocktail of siRNA may comprise sequences which are directed to the same region or domain (*e.g.*, a "hot spot") and/or to different regions or domains of one or more target genes. In certain instances, one or more (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) modified siRNA that silence target gene expression are present in a cocktail. In certain other instances, one or more (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) unmodified siRNA sequences that silence target gene expression are present in a cocktail.

[0112] In some embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to the target sequence or a portion thereof. In other embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is 100% complementary to the target sequence or a portion thereof. In further embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that specifically hybridizes to the target sequence or a portion thereof.

[0113] In further embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the target sequence or a portion thereof. In additional embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is 100% identical to the target sequence or a portion thereof.

[0114] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the cationic lipid may comprise, *e.g.*, one or more of the following: 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-

dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), or mixtures thereof. In certain preferred embodiments, the cationic lipid is DLinDMA, DLin-K-C2-DMA ("XTC2"), or mixtures thereof.

[0115] The synthesis of cationic lipids such as DLin-K-C2-DMA ("XTC2"), DLin-K-C3-DMA, DLin-K-C4-DMA, DLin-K6-DMA, and DLin-K-MPZ, as well as additional cationic lipids, is described in U.S. Provisional Application No. 61/104,212, filed October 9, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as DLin-K-DMA, DLin-C-DAP, DLin-DAC, DLin-MA, DLinDAP, DLin-S-DMA, DLin-2-DMAP, DLin-TMA.Cl, DLin-TAP.Cl, DLin-MPZ,

DLinAP, DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Application No. PCT/US08/88676, filed December 31, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as CLinDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060240554, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0116] In some embodiments, the cationic lipid may comprise from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 50 mol % to about 60 mol % of the total lipid present in the particle.

[0117] In other embodiments, the cationic lipid may comprise from about 55 mol % to about 90 mol %, from about 55 mol % to about 85 mol %, from about 55 mol % to about 80 mol %, from about 55 mol % to about 75 mol %, from about 55 mol % to about 70 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0118] In yet other embodiments, the cationic lipid may comprise from about 60 mol % to about 90 mol %, from about 60 mol % to about 85 mol %, from about 60 mol % to about 80 mol %, from about 60 mol % to about 75 mol %, or from about 60 mol % to about 70 mol % of the total lipid present in the particle.

[0119] In still yet other embodiments, the cationic lipid may comprise from about 65 mol % to about 90 mol %, from about 65 mol % to about 85 mol %, from about 65 mol % to about 80 mol %, or from about 65 mol % to about 75 mol % of the total lipid present in the particle.

[0120] In further embodiments, the cationic lipid may comprise from about 70 mol % to about 90 mol %, from about 70 mol % to about 85 mol %, from about 70 mol % to about 80 mol %, from about 75 mol % to about 90 mol %, from about 75 mol % to about 85 mol %, or from about 80 mol % to about 90 mol % of the total lipid present in the particle.

[0121] In additional embodiments, the cationic lipid may comprise (at least) about 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0122] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the non-cationic lipid may comprise, *e.g.*, one or more anionic lipids and/or neutral lipids. In preferred embodiments, the non-cationic lipid comprises one of the

following neutral lipid components: (1) cholesterol or a derivative thereof; (2) a phospholipid; or (3) a mixture of a phospholipid and cholesterol or a derivative thereof.

[0123] Examples of cholesterol derivatives include, but are not limited to, cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof. The synthesis of cholesteryl-2'-hydroxyethyl ether is described herein.

[0124] The phospholipid may be a neutral lipid including, but not limited to, dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), egg phosphatidylcholine (EPC), and mixtures thereof. In certain preferred embodiments, the phospholipid is DPPC, DSPC, or mixtures thereof.

[0125] In some embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 60 mol %, from about 15 mol % to about 60 mol %, from about 20 mol % to about 60 mol %, from about 25 mol % to about 60 mol %, from about 30 mol % to about 60 mol %, from about 10 mol % to about 55 mol %, from about 15 mol % to about 55 mol %, from about 20 mol % to about 55 mol %, from about 25 mol % to about 55 mol %, from about 30 mol % to about 55 mol %, from about 13 mol % to about 50 mol %, from about 15 mol % to about 50 mol % or from about 20 mol % to about 50 mol % of the total lipid present in the particle. When the non-cationic lipid is a mixture of a phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 50, or 60 mol % of the total lipid present in the particle.

[0126] In other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 49.5 mol %, from about 13 mol % to about 49.5 mol %, from about 15 mol % to about 49.5 mol %, from about 20 mol % to about 49.5 mol %, from about 25 mol % to about 49.5 mol %, from about 30 mol % to about 49.5 mol %, from about 35 mol % to about 49.5 mol %, or from about 40 mol % to about 49.5 mol % of the total lipid present in the particle.

[0127] In yet other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 45 mol %, from about 13

mol % to about 45 mol %, from about 15 mol % to about 45 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, or from about 35 mol % to about 45 mol % of the total lipid present in the particle.

[0128] In still yet other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 40 mol %, from about 13 mol % to about 40 mol %, from about 15 mol % to about 40 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0129] In further embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 35 mol %, from about 13 mol % to about 35 mol %, from about 15 mol % to about 35 mol %, from about 20 mol % to about 35 mol %, or from about 25 mol % to about 35 mol % of the total lipid present in the particle.

[0130] In yet further embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 30 mol %, from about 13 mol % to about 30 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 13 mol % to about 25 mol %, or from about 15 mol % to about 25 mol % of the total lipid present in the particle.

[0131] In additional embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise (at least) about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0132] In certain preferred embodiments, the non-cationic lipid comprises cholesterol or a derivative thereof of from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle of the invention may comprise cholesterol or a derivative thereof at about 37 mol % of the total lipid present in the particle. In other preferred embodiments, a phospholipid-free lipid particle of the invention may comprise cholesterol or a derivative thereof of from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 32 mol % to about 45 mol %, from about 32 mol % to about 42 mol %, from about 32 mol % to about 40 mol %, from about 34 mol % to about 45 mol %, from about 34 mol % to about 42 mol %, from about 34 mol % to about 40 mol %, or about 30, 31, 32, 33, 34, 35, 36,

37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0133] In certain other preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 4 mol % to about 10 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 30 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 7 mol % and cholesterol at about 34 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 3 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 4 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 4 mol % to about 8 mol %, from about 5 mol % to about 12 mol %, from about 5 mol % to about 9 mol %, from about 6 mol % to about 12 mol %, from about 6 mol % to about 10 mol %, or about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 40 mol %, from about 25 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 28 mol % to about 40 mol %, from about 28 mol % to about 38 mol %, from about 30 mol % to about 38 mol %, from about 32 mol % to about 36 mol %, or about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0134] In further preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 20 mol % and cholesterol at about 20 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20,

21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0135] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the conjugated lipid that inhibits aggregation of particles may comprise, *e.g.*, one or more of the following: a polyethyleneglycol (PEG)-lipid conjugate, a polyamide (ATTA)-lipid conjugate, a cationic-polymer-lipid conjugates (CPLs), or mixtures thereof. In one preferred embodiment, the nucleic acid-lipid particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate. In certain embodiments, the PEG-lipid conjugate or ATTA-lipid conjugate is used together with a CPL. The conjugated lipid that inhibits aggregation of particles may comprise a PEG-lipid including, *e.g.*, a PEG-diacylglycerol (DAG), a PEG dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or mixtures thereof. The PEG-DAA conjugate may be PEG-dilauryloxypropyl (C12), a PEG-dimyristyloxypropyl (C14), a PEG-dipalmityloxypropyl (C16), a PEG-distearyloxypropyl (C18), or mixtures thereof.

[0136] Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-*sn*3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed December 31, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Yet additional PEG-lipid conjugates suitable for use in the invention include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol) (2KPEG-DMG). The synthesis of 2KPEG-DMG is described in U.S. Patent No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0137] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about

750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

[0138] In some embodiments, the conjugated lipid that inhibits aggregation of particles is a CPL that has the formula: A-W-Y, wherein A is a lipid moiety, W is a hydrophilic polymer, and Y is a polycationic moiety. W may be a polymer selected from the group consisting of polyethyleneglycol (PEG), polyamide, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, or combinations thereof, the polymer having a molecular weight of from about 250 to about 7000 daltons. In some embodiments, Y has at least 4 positive charges at a selected pH. In some embodiments, Y may be lysine, arginine, asparagine, glutamine, derivatives thereof, or combinations thereof.

[0139] In certain instances, the conjugated lipid that inhibits aggregation of particles (*e.g.*, PEG-lipid conjugate) may comprise from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 1 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, from about 1.4 mol % to about 1.5 mol %, or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0140] In the lipid particles of the invention, the active agent or therapeutic agent may be fully encapsulated within the lipid portion of the particle, thereby protecting the active agent or therapeutic agent from enzymatic degradation. In preferred embodiments, a SNALP comprising a nucleic acid such as an interfering RNA (*e.g.*, siRNA) is fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the nucleic acid in the SNALP is not substantially degraded after exposure of the particle to a nuclease at 37°C for at least about 20, 30, 45, or 60 minutes. In certain other instances, the nucleic acid in the SNALP is not substantially degraded after incubation of the particle in serum at 37°C for at least about 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In other embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid such as siRNA) is complexed with the lipid portion of the particle. One of the benefits of the formulations of the present invention is that the lipid particle compositions are substantially non-toxic to mammals such as humans.

[0141] The term "fully encapsulated" indicates that the active agent or therapeutic agent in the lipid particle is not significantly degraded after exposure to serum or a nuclease or

protease assay that would significantly degrade free DNA, RNA, or protein. In a fully encapsulated system, preferably less than about 25% of the active agent or therapeutic agent in the particle is degraded in a treatment that would normally degrade 100% of free active agent or therapeutic agent, more preferably less than about 10%, and most preferably less than about 5% of the active agent or therapeutic agent in the particle is degraded. In the context of nucleic acid therapeutic agents, full encapsulation may be determined by an Oligreen® assay. Oligreen® is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA or RNA in solution (available from Invitrogen Corporation; Carlsbad, CA). "Fully encapsulated" also indicates that the lipid particles are serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

[0142] In another aspect, the present invention provides a lipid particle (*e.g.*, SNALP) composition comprising a plurality of lipid particles. In preferred embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid) is fully encapsulated within the lipid portion of the lipid particles (*e.g.*, SNALP), such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, %, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the lipid particles (*e.g.*, SNALP) have the active agent or therapeutic agent encapsulated therein.

[0143] Typically, the lipid particles (*e.g.*, SNALP) of the invention have a lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 1 to about 100. In some instances, the lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) ranges from about 1 to about 50, from about 2 to about 25, from about 3 to about 20, from about 4 to about 15, or from about 5 to about 10. In preferred embodiments, the lipid particles of the invention have a lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 5 to about 15, *e.g.*, about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 (or any fraction thereof or range therein).

[0144] Typically, the lipid particles (*e.g.*, SNALP) of the invention have a mean diameter of from about 40 nm to about 150 nm. In preferred embodiments, the lipid particles (*e.g.*, SNALP) of the invention have a mean diameter of from about 40 nm to about 130 nm, from about 40 nm to about 120 nm, from about 40 nm to about 100 nm, from about 50 nm to about 120 nm, from about 50 nm to about 100 nm, from about 60 nm to about 120 nm, from about 60 nm to about 110 nm, from about 60 nm to about 100 nm, from about 60 nm to about 90 nm, from about 60 nm to about 80 nm, from about 70 nm to about 120 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 70 nm to about 90 nm, from about 70 nm to about 80 nm, or less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm (or any fraction thereof or range therein).

[0145] In one specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:62" formulation. In a preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is cholesterol, and the conjugated lipid is a PEG-DAA conjugate. Although these are preferred embodiments of the 1:62 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic lipids (including other cholesterol derivatives), and conjugated lipids can be used in the 1:62 formulation as described herein.

[0146] In another specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:57" formulation. In one preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 5 mol % to about 9 mol % of the total lipid present in the particle (*e.g.*,

about 7.1 mol %) and the cholesterol (or cholesterol derivative) comprises from about 32 mol % to about 37 mol % of the total lipid present in the particle (*e.g.*, about 34.3 mol %), and the PEG-lipid is a PEG-DAA (*e.g.*, PEG-cDMA). In another preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.*, about 20 mol %) and the cholesterol (or cholesterol derivative) comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.*, about 20 mol %), and the PEG-lipid is a PEG-DAA (*e.g.*, PEG-cDMA). Although these are preferred embodiments of the 1:57 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic lipids (including other phospholipids and other cholesterol derivatives), and conjugated lipids can be used in the 1:57 formulation as described herein.

[0147] In preferred embodiments, the 1:62 SNALP formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-cDMA (or PEG-cDSA), about 61.5 mol % DLinDMA (or XTC2), and about 36.9 mol % cholesterol (or derivative thereof). In other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 7.1 mol % DPPC, and about 34.3 mol % cholesterol (or derivative thereof). In yet other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 20 mol % DPPC, and about 20 mol % cholesterol (or derivative thereof). It should be understood that these SNALP formulations are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the SNALP formulations may vary.

[0148] The present invention also provides a pharmaceutical composition comprising a lipid particle (*e.g.*, SNALP) described herein and a pharmaceutically acceptable carrier.

[0149] In a further aspect, the present invention provides a method for introducing one or more active agents or therapeutic agents (*e.g.*, nucleic acid) into a cell, comprising contacting the cell with a lipid particle (*e.g.*, SNALP) described herein. In one embodiment, the cell is in a mammal and the mammal is a human. In another embodiment, the present invention provides a method for the *in vivo* delivery of one or more active agents or therapeutic agents (*e.g.*, nucleic acid), comprising administering to a mammalian subject a lipid particle (*e.g.*, SNALP) described herein. In a preferred embodiment, the mode of administration includes,

but is not limited to, oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. Preferably, the mammalian subject is a human.

[0150] In one embodiment, at least about 5%, 10%, 15%, 20%, or 25% of the total injected dose of the lipid particles (*e.g.*, SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In other embodiments, more than about 20%, 30%, 40% and as much as about 60%, 70% or 80% of the total injected dose of the lipid particles (*e.g.*, SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In certain instances, more than about 10% of a plurality of the particles is present in the plasma of a mammal about 1 hour after administration. In certain other instances, the presence of the lipid particles (*e.g.*, SNALP) is detectable at least about 1 hour after administration of the particle. In certain embodiments, the presence of an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is detectable in cells of the lung, liver, tumor, or at a site of inflammation at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is detectable at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In yet other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) occurs preferentially in tumor cells or in cells at a site of inflammation. In further embodiments, the presence or effect of an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) in cells at a site proximal or distal to the site of administration or in cells of the lung, liver, or a tumor is detectable at about 12, 24, 48, 72, or 96 hours, or at about 6, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24, 26, or 28 days after administration. In additional embodiments, the lipid particles (*e.g.*, SNALP) of the invention are administered parenterally or intraperitoneally.

[0151] In some embodiments, the lipid particles (*e.g.*, SNALP) of the invention are particularly useful in methods for the therapeutic delivery of one or more nucleic acids comprising an interfering RNA sequence (*e.g.*, siRNA). In particular, it is an object of this invention to provide *in vitro* and *in vivo* methods for treatment of a disease or disorder in a mammal (*e.g.*, a rodent such as a mouse or a primate such as a human, chimpanzee, or monkey) by downregulating or silencing the transcription and/or translation of one or more target nucleic acid sequences or genes of interest. As a non-limiting example, the methods of the invention are useful for *in vivo* delivery of interfering RNA (*e.g.*, siRNA) to the liver and/or tumor of a mammalian subject. In certain embodiments, the disease or disorder is

associated with expression and/or overexpression of a gene and expression or overexpression of the gene is reduced by the interfering RNA (*e.g.*, siRNA). In certain other embodiments, a therapeutically effective amount of the lipid particle (*e.g.*, SNALP) may be administered to the mammal. In some instances, an interfering RNA (*e.g.*, siRNA) is formulated into a SNALP, and the particles are administered to patients requiring such treatment. In other instances, cells are removed from a patient, the interfering RNA (*e.g.*, siRNA) is delivered *in vitro* (*e.g.*, using a SNALP described herein), and the cells are reinjected into the patient.

[0152] In an additional aspect, the present invention provides lipid particles (*e.g.*, SNALP) comprising asymmetrical interfering RNA (aiRNA) molecules that silence the expression of a target gene and methods of using such particles to silence target gene expression.

[0153] In one embodiment, the aiRNA molecule comprises a double-stranded (duplex) region of about 10 to about 25 (base paired) nucleotides in length, wherein the aiRNA molecule comprises an antisense strand comprising 5' and 3' overhangs, and wherein the aiRNA molecule is capable of silencing target gene expression.

[0154] In certain instances, the aiRNA molecule comprises a double-stranded (duplex) region of about 12-20, 12-19, 12-18, 13-17, or 14-17 (base paired) nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 (base paired) nucleotides in length. In certain other instances, the 5' and 3' overhangs on the antisense strand comprise sequences that are complementary to the target RNA sequence, and may optionally further comprise nontargeting sequences. In some embodiments, each of the 5' and 3' overhangs on the antisense strand comprises or consists of one, two, three, four, five, six, seven, or more nucleotides.

[0155] In other embodiments, the aiRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0156] In a related aspect, the present invention provides lipid particles (*e.g.*, SNALP) comprising microRNA (miRNA) molecules that silence the expression of a target gene and methods of using such compositions to silence target gene expression.

[0157] In one embodiment, the miRNA molecule comprises about 15 to about 60 nucleotides in length, wherein the miRNA molecule is capable of silencing target gene expression.

[0158] In certain instances, the miRNA molecule comprises about 15-50, 15-40, or 15-30 nucleotides in length, more typically about 15-25 or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In a preferred embodiment, the miRNA molecule is a mature miRNA molecule targeting an RNA sequence of interest.

[0159] In some embodiments, the miRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0160] As such, the lipid particles of the invention (*e.g.*, SNALP) are advantageous and suitable for use in the administration of active agents or therapeutic agents such as nucleic acid (*e.g.*, interfering RNA such as siRNA, aiRNA, and/or miRNA) to a subject (*e.g.*, a mammal such as a human) because they are stable in circulation, of a size required for pharmacodynamic behavior resulting in access to extravascular sites, and are capable of reaching target cell populations.

IV. Active Agents

[0161] Active agents (*e.g.*, therapeutic agents) include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be, *e.g.*, biological, physiological, and/or cosmetic. Active agents may be any type of molecule or compound including, but not limited to, nucleic acids, peptides, polypeptides, small molecules, and mixtures thereof. Non-limiting examples of nucleic acids include interfering RNA molecules (*e.g.*, siRNA, aiRNA, miRNA), antisense oligonucleotides, plasmids, ribozymes, immunostimulatory oligonucleotides, and mixtures thereof. Examples of peptides or polypeptides include, without limitation, antibodies (*e.g.*, polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, Primatized™ antibodies), cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell-surface receptors and their ligands, hormones, and mixtures thereof. Examples of small molecules include, but are not limited to, small organic molecules or compounds such as any conventional agent or drug known to those of skill in the art.

[0162] In some embodiments, the active agent is a therapeutic agent, or a salt or derivative thereof. Therapeutic agent derivatives may be therapeutically active themselves or they may

be prodrugs, which become active upon further modification. Thus, in one embodiment, a therapeutic agent derivative retains some or all of the therapeutic activity as compared to the unmodified agent, while in another embodiment, a therapeutic agent derivative is a prodrug that lacks therapeutic activity, but becomes active upon further modification.

A. Nucleic Acids

[0163] In certain embodiments, lipid particles of the present invention are associated with a nucleic acid, resulting in a nucleic acid-lipid particle (*e.g.*, SNALP). In some embodiments, the nucleic acid is fully encapsulated in the lipid particle. As used herein, the term "nucleic acid" includes any oligonucleotide or polynucleotide, with fragments containing up to 60 nucleotides generally termed oligonucleotides, and longer fragments termed polynucleotides. In particular embodiments, oligonucletoides of the invention are from about 15 to about 60 nucleotides in length. Nucleic acid may be administered alone in the lipid particles of the invention, or in combination (*e.g.*, co-administered) with lipid particles of the invention comprising peptides, polypeptides, or small molecules such as conventional drugs.

[0164] In the context of this invention, the terms "polynucleotide" and "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally-occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also include polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake, reduced immunogenicity, and increased stability in the presence of nucleases.

[0165] Oligonucleotides are generally classified as deoxyribooligonucleotides or ribooligonucleotides. A deoxyribooligonucleotide consists of a 5-carbon sugar called deoxyribose joined covalently to phosphate at the 5′ and 3′ carbons of this sugar to form an alternating, unbranched polymer. A ribooligonucleotide consists of a similar repeating structure where the 5-carbon sugar is ribose.

[0166] The nucleic acid that is present in a lipid-nucleic acid particle according to this invention includes any form of nucleic acid that is known. The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Examples of double-stranded DNA are described herein and include, *e.g.*, structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA are described herein and include, *e.g.*,

siRNA and other RNAi agents such as aiRNA and pre-miRNA. Single-stranded nucleic acids include, *e.g.*, antisense oligonucleotides, ribozymes, mature miRNA, and triplex-forming oligonucleotides.

[0167] Nucleic acids of the invention may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to about 100,000 nucleotide residues in length. In particular embodiments, oligonucleotides may range from about 10 to about 100 nucleotides in length. In various related embodiments, oligonucleotides, both single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 60 nucleotides, from about 15 to about 60 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, or from about 20 to about 30 nucleotides in length.

[0168] In particular embodiments, an oligonucleotide (or a strand thereof) of the invention specifically hybridizes to or is complementary to a target polynucleotide sequence. The terms "specifically hybridizable" and "complementary" as used herein indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. In preferred embodiments, an oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target sequence interferes with the normal function of the target sequence to cause a loss of utility or expression therefrom, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or, in the case of *in vitro* assays, under conditions in which the assays are conducted. Thus, the oligonucleotide may include 1, 2, 3, or more base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

1. siRNA

[0169] The siRNA component of the nucleic acid-lipid particles of the present invention is capable of silencing the expression of a target gene of interest. Each strand of the siRNA duplex is typically about 15 to about 60 nucleotides in length, preferably about 15 to about 30 nucleotides in length. In certain embodiments, the siRNA comprises at least one modified nucleotide. The modified siRNA is generally less immunostimulatory than a corresponding unmodified siRNA sequence and retains RNAi activity against the target gene of interest. In

some embodiments, the modified siRNA contains at least one 2'OMe purine or pyrimidine nucleotide such as a 2'OMe-guanosine, 2'OMe-uridine, 2'OMe-adenosine, and/or 2'OMe-cytosine nucleotide. In preferred embodiments, one or more of the uridine and/or guanosine nucleotides are modified. The modified nucleotides can be present in one strand (*i.e.*, sense or antisense) or both strands of the siRNA. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell,* 107:309 (2001)), or may lack overhangs (*i.e.*, have blunt ends).

[0170] The modified siRNA generally comprises from about 1% to about 100% (*e.g.*, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region of the siRNA duplex. In certain embodiments, one, two, three, four, five, six, seven, eight, nine, ten, or more of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0171] In some embodiments, less than about 25% (*e.g.*, less than about 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0172] In other embodiments, from about 1% to about 25% (*e.g.*, from about 1%-25%, 2%-25%, 3%-25%, 4%-25%, 5%-25%, 6%-25%, 7%-25%, 8%-25%, 9%-25%, 10%-25%, 11%-25%, 12%-25%, 13%-25%, 14%-25%, 15%-25%, 16%-25%, 17%-25%, 18%-25%, 19%-25%, 20%-25%, 21%-25%, 22%-25%, 23%-25%, 24%-25%, *etc.*) or from about 1% to about 20% (*e.g.*, from about 1%-20%, 2%-20%, 3%-20%, 4%-20%, 5%-20%, 6%-20%, 7%-20%, 8%-20%, 9%-20%, 10%-20%, 11%-20%, 12%-20%, 13%-20%, 14%-20%, 15%-20%, 16%-20%, 17%-20%, 18%-20%, 19%-20%, 1%-19%, 2%-19%, 3%-19%, 4%-19%, 5%-19%, 6%-19%, 7%-19%, 8%-19%, 9%-19%, 10%-19%, 11%-19%, 12%-19%, 13%-19%, 14%-19%, 15%-19%, 16%-19%, 17%-19%, 18%-19%, 1%-18%, 2%-18%, 3%-18%, 4%-18%, 5%-18%, 6%-18%, 7%-18%, 8%-18%, 9%-18%, 10%-18%, 11%-18%, 12%-18%, 13%-18%, 14%-18%, 15%-18%, 16%-18%, 17%-18%, 1%-17%, 2%-17%, 3%-17%, 4%-17%, 5%-17%, 6%-17%, 7%-17%, 8%-17%, 9%-17%, 10%-17%, 11%-17%, 12%-17%, 13%-17%, 14%-17%, 15%-17%, 16%-17%, 1%-16%, 2%-16%, 3%-16%, 4%-16%, 5%-16%, 6%-16%, 7%-16%, 8%-16%, 9%-16%, 10%-16%, 11%-16%, 12%-16%, 13%-16%, 14%-16%, 15%-16%, 1%-15%, 2%-15%, 3%-15%, 4%-15%, 5%-15%, 6%-15%, 7%-15%, 8%-15%, 9%-

15%, 10%-15%, 11%-15%, 12%-15%, 13%-15%, 14%-15%, *etc.*) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0173] In further embodiments, *e.g.*, when one or both strands of the siRNA are selectively modified at uridine and/or guanosine nucleotides, the resulting modified siRNA can comprise less than about 30% modified nucleotides (*e.g.*, less than about 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% modified nucleotides) or from about 1% to about 30% modified nucleotides (*e.g.*, from about 1%-30%, 2%-30%, 3%-30%, 4%-30%, 5%-30%, 6%-30%, 7%-30%, 8%-30%, 9%-30%, 10%-30%, 11%-30%, 12%-30%, 13%-30%, 14%-30%, 15%-30%, 16%-30%, 17%-30%, 18%-30%, 19%-30%, 20%-30%, 21%-30%, 22%-30%, 23%-30%, 24%-30%, 25%-30%, 26%-30%, 27%-30%, 28%-30%, or 29%-30% modified nucleotides).

a. Selection of siRNA Sequences

[0174] Suitable siRNA sequences can be identified using any means known in the art. Typically, the methods described in Elbashir *et al.*, *Nature*, 411:494-498 (2001) and Elbashir *et al.*, *EMBO J.*, 20:6877-6888 (2001) are combined with rational design rules set forth in Reynolds *et al.*, *Nature Biotech.*, 22(3):326-330 (2004).

[0175] Generally, the nucleotide sequence 3' of the AUG start codon of a transcript from the target gene of interest is scanned for dinucleotide sequences (*e.g.*, AA, NA, CC, GG, or UU, wherein N = C, G, or U) (*see*, *e.g.*, Elbashir *et al.*, *EMBO J.*, 20:6877-6888 (2001)). The nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences (*i.e.*, a target sequence or a sense strand sequence). Typically, the 19, 21, 23, 25, 27, 29, 31, 33, 35, or more nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences. In some embodiments, the dinucleotide sequence is an AA or NA sequence and the 19 nucleotides immediately 3' to the AA or NA dinucleotide are identified as potential siRNA sequences. siRNA sequences are usually spaced at different positions along the length of the target gene. To further enhance silencing efficiency of the siRNA sequences, potential siRNA sequences may be analyzed to identify sites that do not contain regions of homology to other coding sequences, *e.g.*, in the target cell or organism. For example, a suitable siRNA sequence of about 21 base pairs typically will not have more than 16-17 contiguous base pairs of homology to coding sequences in the target cell or organism. If the siRNA sequences are to be expressed from an RNA Pol III promoter, siRNA sequences lacking more than 4 contiguous A's or T's are selected.

[0176] Once a potential siRNA sequence has been identified, a complementary sequence (*i.e.*, an antisense strand sequence) can be designed. A potential siRNA sequence can also be analyzed using a variety of criteria known in the art. For example, to enhance their silencing efficiency, the siRNA sequences may be analyzed by a rational design algorithm to identify sequences that have one or more of the following features: (1) G/C content of about 25% to about 60% G/C; (2) at least 3 A/Us at positions 15-19 of the sense strand; (3) no internal repeats; (4) an A at position 19 of the sense strand; (5) an A at position 3 of the sense strand; (6) a U at position 10 of the sense strand; (7) no G/C at position 19 of the sense strand; and (8) no G at position 13 of the sense strand. siRNA design tools that incorporate algorithms that assign suitable values of each of these features and are useful for selection of siRNA can be found at, *e.g.*, http://boz094.ust.hk/RNAi/siRNA. One of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may be selected for further analysis and testing as potential siRNA sequences.

[0177] Additionally, potential siRNA sequences with one or more of the following criteria can often be eliminated as siRNA: (1) sequences comprising a stretch of 4 or more of the same base in a row; (2) sequences comprising homopolymers of Gs (*i.e.*, to reduce possible non-specific effects due to structural characteristics of these polymers; (3) sequences comprising triple base motifs (*e.g.*, GGG, CCC, AAA, or TTT); (4) sequences comprising stretches of 7 or more G/Cs in a row; and (5) sequences comprising direct repeats of 4 or more bases within the candidates resulting in internal fold-back structures. However, one of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may still be selected for further analysis and testing as potential siRNA sequences.

[0178] In some embodiments, potential siRNA sequences may be further analyzed based on siRNA duplex asymmetry as described in, *e.g.*, Khvorova *et al.*, *Cell*, 115:209-216 (2003); and Schwarz *et al.*, *Cell*, 115:199-208 (2003). In other embodiments, potential siRNA sequences may be further analyzed based on secondary structure at the target site as described in, *e.g.*, Luo *et al.*, *Biophys. Res. Commun.*, 318:303-310 (2004). For example, secondary structure at the target site can be modeled using the Mfold algorithm (available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) to select siRNA sequences which favor accessibility at the target site where less secondary structure in the form of base-pairing and stem-loops is present.

[0179] Once a potential siRNA sequence has been identified, the sequence can be analyzed for the presence of any immunostimulatory properties, *e.g.*, using an *in vitro* cytokine assay or an *in vivo* animal model. Motifs in the sense and/or antisense strand of the siRNA

sequence such as GU-rich motifs (*e.g.*, 5'-GU-3', 5'-UGU-3', 5'-GUGU-3', 5'-UGUGU-3', *etc.*) can also provide an indication of whether the sequence may be immunostimulatory. Once an siRNA molecule is found to be immunostimulatory, it can then be modified to decrease its immunostimulatory properties as described herein. As a non-limiting example, an siRNA sequence can be contacted with a mammalian responder cell under conditions such that the cell produces a detectable immune response to determine whether the siRNA is an immunostimulatory or a non-immunostimulatory siRNA. The mammalian responder cell may be from a naïve mammal (*i.e.*, a mammal that has not previously been in contact with the gene product of the siRNA sequence). The mammalian responder cell may be, *e.g.*, a peripheral blood mononuclear cell (PBMC), a macrophage, and the like. The detectable immune response may comprise production of a cytokine or growth factor such as, *e.g.*, TNF-α, IFN-α, IFN-β, IFN-γ, IL-6, IL-12, or a combination thereof. An siRNA molecule identified as being immunostimulatory can then be modified to decrease its immunostimulatory properties by replacing at least one of the nucleotides on the sense and/or antisense strand with modified nucleotides. For example, less than about 30% (*e.g.*, less than about 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA duplex can be replaced with modified nucleotides such as 2'OMe nucleotides. The modified siRNA can then be contacted with a mammalian responder cell as described above to confirm that its immunostimulatory properties have been reduced or abrogated.

[0180] Suitable *in vitro* assays for detecting an immune response include, but are not limited to, the double monoclonal antibody sandwich immunoassay technique of David *et al.* (U.S. Patent No. 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide *et al.*, in Kirkham and Hunter, eds., *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh (1970)); the "Western blot" method of Gordon *et al.* (U.S. Patent No. 4,452,901); immunoprecipitation of labeled ligand (Brown *et al.*, *J. Biol. Chem.*, 255:4980-4983 (1980)); enzyme-linked immunosorbent assays (ELISA) as described, for example, by Raines *et al.*, *J. Biol. Chem.*, 257:5154-5160 (1982); immunocytochemical techniques, including the use of fluorochromes (Brooks *et al.*, *Clin. Exp. Immunol.*, 39:477 (1980)); and neutralization of activity (Bowen-Pope *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:2396-2400 (1984)). In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S. Patent Nos. 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0181] A non-limiting example of an *in vivo* model for detecting an immune response includes an *in vivo* mouse cytokine induction assay as described in, *e.g.*, Judge *et al.*, *Mol. Ther.*, 13:494-505 (2006). In certain embodiments, the assay that can be performed as follows: (1) siRNA can be administered by standard intravenous injection in the lateral tail vein; (2) blood can be collected by cardiac puncture about 6 hours after administration and processed as plasma for cytokine analysis; and (3) cytokines can be quantified using sandwich ELISA kits according to the manufacturer's instructions (*e.g.*, mouse and human IFN-α (PBL Biomedical; Piscataway, NJ); human IL-6 and TNF-α (eBioscience; San Diego, CA); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences; San Diego, CA)).

[0182] Monoclonal antibodies that specifically bind cytokines and growth factors are commercially available from multiple sources and can be generated using methods known in the art (*see*, *e.g.*, Kohler *et al.*, *Nature*, 256: 495-497 (1975) and Harlow and Lane, ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publication, New York (1999)). Generation of monoclonal antibodies has been previously described and can be accomplished by any means known in the art (Buhring *et al.*, in Hybridoma, Vol. 10, No. 1, pp. 77-78 (1991)). In some methods, the monoclonal antibody is labeled (*e.g.*, with any composition detectable by spectroscopic, photochemical, biochemical, electrical, optical, or chemical means) to facilitate detection.

b. Generating siRNA Molecules

[0183] siRNA can be provided in several forms including, *e.g.*, as one or more isolated small-interfering RNA (siRNA) duplexes, as longer double-stranded RNA (dsRNA), or as siRNA or dsRNA transcribed from a transcriptional cassette in a DNA plasmid. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell*, 107:309 (2001), or may lack overhangs (*i.e.*, to have blunt ends).

[0184] An RNA population can be used to provide long precursor RNAs, or long precursor RNAs that have substantial or complete identity to a selected target sequence can be used to make the siRNA. The RNAs can be isolated from cells or tissue, synthesized, and/or cloned according to methods well known to those of skill in the art. The RNA can be a mixed population (obtained from cells or tissue, transcribed from cDNA, subtracted, selected, *etc.*), or can represent a single target sequence. RNA can be naturally occurring (*e.g.*, isolated from tissue or cell samples), synthesized *in vitro (e.g.*, using T7 or SP6 polymerase and PCR products or a cloned cDNA), or chemically synthesized.

[0185] To form a long dsRNA, for synthetic RNAs, the complement is also transcribed *in vitro* and hybridized to form a dsRNA. If a naturally occuring RNA population is used, the RNA complements are also provided (*e.g.*, to form dsRNA for digestion by *E. coli* RNAse III or Dicer), *e.g.*, by transcribing cDNAs corresponding to the RNA population, or by using RNA polymerases. The precursor RNAs are then hybridized to form double stranded RNAs for digestion. The dsRNAs can be directly administered to a subject or can be digested *in vitro* prior to administration.

[0186] Methods for isolating RNA, synthesizing RNA, hybridizing nucleic acids, making and screening cDNA libraries, and performing PCR are well known in the art (*see, e.g.,* Gubler and Hoffman, *Gene*, 25:263-269 (1983); Sambrook *et al., supra*; Ausubel *et al., supra*), as are PCR methods (*see,* U.S. Patent Nos. 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Expression libraries are also well known to those of skill in the art. Additional basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0187] Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules of the invention can be synthesized using any of a variety of techniques known in the art, such as those described in Usman *et al.*, *J. Am. Chem. Soc.*, 109:7845 (1987); Scaringe *et al.*, *Nucl. Acids Res.*, 18:5433 (1990); Wincott *et al.*, *Nucl. Acids Res.*, 23:2677-2684 (1995); and Wincott *et al., Methods Mol. Bio.*, 74:59 (1997). The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example, small scale syntheses can be conducted on an Applied Biosystems synthesizer using a 0.2 μmol scale protocol. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer from Protogene (Palo Alto, CA). However, a larger or smaller scale of synthesis is also within the scope of this invention. Suitable reagents for oligonucleotide synthesis, methods for RNA deprotection, and methods for RNA purification are known to those of skill in the art.

[0188] siRNA molecules can also be synthesized via a tandem synthesis technique, wherein both strands are synthesized as a single continuous oligonucleotide fragment or strand separated by a cleavable linker that is subsequently cleaved to provide separate fragments or

strands that hybridize to form the siRNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siRNA can be readily adapted to both multiwell/multiplate synthesis platforms as well as large scale synthesis platforms employing batch reactors, synthesis columns, and the like. Alternatively, siRNA molecules can be assembled from two distinct oligonucleotides, wherein one oligonucleotide comprises the sense strand and the other comprises the antisense strand of the siRNA. For example, each strand can be synthesized separately and joined together by hybridization or ligation following synthesis and/or deprotection. In certain other instances, siRNA molecules can be synthesized as a single continuous oligonucleotide fragment, where the self-complementary sense and antisense regions hybridize to form an siRNA duplex having hairpin secondary structure.

c. Modifying siRNA Sequences

[0189] In certain aspects, siRNA molecules comprise a duplex having two strands and at least one modified nucleotide in the double-stranded region, wherein each strand is about 15 to about 60 nucleotides in length. Advantageously, the modified siRNA is less immunostimulatory than a corresponding unmodified siRNA sequence, but retains the capability of silencing the expression of a target sequence. In preferred embodiments, the degree of chemical modifications introduced into the siRNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the siRNA and retention of RNAi activity. As a non-limiting example, an siRNA molecule that targets a gene of interest can be minimally modified (*e.g.*, less than about 30%, 25%, 20%, 15%, 10%, or 5% modified) at selective uridine and/or guanosine nucleotides within the siRNA duplex to eliminate the immune response generated by the siRNA while retaining its capability to silence target gene expression.

[0190] Examples of modified nucleotides suitable for use in the invention include, but are not limited to, ribonucleotides having a 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl group. Modified nucleotides having a Northern conformation such as those described in, *e.g.*, Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag Ed. (1984), are also suitable for use in siRNA molecules. Such modified nucleotides include, without limitation, locked nucleic acid (LNA) nucleotides (*e.g.*, 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides),

2'-O-(2-methoxyethyl) (MOE) nucleotides, 2'-methyl-thio-ethyl nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy-2'-chloro (2'Cl) nucleotides, and 2'-azido nucleotides. In certain instances, the siRNA molecules described herein include one or more G-clamp nucleotides. A G-clamp nucleotide refers to a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine nucleotide within a duplex (*see, e.g.,* Lin *et al., J. Am. Chem. Soc.,* 120:8531-8532 (1998)). In addition, nucleotides having a nucleotide base analog such as, for example, C-phenyl, C-naphthyl, other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole (*see, e.g.,* Loakes, *Nucl. Acids Res.,* 29:2437-2447 (2001)) can be incorporated into siRNA molecules.

[0191] In certain embodiments, siRNA molecules may further comprise one or more chemical modifications such as terminal cap moieties, phosphate backbone modifications, and the like. Examples of terminal cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4',5'-methylene nucleotides, 1-(β-D-erythrofuranosyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α-nucleotides, modified base nucleotides, *threo*-pentofuranosyl nucleotides, acyclic 3',4'-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3'-3'-inverted nucleotide moieties, 3'-3'-inverted abasic moieties, 3'-2'-inverted nucleotide moieties, 3'-2'-inverted abasic moieties, 5'-5'-inverted nucleotide moieties, 5'-5'-inverted abasic moieties, 3'-5'-inverted deoxy abasic moieties, 5'-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, 6-aminohexyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3'-phosphoramidate, 5'-phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 5'-amino, 3'-phosphorothioate, 5'-phosphorothioate, phosphorodithioate, and bridging or non-bridging methylphosphonate or 5'-mercapto moieties (*see, e.g.,* U.S. Patent No. 5,998,203; Beaucage *et al., Tetrahedron* 49:1925 (1993)). Non-limiting examples of phosphate backbone modifications (*i.e.,* resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and alkylsilyl substitutions (*see, e.g.,* Hunziker *et al., Nucleic Acid Analogues: Synthesis and Properties,* in *Modern Synthetic Methods,* VCH, 331-417 (1995);

Mesmaeker *et al.*, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39 (1994)). Such chemical modifications can occur at the 5'-end and/or 3'-end of the sense strand, antisense strand, or both strands of the siRNA. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0192] In some embodiments, the sense and/or antisense strand of the siRNA molecule can further comprise a 3'-terminal overhang having about 1 to about 4 (*e.g.*, 1, 2, 3, or 4) 2'-deoxy ribonucleotides and/or any combination of modified and unmodified nucleotides. Additional examples of modified nucleotides and types of chemical modifications that can be introduced into siRNA molecules are described, *e.g.*, in UK Patent No. GB 2,397,818 B and U.S. Patent Publication Nos. 20040192626, 20050282188, and 20070135372, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0193] The siRNA molecules described herein can optionally comprise one or more non-nucleotides in one or both strands of the siRNA. As used herein, the term "non-nucleotide" refers to any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base such as adenosine, guanine, cytosine, uracil, or thymine and therefore lacks a base at the 1'-position.

[0194] In other embodiments, chemical modification of the siRNA comprises attaching a conjugate to the siRNA molecule. The conjugate can be attached at the 5' and/or 3'-end of the sense and/or antisense strand of the siRNA via a covalent attachment such as, *e.g.*, a biodegradable linker. The conjugate can also be attached to the siRNA, *e.g.*, through a carbamate group or other linking group (*see, e.g.*, U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). In certain instances, the conjugate is a molecule that facilitates the delivery of the siRNA into a cell. Examples of conjugate molecules suitable for attachment to siRNA include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (*e.g.*, folic acid, folate analogs and derivatives thereof), sugars (*e.g.*, galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, *etc.*), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof (*see, e.g.*, U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Patent No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule,

oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Yet other examples include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidininium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples include the hydrophobic group, membrane active compound, cell penetrating compound, cell targeting signal, interaction modifier, and steric stabilizer conjugate molecules described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described in U.S. Patent Publication No. 20050239739. The type of conjugate used and the extent of conjugation to the siRNA molecule can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the siRNA while retaining RNAi activity. As such, one skilled in the art can screen siRNA molecules having various conjugates attached thereto to identify ones having improved properties and full RNAi activity using any of a variety of well-known *in vitro* cell culture or *in vivo* animal models. The disclosures of the above-described patent documents are herein incorporated by reference in their entirety for all purposes.

d. Target Genes

[0195] The siRNA component of the nucleic acid-lipid particles described herein can be used to downregulate or silence the translation (*i.e.*, expression) of a gene of interest. Genes of interest include, but are not limited to, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders (*e.g.*, liver diseases and disorders), genes associated with tumorigenesis and cell transformation (*e.g.*, cancer), angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0196] Genes associated with viral infection and survival include those expressed by a virus in order to bind, enter, and replicate in a cell. Of particular interest are viral sequences associated with chronic viral diseases. Viral sequences of particular interest include sequences of Filoviruses such as Ebola virus and Marburg virus (*see, e.g.,* Geisbert *et al., J. Infect. Dis.*, 193:1650-1657 (2006)); Arenaviruses such as Lassa virus, Junin virus, Machupo virus, Guanarito virus, and Sabia virus (Buchmeier *et al., Arenaviridae*: the viruses and their replication, In: FIELDS VIROLOGY, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia, (2001)); Influenza viruses such as Influenza A, B, and C viruses, (*see, e.g.,*

Steinhauer *et al.*, *Annu Rev Genet.*, 36:305-332 (2002); and Neumann *et al.*, *J Gen Virol.*, 83:2635-2662 (2002)); Hepatitis viruses (*see, e.g.*, Hamasaki *et al.*, *FEBS Lett.*, 543:51 (2003); Yokota *et al.*, *EMBO Rep.*, 4:602 (2003); Schlomai *et al.*, *Hepatology*, 37:764 (2003); Wilson *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2783 (2003); Kapadia *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2014 (2003); and FIELDS VIROLOGY, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia (2001)); Human Immunodeficiency Virus (HIV) (Banerjea *et al.*, *Mol. Ther.*, 8:62 (2003); Song *et al.*, *J. Virol.*, 77:7174 (2003); Stephenson, *JAMA*, 289:1494 (2003); Qin *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:183 (2003)); Herpes viruses (Jia *et al.*, *J. Virol.*, 77:3301 (2003)); and Human Papilloma Viruses (HPV) (Hall *et al.*, *J. Virol.*, 77:6066 (2003); Jiang *et al.*, *Oncogene*, 21:6041 (2002)).

[0197] Exemplary Filovirus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding structural proteins (*e.g.*, VP30, VP35, nucleoprotein (NP), polymerase protein (L-pol)) and membrane-associated proteins (*e.g.*, VP40, glycoprotein (GP), VP24). Complete genome sequences for Ebola virus are set forth in, *e.g.*, Genbank Accession Nos. NC_002549; AY769362; NC_006432; NC_004161; AY729654; AY354458; AY142960; AB050936; AF522874; AF499101; AF272001; and AF086833. Ebola virus VP24 sequences are set forth in, *e.g.*, Genbank Accession Nos. U77385 and AY058897. Ebola virus L-pol sequences are set forth in, *e.g.*, Genbank Accession No. X67110. Ebola virus VP40 sequences are set forth in, *e.g.*, Genbank Accession No. AY058896. Ebola virus NP sequences are set forth in, *e.g.*, Genbank Accession No. AY058895. Ebola virus GP sequences are set forth in, *e.g.*, Genbank Accession No. AY058898; Sanchez *et al.*, *Virus Res.*, 29:215-240 (1993); Will *et al.*, *J. Virol.*, 67:1203-1210 (1993); Volchkov *et al.*, *FEBS Lett.*, 305:181-184 (1992); and U.S. Patent No. 6,713,069. Additional Ebola virus sequences are set forth in, *e.g.*, Genbank Accession Nos. L11365 and X61274. Complete genome sequences for Marburg virus are set forth in, *e.g.*, Genbank Accession Nos. NC_001608; AY430365; AY430366; and AY358025. Marburg virus GP sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005734; AF005733; and AF005732. Marburg virus VP35 sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005731 and AF005730. Additional Marburg virus sequences are set forth in, *e.g.*, Genbank Accession Nos. X64406; Z29337; AF005735; and Z12132. Non-limiting examples of siRNA molecules targeting Ebola virus and Marburg virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070135370, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0198] Exemplary Influenza virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding nucleoprotein (NP), matrix proteins (M1 and M2), nonstructural proteins (NS1 and NS2), RNA polymerase (PA, PB1, PB2), neuraminidase (NA), and haemagglutinin (HA). Influenza A NP sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004522; AY818138; AB166863; AB188817; AB189046; AB189054; AB189062; AY646169; AY646177; AY651486; AY651493; AY651494; AY651495; AY651496; AY651497; AY651498; AY651499; AY651500; AY651501; AY651502; AY651503; AY651504; AY651505; AY651506; AY651507; AY651509; AY651528; AY770996; AY790308; AY818138; and AY818140. Influenza A PA sequences are set forth in, *e.g.*, Genbank Accession Nos. AY818132; AY790280; AY646171; AY818132;AY818133; AY646179; AY818134; AY551934; AY651613; AY651610; AY651620; AY651617; AY651600; AY651611; AY651606; AY651618; AY651608; AY651607; AY651605; AY651609; AY651615; AY651616; AY651640; AY651614; AY651612; AY651621; AY651619; AY770995; and AY724786. Non-limiting examples of siRNA molecules targeting Influenza virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070218122, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0199] Exemplary hepatitis virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences involved in transcription and translation (*e.g.*, En1, En2, X, P) and nucleic acid sequences encoding structural proteins (*e.g.*, core proteins including C and C-related proteins, capsid and envelope proteins including S, M, and/or L proteins, or fragments thereof) (*see*, *e.g.*, FIELDS VIROLOGY, *supra*). Exemplary Hepatits C virus (HCV) nucleic acid sequences that can be silenced include, but are not limited to, the 5'-untranslated region (5'-UTR), the 3'-untranslated region (3'-UTR), the polyprotein translation initiation codon region, the internal ribosome entry site (IRES) sequence, and/or nucleic acid sequences encoding the core protein, the E1 protein, the E2 protein, the p7 protein, the NS2 protein, the NS3 protease/helicase, the NS4A protein, the NS4B protein, the NS5A protein, and/or the NS5B RNA-dependent RNA polymerase. HCV genome sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004102 (HCV genotype 1a), AJ238799 (HCV genotype 1b), NC_009823 (HCV genotype 2), NC_009824 (HCV genotype 3), NC_009825 (HCV genotype 4), NC_009826 (HCV genotype 5), and NC_009827 (HCV genotype 6). Hepatitis A virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001489; Hepatitis B virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_003977; Hepatitis D virus nucleic acid sequence are set forth in,

e.g., Genbank Accession No. NC_001653; Hepatitis E virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001434; and Hepatitis G virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001710. Silencing of sequences that encode genes associated with viral infection and survival can conveniently be used in combination with the administration of conventional agents used to treat the viral condition. Non-limiting examples of siRNA molecules targeting hepatitis virus nucleic acid sequences include those described in U.S. Patent Publication Nos. 20060281175, 20050058982, and 20070149470; U.S. Patent No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed March 20, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0200] Genes associated with metabolic diseases and disorders (*e.g.*, disorders in which the liver is the target and liver diseases and disorders) include, for example, genes expressed in dyslipidemia (*e.g.*, liver X receptors such as LXRα and LXRβ (Genback Accession No. NM_007121), farnesoid X receptors (FXR) (Genbank Accession No. NM_005123), sterol-regulatory element binding protein (SREBP), site-1 protease (S1P), 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG coenzyme-A reductase), apolipoprotein B (ApoB) (Genbank Accession No. NM_000384), apolipoprotein CIII (ApoC3) (Genbank Accession Nos. NM_000040 and NG_008949 REGION: 5001..8164), and apolipoprotein E (ApoE) (Genbank Accession Nos. NM_000041 and NG_007084 REGION: 5001..8612)); and diabetes (*e.g.*, glucose 6-phosphatase) (*see*, *e.g.*, Forman *et al.*, *Cell*, 81:687 (1995); Seol *et al.*, *Mol. Endocrinol.*, 9:72 (1995), Zavacki *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:7909 (1997); Sakai *et al.*, *Cell*, 85:1037-1046 (1996); Duncan *et al.*, *J. Biol. Chem.*, 272:12778-12785 (1997); Willy *et al.*, *Genes Dev.*, 9:1033-1045 (1995); Lehmann *et al.*, *J. Biol. Chem.*, 272:3137-3140 (1997); Janowski *et al.*, *Nature*, 383:728-731 (1996); and Peet *et al.*, *Cell*, 93:693-704 (1998)). One of skill in the art will appreciate that genes associated with metabolic diseases and disorders (*e.g.*, diseases and disorders in which the liver is a target and liver diseases and disorders) include genes that are expressed in the liver itself as well as and genes expressed in other organs and tissues. Silencing of sequences that encode genes associated with metabolic diseases and disorders can conveniently be used in combination with the administration of conventional agents used to treat the disease or disorder. Non-limiting examples of siRNA molecules targeting the ApoB gene include those described in U.S. Patent Publication No. 20060134189, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules

targeting the ApoC3 gene include those described in U.S. Provisional Application No. 61/147,235, filed January 26, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0201] Examples of gene sequences associated with tumorigenesis and cell transformation (*e.g.*, cancer or other neoplasia) include mitotic kinesins such as Eg5 (KSP, KIF11; Genbank Accession No. NM_004523); serine/threonine kinases such as polo-like kinase 1 (PLK-1) (Genbank Accession No. NM_005030; Barr *et al.*, *Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)); tyrosine kinases such as WEE1 (Genbank Accession Nos. NM_003390 and NM_001143976); inhibitors of apoptosis such as XIAP (Genbank Accession No. NM_001167); COP9 signalosome subunits such as CSN1, CSN2, CSN3, CSN4, CSN5 (JAB1; Genbank Accession No. NM_006837); CSN6, CSN7A, CSN7B, and CSN8; ubiquitin ligases such as COP1 (RFWD2; Genbank Accession Nos. NM_022457 and NM_001001740); and histone deacetylases such as HDAC1, HDAC2 (Genbank Accession No. NM_001527), HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, *etc.* Non-limiting examples of siRNA molecules targeting the Eg5 and XIAP genes include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the PLK-1 gene include those described in U.S. Patent Publication Nos. 20050107316 and 20070265438; and U.S. Patent Application No. 12/343,342, filed December 23, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of siRNA molecules targeting the CSN5 gene include those described in U.S. Provisional Application No. 61/045,251, filed April 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0202] Additional examples of gene sequences associated with tumorigenesis and cell transformation include translocation sequences such as MLL fusion genes, BCR-ABL (Wilda *et al.*, *Oncogene*, 21:5716 (2002); Scherr *et al.*, *Blood*, 101:1566 (2003)), TEL-AML1, EWS-FLI1, TLS-FUS, PAX3-FKHR, BCL-2, AML1-ETO, and AML1-MTG8 (Heidenreich *et al.*, *Blood*, 101:3157 (2003)); overexpressed sequences such as multidrug resistance genes (Nieth *et al.*, *FEBS Lett.*, 545:144 (2003); Wu *et al*, *Cancer Res.* 63:1515 (2003)), cyclins (Li *et al.*, *Cancer Res.*, 63:3593 (2003); Zou *et al.*, *Genes Dev.*, 16:2923 (2002)), beta-catenin (Verma *et al.*, *Clin Cancer Res.*, 9:1291 (2003)), telomerase genes (Kosciolek *et al.*, *Mol Cancer Ther.*, 2:209 (2003)), c-MYC, N-MYC, BCL-2, growth factor receptors (*e.g.*, EGFR/ErbB1 (Genbank Accession Nos. NM_005228, NM_201282, NM_201283, and NM_201284; *see*

also, Nagy *et al. Exp. Cell Res.,* 285:39-49 (2003), ErbB2/HER-2 (Genbank Accession Nos. NM_004448 and NM_001005862), ErbB3 (Genbank Accession Nos. NM_001982 and NM_001005915), and ErbB4 (Genbank Accession Nos. NM_005235 and NM_001042599); and mutated sequences such as RAS (reviewed in Tuschl and Borkhardt, *Mol. Interventions,* 2:158 (2002)). Non-limiting examples of siRNA molecules targeting the EGFR gene include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0203] Silencing of sequences that encode DNA repair enzymes find use in combination with the administration of chemotherapeutic agents (Collis *et al.*, *Cancer Res.*, 63:1550 (2003)). Genes encoding proteins associated with tumor migration are also target sequences of interest, for example, integrins, selectins, and metalloproteinases. The foregoing examples are not exclusive. Those of skill in the art will understand that any whole or partial gene sequence that facilitates or promotes tumorigenesis or cell transformation, tumor growth, or tumor migration can be included as a template sequence.

[0204] Angiogenic genes are able to promote the formation of new vessels. Of particular interest is vascular endothelial growth factor (VEGF) (Reich *et al.*, *Mol. Vis.*, 9:210 (2003)) or VEGFR. siRNA sequences that target VEGFR are set forth in, *e.g.*, GB 2396864; U.S. Patent Publication No. 20040142895; and CA 2456444, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0205] Anti-angiogenic genes are able to inhibit neovascularization. These genes are particularly useful for treating those cancers in which angiogenesis plays a role in the pathological development of the disease. Examples of anti-angiogenic genes include, but are not limited to, endostatin (*see, e.g.,* U.S. Patent No. 6,174,861), angiostatin (*see, e.g.,* U.S. Patent No. 5,639,725), and VEGFR2 (*see, e.g.,* Decaussin *et al.*, *J. Pathol.*, 188: 369-377 (1999)), the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0206] Immunomodulator genes are genes that modulate one or more immune responses. Examples of immunomodulator genes include, without limitation, cytokines such as growth factors (*e.g.*, TGF-α, TGF-□β, EGF, FGF, IGF, NGF, PDGF, CGF, GM-CSF, SCF, *etc.*), interleukins (*e.g.*, IL-2, IL-4, IL-12 (Hill *et al.*, *J. Immunol.*, 171:691 (2003)), IL-15, IL-18, IL-20, *etc.*), interferons (*e.g.*, IFN-α, IFN-β, IFN-γ, *etc.*) and TNF. Fas and Fas ligand genes are also immunomodulator target sequences of interest (Song *et al.*, *Nat. Med.,* 9:347 (2003)). Genes encoding secondary signaling molecules in hematopoietic and lymphoid cells are also

included in the present invention, for example, Tec family kinases such as Bruton's tyrosine kinase (Btk) (Heinonen *et al.*, *FEBS Lett.*, 527:274 (2002)).

[0207] Cell receptor ligands include ligands that are able to bind to cell surface receptors (*e.g.*, insulin receptor, EPO receptor, G-protein coupled receptors, receptors with tyrosine kinase activity, cytokine receptors, growth factor receptors, *etc.*), to modulate (*e.g.*, inhibit, activate, *etc.*) the physiological pathway that the receptor is involved in (*e.g.*, glucose level modulation, blood cell development, mitogenesis, *etc.*). Examples of cell receptor ligands include, but are not limited to, cytokines, growth factors, interleukins, interferons, erythropoietin (EPO), insulin, glucagon, G-protein coupled receptor ligands, *etc.* Templates coding for an expansion of trinucleotide repeats (*e.g.*, CAG repeats) find use in silencing pathogenic sequences in neurodegenerative disorders caused by the expansion of trinucleotide repeats, such as spinobulbular muscular atrophy and Huntington's Disease (Caplen *et al.*, *Hum. Mol. Genet.*, 11:175 (2002)).

[0208] In addition to its utility in silencing the expression of any of the above-described genes for therapeutic purposes, the siRNA described herein are also useful in research and development applications as well as diagnostic, prophylactic, prognostic, clinical, and other healthcare applications. As a non-limiting example, the siRNA can be used in target validation studies directed at testing whether a gene of interest has the potential to be a therapeutic target. The siRNA can also be used in target identification studies aimed at discovering genes as potential therapeutic targets.

2. aiRNA

[0209] Like siRNA, asymmetrical interfering RNA (aiRNA) can recruit the RNA-induced silencing complex (RISC) and lead to effective silencing of a variety of genes in mammalian cells by mediating sequence-specific cleavage of the target sequence between nucleotide 10 and 11 relative to the 5' end of the antisense strand (Sun *et al.*, *Nat. Biotech.*, 26:1379-1382 (2008)). Typically, an aiRNA molecule comprises a short RNA duplex having a sense strand and an antisense strand, wherein the duplex contains overhangs at the 3' and 5' ends of the antisense strand. The aiRNA is generally asymmetric because the sense strand is shorter on both ends when compared to the complementary antisense strand. In some aspects, aiRNA molecules may be designed, synthesized, and annealed under conditions similar to those used for siRNA molecules. As a non-limiting example, aiRNA sequences may be selected and generated using the methods described above for selecting siRNA sequences.

[0210] In another embodiment, aiRNA duplexes of various lengths (*e.g.*, about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 base pairs, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 base pairs) may be designed with overhangs at the 3' and 5' ends of the antisense strand to target an mRNA of interest. In certain instances, the sense strand of the aiRNA molecule is about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length. In certain other instances, the antisense strand of the aiRNA molecule is about 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 nucleotides in length.

[0211] In some embodiments, the 5' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*, "AA", "UU", "dTdT", *etc.*). In other embodiments, the 3' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*, "AA", "UU", "dTdT", *etc.*). In certain aspects, the aiRNA molecules described herein may comprise one or more modified nucleotides, *e.g.*, in the double-stranded (duplex) region and/or in the antisense overhangs. As a non-limiting example, aiRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0212] In certain embodiments, aiRNA molecules may comprise an antisense strand which corresponds to the antisense strand of an siRNA molecule, *e.g.*, one of the siRNA molecules described herein. In other embodiments, aiRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.*, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

3. miRNA

[0213] Generally, microRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. miRNAs are encoded by genes from whose DNA they are transcribed, but miRNAs are not translated into protein (non-coding RNA); instead, each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional mature miRNA. Mature miRNA molecules are either partially or completely complementary to one or more

messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression. The identification of miRNA molecules is described, *e.g.*, in Lagos-Quintana *et al.*, *Science*, 294:853-858; Lau *et al.*, *Science*, 294:858-862; and Lee *et al.*, *Science*, 294:862-864.

[0214] The genes encoding miRNA are much longer than the processed mature miRNA molecule. miRNA are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail and processed to short, ~70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha (Denli *et al.*, *Nature*, 432:231-235 (2004)). These pre-miRNA are then processed to mature miRNA in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC) (Bernstein *et al.*, *Nature*, 409:363-366 (2001). Either the sense strand or antisense strand of DNA can function as templates to give rise to miRNA.

[0215] When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC complex. This strand is known as the guide strand and is selected by the argonaute protein, the catalytically active RNase in the RISC complex, on the basis of the stability of the 5' end (Preall *et al.*, *Curr. Biol.*, 16:530-535 (2006)). The remaining strand, known as the anti-guide or passenger strand, is degraded as a RISC complex substrate (Gregory *et al.*, *Cell*, 123:631-640 (2005)). After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and induce target mRNA degradation and/or translational silencing.

[0216] Mammalian miRNA molecules are usually complementary to a site in the 3' UTR of the target mRNA sequence. In certain instances, the annealing of the miRNA to the target mRNA inhibits protein translation by blocking the protein translation machinery. In certain other instances, the annealing of the miRNA to the target mRNA facilitates the cleavage and degradation of the target mRNA through a process similar to RNA interference (RNAi). miRNA may also target methylation of genomic sites which correspond to targeted mRNA. Generally, miRNA function in association with a complement of proteins collectively termed the miRNP.

[0217] In certain aspects, the miRNA molecules described herein are about 15-100, 15-90, 15-80, 15-75, 15-70, 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In certain other aspects, miRNA molecules may comprise one or more

modified nucleotides. As a non-limiting example, miRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0218] In some embodiments, miRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.*, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0219] In other embodiments, one or more agents that block the activity of a miRNA targeting an mRNA of interest are administered using a lipid particle of the invention (*e.g.*, a nucleic acid-lipid particle). Examples of blocking agents include, but are not limited to, steric blocking oligonucleotides, locked nucleic acid oligonucleotides, and Morpholino oligonucleotides. Such blocking agents may bind directly to the miRNA or to the miRNA binding site on the target mRNA.

4. Antisense Oligonucleotides

[0220] In one embodiment, the nucleic acid is an antisense oligonucleotide directed to a target gene or sequence of interest. The terms "antisense oligonucleotide" or "antisense" include oligonucleotides that are complementary to a targeted polynucleotide sequence. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence. Antisense RNA oligonucleotides prevent the translation of complementary RNA strands by binding to the RNA. Antisense DNA oligonucleotides can be used to target a specific, complementary (coding or non-coding) RNA. If binding occurs, this DNA/RNA hybrid can be degraded by the enzyme RNase H. In a particular embodiment, antisense oligonucleotides comprise from about 10 to about 60 nucleotides, more preferably from about 15 to about 30 nucleotides. The term also encompasses antisense oligonucleotides that may not be exactly complementary to the desired target gene. Thus, the invention can be utilized in instances where non-target specific-activities are found with antisense, or where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use.

[0221] Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein

synthesis by a targeted gene. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (*see,* U.S. Patent Nos. 5,739,119 and 5,759,829). Furthermore, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDR1), ICAM-1, E-selectin, STK-1, striatal GABAA receptor, and human EGF (*see,* Jaskulski *et al.*, *Science*, 240:1544-6 (1988); Vasanthakumar *et al.*, *Cancer Commun.*, 1:225-32 (1989); Peris *et al.*, *Brain Res Mol Brain Res.*, 15;57:310-20 (1998); and U.S. Patent Nos. 5,801,154; 5,789,573; 5,718,709 and 5,610,288). Moreover, antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.*, cancer (*see,* U.S. Patent Nos. 5,747,470; 5,591,317; and 5,783,683). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0222] Methods of producing antisense oligonucleotides are known in the art and can be readily adapted to produce an antisense oligonucleotide that targets any polynucleotide sequence. Selection of antisense oligonucleotide sequences specific for a given target sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense oligonucleotides may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-402 (1997)).

5. Ribozymes

[0223] According to another embodiment of the invention, nucleic acid-lipid particles are associated with ribozymes. Ribozymes are RNA-protein complexes having specific catalytic domains that possess endonuclease activity (*see,* Kim *et al.*, *Proc. Natl. Acad. Sci. USA.*, 84:8788-92 (1987); and Forster *et al.*, *Cell*, 49:211-20 (1987)). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (*see,* Cech *et*

al., *Cell*, 27:487-96 (1981); Michel *et al.*, *J. Mol. Biol.*, 216:585-610 (1990); Reinhold-Hurek *et al.*, *Nature*, 357:173-6 (1992)). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

[0224] At least six basic varieties of naturally-occurring enzymatic RNA molecules are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

[0225] The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence), or Neurospora VS RNA motif, for example. Specific examples of hammerhead motifs are described in, *e.g.*, Rossi *et al.*, *Nucleic Acids Res.*, 20:4559-65 (1992). Examples of hairpin motifs are described in, *e.g.*, EP 0360257, Hampel *et al.*, *Biochemistry*, 28:4929-33 (1989); Hampel *et al.*, *Nucleic Acids Res.*, 18:299-304 (1990); and U.S. Patent No. 5,631,359. An example of the hepatitis δ virus motif is described in, *e.g.*, Perrotta *et al.*, *Biochemistry*, 31:11843-52 (1992). An example of the RNaseP motif is described in, *e.g.*, Guerrier-Takada *et al.*, *Cell*, 35:849-57 (1983). Examples of the Neurospora VS RNA ribozyme motif is described in, *e.g.*, Saville *et al.*, *Cell*, 61:685-96 (1990); Saville *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:8826-30 (1991); Collins *et al.*, *Biochemistry*, 32:2795-9 (1993). An example of the Group I intron is described in, *e.g.*, U.S. Patent No. 4,987,071. Important characteristics of enzymatic nucleic acid molecules used according to the invention are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus, the ribozyme constructs need not be limited to specific motifs mentioned herein. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0226] Methods of producing a ribozyme targeted to any polynucleotide sequence are known in the art. Ribozymes may be designed as described in, *e.g.*, PCT Publication Nos. WO 93/23569 and WO 94/02595, and synthesized to be tested *in vitro* and/or *in vivo* as described therein. The disclosures of these PCT publications are herein incorporated by reference in their entirety for all purposes.

[0227] Ribozyme activity can be optimized by altering the length of the ribozyme binding arms or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (*see, e.g.*, PCT Publication Nos. WO 92/07065, WO 93/15187, WO 91/03162, and WO 94/13688; EP 92110298.4; and U.S. Patent No. 5,334,711, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules, the disclosures of which are each herein incorporated by reference in their entirety for all purposes), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

6. Immunostimulatory Oligonucleotides

[0228] Nucleic acids associated with lipid paticles of the present invention may be immunostimulatory, including immunostimulatory oligonucleotides (ISS; single-or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal such as a human. ISS include, *e.g.*, certain palindromes leading to hairpin secondary structures (*see*, Yamamoto *et al.*, *J. Immunol.*, 148:4072-6 (1992)), or CpG motifs, as well as other known ISS features (such as multi-G domains; *see;* PCT Publication No. WO 96/11266, the disclosure of which is herein incorporated by reference in its entirety for all purposes).

[0229] Immunostimulatory nucleic acids are considered to be non-sequence specific when it is not required that they specifically bind to and reduce the expression of a target sequence in order to provoke an immune response. Thus, certain immunostimulatory nucleic acids may comprise a sequence corresponding to a region of a naturally-occurring gene or mRNA, but they may still be considered non-sequence specific immunostimulatory nucleic acids.

[0230] In one embodiment, the immunostimulatory nucleic acid or oligonucleotide comprises at least one CpG dinucleotide. The oligonucleotide or CpG dinucleotide may be unmethylated or methylated. In another embodiment, the immunostimulatory nucleic acid comprises at least one CpG dinucleotide having a methylated cytosine. In one embodiment, the nucleic acid comprises a single CpG dinucleotide, wherein the cytosine in the CpG dinucleotide is methylated. In an alternative embodiment, the nucleic acid comprises at least

two CpG dinucleotides, wherein at least one cytosine in the CpG dinucleotides is methylated. In a further embodiment, each cytosine in the CpG dinucleotides present in the sequence is methylated. In another embodiment, the nucleic acid comprises a plurality of CpG dinucleotides, wherein at least one of the CpG dinucleotides comprises a methylated cytosine. Examples of immunostimulatory oligonucleotides suitable for use in the compositions and methods of the present invention are described in PCT Application No. PCT/US08/88676, filed December 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Patent No. 6,406,705, and Raney *et al.*, *J. Pharm. Exper. Ther.*, 298:1185-92 (2001), the disclosures of which are each herein incorporated by reference in their entirety for all purposes. In certain embodiments, the oligonucleotides used in the compositions and methods of the invention have a phosphodiester ("PO") backbone or a phosphorothioate ("PS") backbone, and/or at least one methylated cytosine residue in a CpG motif.

B. Other Active Agents

[0231] In certain embodiments, the active agent associated with the lipid paticles of the invention may comprise one or more therapeutic proteins, polypeptides, or small organic molecules or compounds. Non-limiting examples of such therapeutically effective agents or drugs include oncology drugs (*e.g.*, chemotherapy drugs, hormonal therapaeutic agents, immunotherapeutic agents, radiotherapeutic agents, *etc.*), lipid-lowering agents, anti-viral drugs, anti-inflammatory compounds, antidepressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs such as anti-arrhythmic agents, hormones, vasoconstrictors, and steroids. These active agents may be administered alone in the lipid particles of the invention, or in combination (*e.g.*, co-administered) with lipid particles of the invention comprising nucleic acid such as interfering RNA.

[0232] Non-limiting examples of chemotherapy drugs include platinum-based drugs (*e.g.*, oxaliplatin, cisplatin, carboplatin, spiroplatin, iproplatin, satraplatin, *etc.*), alkylating agents (*e.g.*, cyclophosphamide, ifosfamide, chlorambucil, busulfan, melphalan, mechlorethamine, uramustine, thiotepa, nitrosoureas, *etc.*), anti-metabolites (*e.g.*, 5-fluorouracil (5-FU), azathioprine, methotrexate, leucovorin, capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, pemetrexed, raltitrexed, *etc.*), plant alkaloids (*e.g.*, vincristine, vinblastine, vinorelbine, vindesine, podophyllotoxin, paclitaxel (taxol), docetaxel, *etc.*), topoisomerase inhibitors (*e.g.*, irinotecan (CPT-11; Camptosar), topotecan, amsacrine, etoposide (VP16), etoposide phosphate, teniposide, *etc.*), antitumor antibiotics (*e.g.*, doxorubicin, adriamycin,

daunorubicin, epirubicin, actinomycin, bleomycin, mitomycin, mitoxantrone, plicamycin, *etc.*), tyrosine kinase inhibitors (*e.g.*, gefitinib (Iressa®), sunitinib (Sutent®; SU11248), erlotinib (Tarceva®; OSI-1774), lapatinib (GW572016; GW2016), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), imatinib (Gleevec®; STI571), dasatinib (BMS-354825), leflunomide (SU101), vandetanib (Zactima™; ZD6474), *etc.*), pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and combinations thereof.

[0233] Examples of conventional hormonal therapaeutic agents include, without limitation, steroids (*e.g.*, dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

[0234] Examples of conventional immunotherapeutic agents include, but are not limited to, immunostimulants (*e.g.*, Bacillus Calmette-Guérin (BCG), levamisole, interleukin-2, alpha-interferon, *etc.*), monoclonal antibodies (*e.g.*, anti-CD20, anti-HER2, anti-CD52, anti-HLA-DR, and anti-VEGF monoclonal antibodies), immunotoxins (*e.g.*, anti-CD33 monoclonal antibody-calicheamicin conjugate, anti-CD22 monoclonal antibody-pseudomonas exotoxin conjugate, *etc.*), and radioimmunotherapy (*e.g.*, anti-CD20 monoclonal antibody conjugated to ^{111}In, ^{90}Y, or ^{131}I, *etc.*).

[0235] Examples of conventional radiotherapeutic agents include, but are not limited to, radionuclides such as ^{47}Sc, ^{64}Cu, ^{67}Cu, ^{89}Sr, ^{86}Y, ^{87}Y, ^{90}Y, ^{105}Rh, ^{111}Ag, ^{111}In, ^{117m}Sn, ^{149}Pm, ^{153}Sm, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi, optionally conjugated to antibodies directed against tumor antigens.

[0236] Additional oncology drugs that may be used according to the invention include, but are not limited to, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, bexarotene, biCNU, carmustine, CCNU, celecoxib, cladribine, cyclosporin A, cytosine arabinoside, cytoxan, dexrazoxane, DTIC, estramustine, exemestane, FK506, gemtuzumab-ozogamicin, hydrea, hydroxyurea, idarubicin, interferon, letrozole, leustatin, leuprolide, litretinoin, megastrol, L-PAM, mesna, methoxsalen, mithramycin, nitrogen mustard, pamidronate, Pegademase, pentostatin, porfimer sodium, prednisone, rituxan, streptozocin, STI-571, taxotere, temozolamide, VM-26, toremifene, tretinoin, ATRA, valrubicin, and velban. Other examples of oncology drugs that may be used according to the invention are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors, and camptothecins.

[0237] Non-limiting examples of lipid-lowering agents for treating a lipid disease or disorder associated with elevated triglycerides, cholesterol, and/or glucose include statins, fibrates, ezetimibe, thiazolidinediones, niacin, beta-blockers, nitroglycerin, calcium antagonists, fish oil, and mixtures thereof.

[0238] Examples of anti-viral drugs include, but are not limited to, abacavir, aciclovir, acyclovir, adefovir, amantadine, amprenavir, arbidol, atazanavir, atripla, cidofovir, combivir, darunavir, delavirdine, didanosine, docosanol, edoxudine, efavirenz, emtricitabine, enfuvirtide, entecavir, entry inhibitors, famciclovir, fixed dose combinations, fomivirsen, fosamprenavir, foscarnet, fosfonet, fusion inhibitors, ganciclovir, ibacitabine, imunovir, idoxuridine, imiquimod, indinavir, inosine, integrase inhibitors, interferon type III (*e.g.*, IFN-λ molecules such as IFN-λ1, IFN-λ2, and IFN-λ3), interferon type II (*e.g.*, IFN-γ), interferon type I (*e.g.*, IFN-α such as PEGylated IFN-α, IFN-β, IFN-κ, IFN-δ, IFN-ε, IFN-τ, IFN-ω, and IFN-ζ), interferon, lamivudine, lopinavir, loviride, MK-0518, maraviroc, moroxydine, nelfinavir, nevirapine, nexavir, nucleoside analogues, oseltamivir, penciclovir, peramivir, pleconaril, podophyllotoxin, protease inhibitors, reverse transcriptase inhibitors, ribavirin, rimantadine, ritonavir, saquinavir, stavudine, synergistic enhancers, tenofovir, tenofovir disoproxil, tipranavir, trifluridine, trizivir, tromantadine, truvada, valaciclovir, valganciclovir, vicriviroc, vidarabine, viramidine, zalcitabine, zanamivir, zidovudine, pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and mixtures thereof.

V. Lipid Particles

[0239] The lipid particles of the invention typically comprise an active agent or therapeutic agent, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans. The lipid particles of the invention typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm.

[0240] In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (*e.g.*, siRNA,

aiRNA, and/or miRNA), a cationic lipid (*e.g.*, a cationic lipid of Formulas I, II, and/or III), a non-cationic lipid (*e.g.*, cholesterol alone or mixtures of one or more phospholipids and cholesterol), and a conjugated lipid that inhibits aggregation of the particles (*e.g.*, one or more PEG-lipid conjugates). The SNALP may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more unmodified and/or modified interfering RNA molecules. Nucleic acid-lipid particles and their method of preparation are described in, *e.g.*, U.S. Patent Nos. 5,753,613; 5,785,992; 5,705,385; 5,976,567; 5,981,501; 6,110,745; and 6,320,017; and PCT Publication No. WO 96/40964, the disclosures of which are each herein incorporated by reference in their entirety for all purposes.

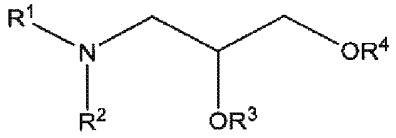
A. Cationic Lipids

[0241] Any of a variety of cationic lipids may be used in the lipid particles of the invention (*e.g.*, SNALP), either alone or in combination with one or more other cationic lipid species or non-cationic lipid species.

[0242] Cationic lipids which are useful in the present invention can be any of a number of lipid species which carry a net positive charge at physiological pH. Such lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3.beta.-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-Dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), 1,2-Dilinoleoylcarbamyl-3-dimethylaminopropane (DLinCDAP), and mixtures thereof. A number of these lipids and related analogs have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are each herein incorporated by reference in their

entirety for all purposes. Additionally, a number of commercial preparations of cationic lipids are available and can be used in the present invention. These include, *e.g.*, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic liposomes comprising DOGS from Promega Corp., Madison, Wisconsin, USA).

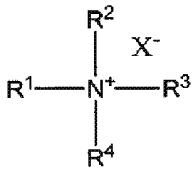
[0243] Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.



(I),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.*, R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.*, R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipid of Formula I is symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4comprise at least three sites of unsaturation and are independently selected from, *e.g.*, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl. In particularly preferred embodiments, the cationic lipid of Formula I is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) or 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA).

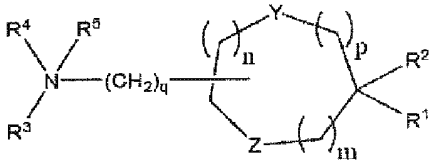
[0244] Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.



(II),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.*, R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.*, R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipids of the present invention are symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4comprise at least three sites of unsaturation and are independently selected from, *e.g.*, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl.

[0245] Moreover, cationic lipids of Formula III having the following structures (or salts thereof) are useful in the present invention.



(III),

Wherein R^1 and R^2 are either the same or different and independently optionally substituted C_{12}-C_{24} alkyl, optionally substituted C_{12}-C_{24} alkenyl, optionallysubstituted C_{12}-C_{24} alkynyl, or optionally substituted C_{12}-C_{24} acyl; R^3 and R^4 are either the same or different and independently optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 alkenyl, or optionally substituted C_1-C_6 alkynyl or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen; R^5 is either absent or hydrogen or C_1-C_6 alkyl to provide a quaternary amine; m, n, and p are either the same or different and independently either 0 or 1 with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; and Y and Z are either the same or different and independently O, S, or NH.

[0246] In some embodiments, the cationic lipid of Formula III is 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-

[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), or mixtures thereof. In preferred embodiments, the cationic lipid of Formula III is DLin-K-C2-DMA (XTC2).

[0247] The cationic lipid typically comprises from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0248] It will be readily apparent to one of skill in the art that depending on the intended use of the particles, the proportions of the components can be varied and the delivery efficiency of a particular formulation can be measured using, *e.g.*, an endosomal release parameter (ERP) assay.

B. Non-Cationic Lipids

[0249] The non-cationic lipids used in the lipid particles of the invention (*e.g.*, SNALP) can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

[0250] Non-limiting examples of non-cationic lipids include phospholipids such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol

(POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C_{10}-C_{24} carbon chains, *e.g.*, lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

[0251] Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof such as cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof.

[0252] In some embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of cholesterol or a derivative thereof, *e.g.*, a phospholipid-free lipid particle formulation. In other embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of one or more phospholipids, *e.g.*, a cholesterol-free lipid particle formulation. In further embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof.

[0253] Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, *e.g.*, stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

[0254] In some embodiments, the non-cationic lipid comprises from about 13 mol % to about 49.5 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 35 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0255] In certain embodiments, the cholesterol present in phospholipid-free lipid particles comprises from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, or from about 35 mol % to about 40 mol % of the

total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle may comprise cholesterol at about 37 mol % of the total lipid present in the particle.

[0256] In certain other embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, or from about 35 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 34 mol % of the total lipid present in the particle.

[0257] In further embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 10 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 20 mol % of the total lipid present in the particle.

[0258] In embodiments where the lipid particles contain a mixture of phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 45, 50, 55, or 60 mol % of the total lipid present in the particle. In certain instances, the phospholipid component in the mixture may comprise from about 2 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, or from about 6 mol % to about 8 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (*e.g.*, in a mixture with about 34 mol % cholesterol) of the total lipid present in the particle. In certain other instances, the phospholipid component in the mixture may comprise from about 10 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As another non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 20 mol % (*e.g.*, in a mixture with about 20 mol % cholesterol) of the total lipid present in the particle.

C. Lipid Conjugate

[0259] In addition to cationic and non-cationic lipids, the lipid particles of the invention (*e.g.*, SNALP) comprise a lipid conjugate. The conjugated lipid is useful in that it prevents the aggregation of particles. Suitable conjugated lipids include, but are not limited to, PEG-

lipid conjugates, ATTA-lipid conjugates, cationic-polymer-lipid conjugates (CPLs), and mixtures thereof. In certain embodiments, the particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate together with a CPL.

[0260] In a preferred embodiment, the lipid conjugate is a PEG-lipid. Examples of PEG-lipids include, but are not limited to, PEG coupled to dialkyloxypropyls (PEG-DAA) as described in, *e.g.*, PCT Publication No. WO 05/026372, PEG coupled to diacylglycerol (PEG-DAG) as described in, *e.g.*, U.S. Patent Publication Nos. 20030077829 and 2005008689, PEG coupled to phospholipids such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides as described in, *e.g.*, U.S. Patent No. 5,885,613, PEG conjugated to cholesterol or a derivative thereof, and mixtures thereof. The disclosures of these patent documents are herein incorporated by reference in their entirety for all purposes. Additional PEG-lipids include, without limitation, PEG-C-DOMG, 2KPEG-DMG, and a mixture thereof.

[0261] PEG is a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG 2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, for example, the following: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH_2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM). Other PEGs such as those described in U.S. Patent Nos. 6,774,180 and 7,053,150 (*e.g.*, mPEG (20 KDa) amine) are also useful for preparing the PEG-lipid conjugates of the present invention. The disclosures of these patents are herein incorporated by reference in their entirety for all purposes. In addition, monomethoxypolyethyleneglycol-acetic acid (MePEG-CH_2COOH) is particularly useful for preparing PEG-lipid conjugates including, *e.g.*, PEG-DAA conjugates.

[0262] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about

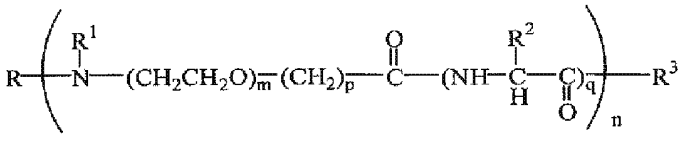
750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

[0263] In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In a preferred embodiment, the linker moiety is a non-ester containing linker moiety. As used herein, the term "non-ester containing linker moiety" refers to a linker moiety that does not contain a carboxylic ester bond (-OC(O)-). Suitable non-ester containing linker moieties include, but are not limited to, amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulphide (-S-S-), ether (-O-), succinyl (-(O)CCH$_2$CH$_2$C(O)-), succinamidyl (-NHC(O)CH$_2$CH$_2$C(O)NH-), ether, disulphide, as well as combinations thereof (such as a linker containing both a carbamate linker moiety and an amido linker moiety). In a preferred embodiment, a carbamate linker is used to couple the PEG to the lipid.

[0264] In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, *e.g.*, carbonate (-OC(O)O-), succinoyl, phosphate esters (-O-(O)POH-O-), sulfonate esters, and combinations thereof.

[0265] Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to PEG to form the lipid conjugate. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skilled in the art. Phosphatidyl-ethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), and distearoyl-phosphatidylethanolamine (DSPE).

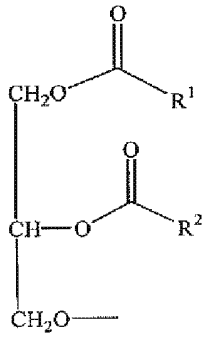
[0266] The term "ATTA" or "polyamide" refers to, without limitation, compounds described in U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes. These compounds include a compound having the formula:



(IV),

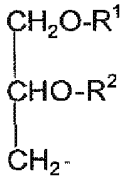
wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R^1 is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R^1 and the nitrogen to which they are bound form an azido moiety; R^2 is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; R^3 is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and NR^4R^5, wherein R^4 and R^5 are independently hydrogen or alkyl; n is 4 to 80; m is 2 to 6; p is 1 to 4; and q is 0 or 1. It will be apparent to those of skill in the art that other polyamides can be used in the compounds of the present invention.

[0267] The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.* Diacylglycerols have the following general formula:



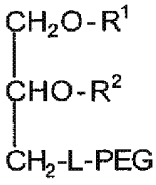
(V).

[0268] The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:



(VI).

[0269] In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:



(VII),

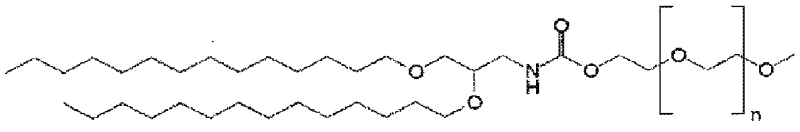
wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.*

[0270] In Formula VII above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG has an average molecular weight of about 2,000 daltons or about 750 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl, or aryl. In certain embodiments, the terminal hydroxyl group is substituted with a methoxy or methyl group.

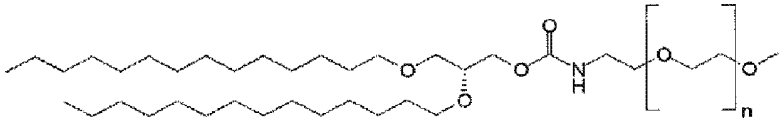
[0271] In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, a succinamidyl linker moiety, and combinations thereof. In a preferred embodiment, the non-ester containing linker moiety is a carbamate linker moiety (*i.e.*, a PEG-*C*-DAA conjugate). In another preferred embodiment,

the non-ester containing linker moiety is an amido linker moiety (*i.e.*, a PEG-*A*-DAA conjugate). In yet another preferred embodiment, the non-ester containing linker moiety is a succinamidyl linker moiety (*i.e.*, a PEG-*S*-DAA conjugate).

[0272] In particular embodiments, the PEG-lipid conjugate is selected from:



(PEG-C-DMA); and



(PEG-C-DOMG).

[0273] The PEG-DAA conjugates are synthesized using standard techniques and reagents known to those of skill in the art. It will be recognized that the PEG-DAA conjugates will contain various amide, amine, ether, thio, carbamate, and urea linkages. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See, e.g.*, March, ADVANCED ORGANIC CHEMISTRY (Wiley 1992); Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY, 5th ed. (Longman 1989). It will also be appreciated that any functional groups present may require protection and deprotection at different points in the synthesis of the PEG-DAA conjugates. Those of skill in the art will recognize that such techniques are well known. *See, e.g.*, Green and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS (Wiley 1991).

[0274] Preferably, the PEG-DAA conjugate is a dilauryloxypropyl (C_{12})-PEG conjugate, dimyristyloxypropyl (C_{14})-PEG conjugate, a dipalmityloxypropyl (C_{16})-PEG conjugate, or a distearyloxypropyl (C_{18})-PEG conjugate. Those of skill in the art will readily appreciate that other dialkyloxypropyls can be used in the PEG-DAA conjugates of the present invention.

[0275] In addition to the foregoing, it will be readily apparent to those of skill in the art that other hydrophilic polymers can be used in place of PEG. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

[0276] In addition to the foregoing components, the particles (*e.g.*, SNALP or SPLP) of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids or CPLs (*see, e.g.*, Chen *et al.*, *Bioconj. Chem.*, 11:433-437 (2000)). Suitable SPLPs and SPLP-CPLs for use in the present invention, and methods of making and using SPLPs and SPLP-CPLs, are disclosed, *e.g.*, in U.S. Patent No. 6,852,334 and PCT Publication No. WO 00/62813, , the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0277] Suitable CPLs include compounds of Formula VIII:

A-W-Y (VIII),

wherein A, W, and Y are as described below.

[0278] With reference to Formula VIII, "A" is a lipid moiety such as an amphipathic lipid, a neutral lipid, or a hydrophobic lipid that acts as a lipid anchor. Suitable lipid examples include, but are not limited to, diacylglycerolyls, dialkylglycerolyls, N-N-dialkylaminos, 1,2-diacyloxy-3-aminopropanes, and 1,2-dialkyl-3-aminopropanes.

[0279] "W" is a polymer or an oligomer such as a hydrophilic polymer or oligomer. Preferably, the hydrophilic polymer is a biocompatable polymer that is nonimmunogenic or possesses low inherent immunogenicity. Alternatively, the hydrophilic polymer can be weakly antigenic if used with appropriate adjuvants. Suitable nonimmunogenic polymers include, but are not limited to, PEG, polyamides, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, and combinations thereof. In a preferred embodiment, the polymer has a molecular weight of from about 250 to about 7,000 daltons.

[0280] "Y" is a polycationic moiety. The term polycationic moiety refers to a compound, derivative, or functional group having a positive charge, preferably at least 2 positive charges at a selected pH, preferably physiological pH. Suitable polycationic moieties include basic amino acids and their derivatives such as arginine, asparagine, glutamine, lysine, and histidine; spermine; spermidine; cationic dendrimers; polyamines; polyamine sugars; and amino polysaccharides. The polycationic moieties can be linear, such as linear tetralysine, branched or dendrimeric in structure. Polycationic moieties have between about 2 to about 15 positive charges, preferably between about 2 to about 12 positive charges, and more preferably between about 2 to about 8 positive charges at selected pH values. The selection of which polycationic moiety to employ may be determined by the type of particle application which is desired.

[0281] The charges on the polycationic moieties can be either distributed around the entire particle moiety, or alternatively, they can be a discrete concentration of charge density in one particular area of the particle moiety *e.g.*, a charge spike. If the charge density is distributed

on the particle, the charge density can be equally distributed or unequally distributed. All variations of charge distribution of the polycationic moiety are encompassed by the present invention.

[0282] The lipid "A" and the nonimmunogenic polymer "W" can be attached by various methods and preferably by covalent attachment. Methods known to those of skill in the art can be used for the covalent attachment of "A" and "W." Suitable linkages include, but are not limited to, amide, amine, carboxyl, carbonate, carbamate, ester, and hydrazone linkages. It will be apparent to those skilled in the art that "A" and "W" must have complementary functional groups to effectuate the linkage. The reaction of these two groups, one on the lipid and the other on the polymer, will provide the desired linkage. For example, when the lipid is a diacylglycerol and the terminal hydroxyl is activated, for instance with NHS and DCC, to form an active ester, and is then reacted with a polymer which contains an amino group, such as with a polyamide (*see, e.g.*, U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes), an amide bond will form between the two groups.

[0283] In certain instances, the polycationic moiety can have a ligand attached, such as a targeting ligand or a chelating moiety for complexing calcium. Preferably, after the ligand is attached, the cationic moiety maintains a positive charge. In certain instances, the ligand that is attached has a positive charge. Suitable ligands include, but are not limited to, a compound or device with a reactive functional group and include lipids, amphipathic lipids, carrier compounds, bioaffinity compounds, biomaterials, biopolymers, biomedical devices, analytically detectable compounds, therapeutically active compounds, enzymes, peptides, proteins, antibodies, immune stimulators, radiolabels, fluorogens, biotin, drugs, haptens, DNA, RNA, polysaccharides, liposomes, virosomes, micelles, immunoglobulins, functional groups, other targeting moieties, or toxins.

[0284] The lipid conjugate (*e.g.*, PEG-lipid) typically comprises from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 0.9 mol % to about 1.6 mol %, from about 0.9 mol % to about 1.8 mol %, from about 1 mol % to about 1.8 mol %, from about 1 mol % to about 1.7 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, or from about 1.4 mol % to about 1.5 mol % of the total lipid present in the particle.

[0285] One of ordinary skill in the art will appreciate that the concentration of the lipid conjugate can be varied depending on the lipid conjugate employed and the rate at which the nucleic acid-lipid particle is to become fusogenic.

[0286] By controlling the composition and concentration of the lipid conjugate, one can control the rate at which the lipid conjugate exchanges out of the nucleic acid-lipid particle and, in turn, the rate at which the nucleic acid-lipid particle becomes fusogenic. For instance, when a PEG-phosphatidylethanolamine conjugate or a PEG-ceramide conjugate is used as the lipid conjugate, the rate at which the nucleic acid-lipid particle becomes fusogenic can be varied, for example, by varying the concentration of the lipid conjugate, by varying the molecular weight of the PEG, or by varying the chain length and degree of saturation of the acyl chain groups on the phosphatidylethanolamine or the ceramide. In addition, other variables including, for example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the nucleic acid-lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the nucleic acid-lipid particle becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure.

VI. Preparation of Lipid Particles

[0287] The lipid particles of the present invention, *e.g.,* SNALP, in which an active agent or therapeutic agent such as an interfering RNA is encapsulated in a lipid bilayer and is protected from degradation, can be formed by any method known in the art including, but not limited to, a continuous mixing method or a direct dilution process.

[0288] In preferred embodiments, the cationic lipids are lipids of Formula I, II, and III, or combinations thereof. In other preferred embodiments, the non-cationic lipids are egg sphingomyelin (ESM), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dipalmitoyl-phosphatidylcholine (DPPC), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, 14:0 PE (1,2-dimyristoyl-phosphatidylethanolamine (DMPE)), 16:0 PE (1,2-dipalmitoyl-phosphatidylethanolamine (DPPE)), 18:0 PE (1,2-distearoyl-phosphatidylethanolamine (DSPE)), 18:1 PE (1,2-dioleoyl-phosphatidylethanolamine (DOPE)), 18:1 trans PE (1,2-dielaidoyl-phosphatidylethanolamine (DEPE)), 18:0-18:1 PE (1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE)), 16:0-18:1 PE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)), polyethylene glycol-based polymers (*e.g.,* PEG 2000,

PEG 5000, PEG-modified diacylglycerols, or PEG-modified dialkyloxypropyls), cholesterol, or combinations thereof.

[0289] In certain embodiments, the present invention provides for SNALP produced via a continuous mixing method, *e.g.*, a process that includes providing an aqueous solution comprising a nucleic acid such as an interfering RNA in a first reservoir, providing an organic lipid solution in a second reservoir, and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a liposome encapsulating the nucleic acid (*e.g.*, interfering RNA). This process and the apparatus for carrying this process are described in detail in U.S. Patent Publication No. 20040142025, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0290] The action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a liposome substantially instantaneously upon mixing. As used herein, the phrase "continuously diluting a lipid solution with a buffer solution" (and variations) generally means that the lipid solution is diluted sufficiently rapidly in a hydration process with sufficient force to effectuate vesicle generation. By mixing the aqueous solution comprising a nucleic acid with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution in the presence of the buffer solution (*i.e.*, aqueous solution) to produce a nucleic acid-lipid particle.

[0291] The SNALP formed using the continuous mixing method typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0292] In another embodiment, the present invention provides for SNALP produced via a direct dilution process that includes forming a liposome solution and immediately and directly introducing the liposome solution into a collection vessel containing a controlled amount of dilution buffer. In preferred aspects, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In one aspect, the amount of dilution buffer present in the collection vessel is substantially equal to the volume of liposome solution introduced thereto. As a non-limiting example, a liposome solution in 45% ethanol when introduced into the collection vessel containing an equal volume of dilution buffer will advantageously yield smaller particles.

[0293] In yet another embodiment, the present invention provides for SNALP produced via a direct dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the liposome solution formed in a first mixing region is immediately and directly mixed with dilution buffer in the second mixing region. In preferred aspects, the second mixing region includes a T-connector arranged so that the liposome solution and the dilution buffer flows meet as opposing 180° flows; however, connectors providing shallower angles can be used, *e.g.*, from about 27° to about 180°. A pump mechanism delivers a controllable flow of buffer to the second mixing region. In one aspect, the flow rate of dilution buffer provided to the second mixing region is controlled to be substantially equal to the flow rate of liposome solution introduced thereto from the first mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the liposome solution in the second mixing region, and therefore also the concentration of liposome solution in buffer throughout the second mixing process. Such control of the dilution buffer flow rate advantageously allows for small particle size formation at reduced concentrations.

[0294] These processes and the apparatuses for carrying out these direct dilution processes are described in detail in U.S. Patent Publication No. 20070042031, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0295] The SNALP formed using the direct dilution process typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0296] If needed, the lipid particles of the invention (*e.g.*, SNALP) can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

[0297] Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles, is described in U.S. Patent No. 4,737,323, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between

about 60 and about 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

[0298] Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

[0299] In some embodiments, the nucleic acids in the SNALP are precondensed as described in, *e.g.*, U.S. Patent Application No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0300] In other embodiments, the methods will further comprise adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions. Examples of suitable non-lipid polycations include, hexadimethrine bromide (sold under the brandname POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine, and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

[0301] In some embodiments, the nucleic acid to lipid ratios (mass/mass ratios) in a formed SNALP will range from about 0.01 to about 0.2, from about 0.02 to about 0.1, from about 0.03 to about 0.1, or from about 0.01 to about 0.08. The ratio of the starting materials also falls within this range. In other embodiments, the SNALP preparation uses about 400 µg nucleic acid per 10 mg total lipid or a nucleic acid to lipid mass ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 µg of nucleic acid. In other preferred embodiments, the particle has a nucleic acid:lipid mass ratio of about 0.08.

[0302] In other embodiments, the lipid to nucleic acid ratios (mass/mass ratios) in a formed SNALP will range from about 1 (1:1) to about 100 (100:1), from about 5 (5:1) to about 100 (100:1), from about 1 (1:1) to about 50 (50:1), from about 2 (2:1) to about 50 (50:1), from about 3 (3:1) to about 50 (50:1), from about 4 (4:1) to about 50 (50:1), from about 5 (5:1) to about 50 (50:1), from about 1 (1:1) to about 25 (25:1), from about 2 (2:1) to about 25 (25:1), from about 3 (3:1) to about 25 (25:1), from about 4 (4:1) to about 25 (25:1), from about 5 (5:1) to about 25 (25:1), from about 5 (5:1) to about 20 (20:1), from about 5 (5:1) to about 15 (15:1), from about 5 (5:1) to about 10 (10:1), about 5 (5:1), 6 (6:1), 7 (7:1), 8 (8:1), 9 (9:1),

10 (10:1), 11 (11:1), 12 (12:1), 13 (13:1), 14 (14:1), or 15 (15:1). The ratio of the starting materials also falls within this range.

[0303] As previously discussed, the conjugated lipid may further include a CPL. A variety of general methods for making SNALP-CPLs (CPL-containing SNALP) are discussed herein. Two general techniques include "post-insertion" technique, that is, insertion of a CPL into, for example, a pre-formed SNALP, and the "standard" technique, wherein the CPL is included in the lipid mixture during, for example, the SNALP formation steps. The post-insertion technique results in SNALP having CPLs mainly in the external face of the SNALP bilayer membrane, whereas standard techniques provide SNALP having CPLs on both internal and external faces. The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAAs and PEG-DAGs). Methods of making SNALP-CPL, are taught, for example, in U.S. Patent Nos. 5,705,385; 6,586,410; 5,981,501; 6,534,484; and 6,852,334; U.S. Patent Publication No. 20020072121; and PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

VII. Kits

[0304] The present invention also provides lipid particles (*e.g.*, SNALP) in kit form. The kit may comprise a container which is compartmentalized for holding the various elements of the lipid particles (*e.g.*, the active agents or therapeutic agents such as nucleic acids and the individual lipid components of the particles). In some embodiments, the kit may further comprise an endosomal membrane destabilizer (*e.g.*, calcium ions). The kit typically contains the lipid particle compositions of the present invention, preferably in dehydrated form, with instructions for their rehydration and administration.

[0305] As explained herein, the lipid particles of the invention (*e.g.*, SNALP) can be tailored to preferentially target particular tissues, organs, or tumors of interest. In certain instances, preferential targeting of lipid particles such as SNALP may be carried out by controlling the composition of the particle itself. For instance, as set forth in Example 11, it has been found that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP formulation can be used to preferentially target the liver (including liver tumors).

[0306] In certain other instances, it may be desirable to have a targeting moiety attached to the surface of the lipid particle to further enhance the targeting of the particle. Methods of

attaching targeting moieties (*e.g.*, antibodies, proteins, *etc.*) to lipids (such as those used in the present particles) are known to those of skill in the art.

VII. Administration of Lipid Particles

[0307] Once formed, the lipid particles of the invention (*e.g.*, SNALP) are useful for the introduction of active agents or therapeutic agents (*e.g.*, nucleic acids such as interfering RNA) into cells. Accordingly, the present invention also provides methods for introducing an active agent or therapeutic agent such as a nucleic acid (*e.g.*, interfering RNA) into a cell. The methods are carried out *in vitro* or *in vivo* by first forming the particles as described above and then contacting the particles with the cells for a period of time sufficient for delivery of the active agent or therapeutic agent to the cells to occur.

[0308] The lipid particles of the invention (*e.g.*, SNALP) can be adsorbed to almost any cell type with which they are mixed or contacted. Once adsorbed, the particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the active agent or therapeutic agent (*e.g.*, nucleic acid) portion of the particle can take place via any one of these pathways. In particular, when fusion takes place, the particle membrane is integrated into the cell membrane and the contents of the particle combine with the intracellular fluid.

[0309] The lipid particles of the invention (*e.g.*, SNALP) can be administered either alone or in a mixture with a pharmaceutically-acceptable carrier (*e.g.*, physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice. Generally, normal buffered saline (*e.g.*, 135-150 mM NaCl) will be employed as the pharmaceutically-acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Additional suitable carriers are described in, *e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

[0310] The pharmaceutically-acceptable carrier is generally added following particle formation. Thus, after the particle is formed, the particle can be diluted into pharmaceutically-acceptable carriers such as normal buffered saline.

[0311] The concentration of particles in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2 to 5%, to as much as about 10 to 90% by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, particles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

[0312] The pharmaceutical compositions of the present invention may be sterilized by conventional, well-known sterilization techniques. Aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically-acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, and calcium chloride. Additionally, the particle suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

A. *In vivo* Administration

[0313] Systemic delivery for *in vivo* therapy, *e.g.*, delivery of a therapeutic nucleic acid to a distal target cell via body systems such as the circulation, has been achieved using nucleic acid-lipid particles such as those described in PCT Publication Nos. WO 05/007196, WO 05/121348, WO 05/120152, and WO 04/002453, the disclosures of which are herein incorporated by reference in their entirety for all purposes. The present invention also provides fully encapsulated lipid particles that protect the nucleic acid from nuclease degradation in serum, are nonimmunogenic, are small in size, and are suitable for repeat dosing.

[0314] For *in vivo* administration, administration can be in any manner known in the art, *e.g.*, by injection, oral administration, inhalation (*e.g.*, intransal or intratracheal), transdermal

application, or rectal administration. Administration can be accomplished via single or divided doses. The pharmaceutical compositions can be administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In some embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection (*see, e.g.*, U.S. Patent No. 5,286,634). Intracellular nucleic acid delivery has also been discussed in Straubringer *et al.*, *Methods Enzymol.*, 101:512 (1983); Mannino *et al.*, *Biotechniques,* 6:682 (1988); Nicolau *et al.*, *Crit. Rev. Ther. Drug Carrier Syst.*, 6:239 (1989); and Behr, *Acc. Chem. Res.*, 26:274 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, U.S. Patent Nos. 3,993,754; 4,145,410; 4,235,871; 4,224,179; 4,522,803; and 4,588,578. The lipid particles can be administered by direct injection at the site of disease or by injection at a site distal from the site of disease (*see, e.g.*, Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71(1994)). The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0315] The compositions of the present invention, either alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation (*e.g.*, intranasally or intratracheally) (*see*, Brigham *et al.*, *Am. J. Sci.*, 298:278 (1989)). Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0316] In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering nucleic acid compositions directly to the lungs via nasal aerosol sprays have been described, *e.g.*, in U.S. Patent Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Similarly, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Patent No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

[0317] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers,

and preservatives. In the practice of this invention, compositions are preferably administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically, or intrathecally.

[0318] Generally, when administered intravenously, the lipid particle formulations are formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc*. Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, *etc*. These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

[0319] In certain applications, the lipid particles disclosed herein may be delivered via oral administration to the individual. The particles may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, pills, lozenges, elixirs, mouthwash, suspensions, oral sprays, syrups, wafers, and the like (*see, e.g.,* U.S. Patent Nos. 5,641,515, 5,580,579, and 5,792,451, the disclosures of which are herein incorporated by reference in their entirety for all purposes). These oral dosage forms may also contain the following: binders, gelatin; excipients, lubricants, and/or flavoring agents. When the unit dosage form is a capsule, it may contain, in addition to the materials described above, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. Of course, any material used in preparing any unit dosage form should be pharmaceutically pure and substantially non-toxic in the amounts employed.

[0320] Typically, these oral formulations may contain at least about 0.1% of the lipid particles or more, although the percentage of the particles may, of course, be varied and may conveniently be between about 1% or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of particles in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0321] Formulations suitable for oral administration can consist of: (a) liquid solutions, such as an effective amount of a packaged therapeutic agent such as nucleic acid (*e.g.*, interfering RNA) suspended in diluents such as water, saline, or PEG 400; (b) capsules, sachets, or tablets, each containing a predetermined amount of a therapeutic agent such as nucleic acid (*e.g.*, interfering RNA), as liquids, solids, granules, or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise a therapeutic agent such as nucleic acid (*e.g.*, interfering RNA) in a flavor, *e.g.*, sucrose, as well as pastilles comprising the therapeutic agent in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the therapeutic agent, carriers known in the art.

[0322] In another example of their use, lipid particles can be incorporated into a broad range of topical dosage forms. For instance, a suspension containing nucleic acid-lipid particles such as SNALP can be formulated and administered as gels, oils, emulsions, topical creams, pastes, ointments, lotions, foams, mousses, and the like.

[0323] When preparing pharmaceutical preparations of the lipid particles of the invention, it is preferable to use quantities of the particles which have been purified to reduce or eliminate empty particles or particles with therapeutic agents such as nucleic acid associated with the external surface.

[0324] The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as primates (*e.g.*, humans and chimpanzees as well as other nonhuman primates), canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, rats and mice), lagomorphs, and swine.

[0325] The amount of particles administered will depend upon the ratio of therapeutic agent (*e.g.*, nucleic acid) to lipid, the particular therapeutic agent (*e.g.*, nucleic acid) used, the disease or disorder being treated, the age, weight, and condition of the patient, and the judgment of the clinician, but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight, or about 10^8-10^{10} particles per administration (*e.g.*, injection).

B. *In vitro* Administration

[0326] For *in vitro* applications, the delivery of therapeutic agents such as nucleic acids (*e.g.*, interfering RNA) can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells are animal cells, more preferably mammalian cells, and most preferably human cells.

[0327] Contact between the cells and the lipid particles, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of particles varies widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. Treatment of the cells with the lipid particles is generally carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

[0328] In one group of preferred embodiments, a lipid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/ml, more preferably about 2 x 10^4 cells/ml. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/ml, more preferably about 0.1 μg/ml.

[0329] Using an Endosomal Release Parameter (ERP) assay, the delivery efficiency of the SNALP or other lipid particle of the invention can be optimized. An ERP assay is described in detail in U.S. Patent Publication No. 20030077829, the disclosure of which is herein incorporated by reference in its entirety for all purposes. More particularly, the purpose of an ERP assay is to distinguish the effect of various cationic lipids and helper lipid components of SNALP based on their relative effect on binding/uptake or fusion with/destabilization of the endosomal membrane. This assay allows one to determine quantitatively how each component of the SNALP or other lipid particle affects delivery efficiency, thereby

optimizing the SNALP or other lipid particle. Usually, an ERP assay measures expression of a reporter protein (*e.g.*, luciferase, β-galactosidase, green fluorescent protein (GFP), *etc.*), and in some instances, a SNALP formulation optimized for an expression plasmid will also be appropriate for encapsulating an interfering RNA. In other instances, an ERP assay can be adapted to measure downregulation of transcription or translation of a target sequence in the presence or absence of an interfering RNA (*e.g.*, siRNA). By comparing the ERPs for each of the various SNALP or other lipid particles, one can readily determine the optimized system, *e.g.*, the SNALP or other lipid particle that has the greatest uptake in the cell.

C. Cells for Delivery of Lipid Particles

[0330] The compositions and methods of the present invention are used to treat a wide variety of cell types, *in vivo* and *in vitro*. Suitable cells include, *e.g.*, hematopoietic precursor (stem) cells, fibroblasts, keratinocytes, hepatocytes, endothelial cells, skeletal and smooth muscle cells, osteoblasts, neurons, quiescent lymphocytes, terminally differentiated cells, slow or noncycling primary cells, parenchymal cells, lymphoid cells, epithelial cells, bone cells, and the like. In preferred embodiments, an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is delivered to cancer cells such as, *e.g.*, lung cancer cells, colon cancer cells, rectal cancer cells, anal cancer cells, bile duct cancer cells, small intestine cancer cells, stomach (gastric) cancer cells, esophageal cancer cells, gallbladder cancer cells, liver cancer cells, pancreatic cancer cells, appendix cancer cells, breast cancer cells, ovarian cancer cells, cervical cancer cells, prostate cancer cells, renal cancer cells, cancer cells of the central nervous system, glioblastoma tumor cells, skin cancer cells, lymphoma cells, choriocarcinoma tumor cells, head and neck cancer cells, osteogenic sarcoma tumor cells, and blood cancer cells.

[0331] *In vivo* delivery of lipid particles such as SNALP encapsulating an interfering RNA (*e.g.*, siRNA) is suited for targeting cells of any cell type. The methods and compositions can be employed with cells of a wide variety of vertebrates, including mammals, such as, *e.g*, canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, mice, rats, and guinea pigs), lagomorphs, swine, and primates (*e.g.* monkeys, chimpanzees, and humans).

[0332] To the extent that tissue culture of cells may be required, it is well-known in the art. For example, Freshney, Culture of Animal Cells, a Manual of Basic Technique, 3rd Ed., Wiley-Liss, New York (1994), Kuchler *et al.*, Biochemical Methods in Cell Culture and Virology, Dowden, Hutchinson and Ross, Inc. (1977), and the references cited therein

provide a general guide to the culture of cells. Cultured cell systems often will be in the form of monolayers of cells, although cell suspensions are also used.

D. Detection of Lipid Particles

[0333] In some embodiments, the lipid particles of the present invention (*e.g.*, SNALP) are detectable in the subject at about 1, 2, 3, 4, 5, 6, 7, 8 or more hours. In other embodiments, the lipid particles of the present invention (*e.g.*, SNALP) are detectable in the subject at about 8, 12, 24, 48, 60, 72, or 96 hours, or about 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 25, or 28 days after administration of the particles. The presence of the particles can be detected in the cells, tissues, or other biological samples from the subject. The particles may be detected, *e.g.*, by direct detection of the particles, detection of a therapeutic nucleic acid such as an interfering RNA (*e.g.*, siRNA) sequence, detection of the target sequence of interest (*i.e.*, by detecting expression or reduced expression of the sequence of interest), or a combination thereof.

1. Detection of Particles

[0334] Lipid particles of the invention such as SNALP can be detected using any method known in the art. For example, a label can be coupled directly or indirectly to a component of the lipid particle using methods well-known in the art. A wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the lipid particle component, stability requirements, and available instrumentation and disposal provisions. Suitable labels include, but are not limited to, spectral labels such as fluorescent dyes (*e.g.*, fluorescein and derivatives, such as fluorescein isothiocyanate (FITC) and Oregon Green™; rhodamine and derivatives such Texas red, tetrarhodimine isothiocynate (TRITC), *etc.*, digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like; radiolabels such as ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, *etc.*; enzymes such as horse radish peroxidase, alkaline phosphatase, *etc.*; spectral colorimetric labels such as colloidal gold or colored glass or plastic beads such as polystyrene, polypropylene, latex, *etc.* The label can be detected using any means known in the art.

2. Detection of Nucleic Acids

[0335] Nucleic acids (*e.g.*, interfering RNA) are detected and quantified herein by any of a number of means well-known to those of skill in the art. The detection of nucleic acids may proceed by well-known methods such as Southern analysis, Northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography. Additional analytic biochemical methods such as spectrophotometry, radiography,

electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography may also be employed.

[0336] The selection of a nucleic acid hybridization format is not critical. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in, *e.g.*, "Nucleic Acid Hybridization, A Practical Approach," Eds. Hames and Higgins, IRL Press (1985).

[0337] The sensitivity of the hybridization assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBA™) are found in Sambrook *et al.*, In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (2000); and Ausubel *et al.*, SHORT PROTOCOLS IN MOLECULAR BIOLOGY, eds., Current Protocols, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (2002); as well as U.S. Patent No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis *et al.* eds.) Academic Press Inc. San Diego, CA (1990); Arnheim & Levinson (October 1, 1990), *C&EN* 36; The *Journal Of NIH Research*, 3:81 (1991); Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173 (1989); Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874 (1990); Lomell *et al.*, *J. Clin. Chem.*, 35:1826 (1989); Landegren *et al.*, *Science*, 241:1077 (1988); Van Brunt, *Biotechnology*, 8:291 (1990); Wu and Wallace, *Gene*, 4:560 (1989); Barringer *et al.*, *Gene*, 89:117 (1990); and Sooknanan and Malek, *Biotechnology*, 13:563 (1995). Improved methods of cloning *in vitro* amplified nucleic acids are described in U.S. Pat. No. 5,426,039. Other methods described in the art are the nucleic acid sequence based amplification (NASBA™, Cangene, Mississauga, Ontario) and Qβ-replicase systems. These systems can be used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using, for example, nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation. The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0338] Nucleic acids for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as inhibitor components are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage *et al.*, *Tetrahedron Letts.*, 22:1859 1862 (1981), *e.g.*, using an automated synthesizer, as described in Needham VanDevanter *et al.*, *Nucleic Acids Res.*, 12:6159 (1984). Purification of ploynucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion exchange HPLC as described in Pearson *et al.*, *J. Chrom.*, 255:137 149 (1983). The sequence of the synthetic poluyucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology*, 65:499.

[0339] An alternative means for determining the level of transcription is *in situ* hybridization. *In situ* hybridization assays are well-known and are generally described in Angerer *et al.*, *Methods Enzymol.*, 152:649 (1987). In an *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

VIII. Examples

[0340] The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

Example 1. Materials and Methods.

[0341] ***siRNA***: All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, CO). The siRNAs were desalted and annealed using standard procedures.

[0342] ***Lipid Encapsulation of siRNA***: In some embodiments, siRNA molecules were encapsulated into nucleic acid-lipid particles composed of the following lipids: the lipid conjugate PEG-cDMA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropylamine); the cationic lipid DLinDMA (1,2-Dilinoleyloxy-3-(N,N-dimethyl)aminopropane); the phospholipid DPPC (1,2-Dipalmitoyl-sn-glycero-3-

phosphocholine; Avanti Polar Lipids; Alabaster, AL); and synthetic cholesterol (Sigma-Aldrich Corp.; St. Louis, MO) in the molar ratio 1.4:57.1:7.1:34.3, respectively. In other words, siRNAs were encapsulated into SNALP of the following "1:57" formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol. In other embodiments, siRNA molecules were encapsulated into phospholipid-free SNALP composed of the following lipids: the lipid conjugate PEG-cDMA; the cationic lipid DLinDMA; and synthetic cholesterol in the molar ratio 1.5:61.5:36.9, respectively. In other words, siRNAs were encapsulated into phospholipid-free SNALP of the following "1:62" formulation: 1.5% PEG-cDMA; 61.5% DLinDMA; and 36.9% cholesterol. For vehicle controls, empty particles with identical lipid composition were formed in the absence of siRNA. It should be understood that the 1:57 formulation and 1:62 formulation are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the formulation may vary. Typically, in the 1:57 formulation, the amount of cationic lipid will be 57 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:57 formulation being made up of non-cationic lipid (*e.g.*, phospholipid, cholesterol, or a mixture of the two). Similarly, in the 1:62 formulation, the amount of cationic lipid will be 62 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:62 formulation being made up of the non-cationic lipid (*e.g.*, cholesterol).

Example 2. Eg5 siRNA Formulated as 1:57 SNALP Are Potent Inhibitors of Cell Growth *in vitro*.

[0343] SNALP formulations were prepared with an siRNA targeting Eg5 as the nucleic acid component. Eg5 is a member of kinesin-related proteins that are involved in functions related to movements of organelles, microtubules, or chromosomes along microtubules. These functions include axonal transport, microtubule sliding during nuclear fusion or division, and chromosome disjunction during meiosis and early mitosis. Eg5 plays a critical role in mitosis of mammalian cells. The Eg5 siRNA used in this study is provided in Table 1. The modifications involved introducing 2'OMe-uridine at selected positions in the sense and antisense strands of the Eg5 2263 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 1. siRNA duplex comprising sense and antisense Eg5 RNA polynucleotides.

Modification	Eg5 2263 siRNA sequence	SEQ ID NO:	% 2'OMe-Modified	% Modified in DS Region
U/U	5'-CUGAAGACCUGAAGACAAUdTdT-3' 3'-dTdTGACUUCUGGACUUCUGUUA-5'	1 2	6/42 = 14.3%	6/38 = 15.8%

Column 1: "U/U" = 2'OMe-uridine modified siRNA duplex; Column 2: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. "dT" = deoxythymidine. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

[0344] The lipid components and physical characteristics of the SNALP formulations are summarized in Table 2. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 2. Characteristics of the SNALP formulations used in this study.

Sample No.	Formulation Composition, Mole % PEG(2000)-C-DMA I DLinDMA I DPPC I Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
1	2 I 40 I 10 I 48	12.4	57	0.07	90
2	1.8 I 36.4 I 18.2 I 43.6	14.0	72	0.12	89
3	1.4 I 27.0 I 6.8 I 64.9	16.5	70	0.12	92
4	1.3 I 25.3 I 12.7 I 60.8	18.1	76	0.07	93
5	3.9 I 39.2 I 9.8 I 47.1	13.5	53	0.27	86
6	3.6 I 35.7 I 17.9 I 42.9	15.1	58	0.18	87
7	2.7 I 26.7 I 6.7 I 64.0	17.6	56	0.17	92
8	2.5 I 25.0 I 12.5 I 60.0	19.2	61	0.13	92
9	1.4 I 57.1 I 7.1 I 34.3	17.8	64	0.10	88
10	1.3 I 53.3 I 13.3 I 32.0	19.5	63	0.10	89
11	1.1 I 42.6 I 5.3 I 51.1	22.0	80	0.10	93
12	1.0 I 40.4 I 10.1 I 48.5	23.6	78	0.11	88
13	2.8 I 56.3 I 7.0 I 33.8	19.0	62	0.14	80
14	2.6 I 52.6 I 13.2 I 31.6	20.6	66	0.14	82
15	2.1 I 42.1 I 5.3 I 50.5	23.1	71	0.16	91
16	2 I 40 I 10 I 48	24.7	67	0.14	92

[0345] Silencing of Eg5 by siRNA transfection causes mitotic arrest and apoptosis in mammalian cells. Cell viability following transfection with SNALP containing an siRNA targeting Eg5 therefore provides a simple biological readout of *in vitro* transfection efficiency. Cell viability of *in vitro* cell cultures was assessed using the commercial reagent CellTiter-Blue® (Promega Corp.; Madison, WI), a resazurin dye that is reduced by metabolically active cells to the flourogenic product resorufin. The human colon cancer cell

line HT29 was cultured using standard tissue culture techniques. 72 hours after SNALP application, CellTiter-Blue® reagent was added to the culture to quantify the metabolic activity of the cells, which is a measure of cell viability. Data are presented as a percent of cell viability relative to ("untreated") control cells that received phosphate buffered saline (PBS) vehicle only.

[0346] Figure 1 shows that the 1:57 SNALP formulation containing Eg5 2263 U/U siRNA was among the most potent inhibitors of tumor cell growth at all siRNA concentrations tested (*see,* Figure 1B, Sample 9).

Example 3. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo*.

[0347] SNALP formulations were prepared with an siRNA targeting apolipoprotein B (ApoB) as the nucleic acid component. ApoB is the main apolipoprotein of chylomicrons and low density lipoproteins (LDL). Mutations in ApoB are associated with hypercholesterolemia. ApoB occurs in the plasma in 2 main forms, ApoB48 and ApoB100, which are synthesized in the intestine and liver, respectively, due to an organ-specific stop codon. The ApoB siRNA used in this study is provided in Table 3. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the ApoB siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 3. siRNA duplex comprising sense and antisense ApoB RNA polynucleotides.

Position	Modification	ApoB siRNA sequence	SEQ ID NO:	% 2'OMe-Modified	% Modified in DS Region
10048	U2/2 G1/2	5'-AGUGUCAUCACACUGAAUACC-3' 3'-GUUCACAGUAGUGUGACUUAU-5'	3 4	7/42 = 16.7%	7/38 = 18.4%

Column 1: The number refers to the nucleotide position of the 5' base of the sense strand relative to the mouse ApoB mRNA sequence XM_137955. Column 2: The numbers refer to the distribution of 2'OMe chemical modifications in each strand. Column 3: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. Column 4: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 5: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

[0348] The lipid components and physical characteristics of the formulations are summarized in Table 4. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments

Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 4. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2 | 40 | 10 | 48	12.4	59	0.15	93
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 2.2 | 44.4 | 53.3	10.7	55	0.17	91
4	PEG(2000)-C-DMA | DLinDMA | DOPC | Cholesterol 2 | 40 | 10 | 48	12.5	59	0.16	92
5	PEG(2000)-C-DMA | DLinDMA | DMPC | Cholesterol 2 | 40 | 10 | 48	12.2	56	0.11	92
6	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.8 | 36.4 | 18.2 | 43.6	13.8	66	0.16	93
7	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 2 | 40 | 10 | 48	12.4	56	0.12	92
8	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 27.0 | 6.8 | 64.9	16.5	60	0.10	93
9	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.3 | 25.3 | 12.7 | 60.8	18.1	74	0.13	92
10	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2.5 | 25.0 | 12.5 | 60.0	19.2	60	0.13	93
11	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.4 | 34.3	17.8	79	0.09	94
12	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.0 | 40.4 | 10.1 | 48.5	23.6	72	0.11	93
13	PEG(2000)-C-DMA | DLinDMA | DPPC 2 | 70 | 28	8.7	73	0.09	87
14	PEG(2000)-C-DMA | DLinDMA | DPPC 1.6 | 54.7 | 43.8	11.3	65	0.11	87

[0349] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0350] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0351] Figure 2 shows that the 1:57 SNALP formulation containing ApoB 10048 U2/2 G1/2 siRNA was the most potent at reducing ApoB expression *in vivo* (*see*, Group 11).

Example 4. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo*.

[0352] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 5. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 5. Characteristics of the SNALP formulations used in this study.

SNALP (L:D ratio)	siRNA Payload	Particle Size (Polydispersity)	% Encapsulation
2:30 (13)	ApoB-10048 U2/2 G1/2	65 nm (0.16)	88
1:57 (9)	ApoB-10048 U2/2 G1/2	74 nm (0.10)	89

[0353] The 2:30 SNALP formulation used in this study is lipid composition 2:30:20:48 as described in molar percentages of PEG-C-DMA, DLinDMA, DSPC, and cholesterol (in that order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 13:1.

[0354] The 1:57 SNALP formulation used in this study is lipid composition 1.5:57.1:7:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 9:1.

[0355] BALB/c mice (female, 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Days 0, 1, 2, 3 & 4 for a total of 5 doses per animal. Daily dosage was either 1.0 (for 2:30 SNALP) or 0.1 (for 1:57 SNALP) mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 µl). As a negative control, one group of animals was given IV injections of phosphate buffered saline (PBS) vehicle. On Study Day 7, 72 h after the last treatment, animals were euthanized and liver tissue was collected in RNAlater.

[0356] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0357] Figure 3 shows that the 1:57 SNALP containing ApoB 10048 U2/2 G1/2 siRNA was more than 10 times as efficacious as the 2:30 SNALP in mediating ApoB gene silencing in mouse liver at a 10-fold lower dose.

Example 5. ApoB siRNA Formulated as 1:57 or 1:62 SNALP Have Potent Silencing Activity *in vivo*.

[0358] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 6. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 6. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	76	0.06	89
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 1.5 | 61.5 | 36.9	8.1	76	0.04	86
4	PEG(2000)-C-DMA | DODMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.0	72	0.05	95
5	PEG(5000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.6	52	0.16	89
6	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 1.4 | 57.1 | 7.1 | 34.3	8.9	68	0.10	94
7	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	72	0.07	95
8	PEG(2000)-C-DMA | DLinDMA | DPPC 1.8 | 70.2 | 28.1	8.6	74	0.13	86

[0359] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.75 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0360] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0361] Figure 4 shows that the 1:57 and 1:62 SNALP formulations had comparable ApoB silencing activity *in vivo* (*see, e.g.*, Groups 2 & 3).

Example 6. ApoB siRNA Formulated as 1:62 SNALP Have Potent Silencing Activity *in vivo*.

[0362] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 7. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 7. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition, Mole % PEG(2000)-C-DMA I DLinDMA I Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	1.5 I 61.5 I 36.9	6.1	80	0.07	92
3	1.4 I 54.8 I 43.8	6.6	74	0.05	89
4	2.0 I 61.2 I 36.7	6.2	71	0.11	91
5	1.8 I 54.5 I 43.6	6.7	67	0.09	91
6	1.3 I 68.1 I 30.6	7.4	91	0.08	89
7	1.2 I 61.8 I 37.1	8.0	87	0.10	90
8	1.7 I 67.8 I 30.5	7.6	81	0.07	91
9	1.4 I 56.3 I 42.3	8.6	75	0.11	92
10	1.9 I 61.3 I 36.8	8.2	72	0.10	91
11	1.8 I 56.1 I 42.1	8.8	70	0.10	90
12	1.3 I 66.7 I 32.0	9.5	89	0.09	89
13	1.2 I 61.7 I 37.0	10.0	87	0.10	91
14	1.7 I 66.4 I 31.9	9.6	82	0.11	90
15	1.5 I 61.5 I 36.9	10.1	79	0.10	91

[0363] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0364] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene

assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0365] Figure 5 shows that the 1:62 SNALP formulation was one of the most potent inhibitors of ApoB expression at two different lipid:drug ratios (*i.e.*, 6.1 & 10.1) among the phospholipid-free SNALP formulations tested (*see*, Groups 2 & 15).

Example 7. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Syringe Press or Gear Pump Process.

[0366] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by various manufacturing processes. In particular, 1:57 SNALP was prepared by a syringe press or gear pump process using either PBS or citrate buffer (post-blend dilution) and administered intravenously in mice.

Experimental Design

[0367] Animal Model: Female BALB/c mice, 5 wks old, n=4 per group/cage.

[0368] siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

Tolerability:

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
1	PBS vehicle	Standard 10 mL/kg volume	
2	1|57 Citrate Direct Dil, Syringe Press	7	77
3	1|57 PBS Direct Dil, Syringe Press	7	96
4	1|57 PBS Direct Dil, Gear Pump	7	79
5	1|57 Citrate Direct Dil, Syringe Press	9	99
6	1|57 PBS Direct Dil, Syringe Press	9	123
7	1|57 PBS Direct Dil, Gear Pump	9	102

Efficacy:

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
8	PBS vehicle	Standard 10 mL/kg volume	
9	1|57 PBS Direct Dil, Syringe Press	0.05	0.68
10	1|57 PBS Direct Dil, Gear Pump	0.05	0.57
11	1|57 PBS Direct Dil, Syringe Press	0.1	1.36
12	1|57 PBS Direct Dil, Gear Pump	0.1	1.13

Formulation:

[0369] Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

[0370] Formulation Details:

1. Lipid composition "1|57 Citrate blend" used in this study is 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation has an input lipid to drug ratio of 8.9.
2. Gear pump set up included 0.8 mm T-connector and 400 mL/min speed.
3. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0371] Formulation Summary:

		Particle Size			Final L:D
	1:57 (9:1) + DOW siRNA	Zavg (nm)	Poly	% Encap	(mg:mg)
322-050807-1	Syringe PBS Blend	79	0.12	92	13.6
322-050807-2	Syringe Citrate Blend	86	0.11	91	11.0
322-050807-3	Gear PBS Blend	80	0.09	93	11.3

Procedures

[0372] **Treatment:** Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 μl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0373] **Group 1-7 Endpoint:** Animals are sacrificed on Day 1, 24 h after test article administration. Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein.

[0374] **Group 8-12 Endpoint:** Animals are sacrificed on Day 2, 48 h after test article administration. Blood is collected by cardiac puncture and processed for plasma. Immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed and weighed separately: liver and spleen.

The bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at -20°C or -80°C for archival purposes. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0375] Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0376] Data Analysis: Tolerability of treatment regime is monitored by animal appearance and behavior as well as body weight. Blood clinical chemistry is measured by automated analyzer. ApoB and GAPDH mRNA levels in liver are measured via QG assay. ApoB protein in plasma is measured via ELISA. Total cholesterol in plasma is measured via standard enzymatic/colorimetric assay.

Results

[0377] There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. Figure 6 shows that the tolerability of SNALP prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters. There was a tolerability difference between syringe citrate and syringe PBS at constant siRNA dosage, but that was likely an artifact dependent on the different final lipid:drug (L:D) ratios of these two preparations.

[0378] Figure 7 shows that the efficacy of the 1:57 SNALP prepared by gear pump was similar to the same SNALP prepared by syringe press. The tolerability profile was improved with the gear pump process, which could be attributed to increased initial encapsulation rate and decreased final L:D ratio.

Example 8. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Direct Dilution or In-Line Dilution Process.

[0379] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by a direct dilution or in-line dilution process at an input lipid to drug ratio of 6:1 or 9:1.

Experimental Design

[0380] Animal Model: Female BALB/c mice, 7 wks old.

[0381] siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

CBC/Diff:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
1	3	PBS	-	-
2	3	1|57 SNALP (9:1)	7 mg/kg	71 mg/kg
3	3	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Clinical Chemistry:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
4	4	PBS	-	-
5	4	1|57 SNALP (9:1)	9 mg/kg	92 mg/kg
6	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg
7	4	(6:1) New 1|57 SNALP	11 mg/kg	78 mg/kg
8	4	(6:1) New 1|57 SNALP	13 mg/kg	93 mg/kg
9	4	(6:1) New 1|57 SNALP	15 mg/kg	107 mg/kg
10	4	(6:1) New 1|57 SNALP	17 mg/kg	121 mg/kg
11	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Activity:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
12	4	PBS	-	-
13	4	1|57 SNALP (9:1)	0.05 mg/kg	0.51 mg/kg
14	4	1|57 SNALP (9:1)	0.1 mg/kg	1.02 mg/kg
15	4	1|57 SNALP (9:1)	0.2 mg/kg	2.04 mg/kg
16	4	(6:1) New 1|57 SNALP	0.05 mg/kg	0.36 mg/kg
17	4	(6:1) New 1|57 SNALP	0.1 mg/kg	0.71 mg/kg
18	4	(6:1) New 1|57 SNALP	0.2 mg/kg	1.42 mg/kg
19	4	(6:1) New 1|57 SNALP	0.4 mg/kg	2.85 mg/kg

Formulation:

[0382] Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

[0383] Formulation Details:

1. "1|57 SNALP" used in this study is lipid composition 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by gear pump at an input lipid to drug ratio of 9:1 (28 mM lipids) or 6:1 (14 mM lipids).
2. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0384] Formulation Summary:

	1|57 SNALP Gear PBS In-Line	Particle Size			Final L:D
		Zavg (nm)	Poly	% Encap	(mg:mg)
322-051407-1	Input 9:1	78	0.07	93	10.2
322-051407-2	Input 6:1	81	0.05	92	7.1

Procedures

[0385] Treatment: Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 µl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0386] Endpoint: Animals are sacrificed on Day 1, 24 h after test article administration (Grps 1-10) or on Day 2, 48 h after test article administration (Grps 11-19).

[0387] Groups 1-3: Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into an EDTA microtainer, mixed immediately to prevent coagulation, and sent for analysis of CBC/Diff profile. Perform brief necropsy.

[0388] Groups 4-11: Blood is collected by cardiac puncture into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein. Perform brief necropsy.

[0389] Groups 12-19: Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed: liver. The liver is not weighed; the bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at –80°C. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0390] Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0391] Data Analysis: Tolerability of treatment regime is monitored by animal appearance and behavior, and body weight. Blood clinical chemistry and CBC/Diff profile is measured

by automated analyzer. Liver ApoB mRNA is measured using the QuantiGene Assay. Plasma ApoB-100 is measured using ELISA. Plasma total cholesterol is measured using a standard enzymatic assay.

Results

Tolerability:

[0392] Figure 8 shows that there was very little effect on body weight 24 hours after 1:57 SNALP administration. The maximum weight loss of 3.6 ± 0.7% was observed at the highest drug dose of 17 mg/kg. There was also no obvious change in animal appearance/behavior at any of the dosages tested.

[0393] Figure 9 shows that there were no obvious changes in platelet count. Reduction of platelets can cause the mean platelet volume to increase as the body produces new platelets in compensation for the treatment-related decrease. Under the conditions of this study, the mean platelet volume did not change in SNALP-treated groups.

[0394] Figure 10 shows that clinically significant liver enzyme elevations (3xULN) occurred at drug dosages of 11 mg/kg for 1:57 SNALP at a lipid:drug (L:D) ratio of 10, and at 13 mg/kg at a L:D of 7. A slight dose response trend upwards in plasma total protein and globulin was also observed.

Efficacy:

[0395] Figure 11 shows that based on the liver mRNA QuantiGene analysis, the potency of the lower L:D SNALP was as good as that of the higher L:D SNALP at the tested drug dosages. In fact, the ApoB silencing activity was identical at the 0.05 and 0.1 mg/kg dosages. As such, the potency of the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) was similar to the potency of the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) at reducing ApoB expression.

[0396] Figure 12 shows that ApoB protein and total cholesterol levels were reduced to a similar extent by the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

Therapeutic Index:

[0397] This study demonstrates that both the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) caused about 60% ApoB liver mRNA silencing with a drug dose of 0.1 mg/kg. Interpolating from the available data points in Figure 10, a 10:1 final L:D ratio at 10 mg/kg may cause a similar

degree of enzyme elevation as a 7:1 final L:D ratio at 13 mg/kg. Using these activity and toxicity points, the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio is (10 mg/kg) / (0.1 mg/kg) = 100 and the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is (13 mg/kg) / (0.1 mg/kg) = 130. Using this dataset, the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is 30% greater than the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio.

Example 9. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Increases Survival of Hep3B Tumor-Bearing Mice.

[0398] SNALP containing polo-like kinase 1 (PLK-1) siRNA (1:57 SNALP formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) were tested for their effects on the survival of CD1 nu/nu mice bearing Hep3B liver tumors. PLK-1 is a serine/threonine kinase containing two functional domains: (1) a kinase domain; and (2) a polo-box domain (*see, e.g.*, Barr *et al.*, *Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)). The activity and cellular concentration of PLK-1 are crucial for the precise regulation of cell division. PLK-1 is overexpressed in many cancer types including hepatoma and colon cancer, and PLK-1 expression often correlates with poor patient prognosis. Overexpression of PLK-1 (wild-type or kinase inactive) results in multinucleation (genetic instability). Hyperactive PLK-1 overrides the DNA damage checkpoint. Constitutive PLK-1 expression causes transformation of NIH 3T3 cells. PLK-1 phosphorylates the p53 tumor suppressor, thereby inhibiting the pro-apoptotic effects of p53. The PLK-1 siRNA used in this study are provided in Table 8. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the PLK-1 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 8. siRNA duplexes comprising sense and antisense PLK-1 RNA polynucleotides.

siRNA	PLK-1 siRNA Sequence	SEQ ID NO:	% Modified in DS Region
PLK1424 U4/GU	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' ~~(SEQ ID NO.57)~~ 3'-NNUC**U**AGUGGGAG**G**AAUUUAU-5' ~~(SEQ ID NO.54)~~	5 6	6/38 = 15.8%
PLK1424 U4/G	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3' ~~(SEQ ID NO.57)~~ 3'-NNUCUAGUGGGAGGAAUUUAU-5' ~~(SEQ ID NO.56)~~	5 7	7/38 = 18.4%

Column 1: The number after "PLK" refers to the nucleotide position of the 5' base of the sense strand relative to the start codon (ATG) of the human PLK-1 mRNA sequence NM_005030. Column 2: 2'-O-methyl (2'OMe) nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. N = deoxythymidine (dT) nucleotide, uridine (U) ribonucleotide, or

ribonucleotide having complementarity to the target sequence (antisense strand) or the complementary strand thereof (sense strand). Column 3: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

Experimental Groups

[0399] 20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay
A	20 to seed	I.H. $1.5x10^6$ Hep3B	Luc 1:57	9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10 x 2 mg/kg	When moribund	Survival Body Weights
B			PLK 1424 1:57	9				

Test Articles

[0400] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 20 ml of each SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	Luc U/U SNALP 1:57 (28mM lipid)
B	PLK1424 U4/GU SNALP 1:57 (28mM lipid) PLK1424 U4/G SNALP 1:57 (28mM lipid)

Procedures

Day 0 Mice will receive Anafen by SC injection (100 μg in 20 μl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25 μl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 μl) and 30G (3/8") needle. Cells will be injected slowly (~ 30 s) and a swab applied to the puncture wound immediately after

needle withdrawal. After any bleeding has stopped (~1 min), the incision is closed with 5-6 sutures in the muscle wall and 3-4 skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.

Day 1 All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 μg in 20 μl saline).

Day 10 Mice will be randomized into the appropriate treatment groups.

Day 11 **Groups A, B – Day 11:** All Animals will be administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg). Dosing will be repeated for 5 consecutive days based on initial weight.

Day 14-35 **Groups A, B – Days 14, 17, 21, 25, 28, 32, 35:** All Animals will be re-administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg).

Body weights Groups: Mice will be weighed on the day of dosing for 5 weeks, then twice weekly until close of the study.

Endpoint: Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the treatment or tumor burden are terminated at the discretion of the vivarium staff.

Termination: Mice are anesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.

Data Analysis: Survival and body weights are assayed.

Results

[0401] Figure 13 shows the mean body weights of mice during therapeutic dosing of PLK1424 SNALP in the Hep3B intrahepatic (I.H.) tumor model. The treatment regimen was well tolerated with no apparent signs of treatment-related toxicity.

[0402] Figure 14 shows that treatment with 1:57 SNALP-formulated PLK1424 caused a significant increase in the survival of Hep3B tumor-bearing mice. This *in vivo* anti-tumor effect was observed in the absence of any apparent toxicity or immune stimulation.

Example 10. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Induces Tumor Cell Apoptosis in Hep3B Tumor-Bearing Mice.

[0403] The objectives of this study were as follows:

1. To determine the level of mRNA silencing in established Hep3B liver tumors following a single IV administration of PLK1424 SNALP.
2. To confirm the mechanism of mRNA silencing by detecting specific RNA cleavage products using RACE-PCR.
3. To confirm induction of tumor cell apoptosis by histopathology.

[0404] The 1:57 SNALP formulation (1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) was used for this study.

Experimental Groups

[0405] 20 SCID/beige mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	Sacrifice	Assay
A	20 to seed	I.H. $1x10^6$ Hep3B	PBS	6	1 x 2 mg/kg Day 20	24 h after treatment	Tumor QG Tumor RACE-PCR Histopathology
			Luc 1:57	7			
B			PLK 1424 1:57	7			
C							

Test Articles

[0406] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 2 ml of SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	PBS
B	Luc U/U 1:57 SNALP
C	PLK1424 U4/GU 1:57 SNALP

Procedures

Day 0 Mice will receive Anafen by SC injection (100 µg in 20 µl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25µl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 µl) and 30G (3/8") needle. Cells will be injected slowly (~30 s) and a swab applied to the puncture wound immediately after needle withdrawal. After any bleeding has stopped (~1 min), the muscle wall incision is closed with 5-6 sutures. The skin incision is then closed with 3-4 metal skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.

Day 1 All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 µg in 20 µl saline).

Day 7 Mice will be randomized into the appropriate treatment groups.

Day 20 **Groups A-C:** Mice will be weighed and then administered either PBS, Luc, or PLK1424 SNALP by IV injection via the lateral tail vein. SNALP will be dosed at 2 mg/kg or equivalent volume (10 ml/kg) according to body weight.

Day 21 **Groups A-C:** All mice will be weighed and then euthanized by lethal anesthesia.

Tumor bearing liver lobes from all mice in each group will be weighed and collected into RNALater for RNA analysis.

Endpoint: Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the

	treatment or tumor burden are terminated at the discretion of the vivarium staff.
Termination:	Mice are anaesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.
Data Analysis:	mRNA analysis of liver tumors by bDNA (QG) assay and RACE-PCR. Tumor cell apoptosis by histopathology.

Results

[0407] Body weights were monitored from Day 14 onwards to assess tumor progression. On Day 20, 6 mice showing greatest weight loss were randomized into each of the 3 groups and treated. All six mice had substantial-large I.H. tumors at sacrifice (Day 21). Treatment of the remaining 14 mice was therefore initiated on the Day 21 (sacrifice Day 22). 10/14 mice had substantial tumors; 2/14 mice had small/probable tumors; and 2/14 mice had no visible tumor burden.

[0408] Figure 15 shows data from Quantigene assays used to measure human (tumor)-specific PLK-1 mRNA levels. A single 2 mg/kg dose of 1:57 SNALP reduced PLK-1 mRNA levels by about 50% in intrahepatic Hep3B tumors growing in mice.

[0409] Figure 16 shows that a specific cleavage product of PLK-1 mRNA was detectable in mice treated with PLK1424 SNALP by 5' RACE-PCR. No specific PCR product was detectable in mice treated with either PBS or control (Luc) SNALP. Nucleotide sequencing of the PCR product confirmed the predicted cleavage site by PLK1424 siRNA-mediated RNA interference in the PLK-1 mRNA.

[0410] Figure 17 shows Hep3B tumor histology in mice treated with either Luc SNALP (top) or PLK1424 SNALP (bottom). Luc SNALP-treated mice displayed normal mitoses in Hep3B tumors, whereas PLK1424 SNALP-treated mice exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors.

Conclusion

[0411] This example illustrates that a single administration of PLK1424 1:57 SNALP to Hep3B tumor-bearing mice induced significant *in vivo* silencing of PLK-1 mRNA. This reduction in PLK-1 mRNA was confirmed to be mediated by RNA interference using 5' RACE-PCR analysis. Importantly, PLK-1 mRNA silencing by the 1:57 SNALP formulation profoundly disrupted tumor cell proliferation (mitosis), causing subsequent apoptosis of

tumor cells. As demonstrated in the previous example, this anti-tumor effect translated into extended survival times in the tumor-bearing mice.

Example 11. Comparison of 1:57 PLK-1 SNALP Containing Either PEG-cDMA or PEG-cDSA in a Subcutaneous Hep3B Tumor Model.

[0412] This example demonstrates the utility of the PEG-lipid PEG-cDSA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-distearyloxypropylamine) in the 1:57 formulation for systemically targeting distal (*e.g.,* subcutaneous) tumors. In particular, this example compares the tumor targeting ability of 1:57 PLK-1 SNALPs containing either PEG-cDMA (C_{14}) or PEG-cDSA (C_{18}). Readouts are tumor growth inhibition and PLK1 mRNA silencing. The PLK-1 siRNA used was PLK1424 U4/GU, the sequence of which is provided in Table 8.

[0413] Subcutaneous (S.C.) Hep3B tumors were established in scid/beige mice. Multidose anti-tumor efficacy of 1:57 PLK-1 SNALP was evaluated for the following groups (n=5 for each group): (1) "Luc-cDMA" - PEG-cDMA Luc SNALP; (2) "PLK-cDMA" - PEG-cDMA PLK-1 SNALP; and (3) "PLK-cDSA" - PEG-cDSA PLK-1 SNALP. Administration of 6 x 2mg/kg siRNA was initiated once tumors reached about 5 mm in diameter (Day 10). Dosing was performed on Days 10, 12, 14, 17, 19, and 21. Tumors were measured by caliper twice weekly.

[0414] Figure 18 shows that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B S.C. tumors. In particular, 5/5 tumors in the PLK1-cDSA treated mice appeared flat, measurable only by discoloration at the tumor site.

[0415] Figure 19 shows the mRNA silencing of 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration. The extent of silencing observed with the PLK1-cDSA SNALP correlated with the anti-tumor activity in the multi-dose study shown in Figure 18.

[0416] The Luc-cDMA SNALP-treated group, which had developed large S.C. tumors at Day 24, were then administered PLK-cDSA SNALP on Days 24, 26, 28, 31, 33, and 35. There was no additional dosing of the original PLK-1 SNALP-treated groups. The results from this crossover dosing study with large established tumors is provided in Figure 20, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0417] A comparison of the effect of PEG-cDMA and PEG-cDSA 1:57 SNALPs on PLK-1 mRNA silencing was performed using established intrahepatic Hep3B tumors in scid/beige mice. A single 2 mg/kg dose of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-

cDSA was administered intravenously. Liver/tumor samples were collected at 24 and 96 hours after SNALP treatment. Control = 2 mg/kg Luc-cDMA SNALP at 24 hours.

[0418] Figure 21 shows that PLK-cDMA SNALP and PLK-cDSA SNALP had similar silencing activities after 24 hours, but that the PLK-cDSA SNALP may increase the duration of mRNA silencing in intrahepatic tumors.

[0419] Figure 22 shows the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA. The extended blood circulation times observed for the PLK-cDSA SNALP may enable the increased accumulation and activity at distal (*e.g.*, subcutaneous) tumor sites.

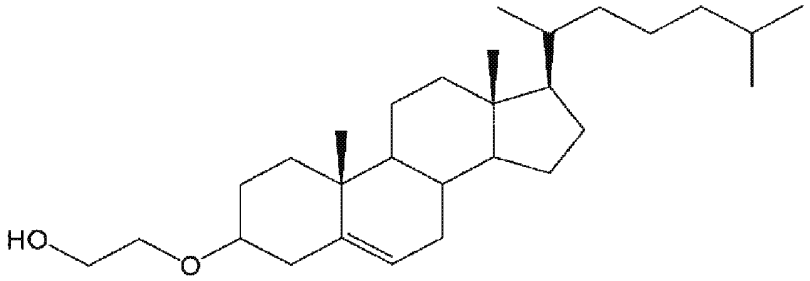
[0420] Thus, this study shows that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP can be used to preferentially target the liver.

Example 12. Synthesis of Cholesteryl-2'-Hydroxyethyl Ether.

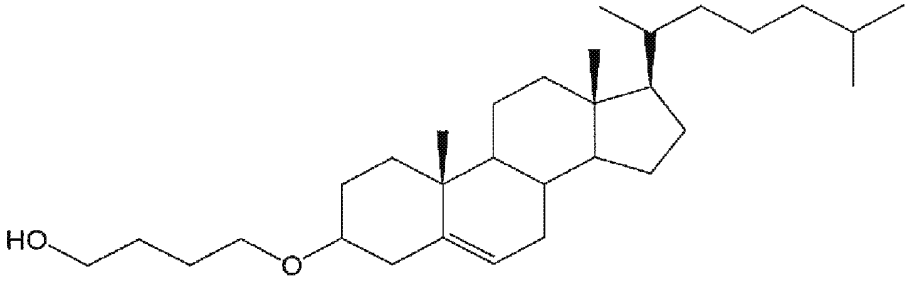
[0421] Step 1: A 250 ml round bottom flask containing cholesterol (5.0 g, 12.9 mmol) and a stir bar was sealed and flushed with nitrogen. Toluenesulphonyl chloride (5.0 g, 26.2 mmol) was weighed into a separate 100-mL round bottom flask, also sealed and flushed with nitrogen. Anhydrous pyridine (2 x 50 ml) was delivered to each flask. The toluenesulphonyl chloride solution was then transferred, via cannula, into the 250 ml flask, and the reaction stirred overnight. The pyridine was removed by rotovap, and methanol (80 ml) added to the residue. This was then stirred for 1 hour until a homogeneous suspension was obtained. The suspension was filtered, washed with acetonitrile (50 ml), and dried under vacuum to yield cholesteryl tosylate as a fluffy white solid (6.0 g, 86%).

[0422] Step 2: Cholesteryl tosylate (2.0 g, 3.7 mmol), 1,4-dioxane (50 mL), and ethylene glycol (4.6 g, 74 mmol) were added to a 100 ml flask containing a stir bar. The flask was fitted with a condenser, and refluxed overnight. The dioxane was then removed by rotovap, and the reaction mixture suspended in water (100 ml). The solution was transferred to a separating funnel and extracted with chloroform (3 x 100 ml). The organic phases were combined, washed with water (2 x 150 ml), dried over magnesium sulphate, and the solvent removed. The crude product was purified by column chromatography (5% acetone/hexane) to yield the product as a white solid (1.1 g, 69%).

[0423] The structures of the cholesterol derivatives cholesteryl-2'-hydroxyethyl ether and cholesteryl-4'-hydroxybutyl ether are as follows:



Cholesteryl-2'-hydroxyethyl ether



Cholesteryl-4'-hydroxybutyl ether

[0424] It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent applications, patents, PCT publications, and Genbank Accession Nos., are incorporated herein by reference for all purposes.

WHAT IS CLAIMED IS:

1. A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

2. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid comprises a small interfering RNA (siRNA).

3. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises from about 15 to about 60 nucleotides.

4. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one modified nucleotide.

5. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

6. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), or a mixture thereof.

7. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA).

8. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle.

9. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 52 mol % to about 62 mol % of the total lipid present in the particle.

10. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises cholesterol or a derivative thereof.

11. The nucleic acid-lipid particle of claim 10, wherein the cholesterol or derivative thereof comprises from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle.

12. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a phospholipid.

13. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.

14. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

15. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises from about 4 mol % to about 10 mol % of the total lipid present in the particle and the cholesterol comprises from about 30 mol % to about 40 mol % of the total lipid present in the particle.

16. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises from about 10 mol % to about 30 mol % of the total lipid present in the particle and the cholesterol comprises from about 10 mol % to about 30 mol % of the total lipid present in the particle.

17. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

18. The nucleic acid-lipid particle of claim 17, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

19. The nucleic acid-lipid particle of claim 18, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

20. The nucleic acid-lipid particle of claim 19, wherein the PEG has an average molecular weight of about 2,000 daltons.

21. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from about 1 mol % to about 2 mol % of the total lipid present in the particle.

22. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid in the nucleic acid-lipid particle is not substantially degraded after incubation of the particle in serum at 37°C for 30 minutes.

23. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

24. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a lipid:nucleic acid mass ratio of from about 5 to about 15.

25. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a median diameter of from about 40 nm to about 150 nm.

26. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.

27. A nucleic acid-lipid particle comprising:

(a) an siRNA;

(b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle;

(c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and

(d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

28. The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises DLinDMA.

29. The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises DLin-K-C2-DMA.

30. The nucleic acid-lipid particle of claim 27, wherein the PEG-lipid conjugate comprises a PEG-DAA conjugate.

31. The nucleic acid-lipid particle of claim 27, wherein the nucleic acid-lipid particle comprises about 61.5 mol % cationic lipid, about 36.9% cholesterol or a derivative thereof, and about 1.5 mol % PEG-lipid conjugate.

32. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 27 and a pharmaceutically acceptable carrier.

33. A nucleic acid-lipid particle, comprising:

(a) an siRNA;

(b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle;

(c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and

(d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

34. The nucleic acid-lipid particle of claim 33, wherein the cationic lipid comprises DLinDMA.

35. The nucleic acid-lipid particle of claim 33, wherein the cationic lipid comprises DLin-K-C2-DMA.

36. The nucleic acid-lipid particle of claim 33, wherein the phospholipid comprises DPPC.

37. The nucleic acid-lipid particle of claim 33, wherein the PEG-lipid conjugate comprises a PEG-DAA conjugate.

38. The nucleic acid-lipid particle of claim 33, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 7.1 mol % phospholipid, about 34.3 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

39. The nucleic acid-lipid particle of claim 33, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 20 mol % phospholipid, about 20 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

40. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 33 and a pharmaceutically acceptable carrier.

41. A method for introducing a nucleic acid into a cell, the method comprising:

contacting the cell with a nucleic acid-lipid particle of claim 1, 27, or 33.

42. The method of claim 41, wherein the cell is in a mammal.

43. A method for the *in vivo* delivery of a nucleic acid, the method comprising:

administering to a mammalian subject a nucleic acid-lipid particle of claim 1, 27, or 33.

44. The method of claim 43, wherein the administration is selected from the group consisting of oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal.

45. A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising:

administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 1, 27, or 33.

46. The method of claim 45, wherein the disease or disorder is selected from the group consisting of a viral infection, a liver disease or disorder, and cancer.

ABSTRACT OF THE DISCLOSURE

The present invention provides novel, stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles. More particularly, the present invention provides stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (such as one or more interfering RNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP.

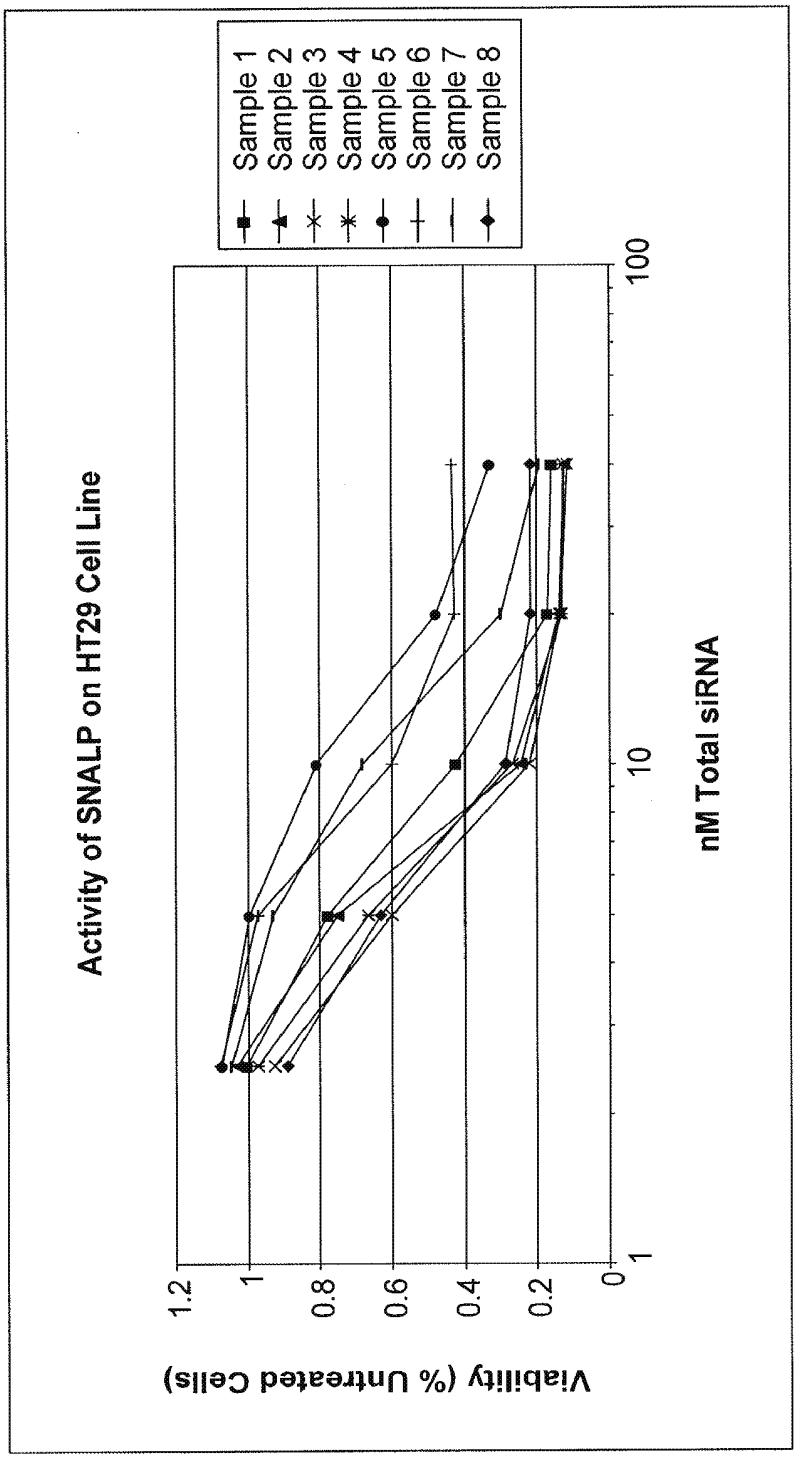

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8

FIG. 1A

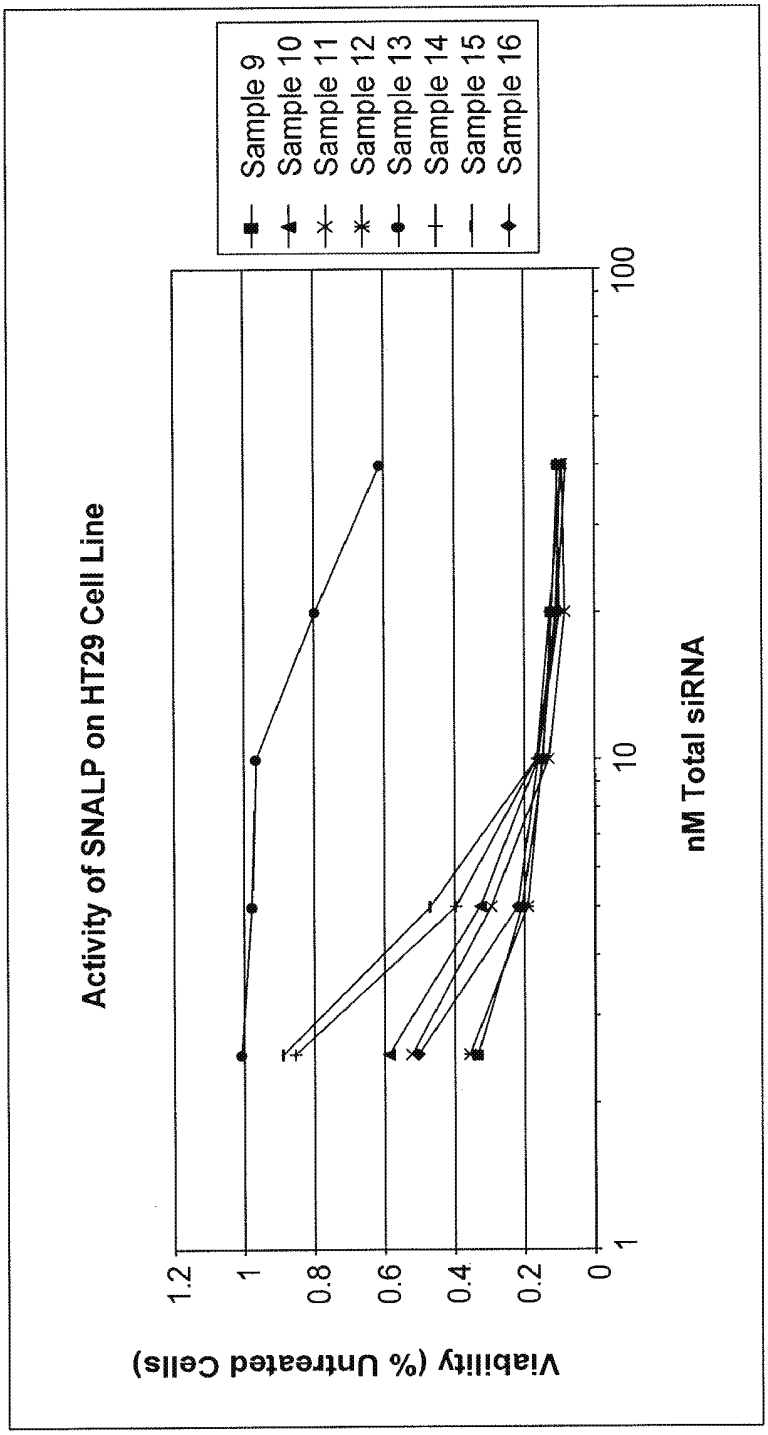

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 9
Sample 10
Sample 11
Sample 12
Sample 13
Sample 14
Sample 15
Sample 16

FIG. 1B

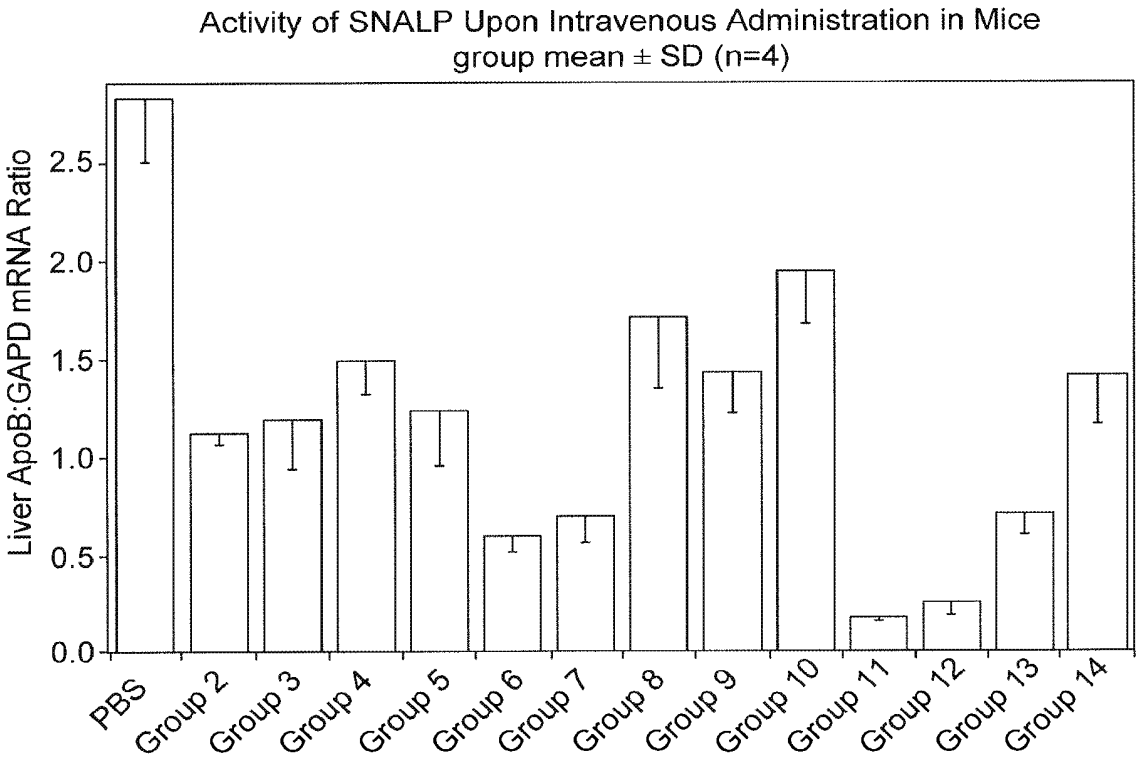

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
2.5
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

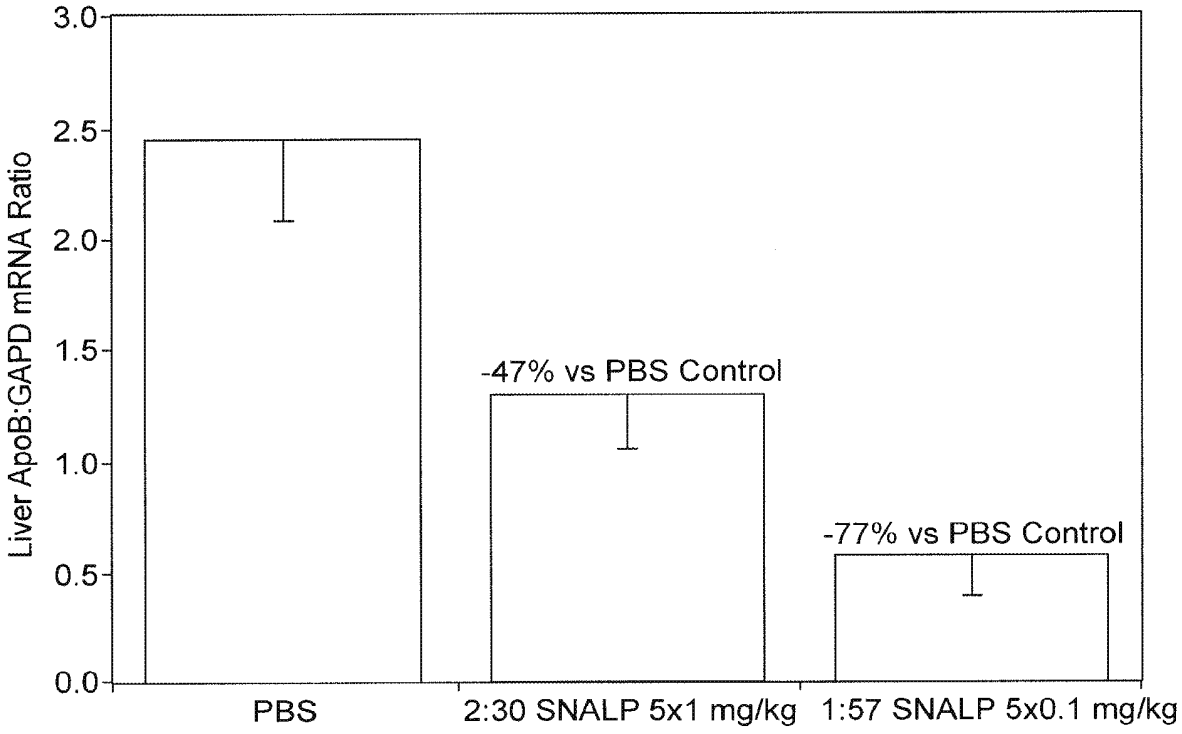

Liver ApoB:GAPD mRNA Ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5x1 mg/kg
1:57 SNALP 5x0.1 mg/kg

FIG. 3

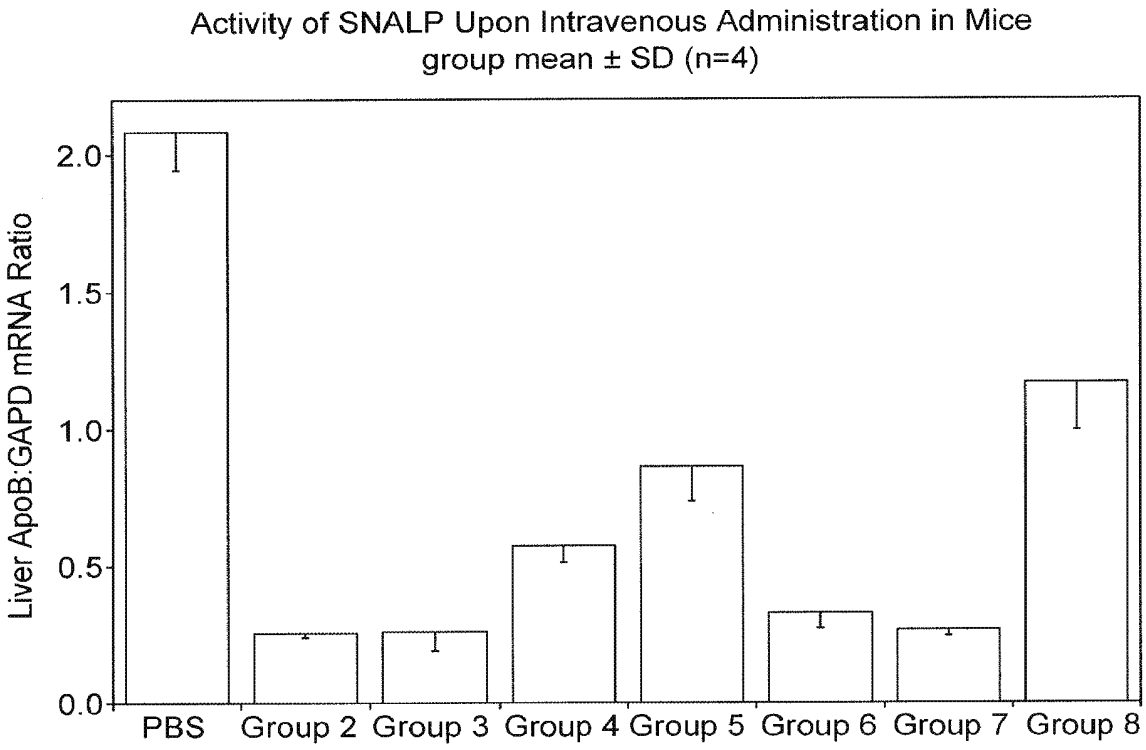

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

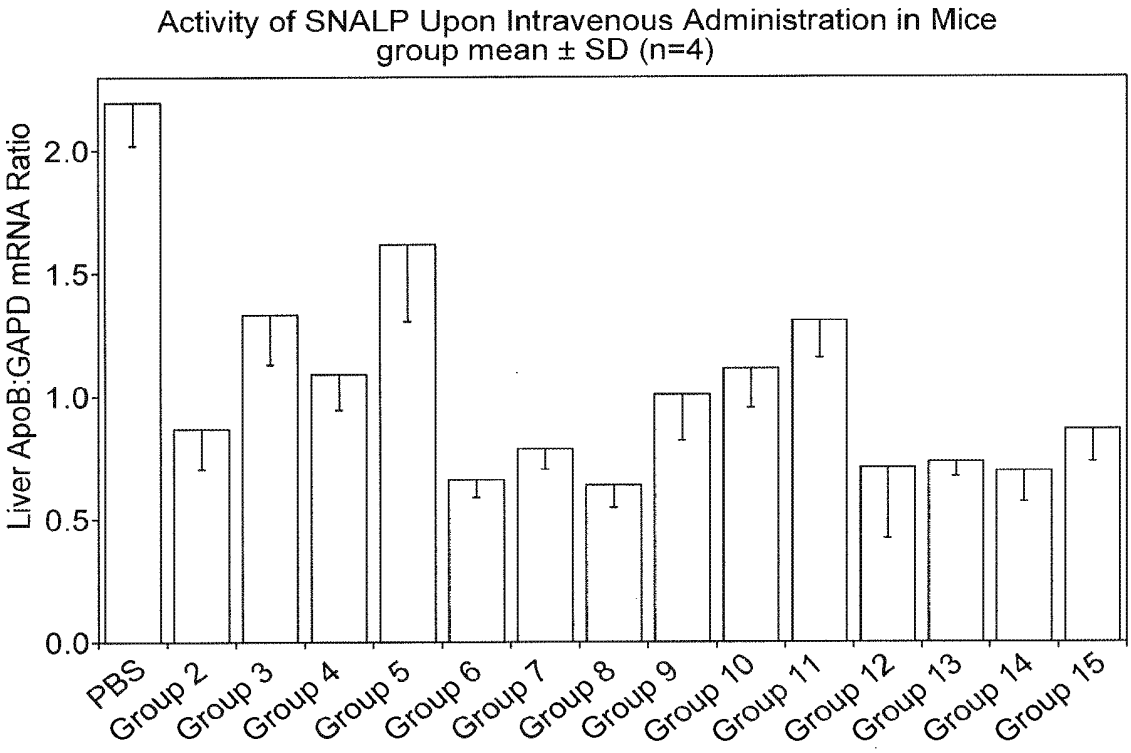

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

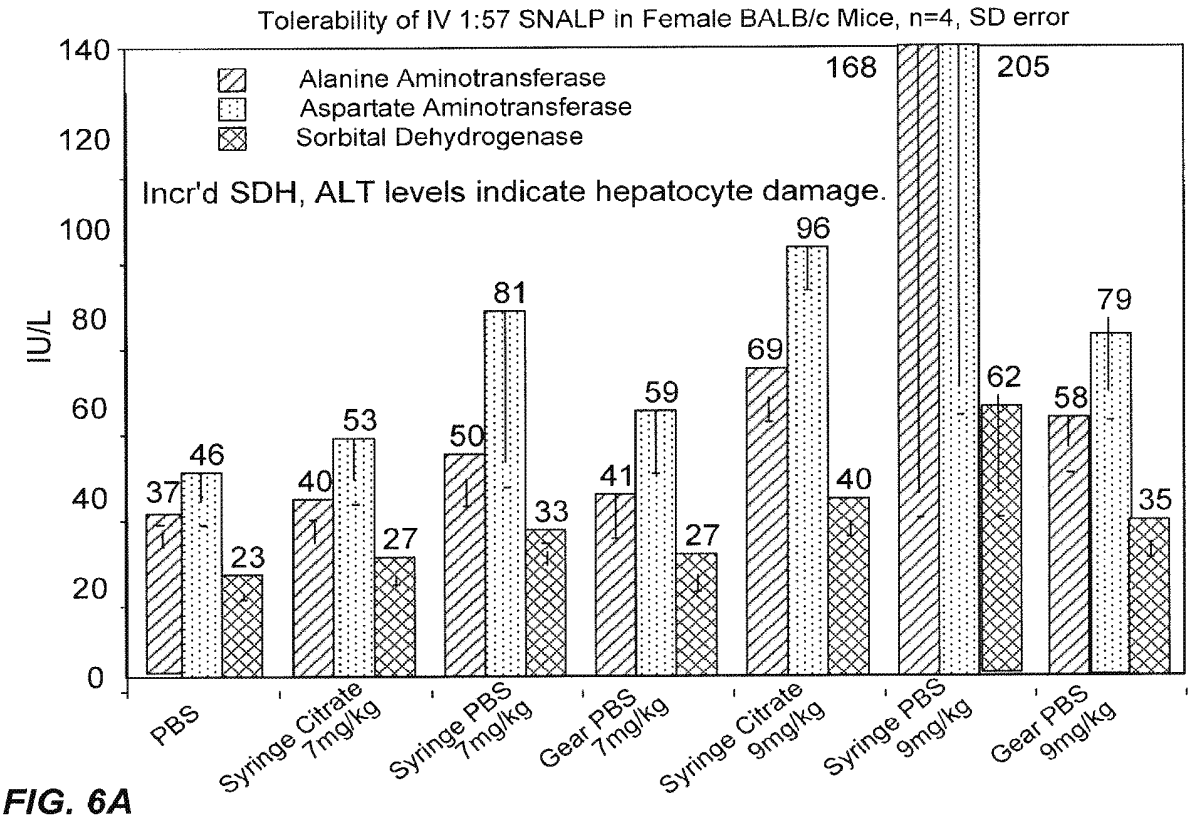

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
0
20
40
60
80
100
120
140
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
168
205
62
58
79
35
PBS
Syringe Citrate 7mg/kg
Syringe PBS 7mg/kg
Gear PBS 7mg/kg
Syringe Citrate 9mg/kg
Syringe PBS 9mg/kg
Gear PBS 9mg/kg

FIG. 6A

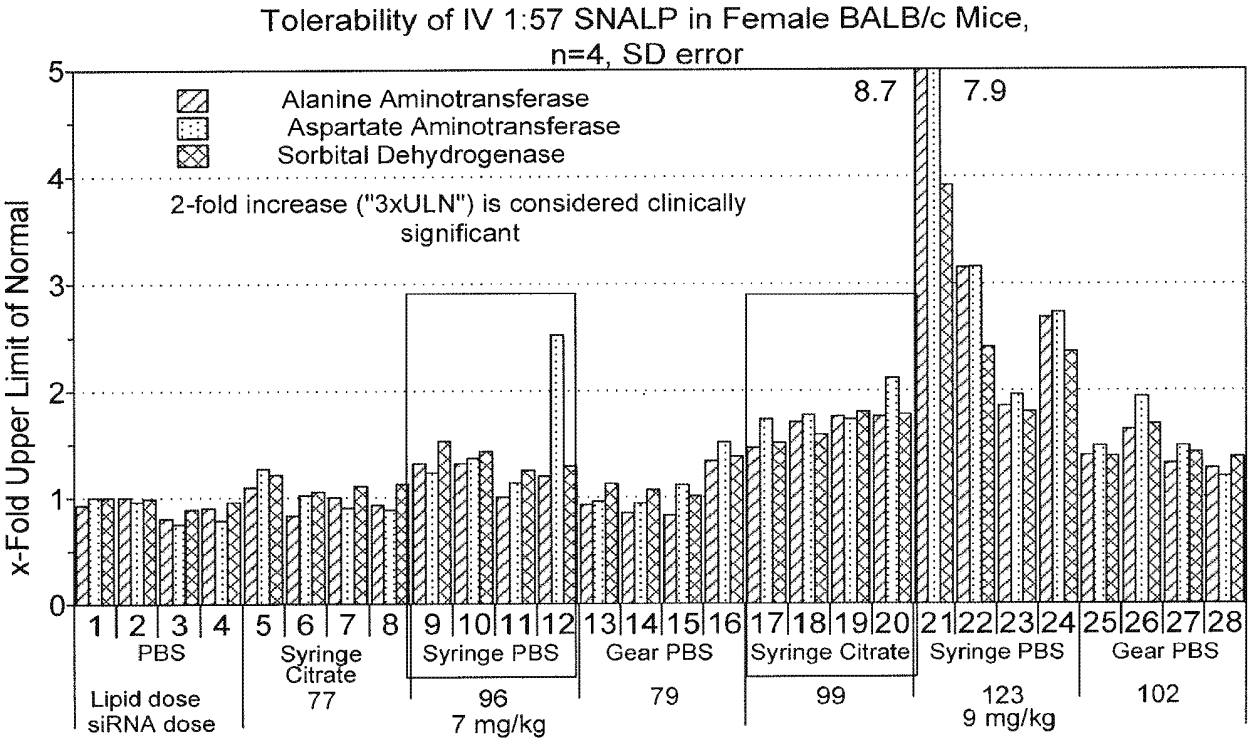

Tolerability of IV 1:57 SNALP in Female BALB/c Mice,
n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
1
2
3
4
5
8.7
7.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
PBS
Syringe Citrate
Syringe PBS
Gear PBS
Syringe Citrate
Syringe PBS
Gear PBS
Lipid dose
siRNA dose
77
96
7 mg/kg
79
99
123
9 mg/kg
102

FIG. 6B

FIG. 7A

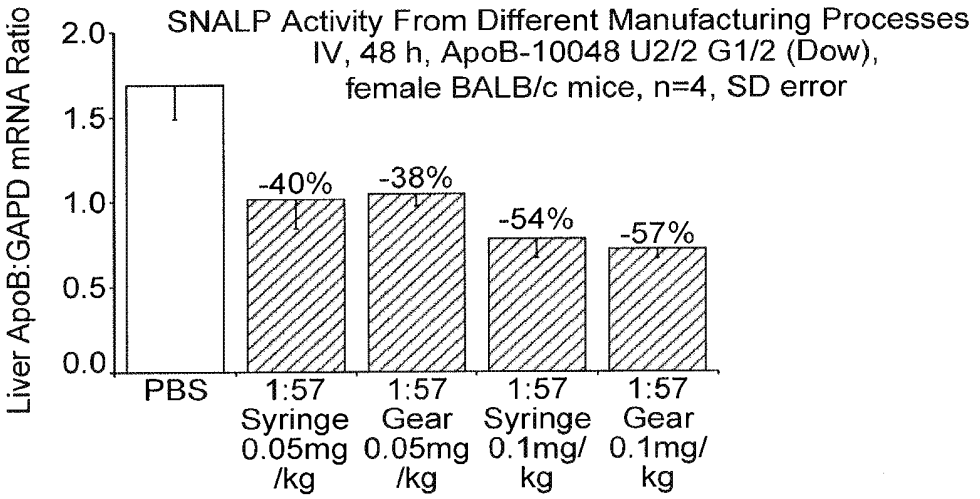


FIG. 7B

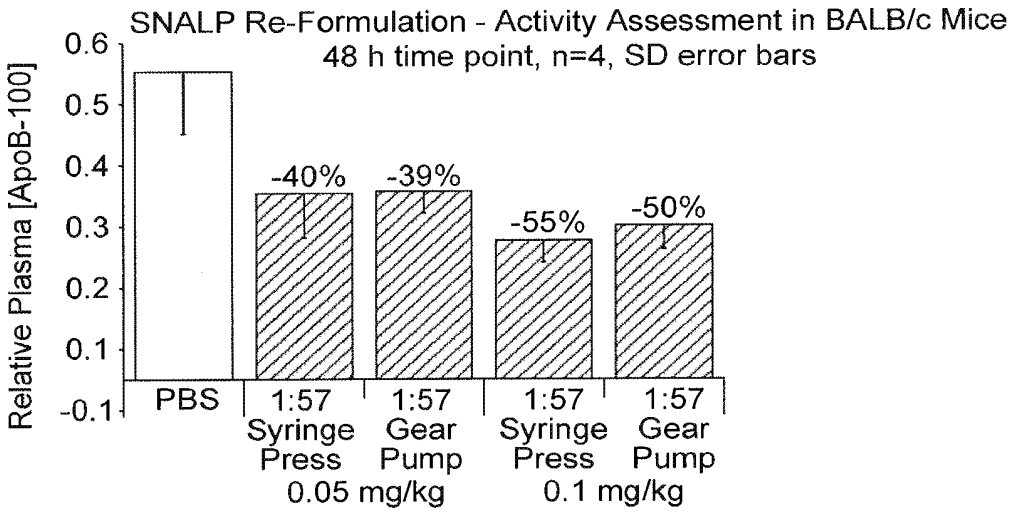
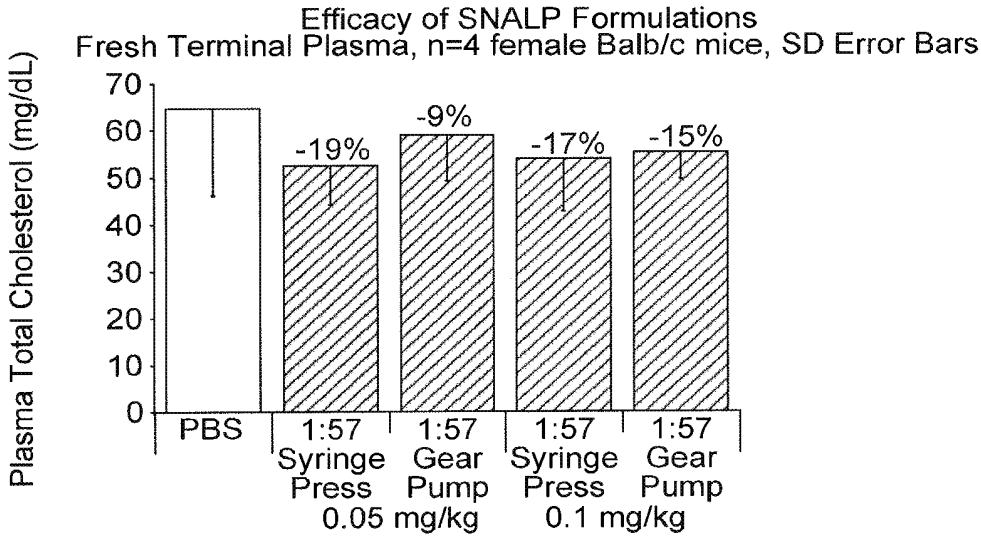


FIG. 7C



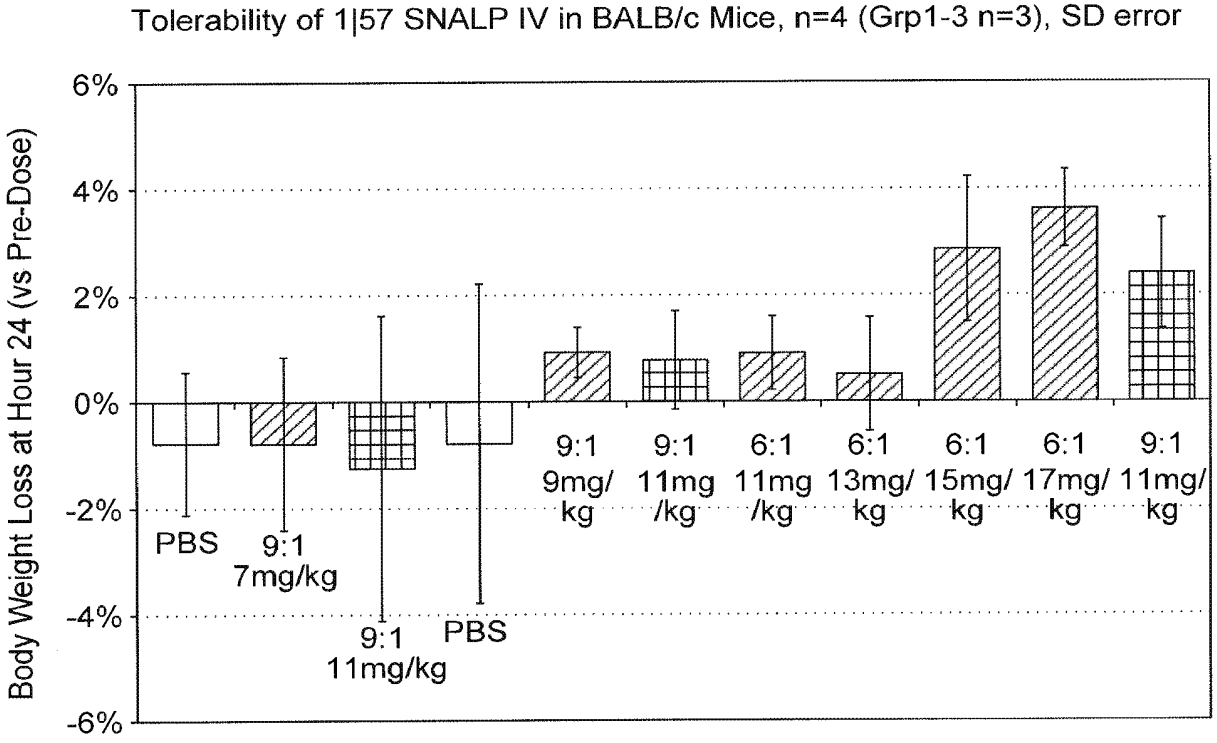

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

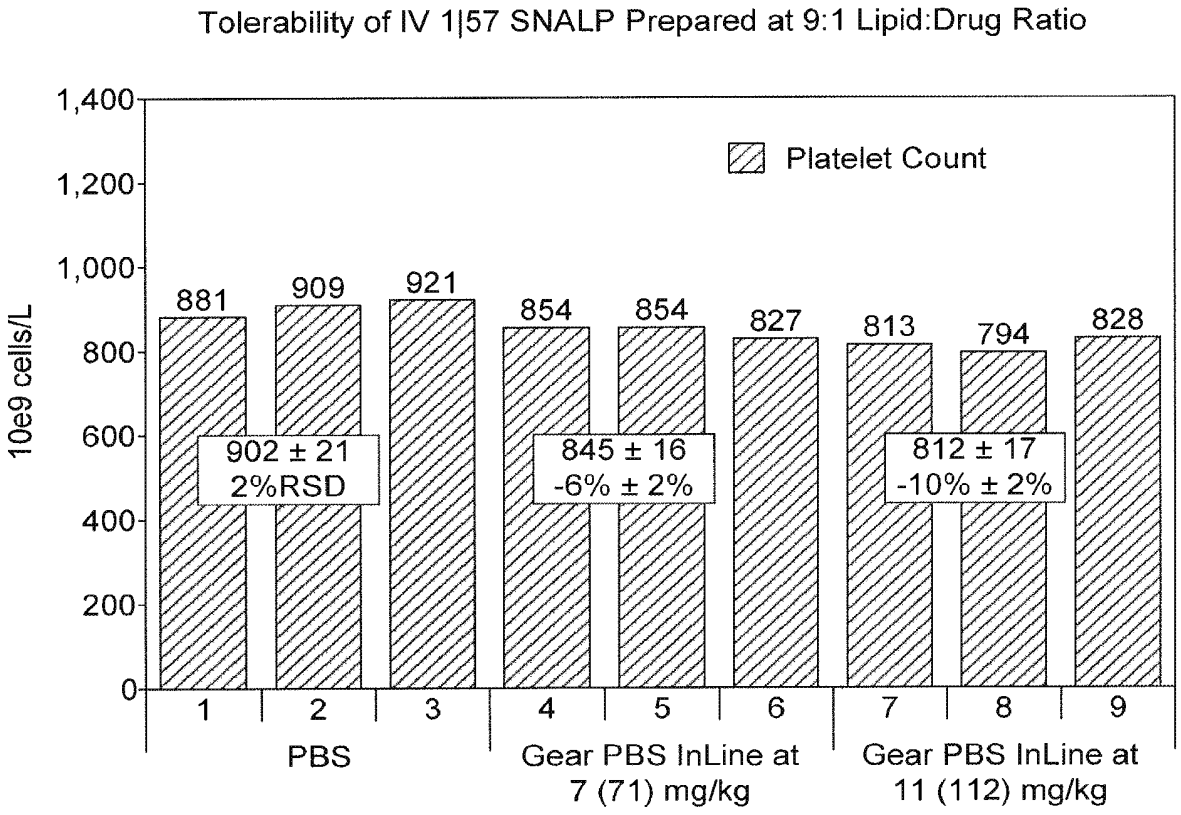

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
Platelet Count
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at
7 (71) mg/kg
Gear PBS InLine at
11 (112) mg/kg

FIG. 9

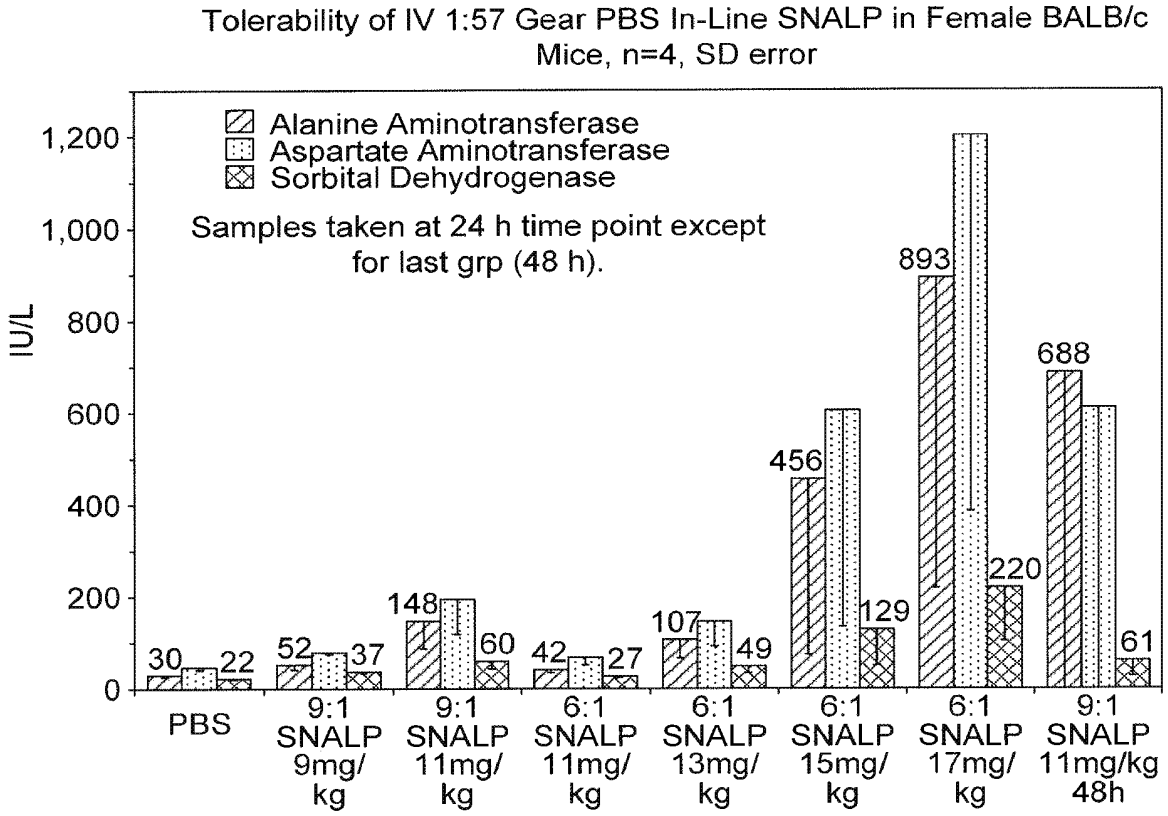

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
688
61
PBS
9:1 SNALP 9mg/kg
9:1 SNALP 11mg/kg
6:1 SNALP 11mg/kg
6:1 SNALP 13mg/kg
6:1 SNALP 15mg/kg
6:1 SNALP 17mg/kg
9:1 SNALP 11mg/kg 48h

FIG. 10A

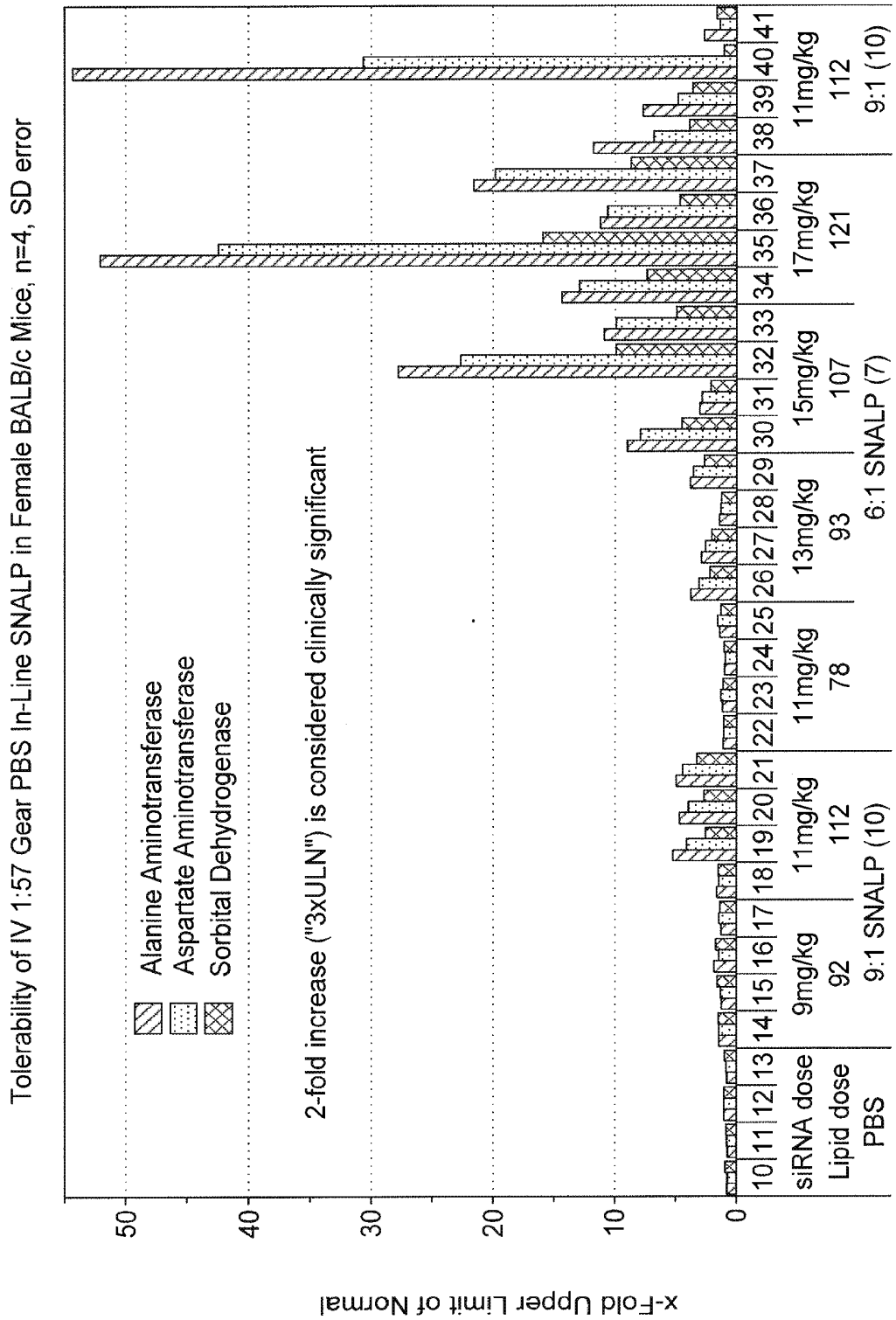

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
10
20
30
40
50
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
siRNA dose
Lipid dose
PBS
9mg/kg
92
9:1 SNALP (10)
11mg/kg
112
9:1 SNALP (10)
11mg/kg
78
6:1 SNALP (7)
13mg/kg
93
6:1 SNALP (7)
15mg/kg
107
6:1 SNALP (7)
17mg/kg
121
6:1 SNALP (7)
11mg/kg
112
9:1 (10)

FIG. 10B

FIG. 11A

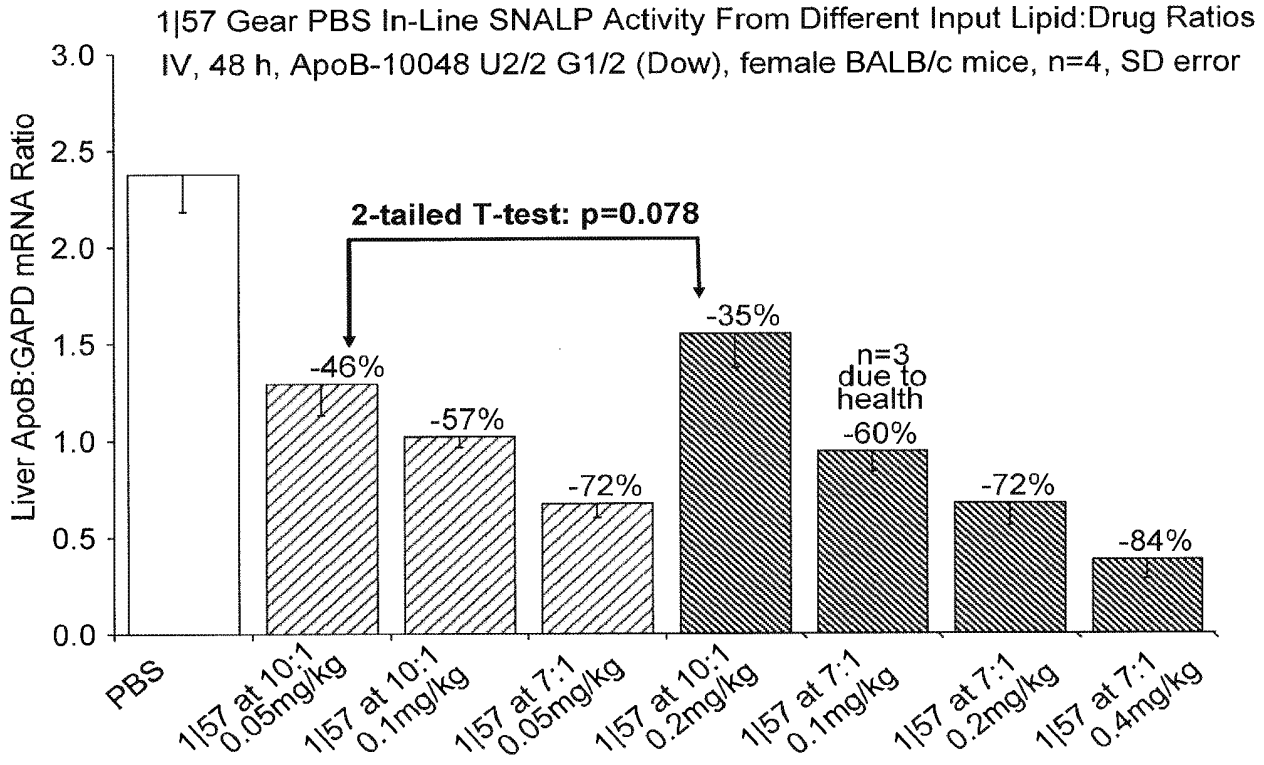
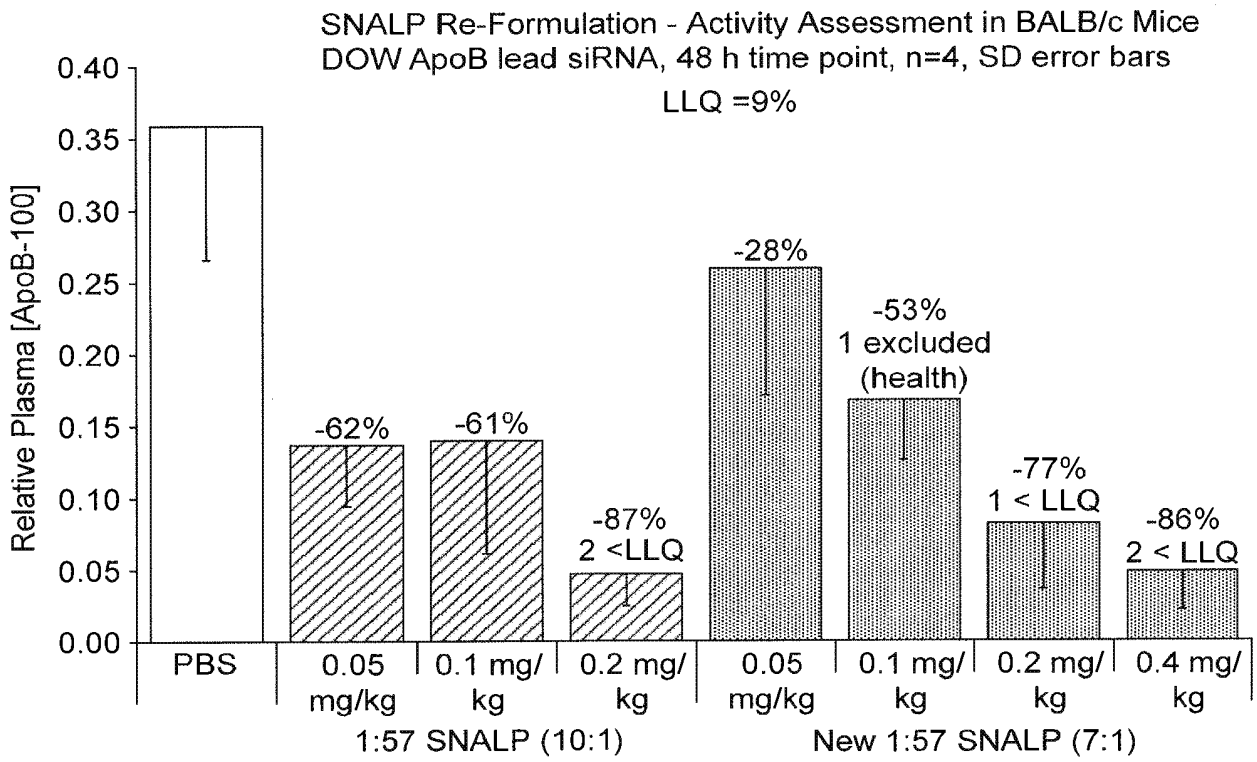


FIG. 11B



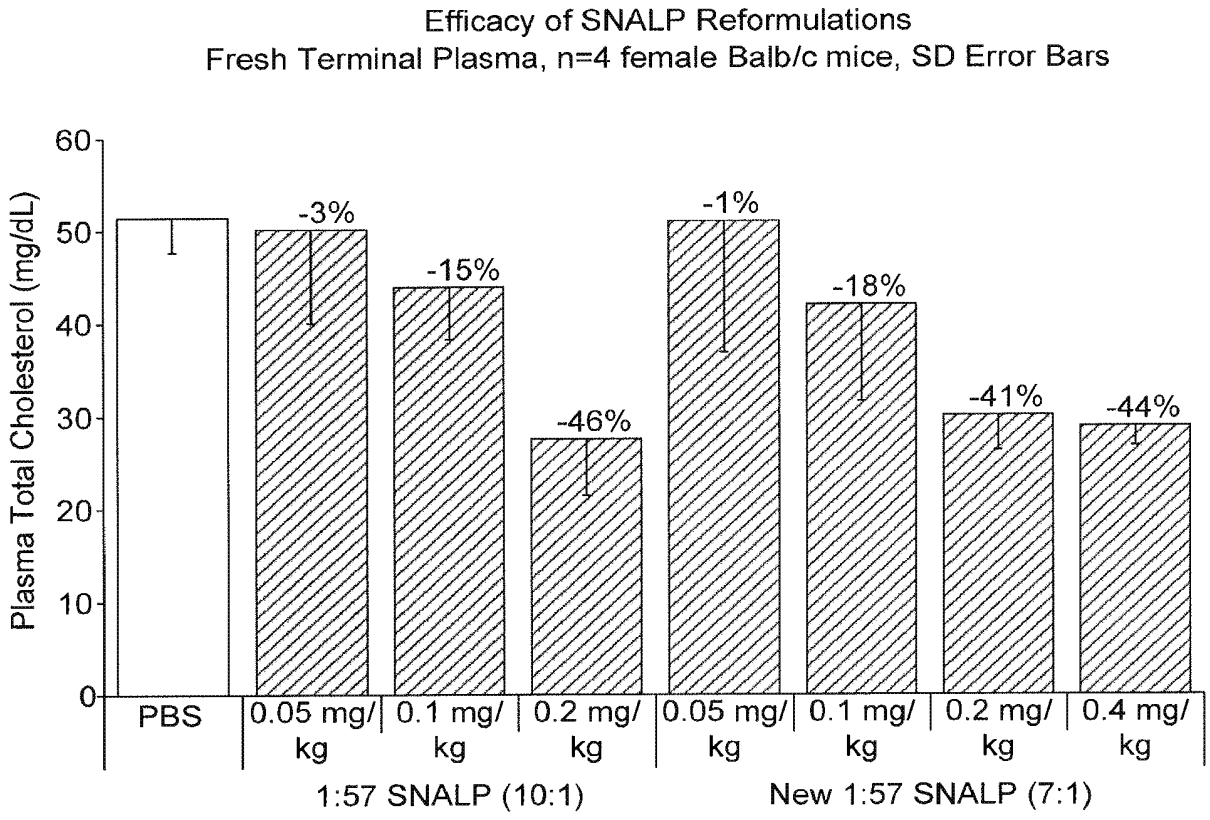

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 12

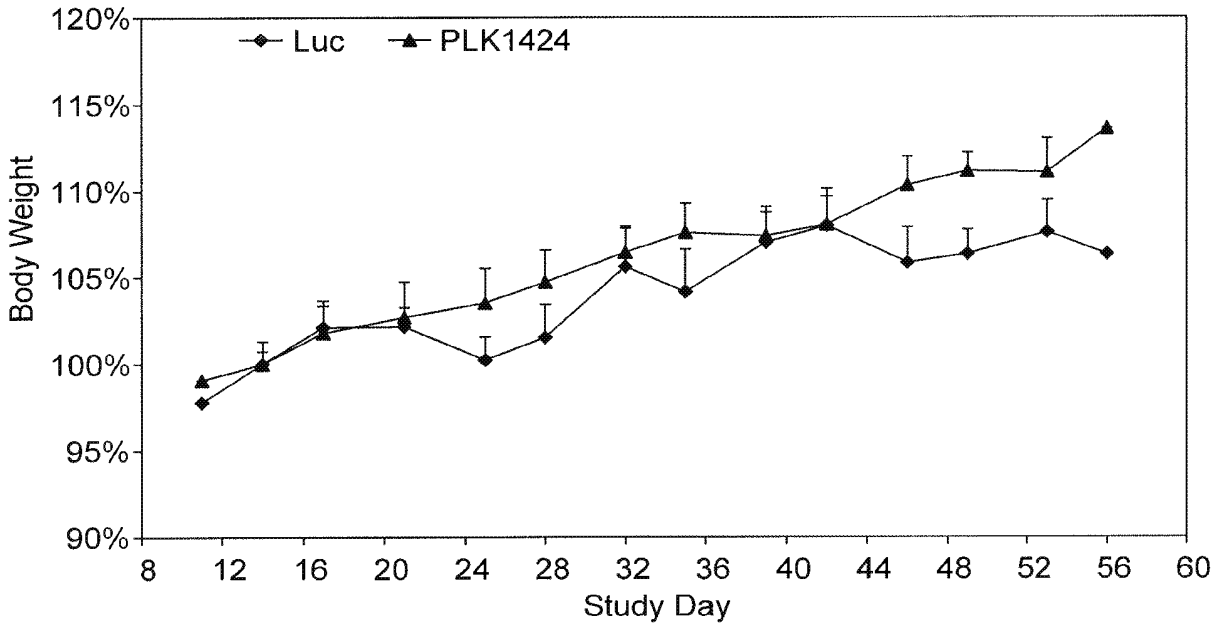

120%
115%
110%
105%
100%
95%
90%
Body Weight
Luc
PLK1424
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

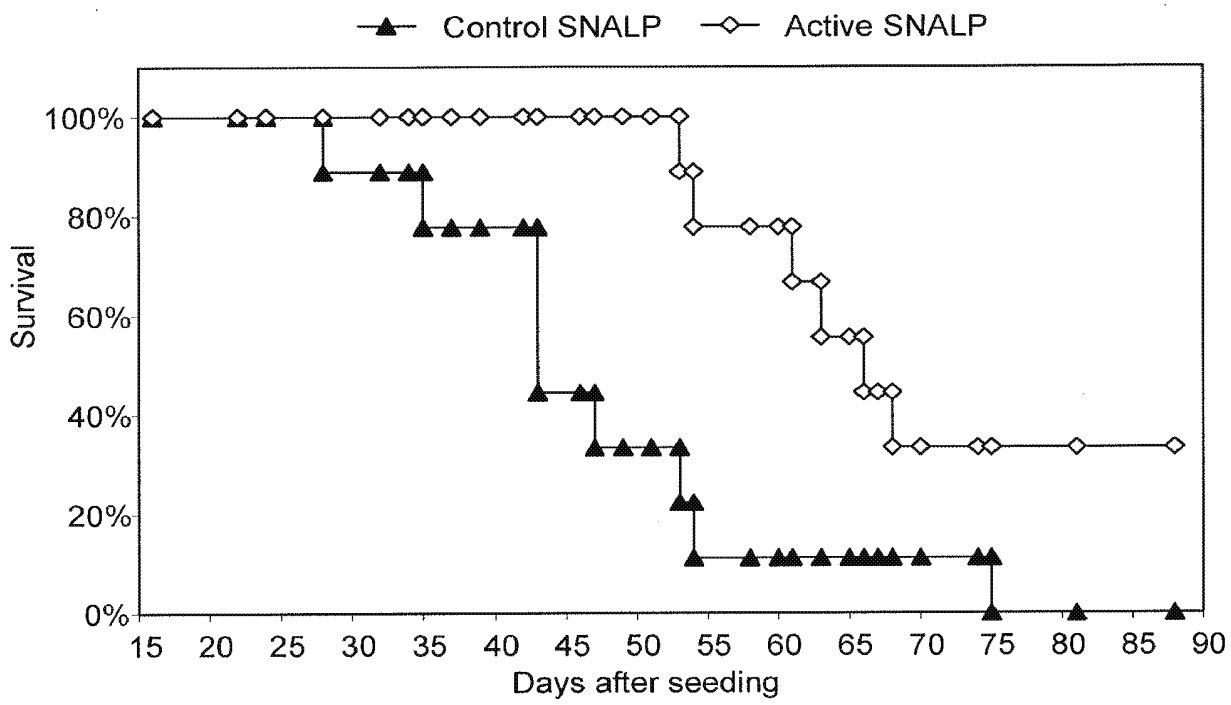

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
Days after seeding

FIG. 14

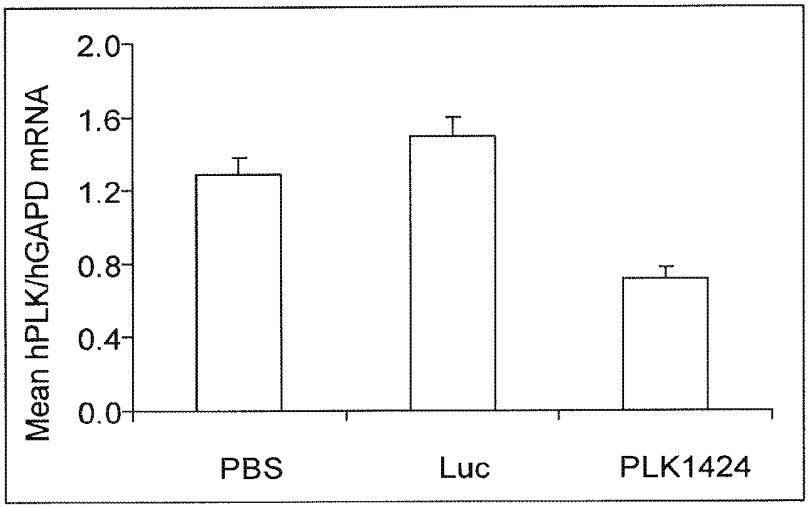


FIG. 15

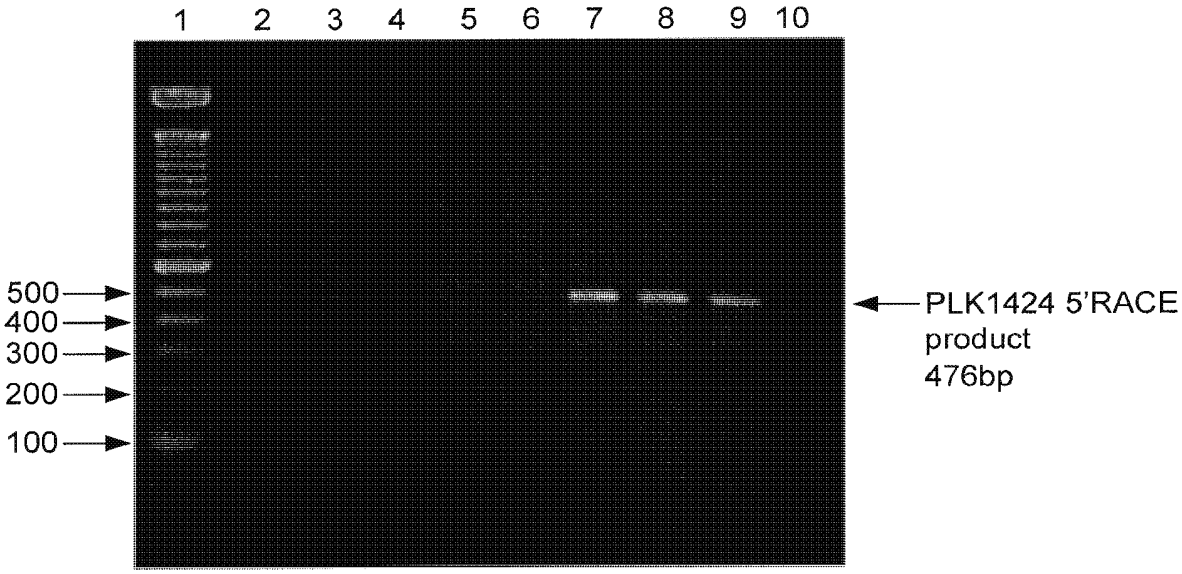
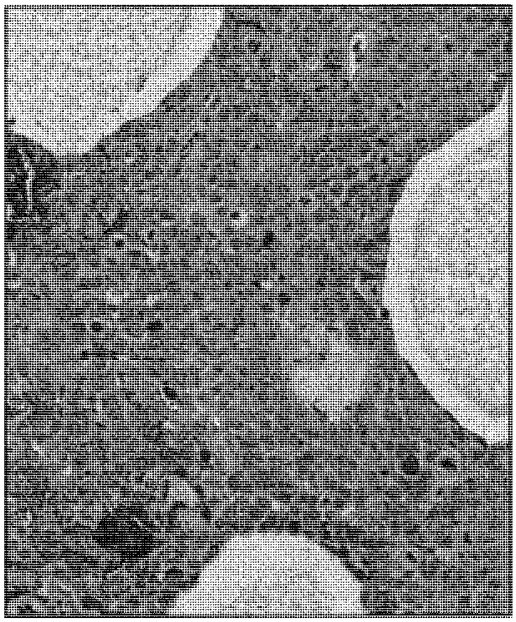
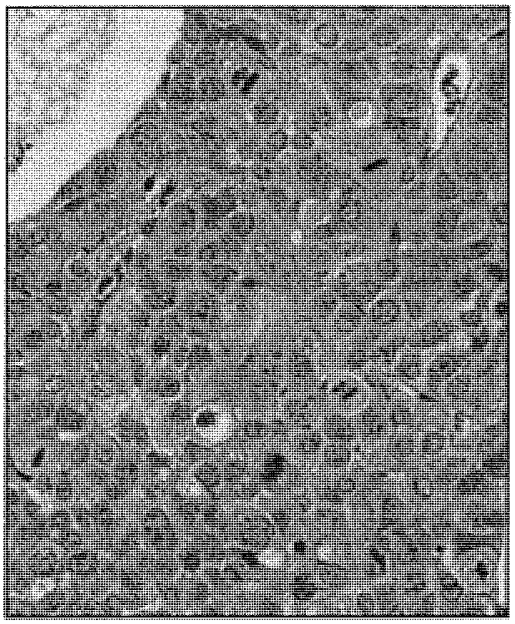

1 2 3 4 5 6 7 8 9 10
500
400
300
200
100
PLK1424 5'RACE
product
476bp

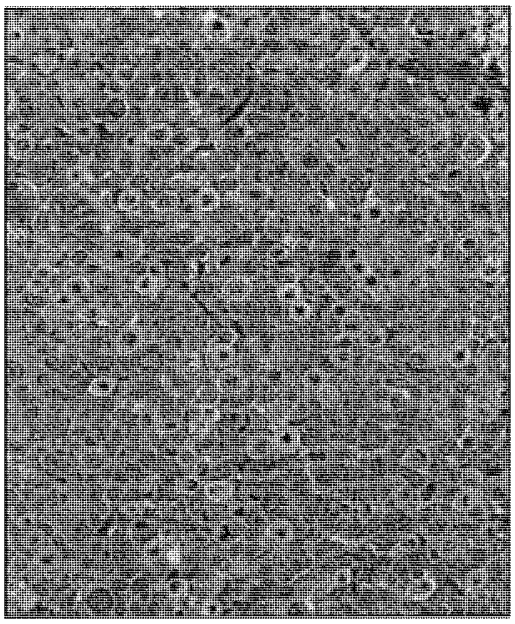
FIG. 16



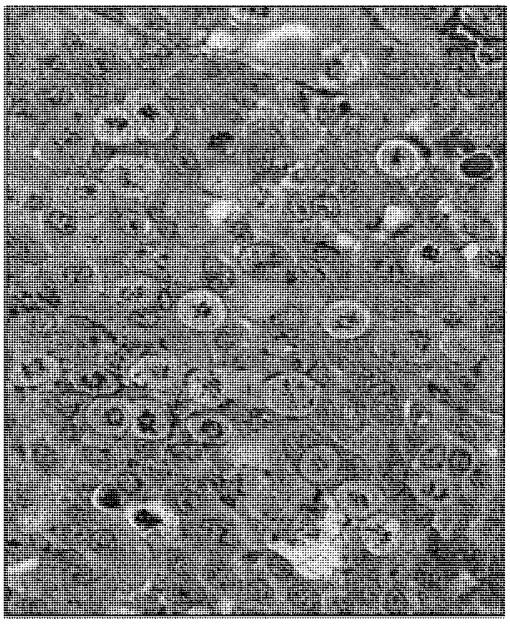
x200 mag



x400 mag



x200 mag



x400 mag

FIG. 17

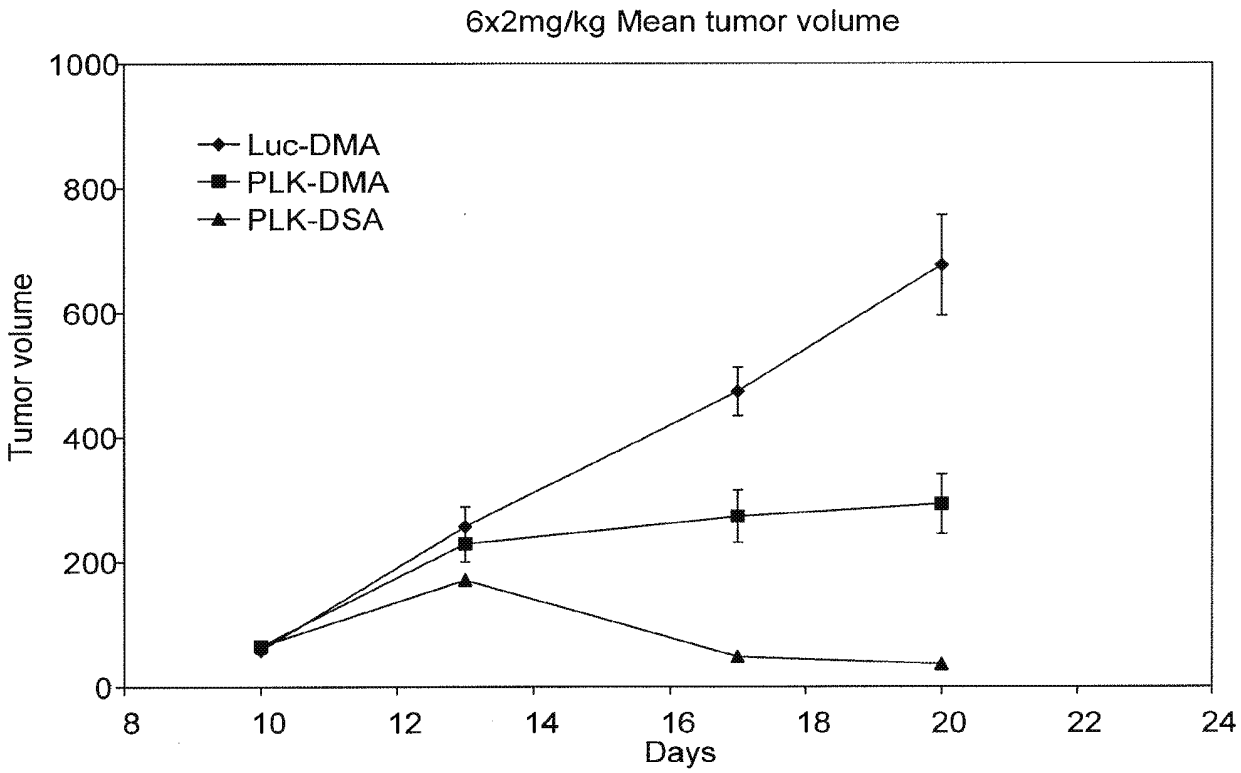

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

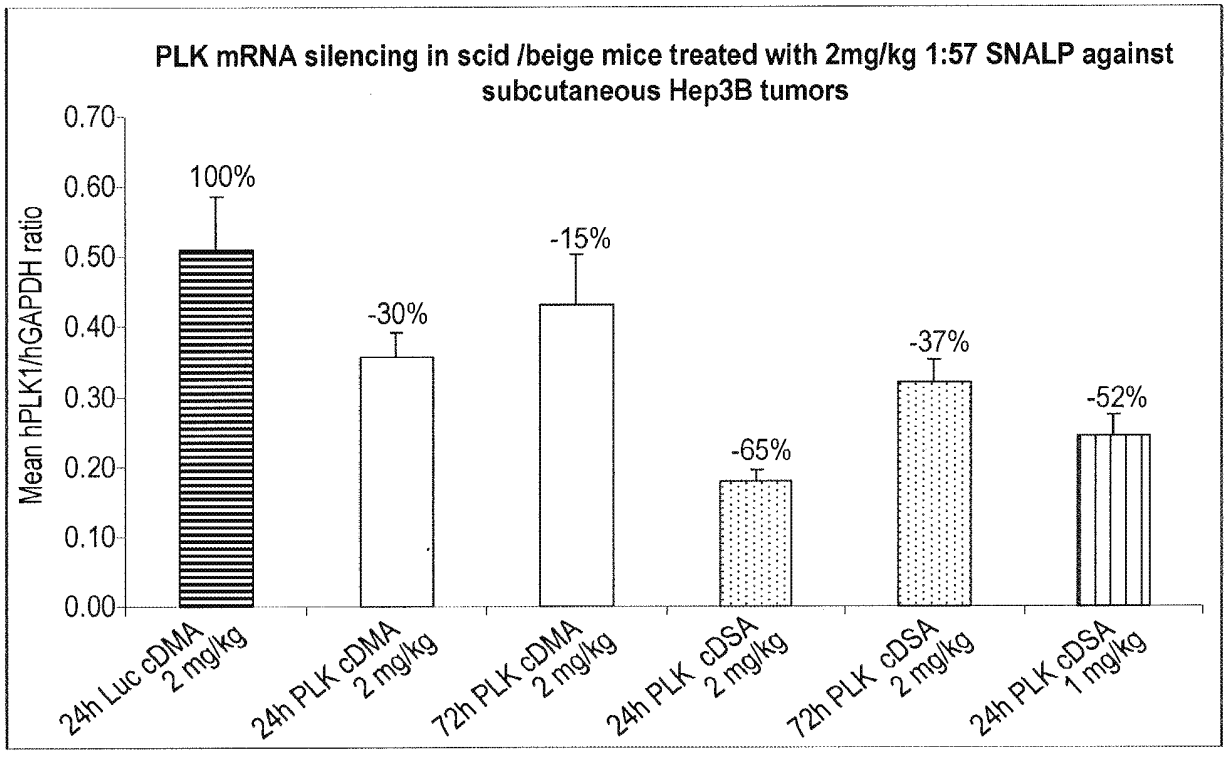

PLK mRNA silencing in scid /beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

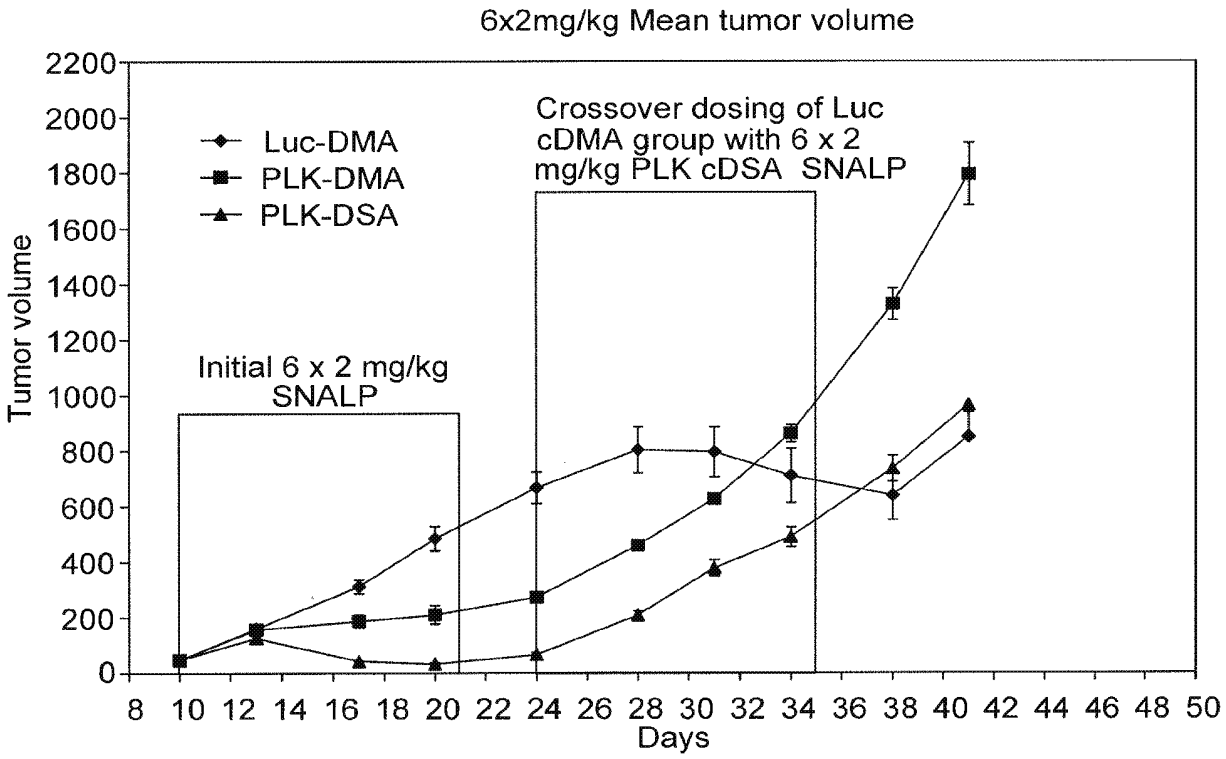

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Initial 6 x 2 mg/kg SNALP
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

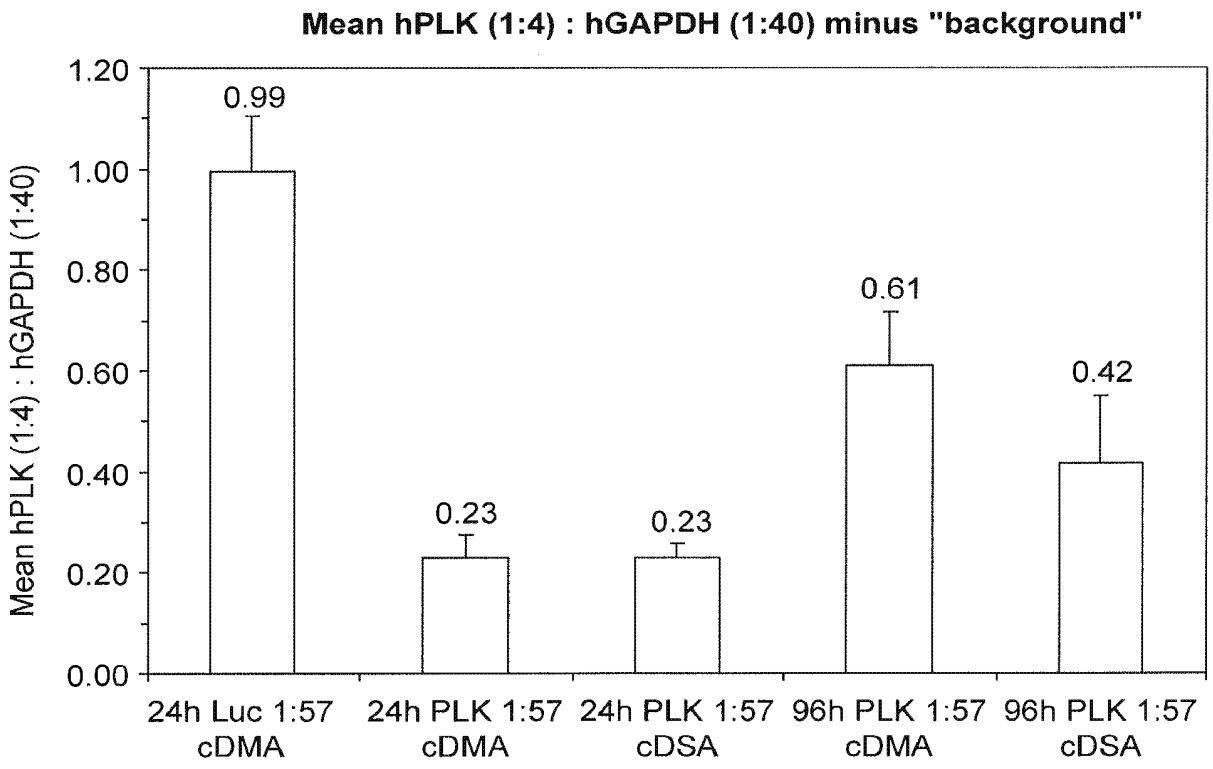

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

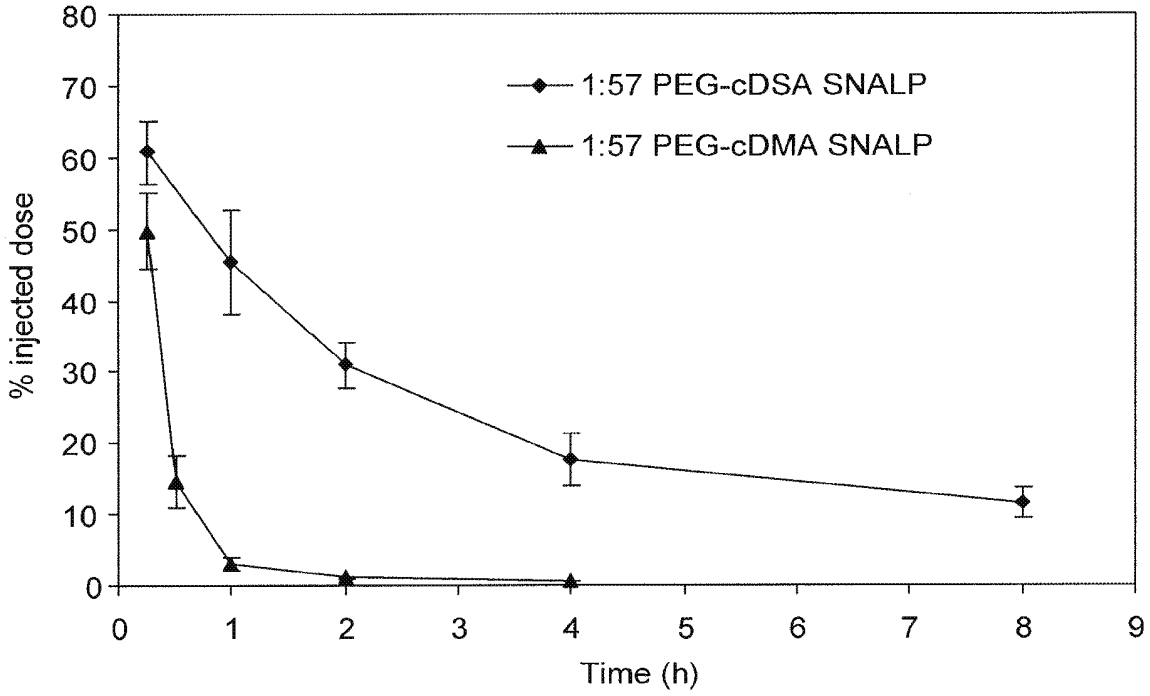

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

JOINT APPENDIX 62

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on February 26, 2015.

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Judith Cotham/
Judith Cotham

PATENT
Attorney Docket No.: 86399-007740US-913296

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Yaworski *et al.*

Application No.: 14/462,441

Filed: August 18, 2014

For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Customer No.: 20350

Confirmation No. 6562

Examiner: Not yet assigned

Art Unit: Not yet assigned

PRELIMINARY AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

Prior to examination of the above-referenced application, please enter the following amendments and remarks.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks begin on page 5 of this paper.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-46. (Canceled)

47. (New) A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from 50 mol % to 85 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising from 13 mol % to 49.5 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle.

48. (New) The nucleic acid-lipid particle of claim 47, wherein the nucleic acid comprises an interfering RNA, mRNA, an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

49. (New) The nucleic acid-lipid particle of claim 48, wherein the interfering RNA comprises a small interfering RNA (siRNA), an asymmetrical interfering RNA (aiRNA), a microRNA (miRNA), or mixtures thereof.

50. (New) The nucleic acid-lipid particle of claim 47, wherein the cationic lipid comprises from 50 mol % to 65 mol % of the total lipid present in the particle.

51. (New) The nucleic acid-lipid particle of claim 47, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.

52. (New) The nucleic acid-lipid particle of claim 51, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

53. (New) The nucleic acid-lipid particle of claim 51, wherein the phospholipid comprises from 3 mol % to 15 mol % of the total lipid present in the particle.

54. (New) The nucleic acid-lipid particle of claim 51, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle.

55. (New) The nucleic acid-lipid particle of claim 47, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

56. (New) The nucleic acid-lipid particle of claim 55, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

57. (New) The nucleic acid-lipid particle of claim 56, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

58. (New) The nucleic acid-lipid particle of claim 47, wherein the conjugated lipid that inhibits aggregation of particles comprises from 1 mol % to 2 mol % of the total lipid present in the particle.

59. (New) The nucleic acid-lipid particle of claim 47, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

60. (New) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 47 and a pharmaceutically acceptable carrier.

61. (New) A method for introducing a nucleic acid into a cell, the method comprising:

contacting the cell with a nucleic acid-lipid particle of claim 47.

62. (New) A method for the *in vivo* delivery of a nucleic acid, the method comprising:

administering to a mammalian subject a nucleic acid-lipid particle of claim 47.

63. (New) A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising:

administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 47.

64. (New) The method of claim 63, wherein the disease or disorder is a viral infection.

65. (New) The method of claim 63, wherein the disease or disorder is a liver disease or disorder.

66. (New) The method of claim 63, wherein the disease or disorder is cancer.

REMARKS

After entry of this amendment, claims 47-66 are pending in this application and are presented for examination. Claims 1-46 have been canceled without prejudice to future prosecution. Claims 47-66 are newly added.

New claim 47 finds support, for example, in original claim 1. New claims 48 and 49 find support, for example, on page 13, lines 25-32 and on page 36, lines 22-24 of the instant specification. New claim 50 finds support, for example, on page 25, lines 7-11 of the instant specification. New claim 51 finds support, for example, in original claim 13. New claim 52 finds support, for example, in original claim 14. New claim 53 finds support, for example, on page 28, lines 9-10 of the instant specification. New claim 54 finds support, for example, on page 69, lines 3-4 of the instant specification. New claim 55 finds support, for example, in original claim 17. New claim 56 finds support, for example, in original claim 18. New claim 57 finds support, for example, in original claim 19. New claim 58 finds support, for example, in original claim 21. New claim 59 finds support, for example, in original claim 23. New claim 60 finds support, for example, in original claim 26. New claim 61 finds support, for example, in original claim 41. New claim 62 finds support, for example, in original claim 43. New claim 63 finds support, for example, in original claim 45. New claims 64-66 find support, for example, in original claim 46. As such, no new matter has been introduced with the foregoing amendments.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,

/Joe C. Hao/

Joe C. Hao
Reg. No. 55,246

KILPATRICK TOWNSEND & STOCKTON LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 925-472-5000
Fax: 415-576-0300
KILPATRICK TOWNSEND 66942357 1

JOINT APPENDIX 63

NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] The present application is a continuation of U.S. Application No. 13/253,917, filed October 5, 2011, which application is a continuation of 12/424,367 filed April 15, 2009, now U.S. Patent No. 8,058,069, which application claims priority to U.S. Provisional Application No. 61/045,228, filed April 15, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] Not applicable.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

[0003] Not applicable.

REFERENCE TO A "SEQUENCE LISTING"

[0004] Not applicable.

BACKGROUND OF THE INVENTION

[0005] RNA interference (RNAi) is an evolutionarily conserved process in which recognition of double-stranded RNA (dsRNA) ultimately leads to posttranscriptional suppression of gene expression. This suppression is mediated by short dsRNA, also called small interfering RNA (siRNA), which induces specific degradation of mRNA through complementary base pairing. In several model systems, this natural response has been developed into a powerful tool for the investigation of gene function (*see, e.g.,* Elbashir *et al.*, *Genes Dev.*, 15:188-200 (2001); Hammond *et al.*, *Nat. Rev. Genet.*, 2:110-119 (2001)). More recently, it was discovered that introducing synthetic 21-nucleotide dsRNA duplexes into mammalian cells could efficiently silence gene expression.

[0006] Although the precise mechanism is still unclear, RNAi provides a potential new approach to downregulate or silence the transcription and translation of a gene of interest. For example, it is desirable to modulate (*e.g.*, reduce) the expression of certain genes for the treatment of neoplastic disorders such as cancer. It is also desirable to silence the expression of genes associated with liver diseases and disorders such as hepatitis. It is further desirable

to reduce the expression of certain genes for the treatment of atherosclerosis and its manifestations, *e.g.*, hypercholesterolemia, myocardial infarction, and thrombosis.

[0007] A safe and effective nucleic acid delivery system is required for RNAi to be therapeutically useful. Viral vectors are relatively efficient gene delivery systems, but suffer from a variety of limitations, such as the potential for reversion to the wild-type as well as immune response concerns. As a result, nonviral gene delivery systems are receiving increasing attention (Worgall *et al.*, *Human Gene Therapy*, 8:37 (1997); Peeters *et al.*, *Human Gene Therapy*, 7:1693 (1996); Yei *et al.*, *Gene Therapy*, 1:192 (1994); Hope *et al.*, *Molecular Membrane Biology*, 15:1 (1998)). Furthermore, viral systems are rapidly cleared from the circulation, limiting transfection to "first-pass" organs such as the lungs, liver, and spleen. In addition, these systems induce immune responses that compromise delivery with subsequent injections.

[0008] Plasmid DNA-cationic liposome complexes are currently the most commonly employed nonviral gene delivery vehicles (Felgner, *Scientific American*, 276:102 (1997); Chonn *et al.*, *Current Opinion in Biotechnology*, 6:698 (1995)). For instance, cationic liposome complexes made of an amphipathic compound, a neutral lipid, and a detergent for transfecting insect cells are disclosed in U.S. Patent No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Publication No. 20030073640.

[0009] Cationic liposome complexes are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects (Harrison *et al.*, *Biotechniques*, 19:816 (1995); Li *et al.*, *The Gene*, 4:891 (1997); Tam *et al*, *Gene Ther.*, 7:1867 (2000)). As large, positively charged aggregates, lipoplexes are rapidly cleared when administered *in vivo*, with highest expression levels observed in first-pass organs, particularly the lungs (Huang *et al.*, *Nature Biotechnology*, 15:620 (1997); Templeton *et al.*, *Nature Biotechnology*, 15:647 (1997); Hofland *et al.*, *Pharmaceutical Research*, 14:742 (1997)).

[0010] Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Patent No. 6,429,200. Anionic liposomes are disclosed in U.S. Patent Publication No. 20030026831. Polymer liposomes that incorporate dextrin or glycerol-phosphocholine polymers are disclosed in U.S. Patent Publication Nos. 20020081736 and 20030082103, respectively.

[0011] A gene delivery system containing an encapsulated nucleic acid for systemic delivery should be small (*i.e.*, less than about 100 nm diameter) and should remain intact in the circulation for an extended period of time in order to achieve delivery to affected tissues. This requires a highly stable, serum-resistant nucleic acid-containing particle that does not

interact with cells and other components of the vascular compartment. The particle should also readily interact with target cells at a disease site in order to facilitate intracellular delivery of a desired nucleic acid.

[0012] Recent work has shown that nucleic acids can be encapsulated in small (*e.g.*, about 70 nm diameter) "stabilized plasmid-lipid particles" (SPLP) that consist of a single plasmid encapsulated within a bilayer lipid vesicle (Wheeler *et al.*, *Gene Therapy*, 6:271 (1999)). These SPLPs typically contain the "fusogenic" lipid dioleoylphosphatidylethanolamine (DOPE), low levels of cationic lipid, and are stabilized in aqueous media by the presence of a poly(ethylene glycol) (PEG) coating. SPLPs have systemic application as they exhibit extended circulation lifetimes following intravenous (i.v.) injection, accumulate preferentially at distal tumor sites due to the enhanced vascular permeability in such regions, and can mediate transgene expression at these tumor sites. The levels of transgene expression observed at the tumor site following i.v. injection of SPLPs containing the luciferase marker gene are superior to the levels that can be achieved employing plasmid DNA-cationic liposome complexes (lipoplexes) or naked DNA.

[0013] Thus, there remains a strong need in the art for novel and more efficient methods and compositions for introducing nucleic acids such as siRNA into cells. In addition, there is a need in the art for methods of downregulating the expression of genes of interest to treat or prevent diseases and disorders such as cancer and atherosclerosis. The present invention addresses these and other needs.

BRIEF SUMMARY OF THE INVENTION

[0014] The present invention provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.*, for the treatment of a disease or disorder).

[0015] In preferred embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In other preferred embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0016] In one aspect, the present invention provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more

non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0017] More particularly, the present invention provides serum-stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (*e.g.*, one or more interfering RNA molecules such as siRNA, aiRNA, and/or miRNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP (*e.g.*, for the treatment of a disease or disorder).

[0018] In certain embodiments, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) a nucleic acid (*e.g.*, an interfering RNA); (b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0019] In one preferred embodiment, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:62" formulation.

[0020] In another preferred embodiment, the nucleic acid-lipid particle (*e.g.*, SNALP) comprises: (a) an siRNA; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This preferred embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:57" formulation.

[0021] The present invention also provides pharmaceutical compositions comprising a lipid particle described herein (*e.g.*, SNALP) and a pharmaceutically acceptable carrier.

[0022] In another aspect, the present invention provides methods for introducing an active agent or therapeutic agent (*e.g.*, nucleic acid) into a cell, the method comprising contacting the cell with a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.*, SNALP).

[0023] In yet another aspect, the present invention provides methods for the *in vivo* delivery of an active agent or therapeutic agent (*e.g.,* nucleic acid), the method comprising administering to a mammalian subject a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.,* SNALP).

[0024] In a further aspect, the present invention provides methods for treating a disease or disorder in a mammalian subject in need thereof, the method comprising administering to the mammalian subject a therapeutically effective amount of a lipid particle described herein such as a nucleic acid-lipid particle (*e.g.,* SNALP).

[0025] Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] Figure 1 illustrates data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

[0027] Figure 2 illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

[0028] Figure 3 illustrates additional data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice. Each bar represents the group mean of five animals. Error bars indicate the standard deviation.

[0029] Figure 4 illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

[0030] Figure 5 illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

[0031] Figure 6 illustrates data demonstrating that the tolerability of 1:57 SNALP containing ApoB siRNA prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters.

[0032] Figure 7 illustrates data demonstrating that the efficacy of 1:57 SNALP containing ApoB siRNA prepared by gear pump was similar to the same SNALP prepared by syringe press.

[0033] Figure 8 illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

[0034] Figure 9 illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

[0035] Figure 10 illustrates data demonstrating that clinically significant liver enzyme elevations (3xULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

[0036] Figure 11 illustrates data demonstrating that the potency of the lower lipid:drug (L:D) 1:57 SNALP containing ApoB siRNA was as good as that of the higher L:D SNALP at the tested drug dosages.

[0037] Figure 12 illustrates data demonstrating that ApoB protein and total cholesterol levels were reduced to a similar extent by 1:57 SNALP containing ApoB siRNA at a 6:1 input L:D ratio (final ratio of 7:1) and 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

[0038] Figure 13 illustrates data demonstrating that a treatment regimen of 1:57 SNALP with siRNA targeting PLK-1 is well tolerated with no apparent signs of treatment related toxicity in mice bearing Hep3B liver tumors.

[0039] Figure 14 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA caused a significant increase in the survival of Hep3B tumor-bearing mice.

[0040] Figure 15 illustrates data demonstrating that treatment with 1:57 SNALP containing PLK-1 siRNA reduced PLK-1 mRNA levels by 50% in intrahepatic Hep3B tumors growing in mice 24 hours after SNALP administration.

[0041] Figure 16 illustrates data demonstrating that a specific cleavage product of PLK-1 mRNA was detectable by 5' RACE-PCR in mice treated with 1:57 SNALP containing PLK-1 siRNA. 10 μl PCR product/well were loaded onto a 1.5% agarose gel. Lane Nos.: (1) molecular weight (MW) marker; (2) PBS mouse 1; (3) PBS mouse 2; (4) PBS mouse 3; (5) Luc SNALP mouse 1; (6) Luc SNALP mouse 2; (7) PLK SNALP mouse 1; (8) PLK SNALP mouse 2; (9) PLK SNALP mouse 3; and (10) no template control.

[0042] Figure 17 illustrates data demonstrating that control (Luc) 1:57 SNALP-treated mice displayed normal mitoses in Hep3B tumors (top panels), whereas mice treated with 1:57 SNALP containing PLK-1 siRNA exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors (bottom panels).

[0043] Figure 18 illustrates data demonstrating that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B subcutaneous (S.C.) tumors.

[0044] Figure 19 illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

[0045] Figure 20 illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0046] Figure 21 illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

[0047] Figure 22 illustrates data demonstrating the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA.

DETAILED DESCRIPTION OF THE INVENTION

I. Introduction

[0048] The present invention is based, in part, upon the surprising discovery that lipid particles comprising from about 50 mol % to about 85 mol % of a cationic lipid, from about 13 mol % to about 49.5 mol % of a non-cationic lipid, and from about 0.5 mol % to about 2 mol % of a lipid conjugate provide advantages when used for the *in vitro* or *in vivo* delivery of an active agent, such as a therapeutic nucleic acid (*e.g.*, an interfering RNA). In particular, as illustrated by the Examples herein, the present invention provides stable nucleic acid-lipid particles (SNALP) that advantageously impart increased activity of the encapsulated nucleic acid (*e.g.*, an interfering RNA such as siRNA) and improved tolerability of the formulations *in vivo*, resulting in a significant increase in the therapeutic index as compared to nucleic acid-lipid particle compositions previously described. Additionally, the SNALP of the invention are stable in circulation, *e.g.*, resistant to degradation by nucleases in serum, and are substantially non-toxic to mammals such as humans. As a non-limiting example, Figure 3 of Example 4 shows that one SNALP embodiment of the invention ("1:57 SNALP") was more than 10 times as efficacious as compared to a nucleic acid-lipid particle previously described ("2:30 SNALP") in mediating target gene silencing at a 10-fold lower dose. Similarly, Figure 2 of Example 3 shows that the "1:57 SNALP" formulation was substantially more effective at silencing the expression of a target gene as compared to nucleic acid-lipid particles previously described ("2:40 SNALP").

[0049] In certain embodiments, the present invention provides improved compositions for the delivery of interfering RNA such as siRNA molecules. In particular, the Examples herein illustrate that the improved lipid particle formulations of the invention are highly effective in downregulating the mRNA and/or protein levels of target genes. Furthermore, the Examples herein illustrate that the presence of certain molar ratios of lipid components results in improved or enhanced activity of these lipid particle formulations of the present invention. For instance, the "1:57 SNALP" and "1:62 SNALP" formulations described herein are

exemplary formulations of the present invention that are particularly advantageous because they provide improved efficacy and tolerability *in vivo*, are serum-stable, are substantially non-toxic, are capable of accessing extravascular sites, and are capable of reaching target cell populations.

[0050] The lipid particles and compositions of the present invention may be used for a variety of purposes, including the delivery of associated or encapsulated therapeutic agents to cells, both *in vitro* and *in vivo*. Accordingly, the present invention provides methods for treating diseases or disorders in a subject in need thereof, by contacting the subject with a lipid particle described herein comprising one or more suitable therapeutic agents.

[0051] Various exemplary embodiments of the lipid particles of the invention, as well as compositions and formulations comprising the same, and their use to deliver therapeutic agents and modulate target gene and protein expression, are described in further detail below.

II. Definitions

[0052] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0053] The term "interfering RNA" or "RNAi" or "interfering RNA sequence" refers to single-stranded RNA (*e.g.*, mature miRNA) or double-stranded RNA (*i.e.*, duplex RNA such as siRNA, aiRNA, or pre-miRNA) that is capable of reducing or inhibiting the expression of a target gene or sequence (*e.g.*, by mediating the degradation or inhibiting the translation of mRNAs which are complementary to the interfering RNA sequence) when the interfering RNA is in the same cell as the target gene or sequence. Interfering RNA thus refers to the single-stranded RNA that is complementary to a target mRNA sequence or to the double-stranded RNA formed by two complementary strands or by a single, self-complementary strand. Interfering RNA may have substantial or complete identity to the target gene or sequence, or may comprise a region of mismatch (*i.e.*, a mismatch motif). The sequence of the interfering RNA can correspond to the full-length target gene, or a subsequence thereof.

[0054] Interfering RNA includes "small-interfering RNA" or "siRNA," *e.g.*, interfering RNA of about 15-60, 15-50, or 15-40 (duplex) nucleotides in length, more typically about 15-30, 15-25, or 19-25 (duplex) nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (*e.g.*, each complementary sequence of the double-stranded siRNA is 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, preferably about 20-24, 21-22, or 21-23 nucleotides in length, and the double-stranded siRNA is about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 base pairs in length, preferably

about 18-22, 19-20, or 19-21 base pairs in length). siRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides and 5' phosphate termini. Examples of siRNA include, without limitation, a double-stranded polynucleotide molecule assembled from two separate stranded molecules, wherein one strand is the sense strand and the other is the complementary antisense strand; a double-stranded polynucleotide molecule assembled from a single stranded molecule, where the sense and antisense regions are linked by a nucleic acid-based or non-nucleic acid-based linker; a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions; and a circular single-stranded polynucleotide molecule with two or more loop structures and a stem having self-complementary sense and antisense regions, where the circular polynucleotide can be processed *in vivo* or *in vitro* to generate an active double-stranded siRNA molecule.

[0055] Preferably, siRNA are chemically synthesized. siRNA can also be generated by cleavage of longer dsRNA (*e.g.*, dsRNA greater than about 25 nucleotides in length) with the *E. coli* RNase III or Dicer. These enzymes process the dsRNA into biologically active siRNA (*see*, *e.g.*, Yang *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:9942-9947 (2002); Calegari *et al.*, *Proc. Natl. Acad. Sci. USA,* 99:14236 (2002); Byrom *et al.*, *Ambion TechNotes,* 10(1):4-6 (2003); Kawasaki *et al.*, *Nucleic Acids Res.,* 31:981-987 (2003); Knight *et al.*, *Science,* 293:2269-2271 (2001); and Robertson *et al.*, *J. Biol. Chem.,* 243:82 (1968)). Preferably, dsRNA are at least 50 nucleotides to about 100, 200, 300, 400, or 500 nucleotides in length. A dsRNA may be as long as 1000, 1500, 2000, 5000 nucleotides in length, or longer. The dsRNA can encode for an entire gene transcript or a partial gene transcript. In certain instances, siRNA may be encoded by a plasmid (*e.g.*, transcribed as sequences that automatically fold into duplexes with hairpin loops).

[0056] As used herein, the term "mismatch motif" or "mismatch region" refers to a portion of an interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) sequence that does not have 100 % complementarity to its target sequence. An interfering RNA may have at least one, two, three, four, five, six, or more mismatch regions. The mismatch regions may be contiguous or may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides. The mismatch motifs or regions may comprise a single nucleotide or may comprise two, three, four, five, or more nucleotides.

[0057] An "effective amount" or "therapeutically effective amount" of an active agent or therapeutic agent such as an interfering RNA is an amount sufficient to produce the desired effect, *e.g.*, an inhibition of expression of a target sequence in comparison to the normal

expression level detected in the absence of an interfering RNA. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with an interfering RNA relative to the control is about 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, *e.g.,* examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, *in situ* hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

[0058] By "decrease," "decreasing," "reduce," or "reducing" of an immune response by an interfering RNA is intended to mean a detectable decrease of an immune response to a given interfering RNA (*e.g.*, a modified interfering RNA). The amount of decrease of an immune response by a modified interfering RNA may be determined relative to the level of an immune response in the presence of an unmodified interfering RNA. A detectable decrease can be about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, or more lower than the immune response detected in the presence of the unmodified interfering RNA. A decrease in the immune response to interfering RNA is typically measured by a decrease in cytokine production (*e.g.*, IFNγ, IFNα, TNFα, IL-6, or IL-12) by a responder cell *in vitro* or a decrease in cytokine production in the sera of a mammalian subject after administration of the interfering RNA.

[0059] As used herein, the term "responder cell" refers to a cell, preferably a mammalian cell, that produces a detectable immune response when contacted with an immunostimulatory interfering RNA such as an unmodified siRNA. Exemplary responder cells include, *e.g.*, dendritic cells, macrophages, peripheral blood mononuclear cells (PBMCs), splenocytes, and the like. Detectable immune responses include, *e.g.*, production of cytokines or growth factors such as TNF-α, IFN-α, IFN-β, IFN-γ, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TGF, and combinations thereof.

[0060] "Substantial identity" refers to a sequence that hybridizes to a reference sequence under stringent conditions, or to a sequence that has a specified percent identity over a specified region of a reference sequence.

[0061] The phrase "stringent hybridization conditions" refers to conditions under which a nucleic acid will hybridize to its target sequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen,

Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0062] Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C-95°C for 30 sec.-2 min., an annealing phase lasting 30 sec.-2 min., and an extension phase of about 72°C for 1-2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, *e.g.*, in Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y. (1990).

[0063] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional

guidelines for determining hybridization parameters are provided in numerous references, *e.g.*, Current Protocols in Molecular Biology, Ausubel *et al.*, eds.

[0064] The terms "substantially identical" or "substantial identity," in the context of two or more nucleic acids, refer to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides that are the same (*i.e.*, at least about 60%, preferably at least about 65%, 70%, 75%, 80%, 85%, 90%, or 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. This definition, when the context indicates, also refers analogously to the complement of a sequence. Preferably, the substantial identity exists over a region that is at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 nucleotides in length.

[0065] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[0066] A "comparison window," as used herein, includes reference to a segment of any one of a number of contiguous positions selected from the group consisting of from about 5 to about 60, usually about 10 to about 45, more usually about 15 to about 30, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.*, 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.*, 48:443 (1970), by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA*, 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology*, Ausubel *et al.*, eds. (1995 supplement)).

[0067] A preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al., Nuc. Acids Res.,* 25:3389-3402 (1977) and Altschul *et al., J. Mol. Biol.*, 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

[0068] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin and Altschul, *Proc. Natl. Acad. Sci. USA,* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

[0069] The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA and RNA. DNA may be in the form of, *e.g.*, antisense molecules, plasmid DNA, pre-condensed DNA, a PCR product, vectors (P1, PAC, BAC, YAC, artificial chromosomes), expression cassettes, chimeric sequences, chromosomal DNA, or derivatives and combinations of these groups. RNA may be in the form of siRNA, asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, tRNA, viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.,* degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third

position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.*, 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.*, 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes*, 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

[0070] The term "gene" refers to a nucleic acid (*e.g.*, DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

[0071] "Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

[0072] The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are characterized by being insoluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) "simple lipids," which include fats and oils as well as waxes; (2) "compound lipids," which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids.

[0073] A "lipid particle" is used herein to refer to a lipid formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), to a target site of interest. In the lipid particle of the invention, which is typically formed from a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of the particle, the active agent or therapeutic agent may be encapsulated in the lipid, thereby protecting the agent from enzymatic degradation.

[0074] As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a particle made from lipids (*e.g.*, a cationic lipid, a non-cationic lipid, and a conjugated lipid that prevents aggregation of the particle), wherein the nucleic acid (*e.g.*, siRNA, aiRNA, miRNA, ssDNA, dsDNA, ssRNA, short hairpin RNA (shRNA), dsRNA, or a plasmid, including plasmids from which an interfering RNA is transcribed) is fully encapsulated within the lipid. As used herein, the term "SNALP" includes an SPLP, which is the term used to refer to a nucleic acid-lipid particle comprising a nucleic acid (*e.g.*, a plasmid) encapsulated within the lipid. SNALP and SPLP typically contain a cationic lipid, a

non-cationic lipid, and a lipid conjugate (*e.g.*, a PEG-lipid conjugate). SNALP and SPLP are extremely useful for systemic applications, as they can exhibit extended circulation lifetimes following intravenous (i.v.) injection, they can accumulate at distal sites (*e.g.*, sites physically separated from the administration site), and they can mediate expression of the transfected gene or silencing of target gene expression at these distal sites. SPLP include "pSPLP," which comprise an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0075] The lipid particles of the invention (*e.g.*, SNALP) typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm, and are substantially non-toxic. In addition, nucleic acids, when present in the lipid particles of the invention, are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Publication Nos. 20040142025 and 20070042031, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0076] As used herein, "lipid encapsulated" can refer to a lipid particle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, an interfering RNA), with full encapsulation, partial encapsulation, or both. In a preferred embodiment, the nucleic acid is fully encapsulated in the lipid particle (*e.g.*, to form an SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle).

[0077] The term "lipid conjugate" refers to a conjugated lipid that inhibits aggregation of lipid particles. Such lipid conjugates include, but are not limited to, polyamide oligomers (*e.g.*, ATTA-lipid conjugates), PEG-lipid conjugates, such as PEG coupled to dialkyloxypropyls, PEG coupled to diacylglycerols, PEG coupled to cholesterol, PEG coupled to phosphatidylethanolamines, PEG conjugated to ceramides (*see, e.g.*, U.S. Patent No. 5,885,613, the disclosure of which is herein incorporated by reference in its entirety for all purposes), cationic PEG lipids, and mixtures thereof. PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In preferred embodiments, non-ester containing linker moieties are used.

[0078] The term "amphipathic lipid" refers, in part, to any suitable material wherein the hydrophobic portion of the lipid material orients into a hydrophobic phase, while the

hydrophilic portion orients toward the aqueous phase. Hydrophilic characteristics derive from the presence of polar or charged groups such as carbohydrates, phosphate, carboxylic, sulfato, amino, sulfhydryl, nitro, hydroxyl, and other like groups. Hydrophobicity can be conferred by the inclusion of apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Examples of amphipathic compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids.

[0079] Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine. Other compounds lacking in phosphorus, such as sphingolipid, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, are also within the group designated as amphipathic lipids. Additionally, the amphipathic lipids described above can be mixed with other lipids including triglycerides and sterols.

[0080] The term "neutral lipid" refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides, and diacylglycerols.

[0081] The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

[0082] The term "anionic lipid" refers to any lipid that is negatively charged at physiological pH. These lipids include, but are not limited to, phosphatidylglycerols, cardiolipins, diacylphosphatidylserines, diacylphosphatidic acids, N-dodecanoyl phosphatidylethanolamines, N-succinyl phosphatidylethanolamines, N-glutarylphosphatidylethanolamines, lysylphosphatidylglycerols, palmitoyloleyolphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

[0083] The term "cationic lipid" refers to any of a number of lipid species that carry a net positive charge at a selected pH, such as physiological pH (*e.g.*, pH of about 7.0). It has been surprisingly found that cationic lipids comprising alkyl chains with multiple sites of unsaturation, *e.g.*, at least two or three sites of unsaturation, are particularly useful for

forming lipid particles with increased membrane fluidity. A number of cationic lipids and related analogs, which are also useful in the present invention, have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of cationic lipids are described in detail herein. In some cases, the cationic lipids comprise a protonatable tertiary amine (*e.g.*, pH titratable) head group, C18 alkyl chains, ether linkages between the head group and alkyl chains, and 0 to 3 double bonds. Such lipids include, *e.g.*, DSDMA, DLinDMA, DLenDMA, and DODMA.

[0084] The term "hydrophobic lipid" refers to compounds having apolar groups that include, but are not limited to, long-chain saturated and unsaturated aliphatic hydrocarbon groups and such groups optionally substituted by one or more aromatic, cycloaliphatic, or heterocyclic group(s). Suitable examples include, but are not limited to, diacylglycerol, dialkylglycerol, N-N-dialkylamino, 1,2-diacyloxy-3-aminopropane, and 1,2-dialkyl-3-aminopropane.

[0085] The term "fusogenic" refers to the ability of a lipid particle, such as a SNALP, to fuse with the membranes of a cell. The membranes can be either the plasma membrane or membranes surrounding organelles, *e.g.*, endosome, nucleus, *etc.*

[0086] As used herein, the term "aqueous solution" refers to a composition comprising in whole, or in part, water.

[0087] As used herein, the term "organic lipid solution" refers to a composition comprising in whole, or in part, an organic solvent having a lipid.

[0088] "Distal site," as used herein, refers to a physically separated site, which is not limited to an adjacent capillary bed, but includes sites broadly distributed throughout an organism.

[0089] "Serum-stable" in relation to nucleic acid-lipid particles such as SNALP means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNAse assay, or an RNAse assay.

[0090] "Systemic delivery," as used herein, refers to delivery of lipid particles that leads to a broad biodistribution of an active agent or therapeutic agent such as an interfering RNA within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. To obtain broad biodistribution

generally requires a blood lifetime such that the agent is not rapidly degraded or cleared (such as by first pass organs (liver, lung, *etc.*) or by rapid, nonspecific cell binding) before reaching a disease site distal to the site of administration. Systemic delivery of lipid particles can be by any means known in the art including, for example, intravenous, subcutaneous, and intraperitoneal. In a preferred embodiment, systemic delivery of lipid particles is by intravenous delivery.

[0091] "Local delivery," as used herein, refers to delivery of an active agent or therapeutic agent such as an interfering RNA directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site such as a tumor or other target site such as a site of inflammation or a target organ such as the liver, heart, pancreas, kidney, and the like.

[0092] The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

[0093] The term "cancer" refers to any member of a class of diseases characterized by the uncontrolled growth of aberrant cells. The term includes all known cancers and neoplastic conditions, whether characterized as malignant, benign, soft tissue, or solid, and cancers of all stages and grades including pre- and post-metastatic cancers. Examples of different types of cancer include, but are not limited to, lung cancer, colon cancer, rectal cancer, anal cancer, bile duct cancer, small intestine cancer, stomach (gastric) cancer, esophageal cancer; gallbladder cancer, liver cancer, pancreatic cancer, appendix cancer, breast cancer, ovarian cancer; cervical cancer, prostate cancer, renal cancer (*e.g.*, renal cell carcinoma), cancer of the central nervous system, glioblastoma, skin cancer, lymphomas, choriocarcinomas, head and neck cancers, osteogenic sarcomas, and blood cancers. Non-limiting examples of specific types of liver cancer include hepatocellular carcinoma (HCC), secondary liver cancer (*e.g.*, caused by metastasis of some other non-liver cancer cell type), and hepatoblastoma. As used herein, a "tumor" comprises one or more cancerous cells.

III. Description of the Embodiments

[0094] The present invention provides novel, serum-stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles (*e.g.*, for the treatment of a disease or disorder).

[0095] In one aspect, the present invention provides lipid particles comprising: (a) one or more active agents or therapeutic agents; (b) one or more cationic lipids comprising from

about 50 mol % to about 85 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

[0096] In certain embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.,* by a nuclease or protease. In certain other embodiments, the lipid particles are substantially non-toxic to mammals such as humans.

[0097] In some embodiments, the active agent or therapeutic agent comprises a nucleic acid. In certain instances, the nucleic acid comprises an interfering RNA molecule such as, *e.g.,* an siRNA, aiRNA, miRNA, or mixtures thereof. In certain other instances, the nucleic acid comprises single-stranded or double-stranded DNA, RNA, or a DNA/RNA hybrid such as, *e.g.,* an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

[0098] In other embodiments, the active agent or therapeutic agent comprises a peptide or polypeptide. In certain instances, the peptide or polypeptide comprises an antibody such as, *e.g.,* a polyclonal antibody, a monoclonal antibody, an antibody fragment; a humanized antibody, a recombinant antibody, a recombinant human antibody, a Primatized™ antibody, or mixtures thereof. In certain other instances, the peptide or polypeptide comprises a cytokine, a growth factor, an apoptotic factor, a differentiation-inducing factor, a cell-surface receptor, a ligand, a hormone, a small molecule (*e.g.,* small organic molecule or compound), or mixtures thereof.

[0099] In preferred embodiments, the active agent or therapeutic agent comprises an siRNA. In one embodiment, the siRNA molecule comprises a double-stranded region of about 15 to about 60 nucleotides in length (*e.g.,* about 15-60, 15-50, 15-40, 15-30, 15-25, or 19-25 nucleotides in length, or 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length). The siRNA molecules of the invention are capable of silencing the expression of a target sequence *in vitro* and/or *in vivo*.

[0100] In some embodiments, the siRNA molecule comprises at least one modified nucleotide. In certain preferred embodiments, the siRNA molecule comprises one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides in the double-stranded region. In certain instances, the siRNA comprises from about 1% to about 100%

(*e.g.*, about 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region. In preferred embodiments, less than about 25% (*e.g.*, less than about 25%, 20%, 15%, 10%, or 5%) or from about 1% to about 25% (*e.g.*, from about 1%-25%, 5%-25%, 10%-25%, 15%-25%, 20%-25%, or 10%-20%) of the nucleotides in the double-stranded region comprise modified nucleotides.

[0101] In other embodiments, the siRNA molecule comprises modified nucleotides including, but not limited to, 2'-O-methyl (2'OMe) nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, locked nucleic acid (LNA) nucleotides, and mixtures thereof. In preferred embodiments, the siRNA comprises 2'OMe nucleotides (*e.g.*, 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof. In certain instances, the siRNA does not comprise 2'OMe-cytosine nucleotides. In other embodiments, the siRNA comprises a hairpin loop structure.

[0102] The siRNA may comprise modified nucleotides in one strand (*i.e.*, sense or antisense) or both strands of the double-stranded region of the siRNA molecule. Preferably, uridine and/or guanosine nucleotides are modified at selective positions in the double-stranded region of the siRNA duplex. With regard to uridine nucleotide modifications, at least one, two, three, four, five, six, or more of the uridine nucleotides in the sense and/or antisense strand can be a modified uridine nucleotide such as a 2'OMe-uridine nucleotide. In some embodiments, every uridine nucleotide in the sense and/or antisense strand is a 2'OMe-uridine nucleotide. With regard to guanosine nucleotide modifications, at least one, two, three, four, five, six, or more of the guanosine nucleotides in the sense and/or antisense strand can be a modified guanosine nucleotide such as a 2'OMe-guanosine nucleotide. In some embodiments, every guanosine nucleotide in the sense and/or antisense strand is a 2'OMe-guanosine nucleotide.

[0103] In certain embodiments, at least one, two, three, four, five, six, seven, or more 5'-GU-3' motifs in an siRNA sequence may be modified, *e.g.*, by introducing mismatches to eliminate the 5'-GU-3' motifs and/or by introducing modified nucleotides such as 2'OMe nucleotides. The 5'-GU-3' motif can be in the sense strand, the antisense strand, or both strands of the siRNA sequence. The 5'-GU-3' motifs may be adjacent to each other or, alternatively, they may be separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more nucleotides.

[0104] In some preferred embodiments, a modified siRNA molecule is less immunostimulatory than a corresponding unmodified siRNA sequence. In such embodiments, the modified siRNA molecule with reduced immunostimulatory properties advantageously retains RNAi activity against the target sequence. In another embodiment, the immunostimulatory properties of the modified siRNA molecule and its ability to silence target gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the siRNA sequence such as, *e.g.,* within the double-stranded region of the siRNA duplex. In certain instances, the modified siRNA is at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% less immunostimulatory than the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that the immunostimulatory properties of the modified siRNA molecule and the corresponding unmodified siRNA molecule can be determined by, for example, measuring INF-α and/or IL-6 levels from about two to about twelve hours after systemic administration in a mammal or transfection of a mammalian responder cell using an appropriate lipid-based delivery system (such as the SNALP delivery system disclosed herein).

[0105] In certain embodiments, a modified siRNA molecule has an IC_{50} (*i.e.,* half-maximal inhibitory concentration) less than or equal to ten-fold that of the corresponding unmodified siRNA (*i.e.*, the modified siRNA has an IC_{50} that is less than or equal to ten-times the IC_{50} of the corresponding unmodified siRNA). In other embodiments, the modified siRNA has an IC_{50} less than or equal to three-fold that of the corresponding unmodified siRNA sequence. In yet other embodiments, the modified siRNA has an IC_{50} less than or equal to two-fold that of the corresponding unmodified siRNA. It will be readily apparent to those of skill in the art that a dose-response curve can be generated and the IC_{50} values for the modified siRNA and the corresponding unmodified siRNA can be readily determined using methods known to those of skill in the art.

[0106] In yet another embodiment, a modified siRNA molecule is capable of silencing at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% of the expression of the target sequence relative to the corresponding unmodified siRNA sequence.

[0107] In some embodiments, the siRNA molecule does not comprise phosphate backbone modifications, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In other embodiments, the siRNA comprises one, two, three, four, or more phosphate backbone

modifications, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise phosphate backbone modifications.

[0108] In further embodiments, the siRNA does not comprise 2'-deoxy nucleotides, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In yet further embodiments, the siRNA comprises one, two, three, four, or more 2'-deoxy nucleotides, *e.g.,* in the sense and/or antisense strand of the double-stranded region. In preferred embodiments, the siRNA does not comprise 2'-deoxy nucleotides.

[0109] In certain instances, the nucleotide at the 3'-end of the double-stranded region in the sense and/or antisense strand is not a modified nucleotide. In certain other instances, the nucleotides near the 3'-end (*e.g.,* within one, two, three, or four nucleotides of the 3'-end) of the double-stranded region in the sense and/or antisense strand are not modified nucleotides.

[0110] The siRNA molecules described herein may have 3' overhangs of one, two, three, four, or more nucleotides on one or both sides of the double-stranded region, or may lack overhangs (*i.e.*, have blunt ends) on one or both sides of the double-stranded region. Preferably, the siRNA has 3' overhangs of two nucleotides on each side of the double-stranded region. In certain instances, the 3' overhang on the antisense strand has complementarity to the target sequence and the 3' overhang on the sense strand has complementarity to a complementary strand of the target sequence. Alternatively, the 3' overhangs do not have complementarity to the target sequence or the complementary strand thereof. In some embodiments, the 3' overhangs comprise one, two, three, four, or more nucleotides such as 2'-deoxy (2'H) nucleotides. In certain preferred embodiments, the 3' overhangs comprise deoxythymidine (dT) and/or uridine nucleotides. In other embodiments, one or more of the nucleotides in the 3' overhangs on one or both sides of the double-stranded region comprise modified nucleotides. Non-limiting examples of modified nucleotides are described above and include 2'OMe nucleotides, 2'-deoxy-2'F nucleotides, 2'-deoxy nucleotides, 2'-O-2-MOE nucleotides, LNA nucleotides, and mixtures thereof. In preferred embodiments, one, two, three, four, or more nucleotides in the 3' overhangs present on the sense and/or antisense strand of the siRNA comprise 2'OMe nucleotides (*e.g.,* 2'OMe purine and/or pyrimidine nucleotides) such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, and mixtures thereof.

[0111] The siRNA may comprise at least one or a cocktail (*e.g.,* at least two, three, four, five, six, seven, eight, nine, ten, or more) of unmodified and/or modified siRNA sequences that silence target gene expression. The cocktail of siRNA may comprise sequences which

are directed to the same region or domain (*e.g.*, a "hot spot") and/or to different regions or domains of one or more target genes. In certain instances, one or more (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) modified siRNA that silence target gene expression are present in a cocktail. In certain other instances, one or more (*e.g.*, at least two, three, four, five, six, seven, eight, nine, ten, or more) unmodified siRNA sequences that silence target gene expression are present in a cocktail.

[0112] In some embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% complementary to the target sequence or a portion thereof. In other embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that is 100% complementary to the target sequence or a portion thereof. In further embodiments, the antisense strand of the siRNA molecule comprises or consists of a sequence that specifically hybridizes to the target sequence or a portion thereof.

[0113] In further embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the target sequence or a portion thereof. In additional embodiments, the sense strand of the siRNA molecule comprises or consists of a sequence that is 100% identical to the target sequence or a portion thereof.

[0114] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the cationic lipid may comprise, *e.g.*, one or more of the following: 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP),

3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), or mixtures thereof. In certain preferred embodiments, the cationic lipid is DLinDMA, DLin-K-C2-DMA ("XTC2"), or mixtures thereof.

[0115] The synthesis of cationic lipids such as DLin-K-C2-DMA ("XTC2"), DLin-K-C3-DMA, DLin-K-C4-DMA, DLin-K6-DMA, and DLin-K-MPZ, as well as additional cationic lipids, is described in U.S. Provisional Application No. 61/104,212, filed October 9, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as DLin-K-DMA, DLin-C-DAP, DLin-DAC, DLin-MA, DLinDAP, DLin-S-DMA, DLin-2-DMAP, DLin-TMA.Cl, DLin-TAP.Cl, DLin-MPZ, DLinAP, DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Application No. PCT/US08/88676, filed December 31, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. The synthesis of cationic lipids such as CLinDMA, as well as additional cationic lipids, is described in U.S. Patent Publication No. 20060240554, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0116] In some embodiments, the cationic lipid may comprise from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 50 mol % to about 60 mol % of the total lipid present in the particle.

[0117] In other embodiments, the cationic lipid may comprise from about 55 mol % to about 90 mol %, from about 55 mol % to about 85 mol %, from about 55 mol % to about 80 mol %, from about 55 mol % to about 75 mol %, from about 55 mol % to about 70 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0118] In yet other embodiments, the cationic lipid may comprise from about 60 mol % to about 90 mol %, from about 60 mol % to about 85 mol %, from about 60 mol % to about 80 mol %, from about 60 mol % to about 75 mol %, or from about 60 mol % to about 70 mol % of the total lipid present in the particle.

[0119] In still yet other embodiments, the cationic lipid may comprise from about 65 mol % to about 90 mol %, from about 65 mol % to about 85 mol %, from about 65 mol % to about 80 mol %, or from about 65 mol % to about 75 mol % of the total lipid present in the particle.

[0120] In further embodiments, the cationic lipid may comprise from about 70 mol % to about 90 mol %, from about 70 mol % to about 85 mol %, from about 70 mol % to about 80 mol %, from about 75 mol % to about 90 mol %, from about 75 mol % to about 85 mol %, or from about 80 mol % to about 90 mol % of the total lipid present in the particle.

[0121] In additional embodiments, the cationic lipid may comprise (at least) about 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0122] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the non-cationic lipid may comprise, *e.g.*, one or more anionic lipids and/or neutral lipids. In preferred embodiments, the non-cationic lipid comprises one of the following neutral lipid components: (1) cholesterol or a derivative thereof; (2) a phospholipid; or (3) a mixture of a phospholipid and cholesterol or a derivative thereof.

[0123] Examples of cholesterol derivatives include, but are not limited to, cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof. The synthesis of cholesteryl-2'-hydroxyethyl ether is described herein.

[0124] The phospholipid may be a neutral lipid including, but not limited to, dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-

phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), egg phosphatidylcholine (EPC), and mixtures thereof. In certain preferred embodiments, the phospholipid is DPPC, DSPC, or mixtures thereof.

[0125] In some embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 60 mol %, from about 15 mol % to about 60 mol %, from about 20 mol % to about 60 mol %, from about 25 mol % to about 60 mol %, from about 30 mol % to about 60 mol %, from about 10 mol % to about 55 mol %, from about 15 mol % to about 55 mol %, from about 20 mol % to about 55 mol %, from about 25 mol % to about 55 mol %, from about 30 mol % to about 55 mol %, from about 13 mol % to about 50 mol %, from about 15 mol % to about 50 mol % or from about 20 mol % to about 50 mol % of the total lipid present in the particle. When the non-cationic lipid is a mixture of a phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 50, or 60 mol % of the total lipid present in the particle.

[0126] In other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 49.5 mol %, from about 13 mol % to about 49.5 mol %, from about 15 mol % to about 49.5 mol %, from about 20 mol % to about 49.5 mol %, from about 25 mol % to about 49.5 mol %, from about 30 mol % to about 49.5 mol %, from about 35 mol % to about 49.5 mol %, or from about 40 mol % to about 49.5 mol % of the total lipid present in the particle.

[0127] In yet other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 45 mol %, from about 13 mol % to about 45 mol %, from about 15 mol % to about 45 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, or from about 35 mol % to about 45 mol % of the total lipid present in the particle.

[0128] In still yet other embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 40 mol %, from about 13 mol % to about 40 mol %, from about 15 mol % to about 40 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0129] In further embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 35 mol %, from about 13 mol % to about 35 mol %, from about 15 mol % to about 35 mol %, from about 20 mol % to

about 35 mol %, or from about 25 mol % to about 35 mol % of the total lipid present in the particle.

[0130] In yet further embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise from about 10 mol % to about 30 mol %, from about 13 mol % to about 30 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 13 mol % to about 25 mol %, or from about 15 mol % to about 25 mol % of the total lipid present in the particle.

[0131] In additional embodiments, the non-cationic lipid (*e.g.*, one or more phospholipids and/or cholesterol) may comprise (at least) about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, or 60 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0132] In certain preferred embodiments, the non-cationic lipid comprises cholesterol or a derivative thereof of from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle of the invention may comprise cholesterol or a derivative thereof at about 37 mol % of the total lipid present in the particle. In other preferred embodiments, a phospholipid-free lipid particle of the invention may comprise cholesterol or a derivative thereof of from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 32 mol % to about 45 mol %, from about 32 mol % to about 42 mol %, from about 32 mol % to about 40 mol %, from about 34 mol % to about 45 mol %, from about 34 mol % to about 42 mol %, from about 34 mol % to about 40 mol %, or about 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0133] In certain other preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 4 mol % to about 10 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 30 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 7 mol % and cholesterol at about 34 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 3 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 4 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 4 mol % to about 8 mol

%, from about 5 mol % to about 12 mol %, from about 5 mol % to about 9 mol %, from about 6 mol % to about 12 mol %, from about 6 mol % to about 10 mol %, or about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 40 mol %, from about 25 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 45 mol %, from about 40 mol % to about 45 mol %, from about 28 mol % to about 40 mol %, from about 28 mol % to about 38 mol %, from about 30 mol % to about 38 mol %, from about 32 mol % to about 36 mol %, or about 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0134] In further preferred embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of a phospholipid and cholesterol may comprise DPPC at about 20 mol % and cholesterol at about 20 mol % of the total lipid present in the particle. In other embodiments, the non-cationic lipid comprises a mixture of: (i) a phospholipid of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 10 mol % to about 30 mol %, from about 10 mol % to about 25 mol %, from about 10 mol % to about 20 mol %, from about 15 mol % to about 30 mol %, from about 20 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, from about 12 mol % to about 28 mol %, from about 14 mol % to about 26 mol %, or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0135] In the lipid particles of the invention (*e.g.*, SNALP comprising an interfering RNA such as siRNA), the conjugated lipid that inhibits aggregation of particles may comprise, *e.g.*, one or more of the following: a polyethyleneglycol (PEG)-lipid conjugate, a polyamide

(ATTA)-lipid conjugate, a cationic-polymer-lipid conjugates (CPLs), or mixtures thereof. In one preferred embodiment, the nucleic acid-lipid particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate. In certain embodiments, the PEG-lipid conjugate or ATTA-lipid conjugate is used together with a CPL. The conjugated lipid that inhibits aggregation of particles may comprise a PEG-lipid including, *e.g.*, a PEG-diacylglycerol (DAG), a PEG dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or mixtures thereof. The PEG-DAA conjugate may be PEG-dilauryloxypropyl (C12), a PEG-dimyristyloxypropyl (C14), a PEG-dipalmityloxypropyl (C16), a PEG-distearyloxypropyl (C18), or mixtures thereof.

[0136] Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-*sn*3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed December 31, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Yet additional PEG-lipid conjugates suitable for use in the invention include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxaoctanyl]carbamoyl-ω-methyl-poly(ethylene glycol) (2KPEG-DMG). The synthesis of 2KPEG-DMG is described in U.S. Patent No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0137] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

[0138] In some embodiments, the conjugated lipid that inhibits aggregation of particles is a CPL that has the formula: A-W-Y, wherein A is a lipid moiety, W is a hydrophilic polymer, and Y is a polycationic moiety. W may be a polymer selected from the group consisting of polyethyleneglycol (PEG), polyamide, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, or combinations thereof, the polymer having a molecular weight of from about 250 to about 7000 daltons. In some embodiments, Y has at least 4 positive charges at a selected pH. In some embodiments, Y may be lysine, arginine, asparagine, glutamine, derivatives thereof, or combinations thereof.

[0139] In certain instances, the conjugated lipid that inhibits aggregation of particles (*e.g.*, PEG-lipid conjugate) may comprise from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 1 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, from about 1.4 mol % to about 1.5 mol %, or about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

[0140] In the lipid particles of the invention, the active agent or therapeutic agent may be fully encapsulated within the lipid portion of the particle, thereby protecting the active agent or therapeutic agent from enzymatic degradation. In preferred embodiments, a SNALP comprising a nucleic acid such as an interfering RNA (*e.g.*, siRNA) is fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the nucleic acid in the SNALP is not substantially degraded after exposure of the particle to a nuclease at 37°C for at least about 20, 30, 45, or 60 minutes. In certain other instances, the nucleic acid in the SNALP is not substantially degraded after incubation of the particle in serum at 37°C for at least about 30, 45, or 60 minutes or at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, or 36 hours. In other embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid such as siRNA) is complexed with the lipid portion of the particle. One of the benefits of the formulations of the present invention is that the lipid particle compositions are substantially non-toxic to mammals such as humans.

[0141] The term "fully encapsulated" indicates that the active agent or therapeutic agent in the lipid particle is not significantly degraded after exposure to serum or a nuclease or protease assay that would significantly degrade free DNA, RNA, or protein. In a fully encapsulated system, preferably less than about 25% of the active agent or therapeutic agent in the particle is degraded in a treatment that would normally degrade 100% of free active agent or therapeutic agent, more preferably less than about 10%, and most preferably less than about 5% of the active agent or therapeutic agent in the particle is degraded. In the context of nucleic acid therapeutic agents, full encapsulation may be determined by an Oligreen® assay. Oligreen® is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA or RNA in solution (available from Invitrogen Corporation; Carlsbad, CA). "Fully encapsulated" also indicates that the lipid particles are

serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

[0142] In another aspect, the present invention provides a lipid particle (*e.g.*, SNALP) composition comprising a plurality of lipid particles. In preferred embodiments, the active agent or therapeutic agent (*e.g.*, nucleic acid) is fully encapsulated within the lipid portion of the lipid particles (*e.g.*, SNALP), such that from about 30% to about 100%, from about 40% to about 100%, from about 50% to about 100%, from about 60% to about 100%, from about 70% to about 100%, from about 80% to about 100%, from about 90% to about 100%, from about 30% to about 95%, from about 40% to about 95%, from about 50% to about 95%, from about 60% to about 95%, %, from about 70% to about 95%, from about 80% to about 95%, from about 85% to about 95%, from about 90% to about 95%, from about 30% to about 90%, from about 40% to about 90%, from about 50% to about 90%, from about 60% to about 90%, from about 70% to about 90%, from about 80% to about 90%, or at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% (or any fraction thereof or range therein) of the lipid particles (*e.g.*, SNALP) have the active agent or therapeutic agent encapsulated therein.

[0143] Typically, the lipid particles (*e.g.*, SNALP) of the invention have a lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 1 to about 100. In some instances, the lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) ranges from about 1 to about 50, from about 2 to about 25, from about 3 to about 20, from about 4 to about 15, or from about 5 to about 10. In preferred embodiments, the lipid particles of the invention have a lipid:active agent (*e.g.*, lipid:nucleic acid) ratio (mass/mass ratio) of from about 5 to about 15, *e.g.*, about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 (or any fraction thereof or range therein).

[0144] Typically, the lipid particles (*e.g.*, SNALP) of the invention have a mean diameter of from about 40 nm to about 150 nm. In preferred embodiments, the lipid particles (*e.g.*, SNALP) of the invention have a mean diameter of from about 40 nm to about 130 nm, from about 40 nm to about 120 nm, from about 40 nm to about 100 nm, from about 50 nm to about 120 nm, from about 50 nm to about 100 nm, from about 60 nm to about 120 nm, from about 60 nm to about 110 nm, from about 60 nm to about 100 nm, from about 60 nm to about 90 nm, from about 60 nm to about 80 nm, from about 70 nm to about 120 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 70 nm to about 90 nm, from about 70 nm to about 80 nm, or less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm (or any fraction thereof or range therein).

[0145] In one specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:62" formulation. In a preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is cholesterol, and the conjugated lipid is a PEG-DAA conjugate. Although these are preferred embodiments of the 1:62 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic lipids (including other cholesterol derivatives), and conjugated lipids can be used in the 1:62 formulation as described herein.

[0146] In another specific embodiment of the invention, the SNALP comprises: (a) one or more unmodified and/or modified interfering RNA (*e.g.*, siRNA, aiRNA, miRNA) that silence target gene expression; (b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle; (c) a non-cationic lipid comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and (d) a conjugated lipid that inhibits aggregation of particles comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle. This specific embodiment of SNALP is generally referred to herein as the "1:57" formulation. In one preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 5 mol % to about 9 mol % of the total lipid present in the particle (*e.g.*, about 7.1 mol %) and the cholesterol (or cholesterol derivative) comprises from about 32 mol % to about 37 mol % of the total lipid present in the particle (*e.g.*, about 34.3 mol %), and the PEG-lipid is a PEG-DAA (*e.g.*, PEG-cDMA). In another preferred embodiment, the cationic lipid is DLinDMA or DLin-K-C2-DMA ("XTC2"), the non-cationic lipid is a mixture of a phospholipid (such as DPPC) and cholesterol, wherein the phospholipid comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.*, about 20 mol %) and the cholesterol (or cholesterol derivative) comprises from about 15 mol % to about 25 mol % of the total lipid present in the particle (*e.g.*, about 20 mol %), and the PEG-lipid is a PEG-DAA (*e.g.*, PEG-cDMA). Although these are preferred embodiments of the 1:57 formulation, those of skill in the art will appreciate that other cationic lipids, non-cationic

lipids (including other phospholipids and other cholesterol derivatives), and conjugated lipids can be used in the 1:57 formulation as described herein.

[0147] In preferred embodiments, the 1:62 SNALP formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-cDMA (or PEG-cDSA), about 61.5 mol % DLinDMA (or XTC2), and about 36.9 mol % cholesterol (or derivative thereof). In other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 7.1 mol % DPPC, and about 34.3 mol % cholesterol (or derivative thereof). In yet other preferred embodiments, the 1:57 SNALP formulation is a four-component system which comprises about 1.4 mol % PEG-cDMA (or PEG-cDSA), about 57.1 mol % DLinDMA (or XTC2), about 20 mol % DPPC, and about 20 mol % cholesterol (or derivative thereof). It should be understood that these SNALP formulations are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the SNALP formulations may vary.

[0148] The present invention also provides a pharmaceutical composition comprising a lipid particle (*e.g.*, SNALP) described herein and a pharmaceutically acceptable carrier.

[0149] In a further aspect, the present invention provides a method for introducing one or more active agents or therapeutic agents (*e.g.*, nucleic acid) into a cell, comprising contacting the cell with a lipid particle (*e.g.*, SNALP) described herein. In one embodiment, the cell is in a mammal and the mammal is a human. In another embodiment, the present invention provides a method for the *in vivo* delivery of one or more active agents or therapeutic agents (*e.g.*, nucleic acid), comprising administering to a mammalian subject a lipid particle (*e.g.*, SNALP) described herein. In a preferred embodiment, the mode of administration includes, but is not limited to, oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. Preferably, the mammalian subject is a human.

[0150] In one embodiment, at least about 5%, 10%, 15%, 20%, or 25% of the total injected dose of the lipid particles (*e.g.*, SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In other embodiments, more than about 20%, 30%, 40% and as much as about 60%, 70% or 80% of the total injected dose of the lipid particles (*e.g.*, SNALP) is present in plasma about 8, 12, 24, 36, or 48 hours after injection. In certain instances, more than about 10% of a plurality of the particles is present in the plasma of a mammal about 1 hour after administration. In certain other instances, the presence of the lipid particles (*e.g.*,

SNALP) is detectable at least about 1 hour after administration of the particle. In certain embodiments, the presence of an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is detectable in cells of the lung, liver, tumor, or at a site of inflammation at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is detectable at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In yet other embodiments, downregulation of expression of a target sequence by an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) occurs preferentially in tumor cells or in cells at a site of inflammation. In further embodiments, the presence or effect of an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) in cells at a site proximal or distal to the site of administration or in cells of the lung, liver, or a tumor is detectable at about 12, 24, 48, 72, or 96 hours, or at about 6, 8, 10, 12, 14, 16, 18, 19, 20, 22, 24, 26, or 28 days after administration. In additional embodiments, the lipid particles (*e.g.*, SNALP) of the invention are administered parenterally or intraperitoneally.

[0151] In some embodiments, the lipid particles (*e.g.*, SNALP) of the invention are particularly useful in methods for the therapeutic delivery of one or more nucleic acids comprising an interfering RNA sequence (*e.g.*, siRNA). In particular, it is an object of this invention to provide *in vitro* and *in vivo* methods for treatment of a disease or disorder in a mammal (*e.g.*, a rodent such as a mouse or a primate such as a human, chimpanzee, or monkey) by downregulating or silencing the transcription and/or translation of one or more target nucleic acid sequences or genes of interest. As a non-limiting example, the methods of the invention are useful for *in vivo* delivery of interfering RNA (*e.g.*, siRNA) to the liver and/or tumor of a mammalian subject. In certain embodiments, the disease or disorder is associated with expression and/or overexpression of a gene and expression or overexpression of the gene is reduced by the interfering RNA (*e.g.*, siRNA). In certain other embodiments, a therapeutically effective amount of the lipid particle (*e.g.*, SNALP) may be administered to the mammal. In some instances, an interfering RNA (*e.g.*, siRNA) is formulated into a SNALP, and the particles are administered to patients requiring such treatment. In other instances, cells are removed from a patient, the interfering RNA (*e.g.*, siRNA) is delivered *in vitro* (*e.g.*, using a SNALP described herein), and the cells are reinjected into the patient.

[0152] In an additional aspect, the present invention provides lipid particles (*e.g.*, SNALP) comprising asymmetrical interfering RNA (aiRNA) molecules that silence the expression of a target gene and methods of using such particles to silence target gene expression.

[0153] In one embodiment, the aiRNA molecule comprises a double-stranded (duplex) region of about 10 to about 25 (base paired) nucleotides in length, wherein the aiRNA molecule comprises an antisense strand comprising 5' and 3' overhangs, and wherein the aiRNA molecule is capable of silencing target gene expression.

[0154] In certain instances, the aiRNA molecule comprises a double-stranded (duplex) region of about 12-20, 12-19, 12-18, 13-17, or 14-17 (base paired) nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 (base paired) nucleotides in length. In certain other instances, the 5' and 3' overhangs on the antisense strand comprise sequences that are complementary to the target RNA sequence, and may optionally further comprise nontargeting sequences. In some embodiments, each of the 5' and 3' overhangs on the antisense strand comprises or consists of one, two, three, four, five, six, seven, or more nucleotides.

[0155] In other embodiments, the aiRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0156] In a related aspect, the present invention provides lipid particles (*e.g.,* SNALP) comprising microRNA (miRNA) molecules that silence the expression of a target gene and methods of using such compositions to silence target gene expression.

[0157] In one embodiment, the miRNA molecule comprises about 15 to about 60 nucleotides in length, wherein the miRNA molecule is capable of silencing target gene expression.

[0158] In certain instances, the miRNA molecule comprises about 15-50, 15-40, or 15-30 nucleotides in length, more typically about 15-25 or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In a preferred embodiment, the miRNA molecule is a mature miRNA molecule targeting an RNA sequence of interest.

[0159] In some embodiments, the miRNA molecule comprises modified nucleotides selected from the group consisting of 2'OMe nucleotides, 2'F nucleotides, 2'-deoxy nucleotides, 2'-O-MOE nucleotides, LNA nucleotides, and mixtures thereof. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides. As a non-limiting example, the 2'OMe nucleotides may be selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, and mixtures thereof.

[0160] As such, the lipid particles of the invention (*e.g.*, SNALP) are advantageous and suitable for use in the administration of active agents or therapeutic agents such as nucleic acid (*e.g.*, interfering RNA such as siRNA, aiRNA, and/or miRNA) to a subject (*e.g.*, a mammal such as a human) because they are stable in circulation, of a size required for pharmacodynamic behavior resulting in access to extravascular sites, and are capable of reaching target cell populations.

IV. Active Agents

[0161] Active agents (*e.g.*, therapeutic agents) include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be, *e.g.*, biological, physiological, and/or cosmetic. Active agents may be any type of molecule or compound including, but not limited to, nucleic acids, peptides, polypeptides, small molecules, and mixtures thereof. Non-limiting examples of nucleic acids include interfering RNA molecules (*e.g.*, siRNA, aiRNA, miRNA), antisense oligonucleotides, plasmids, ribozymes, immunostimulatory oligonucleotides, and mixtures thereof. Examples of peptides or polypeptides include, without limitation, antibodies (*e.g.*, polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, Primatized™ antibodies), cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell-surface receptors and their ligands, hormones, and mixtures thereof. Examples of small molecules include, but are not limited to, small organic molecules or compounds such as any conventional agent or drug known to those of skill in the art.

[0162] In some embodiments, the active agent is a therapeutic agent, or a salt or derivative thereof. Therapeutic agent derivatives may be therapeutically active themselves or they may be prodrugs, which become active upon further modification. Thus, in one embodiment, a therapeutic agent derivative retains some or all of the therapeutic activity as compared to the unmodified agent, while in another embodiment, a therapeutic agent derivative is a prodrug that lacks therapeutic activity, but becomes active upon further modification.

A. Nucleic Acids

[0163] In certain embodiments, lipid particles of the present invention are associated with a nucleic acid, resulting in a nucleic acid-lipid particle (*e.g.*, SNALP). In some embodiments, the nucleic acid is fully encapsulated in the lipid particle. As used herein, the term "nucleic acid" includes any oligonucleotide or polynucleotide, with fragments containing up to 60

nucleotides generally termed oligonucleotides, and longer fragments termed polynucleotides. In particular embodiments, oligonucletoides of the invention are from about 15 to about 60 nucleotides in length. Nucleic acid may be administered alone in the lipid particles of the invention, or in combination (*e.g.*, co-administered) with lipid particles of the invention comprising peptides, polypeptides, or small molecules such as conventional drugs.

[0164] In the context of this invention, the terms "polynucleotide" and "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally-occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also include polymers or oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake, reduced immunogenicity, and increased stability in the presence of nucleases.

[0165] Oligonucleotides are generally classified as deoxyribooligonucleotides or ribooligonucleotides. A deoxyribooligonucleotide consists of a 5-carbon sugar called deoxyribose joined covalently to phosphate at the 5′ and 3′ carbons of this sugar to form an alternating, unbranched polymer. A ribooligonucleotide consists of a similar repeating structure where the 5-carbon sugar is ribose.

[0166] The nucleic acid that is present in a lipid-nucleic acid particle according to this invention includes any form of nucleic acid that is known. The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Examples of double-stranded DNA are described herein and include, *e.g.*, structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA are described herein and include, *e.g.*, siRNA and other RNAi agents such as aiRNA and pre-miRNA. Single-stranded nucleic acids include, *e.g.*, antisense oligonucleotides, ribozymes, mature miRNA, and triplex-forming oligonucleotides.

[0167] Nucleic acids of the invention may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to about 100,000 nucleotide residues in length. In particular embodiments, oligonucleotides may range from about 10 to about 100 nucleotides in length. In various related embodiments, oligonucleotides, both single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 60 nucleotides, from about 15 to

about 60 nucleotides, from about 20 to about 50 nucleotides, from about 15 to about 30 nucleotides, or from about 20 to about 30 nucleotides in length.

[0168] In particular embodiments, an oligonucleotide (or a strand thereof) of the invention specifically hybridizes to or is complementary to a target polynucleotide sequence. The terms "specifically hybridizable" and "complementary" as used herein indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. In preferred embodiments, an oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target sequence interferes with the normal function of the target sequence to cause a loss of utility or expression therefrom, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or, in the case of *in vitro* assays, under conditions in which the assays are conducted. Thus, the oligonucleotide may include 1, 2, 3, or more base substitutions as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

1. siRNA

[0169] The siRNA component of the nucleic acid-lipid particles of the present invention is capable of silencing the expression of a target gene of interest. Each strand of the siRNA duplex is typically about 15 to about 60 nucleotides in length, preferably about 15 to about 30 nucleotides in length. In certain embodiments, the siRNA comprises at least one modified nucleotide. The modified siRNA is generally less immunostimulatory than a corresponding unmodified siRNA sequence and retains RNAi activity against the target gene of interest. In some embodiments, the modified siRNA contains at least one 2'OMe purine or pyrimidine nucleotide such as a 2'OMe-guanosine, 2'OMe-uridine, 2'OMe-adenosine, and/or 2'OMe-cytosine nucleotide. In preferred embodiments, one or more of the uridine and/or guanosine nucleotides are modified. The modified nucleotides can be present in one strand (*i.e.*, sense or antisense) or both strands of the siRNA. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell*, 107:309 (2001)), or may lack overhangs (*i.e.*, have blunt ends).

[0170] The modified siRNA generally comprises from about 1% to about 100% (*e.g.*, about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%,

19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) modified nucleotides in the double-stranded region of the siRNA duplex. In certain embodiments, one, two, three, four, five, six, seven, eight, nine, ten, or more of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0171] In some embodiments, less than about 25% (*e.g.*, less than about 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0172] In other embodiments, from about 1% to about 25% (*e.g.*, from about 1%-25%, 2%-25%, 3%-25%, 4%-25%, 5%-25%, 6%-25%, 7%-25%, 8%-25%, 9%-25%, 10%-25%, 11%-25%, 12%-25%, 13%-25%, 14%-25%, 15%-25%, 16%-25%, 17%-25%, 18%-25%, 19%-25%, 20%-25%, 21%-25%, 22%-25%, 23%-25%, 24%-25%, *etc.*) or from about 1% to about 20% (*e.g.*, from about 1%-20%, 2%-20%, 3%-20%, 4%-20%, 5%-20%, 6%-20%, 7%-20%, 8%-20%, 9%-20%, 10%-20%, 11%-20%, 12%-20%, 13%-20%, 14%-20%, 15%-20%, 16%-20%, 17%-20%, 18%-20%, 19%-20%, 1%-19%, 2%-19%, 3%-19%, 4%-19%, 5%-19%, 6%-19%, 7%-19%, 8%-19%, 9%-19%, 10%-19%, 11%-19%, 12%-19%, 13%-19%, 14%-19%, 15%-19%, 16%-19%, 17%-19%, 18%-19%, 1%-18%, 2%-18%, 3%-18%, 4%-18%, 5%-18%, 6%-18%, 7%-18%, 8%-18%, 9%-18%, 10%-18%, 11%-18%, 12%-18%, 13%-18%, 14%-18%, 15%-18%, 16%-18%, 17%-18%, 1%-17%, 2%-17%, 3%-17%, 4%-17%, 5%-17%, 6%-17%, 7%-17%, 8%-17%, 9%-17%, 10%-17%, 11%-17%, 12%-17%, 13%-17%, 14%-17%, 15%-17%, 16%-17%, 1%-16%, 2%-16%, 3%-16%, 4%-16%, 5%-16%, 6%-16%, 7%-16%, 8%-16%, 9%-16%, 10%-16%, 11%-16%, 12%-16%, 13%-16%, 14%-16%, 15%-16%, 1%-15%, 2%-15%, 3%-15%, 4%-15%, 5%-15%, 6%-15%, 7%-15%, 8%-15%, 9%-15%, 10%-15%, 11%-15%, 12%-15%, 13%-15%, 14%-15%, *etc.*) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

[0173] In further embodiments, *e.g.*, when one or both strands of the siRNA are selectively modified at uridine and/or guanosine nucleotides, the resulting modified siRNA can comprise less than about 30% modified nucleotides (*e.g.*, less than about 30%, 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% modified nucleotides) or from about 1% to about 30% modified nucleotides (*e.g.*, from about 1%-30%, 2%-30%, 3%-30%, 4%-30%, 5%-30%, 6%-30%, 7%-30%, 8%-30%, 9%-30%, 10%-30%, 11%-30%, 12%-30%, 13%-30%, 14%-30%, 15%-30%, 16%-30%, 17%-30%, 18%-30%, 19%-30%, 20%-30%, 21%-30%, 22%-

30%, 23%-30%, 24%-30%, 25%-30%, 26%-30%, 27%-30%, 28%-30%, or 29%-30% modified nucleotides).

a. Selection of siRNA Sequences

[0174] Suitable siRNA sequences can be identified using any means known in the art. Typically, the methods described in Elbashir *et al.*, *Nature,* 411:494-498 (2001) and Elbashir *et al.*, *EMBO J.,* 20:6877-6888 (2001) are combined with rational design rules set forth in Reynolds *et al.*, *Nature Biotech.,* 22(3):326-330 (2004).

[0175] Generally, the nucleotide sequence 3' of the AUG start codon of a transcript from the target gene of interest is scanned for dinucleotide sequences (*e.g.*, AA, NA, CC, GG, or UU, wherein N = C, G, or U) (*see*, *e.g.*, Elbashir *et al.*, *EMBO J.,* 20:6877-6888 (2001)). The nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences (*i.e.*, a target sequence or a sense strand sequence). Typically, the 19, 21, 23, 25, 27, 29, 31, 33, 35, or more nucleotides immediately 3' to the dinucleotide sequences are identified as potential siRNA sequences. In some embodiments, the dinucleotide sequence is an AA or NA sequence and the 19 nucleotides immediately 3' to the AA or NA dinucleotide are identified as potential siRNA sequences. siRNA sequences are usually spaced at different positions along the length of the target gene. To further enhance silencing efficiency of the siRNA sequences, potential siRNA sequences may be analyzed to identify sites that do not contain regions of homology to other coding sequences, *e.g.,* in the target cell or organism. For example, a suitable siRNA sequence of about 21 base pairs typically will not have more than 16-17 contiguous base pairs of homology to coding sequences in the target cell or organism. If the siRNA sequences are to be expressed from an RNA Pol III promoter, siRNA sequences lacking more than 4 contiguous A's or T's are selected.

[0176] Once a potential siRNA sequence has been identified, a complementary sequence (*i.e.*, an antisense strand sequence) can be designed. A potential siRNA sequence can also be analyzed using a variety of criteria known in the art. For example, to enhance their silencing efficiency, the siRNA sequences may be analyzed by a rational design algorithm to identify sequences that have one or more of the following features: (1) G/C content of about 25% to about 60% G/C; (2) at least 3 A/Us at positions 15-19 of the sense strand; (3) no internal repeats; (4) an A at position 19 of the sense strand; (5) an A at position 3 of the sense strand; (6) a U at position 10 of the sense strand; (7) no G/C at position 19 of the sense strand; and (8) no G at position 13 of the sense strand. siRNA design tools that incorporate algorithms that assign suitable values of each of these features and are useful for selection of siRNA can

be found at, *e.g.*, http://boz094.ust.hk/RNAi/siRNA. One of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may be selected for further analysis and testing as potential siRNA sequences.

[0177] Additionally, potential siRNA sequences with one or more of the following criteria can often be eliminated as siRNA: (1) sequences comprising a stretch of 4 or more of the same base in a row; (2) sequences comprising homopolymers of Gs (*i.e.*, to reduce possible non-specific effects due to structural characteristics of these polymers; (3) sequences comprising triple base motifs (*e.g.*, GGG, CCC, AAA, or TTT); (4) sequences comprising stretches of 7 or more G/Cs in a row; and (5) sequences comprising direct repeats of 4 or more bases within the candidates resulting in internal fold-back structures. However, one of skill in the art will appreciate that sequences with one or more of the foregoing characteristics may still be selected for further analysis and testing as potential siRNA sequences.

[0178] In some embodiments, potential siRNA sequences may be further analyzed based on siRNA duplex asymmetry as described in, *e.g.*, Khvorova *et al.*, *Cell*, 115:209-216 (2003); and Schwarz *et al.*, *Cell*, 115:199-208 (2003). In other embodiments, potential siRNA sequences may be further analyzed based on secondary structure at the target site as described in, *e.g.*, Luo *et al.*, *Biophys. Res. Commun.*, 318:303-310 (2004). For example, secondary structure at the target site can be modeled using the Mfold algorithm (available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) to select siRNA sequences which favor accessibility at the target site where less secondary structure in the form of base-pairing and stem-loops is present.

[0179] Once a potential siRNA sequence has been identified, the sequence can be analyzed for the presence of any immunostimulatory properties, *e.g.*, using an *in vitro* cytokine assay or an *in vivo* animal model. Motifs in the sense and/or antisense strand of the siRNA sequence such as GU-rich motifs (*e.g.*, 5'-GU-3', 5'-UGU-3', 5'-GUGU-3', 5'-UGUGU-3', *etc.*) can also provide an indication of whether the sequence may be immunostimulatory. Once an siRNA molecule is found to be immunostimulatory, it can then be modified to decrease its immunostimulatory properties as described herein. As a non-limiting example, an siRNA sequence can be contacted with a mammalian responder cell under conditions such that the cell produces a detectable immune response to determine whether the siRNA is an immunostimulatory or a non-immunostimulatory siRNA. The mammalian responder cell may be from a naïve mammal (*i.e.*, a mammal that has not previously been in contact with the gene product of the siRNA sequence). The mammalian responder cell may be, *e.g.*, a peripheral blood mononuclear cell (PBMC), a macrophage, and the like. The detectable

immune response may comprise production of a cytokine or growth factor such as, *e.g.*, TNF-α, IFN-α, IFN-β, IFN-γ, IL-6, IL-12, or a combination thereof. An siRNA molecule identified as being immunostimulatory can then be modified to decrease its immunostimulatory properties by replacing at least one of the nucleotides on the sense and/or antisense strand with modified nucleotides. For example, less than about 30% (*e.g.*, less than about 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA duplex can be replaced with modified nucleotides such as 2'OMe nucleotides. The modified siRNA can then be contacted with a mammalian responder cell as described above to confirm that its immunostimulatory properties have been reduced or abrogated.

[0180] Suitable *in vitro* assays for detecting an immune response include, but are not limited to, the double monoclonal antibody sandwich immunoassay technique of David *et al.* (U.S. Patent No. 4,376,110); monoclonal-polyclonal antibody sandwich assays (Wide *et al.*, in Kirkham and Hunter, eds., *Radioimmunoassay Methods*, E. and S. Livingstone, Edinburgh (1970)); the "Western blot" method of Gordon *et al.* (U.S. Patent No. 4,452,901); immunoprecipitation of labeled ligand (Brown *et al.*, *J. Biol. Chem.*, 255:4980-4983 (1980)); enzyme-linked immunosorbent assays (ELISA) as described, for example, by Raines *et al.*, *J. Biol. Chem.*, 257:5154-5160 (1982); immunocytochemical techniques, including the use of fluorochromes (Brooks *et al.*, *Clin. Exp. Immunol.*, 39:477 (1980)); and neutralization of activity (Bowen-Pope *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:2396-2400 (1984)). In addition to the immunoassays described above, a number of other immunoassays are available, including those described in U.S. Patent Nos. 3,817,827; 3,850,752; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0181] A non-limiting example of an *in vivo* model for detecting an immune response includes an *in vivo* mouse cytokine induction assay as described in, *e.g.*, Judge *et al.*, *Mol. Ther.*, 13:494-505 (2006). In certain embodiments, the assay that can be performed as follows: (1) siRNA can be administered by standard intravenous injection in the lateral tail vein; (2) blood can be collected by cardiac puncture about 6 hours after administration and processed as plasma for cytokine analysis; and (3) cytokines can be quantified using sandwich ELISA kits according to the manufacturer's instructions (*e.g.*, mouse and human IFN-α (PBL Biomedical; Piscataway, NJ); human IL-6 and TNF-α (eBioscience; San Diego, CA); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences; San Diego, CA)).

[0182] Monoclonal antibodies that specifically bind cytokines and growth factors are commercially available from multiple sources and can be generated using methods known in

the art (*see, e.g.*, Kohler *et al.*, *Nature*, 256: 495-497 (1975) and Harlow and Lane, ANTIBODIES, A LABORATORY MANUAL, Cold Spring Harbor Publication, New York (1999)). Generation of monoclonal antibodies has been previously described and can be accomplished by any means known in the art (Buhring *et al.*, in Hybridoma, Vol. 10, No. 1, pp. 77-78 (1991)). In some methods, the monoclonal antibody is labeled (*e.g.*, with any composition detectable by spectroscopic, photochemical, biochemical, electrical, optical, or chemical means) to facilitate detection.

b. Generating siRNA Molecules

[0183] siRNA can be provided in several forms including, *e.g.*, as one or more isolated small-interfering RNA (siRNA) duplexes, as longer double-stranded RNA (dsRNA), or as siRNA or dsRNA transcribed from a transcriptional cassette in a DNA plasmid. The siRNA sequences may have overhangs (*e.g.*, 3' or 5' overhangs as described in Elbashir *et al.*, *Genes Dev.*, 15:188 (2001) or Nykänen *et al.*, *Cell*, 107:309 (2001), or may lack overhangs (*i.e.*, to have blunt ends).

[0184] An RNA population can be used to provide long precursor RNAs, or long precursor RNAs that have substantial or complete identity to a selected target sequence can be used to make the siRNA. The RNAs can be isolated from cells or tissue, synthesized, and/or cloned according to methods well known to those of skill in the art. The RNA can be a mixed population (obtained from cells or tissue, transcribed from cDNA, subtracted, selected, *etc.*), or can represent a single target sequence. RNA can be naturally occurring (*e.g.*, isolated from tissue or cell samples), synthesized *in vitro* (*e.g.*, using T7 or SP6 polymerase and PCR products or a cloned cDNA), or chemically synthesized.

[0185] To form a long dsRNA, for synthetic RNAs, the complement is also transcribed *in vitro* and hybridized to form a dsRNA. If a naturally occuring RNA population is used, the RNA complements are also provided (*e.g.*, to form dsRNA for digestion by *E. coli* RNAse III or Dicer), *e.g.*, by transcribing cDNAs corresponding to the RNA population, or by using RNA polymerases. The precursor RNAs are then hybridized to form double stranded RNAs for digestion. The dsRNAs can be directly administered to a subject or can be digested *in vitro* prior to administration.

[0186] Methods for isolating RNA, synthesizing RNA, hybridizing nucleic acids, making and screening cDNA libraries, and performing PCR are well known in the art (*see, e.g.*, Gubler and Hoffman, *Gene*, 25:263-269 (1983); Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*), as are PCR methods (*see,* U.S. Patent Nos. 4,683,195 and 4,683,202; *PCR Protocols:*

A Guide to Methods and Applications (Innis *et al.*, eds, 1990)). Expression libraries are also well known to those of skill in the art. Additional basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0187] Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules of the invention can be synthesized using any of a variety of techniques known in the art, such as those described in Usman *et al.*, *J. Am. Chem. Soc.*, 109:7845 (1987); Scaringe *et al.*, *Nucl. Acids Res.*, 18:5433 (1990); Wincott *et al.*, *Nucl. Acids Res.*, 23:2677-2684 (1995); and Wincott *et al., Methods Mol. Bio.*, 74:59 (1997). The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end and phosphoramidites at the 3'-end. As a non-limiting example, small scale syntheses can be conducted on an Applied Biosystems synthesizer using a 0.2 μmol scale protocol. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer from Protogene (Palo Alto, CA). However, a larger or smaller scale of synthesis is also within the scope of this invention. Suitable reagents for oligonucleotide synthesis, methods for RNA deprotection, and methods for RNA purification are known to those of skill in the art.

[0188] siRNA molecules can also be synthesized via a tandem synthesis technique, wherein both strands are synthesized as a single continuous oligonucleotide fragment or strand separated by a cleavable linker that is subsequently cleaved to provide separate fragments or strands that hybridize to form the siRNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siRNA can be readily adapted to both multiwell/multiplate synthesis platforms as well as large scale synthesis platforms employing batch reactors, synthesis columns, and the like. Alternatively, siRNA molecules can be assembled from two distinct oligonucleotides, wherein one oligonucleotide comprises the sense strand and the other comprises the antisense strand of the siRNA. For example, each strand can be synthesized separately and joined together by hybridization or ligation following synthesis and/or deprotection. In certain other instances, siRNA molecules can be synthesized as a single continuous oligonucleotide fragment, where the self-complementary sense and antisense regions hybridize to form an siRNA duplex having hairpin secondary structure.

c. Modifying siRNA Sequences

[0189] In certain aspects, siRNA molecules comprise a duplex having two strands and at least one modified nucleotide in the double-stranded region, wherein each strand is about 15 to about 60 nucleotides in length. Advantageously, the modified siRNA is less immunostimulatory than a corresponding unmodified siRNA sequence, but retains the capability of silencing the expression of a target sequence. In preferred embodiments, the degree of chemical modifications introduced into the siRNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the siRNA and retention of RNAi activity. As a non-limiting example, an siRNA molecule that targets a gene of interest can be minimally modified (*e.g.*, less than about 30%, 25%, 20%, 15%, 10%, or 5% modified) at selective uridine and/or guanosine nucleotides within the siRNA duplex to eliminate the immune response generated by the siRNA while retaining its capability to silence target gene expression.

[0190] Examples of modified nucleotides suitable for use in the invention include, but are not limited to, ribonucleotides having a 2'-O-methyl (2'OMe), 2'-deoxy-2'-fluoro (2'F), 2'-deoxy, 5-C-methyl, 2'-O-(2-methoxyethyl) (MOE), 4'-thio, 2'-amino, or 2'-C-allyl group. Modified nucleotides having a Northern conformation such as those described in, *e.g.*, Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag Ed. (1984), are also suitable for use in siRNA molecules. Such modified nucleotides include, without limitation, locked nucleic acid (LNA) nucleotides (*e.g.*, 2'-O, 4'-C-methylene-(D-ribofuranosyl) nucleotides), 2'-O-(2-methoxyethyl) (MOE) nucleotides, 2'-methyl-thio-ethyl nucleotides, 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy-2'-chloro (2'Cl) nucleotides, and 2'-azido nucleotides. In certain instances, the siRNA molecules described herein include one or more G-clamp nucleotides. A G-clamp nucleotide refers to a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine nucleotide within a duplex (*see, e.g.*, Lin *et al.*, *J. Am. Chem. Soc.*, 120:8531-8532 (1998)). In addition, nucleotides having a nucleotide base analog such as, for example, C-phenyl, C-naphthyl, other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole (*see, e.g.*, Loakes, *Nucl. Acids Res.*, 29:2437-2447 (2001)) can be incorporated into siRNA molecules.

[0191] In certain embodiments, siRNA molecules may further comprise one or more chemical modifications such as terminal cap moieties, phosphate backbone modifications, and the like. Examples of terminal cap moieties include, without limitation, inverted deoxy abasic residues, glyceryl modifications, 4',5'-methylene nucleotides, 1-(β-D-erythrofuranosyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides, α-nucleotides, modified base nucleotides, *threo*-pentofuranosyl nucleotides, acyclic 3',4'-seco nucleotides, acyclic 3,4-dihydroxybutyl nucleotides, acyclic 3,5-dihydroxypentyl nucleotides, 3'-3'-inverted nucleotide moieties, 3'-3'-inverted abasic moieties, 3'-2'-inverted nucleotide moieties, 3'-2'-inverted abasic moieties, 5'-5'-inverted nucleotide moieties, 5'-5'-inverted abasic moieties, 3'-5'-inverted deoxy abasic moieties, 5'-amino-alkyl phosphate, 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate, 6-aminohexyl phosphate, 1,2-aminododecyl phosphate, hydroxypropyl phosphate, 1,4-butanediol phosphate, 3'-phosphoramidate, 5'-phosphoramidate, hexylphosphate, aminohexyl phosphate, 3'-phosphate, 5'-amino, 3'-phosphorothioate, 5'-phosphorothioate, phosphorodithioate, and bridging or non-bridging methylphosphonate or 5'-mercapto moieties (*see, e.g.,* U.S. Patent No. 5,998,203; Beaucage *et al.*, *Tetrahedron* 49:1925 (1993)). Non-limiting examples of phosphate backbone modifications (*i.e.,* resulting in modified internucleotide linkages) include phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate, carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and alkylsilyl substitutions (*see, e.g.,* Hunziker *et al.*, *Nucleic Acid Analogues: Synthesis and Properties*, in *Modern Synthetic Methods*, VCH, 331-417 (1995); Mesmaeker *et al.*, *Novel Backbone Replacements for Oligonucleotides*, in *Carbohydrate Modifications in Antisense Research*, ACS, 24-39 (1994)). Such chemical modifications can occur at the 5'-end and/or 3'-end of the sense strand, antisense strand, or both strands of the siRNA. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0192] In some embodiments, the sense and/or antisense strand of the siRNA molecule can further comprise a 3'-terminal overhang having about 1 to about 4 (*e.g.,* 1, 2, 3, or 4) 2'-deoxy ribonucleotides and/or any combination of modified and unmodified nucleotides. Additional examples of modified nucleotides and types of chemical modifications that can be introduced into siRNA molecules are described, *e.g.,* in UK Patent No. GB 2,397,818 B and

U.S. Patent Publication Nos. 20040192626, 20050282188, and 20070135372, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0193] The siRNA molecules described herein can optionally comprise one or more non-nucleotides in one or both strands of the siRNA. As used herein, the term "non-nucleotide" refers to any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base such as adenosine, guanine, cytosine, uracil, or thymine and therefore lacks a base at the 1'-position.

[0194] In other embodiments, chemical modification of the siRNA comprises attaching a conjugate to the siRNA molecule. The conjugate can be attached at the 5' and/or 3'-end of the sense and/or antisense strand of the siRNA via a covalent attachment such as, *e.g.*, a biodegradable linker. The conjugate can also be attached to the siRNA, *e.g.*, through a carbamate group or other linking group (*see, e.g.*, U.S. Patent Publication Nos. 20050074771, 20050043219, and 20050158727). In certain instances, the conjugate is a molecule that facilitates the delivery of the siRNA into a cell. Examples of conjugate molecules suitable for attachment to siRNA include, without limitation, steroids such as cholesterol, glycols such as polyethylene glycol (PEG), human serum albumin (HSA), fatty acids, carotenoids, terpenes, bile acids, folates (*e.g.*, folic acid, folate analogs and derivatives thereof), sugars (*e.g.*, galactose, galactosamine, N-acetyl galactosamine, glucose, mannose, fructose, fucose, *etc.*), phospholipids, peptides, ligands for cellular receptors capable of mediating cellular uptake, and combinations thereof (*see, e.g.*, U.S. Patent Publication Nos. 20030130186, 20040110296, and 20040249178; U.S. Patent No. 6,753,423). Other examples include the lipophilic moiety, vitamin, polymer, peptide, protein, nucleic acid, small molecule, oligosaccharide, carbohydrate cluster, intercalator, minor groove binder, cleaving agent, and cross-linking agent conjugate molecules described in U.S. Patent Publication Nos. 20050119470 and 20050107325. Yet other examples include the 2'-O-alkyl amine, 2'-O-alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidininium group, cationic amino acid conjugate molecules described in U.S. Patent Publication No. 20050153337. Additional examples include the hydrophobic group, membrane active compound, cell penetrating compound, cell targeting signal, interaction modifier, and steric stabilizer conjugate molecules described in U.S. Patent Publication No. 20040167090. Further examples include the conjugate molecules described in U.S. Patent Publication No. 20050239739. The type of conjugate used and the extent of

conjugation to the siRNA molecule can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of the siRNA while retaining RNAi activity. As such, one skilled in the art can screen siRNA molecules having various conjugates attached thereto to identify ones having improved properties and full RNAi activity using any of a variety of well-known *in vitro* cell culture or *in vivo* animal models. The disclosures of the above-described patent documents are herein incorporated by reference in their entirety for all purposes.

d. Target Genes

[0195] The siRNA component of the nucleic acid-lipid particles described herein can be used to downregulate or silence the translation (*i.e.*, expression) of a gene of interest. Genes of interest include, but are not limited to, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders (*e.g.*, liver diseases and disorders), genes associated with tumorigenesis and cell transformation (*e.g.*, cancer), angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0196] Genes associated with viral infection and survival include those expressed by a virus in order to bind, enter, and replicate in a cell. Of particular interest are viral sequences associated with chronic viral diseases. Viral sequences of particular interest include sequences of Filoviruses such as Ebola virus and Marburg virus (*see, e.g.*, Geisbert *et al.*, *J. Infect. Dis.*, 193:1650-1657 (2006)); Arenaviruses such as Lassa virus, Junin virus, Machupo virus, Guanarito virus, and Sabia virus (Buchmeier *et al.*, *Arenaviridae*: the viruses and their replication, In: FIELDS VIROLOGY, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia, (2001)); Influenza viruses such as Influenza A, B, and C viruses, (*see, e.g.*, Steinhauer *et al.*, *Annu Rev Genet.*, 36:305-332 (2002); and Neumann *et al.*, *J Gen Virol.*, 83:2635-2662 (2002)); Hepatitis viruses (*see, e.g.*, Hamasaki *et al.*, *FEBS Lett.*, 543:51 (2003); Yokota *et al.*, *EMBO Rep.*, 4:602 (2003); Schlomai *et al.*, *Hepatology,* 37:764 (2003); Wilson *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2783 (2003); Kapadia *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:2014 (2003); and FIELDS VIROLOGY, Knipe *et al.* (eds.), 4th ed., Lippincott-Raven, Philadelphia (2001)); Human Immunodeficiency Virus (HIV) (Banerjea *et al.*, *Mol. Ther.*, 8:62 (2003); Song *et al.*, *J. Virol.*, 77:7174 (2003); Stephenson, *JAMA,* 289:1494 (2003); Qin *et al.*, *Proc. Natl. Acad. Sci. USA*, 100:183 (2003)); Herpes viruses (Jia *et al.*, *J. Virol.*, 77:3301 (2003)); and Human Papilloma Viruses (HPV) (Hall *et al.*, *J. Virol.*, 77:6066 (2003); Jiang *et al.*, *Oncogene,* 21:6041 (2002)).

[0197] Exemplary Filovirus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding structural proteins (*e.g.*, VP30, VP35, nucleoprotein (NP), polymerase protein (L-pol)) and membrane-associated proteins (*e.g.*, VP40, glycoprotein (GP), VP24). Complete genome sequences for Ebola virus are set forth in, *e.g.*, Genbank Accession Nos. NC_002549; AY769362; NC_006432; NC_004161; AY729654; AY354458; AY142960; AB050936; AF522874; AF499101; AF272001; and AF086833. Ebola virus VP24 sequences are set forth in, *e.g.*, Genbank Accession Nos. U77385 and AY058897. Ebola virus L-pol sequences are set forth in, *e.g.*, Genbank Accession No. X67110. Ebola virus VP40 sequences are set forth in, *e.g.*, Genbank Accession No. AY058896. Ebola virus NP sequences are set forth in, *e.g.*, Genbank Accession No. AY058895. Ebola virus GP sequences are set forth in, *e.g.*, Genbank Accession No. AY058898; Sanchez *et al.*, *Virus Res.*, 29:215-240 (1993); Will *et al.*, *J. Virol.*, 67:1203-1210 (1993); Volchkov *et al.*, *FEBS Lett.*, 305:181-184 (1992); and U.S. Patent No. 6,713,069. Additional Ebola virus sequences are set forth in, *e.g.*, Genbank Accession Nos. L11365 and X61274. Complete genome sequences for Marburg virus are set forth in, *e.g.*, Genbank Accession Nos. NC_001608; AY430365; AY430366; and AY358025. Marburg virus GP sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005734; AF005733; and AF005732. Marburg virus VP35 sequences are set forth in, *e.g.*, Genbank Accession Nos. AF005731 and AF005730. Additional Marburg virus sequences are set forth in, *e.g.*, Genbank Accession Nos. X64406; Z29337; AF005735; and Z12132. Non-limiting examples of siRNA molecules targeting Ebola virus and Marburg virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070135370, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0198] Exemplary Influenza virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences encoding nucleoprotein (NP), matrix proteins (M1 and M2), nonstructural proteins (NS1 and NS2), RNA polymerase (PA, PB1, PB2), neuraminidase (NA), and haemagglutinin (HA). Influenza A NP sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004522; AY818138; AB166863; AB188817; AB189046; AB189054; AB189062; AY646169; AY646177; AY651486; AY651493; AY651494; AY651495; AY651496; AY651497; AY651498; AY651499; AY651500; AY651501; AY651502; AY651503; AY651504; AY651505; AY651506; AY651507; AY651509; AY651528; AY770996; AY790308; AY818138; and AY818140. Influenza A PA sequences are set forth in, *e.g.*, Genbank Accession Nos. AY818132; AY790280; AY646171; AY818132;AY818133; AY646179; AY818134; AY551934; AY651613; AY651610;

AY651620; AY651617; AY651600; AY651611; AY651606; AY651618; AY651608; AY651607; AY651605; AY651609; AY651615; AY651616; AY651640; AY651614; AY651612; AY651621; AY651619; AY770995; and AY724786. Non-limiting examples of siRNA molecules targeting Influenza virus nucleic acid sequences include those described in U.S. Patent Publication No. 20070218122, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0199] Exemplary hepatitis virus nucleic acid sequences that can be silenced include, but are not limited to, nucleic acid sequences involved in transcription and translation (*e.g.*, En1, En2, X, P) and nucleic acid sequences encoding structural proteins (*e.g.*, core proteins including C and C-related proteins, capsid and envelope proteins including S, M, and/or L proteins, or fragments thereof) (*see, e.g.*, FIELDS VIROLOGY, *supra*). Exemplary Hepatits C virus (HCV) nucleic acid sequences that can be silenced include, but are not limited to, the 5'-untranslated region (5'-UTR), the 3'-untranslated region (3'-UTR), the polyprotein translation initiation codon region, the internal ribosome entry site (IRES) sequence, and/or nucleic acid sequences encoding the core protein, the E1 protein, the E2 protein, the p7 protein, the NS2 protein, the NS3 protease/helicase, the NS4A protein, the NS4B protein, the NS5A protein, and/or the NS5B RNA-dependent RNA polymerase. HCV genome sequences are set forth in, *e.g.*, Genbank Accession Nos. NC_004102 (HCV genotype 1a), AJ238799 (HCV genotype 1b), NC_009823 (HCV genotype 2), NC_009824 (HCV genotype 3), NC_009825 (HCV genotype 4), NC_009826 (HCV genotype 5), and NC_009827 (HCV genotype 6). Hepatitis A virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001489; Hepatitis B virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_003977; Hepatitis D virus nucleic acid sequence are set forth in, *e.g.*, Genbank Accession No. NC_001653; Hepatitis E virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001434; and Hepatitis G virus nucleic acid sequences are set forth in, *e.g.*, Genbank Accession No. NC_001710. Silencing of sequences that encode genes associated with viral infection and survival can conveniently be used in combination with the administration of conventional agents used to treat the viral condition. Non-limiting examples of siRNA molecules targeting hepatitis virus nucleic acid sequences include those described in U.S. Patent Publication Nos. 20060281175, 20050058982, and 20070149470; U.S. Patent No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed March 20, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0200] Genes associated with metabolic diseases and disorders (*e.g.*, disorders in which the liver is the target and liver diseases and disorders) include, for example, genes expressed in dyslipidemia (*e.g.*, liver X receptors such as LXRα and LXRβ (Genback Accession No. NM_007121), farnesoid X receptors (FXR) (Genbank Accession No. NM_005123), sterol-regulatory element binding protein (SREBP), site-1 protease (S1P), 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG coenzyme-A reductase), apolipoprotein B (ApoB) (Genbank Accession No. NM_000384), apolipoprotein CIII (ApoC3) (Genbank Accession Nos. NM_000040 and NG_008949 REGION: 5001..8164), and apolipoprotein E (ApoE) (Genbank Accession Nos. NM_000041 and NG_007084 REGION: 5001..8612)); and diabetes (*e.g.*, glucose 6-phosphatase) (*see*, *e.g.*, Forman *et al.*, *Cell*, 81:687 (1995); Seol *et al.*, *Mol. Endocrinol.*, 9:72 (1995), Zavacki *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:7909 (1997); Sakai *et al.*, *Cell*, 85:1037-1046 (1996); Duncan *et al.*, *J. Biol. Chem.*, 272:12778-12785 (1997); Willy *et al.*, *Genes Dev.*, 9:1033-1045 (1995); Lehmann *et al.*, *J. Biol. Chem.*, 272:3137-3140 (1997); Janowski *et al.*, *Nature*, 383:728-731 (1996); and Peet *et al.*, *Cell*, 93:693-704 (1998)). One of skill in the art will appreciate that genes associated with metabolic diseases and disorders (*e.g.*, diseases and disorders in which the liver is a target and liver diseases and disorders) include genes that are expressed in the liver itself as well as and genes expressed in other organs and tissues. Silencing of sequences that encode genes associated with metabolic diseases and disorders can conveniently be used in combination with the administration of conventional agents used to treat the disease or disorder. Non-limiting examples of siRNA molecules targeting the ApoB gene include those described in U.S. Patent Publication No. 20060134189, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the ApoC3 gene include those described in U.S. Provisional Application No. 61/147,235, filed January 26, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0201] Examples of gene sequences associated with tumorigenesis and cell transformation (*e.g.*, cancer or other neoplasia) include mitotic kinesins such as Eg5 (KSP, KIF11; Genbank Accession No. NM_004523); serine/threonine kinases such as polo-like kinase 1 (PLK-1) (Genbank Accession No. NM_005030; Barr *et al.*, *Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)); tyrosine kinases such as WEE1 (Genbank Accession Nos. NM_003390 and NM_001143976); inhibitors of apoptosis such as XIAP (Genbank Accession No. NM_001167); COP9 signalosome subunits such as CSN1, CSN2, CSN3, CSN4, CSN5

(JAB1; Genbank Accession No. NM_006837); CSN6, CSN7A, CSN7B, and CSN8; ubiquitin ligases such as COP1 (RFWD2; Genbank Accession Nos. NM_022457 and NM_001001740); and histone deacetylases such as HDAC1, HDAC2 (Genbank Accession No. NM_001527), HDAC3, HDAC4, HDAC5, HDAC6, HDAC7, HDAC8, HDAC9, *etc.*

Non-limiting examples of siRNA molecules targeting the Eg5 and XIAP genes include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Non-limiting examples of siRNA molecules targeting the PLK-1 gene include those described in U.S. Patent Publication Nos. 20050107316 and 20070265438; and U.S. Patent Application No. 12/343,342, filed December 23, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes. Non-limiting examples of siRNA molecules targeting the CSN5 gene include those described in U.S. Provisional Application No. 61/045,251, filed April 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0202] Additional examples of gene sequences associated with tumorigenesis and cell transformation include translocation sequences such as MLL fusion genes, BCR-ABL (Wilda *et al.*, *Oncogene*, 21:5716 (2002); Scherr *et al.*, *Blood,* 101:1566 (2003)), TEL-AML1, EWS-FLI1, TLS-FUS, PAX3-FKHR, BCL-2, AML1-ETO, and AML1-MTG8 (Heidenreich *et al.*, *Blood,* 101:3157 (2003)); overexpressed sequences such as multidrug resistance genes (Nieth *et al.*, *FEBS Lett.*, 545:144 (2003); Wu *et al*, *Cancer Res.* 63:1515 (2003)), cyclins (Li *et al.*, *Cancer Res.*, 63:3593 (2003); Zou *et al.*, *Genes Dev.,* 16:2923 (2002)), beta-catenin (Verma *et al.*, *Clin Cancer Res.*, 9:1291 (2003)), telomerase genes (Kosciolek *et al.*, *Mol Cancer Ther.*, 2:209 (2003)), c-MYC, N-MYC, BCL-2, growth factor receptors (*e.g.,* EGFR/ErbB1 (Genbank Accession Nos. NM_005228, NM_201282, NM_201283, and NM_201284; *see also,* Nagy *et al. Exp. Cell Res.,* 285:39-49 (2003), ErbB2/HER-2 (Genbank Accession Nos. NM_004448 and NM_001005862), ErbB3 (Genbank Accession Nos. NM_001982 and NM_001005915), and ErbB4 (Genbank Accession Nos. NM_005235 and NM_001042599); and mutated sequences such as RAS (reviewed in Tuschl and Borkhardt, *Mol. Interventions*, 2:158 (2002)). Non-limiting examples of siRNA molecules targeting the EGFR gene include those described in U.S. Patent Application No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0203] Silencing of sequences that encode DNA repair enzymes find use in combination with the administration of chemotherapeutic agents (Collis *et al.*, *Cancer Res.*, 63:1550 (2003)). Genes encoding proteins associated with tumor migration are also target sequences

of interest, for example, integrins, selectins, and metalloproteinases. The foregoing examples are not exclusive. Those of skill in the art will understand that any whole or partial gene sequence that facilitates or promotes tumorigenesis or cell transformation, tumor growth, or tumor migration can be included as a template sequence.

[0204] Angiogenic genes are able to promote the formation of new vessels. Of particular interest is vascular endothelial growth factor (VEGF) (Reich *et al.*, *Mol. Vis.*, 9:210 (2003)) or VEGFR. siRNA sequences that target VEGFR are set forth in, *e.g.*, GB 2396864; U.S. Patent Publication No. 20040142895; and CA 2456444, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0205] Anti-angiogenic genes are able to inhibit neovascularization. These genes are particularly useful for treating those cancers in which angiogenesis plays a role in the pathological development of the disease. Examples of anti-angiogenic genes include, but are not limited to, endostatin (*see, e.g.,* U.S. Patent No. 6,174,861), angiostatin (*see, e.g.*, U.S. Patent No. 5,639,725), and VEGFR2 (*see, e.g.,* Decaussin *et al.*, *J. Pathol.*, 188: 369-377 (1999)), the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0206] Immunomodulator genes are genes that modulate one or more immune responses. Examples of immunomodulator genes include, without limitation, cytokines such as growth factors (*e.g.*, TGF-α, TGF-□β, EGF, FGF, IGF, NGF, PDGF, CGF, GM-CSF, SCF, *etc.*), interleukins (*e.g.*, IL-2, IL-4, IL-12 (Hill *et al.*, *J. Immunol.*, 171:691 (2003)), IL-15, IL-18, IL-20, *etc.*), interferons (*e.g.*, IFN-α, IFN-β, IFN-γ, *etc.*) and TNF. Fas and Fas ligand genes are also immunomodulator target sequences of interest (Song *et al.*, *Nat. Med.*, 9:347 (2003)). Genes encoding secondary signaling molecules in hematopoietic and lymphoid cells are also included in the present invention, for example, Tec family kinases such as Bruton's tyrosine kinase (Btk) (Heinonen *et al.*, *FEBS Lett.*, 527:274 (2002)).

[0207] Cell receptor ligands include ligands that are able to bind to cell surface receptors (*e.g.*, insulin receptor, EPO receptor, G-protein coupled receptors, receptors with tyrosine kinase activity, cytokine receptors, growth factor receptors, *etc.*), to modulate (*e.g.*, inhibit, activate, *etc.*) the physiological pathway that the receptor is involved in (*e.g.*, glucose level modulation, blood cell development, mitogenesis, *etc.*). Examples of cell receptor ligands include, but are not limited to, cytokines, growth factors, interleukins, interferons, erythropoietin (EPO), insulin, glucagon, G-protein coupled receptor ligands, *etc.* Templates coding for an expansion of trinucleotide repeats (*e.g.*, CAG repeats) find use in silencing pathogenic sequences in neurodegenerative disorders caused by the expansion of

trinucleotide repeats, such as spinobulbular muscular atrophy and Huntington's Disease (Caplen *et al.*, *Hum. Mol. Genet.*, 11:175 (2002)).

[0208] In addition to its utility in silencing the expression of any of the above-described genes for therapeutic purposes, the siRNA described herein are also useful in research and development applications as well as diagnostic, prophylactic, prognostic, clinical, and other healthcare applications. As a non-limiting example, the siRNA can be used in target validation studies directed at testing whether a gene of interest has the potential to be a therapeutic target. The siRNA can also be used in target identification studies aimed at discovering genes as potential therapeutic targets.

2. aiRNA

[0209] Like siRNA, asymmetrical interfering RNA (aiRNA) can recruit the RNA-induced silencing complex (RISC) and lead to effective silencing of a variety of genes in mammalian cells by mediating sequence-specific cleavage of the target sequence between nucleotide 10 and 11 relative to the 5' end of the antisense strand (Sun *et al.*, *Nat. Biotech.*, 26:1379-1382 (2008)). Typically, an aiRNA molecule comprises a short RNA duplex having a sense strand and an antisense strand, wherein the duplex contains overhangs at the 3' and 5' ends of the antisense strand. The aiRNA is generally asymmetric because the sense strand is shorter on both ends when compared to the complementary antisense strand. In some aspects, aiRNA molecules may be designed, synthesized, and annealed under conditions similar to those used for siRNA molecules. As a non-limiting example, aiRNA sequences may be selected and generated using the methods described above for selecting siRNA sequences.

[0210] In another embodiment, aiRNA duplexes of various lengths (*e.g.*, about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 base pairs, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 base pairs) may be designed with overhangs at the 3' and 5' ends of the antisense strand to target an mRNA of interest. In certain instances, the sense strand of the aiRNA molecule is about 10-25, 12-20, 12-19, 12-18, 13-17, or 14-17 nucleotides in length, more typically 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length. In certain other instances, the antisense strand of the aiRNA molecule is about 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and is preferably about 20-24, 21-22, or 21-23 nucleotides in length.

[0211] In some embodiments, the 5' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*, "AA", "UU", "dTdT", *etc.*). In other embodiments, the 3' antisense overhang contains one, two, three, four, or more nontargeting nucleotides (*e.g.*,

"AA", "UU", "dTdT", *etc.*). In certain aspects, the aiRNA molecules described herein may comprise one or more modified nucleotides, *e.g.,* in the double-stranded (duplex) region and/or in the antisense overhangs. As a non-limiting example, aiRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the aiRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0212] In certain embodiments, aiRNA molecules may comprise an antisense strand which corresponds to the antisense strand of an siRNA molecule, *e.g.,* one of the siRNA molecules described herein. In other embodiments, aiRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.,* genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

3. miRNA

[0213] Generally, microRNAs (miRNA) are single-stranded RNA molecules of about 21-23 nucleotides in length which regulate gene expression. miRNAs are encoded by genes from whose DNA they are transcribed, but miRNAs are not translated into protein (non-coding RNA); instead, each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional mature miRNA. Mature miRNA molecules are either partially or completely complementary to one or more messenger RNA (mRNA) molecules, and their main function is to downregulate gene expression. The identification of miRNA molecules is described, *e.g.,* in Lagos-Quintana *et al.*, *Science*, 294:853-858; Lau *et al.*, *Science*, 294:858-862; and Lee *et al.*, *Science*, 294:862-864.

[0214] The genes encoding miRNA are much longer than the processed mature miRNA molecule. miRNA are first transcribed as primary transcripts or pri-miRNA with a cap and poly-A tail and processed to short, ~70-nucleotide stem-loop structures known as pre-miRNA in the cell nucleus. This processing is performed in animals by a protein complex known as the Microprocessor complex, consisting of the nuclease Drosha and the double-stranded RNA binding protein Pasha (Denli *et al.*, *Nature*, 432:231-235 (2004)). These pre-miRNA are then processed to mature miRNA in the cytoplasm by interaction with the endonuclease Dicer, which also initiates the formation of the RNA-induced silencing complex (RISC) (Bernstein

et al., *Nature*, 409:363-366 (2001). Either the sense strand or antisense strand of DNA can function as templates to give rise to miRNA.

[0215] When Dicer cleaves the pre-miRNA stem-loop, two complementary short RNA molecules are formed, but only one is integrated into the RISC complex. This strand is known as the guide strand and is selected by the argonaute protein, the catalytically active RNase in the RISC complex, on the basis of the stability of the 5' end (Preall *et al.*, *Curr. Biol.*, 16:530-535 (2006)). The remaining strand, known as the anti-guide or passenger strand, is degraded as a RISC complex substrate (Gregory *et al.*, *Cell*, 123:631-640 (2005)). After integration into the active RISC complex, miRNAs base pair with their complementary mRNA molecules and induce target mRNA degradation and/or translational silencing.

[0216] Mammalian miRNA molecules are usually complementary to a site in the 3' UTR of the target mRNA sequence. In certain instances, the annealing of the miRNA to the target mRNA inhibits protein translation by blocking the protein translation machinery. In certain other instances, the annealing of the miRNA to the target mRNA facilitates the cleavage and degradation of the target mRNA through a process similar to RNA interference (RNAi). miRNA may also target methylation of genomic sites which correspond to targeted mRNA. Generally, miRNA function in association with a complement of proteins collectively termed the miRNP.

[0217] In certain aspects, the miRNA molecules described herein are about 15-100, 15-90, 15-80, 15-75, 15-70, 15-60, 15-50, or 15-40 nucleotides in length, more typically about 15-30, 15-25, or 19-25 nucleotides in length, and are preferably about 20-24, 21-22, or 21-23 nucleotides in length. In certain other aspects, miRNA molecules may comprise one or more modified nucleotides. As a non-limiting example, miRNA sequences may comprise one or more of the modified nucleotides described above for siRNA sequences. In a preferred embodiment, the miRNA molecule comprises 2'OMe nucleotides such as, for example, 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, or mixtures thereof.

[0218] In some embodiments, miRNA molecules may be used to silence the expression of any of the target genes set forth above, such as, *e.g.*, genes associated with viral infection and survival, genes associated with metabolic diseases and disorders, genes associated with tumorigenesis and cell transformation, angiogenic genes, immunomodulator genes such as those associated with inflammatory and autoimmune responses, ligand receptor genes, and genes associated with neurodegenerative disorders.

[0219] In other embodiments, one or more agents that block the activity of a miRNA targeting an mRNA of interest are administered using a lipid particle of the invention (*e.g.*, a

nucleic acid-lipid particle). Examples of blocking agents include, but are not limited to, steric blocking oligonucleotides, locked nucleic acid oligonucleotides, and Morpholino oligonucleotides. Such blocking agents may bind directly to the miRNA or to the miRNA binding site on the target mRNA.

4. Antisense Oligonucleotides

[0220] In one embodiment, the nucleic acid is an antisense oligonucleotide directed to a target gene or sequence of interest. The terms "antisense oligonucleotide" or "antisense" include oligonucleotides that are complementary to a targeted polynucleotide sequence. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence. Antisense RNA oligonucleotides prevent the translation of complementary RNA strands by binding to the RNA. Antisense DNA oligonucleotides can be used to target a specific, complementary (coding or non-coding) RNA. If binding occurs, this DNA/RNA hybrid can be degraded by the enzyme RNase H. In a particular embodiment, antisense oligonucleotides comprise from about 10 to about 60 nucleotides, more preferably from about 15 to about 30 nucleotides. The term also encompasses antisense oligonucleotides that may not be exactly complementary to the desired target gene. Thus, the invention can be utilized in instances where non-target specific-activities are found with antisense, or where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use.

[0221] Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein synthesis by a targeted gene. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (*see,* U.S. Patent Nos. 5,739,119 and 5,759,829). Furthermore, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDR1), ICAM-1, E-selectin, STK-1, striatal GABAA receptor, and human EGF (*see,* Jaskulski *et al.*, *Science*, 240:1544-6 (1988); Vasanthakumar *et al.*, *Cancer Commun.*, 1:225-32 (1989); Peris *et al.*, *Brain Res Mol Brain Res.*, 15;57:310-20 (1998); and U.S. Patent Nos. 5,801,154; 5,789,573; 5,718,709 and 5,610,288). Moreover, antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.,* cancer (*see,* U.S. Patent Nos.

5,747,470; 5,591,317; and 5,783,683). The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0222] Methods of producing antisense oligonucleotides are known in the art and can be readily adapted to produce an antisense oligonucleotide that targets any polynucleotide sequence. Selection of antisense oligonucleotide sequences specific for a given target sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense oligonucleotides may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.*, *Nucleic Acids Res.*, 25:3389-402 (1997)).

5. Ribozymes

[0223] According to another embodiment of the invention, nucleic acid-lipid particles are associated with ribozymes. Ribozymes are RNA-protein complexes having specific catalytic domains that possess endonuclease activity (*see,* Kim *et al.*, *Proc. Natl. Acad. Sci. USA.*, 84:8788-92 (1987); and Forster *et al.*, *Cell*, 49:211-20 (1987)). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (*see,* Cech *et al.*, *Cell*, 27:487-96 (1981); Michel *et al.*, *J. Mol. Biol.*, 216:585-610 (1990); Reinhold-Hurek *et al.*, *Nature*, 357:173-6 (1992)). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

[0224] At least six basic varieties of naturally-occurring enzymatic RNA molecules are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-

pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

[0225] The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence), or Neurospora VS RNA motif, for example. Specific examples of hammerhead motifs are described in, *e.g.,* Rossi *et al.*, *Nucleic Acids Res.*, 20:4559-65 (1992). Examples of hairpin motifs are described in, *e.g.,* EP 0360257, Hampel *et al.*, *Biochemistry*, 28:4929-33 (1989); Hampel *et al.*, *Nucleic Acids Res.*, 18:299-304 (1990); and U.S. Patent No. 5,631,359. An example of the hepatitis δ virus motif is described in, *e.g.,* Perrotta *et al.*, *Biochemistry*, 31:11843-52 (1992). An example of the RNaseP motif is described in, *e.g.,* Guerrier-Takada *et al.*, *Cell*, 35:849-57 (1983). Examples of the Neurospora VS RNA ribozyme motif is described in, *e.g.,* Saville *et al.*, *Cell*, 61:685-96 (1990); Saville *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:8826-30 (1991); Collins *et al.*, *Biochemistry*, 32:2795-9 (1993). An example of the Group I intron is described in, *e.g.,* U.S. Patent No. 4,987,071. Important characteristics of enzymatic nucleic acid molecules used according to the invention are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus, the ribozyme constructs need not be limited to specific motifs mentioned herein. The disclosures of these references are herein incorporated by reference in their entirety for all purposes.

[0226] Methods of producing a ribozyme targeted to any polynucleotide sequence are known in the art. Ribozymes may be designed as described in, *e.g.,* PCT Publication Nos. WO 93/23569 and WO 94/02595, and synthesized to be tested *in vitro* and/or *in vivo* as described therein. The disclosures of these PCT publications are herein incorporated by reference in their entirety for all purposes.

[0227] Ribozyme activity can be optimized by altering the length of the ribozyme binding arms or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (*see, e.g.,* PCT Publication Nos. WO 92/07065, WO 93/15187, WO 91/03162, and WO 94/13688; EP 92110298.4; and U.S. Patent No. 5,334,711, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules, the disclosures of which are each herein incorporated by reference in their entirety

for all purposes), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

6. Immunostimulatory Oligonucleotides

[0228] Nucleic acids associated with lipid paticles of the present invention may be immunostimulatory, including immunostimulatory oligonucleotides (ISS; single-or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal such as a human. ISS include, *e.g.,* certain palindromes leading to hairpin secondary structures (*see,* Yamamoto *et al.*, *J. Immunol.*, 148:4072-6 (1992)), or CpG motifs, as well as other known ISS features (such as multi-G domains; *see;* PCT Publication No. WO 96/11266, the disclosure of which is herein incorporated by reference in its entirety for all purposes).

[0229] Immunostimulatory nucleic acids are considered to be non-sequence specific when it is not required that they specifically bind to and reduce the expression of a target sequence in order to provoke an immune response. Thus, certain immunostimulatory nucleic acids may comprise a sequence corresponding to a region of a naturally-occurring gene or mRNA, but they may still be considered non-sequence specific immunostimulatory nucleic acids.

[0230] In one embodiment, the immunostimulatory nucleic acid or oligonucleotide comprises at least one CpG dinucleotide. The oligonucleotide or CpG dinucleotide may be unmethylated or methylated. In another embodiment, the immunostimulatory nucleic acid comprises at least one CpG dinucleotide having a methylated cytosine. In one embodiment, the nucleic acid comprises a single CpG dinucleotide, wherein the cytosine in the CpG dinucleotide is methylated. In an alternative embodiment, the nucleic acid comprises at least two CpG dinucleotides, wherein at least one cytosine in the CpG dinucleotides is methylated. In a further embodiment, each cytosine in the CpG dinucleotides present in the sequence is methylated. In another embodiment, the nucleic acid comprises a plurality of CpG dinucleotides, wherein at least one of the CpG dinucleotides comprises a methylated cytosine. Examples of immunostimulatory oligonucleotides suitable for use in the compositions and methods of the present invention are described in PCT Application No. PCT/US08/88676, filed December 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Patent No. 6,406,705, and Raney *et al.*, *J. Pharm. Exper. Ther.*, 298:1185-92 (2001), the disclosures of which are each herein incorporated by reference in their entirety for all purposes. In certain embodiments, the oligonucleotides used in the compositions and

methods of the invention have a phosphodiester ("PO") backbone or a phosphorothioate ("PS") backbone, and/or at least one methylated cytosine residue in a CpG motif.

B. Other Active Agents

[0231] In certain embodiments, the active agent associated with the lipid paticles of the invention may comprise one or more therapeutic proteins, polypeptides, or small organic molecules or compounds. Non-limiting examples of such therapeutically effective agents or drugs include oncology drugs (*e.g.,* chemotherapy drugs, hormonal therapaeutic agents, immunotherapeutic agents, radiotherapeutic agents, *etc.*), lipid-lowering agents, anti-viral drugs, anti-inflammatory compounds, antidepressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs such as anti-arrhythmic agents, hormones, vasoconstrictors, and steroids. These active agents may be administered alone in the lipid particles of the invention, or in combination (*e.g.,* co-administered) with lipid particles of the invention comprising nucleic acid such as interfering RNA.

[0232] Non-limiting examples of chemotherapy drugs include platinum-based drugs (*e.g.,* oxaliplatin, cisplatin, carboplatin, spiroplatin, iproplatin, satraplatin, *etc.*), alkylating agents (*e.g.,* cyclophosphamide, ifosfamide, chlorambucil, busulfan, melphalan, mechlorethamine, uramustine, thiotepa, nitrosoureas, *etc.*), anti-metabolites (*e.g.,* 5-fluorouracil (5-FU), azathioprine, methotrexate, leucovorin, capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, pemetrexed, raltitrexed, *etc.*), plant alkaloids (*e.g.,* vincristine, vinblastine, vinorelbine, vindesine, podophyllotoxin, paclitaxel (taxol), docetaxel, *etc.*), topoisomerase inhibitors (*e.g.,* irinotecan (CPT-11; Camptosar), topotecan, amsacrine, etoposide (VP16), etoposide phosphate, teniposide, *etc.*), antitumor antibiotics (*e.g.,* doxorubicin, adriamycin, daunorubicin, epirubicin, actinomycin, bleomycin, mitomycin, mitoxantrone, plicamycin, *etc.*), tyrosine kinase inhibitors (*e.g.,* gefitinib (Iressa®), sunitinib (Sutent®; SU11248), erlotinib (Tarceva®; OSI-1774), lapatinib (GW572016; GW2016), canertinib (CI 1033), semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), imatinib (Gleevec®; STI571), dasatinib (BMS-354825), leflunomide (SU101), vandetanib (Zactima™; ZD6474), *etc.*), pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and combinations thereof.

[0233] Examples of conventional hormonal therapaeutic agents include, without limitation, steroids (*e.g.,* dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

[0234] Examples of conventional immunotherapeutic agents include, but are not limited to, immunostimulants (*e.g.*, Bacillus Calmette-Guérin (BCG), levamisole, interleukin-2, alpha-interferon, *etc.*), monoclonal antibodies (*e.g.*, anti-CD20, anti-HER2, anti-CD52, anti-HLA-DR, and anti-VEGF monoclonal antibodies), immunotoxins (*e.g.*, anti-CD33 monoclonal antibody-calicheamicin conjugate, anti-CD22 monoclonal antibody-pseudomonas exotoxin conjugate, *etc.*), and radioimmunotherapy (*e.g.*, anti-CD20 monoclonal antibody conjugated to ^{111}In, ^{90}Y, or ^{131}I, *etc.*).

[0235] Examples of conventional radiotherapeutic agents include, but are not limited to, radionuclides such as ^{47}Sc, ^{64}Cu, ^{67}Cu, ^{89}Sr, ^{86}Y, ^{87}Y, ^{90}Y, ^{105}Rh, ^{111}Ag, ^{111}In, ^{117m}Sn, ^{149}Pm, ^{153}Sm, ^{166}Ho, ^{177}Lu, ^{186}Re, ^{188}Re, ^{211}At, and ^{212}Bi, optionally conjugated to antibodies directed against tumor antigens.

[0236] Additional oncology drugs that may be used according to the invention include, but are not limited to, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, bexarotene, biCNU, carmustine, CCNU, celecoxib, cladribine, cyclosporin A, cytosine arabinoside, cytoxan, dexrazoxane, DTIC, estramustine, exemestane, FK506, gemtuzumab-ozogamicin, hydrea, hydroxyurea, idarubicin, interferon, letrozole, leustatin, leuprolide, litretinoin, megastrol, L-PAM, mesna, methoxsalen, mithramycin, nitrogen mustard, pamidronate, Pegademase, pentostatin, porfimer sodium, prednisone, rituxan, streptozocin, STI-571, taxotere, temozolamide, VM-26, toremifene, tretinoin, ATRA, valrubicin, and velban. Other examples of oncology drugs that may be used according to the invention are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors, and camptothecins.

[0237] Non-limiting examples of lipid-lowering agents for treating a lipid disease or disorder associated with elevated triglycerides, cholesterol, and/or glucose include statins, fibrates, ezetimibe, thiazolidinediones, niacin, beta-blockers, nitroglycerin, calcium antagonists, fish oil, and mixtures thereof.

[0238] Examples of anti-viral drugs include, but are not limited to, abacavir, aciclovir, acyclovir, adefovir, amantadine, amprenavir, arbidol, atazanavir, atripla, cidofovir, combivir, darunavir, delavirdine, didanosine, docosanol, edoxudine, efavirenz, emtricitabine, enfuvirtide, entecavir, entry inhibitors, famciclovir, fixed dose combinations, fomivirsen, fosamprenavir, foscarnet, fosfonet, fusion inhibitors, ganciclovir, ibacitabine, imunovir, idoxuridine, imiquimod, indinavir, inosine, integrase inhibitors, interferon type III (*e.g.*, IFN-λ molecules such as IFN-λ1, IFN-λ2, and IFN-λ3), interferon type II (*e.g.*, IFN-γ), interferon type I (*e.g.*, IFN-α such as PEGylated IFN-α, IFN-β, IFN-κ, IFN-δ, IFN-ε, IFN-τ, IFN-ω, and

IFN-ζ), interferon, lamivudine, lopinavir, loviride, MK-0518, maraviroc, moroxydine, nelfinavir, nevirapine, nexavir, nucleoside analogues, oseltamivir, penciclovir, peramivir, pleconaril, podophyllotoxin, protease inhibitors, reverse transcriptase inhibitors, ribavirin, rimantadine, ritonavir, saquinavir, stavudine, synergistic enhancers, tenofovir, tenofovir disoproxil, tipranavir, trifluridine, trizivir, tromantadine, truvada, valaciclovir, valganciclovir, vicriviroc, vidarabine, viramidine, zalcitabine, zanamivir, zidovudine, pharmaceutically acceptable salts thereof, stereoisomers thereof, derivatives thereof, analogs thereof, and mixtures thereof.

V. Lipid Particles

[0239] The lipid particles of the invention typically comprise an active agent or therapeutic agent, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the active agent or therapeutic agent is fully encapsulated within the lipid portion of the lipid particle such that the active agent or therapeutic agent in the lipid particle is resistant in aqueous solution to enzymatic degradation, *e.g.*, by a nuclease or protease. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans. The lipid particles of the invention typically have a mean diameter of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 to about 90 nm.

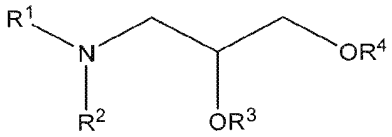
[0240] In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (*e.g.*, siRNA, aiRNA, and/or miRNA), a cationic lipid (*e.g.*, a cationic lipid of Formulas I, II, and/or III), a non-cationic lipid (*e.g.*, cholesterol alone or mixtures of one or more phospholipids and cholesterol), and a conjugated lipid that inhibits aggregation of the particles (*e.g.*, one or more PEG-lipid conjugates). The SNALP may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more unmodified and/or modified interfering RNA molecules. Nucleic acid-lipid particles and their method of preparation are described in, *e.g.*, U.S. Patent Nos. 5,753,613; 5,785,992; 5,705,385; 5,976,567; 5,981,501; 6,110,745; and 6,320,017; and PCT Publication No. WO 96/40964, the disclosures of which are each herein incorporated by reference in their entirety for all purposes.

A. Cationic Lipids

[0241] Any of a variety of cationic lipids may be used in the lipid particles of the invention (*e.g.*, SNALP), either alone or in combination with one or more other cationic lipid species or non-cationic lipid species.

[0242] Cationic lipids which are useful in the present invention can be any of a number of lipid species which carry a net positive charge at physiological pH. Such lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), 3 -(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol (DC-Chol), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA), dioctadecylamidoglycyl spermine (DOGS), 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane (CLinDMA), 2-[5'-(cholest-5-en-3.beta.-oxy)-3'-oxapentoxy)-3-dimethy-1-(cis,cis-9',1-2'-octadecadienoxy)propane (CpLinDMA), N,N-dimethyl-3,4-dioleyloxybenzylamine (DMOBA), 1,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-Dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), 1,2-Dilinoleoylcarbamyl-3-dimethylaminopropane (DLinCDAP), and mixtures thereof. A number of these lipids and related analogs have been described in U.S. Patent Publication Nos. 20060083780 and 20060240554; U.S. Patent Nos. 5,208,036; 5,264,618; 5,279,833; 5,283,185; 5,753,613; and 5,785,992; and PCT Publication No. WO 96/10390, the disclosures of which are each herein incorporated by reference in their entirety for all purposes. Additionally, a number of commercial preparations of cationic lipids are available and can be used in the present invention. These include, *e.g.*, LIPOFECTIN® (commercially available cationic liposomes comprising DOTMA and DOPE, from GIBCO/BRL, Grand Island, New York, USA); LIPOFECTAMINE® (commercially available cationic liposomes comprising DOSPA and DOPE, from GIBCO/BRL); and TRANSFECTAM® (commercially available cationic liposomes comprising DOGS from Promega Corp., Madison, Wisconsin, USA).

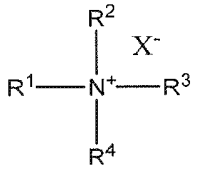
[0243] Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.



(I),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.*, R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.*, R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipid of Formula I is symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments, R^3 and R^4comprise at least three sites of unsaturation and are independently selected from, *e.g.*, dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl. In particularly preferred embodiments, the cationic lipid of Formula I is 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA) or 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA).

[0244] Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.

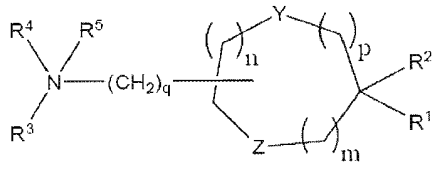


(II),

wherein R^1 and R^2 are independently selected and are H or C_1-C_3 alkyls, R^3 and R^4 are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R^3 and R^4 comprises at least two sites of unsaturation. In certain instances, R^3 and R^4 are both the same, *i.e.*, R^3 and R^4 are both linoleyl (C_{18}), *etc.* In certain other instances, R^3 and R^4 are different, *i.e.*, R^3 is tetradectrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodiment, the cationic lipids of the present invention are symmetrical, *i.e.*, R^3 and R^4 are both the same. In another preferred embodiment, both R^3 and R^4 comprise at least two sites of unsaturation. In some embodiments, R^3 and R^4 are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R^3 and R^4 are both linoleyl. In some embodiments,

R^3 and R^4comprise at least three sites of unsaturation and are independently selected from, *e.g.,* dodecatrienyl, tetradectrienyl, hexadecatrienyl, linolenyl, and icosatrienyl.

[0245] Moreover, cationic lipids of Formula III having the following structures (or salts thereof) are useful in the present invention.



(III),

Wherein R^1 and R^2 are either the same or different and independently optionally substituted C_{12}-C_{24} alkyl, optionally substituted C_{12}-C_{24} alkenyl, optionallysubstituted C_{12}-C_{24} alkynyl, or optionally substituted C_{12}-C_{24} acyl; R^3 and R^4 are either the same or different and independently optionally substituted C_1-C_6 alkyl, optionally substituted C_1-C_6 alkenyl, or optionally substituted C_1-C_6 alkynyl or R^3 and R^4 may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms chosen from nitrogen and oxygen; R^5 is either absent or hydrogen or C_1-C_6 alkyl to provide a quaternary amine; m, n, and p are either the same or different and independently either 0 or 1 with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; and Y and Z are either the same or different and independently O, S, or NH.

[0246] In some embodiments, the cationic lipid of Formula III is 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpepiazino-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanedio (DOAP), 1,2-dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), or mixtures

thereof. In preferred embodiments, the cationic lipid of Formula III is DLin-K-C2-DMA (XTC2).

[0247] The cationic lipid typically comprises from about 50 mol % to about 90 mol %, from about 50 mol % to about 85 mol %, from about 50 mol % to about 80 mol %, from about 50 mol % to about 75 mol %, from about 50 mol % to about 70 mol %, from about 50 mol % to about 65 mol %, or from about 55 mol % to about 65 mol % of the total lipid present in the particle.

[0248] It will be readily apparent to one of skill in the art that depending on the intended use of the particles, the proportions of the components can be varied and the delivery efficiency of a particular formulation can be measured using, *e.g.*, an endosomal release parameter (ERP) assay.

B. Non-Cationic Lipids

[0249] The non-cationic lipids used in the lipid particles of the invention (*e.g.*, SNALP) can be any of a variety of neutral uncharged, zwitterionic, or anionic lipids capable of producing a stable complex.

[0250] Non-limiting examples of non-cationic lipids include phospholipids such as lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleyol-phosphatidylglycerol (POPG), dioleoylphosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl-phosphatidylethanolamine (DPPE), dimyristoyl-phosphatidylethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, dielaidoyl-phosphatidylethanolamine (DEPE), stearoyloleoyl-phosphatidylethanolamine (SOPE), lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof. Other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C_{10}-C_{24} carbon chains, *e.g.*, lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl.

[0251] Additional examples of non-cationic lipids include sterols such as cholesterol and derivatives thereof such as cholestanol, cholestanone, cholestenone, coprostanol, cholesteryl-2'-hydroxyethyl ether, cholesteryl-4'-hydroxybutyl ether, and mixtures thereof.

[0252] In some embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of cholesterol or a derivative thereof, *e.g.*, a phospholipid-free lipid particle formulation. In other embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of one or more phospholipids, *e.g.*, a cholesterol-free lipid particle formulation. In further embodiments, the non-cationic lipid present in the lipid particles (*e.g.*, SNALP) comprises or consists of a mixture of one or more phospholipids and cholesterol or a derivative thereof.

[0253] Other examples of non-cationic lipids suitable for use in the present invention include nonphosphorous containing lipids such as, *e.g.*, stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerolricinoleate, hexadecyl stereate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

[0254] In some embodiments, the non-cationic lipid comprises from about 13 mol % to about 49.5 mol %, from about 20 mol % to about 45 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 45 mol %, from about 35 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 40 mol %, or from about 30 mol % to about 40 mol % of the total lipid present in the particle.

[0255] In certain embodiments, the cholesterol present in phospholipid-free lipid particles comprises from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, or from about 35 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a phospholipid-free lipid particle may comprise cholesterol at about 37 mol % of the total lipid present in the particle.

[0256] In certain other embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 30 mol % to about 40 mol %, from about 30 mol % to about 35 mol %, or from about 35 mol % to about 40 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 34 mol % of the total lipid present in the particle.

[0257] In further embodiments, the cholesterol present in lipid particles containing a mixture of phospholipid and cholesterol comprises from about 10 mol % to about 30 mol %,

from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise cholesterol at about 20 mol % of the total lipid present in the particle.

[0258] In embodiments where the lipid particles contain a mixture of phospholipid and cholesterol or a cholesterol derivative, the mixture may comprise up to about 40, 45, 50, 55, or 60 mol % of the total lipid present in the particle. In certain instances, the phospholipid component in the mixture may comprise from about 2 mol % to about 12 mol %, from about 4 mol % to about 10 mol %, from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, or from about 6 mol % to about 8 mol % of the total lipid present in the particle. As a non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (*e.g.*, in a mixture with about 34 mol % cholesterol) of the total lipid present in the particle. In certain other instances, the phospholipid component in the mixture may comprise from about 10 mol % to about 30 mol %, from about 15 mol % to about 25 mol %, or from about 17 mol % to about 23 mol % of the total lipid present in the particle. As another non-limiting example, a lipid particle comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 20 mol % (*e.g.*, in a mixture with about 20 mol % cholesterol) of the total lipid present in the particle.

C. Lipid Conjugate

[0259] In addition to cationic and non-cationic lipids, the lipid particles of the invention (*e.g.*, SNALP) comprise a lipid conjugate. The conjugated lipid is useful in that it prevents the aggregation of particles. Suitable conjugated lipids include, but are not limited to, PEG-lipid conjugates, ATTA-lipid conjugates, cationic-polymer-lipid conjugates (CPLs), and mixtures thereof. In certain embodiments, the particles comprise either a PEG-lipid conjugate or an ATTA-lipid conjugate together with a CPL.

[0260] In a preferred embodiment, the lipid conjugate is a PEG-lipid. Examples of PEG-lipids include, but are not limited to, PEG coupled to dialkyloxypropyls (PEG-DAA) as described in, *e.g.*, PCT Publication No. WO 05/026372, PEG coupled to diacylglycerol (PEG-DAG) as described in, *e.g.*, U.S. Patent Publication Nos. 20030077829 and 2005008689, PEG coupled to phospholipids such as phosphatidylethanolamine (PEG-PE), PEG conjugated to ceramides as described in, *e.g.*, U.S. Patent No. 5,885,613, PEG conjugated to cholesterol or a derivative thereof, and mixtures thereof. The disclosures of

these patent documents are herein incorporated by reference in their entirety for all purposes. Additional PEG-lipids include, without limitation, PEG-C-DOMG, 2KPEG-DMG, and a mixture thereof.

[0261] PEG is a linear, water-soluble polymer of ethylene PEG repeating units with two terminal hydroxyl groups. PEGs are classified by their molecular weights; for example, PEG 2000 has an average molecular weight of about 2,000 daltons, and PEG 5000 has an average molecular weight of about 5,000 daltons. PEGs are commercially available from Sigma Chemical Co. and other companies and include, for example, the following: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH_2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM). Other PEGs such as those described in U.S. Patent Nos. 6,774,180 and 7,053,150 (*e.g.*, mPEG (20 KDa) amine) are also useful for preparing the PEG-lipid conjugates of the present invention. The disclosures of these patents are herein incorporated by reference in their entirety for all purposes. In addition, monomethoxypolyethyleneglycol-acetic acid (MePEG-CH_2COOH) is particularly useful for preparing PEG-lipid conjugates including, *e.g.*, PEG-DAA conjugates.

[0262] The PEG moiety of the PEG-lipid conjugates described herein may comprise an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG moiety has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

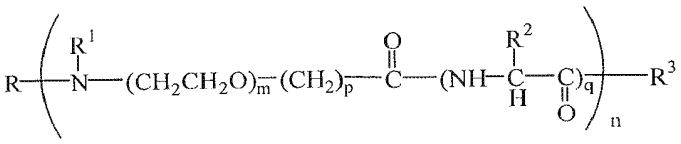
[0263] In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, *e.g.*, non-ester containing linker moieties and ester-containing linker moieties. In a preferred embodiment, the linker moiety is a non-ester containing linker moiety. As used herein, the term "non-ester containing linker moiety" refers to a linker moiety that does not contain a carboxylic ester bond (-OC(O)-). Suitable non-ester containing linker moieties include, but are not limited to, amido (-C(O)NH-), amino (-NR-), carbonyl (-C(O)-), carbamate (-NHC(O)O-), urea (-NHC(O)NH-), disulphide (-S-S-), ether (-O-), succinyl (-

(O)CCH$_2$CH$_2$C(O)-), succinamidyl (-NHC(O)CH$_2$CH$_2$C(O)NH-), ether, disulphide, as well as combinations thereof (such as a linker containing both a carbamate linker moiety and an amido linker moiety). In a preferred embodiment, a carbamate linker is used to couple the PEG to the lipid.

[0264] In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, *e.g.,* carbonate (-OC(O)O-), succinoyl, phosphate esters (-O-(O)POH-O-), sulfonate esters, and combinations thereof.

[0265] Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to PEG to form the lipid conjugate. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skilled in the art. Phosphatidyl-ethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are preferred. Phosphatidylethanolamines with mono- or diunsaturated fatty acids and mixtures of saturated and unsaturated fatty acids can also be used. Suitable phosphatidylethanolamines include, but are not limited to, dimyristoyl-phosphatidylethanolamine (DMPE), dipalmitoyl-phosphatidylethanolamine (DPPE), dioleoylphosphatidylethanolamine (DOPE), and distearoyl-phosphatidylethanolamine (DSPE).

[0266] The term "ATTA" or "polyamide" refers to, without limitation, compounds described in U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes. These compounds include a compound having the formula:

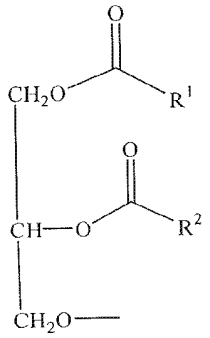


(IV),

wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R^1 is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R^1 and the nitrogen to which they are bound form an azido moiety; R^2 is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; R^3 is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and NR^4R^5, wherein R^4 and R^5 are independently hydrogen or alkyl; n is 4 to 80; m is 2 to 6; p is 1 to 4; and q is 0 or 1. It will

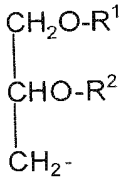
be apparent to those of skill in the art that other polyamides can be used in the compounds of the present invention.

[0267] The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.* Diacylglycerols have the following general formula:



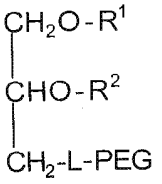
(V).

[0268] The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:



(VI).

[0269] In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:



(VII),

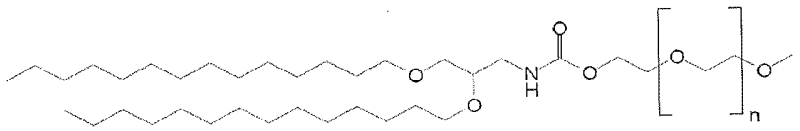
wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester

containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), stearyl (C_{18}), and icosyl (C_{20}). In preferred embodiments, R^1 and R^2 are the same, *i.e.*, R^1 and R^2 are both myristyl (*i.e.*, dimyristyl), R^1 and R^2 are both stearyl (*i.e.*, distearyl), *etc.*

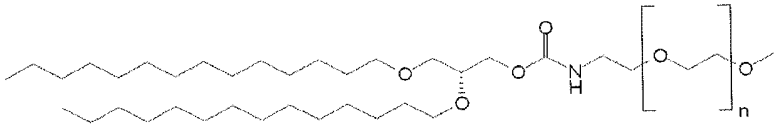
[0270] In Formula VII above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons (*e.g.*, from about 1,000 daltons to about 5,000 daltons, from about 1,500 daltons to about 3,000 daltons, from about 750 daltons to about 3,000 daltons, from about 750 daltons to about 2,000 daltons, *etc.*). In preferred embodiments, the PEG has an average molecular weight of about 2,000 daltons or about 750 daltons. The PEG can be optionally substituted with alkyl, alkoxy, acyl, or aryl. In certain embodiments, the terminal hydroxyl group is substituted with a methoxy or methyl group.

[0271] In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, a succinamidyl linker moiety, and combinations thereof. In a preferred embodiment, the non-ester containing linker moiety is a carbamate linker moiety (*i.e.*, a PEG-*C*-DAA conjugate). In another preferred embodiment, the non-ester containing linker moiety is an amido linker moiety (*i.e.*, a PEG-*A*-DAA conjugate). In yet another preferred embodiment, the non-ester containing linker moiety is a succinamidyl linker moiety (*i.e.*, a PEG-*S*-DAA conjugate).

[0272] In particular embodiments, the PEG-lipid conjugate is selected from:



(PEG-C-DMA); and



(PEG-C-DOMG).

[0273] The PEG-DAA conjugates are synthesized using standard techniques and reagents known to those of skill in the art. It will be recognized that the PEG-DAA conjugates will

contain various amide, amine, ether, thio, carbamate, and urea linkages. Those of skill in the art will recognize that methods and reagents for forming these bonds are well known and readily available. *See, e.g.,* March, ADVANCED ORGANIC CHEMISTRY (Wiley 1992); Larock, COMPREHENSIVE ORGANIC TRANSFORMATIONS (VCH 1989); and Furniss, VOGEL'S TEXTBOOK OF PRACTICAL ORGANIC CHEMISTRY, 5th ed. (Longman 1989). It will also be appreciated that any functional groups present may require protection and deprotection at different points in the synthesis of the PEG-DAA conjugates. Those of skill in the art will recognize that such techniques are well known. *See, e.g.,* Green and Wuts, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS (Wiley 1991).

[0274] Preferably, the PEG-DAA conjugate is a dilauryloxypropyl (C_{12})-PEG conjugate, dimyristyloxypropyl (C_{14})-PEG conjugate, a dipalmityloxypropyl (C_{16})-PEG conjugate, or a distearyloxypropyl (C_{18})-PEG conjugate. Those of skill in the art will readily appreciate that other dialkyloxypropyls can be used in the PEG-DAA conjugates of the present invention.

[0275] In addition to the foregoing, it will be readily apparent to those of skill in the art that other hydrophilic polymers can be used in place of PEG. Examples of suitable polymers that can be used in place of PEG include, but are not limited to, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide and polydimethylacrylamide, polylactic acid, polyglycolic acid, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

[0276] In addition to the foregoing components, the particles (*e.g.,* SNALP or SPLP) of the present invention can further comprise cationic poly(ethylene glycol) (PEG) lipids or CPLs (*see, e.g.,* Chen *et al.*, *Bioconj. Chem.,* 11:433-437 (2000)). Suitable SPLPs and SPLP-CPLs for use in the present invention, and methods of making and using SPLPs and SPLP-CPLs, are disclosed, *e.g.*, in U.S. Patent No. 6,852,334 and PCT Publication No. WO 00/62813, , the disclosures of which are herein incorporated by reference in their entirety for all purposes.

[0277] Suitable CPLs include compounds of Formula VIII:

A-W-Y (VIII),

wherein A, W, and Y are as described below.

[0278] With reference to Formula VIII, "A" is a lipid moiety such as an amphipathic lipid, a neutral lipid, or a hydrophobic lipid that acts as a lipid anchor. Suitable lipid examples include, but are not limited to, diacylglycerolyls, dialkylglycerolyls, N-N-dialkylaminos, 1,2-diacyloxy-3-aminopropanes, and 1,2-dialkyl-3-aminopropanes.

[0279] "W" is a polymer or an oligomer such as a hydrophilic polymer or oligomer. Preferably, the hydrophilic polymer is a biocompatable polymer that is nonimmunogenic or

possesses low inherent immunogenicity. Alternatively, the hydrophilic polymer can be weakly antigenic if used with appropriate adjuvants. Suitable nonimmunogenic polymers include, but are not limited to, PEG, polyamides, polylactic acid, polyglycolic acid, polylactic acid/polyglycolic acid copolymers, and combinations thereof. In a preferred embodiment, the polymer has a molecular weight of from about 250 to about 7,000 daltons.

[0280] "Y" is a polycationic moiety. The term polycationic moiety refers to a compound, derivative, or functional group having a positive charge, preferably at least 2 positive charges at a selected pH, preferably physiological pH. Suitable polycationic moieties include basic amino acids and their derivatives such as arginine, asparagine, glutamine, lysine, and histidine; spermine; spermidine; cationic dendrimers; polyamines; polyamine sugars; and amino polysaccharides. The polycationic moieties can be linear, such as linear tetralysine, branched or dendrimeric in structure. Polycationic moieties have between about 2 to about 15 positive charges, preferably between about 2 to about 12 positive charges, and more preferably between about 2 to about 8 positive charges at selected pH values. The selection of which polycationic moiety to employ may be determined by the type of particle application which is desired.

[0281] The charges on the polycationic moieties can be either distributed around the entire particle moiety, or alternatively, they can be a discrete concentration of charge density in one particular area of the particle moiety *e.g.*, a charge spike. If the charge density is distributed on the particle, the charge density can be equally distributed or unequally distributed. All variations of charge distribution of the polycationic moiety are encompassed by the present invention.

[0282] The lipid "A" and the nonimmunogenic polymer "W" can be attached by various methods and preferably by covalent attachment. Methods known to those of skill in the art can be used for the covalent attachment of "A" and "W." Suitable linkages include, but are not limited to, amide, amine, carboxyl, carbonate, carbamate, ester, and hydrazone linkages. It will be apparent to those skilled in the art that "A" and "W" must have complementary functional groups to effectuate the linkage. The reaction of these two groups, one on the lipid and the other on the polymer, will provide the desired linkage. For example, when the lipid is a diacylglycerol and the terminal hydroxyl is activated, for instance with NHS and DCC, to form an active ester, and is then reacted with a polymer which contains an amino group, such as with a polyamide (*see, e.g.,* U.S. Patent Nos. 6,320,017 and 6,586,559, the disclosures of which are herein incorporated by reference in their entirety for all purposes), an amide bond will form between the two groups.

[0283] In certain instances, the polycationic moiety can have a ligand attached, such as a targeting ligand or a chelating moiety for complexing calcium. Preferably, after the ligand is attached, the cationic moiety maintains a positive charge. In certain instances, the ligand that is attached has a positive charge. Suitable ligands include, but are not limited to, a compound or device with a reactive functional group and include lipids, amphipathic lipids, carrier compounds, bioaffinity compounds, biomaterials, biopolymers, biomedical devices, analytically detectable compounds, therapeutically active compounds, enzymes, peptides, proteins, antibodies, immune stimulators, radiolabels, fluorogens, biotin, drugs, haptens, DNA, RNA, polysaccharides, liposomes, virosomes, micelles, immunoglobulins, functional groups, other targeting moieties, or toxins.

[0284] The lipid conjugate (*e.g.*, PEG-lipid) typically comprises from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 0.6 mol % to about 1.9 mol %, from about 0.7 mol % to about 1.8 mol %, from about 0.8 mol % to about 1.7 mol %, from about 0.9 mol % to about 1.6 mol %, from about 0.9 mol % to about 1.8 mol %, from about 1 mol % to about 1.8 mol %, from about 1 mol % to about 1.7 mol %, from about 1.2 mol % to about 1.8 mol %, from about 1.2 mol % to about 1.7 mol %, from about 1.3 mol % to about 1.6 mol %, or from about 1.4 mol % to about 1.5 mol % of the total lipid present in the particle.

[0285] One of ordinary skill in the art will appreciate that the concentration of the lipid conjugate can be varied depending on the lipid conjugate employed and the rate at which the nucleic acid-lipid particle is to become fusogenic.

[0286] By controlling the composition and concentration of the lipid conjugate, one can control the rate at which the lipid conjugate exchanges out of the nucleic acid-lipid particle and, in turn, the rate at which the nucleic acid-lipid particle becomes fusogenic. For instance, when a PEG-phosphatidylethanolamine conjugate or a PEG-ceramide conjugate is used as the lipid conjugate, the rate at which the nucleic acid-lipid particle becomes fusogenic can be varied, for example, by varying the concentration of the lipid conjugate, by varying the molecular weight of the PEG, or by varying the chain length and degree of saturation of the acyl chain groups on the phosphatidylethanolamine or the ceramide. In addition, other variables including, for example, pH, temperature, ionic strength, *etc.* can be used to vary and/or control the rate at which the nucleic acid-lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the nucleic acid-lipid particle becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure.

VI. Preparation of Lipid Particles

[0287] The lipid particles of the present invention, *e.g.*, SNALP, in which an active agent or therapeutic agent such as an interfering RNA is encapsulated in a lipid bilayer and is protected from degradation, can be formed by any method known in the art including, but not limited to, a continuous mixing method or a direct dilution process.

[0288] In preferred embodiments, the cationic lipids are lipids of Formula I, II, and III, or combinations thereof. In other preferred embodiments, the non-cationic lipids are egg sphingomyelin (ESM), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), dipalmitoyl-phosphatidylcholine (DPPC), monomethyl-phosphatidylethanolamine, dimethyl-phosphatidylethanolamine, 14:0 PE (1,2-dimyristoyl-phosphatidylethanolamine (DMPE)), 16:0 PE (1,2-dipalmitoyl-phosphatidylethanolamine (DPPE)), 18:0 PE (1,2-distearoyl-phosphatidylethanolamine (DSPE)), 18:1 PE (1,2-dioleoyl-phosphatidylethanolamine (DOPE)), 18:1 trans PE (1,2-dielaidoyl-phosphatidylethanolamine (DEPE)), 18:0-18:1 PE (1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE)), 16:0-18:1 PE (1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE)), polyethylene glycol-based polymers (*e.g.*, PEG 2000, PEG 5000, PEG-modified diacylglycerols, or PEG-modified dialkyloxypropyls), cholesterol, or combinations thereof.

[0289] In certain embodiments, the present invention provides for SNALP produced via a continuous mixing method, *e.g.*, a process that includes providing an aqueous solution comprising a nucleic acid such as an interfering RNA in a first reservoir, providing an organic lipid solution in a second reservoir, and mixing the aqueous solution with the organic lipid solution such that the organic lipid solution mixes with the aqueous solution so as to substantially instantaneously produce a liposome encapsulating the nucleic acid (*e.g.*, interfering RNA). This process and the apparatus for carrying this process are described in detail in U.S. Patent Publication No. 20040142025, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0290] The action of continuously introducing lipid and buffer solutions into a mixing environment, such as in a mixing chamber, causes a continuous dilution of the lipid solution with the buffer solution, thereby producing a liposome substantially instantaneously upon mixing. As used herein, the phrase "continuously diluting a lipid solution with a buffer solution" (and variations) generally means that the lipid solution is diluted sufficiently rapidly in a hydration process with sufficient force to effectuate vesicle generation. By mixing the

aqueous solution comprising a nucleic acid with the organic lipid solution, the organic lipid solution undergoes a continuous stepwise dilution in the presence of the buffer solution (*i.e.*, aqueous solution) to produce a nucleic acid-lipid particle.

[0291] The SNALP formed using the continuous mixing method typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0292] In another embodiment, the present invention provides for SNALP produced via a direct dilution process that includes forming a liposome solution and immediately and directly introducing the liposome solution into a collection vessel containing a controlled amount of dilution buffer. In preferred aspects, the collection vessel includes one or more elements configured to stir the contents of the collection vessel to facilitate dilution. In one aspect, the amount of dilution buffer present in the collection vessel is substantially equal to the volume of liposome solution introduced thereto. As a non-limiting example, a liposome solution in 45% ethanol when introduced into the collection vessel containing an equal volume of dilution buffer will advantageously yield smaller particles.

[0293] In yet another embodiment, the present invention provides for SNALP produced via a direct dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the liposome solution formed in a first mixing region is immediately and directly mixed with dilution buffer in the second mixing region. In preferred aspects, the second mixing region includes a T-connector arranged so that the liposome solution and the dilution buffer flows meet as opposing 180° flows; however, connectors providing shallower angles can be used, *e.g.*, from about 27° to about 180°. A pump mechanism delivers a controllable flow of buffer to the second mixing region. In one aspect, the flow rate of dilution buffer provided to the second mixing region is controlled to be substantially equal to the flow rate of liposome solution introduced thereto from the first mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the liposome solution in the second mixing region, and therefore also the concentration of liposome solution in buffer throughout the second mixing process. Such control of the dilution buffer flow rate advantageously allows for small particle size formation at reduced concentrations.

[0294] These processes and the apparatuses for carrying out these direct dilution processes are described in detail in U.S. Patent Publication No. 20070042031, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0295] The SNALP formed using the direct dilution process typically have a size of from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, or from about 70 nm to about 90 nm. The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

[0296] If needed, the lipid particles of the invention (*e.g.*, SNALP) can be sized by any of the methods available for sizing liposomes. The sizing may be conducted in order to achieve a desired size range and relatively narrow distribution of particle sizes.

[0297] Several techniques are available for sizing the particles to a desired size. One sizing method, used for liposomes and equally applicable to the present particles, is described in U.S. Patent No. 4,737,323, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Sonicating a particle suspension either by bath or probe sonication produces a progressive size reduction down to particles of less than about 50 nm in size. Homogenization is another method which relies on shearing energy to fragment larger particles into smaller ones. In a typical homogenization procedure, particles are recirculated through a standard emulsion homogenizer until selected particle sizes, typically between about 60 and about 80 nm, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size discrimination, or QELS.

[0298] Extrusion of the particles through a small-pore polycarbonate membrane or an asymmetric ceramic membrane is also an effective method for reducing particle sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired particle size distribution is achieved. The particles may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in size.

[0299] In some embodiments, the nucleic acids in the SNALP are precondensed as described in, *e.g.*, U.S. Patent Application No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

[0300] In other embodiments, the methods will further comprise adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions. Examples of suitable non-lipid polycations include, hexadimethrine bromide (sold under the brandname POLYBRENE®, from Aldrich Chemical Co., Milwaukee, Wisconsin, USA) or

other salts of hexadimethrine. Other suitable polycations include, for example, salts of poly-L-ornithine, poly-L-arginine, poly-L-lysine, poly-D-lysine, polyallylamine, and polyethyleneimine. Addition of these salts is preferably after the particles have been formed.

[0301] In some embodiments, the nucleic acid to lipid ratios (mass/mass ratios) in a formed SNALP will range from about 0.01 to about 0.2, from about 0.02 to about 0.1, from about 0.03 to about 0.1, or from about 0.01 to about 0.08. The ratio of the starting materials also falls within this range. In other embodiments, the SNALP preparation uses about 400 μg nucleic acid per 10 mg total lipid or a nucleic acid to lipid mass ratio of about 0.01 to about 0.08 and, more preferably, about 0.04, which corresponds to 1.25 mg of total lipid per 50 μg of nucleic acid. In other preferred embodiments, the particle has a nucleic acid:lipid mass ratio of about 0.08.

[0302] In other embodiments, the lipid to nucleic acid ratios (mass/mass ratios) in a formed SNALP will range from about 1 (1:1) to about 100 (100:1), from about 5 (5:1) to about 100 (100:1), from about 1 (1:1) to about 50 (50:1), from about 2 (2:1) to about 50 (50:1), from about 3 (3:1) to about 50 (50:1), from about 4 (4:1) to about 50 (50:1), from about 5 (5:1) to about 50 (50:1), from about 1 (1:1) to about 25 (25:1), from about 2 (2:1) to about 25 (25:1), from about 3 (3:1) to about 25 (25:1), from about 4 (4:1) to about 25 (25:1), from about 5 (5:1) to about 25 (25:1), from about 5 (5:1) to about 20 (20:1), from about 5 (5:1) to about 15 (15:1), from about 5 (5:1) to about 10 (10:1), about 5 (5:1), 6 (6:1), 7 (7:1), 8 (8:1), 9 (9:1), 10 (10:1), 11 (11:1), 12 (12:1), 13 (13:1), 14 (14:1), or 15 (15:1). The ratio of the starting materials also falls within this range.

[0303] As previously discussed, the conjugated lipid may further include a CPL. A variety of general methods for making SNALP-CPLs (CPL-containing SNALP) are discussed herein. Two general techniques include "post-insertion" technique, that is, insertion of a CPL into, for example, a pre-formed SNALP, and the "standard" technique, wherein the CPL is included in the lipid mixture during, for example, the SNALP formation steps. The post-insertion technique results in SNALP having CPLs mainly in the external face of the SNALP bilayer membrane, whereas standard techniques provide SNALP having CPLs on both internal and external faces. The method is especially useful for vesicles made from phospholipids (which can contain cholesterol) and also for vesicles containing PEG-lipids (such as PEG-DAAs and PEG-DAGs). Methods of making SNALP-CPL, are taught, for example, in U.S. Patent Nos. 5,705,385; 6,586,410; 5,981,501; 6,534,484; and 6,852,334; U.S. Patent Publication No. 20020072121; and PCT Publication No. WO 00/62813, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

VII. Kits

[0304] The present invention also provides lipid particles (*e.g.*, SNALP) in kit form. The kit may comprise a container which is compartmentalized for holding the various elements of the lipid particles (*e.g.*, the active agents or therapeutic agents such as nucleic acids and the individual lipid components of the particles). In some embodiments, the kit may further comprise an endosomal membrane destabilizer (*e.g.*, calcium ions). The kit typically contains the lipid particle compositions of the present invention, preferably in dehydrated form, with instructions for their rehydration and administration.

[0305] As explained herein, the lipid particles of the invention (*e.g.*, SNALP) can be tailored to preferentially target particular tissues, organs, or tumors of interest. In certain instances, preferential targeting of lipid particles such as SNALP may be carried out by controlling the composition of the particle itself. For instance, as set forth in Example 11, it has been found that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP formulation can be used to preferentially target the liver (including liver tumors).

[0306] In certain other instances, it may be desirable to have a targeting moiety attached to the surface of the lipid particle to further enhance the targeting of the particle. Methods of attaching targeting moieties (*e.g.*, antibodies, proteins, *etc.*) to lipids (such as those used in the present particles) are known to those of skill in the art.

VII. Administration of Lipid Particles

[0307] Once formed, the lipid particles of the invention (*e.g.*, SNALP) are useful for the introduction of active agents or therapeutic agents (*e.g.*, nucleic acids such as interfering RNA) into cells. Accordingly, the present invention also provides methods for introducing an active agent or therapeutic agent such as a nucleic acid (*e.g.*, interfering RNA) into a cell. The methods are carried out *in vitro* or *in vivo* by first forming the particles as described above and then contacting the particles with the cells for a period of time sufficient for delivery of the active agent or therapeutic agent to the cells to occur.

[0308] The lipid particles of the invention (*e.g.*, SNALP) can be adsorbed to almost any cell type with which they are mixed or contacted. Once adsorbed, the particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the active agent or therapeutic agent (*e.g.*, nucleic acid) portion of the particle can take place via any one of these pathways. In particular, when

fusion takes place, the particle membrane is integrated into the cell membrane and the contents of the particle combine with the intracellular fluid.

[0309] The lipid particles of the invention (*e.g.*, SNALP) can be administered either alone or in a mixture with a pharmaceutically-acceptable carrier (*e.g.*, physiological saline or phosphate buffer) selected in accordance with the route of administration and standard pharmaceutical practice. Generally, normal buffered saline (*e.g.*, 135-150 mM NaCl) will be employed as the pharmaceutically-acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.4% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Additional suitable carriers are described in, *e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

[0310] The pharmaceutically-acceptable carrier is generally added following particle formation. Thus, after the particle is formed, the particle can be diluted into pharmaceutically-acceptable carriers such as normal buffered saline.

[0311] The concentration of particles in the pharmaceutical formulations can vary widely, *i.e.*, from less than about 0.05%, usually at or at least about 2 to 5%, to as much as about 10 to 90% by weight, and will be selected primarily by fluid volumes, viscosities, *etc.*, in accordance with the particular mode of administration selected. For example, the concentration may be increased to lower the fluid load associated with treatment. This may be particularly desirable in patients having atherosclerosis-associated congestive heart failure or severe hypertension. Alternatively, particles composed of irritating lipids may be diluted to low concentrations to lessen inflammation at the site of administration.

[0312] The pharmaceutical compositions of the present invention may be sterilized by conventional, well-known sterilization techniques. Aqueous solutions can be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions can contain pharmaceutically-acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium

chloride, and calcium chloride. Additionally, the particle suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as alphatocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

A. *In vivo* Administration

[0313] Systemic delivery for *in vivo* therapy, *e.g.*, delivery of a therapeutic nucleic acid to a distal target cell via body systems such as the circulation, has been achieved using nucleic acid-lipid particles such as those described in PCT Publication Nos. WO 05/007196, WO 05/121348, WO 05/120152, and WO 04/002453, the disclosures of which are herein incorporated by reference in their entirety for all purposes. The present invention also provides fully encapsulated lipid particles that protect the nucleic acid from nuclease degradation in serum, are nonimmunogenic, are small in size, and are suitable for repeat dosing.

[0314] For *in vivo* administration, administration can be in any manner known in the art, *e.g.*, by injection, oral administration, inhalation (*e.g.*, intransal or intratracheal), transdermal application, or rectal administration. Administration can be accomplished via single or divided doses. The pharmaceutical compositions can be administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. In some embodiments, the pharmaceutical compositions are administered intravenously or intraperitoneally by a bolus injection (*see*, *e.g.*, U.S. Patent No. 5,286,634). Intracellular nucleic acid delivery has also been discussed in Straubringer *et al.*, *Methods Enzymol.*, 101:512 (1983); Mannino *et al.*, *Biotechniques*, 6:682 (1988); Nicolau *et al.*, *Crit. Rev. Ther. Drug Carrier Syst.*, 6:239 (1989); and Behr, *Acc. Chem. Res.*, 26:274 (1993). Still other methods of administering lipid-based therapeutics are described in, for example, U.S. Patent Nos. 3,993,754; 4,145,410; 4,235,871; 4,224,179; 4,522,803; and 4,588,578. The lipid particles can be administered by direct injection at the site of disease or by injection at a site distal from the site of disease (*see*, *e.g.*, Culver, HUMAN GENE THERAPY, MaryAnn Liebert, Inc., Publishers, New York. pp.70-71(1994)). The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0315] The compositions of the present invention, either alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation (*e.g.*, intranasally or intratracheally) (*see*, Brigham *et al.*, *Am.*

J. Sci., 298:278 (1989)). Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

[0316] In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering nucleic acid compositions directly to the lungs via nasal aerosol sprays have been described, *e.g.*, in U.S. Patent Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Similarly, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Patent No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

[0317] Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the practice of this invention, compositions are preferably administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically, or intrathecally.

[0318] Generally, when administered intravenously, the lipid particle formulations are formulated with a suitable pharmaceutical carrier. Many pharmaceutically acceptable carriers may be employed in the compositions and methods of the present invention. Suitable formulations for use in the present invention are found, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). A variety of aqueous carriers may be used, for example, water, buffered water, 0.4% saline, 0.3% glycine, and the like, and may include glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc.* Generally, normal buffered saline (135-150 mM NaCl) will be employed as the pharmaceutically acceptable carrier, but other suitable carriers will suffice. These compositions can be sterilized by conventional liposomal sterilization techniques, such as filtration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium

chloride, sorbitan monolaurate, triethanolamine oleate, *etc*. These compositions can be sterilized using the techniques referred to above or, alternatively, they can be produced under sterile conditions. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

[0319] In certain applications, the lipid particles disclosed herein may be delivered via oral administration to the individual. The particles may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, pills, lozenges, elixirs, mouthwash, suspensions, oral sprays, syrups, wafers, and the like (*see, e.g.*, U.S. Patent Nos. 5,641,515, 5,580,579, and 5,792,451, the disclosures of which are herein incorporated by reference in their entirety for all purposes). These oral dosage forms may also contain the following: binders, gelatin; excipients, lubricants, and/or flavoring agents. When the unit dosage form is a capsule, it may contain, in addition to the materials described above, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. Of course, any material used in preparing any unit dosage form should be pharmaceutically pure and substantially non-toxic in the amounts employed.

[0320] Typically, these oral formulations may contain at least about 0.1% of the lipid particles or more, although the percentage of the particles may, of course, be varied and may conveniently be between about 1% or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of particles in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0321] Formulations suitable for oral administration can consist of: (a) liquid solutions, such as an effective amount of a packaged therapeutic agent such as nucleic acid (*e.g.*, interfering RNA) suspended in diluents such as water, saline, or PEG 400; (b) capsules, sachets, or tablets, each containing a predetermined amount of a therapeutic agent such as nucleic acid (*e.g.*, interfering RNA), as liquids, solids, granules, or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic

acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise a therapeutic agent such as nucleic acid (*e.g.*, interfering RNA) in a flavor, *e.g.*, sucrose, as well as pastilles comprising the therapeutic agent in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the therapeutic agent, carriers known in the art.

[0322] In another example of their use, lipid particles can be incorporated into a broad range of topical dosage forms. For instance, a suspension containing nucleic acid-lipid particles such as SNALP can be formulated and administered as gels, oils, emulsions, topical creams, pastes, ointments, lotions, foams, mousses, and the like.

[0323] When preparing pharmaceutical preparations of the lipid particles of the invention, it is preferable to use quantities of the particles which have been purified to reduce or eliminate empty particles or particles with therapeutic agents such as nucleic acid associated with the external surface.

[0324] The methods of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as primates (*e.g.*, humans and chimpanzees as well as other nonhuman primates), canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, rats and mice), lagomorphs, and swine.

[0325] The amount of particles administered will depend upon the ratio of therapeutic agent (*e.g.*, nucleic acid) to lipid, the particular therapeutic agent (*e.g.*, nucleic acid) used, the disease or disorder being treated, the age, weight, and condition of the patient, and the judgment of the clinician, but will generally be between about 0.01 and about 50 mg per kilogram of body weight, preferably between about 0.1 and about 5 mg/kg of body weight, or about 10^8-10^{10} particles per administration (*e.g.*, injection).

B. *In vitro* Administration

[0326] For *in vitro* applications, the delivery of therapeutic agents such as nucleic acids (*e.g.*, interfering RNA) can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells are animal cells, more preferably mammalian cells, and most preferably human cells.

[0327] Contact between the cells and the lipid particles, when carried out *in vitro*, takes place in a biologically compatible medium. The concentration of particles varies widely depending on the particular application, but is generally between about 1 μmol and about 10

mmol. Treatment of the cells with the lipid particles is generally carried out at physiological temperatures (about 37°C) for periods of time of from about 1 to 48 hours, preferably of from about 2 to 4 hours.

[0328] In one group of preferred embodiments, a lipid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/ml, more preferably about 2×10^4 cells/ml. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2 μg/ml, more preferably about 0.1 μg/ml.

[0329] Using an Endosomal Release Parameter (ERP) assay, the delivery efficiency of the SNALP or other lipid particle of the invention can be optimized. An ERP assay is described in detail in U.S. Patent Publication No. 20030077829, the disclosure of which is herein incorporated by reference in its entirety for all purposes. More particularly, the purpose of an ERP assay is to distinguish the effect of various cationic lipids and helper lipid components of SNALP based on their relative effect on binding/uptake or fusion with/destabilization of the endosomal membrane. This assay allows one to determine quantitatively how each component of the SNALP or other lipid particle affects delivery efficiency, thereby optimizing the SNALP or other lipid particle. Usually, an ERP assay measures expression of a reporter protein (*e.g.*, luciferase, β-galactosidase, green fluorescent protein (GFP), *etc.*), and in some instances, a SNALP formulation optimized for an expression plasmid will also be appropriate for encapsulating an interfering RNA. In other instances, an ERP assay can be adapted to measure downregulation of transcription or translation of a target sequence in the presence or absence of an interfering RNA (*e.g.*, siRNA). By comparing the ERPs for each of the various SNALP or other lipid particles, one can readily determine the optimized system, *e.g.*, the SNALP or other lipid particle that has the greatest uptake in the cell.

C. Cells for Delivery of Lipid Particles

[0330] The compositions and methods of the present invention are used to treat a wide variety of cell types, *in vivo* and *in vitro*. Suitable cells include, *e.g.*, hematopoietic precursor (stem) cells, fibroblasts, keratinocytes, hepatocytes, endothelial cells, skeletal and smooth muscle cells, osteoblasts, neurons, quiescent lymphocytes, terminally differentiated cells, slow or noncycling primary cells, parenchymal cells, lymphoid cells, epithelial cells, bone cells, and the like. In preferred embodiments, an active agent or therapeutic agent such as an interfering RNA (*e.g.*, siRNA) is delivered to cancer cells such as, *e.g.*, lung cancer cells, colon cancer cells, rectal cancer cells, anal cancer cells, bile duct cancer cells, small intestine cancer cells, stomach (gastric) cancer cells, esophageal cancer cells, gallbladder cancer cells,

liver cancer cells, pancreatic cancer cells, appendix cancer cells, breast cancer cells, ovarian cancer cells, cervical cancer cells, prostate cancer cells, renal cancer cells, cancer cells of the central nervous system, glioblastoma tumor cells, skin cancer cells, lymphoma cells, choriocarcinoma tumor cells, head and neck cancer cells, osteogenic sarcoma tumor cells, and blood cancer cells.

[0331] *In vivo* delivery of lipid particles such as SNALP encapsulating an interfering RNA (*e.g.*, siRNA) is suited for targeting cells of any cell type. The methods and compositions can be employed with cells of a wide variety of vertebrates, including mammals, such as, *e.g.* canines, felines, equines, bovines, ovines, caprines, rodents (*e.g.*, mice, rats, and guinea pigs), lagomorphs, swine, and primates (*e.g.* monkeys, chimpanzees, and humans).

[0332] To the extent that tissue culture of cells may be required, it is well-known in the art. For example, Freshney, Culture of Animal Cells, a Manual of Basic Technique, 3rd Ed., Wiley-Liss, New York (1994), Kuchler *et al.*, Biochemical Methods in Cell Culture and Virology, Dowden, Hutchinson and Ross, Inc. (1977), and the references cited therein provide a general guide to the culture of cells. Cultured cell systems often will be in the form of monolayers of cells, although cell suspensions are also used.

D. Detection of Lipid Particles

[0333] In some embodiments, the lipid particles of the present invention (*e.g.*, SNALP) are detectable in the subject at about 1, 2, 3, 4, 5, 6, 7, 8 or more hours. In other embodiments, the lipid particles of the present invention (*e.g.*, SNALP) are detectable in the subject at about 8, 12, 24, 48, 60, 72, or 96 hours, or about 6, 8, 10, 12, 14, 16, 18, 19, 22, 24, 25, or 28 days after administration of the particles. The presence of the particles can be detected in the cells, tissues, or other biological samples from the subject. The particles may be detected, *e.g.*, by direct detection of the particles, detection of a therapeutic nucleic acid such as an interfering RNA (*e.g.*, siRNA) sequence, detection of the target sequence of interest (*i.e.*, by detecting expression or reduced expression of the sequence of interest), or a combination thereof.

1. Detection of Particles

[0334] Lipid particles of the invention such as SNALP can be detected using any method known in the art. For example, a label can be coupled directly or indirectly to a component of the lipid particle using methods well-known in the art. A wide variety of labels can be used, with the choice of label depending on sensitivity required, ease of conjugation with the lipid particle component, stability requirements, and available instrumentation and disposal

provisions. Suitable labels include, but are not limited to, spectral labels such as fluorescent dyes (*e.g.*, fluorescein and derivatives, such as fluorescein isothiocyanate (FITC) and Oregon Green™; rhodamine and derivatives such Texas red, tetrarhodimine isothiocynate (TRITC), *etc.*, digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like; radiolabels such as ^{3}H, ^{125}I, ^{35}S, ^{14}C, ^{32}P, ^{33}P, *etc.*; enzymes such as horse radish peroxidase, alkaline phosphatase, *etc.*; spectral colorimetric labels such as colloidal gold or colored glass or plastic beads such as polystyrene, polypropylene, latex, *etc.* The label can be detected using any means known in the art.

2. Detection of Nucleic Acids

[0335] Nucleic acids (*e.g.*, interfering RNA) are detected and quantified herein by any of a number of means well-known to those of skill in the art. The detection of nucleic acids may proceed by well-known methods such as Southern analysis, Northern analysis, gel electrophoresis, PCR, radiolabeling, scintillation counting, and affinity chromatography. Additional analytic biochemical methods such as spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography may also be employed.

[0336] The selection of a nucleic acid hybridization format is not critical. A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in, *e.g.*, "Nucleic Acid Hybridization, A Practical Approach," Eds. Hames and Higgins, IRL Press (1985).

[0337] The sensitivity of the hybridization assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. *In vitro* amplification techniques suitable for amplifying sequences for use as molecular probes or for generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBA™) are found in Sambrook *et al.*, In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (2000); and Ausubel *et al.*, SHORT PROTOCOLS IN MOLECULAR BIOLOGY, eds., Current Protocols, Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (2002); as well as U.S. Patent No. 4,683,202; PCR Protocols, A Guide to Methods and

Applications (Innis *et al.* eds.) Academic Press Inc. San Diego, CA (1990); Arnheim & Levinson (October 1, 1990), *C&EN* 36; The *Journal Of NIH Research*, 3:81 (1991); Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173 (1989); Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874 (1990); Lomell *et al.*, *J. Clin. Chem.*, 35:1826 (1989); Landegren *et al.*, *Science*, 241:1077 (1988); Van Brunt, *Biotechnology*, 8:291 (1990); Wu and Wallace, *Gene*, 4:560 (1989); Barringer *et al.*, *Gene*, 89:117 (1990); and Sooknanan and Malek, *Biotechnology*, 13:563 (1995). Improved methods of cloning *in vitro* amplified nucleic acids are described in U.S. Pat. No. 5,426,039. Other methods described in the art are the nucleic acid sequence based amplification (NASBA™, Cangene, Mississauga, Ontario) and Qβ-replicase systems. These systems can be used to directly identify mutants where the PCR or LCR primers are designed to be extended or ligated only when a select sequence is present. Alternatively, the select sequences can be generally amplified using, for example, nonspecific PCR primers and the amplified target region later probed for a specific sequence indicative of a mutation. The disclosures of the above-described references are herein incorporated by reference in their entirety for all purposes.

[0338] Nucleic acids for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as inhibitor components are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage *et al.*, *Tetrahedron Letts.*, 22:1859 1862 (1981), *e.g.*, using an automated synthesizer, as described in Needham VanDevanter *et al.*, *Nucleic Acids Res.*, 12:6159 (1984). Purification of ploynucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion exchange HPLC as described in Pearson *et al.*, *J. Chrom.*, 255:137 149 (1983). The sequence of the synthetic poluyucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology*, 65:499.

[0339] An alternative means for determining the level of transcription is *in situ* hybridization. *In situ* hybridization assays are well-known and are generally described in Angerer *et al.*, *Methods Enzymol.*, 152:649 (1987). In an *in situ* hybridization assay, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

VIII. Examples

[0340] The present invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are not intended to limit the invention in any manner. Those of skill in the art will readily recognize a variety of noncritical parameters which can be changed or modified to yield essentially the same results.

Example 1. Materials and Methods.

[0341] ***siRNA***: All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, CO). The siRNAs were desalted and anncaled using standard procedures.

[0342] ***Lipid Encapsulation of siRNA***: In some embodiments, siRNA molecules were encapsulated into nucleic acid-lipid particles composed of the following lipids: the lipid conjugate PEG-cDMA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxypropylamine); the cationic lipid DLinDMA (1,2-Dilinoleyloxy-3-(N,N-dimethyl)aminopropane); the phospholipid DPPC (1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids; Alabaster, AL); and synthetic cholesterol (Sigma-Aldrich Corp.; St. Louis, MO) in the molar ratio 1.4:57.1:7.1:34.3, respectively. In other words, siRNAs were encapsulated into SNALP of the following "1:57" formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol. In other embodiments, siRNA molecules were encapsulated into phospholipid-free SNALP composed of the following lipids: the lipid conjugate PEG-cDMA; the cationic lipid DLinDMA; and synthetic cholesterol in the molar ratio 1.5:61.5:36.9, respectively. In other words, siRNAs were encapsulated into phospholipid-free SNALP of the following "1:62" formulation: 1.5% PEG-cDMA; 61.5% DLinDMA; and 36.9% cholesterol. For vehicle controls, empty particles with identical lipid composition were formed in the absence of siRNA. It should be understood that the 1:57 formulation and 1:62 formulation are target formulations, and that the amount of lipid (both cationic and non-cationic) present and the amount of lipid conjugate present in the formulation may vary. Typically, in the 1:57 formulation, the amount of cationic lipid will be 57 mol % ± 5 mol %, and the amount of lipid conjugate will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:57 formulation being made up of non-cationic lipid (*e.g.*, phospholipid, cholesterol, or a mixture of the two). Similarly, in the 1:62 formulation, the amount of cationic lipid will be 62 mol % ± 5 mol %, and the amount of lipid conjugate

will be 1.5 mol % ± 0.5 mol %, with the balance of the 1:62 formulation being made up of the non-cationic lipid (*e.g.*, cholesterol).

Example 2. Eg5 siRNA Formulated as 1:57 SNALP Are Potent Inhibitors of Cell Growth *in vitro*.

[0343] SNALP formulations were prepared with an siRNA targeting Eg5 as the nucleic acid component. Eg5 is a member of kinesin-related proteins that are involved in functions related to movements of organelles, microtubules, or chromosomes along microtubules. These functions include axonal transport, microtubule sliding during nuclear fusion or division, and chromosome disjunction during meiosis and early mitosis. Eg5 plays a critical role in mitosis of mammalian cells. The Eg5 siRNA used in this study is provided in Table 1. The modifications involved introducing 2'OMe-uridine at selected positions in the sense and antisense strands of the Eg5 2263 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 1. siRNA duplex comprising sense and antisense Eg5 RNA polynucleotides.

Modification	Eg5 2263 siRNA sequence	% 2'OMe-Modified	% Modified in DS Region
U/U	5'-C**U**GAAGACC**U**GAAGACAA**U**dTdT-3' 3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUUA-5'	6/42 = 14.3%	6/38 = 15.8%

Column 1: "U/U" = 2'OMe-uridine modified siRNA duplex; Column 2: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. "dT" = deoxythymidine. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

[0344] The lipid components and physical characteristics of the SNALP formulations are summarized in Table 2. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 2. Characteristics of the SNALP formulations used in this study.

Sample No.	Formulation Composition, Mole % PEG(2000)-C-DMA I DLinDMA I DPPC I Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
1	2 I 40 I 10 I 48	12.4	57	0.07	90
2	1.8 I 36.4 I 18.2 I 43.6	14.0	72	0.12	89
3	1.4 I 27.0 I 6.8 I 64.9	16.5	70	0.12	92
4	1.3 I 25.3 I 12.7 I 60.8	18.1	76	0.07	93
5	3.9 I 39.2 I 9.8 I 47.1	13.5	53	0.27	86
6	3.6 I 35.7 I 17.9 I 42.9	15.1	58	0.18	87
7	2.7 I 26.7 I 6.7 I 64.0	17.6	56	0.17	92
8	2.5 I 25.0 I 12.5 I 60.0	19.2	61	0.13	92
9	1.4 I 57.1 I 7.1 I 34.3	17.8	84	0.10	88
10	1.3 I 53.3 I 13.3 I 32.0	19.5	83	0.10	89
11	1.1 I 42.6 I 5.3 I 51.1	22.0	80	0.10	93
12	1.0 I 40.4 I 10.1 I 48.5	23.6	78	0.11	88
13	2.8 I 56.3 I 7.0 I 33.8	19.0	62	0.14	80
14	2.6 I 52.6 I 13.2 I 31.6	20.6	66	0.14	82
15	2.1 I 42.1 I 5.3 I 50.5	23.1	71	0.16	91
16	2 I 40 I 10 I 48	24.7	67	0.14	92

[0345] Silencing of Eg5 by siRNA transfection causes mitotic arrest and apoptosis in mammalian cells. Cell viability following transfection with SNALP containing an siRNA targeting Eg5 therefore provides a simple biological readout of *in vitro* transfection efficiency. Cell viability of *in vitro* cell cultures was assessed using the commercial reagent CellTiter-Blue® (Promega Corp.; Madison, WI), a resazurin dye that is reduced by metabolically active cells to the flourogenic product resorufin. The human colon cancer cell line HT29 was cultured using standard tissue culture techniques. 72 hours after SNALP application, CellTiter-Blue® reagent was added to the culture to quantify the metabolic activity of the cells, which is a measure of cell viability. Data are presented as a percent of cell viability relative to ("untreated") control cells that received phosphate buffered saline (PBS) vehicle only.

[0346] Figure 1 shows that the 1:57 SNALP formulation containing Eg5 2263 U/U siRNA was among the most potent inhibitors of tumor cell growth at all siRNA concentrations tested (*see,* Figure 1B, Sample 9).

Example 3. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo*.

[0347] SNALP formulations were prepared with an siRNA targeting apolipoprotein B (ApoB) as the nucleic acid component. ApoB is the main apolipoprotein of chylomicrons and low density lipoproteins (LDL). Mutations in ApoB are associated with

hypercholesterolemia. ApoB occurs in the plasma in 2 main forms, ApoB48 and ApoB100, which are synthesized in the intestine and liver, respectively, due to an organ-specific stop codon. The ApoB siRNA used in this study is provided in Table 3. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the ApoB siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 3. siRNA duplex comprising sense and antisense ApoB RNA polynucleotides.

Position	Modification	ApoB siRNA sequence	% 2'OMe-Modified	% Modified in DS Region
10048	U2/2 G1/2	5'-AGU**G**UCA**U**CACAC**U**GAAUACC-3' 3'-GU**U**CACAGUAGU**G**U**G**AC**U**UAU-5'	7/42 = 16.7%	7/38 = 18.4%

Column 1: The number refers to the nucleotide position of the 5' base of the sense strand relative to the mouse ApoB mRNA sequence XM_137955. Column 2: The numbers refer to the distribution of 2'OMe chemical modifications in each strand. Column 3: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. Column 4: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 5: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

[0348] The lipid components and physical characteristics of the formulations are summarized in Table 4. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 4. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2 | 40 | 10 | 48	12.4	59	0.15	93
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 2.2 | 44.4 | 53.3	10.7	55	0.17	91
4	PEG(2000)-C-DMA | DLinDMA | DOPC | Cholesterol 2 | 40 | 10 | 48	12.5	59	0.16	92
5	PEG(2000)-C-DMA | DLinDMA | DMPC | Cholesterol 2 | 40 | 10 | 48	12.2	56	0.11	92
6	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.8 | 36.4 | 18.2 | 43.6	13.8	66	0.16	93
7	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 2 | 40 | 10 | 48	12.4	56	0.12	92
8	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 27.0 | 6.8 | 64.9	16.5	60	0.10	93
9	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.3 | 25.3 | 12.7 | 60.8	18.1	74	0.13	92
10	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2.5 | 25.0 | 12.5 | 60.0	19.2	60	0.13	93
11	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.4 | 34.3	17.8	79	0.09	94

12	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.0 | 40.4 | 10.1 | 48.5	23.6	72	0.11	93
13	PEG(2000)-C-DMA | DLinDMA | DPPC 2 | 70 | 28	8.7	73	0.09	87
14	PEG(2000)-C-DMA | DLinDMA | DPPC 1.6 | 54.7 | 43.8	11.3	65	0.11	87

[0349] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0350] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0351] Figure 2 shows that the 1:57 SNALP formulation containing ApoB 10048 U2/2 G1/2 siRNA was the most potent at reducing ApoB expression *in vivo* (*see,* Group 11).

Example 4. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity *in vivo*.

[0352] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 5. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 5. Characteristics of the SNALP formulations used in this study.

SNALP (L:D ratio)	siRNA Payload	Particle Size (Polydispersity)	% Encapsulation
2:30 (13)	ApoB-10048 U2/2 G1/2	65 nm (0.16)	88
1:57 (9)	ApoB-10048 U2/2 G1/2	74 nm (0.10)	89

[0353] The 2:30 SNALP formulation used in this study is lipid composition 2:30:20:48 as described in molar percentages of PEG-C-DMA, DLinDMA, DSPC, and cholesterol (in that

order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 13:1.

[0354] The 1:57 SNALP formulation used in this study is lipid composition 1.5:57.1:7:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by syringe press at an input lipid to drug (L:D) ratio (mg:mg) of 9:1.

[0355] BALB/c mice (female, 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Days 0, 1, 2, 3 & 4 for a total of 5 doses per animal. Daily dosage was either 1.0 (for 2:30 SNALP) or 0.1 (for 1:57 SNALP) mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given IV injections of phosphate buffered saline (PBS) vehicle. On Study Day 7, 72 h after the last treatment, animals were euthanized and liver tissue was collected in RNAlater.

[0356] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0357] Figure 3 shows that the 1:57 SNALP containing ApoB 10048 U2/2 G1/2 siRNA was more than 10 times as efficacious as the 2:30 SNALP in mediating ApoB gene silencing in mouse liver at a 10-fold lower dose.

Example 5. ApoB siRNA Formulated as 1:57 or 1:62 SNALP Have Potent Silencing Activity *in vivo*.

[0358] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 6. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 6. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	76	0.06	89
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 1.5 | 61.5 | 36.9	8.1	76	0.04	86
4	PEG(2000)-C-DMA | DODMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.0	72	0.05	95
5	PEG(5000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.6	52	0.16	89
6	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 1.4 | 57.1 | 7.1 | 34.3	8.9	68	0.10	94
7	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	72	0.07	95
8	PEG(2000)-C-DMA | DLinDMA | DPPC 1.8 | 70.2 | 28.1	8.6	74	0.13	86

[0359] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.75 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0360] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0361] Figure 4 shows that the 1:57 and 1:62 SNALP formulations had comparable ApoB silencing activity *in vivo* (*see, e.g.*, Groups 2 & 3).

Example 6. ApoB siRNA Formulated as 1:62 SNALP Have Potent Silencing Activity *in vivo*.

[0362] SNALP formulations were prepared with the ApoB siRNA set forth in Table 3. The lipid components and physical characteristics of the formulations are summarized in Table 7. The lipid:drug ratio is described in units of mg total lipid per mg nucleic acid. Mean particle size and polydispersity were measured on a Malvern Instruments Zetasizer. Encapsulation of nucleic acid was measured using a Ribogreen assay essentially as described in Heyes *et al.*, *Journal of Controlled Release*, 107:276-287 (2005).

Table 7. Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition, Mole % PEG(2000)-C-DMA I DLinDMA I Cholesterol	Lipid/Drug Ratio	Finished Product Characterization		
			Size (nm)	Polydispersity	% Encapsulation
2	1.5 I 61.5 I 36.9	6.1	80	0.07	92
3	1.4 I 54.8 I 43.8	6.6	74	0.05	89
4	2.0 I 61.2 I 36.7	6.2	71	0.11	91
5	1.8 I 54.5 I 43.6	6.7	67	0.09	91
6	1.3 I 68.1 I 30.6	7.4	91	0.06	89
7	1.2 I 61.8 I 37.1	8.0	87	0.10	90
8	1.7 I 67.8 I 30.5	7.6	81	0.07	91
9	1.4 I 56.3 I 42.3	8.6	75	0.11	92
10	1.9 I 61.3 I 36.8	8.2	72	0.10	91
11	1.8 I 56.1 I 42.1	8.8	70	0.10	90
12	1.3 I 66.7 I 32.0	9.5	89	0.09	89
13	1.2 I 61.7 I 37.0	10.0	87	0.10	91
14	1.7 I 66.4 I 31.9	9.6	82	0.11	90
15	1.5 I 61.5 I 36.9	10.1	79	0.10	91

[0363] BALB/c mice (female, at least 4 weeks old) were obtained from Harlan Labs. After an acclimation period (of at least 7 days), animals were administered SNALP by intravenous (IV) injection in the lateral tail vein once daily on Study Day 0 (1 dose total per animal). Dosage was 0.1 mg encapsulated siRNA per kg body weight, corresponding to 10 ml/kg (rounded to the nearest 10 μl). As a negative control, one group of animals was given an IV injection of phosphate buffered saline (PBS) vehicle. On Study Day 2, animals were euthanized and liver tissue was collected in RNAlater.

[0364] Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, CA) essentially as described in Judge *et al.*, *Molecular Therapy*, 13:494 (2006).

[0365] Figure 5 shows that the 1:62 SNALP formulation was one of the most potent inhibitors of ApoB expression at two different lipid:drug ratios (*i.e.*, 6.1 & 10.1) among the phospholipid-free SNALP formulations tested (*see*, Groups 2 & 15).

Example 7. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Syringe Press or Gear Pump Process.

[0366] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by various manufacturing processes. In particular, 1:57 SNALP was prepared by a syringe press or gear pump process

using either PBS or citrate buffer (post-blend dilution) and administered intravenously in mice.

Experimental Design

[0367] Animal Model: Female BALB/c mice, 5 wks old, n=4 per group/cage.

[0368] siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

Tolerability:

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
1	PBS vehicle	Standard 10 mL/kg volume	
2	1|57 Citrate Direct Dil, Syringe Press	7	77
3	1|57 PBS Direct Dil, Syringe Press	7	96
4	1|57 PBS Direct Dil, Gear Pump	7	79
5	1|57 Citrate Direct Dil, Syringe Press	9	99
6	1|57 PBS Direct Dil, Syringe Press	9	123
7	1|57 PBS Direct Dil, Gear Pump	9	102

Efficacy:

Group	Formulation	IV Injection	
		siRNA mg/kg	Lipid mg/kg
8	PBS vehicle	Standard 10 mL/kg volume	
9	1|57 PBS Direct Dil, Syringe Press	0.05	0.68
10	1|57 PBS Direct Dil, Gear Pump	0.05	0.57
11	1|57 PBS Direct Dil, Syringe Press	0.1	1.36
12	1|57 PBS Direct Dil, Gear Pump	0.1	1.13

Formulation:

[0369] Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

[0370] Formulation Details:

1. Lipid composition "1|57 Citrate blend" used in this study is 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation has an input lipid to drug ratio of 8.9.
2. Gear pump set up included 0.8 mm T-connector and 400 mL/min speed.
3. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0371] Formulation Summary:

		Particle Size			Final L:D
	1:57 (9:1) + DOW siRNA	Zavg (nm)	Poly	% Encap	(mg:mg)
322-050807-1	Syringe PBS Blend	79	0.12	92	13.6
322-050807-2	Syringe Citrate Blend	86	0.11	91	11.0
322-050807-3	Gear PBS Blend	80	0.09	93	11.3

Procedures

[0372] **Treatment:** Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 µl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0373] **Group 1-7 Endpoint:** Animals are sacrificed on Day 1, 24 h after test article administration. Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein.

[0374] **Group 8-12 Endpoint:** Animals are sacrificed on Day 2, 48 h after test article administration. Blood is collected by cardiac puncture and processed for plasma. Immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed and weighed separately: liver and spleen. The bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at -20°C or -80°C for archival purposes. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0375] **Termination:** Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0376] **Data Analysis:** Tolerability of treatment regime is monitored by animal appearance and behavior as well as body weight. Blood clinical chemistry is measured by automated analyzer. ApoB and GAPDH mRNA levels in liver are measured via QG assay. ApoB

protein in plasma is measured via ELISA. Total cholesterol in plasma is measured via standard enzymatic/colorimetric assay.

Results

[0377] There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. Figure 6 shows that the tolerability of SNALP prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters. There was a tolerability difference between syringe citrate and syringe PBS at constant siRNA dosage, but that was likely an artifact dependent on the different final lipid:drug (L:D) ratios of these two preparations.

[0378] Figure 7 shows that the efficacy of the 1:57 SNALP prepared by gear pump was similar to the same SNALP prepared by syringe press. The tolerability profile was improved with the gear pump process, which could be attributed to increased initial encapsulation rate and decreased final L:D ratio.

Example 8. *In vivo* Silencing of ApoB Expression Using 1:57 SNALP Prepared Via a Direct Dilution or In-Line Dilution Process.

[0379] This study illustrates a comparison of the tolerability and efficacy of the 1:57 SNALP formulation with ApoB-targeting siRNA as prepared by a direct dilution or in-line dilution process at an input lipid to drug ratio of 6:1 or 9:1.

Experimental Design

[0380] Animal Model: Female BALB/c mice, 7 wks old.

[0381] siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

CBC/Diff:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
1	3	PBS	-	-
2	3	1|57 SNALP (9:1)	7 mg/kg	71 mg/kg
3	3	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Clinical Chemistry:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
4	4	PBS	-	-
5	4	1|57 SNALP (9:1)	9 mg/kg	92 mg/kg
6	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg
7	4	(6:1) New 1|57 SNALP	11 mg/kg	78 mg/kg

8	4	(6:1) New 1|57 SNALP	13 mg/kg	93 mg/kg
9	4	(6:1) New 1|57 SNALP	15 mg/kg	107 mg/kg
10	4	(6:1) New 1|57 SNALP	17 mg/kg	121 mg/kg
11	4	1|57 SNALP (9:1)	11 mg/kg	112 mg/kg

Activity:

Group	# Mice	Test Article	IV Dosage	
			Encap. siRNA	Total Lipid
12	4	PBS	-	-
13	4	1|57 SNALP (9:1)	0.05 mg/kg	0.51 mg/kg
14	4	1|57 SNALP (9:1)	0.1 mg/kg	1.02 mg/kg
15	4	1|57 SNALP (9:1)	0.2 mg/kg	2.04 mg/kg
16	4	(6:1) New 1|57 SNALP	0.05 mg/kg	0.36 mg/kg
17	4	(6:1) New 1|57 SNALP	0.1 mg/kg	0.71 mg/kg
18	4	(6:1) New 1|57 SNALP	0.2 mg/kg	1.42 mg/kg
19	4	(6:1) New 1|57 SNALP	0.4 mg/kg	2.85 mg/kg

Formulation:

[0382] Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

[0383] Formulation Details:

1. "1|57 SNALP" used in this study is lipid composition 1.4:57.1:7.1:34.3 as described in molar percentages of PEG-C-DMA, DLinDMA, DPPC, and cholesterol (in that order). This formulation was prepared by gear pump at an input lipid to drug ratio of 9:1 (28 mM lipids) or 6:1 (14 mM lipids).
2. siRNA used in this study is apoB-10048 U2/2 G1/2 siRNA.

[0384] Formulation Summary:

	1|57 SNALP Gear PBS In-Line	Particle Size			Final L:D
		Zavg (nm)	Poly	% Encap	(mg:mg)
322-051407-1	Input 9:1	78	0.07	93	10.2
322-051407-2	Input 6:1	81	0.05	92	7.1

Procedures

[0385] **Treatment:** Just prior to the first treatment, animals are weighed and dose amounts are calculated based on the weight of individual animals (equivalent to 10 mL/kg, rounded to the nearest 10 μl). Test article is administered by IV injection through the tail vein once on Day 0 (1 dose total per animal). Body weight is measured daily (every 24 h) for the duration of the study. Cage-side observations are taken daily in concert with body weight measurements and additionally as warranted.

[0386] **Endpoint:** Animals are sacrificed on Day 1, 24 h after test article administration (Grps 1-10) or on Day 2, 48 h after test article administration (Grps 11-19).

[0387] **Groups 1-3:** Blood is collected by cardiac puncture upon sacrifice. Whole amount is collected into an EDTA microtainer, mixed immediately to prevent coagulation, and sent for analysis of CBC/Diff profile. Perform brief necropsy.

[0388] **Groups 4-11:** Blood is collected by cardiac puncture into a SST microtainer for serum. Clot for 30 (to 60) min at room temp., centrifuge for 5 min at 16,000xg & 16°C, invert to confirm centrifugation is complete, and store at 4°C. Analyze complete small-animal clinical chemistry panel plus AST and SDH. Top priority list: ALT, AST, SDH, Bilirubin, Alkaline Phosphatase, GGT, BUN, CPK, Glucose. Secondary priority list: Creatinine, Albumin, Globulin, Total Protein. Perform brief necropsy.

[0389] **Groups 12-19:** Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16,000xg (at 16°C). Record any observations of unusual plasma appearance. Pipette off clear plasma supernatant into a clean microfuge tube and store at -80°C. The following tissues are removed: liver. The liver is not weighed; the bottom (unattached) half of the left liver lobe is detached and submerged in ≥ 5 volumes of RNAlater (< 0.3 g in 1.5 mL RNAlater in 2.0 mL tube), stored at least 16 hours at 4°C prior to analysis and long term storage at –80°C. Formulations are expected to be well tolerated. Mice which exhibit signs of distress associated with the treatment are terminated at the discretion of the vivarium staff.

[0390] **Termination:** Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

[0391] **Data Analysis:** Tolerability of treatment regime is monitored by animal appearance and behavior, and body weight. Blood clinical chemistry and CBC/Diff profile is measured by automated analyzer. Liver ApoB mRNA is measured using the QuantiGene Assay. Plasma ApoB-100 is measured using ELISA. Plasma total cholesterol is measured using a standard enzymatic assay.

Results

Tolerability:

[0392] Figure 8 shows that there was very little effect on body weight 24 hours after 1:57 SNALP administration. The maximum weight loss of 3.6 ± 0.7% was observed at the highest drug dose of 17 mg/kg. There was also no obvious change in animal appearance/behavior at any of the dosages tested.

[0393] Figure 9 shows that there were no obvious changes in platelet count. Reduction of platelets can cause the mean platelet volume to increase as the body produces new platelets in compensation for the treatment-related decrease. Under the conditions of this study, the mean platelet volume did not change in SNALP-treated groups.

[0394] Figure 10 shows that clinically significant liver enzyme elevations (3xULN) occurred at drug dosages of 11 mg/kg for 1:57 SNALP at a lipid:drug (L:D) ratio of 10, and at 13 mg/kg at a L:D of 7. A slight dose response trend upwards in plasma total protein and globulin was also observed.

Efficacy:

[0395] Figure 11 shows that based on the liver mRNA QuantiGene analysis, the potency of the lower L:D SNALP was as good as that of the higher L:D SNALP at the tested drug dosages. In fact, the ApoB silencing activity was identical at the 0.05 and 0.1 mg/kg dosages. As such, the potency of the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) was similar to the potency of the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) at reducing ApoB expression.

[0396] Figure 12 shows that ApoB protein and total cholesterol levels were reduced to a similar extent by the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1).

Therapeutic Index:

[0397] This study demonstrates that both the 1:57 SNALP at a 6:1 input L:D ratio (final ratio of 7:1) and the 1:57 SNALP at a 9:1 input L:D ratio (final ratio of 10:1) caused about 60% ApoB liver mRNA silencing with a drug dose of 0.1 mg/kg. Interpolating from the available data points in Figure 10, a 10:1 final L:D ratio at 10 mg/kg may cause a similar degree of enzyme elevation as a 7:1 final L:D ratio at 13 mg/kg. Using these activity and toxicity points, the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio is (10 mg/kg) / (0.1 mg/kg) = 100 and the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is (13 mg/kg) / (0.1 mg/kg) = 130. Using this dataset, the therapeutic index for the 1:57 SNALP at a 7:1 final L:D ratio is 30% greater than the therapeutic index for the 1:57 SNALP at a 10:1 final L:D ratio.

Example 9. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Increases Survival of Hep3B Tumor-Bearing Mice.

[0398] SNALP containing polo-like kinase 1 (PLK-1) siRNA (1:57 SNALP formulation: 1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) were tested for their effects on the survival of CD1 nu/nu mice bearing Hep3B liver tumors. PLK-1 is a serine/threonine kinase containing two functional domains: (1) a kinase domain; and (2) a polo-box domain (*see, e.g.*, Barr *et al.*, *Nat. Rev. Mol. Cell Biol.*, 5:429-440 (2004)). The activity and cellular concentration of PLK-1 are crucial for the precise regulation of cell division. PLK-1 is overexpressed in many cancer types including hepatoma and colon cancer, and PLK-1 expression often correlates with poor patient prognosis. Overexpression of PLK-1 (wild-type or kinase inactive) results in multinucleation (genetic instability). Hyperactive PLK-1 overrides the DNA damage checkpoint. Constitutive PLK-1 expression causes transformation of NIH 3T3 cells. PLK-1 phosphorylates the p53 tumor suppressor, thereby inhibiting the pro-apoptotic effects of p53. The PLK-1 siRNA used in this study are provided in Table 8. The modifications involved introducing 2'OMe-uridine or 2'OMe-guanosine at selected positions in the sense and antisense strands of the PLK-1 siRNA sequence, in which the siRNA duplex contained less than about 20% 2'OMe-modified nucleotides.

Table 8. siRNA duplexes comprising sense and antisense PLK-1 RNA polynucleotides.

siRNA	PLK-1 siRNA Sequence		% Modified in DS Region
PLK1424 U4/GU	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3'	(SEQ ID NO.57)	6/38 = 15.8%
	3'-NNUC**U**AGUGGGAG**G**AAUUUAU-5'	(SEQ ID NO.54)	
PLK1424 U4/G	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3'	(SEQ ID NO.57)	7/38 = 18.4%
	3'-NNUCUAGUGG**G**AG**G**AAUUUAU-5'	(SEQ ID NO.56)	

Column 1: The number after "PLK" refers to the nucleotide position of the 5' base of the sense strand relative to the start codon (ATG) of the human PLK-1 mRNA sequence NM_005030. Column 2: 2'-O-methyl (2'OMe) nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. N = deoxythymidine (dT) nucleotide, uridine (U) ribonucleotide, or ribonucleotide having complementarity to the target sequence or the complementary strand thereof. Column 3: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

Experimental Groups

[0399] 20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay

A	20 to seed	I.H. $1.5x10^6$ Hep3B	Luc 1:57	9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10 x 2 mg/kg	When moribund	Survival Body Weights
B			PLK 1424 1:57	9				

Test Articles

[0400] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 20 ml of each SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	Luc U/U SNALP 1:57 (28mM lipid)
B	PLK1424 U4/GU SNALP 1:57 (28mM lipid) PLK1424 U4/G SNALP 1:57 (28mM lipid)

Procedures

Day 0 Mice will receive Anafen by SC injection (100 µg in 20 µl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25 µl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 µl) and 30G (3/8") needle. Cells will be injected slowly (~ 30 s) and a swab applied to the puncture wound immediately after needle withdrawal. After any bleeding has stopped (~1 min), the incision is closed with 5-6 sutures in the muscle wall and 3-4 skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.

Day 1	All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 μg in 20 μl saline).
Day 10	Mice will be randomized into the appropriate treatment groups.
Day 11	**Groups A, B – Day 11:** All Animals will be administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg). Dosing will be repeated for 5 consecutive days based on initial weight.
Day 14-35	**Groups A, B – Days 14, 17, 21, 25, 28, 32, 35:** All Animals will be re-administered SNALP at 2 mg/kg by IV injection via the lateral tail vein. Mice will be dosed according to body weight (10 ml/kg). **Body weights Groups:** Mice will be weighed on the day of dosing for 5 weeks, then twice weekly until close of the study. **Endpoint:** Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the treatment or tumor burden are terminated at the discretion of the vivarium staff.
Termination:	Mice are anesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.
Data Analysis:	Survival and body weights are assayed.

Results

[0401] Figure 13 shows the mean body weights of mice during therapeutic dosing of PLK1424 SNALP in the Hep3B intrahepatic (I.H.) tumor model. The treatment regimen was well tolerated with no apparent signs of treatment-related toxicity.

[0402] Figure 14 shows that treatment with 1:57 SNALP-formulated PLK1424 caused a significant increase in the survival of Hep3B tumor-bearing mice. This *in vivo* anti-tumor effect was observed in the absence of any apparent toxicity or immune stimulation.

Example 10. *In vivo* Silencing of PLK-1 Expression Using 1:57 SNALP Induces Tumor Cell Apoptosis in Hep3B Tumor-Bearing Mice.

[0403] The objectives of this study were as follows:

1. To determine the level of mRNA silencing in established Hep3B liver tumors following a single IV administration of PLK1424 SNALP.
2. To confirm the mechanism of mRNA silencing by detecting specific RNA cleavage products using RACE-PCR.
3. To confirm induction of tumor cell apoptosis by histopathology.

[0404] The 1:57 SNALP formulation (1.4% PEG-cDMA; 57.1% DLinDMA; 7.1% DPPC; and 34.3% cholesterol) was used for this study.

Experimental Groups

[0405] 20 SCID/beige mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	Sacrifice	Assay
A	20 to seed	I.H. $1x10^6$ Hep3B	PBS	6	1 x 2 mg/kg Day 20	24 h after treatment	Tumor QG Tumor RACE-PCR Histopathology
			Luc 1:57	7			
B			PLK 1424 1:57	7			
C							

Test Articles

[0406] All samples were filter-sterilized prior to dilution to working concentration. All tubes were labeled with the formulation date, lipid composition, and nucleic acid concentration. SNALP samples were provided at 0.2 mg/ml nucleic acid. A minimum of 2 ml of SNALP was required to perform the study. Formulations for this study contained:

Group	Test Article Description
A	PBS
B	Luc U/U 1:57 SNALP
C	PLK1424 U4/GU 1:57 SNALP

Procedures

Day 0 Mice will receive Anafen by SC injection (100 µg in 20 µl saline) immediately prior to surgery. Individual mice are anesthetized by isoflourane gas inhalation and eye lube applied to prevent excessive eye drying. While maintained under gas anesthesia from a nose cone, a single 1.5 cm incision across the midline will be made below the

sternum. The left lateral hepatic lobe is then exteriorized using an autoclaved cotton wool bud. 25µl of tumor cells suspended in PBS is injected into the lobe at a shallow angle using a leur tip Hamilton syringe (50 µl) and 30G (3/8") needle. Cells will be injected slowly (~30 s) and a swab applied to the puncture wound immediately after needle withdrawal. After any bleeding has stopped (~1 min), the muscle wall incision is closed with 5-6 sutures. The skin incision is then closed with 3-4 metal skin clips. Cell suspensions will be thoroughly mixed immediately prior to each injection. Mice will recover from anesthesia in a clean cage lined with paper towel and monitored closely for 2-4 hours. Animals are then returned to normal housing.

Day 1 All mice will be lightly anesthetized by isoflourane gas and the sutures examined. Animals will then receive Anafen by SC injection (100 µg in 20 µl saline).

Day 7 Mice will be randomized into the appropriate treatment groups.

Day 20 **Groups A-C:** Mice will be weighed and then administered either PBS, Luc, or PLK1424 SNALP by IV injection via the lateral tail vein. SNALP will be dosed at 2 mg/kg or equivalent volume (10 ml/kg) according to body weight.

Day 21 **Groups A-C**: All mice will be weighed and then euthanized by lethal anesthesia.

Tumor bearing liver lobes from all mice in each group will be weighed and collected into RNALater for RNA analysis.

Endpoint: Tumor burden and formulations are expected to be well tolerated. Mice that exhibit signs of distress associated with the treatment or tumor burden are terminated at the discretion of the vivarium staff.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine followed by cervical dislocation.

Data Analysis: mRNA analysis of liver tumors by bDNA (QG) assay and RACE-PCR.

Tumor cell apoptosis by histopathology.

Results

[0407] Body weights were monitored from Day 14 onwards to assess tumor progression. On Day 20, 6 mice showing greatest weight loss were randomized into each of the 3 groups and treated. All six mice had substantial-large I.H. tumors at sacrifice (Day 21). Treatment of the remaining 14 mice was therefore initiated on the Day 21 (sacrifice Day 22). 10/14 mice had substantial tumors; 2/14 mice had small/probable tumors; and 2/14 mice had no visible tumor burden.

[0408] Figure 15 shows data from Quantigene assays used to measure human (tumor)-specific PLK-1 mRNA levels. A single 2 mg/kg dose of 1:57 SNALP reduced PLK-1 mRNA levels by about 50% in intrahepatic Hep3B tumors growing in mice.

[0409] Figure 16 shows that a specific cleavage product of PLK-1 mRNA was detectable in mice treated with PLK1424 SNALP by 5' RACE-PCR. No specific PCR product was detectable in mice treated with either PBS or control (Luc) SNALP. Nucleotide sequencing of the PCR product confirmed the predicted cleavage site by PLK1424 siRNA-mediated RNA interference in the PLK-1 mRNA.

[0410] Figure 17 shows Hep3B tumor histology in mice treated with either Luc SNALP (top) or PLK1424 SNALP (bottom). Luc SNALP-treated mice displayed normal mitoses in Hep3B tumors, whereas PLK1424 SNALP-treated mice exhibited numerous aberrant mitoses and tumor cell apoptosis in Hep3B tumors.

Conclusion

[0411] This example illustrates that a single administration of PLK1424 1:57 SNALP to Hep3B tumor-bearing mice induced significant *in vivo* silencing of PLK-1 mRNA. This reduction in PLK-1 mRNA was confirmed to be mediated by RNA interference using 5' RACE-PCR analysis. Importantly, PLK-1 mRNA silencing by the 1:57 SNALP formulation profoundly disrupted tumor cell proliferation (mitosis), causing subsequent apoptosis of tumor cells. As demonstrated in the previous example, this anti-tumor effect translated into extended survival times in the tumor-bearing mice.

Example 11. Comparison of 1:57 PLK-1 SNALP Containing Either PEG-cDMA or PEG-cDSA in a Subcutaneous Hep3B Tumor Model.

[0412] This example demonstrates the utility of the PEG-lipid PEG-cDSA (3-N-[(-Methoxypoly(ethylene glycol)2000)carbamoyl]-1,2-distearyloxypropylamine) in the 1:57 formulation for systemically targeting distal (*e.g.*, subcutaneous) tumors. In particular, this example compares the tumor targeting ability of 1:57 PLK-1 SNALPs containing either PEG-cDMA (C_{14}) or PEG-cDSA (C_{18}). Readouts are tumor growth inhibition and PLK1 mRNA silencing. The PLK-1 siRNA used was PLK1424 U4/GU, the sequence of which is provided in Table 8.

[0413] Subcutaneous (S.C.) Hep3B tumors were established in scid/beige mice. Multidose anti-tumor efficacy of 1:57 PLK-1 SNALP was evaluated for the following groups (n=5 for each group): (1) "Luc-cDMA" - PEG-cDMA Luc SNALP; (2) "PLK-cDMA" - PEG-cDMA PLK-1 SNALP; and (3) "PLK-cDSA" - PEG-cDSA PLK-1 SNALP. Administration of 6 x 2mg/kg siRNA was initiated once tumors reached about 5 mm in diameter (Day 10). Dosing was performed on Days 10, 12, 14, 17, 19, and 21. Tumors were measured by caliper twice weekly.

[0414] Figure 18 shows that multiple doses of 1:57 PLK-1 SNALP containing PEG-cDSA induced the regression of established Hep3B S.C. tumors. In particular, 5/5 tumors in the PLK1-cDSA treated mice appeared flat, measurable only by discoloration at the tumor site.

[0415] Figure 19 shows the mRNA silencing of 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration. The extent of silencing observed with the PLK1-cDSA SNALP correlated with the anti-tumor activity in the multi-dose study shown in Figure 18.

[0416] The Luc-cDMA SNALP-treated group, which had developed large S.C. tumors at Day 24, were then administered PLK-cDSA SNALP on Days 24, 26, 28, 31, 33, and 35. There was no additional dosing of the original PLK-1 SNALP-treated groups. The results from this crossover dosing study with large established tumors is provided in Figure 20, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

[0417] A comparison of the effect of PEG-cDMA and PEG-cDSA 1:57 SNALPs on PLK-1 mRNA silencing was performed using established intrahepatic Hep3B tumors in scid/beige mice. A single 2 mg/kg dose of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA was administered intravenously. Liver/tumor samples were collected at 24 and 96 hours after SNALP treatment. Control = 2 mg/kg Luc-cDMA SNALP at 24 hours.

[0418] Figure 21 shows that PLK-cDMA SNALP and PLK-cDSA SNALP had similar silencing activities after 24 hours, but that the PLK-cDSA SNALP may increase the duration of mRNA silencing in intrahepatic tumors.

[0419] Figure 22 shows the blood clearance profile of 1:57 PLK-1 SNALP containing either PEG-cDMA or PEG-cDSA. The extended blood circulation times observed for the PLK-cDSA SNALP may enable the increased accumulation and activity at distal (*e.g.*, subcutaneous) tumor sites.

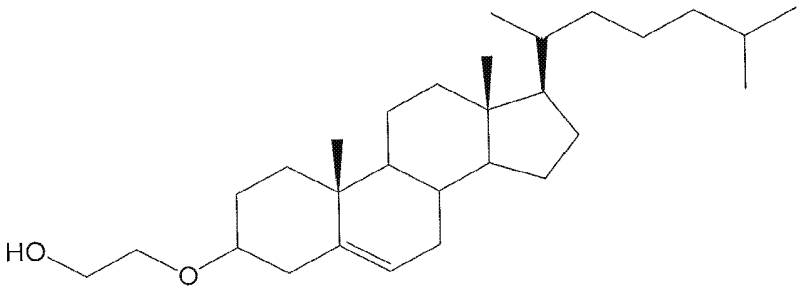
[0420] Thus, this study shows that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP can be used to preferentially target the liver.

Example 12. Synthesis of Cholesteryl-2'-Hydroxyethyl Ether.

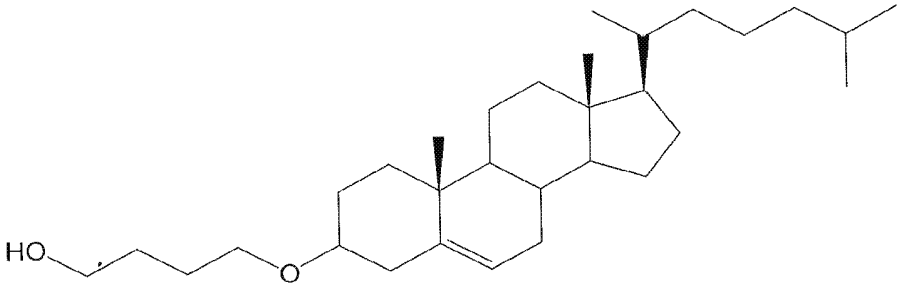
[0421] Step 1: A 250 ml round bottom flask containing cholesterol (5.0 g, 12.9 mmol) and a stir bar was sealed and flushed with nitrogen. Toluenesulphonyl chloride (5.0 g, 26.2 mmol) was weighed into a separate 100-mL round bottom flask, also sealed and flushed with nitrogen. Anhydrous pyridine (2 x 50 ml) was delivered to each flask. The toluenesulphonyl chloride solution was then transferred, via cannula, into the 250 ml flask, and the reaction stirred overnight. The pyridine was removed by rotovap, and methanol (80 ml) added to the residue. This was then stirred for 1 hour until a homogeneous suspension was obtained. The suspension was filtered, washed with acetonitrile (50 ml), and dried under vacuum to yield cholesteryl tosylate as a fluffy white solid (6.0 g, 86%).

[0422] Step 2: Cholesteryl tosylate (2.0 g, 3.7 mmol), 1,4-dioxane (50 mL), and ethylene glycol (4.6 g, 74 mmol) were added to a 100 ml flask containing a stir bar. The flask was fitted with a condenser, and refluxed overnight. The dioxane was then removed by rotovap, and the reaction mixture suspended in water (100 ml). The solution was transferred to a separating funnel and extracted with chloroform (3 x 100 ml). The organic phases were combined, washed with water (2 x 150 ml), dried over magnesium sulphate, and the solvent removed. The crude product was purified by column chromatography (5% acetone/hexane) to yield the product as a white solid (1.1 g, 69%).

[0423] The structures of the cholesterol derivatives cholesteryl-2'-hydroxyethyl ether and cholesteryl-4'-hydroxybutyl ether are as follows:



Cholesteryl-2'-hydroxyethyl ether



Cholesteryl-4'-hydroxybutyl ether

[0424] It is to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reading the above description. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent applications, patents, PCT publications, and Genbank Accession Nos., are incorporated herein by reference for all purposes.

WHAT IS CLAIMED IS:

1. A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from about 50 mol % to about 85 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising from about 13 mol % to about 49.5 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

2. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid comprises a small interfering RNA (siRNA).

3. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises from about 15 to about 60 nucleotides.

4. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one modified nucleotide.

5. The nucleic acid-lipid particle of claim 2, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

6. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), or a mixture thereof.

7. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA).

8. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle.

9. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from about 52 mol % to about 62 mol % of the total lipid present in the particle.

10. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises cholesterol or a derivative thereof.

11. The nucleic acid-lipid particle of claim 10, wherein the cholesterol or derivative thereof comprises from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle.

12. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a phospholipid.

13. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.

14. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

15. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises from about 4 mol % to about 10 mol % of the total lipid present in the particle and the cholesterol comprises from about 30 mol % to about 40 mol % of the total lipid present in the particle.

16. The nucleic acid-lipid particle of claim 13, wherein the phospholipid comprises from about 10 mol % to about 30 mol % of the total lipid present in the particle and the cholesterol comprises from about 10 mol % to about 30 mol % of the total lipid present in the particle.

17. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

18. The nucleic acid-lipid particle of claim 17, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

19. The nucleic acid-lipid particle of claim 18, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

20. The nucleic acid-lipid particle of claim 19, wherein the PEG has an average molecular weight of about 2,000 daltons.

21. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from about 1 mol % to about 2 mol % of the total lipid present in the particle.

22. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid in the nucleic acid-lipid particle is not substantially degraded after incubation of the particle in serum at 37°C for 30 minutes.

23. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

24. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a lipid:nucleic acid mass ratio of from about 5 to about 15.

25. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a median diameter of from about 40 nm to about 150 nm.

26. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.

27. A nucleic acid-lipid particle comprising:

(a) an siRNA;

(b) a cationic lipid comprising from about 56.5 mol % to about 66.5 mol % of the total lipid present in the particle;

(c) cholesterol or a derivative thereof comprising from about 31.5 mol % to about 42.5 mol % of the total lipid present in the particle; and

(d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

28. The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises DLinDMA.

29. The nucleic acid-lipid particle of claim 27, wherein the cationic lipid comprises DLin-K-C2-DMA.

30. The nucleic acid-lipid particle of claim 27, wherein the PEG-lipid conjugate comprises a PEG-DAA conjugate.

31. The nucleic acid-lipid particle of claim 27, wherein the nucleic acid-lipid particle comprises about 61.5 mol % cationic lipid, about 36.9% cholesterol or a derivative thereof, and about 1.5 mol % PEG-lipid conjugate.

32. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 27 and a pharmaceutically acceptable carrier.

33. A nucleic acid-lipid particle, comprising:

(a) an siRNA;

(b) a cationic lipid comprising from about 52 mol % to about 62 mol % of the total lipid present in the particle;

(c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 36 mol % to about 47 mol % of the total lipid present in the particle; and

(d) a PEG-lipid conjugate comprising from about 1 mol % to about 2 mol % of the total lipid present in the particle.

34. The nucleic acid-lipid particle of claim 33, wherein the cationic lipid comprises DLinDMA.

35. The nucleic acid-lipid particle of claim 33, wherein the cationic lipid comprises DLin-K-C2-DMA.

36. The nucleic acid-lipid particle of claim 33, wherein the phospholipid comprises DPPC.

37. The nucleic acid-lipid particle of claim 33, wherein the PEG-lipid conjugate comprises a PEG-DAA conjugate.

38. The nucleic acid-lipid particle of claim 33, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 7.1 mol % phospholipid, about 34.3 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

39. The nucleic acid-lipid particle of claim 33, wherein the nucleic acid-lipid particle comprises about 57.1 mol % cationic lipid, about 20 mol % phospholipid, about 20 mol % cholesterol or a derivative thereof, and about 1.4 mol % PEG-lipid conjugate.

40. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 33 and a pharmaceutically acceptable carrier.

41. A method for introducing a nucleic acid into a cell, the method comprising:

contacting the cell with a nucleic acid-lipid particle of claim 1, 27, or 33.

42. The method of claim 41, wherein the cell is in a mammal.

43. A method for the *in vivo* delivery of a nucleic acid, the method comprising:

administering to a mammalian subject a nucleic acid-lipid particle of claim 1, 27, or 33.

44. The method of claim 43, wherein the administration is selected from the group consisting of oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal.

45. A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising:

administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 1, 27, or 33.

46. The method of claim 45, wherein the disease or disorder is selected from the group consisting of a viral infection, a liver disease or disorder, and cancer.

ABSTRACT OF THE DISCLOSURE

The present invention provides novel, stable lipid particles comprising one or more active agents or therapeutic agents, methods of making the lipid particles, and methods of delivering and/or administering the lipid particles. More particularly, the present invention provides stable nucleic acid-lipid particles (SNALP) comprising a nucleic acid (such as one or more interfering RNA), methods of making the SNALP, and methods of delivering and/or administering the SNALP.

65524864

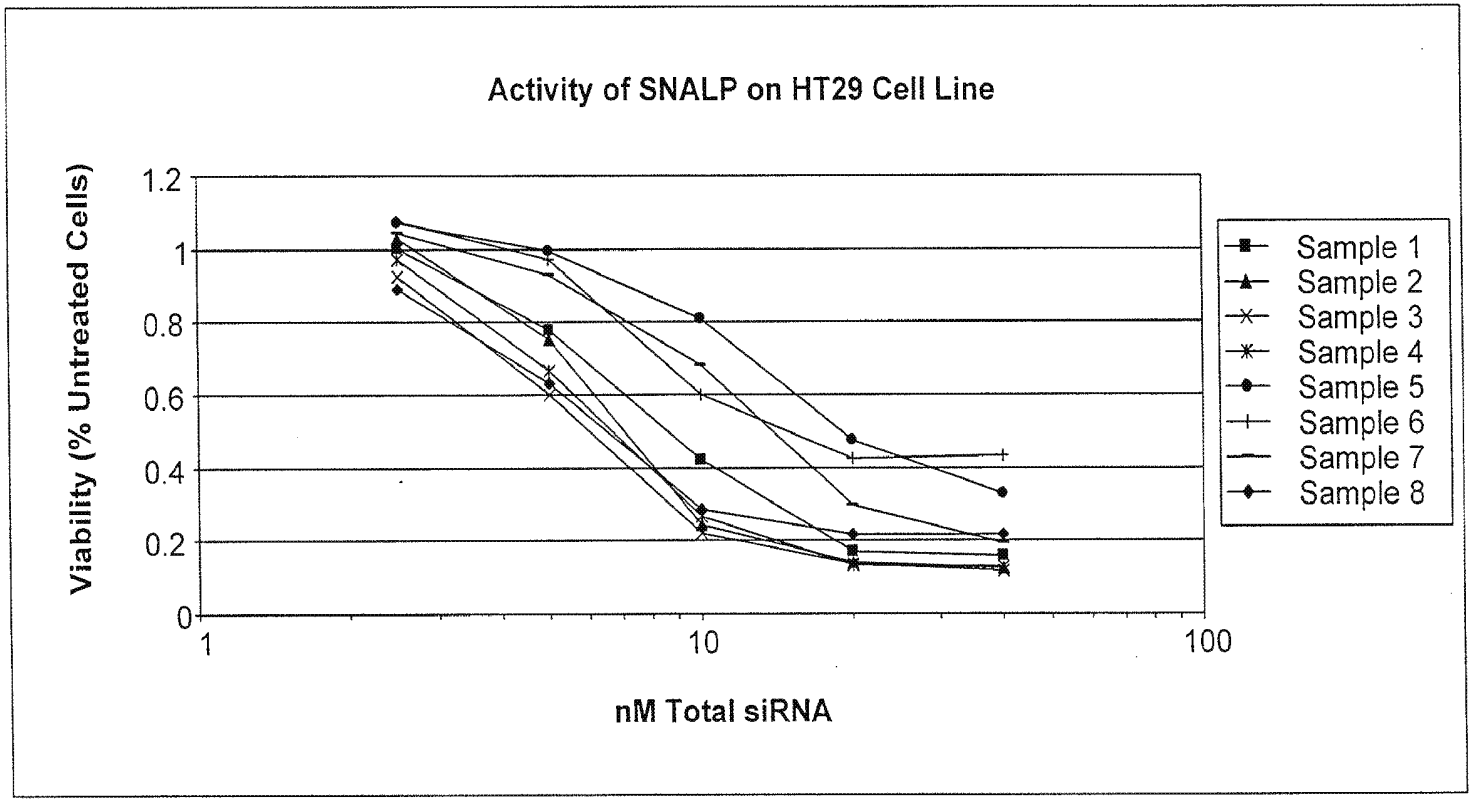


FIG. 1A

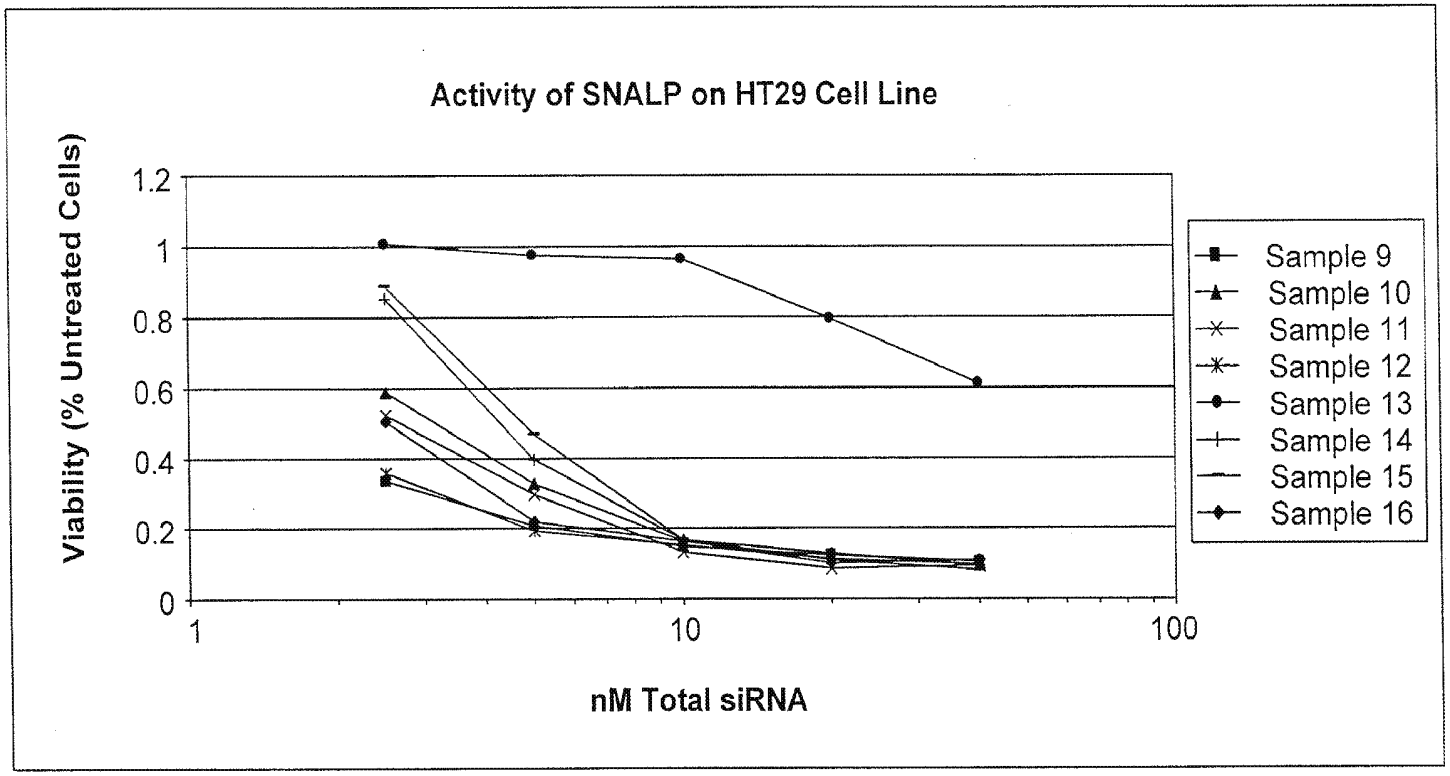

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 9
Sample 10
Sample 11
Sample 12
Sample 13
Sample 14
Sample 15
Sample 16

FIG. 1B

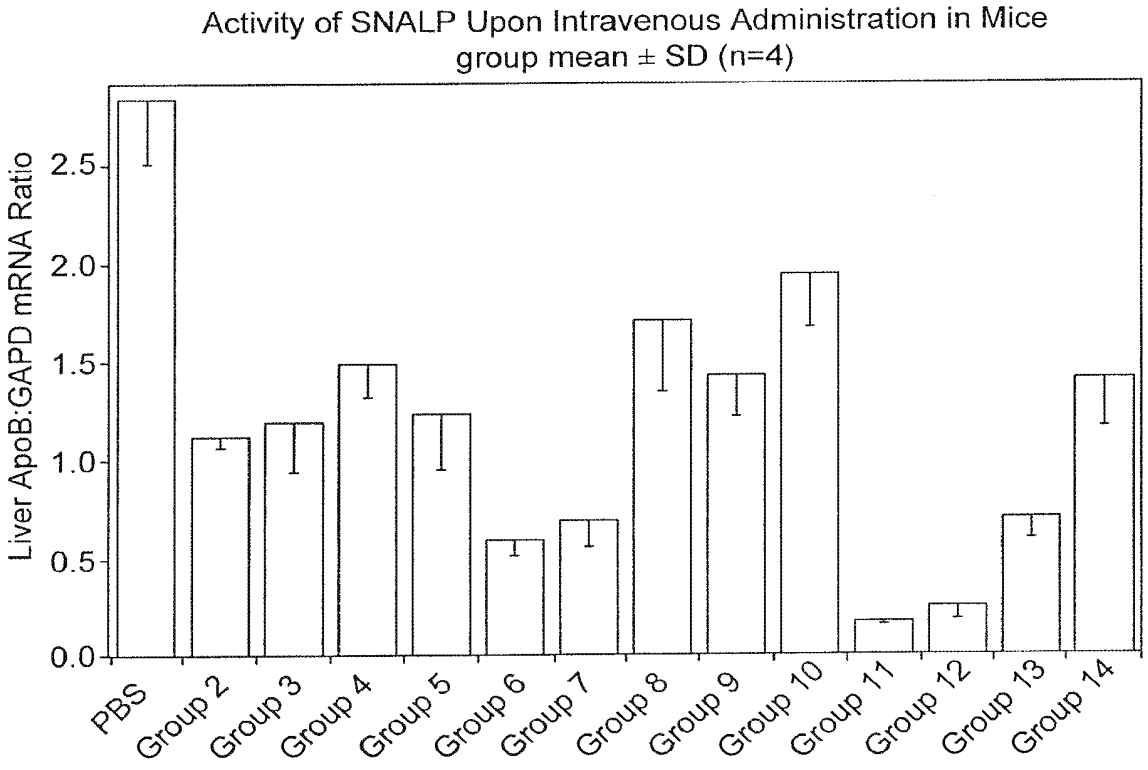

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
2.5
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

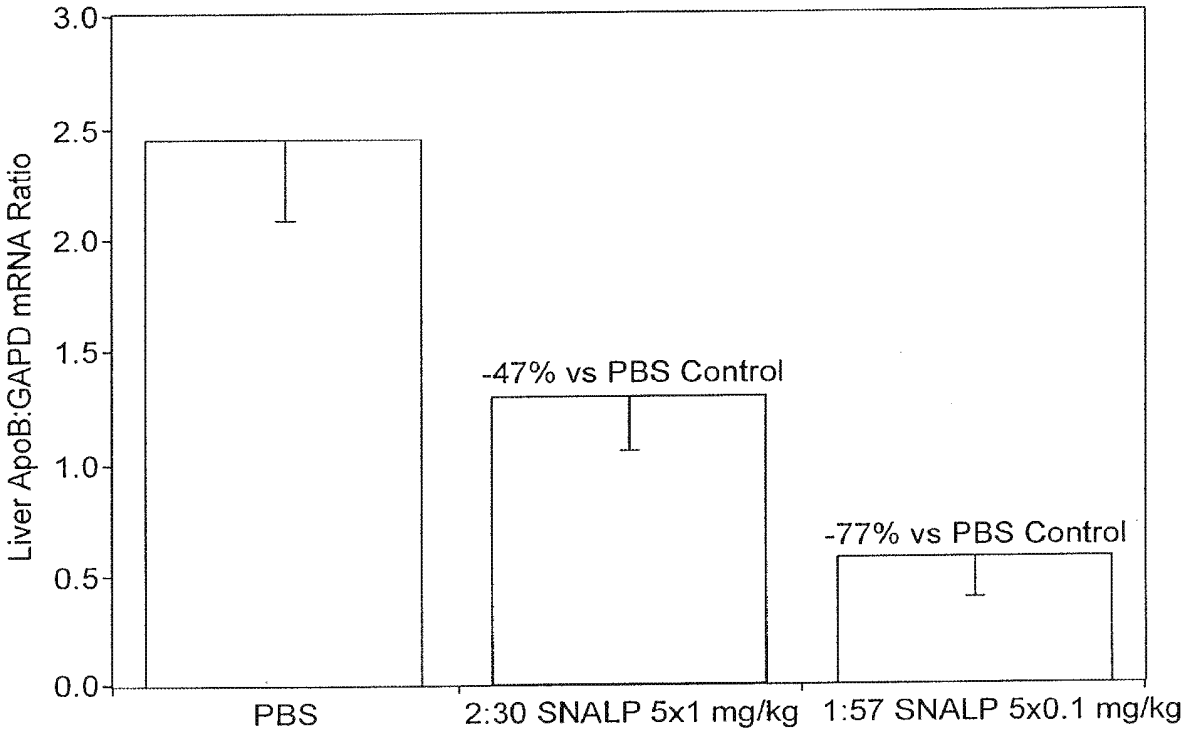

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Liver ApoB:GAPD mRNA Ratio
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5x1 mg/kg
1:57 SNALP 5x0.1 mg/kg

FIG. 3

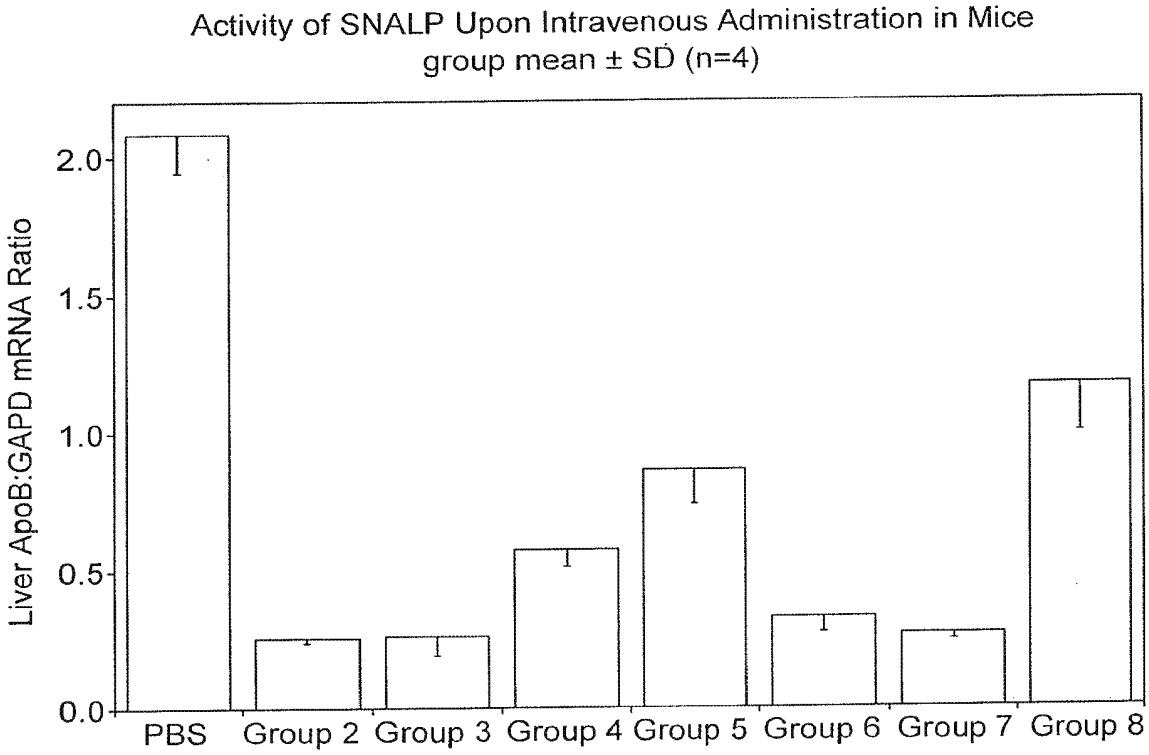

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

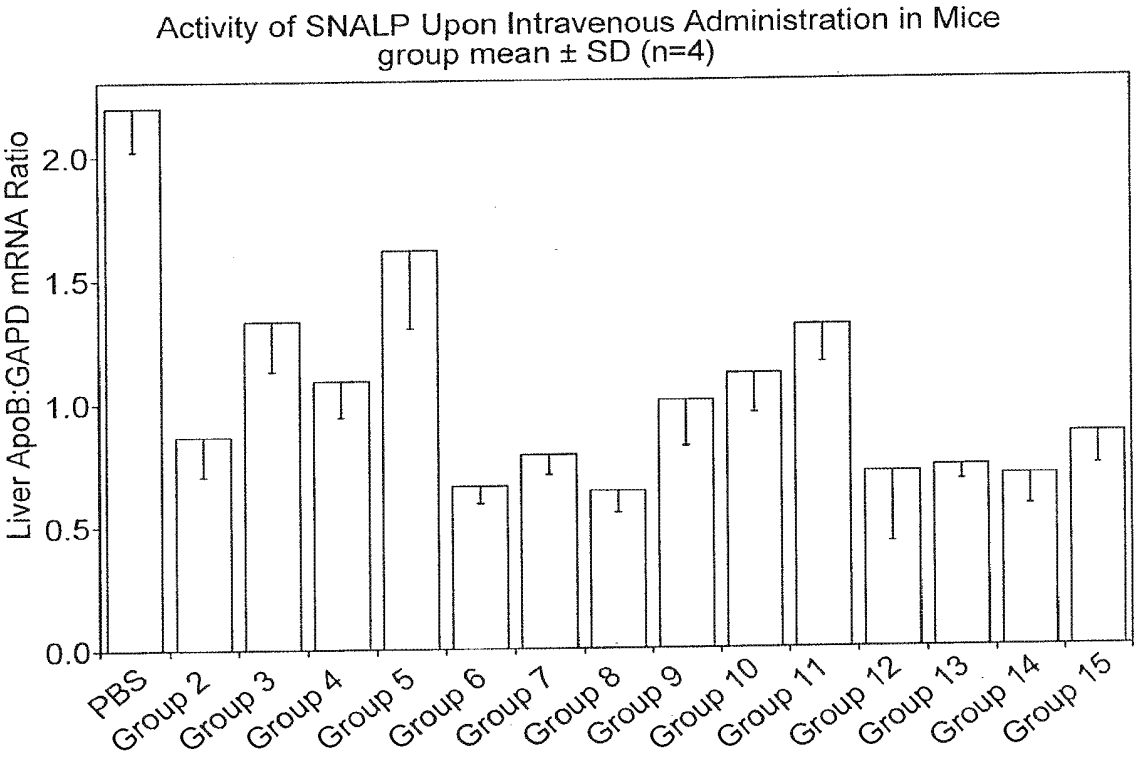

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

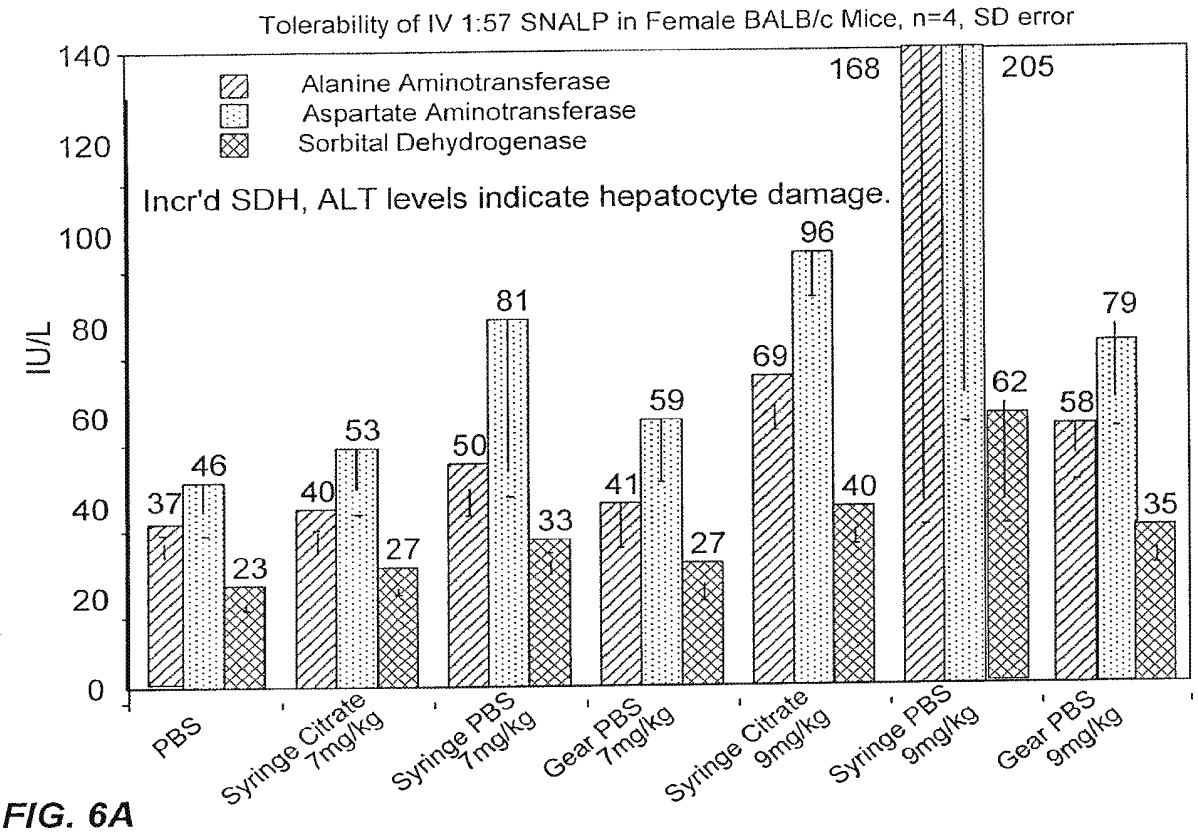

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
140
120
100
80
60
40
20
0
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
168
205
62
58
79
35
PBS
Syringe Citrate 7mg/kg
Syringe PBS 7mg/kg
Gear PBS 7mg/kg
Syringe Citrate 9mg/kg
Syringe PBS 9mg/kg
Gear PBS 9mg/kg

FIG. 6A

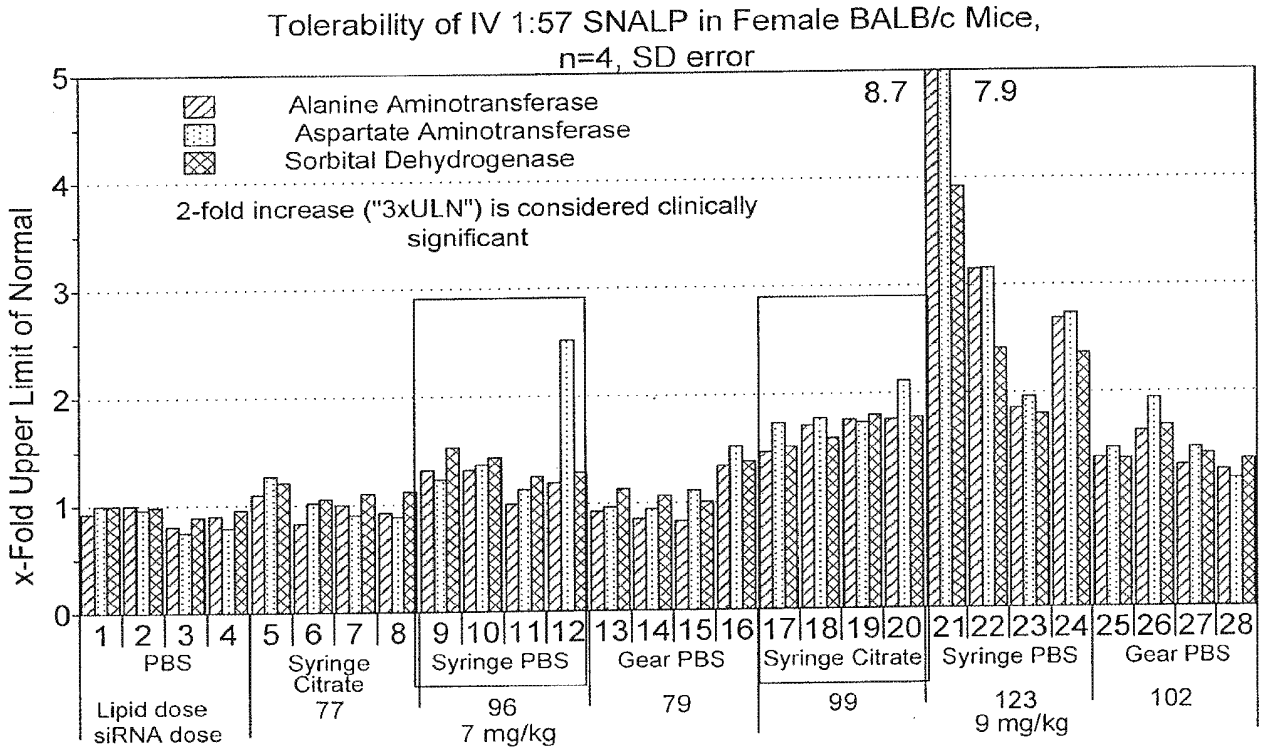

Tolerability of IV 1:57 SNALP in Female BALB/c Mice,
n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
5
4
3
2
1
0
8.7
7.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
PBS
Syringe Citrate
Syringe PBS
Gear PBS
Syringe Citrate
Syringe PBS
Gear PBS
Lipid dose
siRNA dose
77
96
7 mg/kg
79
99
123
9 mg/kg
102

FIG. 6B

FIG. 7A

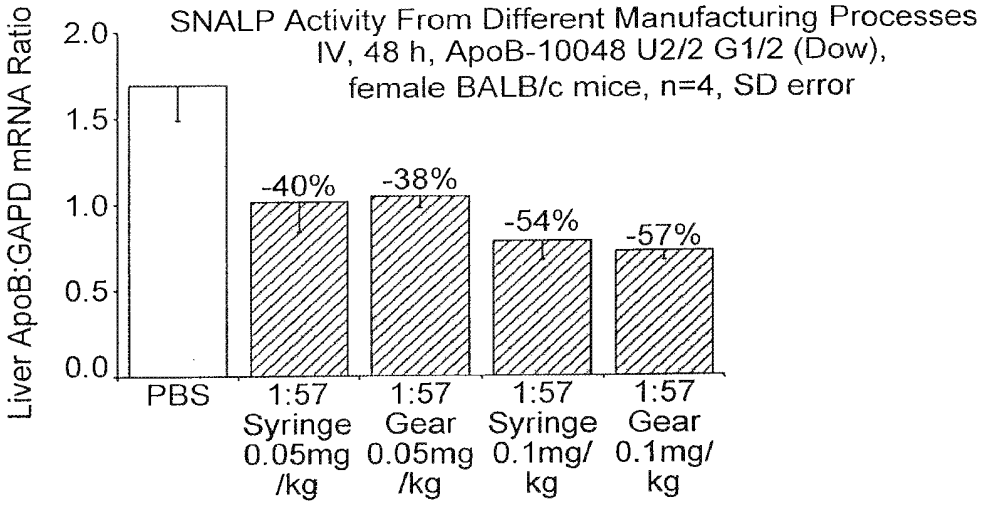


FIG. 7B

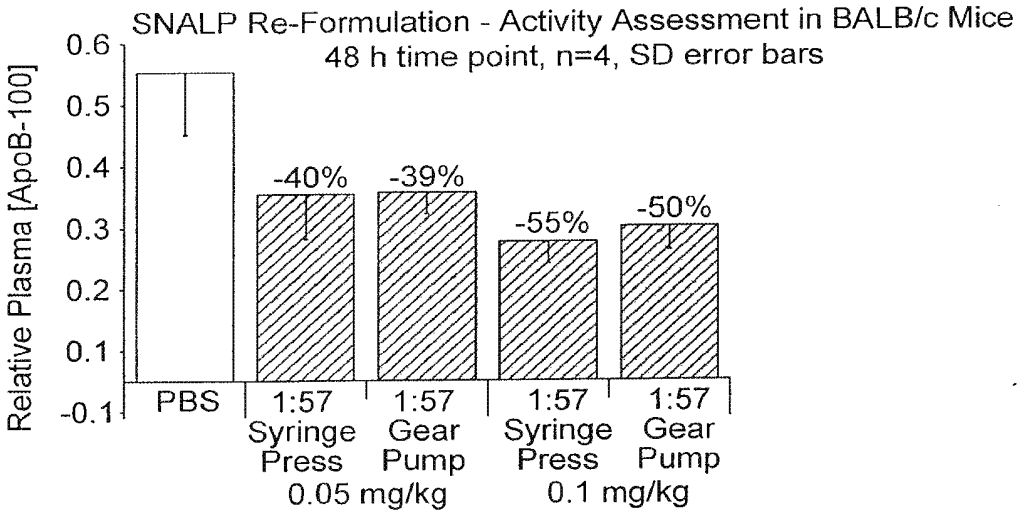
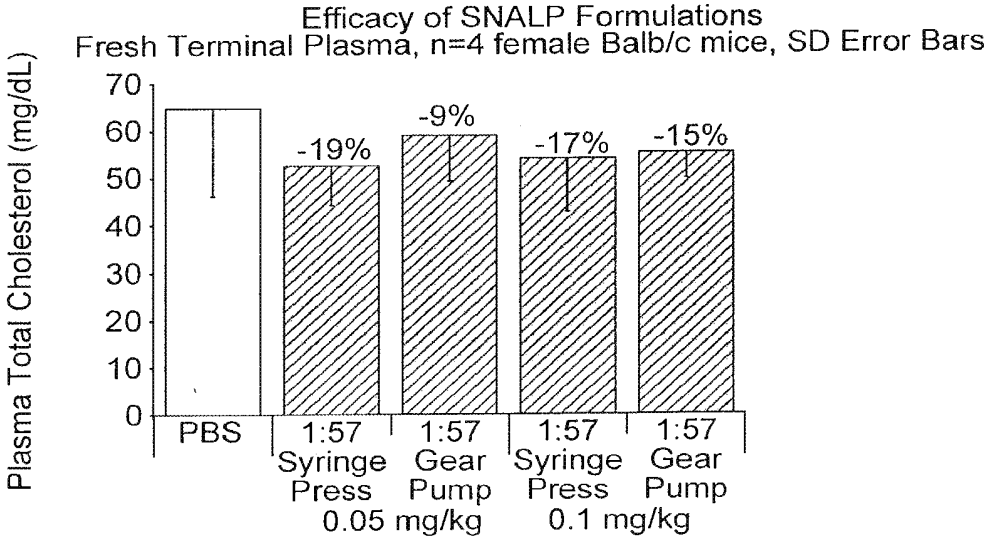


FIG. 7C



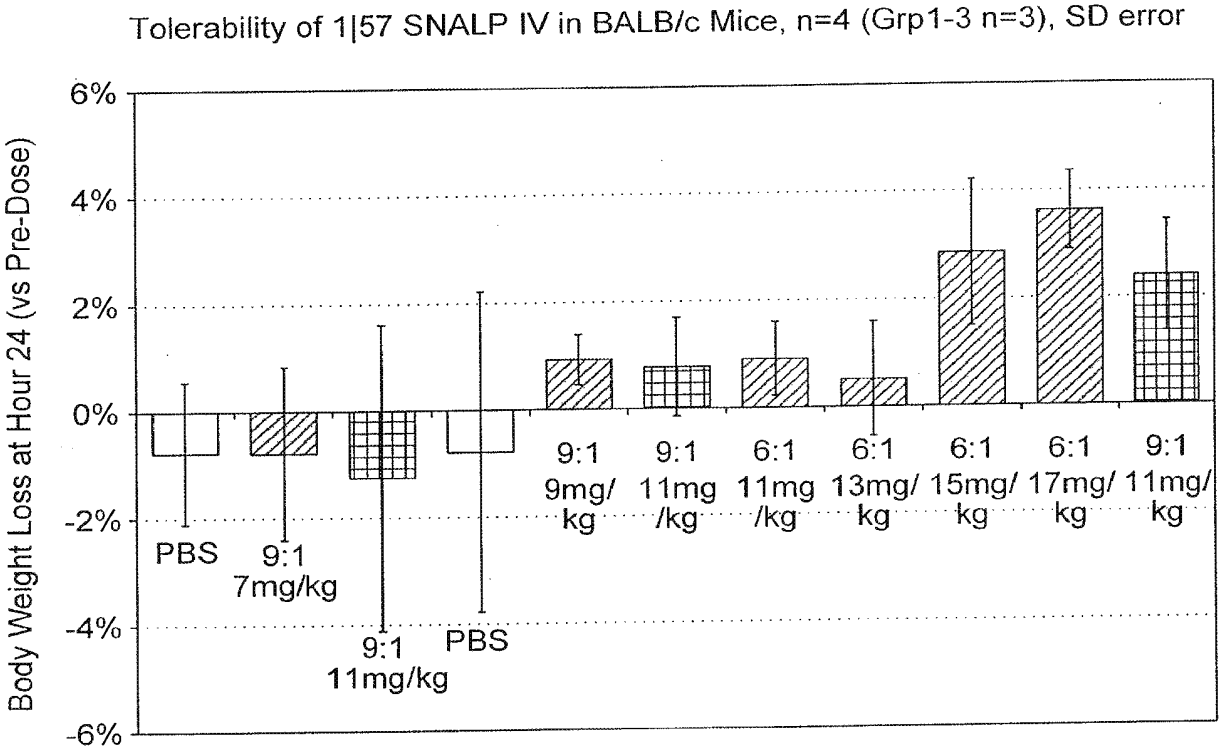

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

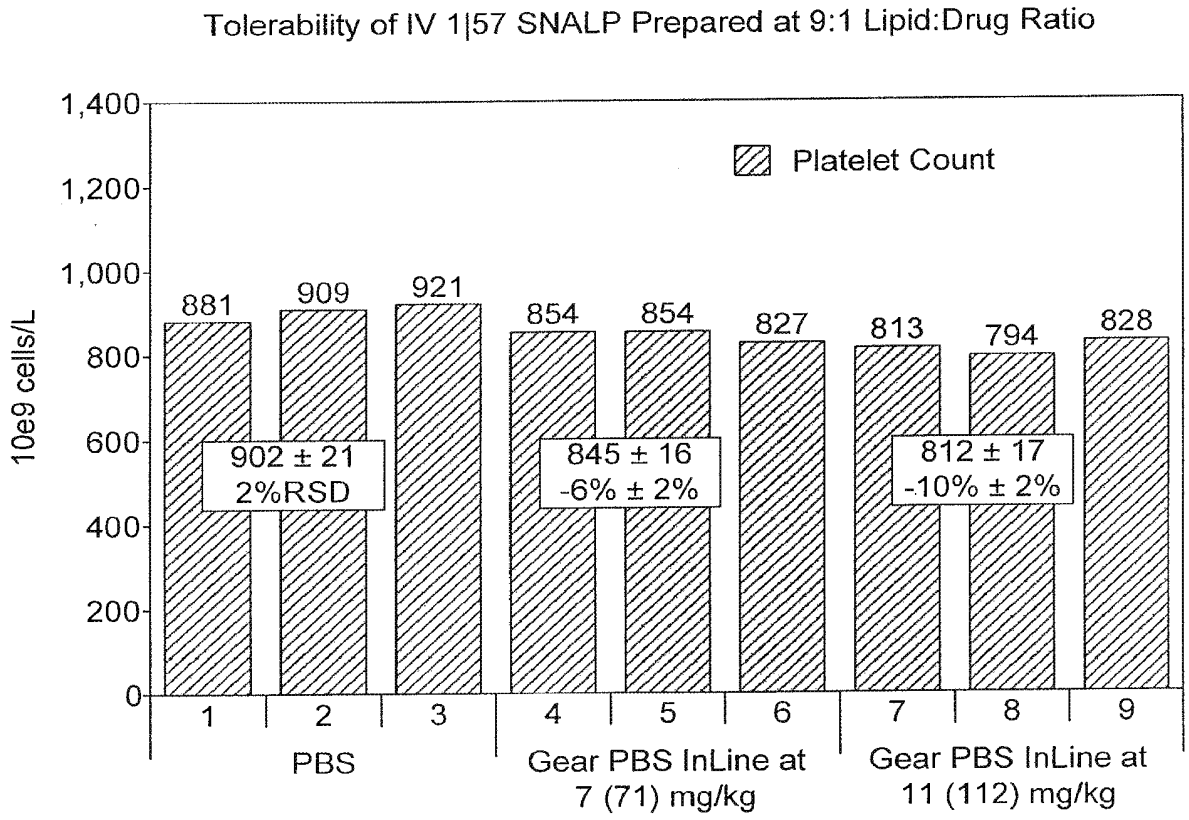

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
Platelet Count
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at
7 (71) mg/kg
Gear PBS InLine at
11 (112) mg/kg

FIG. 9

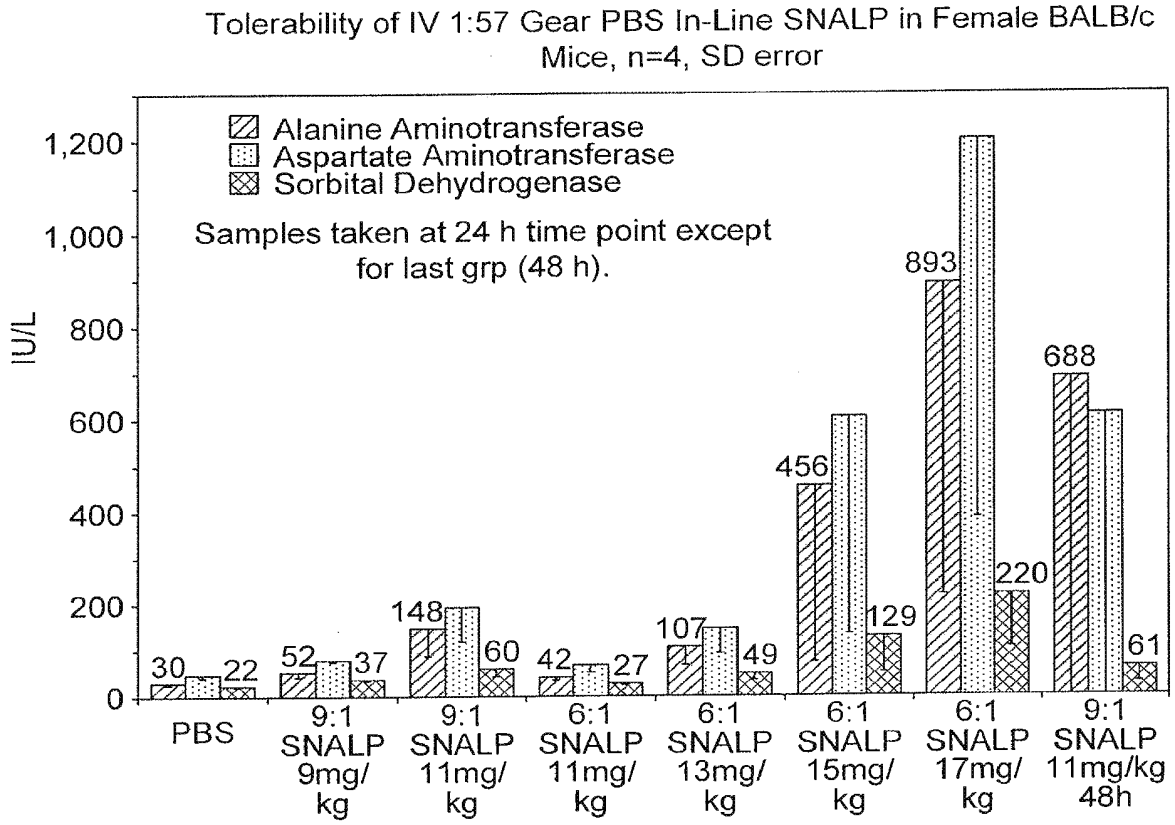

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
688
61
PBS
9:1 SNALP 9mg/kg
9:1 SNALP 11mg/kg
6:1 SNALP 11mg/kg
6:1 SNALP 13mg/kg
6:1 SNALP 15mg/kg
6:1 SNALP 17mg/kg
9:1 SNALP 11mg/kg 48h

FIG. 10A

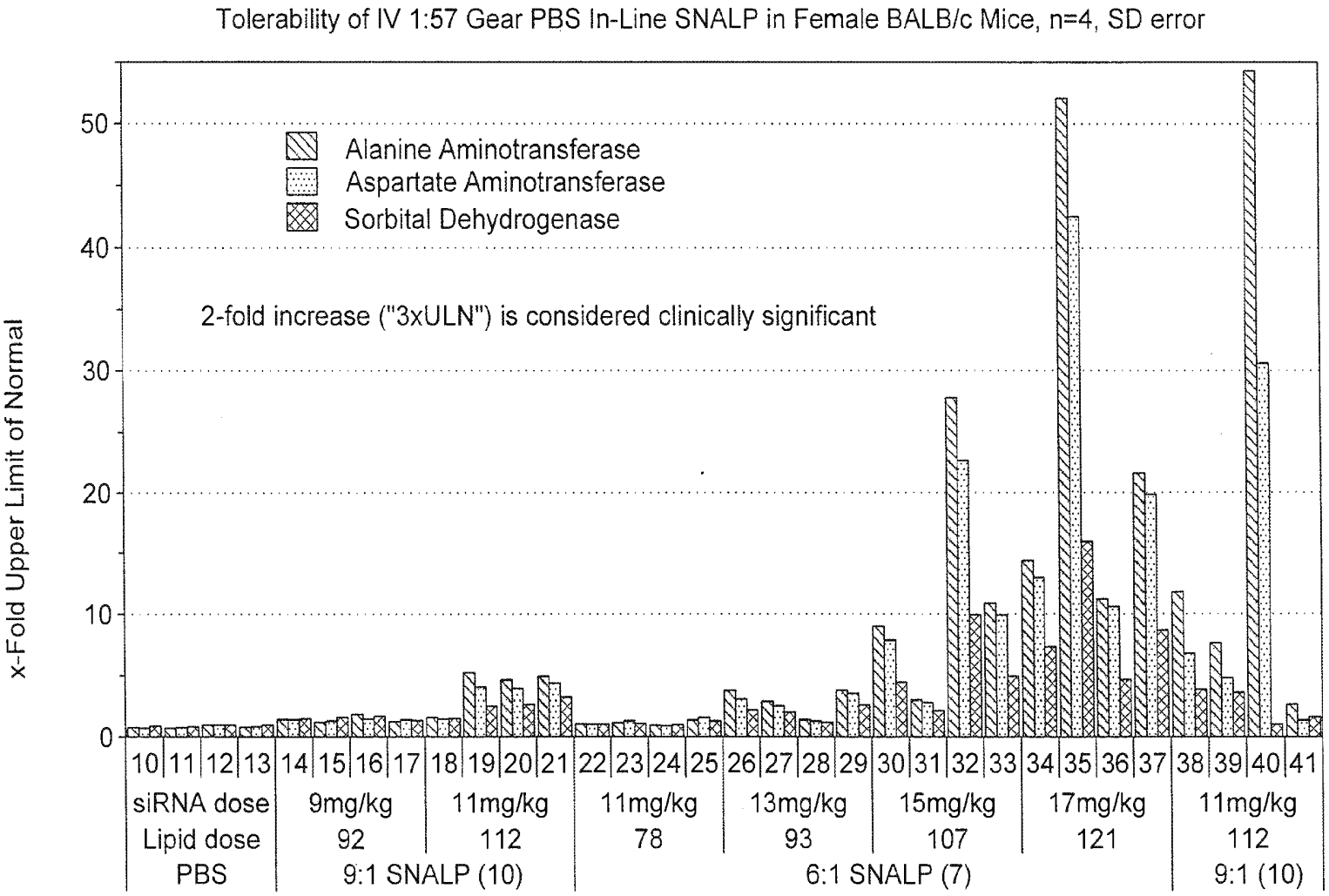

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
x-Fold Upper Limit of Normal
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
0
10
20
30
40
50
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41
siRNA dose
Lipid dose
PBS
9mg/kg
92
9:1 SNALP (10)
11mg/kg
112
11mg/kg
78
13mg/kg
93
15mg/kg
107
17mg/kg
121
6:1 SNALP (7)
11mg/kg
112
9:1 (10)

FIG. 10B

FIG. 11A

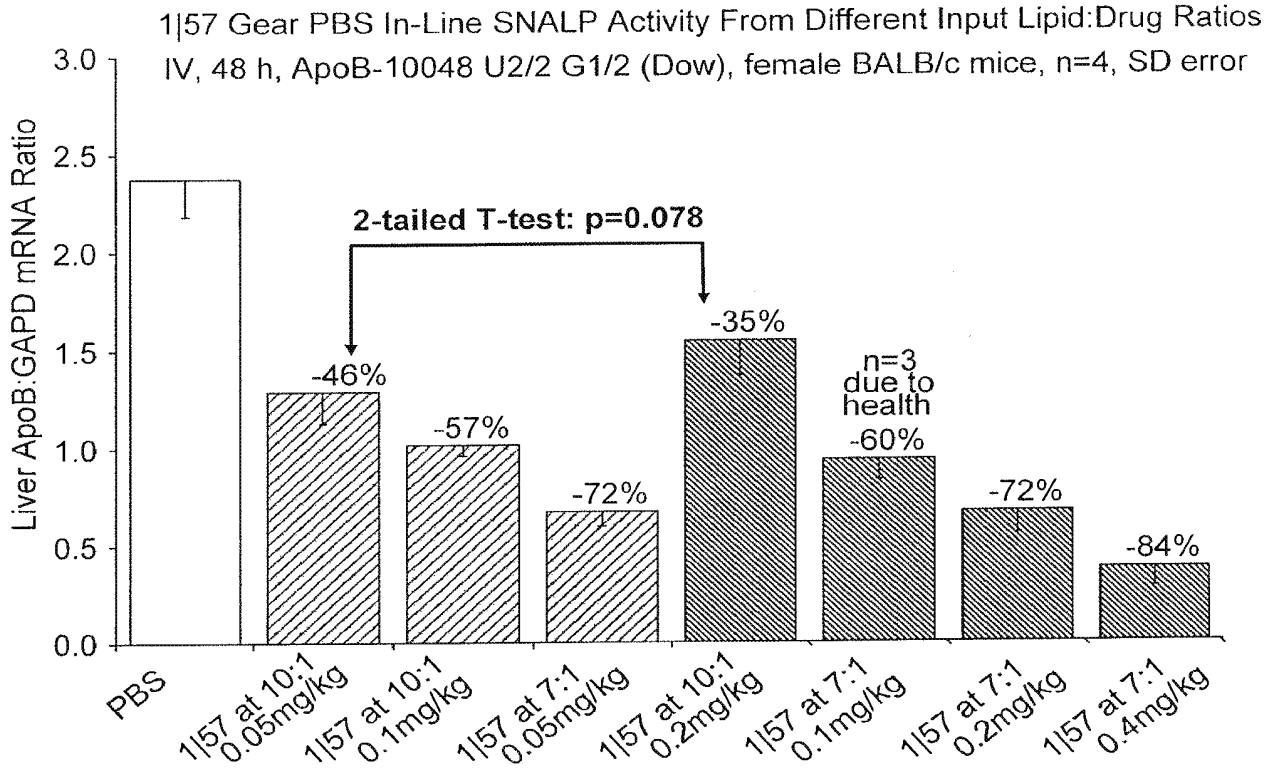
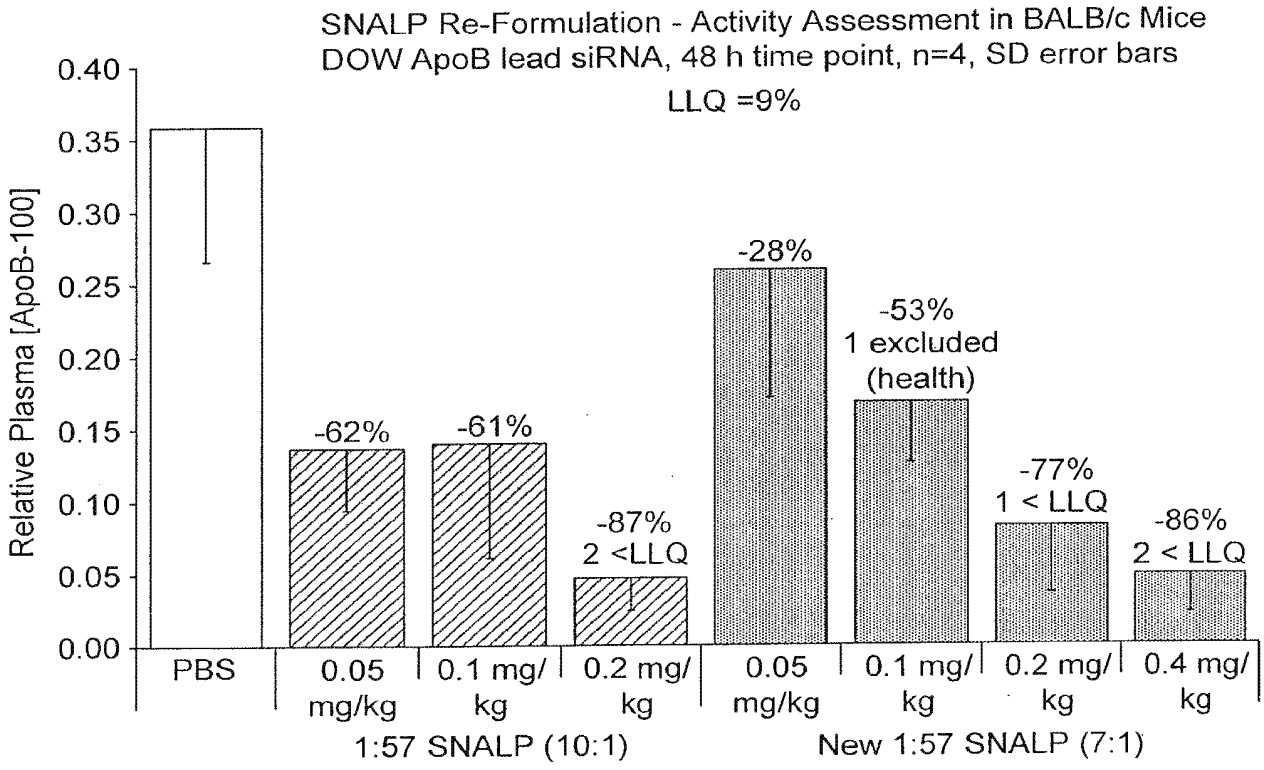


FIG. 11B



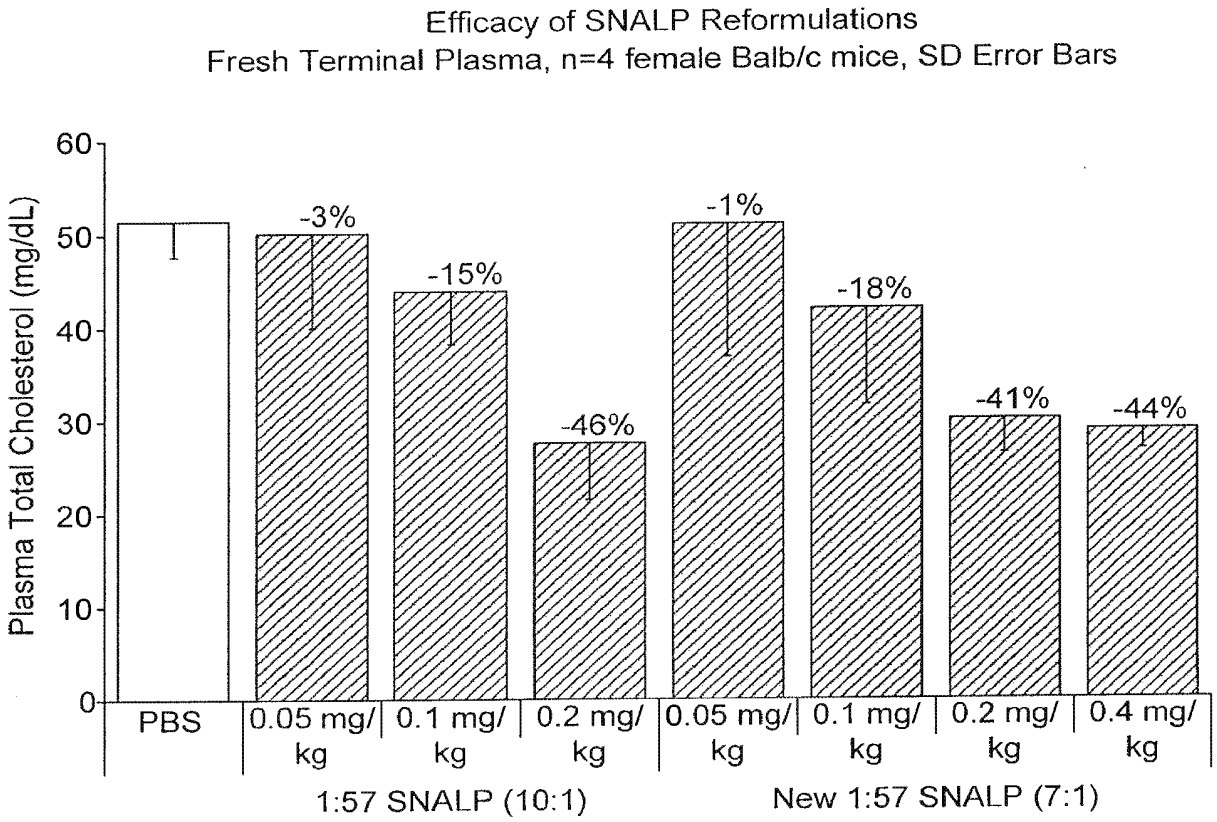

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 12

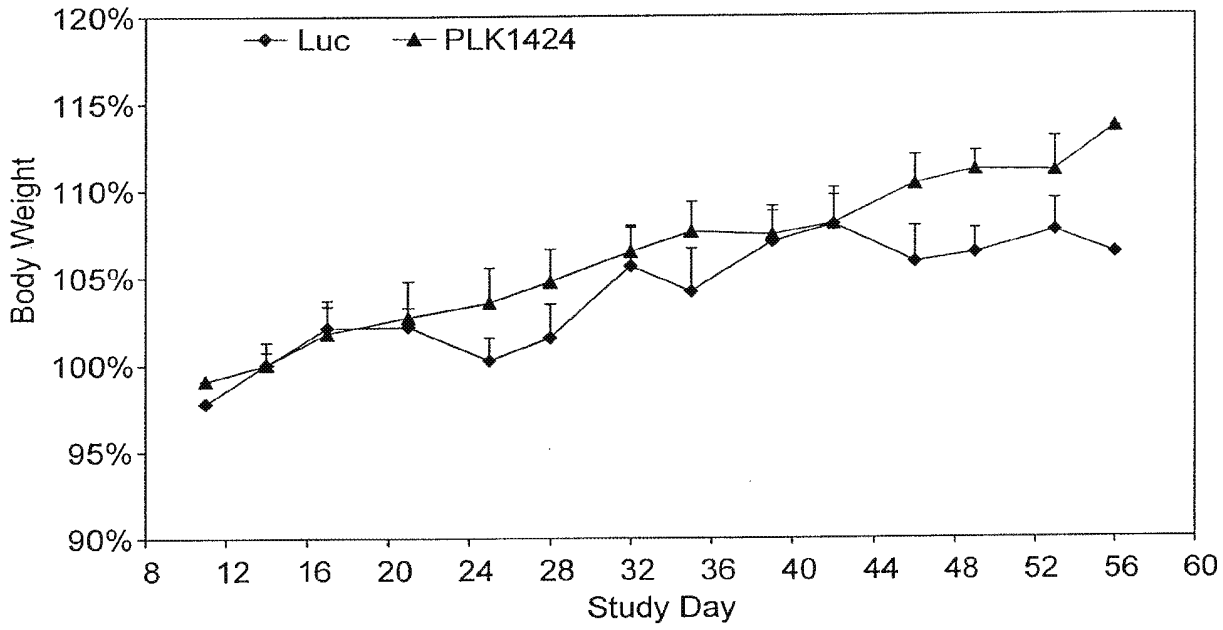

Luc
PLK1424
Body Weight
120%
115%
110%
105%
100%
95%
90%
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

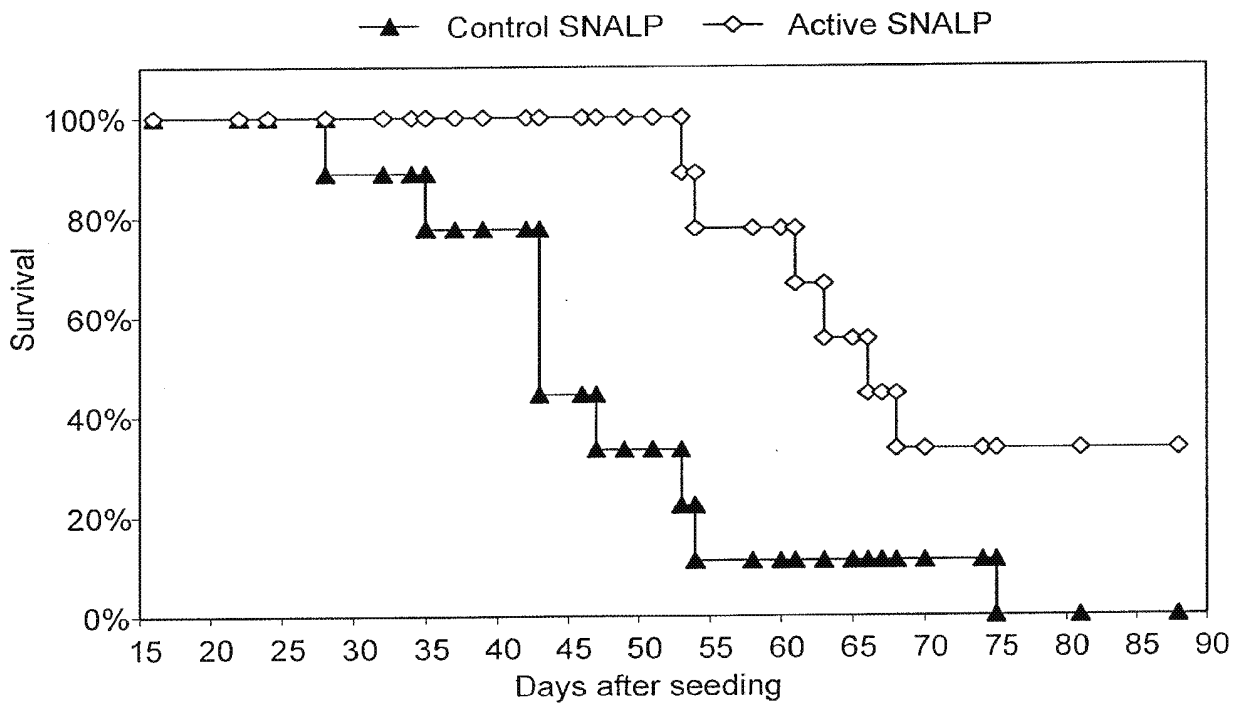

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
Days after seeding

FIG. 14

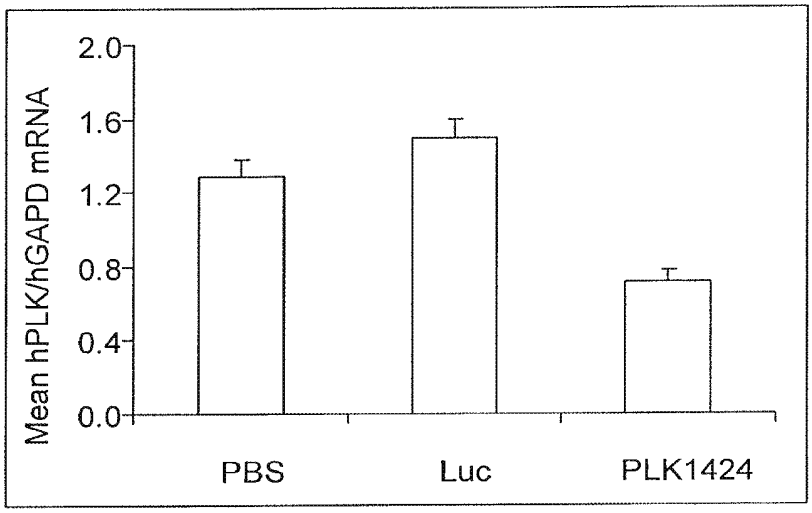


FIG. 15

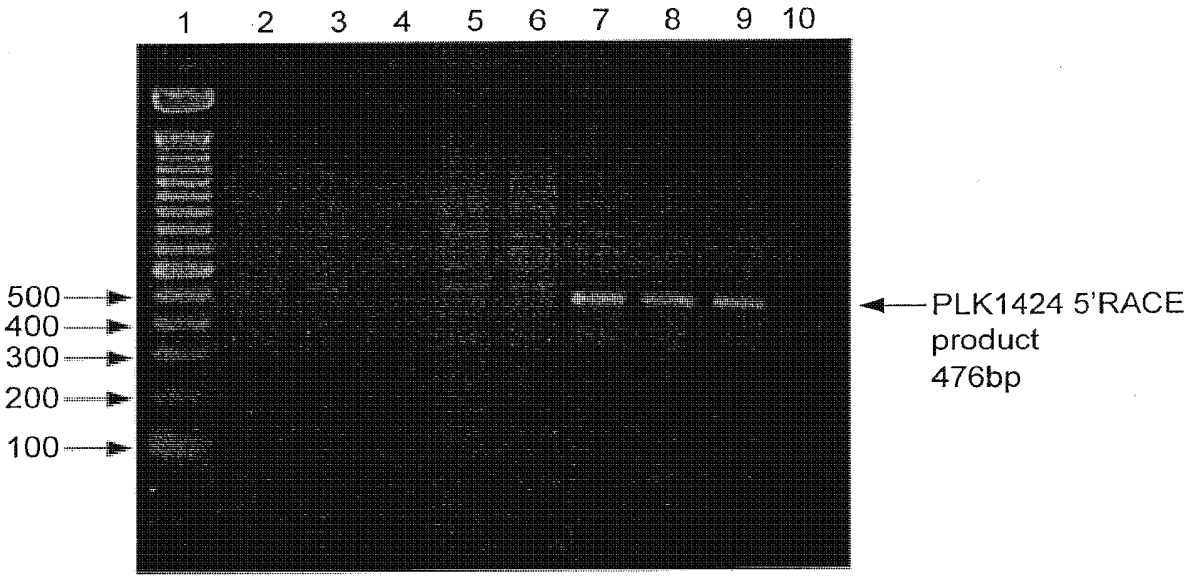
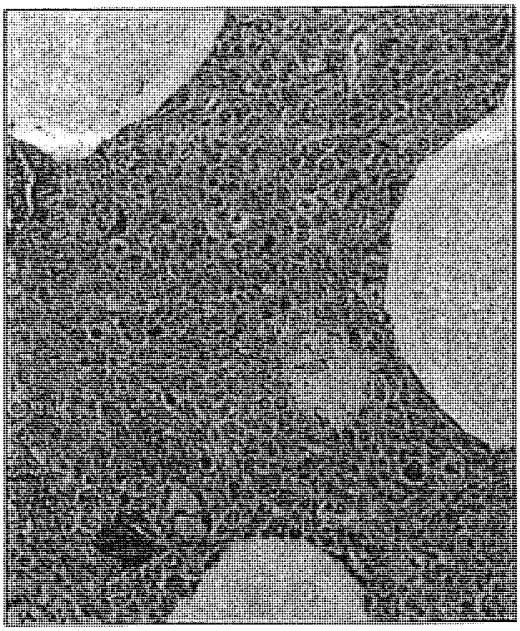
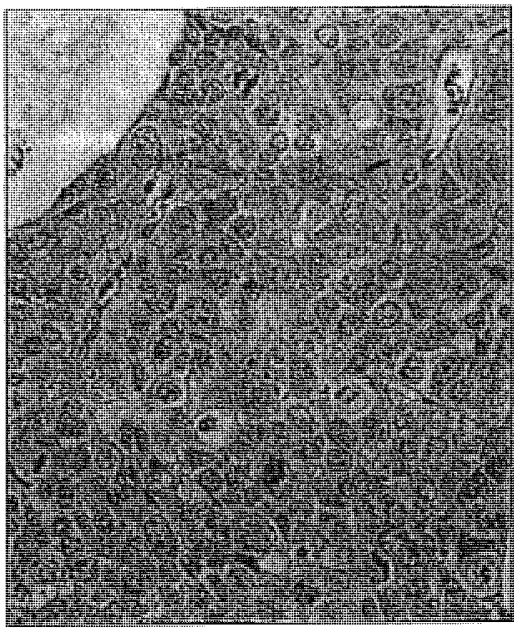

1
2
3
4
5
6
7
8
9
10
500
400
300
200
100
PLK1424 5'RACE
product
476bp

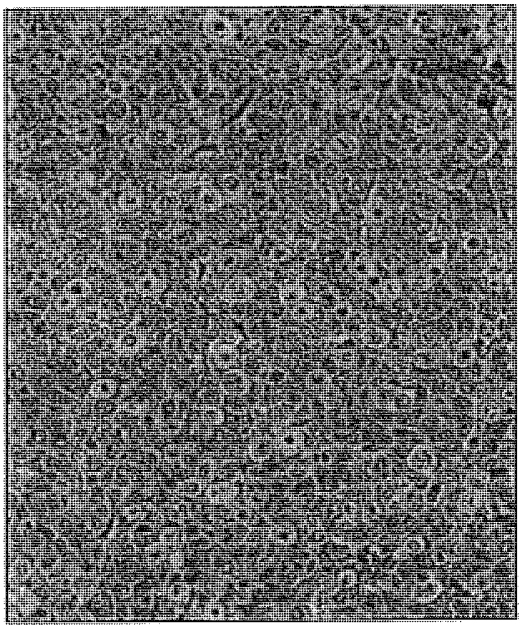
FIG. 16



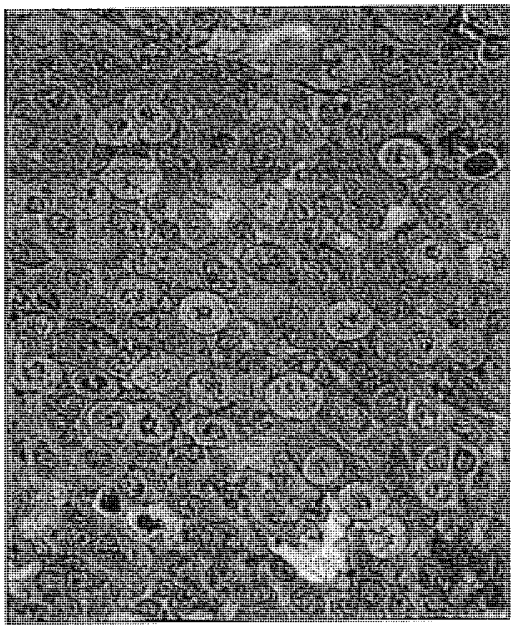
x200 mag



x400 mag



x200 mag



x400 mag

FIG. 17

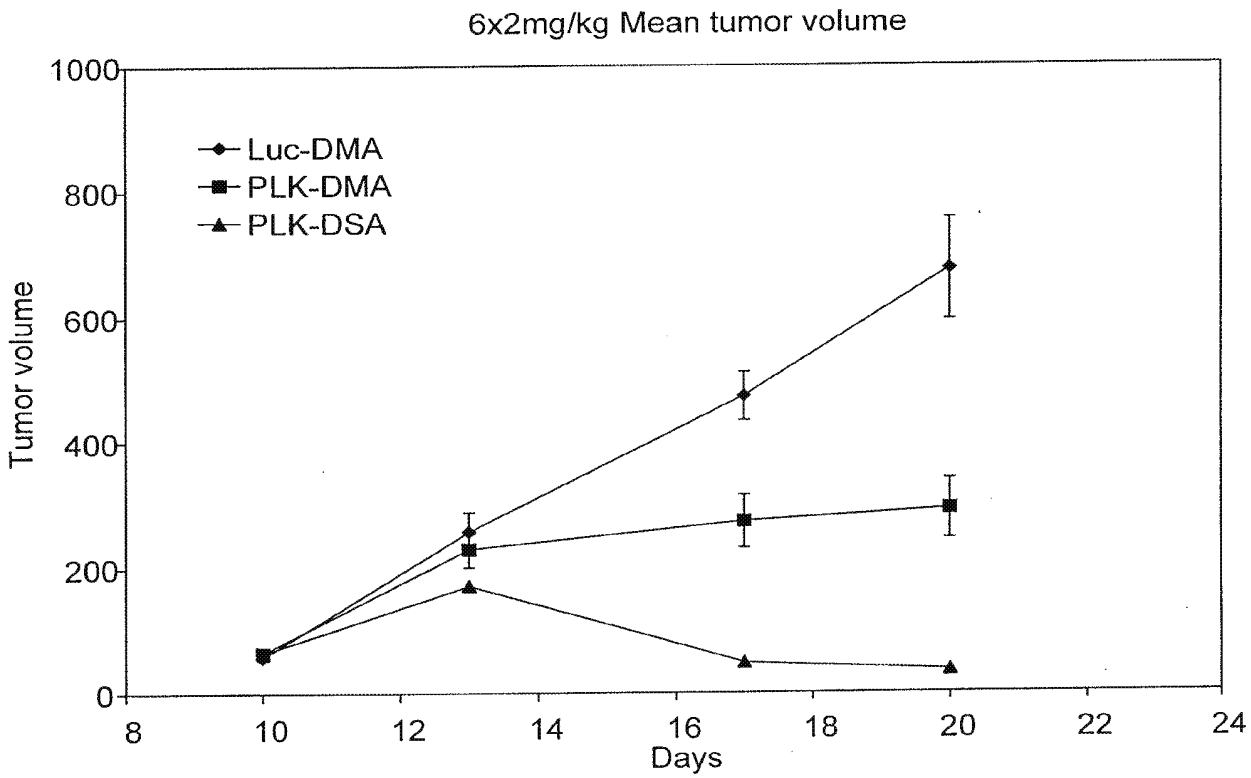

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

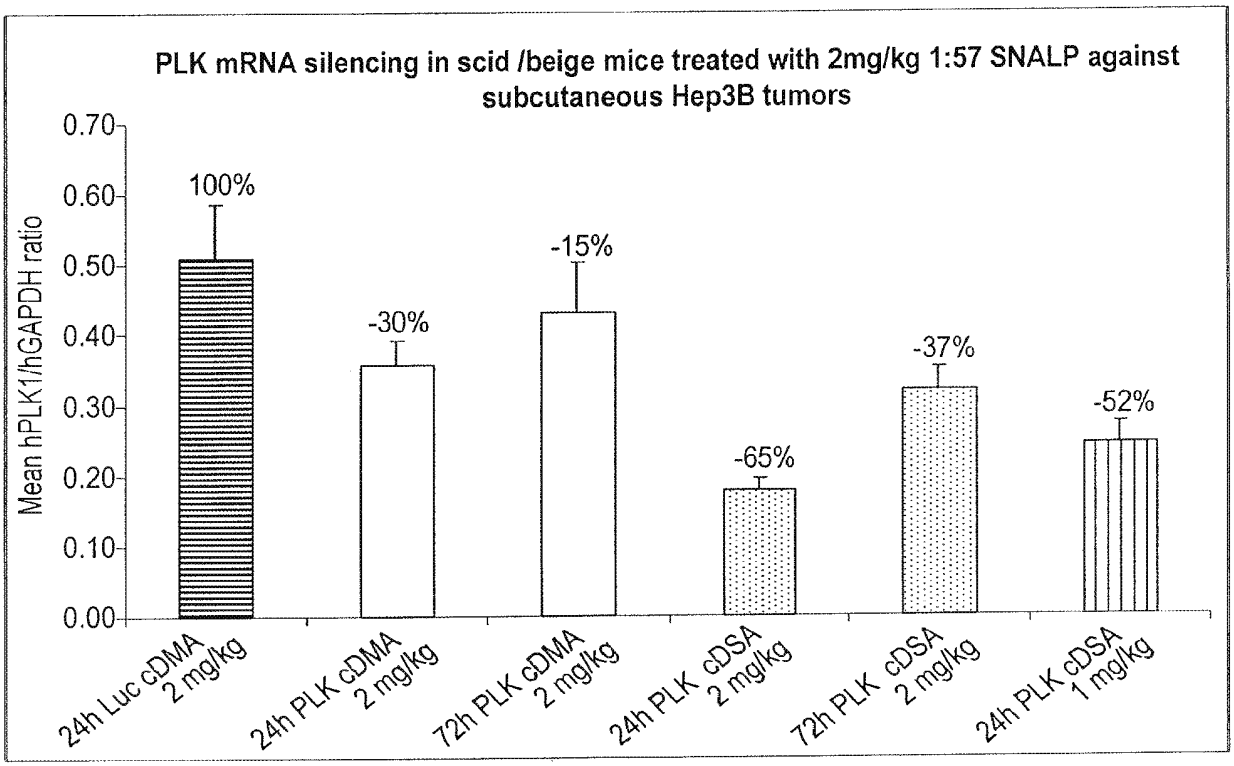

PLK mRNA silencing in scid /beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

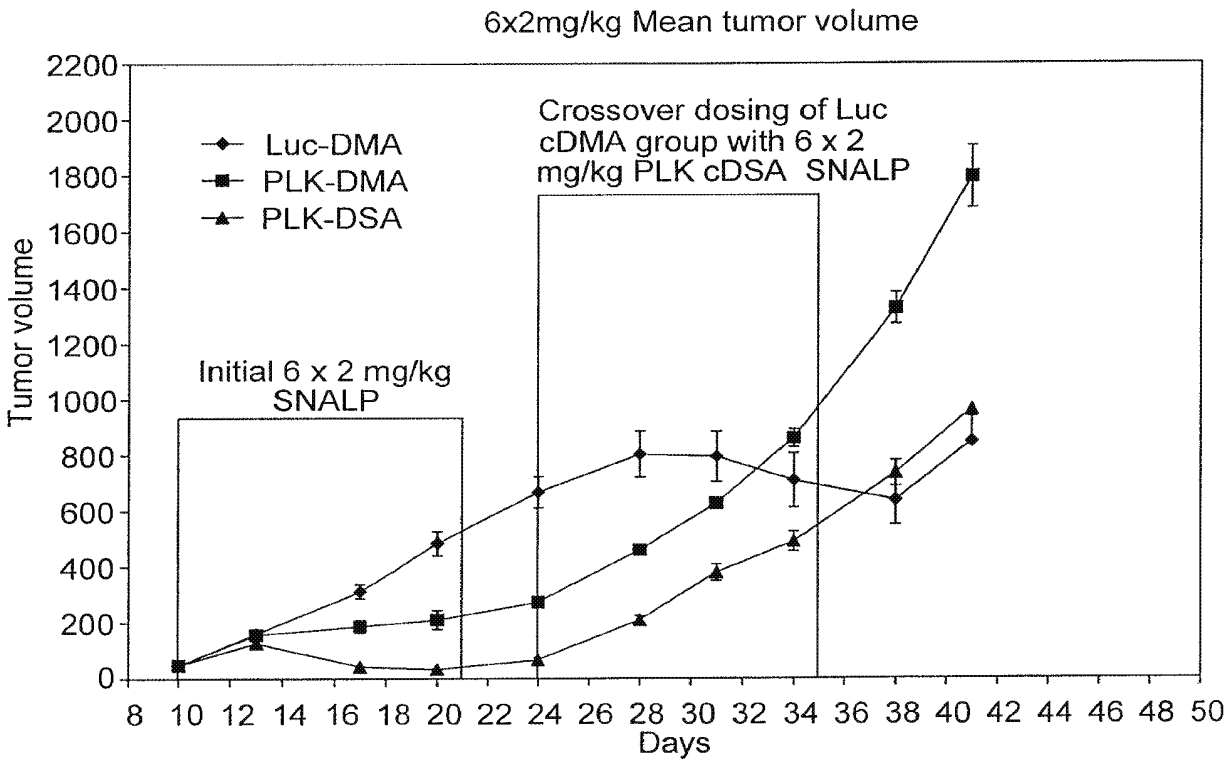

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Initial 6 x 2 mg/kg SNALP
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

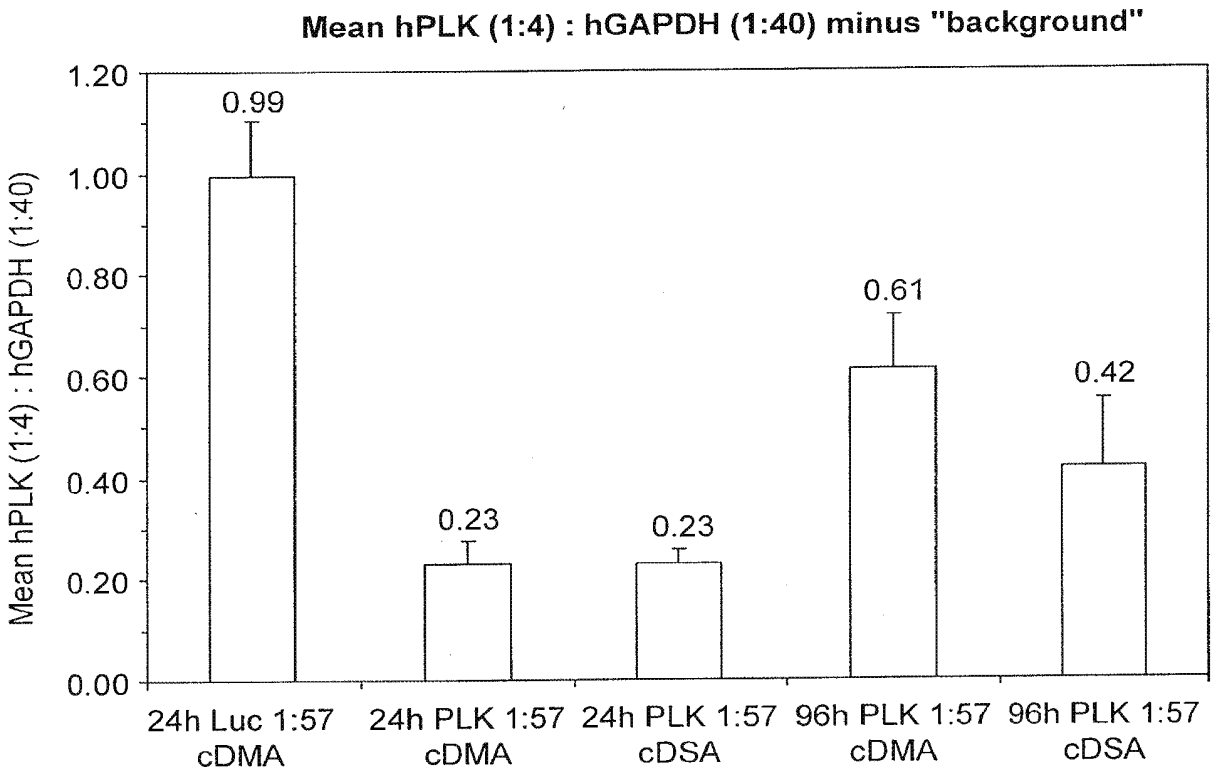

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

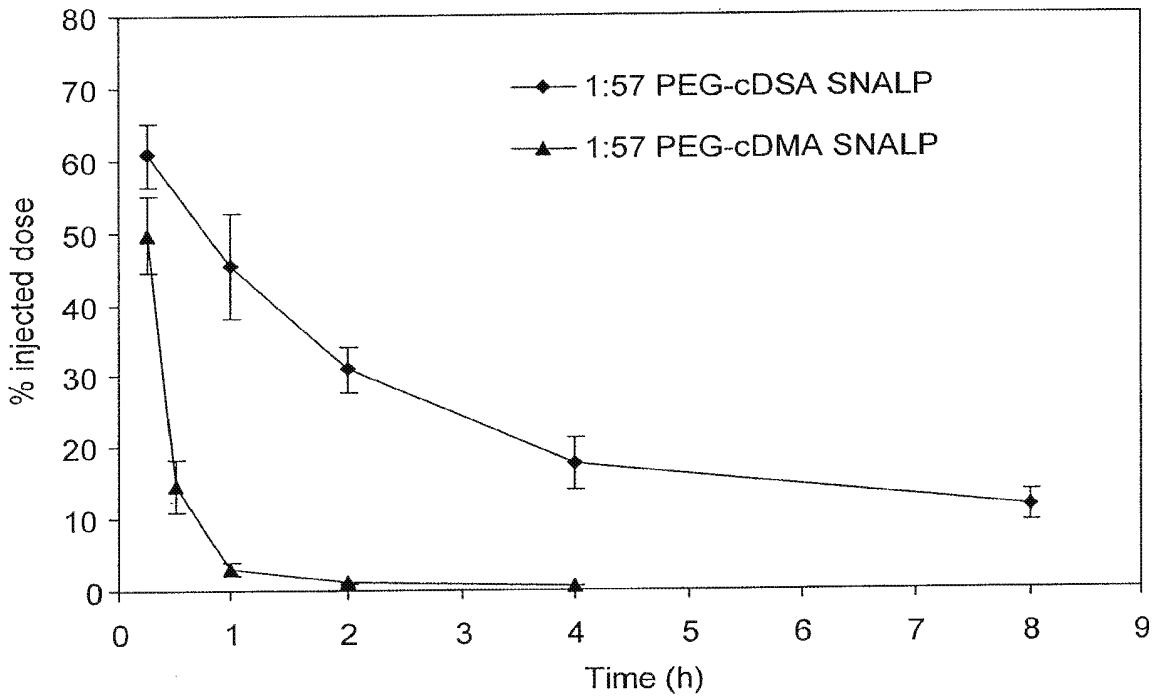

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

SEQUENCE LISTING

<110> Yaworski, Edward
Lam, Kieu
Jeffs, Lloyd
Palmer, Lorne
MacLachlan, Ian

<120> Novel Lipid Formulations for Nucleic Acid Delivery

<130> 020801-007710US

<140> US 12/424,367
<141> 2009-04-15

<150> US 61/045,228
<151> 2008-04-15

<160> 7

<170> FastSEQ for Windows Version 4.0

<210> 1
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic E5 kinesin-related protein 2263 U/U siRNA sense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic E5 kinesin-related protein 2263 U/U siRNA sense strand of siRNA duplex

<221> modified_base
<222> (2)...(19)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT)

<400> 1
cngaagaccn gaagacaann n 21

<210> 2
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic E5 kinesin-related protein 2263 U/U siRNA antisense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic E5 kinesin-related protein 2263 U/U siRNA antisense strand of siRNA duplex

<221> modified_base
<222> (8)...(16)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT)

<400> 2
auugucunca ggncuncagn n 21

<210> 3
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic apolipoprotein B (ApoB) 10048 U2/2 G1/2 siRNA sense strand of siRNA duplex

<221> modified_base
<222> (4)...(4)
<223> n = gm

<221> modified_base
<222> (8)...(14)
<223> n = um

<400> 3
agunucanca cacngaauac c 21

<210> 4
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic apolipoprotein B (ApoB) 10048 U2/2 G1/2 siRNA antisense strand of siRNA duplex

<221> modified_base
<222> (4)...(4)
<223> n = um

<221> modified_base
<222> (7)...(9)
<223> n = gm

<221> modified_base
<222> (19)...(19)
<223> n = um

<400> 4
uauncanunu gaugacacnu g 21

<210> 5
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/GU and PLK1424 U4/G siRNA sense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/GU and PLK1424 U4/G siRNA sense strand of siRNA duplex

<221> modified_base
<222> (4)...(18)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT), u or a ribonucleotide complementary to target complementary sequence

<400> 5
agancacccn ccunaaanan n 21

<210> 6
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/GU siRNA antisense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/GU siRNA antisense strand of siRNA duplex

<221> modified_base
<222> (8)...(8)
<223> n = gm

<221> modified_base
<222> (17)...(17)
<223> n = um

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT), u or a ribonucleotide complementary to target sequence

<400> 6
uauuuaanga gggugancun n 21

<210> 7
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/G siRNA antisense strand of siRNA duplex

<220>
<223> Description of Combined DNA/RNA Molecule:synthetic polo-like kinase 1 (PLK-1) PLK1424 U4/G siRNA antisense strand of siRNA duplex

<221> modified_base
<222> (9)...(15)
<223> n = gm

<221> modified_base
<222> (20)...(21)
<223> n = deoxythimidine (dT), u or a ribonucleotide complementary to target sequence

<400> 7
uauuuaagna gngunaucun n 21

JOINT APPENDIX 64

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on November 6, 2013.

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Judith Cotham/

PATENT

Attorney Docket No.: 86399-007730US-879491

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

MacLACHLAN *et al.*

Application No.: 13/928,309

Filed: June 26, 2013

For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Customer No.: 20350

Confirmation No. 1310

Examiner: Not yet assigned

Art Unit: 1612

COMMUNICATION UNDER

37 C.F.R. §§ 1.821-1.825

AND

PRELIMINARY AMENDMENT

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the request to comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, 37 C.F.R. §§ 1.821-1.825, that accompanied the Notice to File Missing Parts of Nonprovisional Application mailed August 8, 2013, Applicants submit herewith a computer readable copy of the Sequence Listing.

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims which begins on page 5 of this paper.

Remarks begin on page 8 of this paper.

Amendments to the Specification:

Please replace paragraph [0004] beginning at page 1, line 11, with the following:

REFERENCE TO A "SEQUENCE LISTING[["]]," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

[0004] ~~Not applicable.~~ The Sequence Listing written in file -77-3.TXT, created on August 22, 2013, 8,192 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference in its entirety for all purposes.

Please replace paragraph [0026] on page 5 with the following amended paragraph:

[0026] ~~**Figure 1 illustrates**~~ Figure 1A (Samples 1-8) and Figure 1B (Samples 9-16) illustrate data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

Please replace paragraph [0031] on page 5 with the following amended paragraph:

[0031] ~~**Figure 6 illustrates**~~ Figure 6A (expressed as IU/L) and Figure 6B (expressed as x-Fold Upper Limit of Normal) illustrate data demonstrating that the tolerability of 1:57 SNALP containing ApoB siRNA prepared by citrate buffer versus PBS direct dilution did not differ significantly in terms of blood clinical chemistry parameters.

Please replace paragraph [0032] on page 5 with the following amended paragraph:

[0032] ~~**Figure 7 illustrates**~~ Figure 7A (expressed as liver ApoB:GAPD mRNA ratio), Figure 7B (expressed as relative plasma ApoB-100 concentration), and Figure 7C (expressed as plasma total cholesterol illustrate data demonstrating that the efficacy of 1:57 SNALP containing ApoB siRNA prepared by gear pump was similar to the same SNALP prepared by syringe press.

Please replace paragraph [0035] on page 6 with the following amended paragraph:

[0035] ~~**Figure 10 illustrates**~~ Figure 10A (expressed as IU/L) and Figure 10B (expressed as x-Fold Upper Limit of Normal) illustrate data demonstrating that clinically significant liver enzyme elevations (3xULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

Please replace paragraph [0036] on page 6 with the following amended paragraph:

[0036] ~~**Figure 11 illustrates**~~ Figure 11A (expressed as liver ApoB:GAPD mRNA ratio) and Figure 11B (expressed as relative plasma ApoB-100 concentration) illustrate data demonstrating that the potency of the lower lipid:drug (L:D) 1:57 SNALP containing ApoB siRNA was as good as that of the higher L:D SNALP at the tested drug dosages.

Please replace Table 1 beginning at page 92, line 14, with the following:

Table 1. siRNA duplex comprising sense and antisense Eg5 RNA polynucleotides.

Modification	Eg5 2263 siRNA sequence	SEQ ID NO:	% 2'OMe-Modified	% Modified in DS Region
U/U	5'-C**U**GAAGACC**U**GAAGACAA**U**dTdT-3' 3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUUA-5'	1 2	6/42 = 14.3%	6/38 = 15.8%

Column 1: "U/U" = 2'OMe-uridine modified siRNA duplex; Column 2: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. "dT" = deoxythymidine. Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

Please replace Table 3 beginning at page 94, line 7, with the following:

Table 3. siRNA duplex comprising sense and antisense ApoB RNA polynucleotides.

Position	Modification	ApoB siRNA sequence	SEQ ID NO:	% 2'OMe-Modified	% Modified in DS Region
10048	U2/2 G1/2	5'-AGU**G**UCA**U**CACAC**U**GAAUACC-3'	3	7/42 = 16.7%	7/38 = 18.4%

		3′-GUUCACAGUAGUGUGACUUAU-5′	4		

Column 1: The number refers to the nucleotide position of the 5' base of the sense strand relative to the mouse ApoB mRNA sequence XM_137955. Column 2: The numbers refer to the distribution of 2'OMe chemical modifications in each strand. Column 3: 2'OMe-modified nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. Column 4: The number and percentage of 2'OMe-modified nucleotides in the siRNA duplex are provided. Column 5: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

Please replace Table 8 on page 105, beginning at line 19, with the following:

Table 8. siRNA duplexes comprising sense and antisense PLK-1 RNA polynucleotides.

siRNA	PLK-1 siRNA Sequence	SEQ ID NO:	% Modified in DS Region
PLK1424 U4/GU	5′-AGAUCACCCUCCUUAAAUANN-3′ ~~(SEQ ID NO.57)~~ 3′-NNUCUAGUGGGAGGAAUUUAU-5′ ~~(SEQ ID NO.54)~~	5 6	6/38 = 15.8%
PLK1424 U4/G	5′-AGAUCACCCUCCUUAAAUANN-3′ ~~(SEQ ID NO.57)~~ 3′-NNUCUAGUGGGAGGAAUUUAU-5′ ~~(SEQ ID NO.56)~~	5 7	7/38 = 18.4%

Column 1: The number after "PLK" refers to the nucleotide position of the 5' base of the sense strand relative to the start codon (ATG) of the human PLK-1 mRNA sequence NM_005030. Column 2: 2'-O-methyl (2'OMe) nucleotides are indicated in bold and underlined. The siRNA duplex can alternatively or additionally comprise 2'-deoxy-2'-fluoro (2'F) nucleotides, 2'-deoxy nucleotides, 2'-O-(2-methoxyethyl) (MOE) nucleotides, and/or locked nucleic acid (LNA) nucleotides. N = deoxythymidine (dT) nucleotide, uridine (U) ribonucleotide, or ribonucleotide having complementarity to the target sequence (antisense strand) or the complementary strand thereof (sense strand). Column 3: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA duplex are provided.

Please insert the accompanying paper copy of the Sequence Listing, page numbers 1 to 4, at the end of the application.

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings of claims in the application:

Listing of Claims:

1-46. (Canceled)

47. (New) A nucleic acid-lipid particle comprising:

(a) a nucleic acid;

(b) a cationic lipid comprising from 50 mol % to 65 mol % of the total lipid present in the particle;

(c) a non-cationic lipid comprising up to 49.5 mol % of the total lipid present in the particle and comprising a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle; and

(d) a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle.

48. (New) The nucleic acid-lipid particle of claim 47, wherein the nucleic acid comprises an interfering RNA.

49. (New) The nucleic acid-lipid particle of claim 48, wherein the interfering RNA comprises a small interfering RNA (siRNA).

50. (New) The nucleic acid-lipid particle of claim 49, wherein the siRNA comprises at least one modified nucleotide.

51. (New) The nucleic acid-lipid particle of claim 49, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

52. (New) The nucleic acid-lipid particle of claim 49, wherein the siRNA is about 19 to about 25 base pairs in length.

53. (New) The nucleic acid-lipid particle of claim 49, wherein the siRNA comprises 3' overhangs.

54. (New) The nucleic acid-lipid particle of claim 47, wherein the cationic lipid comprises from 50 mol % to 60 mol % of the total lipid present in the particle.

55. (New) The nucleic acid-lipid particle of claim 47, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

56. (New) The nucleic acid-lipid particle of claim 47, wherein the cholesterol or derivative thereof comprises from 30 mol % to 35 mol % of the total lipid present in the particle.

57. (New) The nucleic acid-lipid particle of claim 47, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

58. (New) The nucleic acid-lipid particle of claim 57, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

59. (New) The nucleic acid-lipid particle of claim 58, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

60. (New) The nucleic acid-lipid particle of claim 59, wherein the PEG has an average molecular weight of about 2,000 daltons.

61. (New) The nucleic acid-lipid particle of claim 47, wherein the conjugated lipid that inhibits aggregation of particles comprises from 1 mol % to 2 mol % of the total lipid present in the particle.

62. (New) The nucleic acid-lipid particle of claim 47, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

63. (New) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 47 and a pharmaceutically acceptable carrier.

64. (New) A method for introducing a nucleic acid into a cell, the method comprising:

contacting the cell with a nucleic acid-lipid particle of claim 47.

65. (New) A method for the *in vivo* delivery of a nucleic acid, the method comprising:

administering to a mammalian subject a nucleic acid-lipid particle of claim 47.

66. (New) A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising:

administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 47.

67. (New) The method of claim 66, wherein the disease or disorder is selected from the group consisting of a viral infection, a liver disease or disorder, and cancer.

REMARKS

I. STATUS OF THE CLAIMS

After entry of this amendment, claims 47-67 are pending in this application and are presented for examination. Claims 1-46 have been canceled without prejudice to future prosecution. Claims 47-67 are newly added.

New claim 47 finds support, for example, in original claim 1, in paragraph [0116] on page 24, and in paragraph [0256] on page 68 of the instant specification. New claim 48 finds support, for example, in paragraph [0017] on page 4. New claim 49 finds support, for example, in original claim 2. New claim 50 finds support, for example, in original claim 4. New claim 51 finds support, for example, in original claim 5. New claims 52 and 53 find support, for example, in paragraph [0054] on pages 8-9. New claim 54 finds support, for example, in paragraph [0116] on page 24. New claim 55 finds support, for example, in original claim 14. New claim 56 finds support, for example, in paragraph [0133] on pages 27-28. New claims 57-61 find support, for example, in original claims 17-21, respectively. New claim 62 finds support, for example, in original claim 23. New claim 63 finds support, for example, in original claim 26. New claim 64 finds support, for example, in original claim 41. New claim 65 finds support, for example, in original claim 43. New claim 66 finds support, for example, in original claim 45. New claim 67 finds support, for example, in original claim 46.

As such, no new matter has been introduced with the foregoing amendments.

II. SEQUENCE LISTING

This amendment is accompanied by a computer readable form containing the above named sequences, SEQ ID NOS:1-7. The Sequence Listing in computer readable form was prepared through the use of the software program "FastSEQ" in accordance with 37 C.F.R. §§1.821 to 1.825. The Sequence Listing does not include new matter or matter that goes beyond the disclosure of the application as filed.

According to the Legal Framework for EFS-Web (September 2008), if a sequence listing text file submitted via EFS-Web complies with the requirements of 37 CFR 1.824(a)(2)-(6) and (b), the text file will serve as both the paper copy required by 37 CFR 1.821(c) and the

CRF required by 37 CFR 1.821(e). Therefore, a paper copy of the referenced Sequence Listing is not included with this amendment.

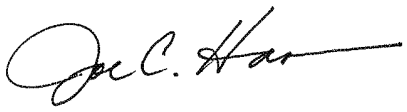
III. DRAWINGS

The Notice to File Missing Parts states that replacement drawings are required as Figures 1, 6, 7, 10, and 11 are not labeled "Fig." with a consecutive Arabic numeral or an Arabic numeral and capital letter in the English alphabet. In response, Applicants respectfully point out that the submitted drawings are in compliance as Figures 1, 6, 7, 10, and 11 are indeed labeled as "Fig." with a consecutive Arabic numeral and capital letter in the English alphabet.

Applicants have amended the instant specification to incorporate amendments to the description of Figures 1, 6, 7, 10, and 11 which were entered in both the parent application (Appl. No. 12/424,367) and the continuation application (Appl. No. 13/253,917). Support for these amendments is found in the specification as filed. No new matter has been added.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,



Joe C. Hao
Reg. No. 55,246

KILPATRICK TOWNSEND & STOCKTON LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 925-472-5000
Fax: 415-576-0300
Attachments
JCH:dmw
KILPATRICK TOWNSEND 65643803 1

JOINT APPENDIX 65

Paper No. ___
Filed: November 13, 2019

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

MODERNA THERAPEUTICS, INC.,
Petitioner,

v.

ARBUTUS BIOPHARMA CORPORATION,
Patent Owner.

Case IPR2019-00554
Patent No. 8,058,069

PATENT OWNER'S RESPONSE PURSUANT TO 37 C.F.R. § 42.107

TABLE OF CONTENTS

Page

I. INTRODUCTION

The nucleic acid-lipid particles claimed by the '069 patent have achieved tremendous recognition in the field of genetic therapy. The '069 patent is now listed in FDA's Orange Book as protecting the patisiran—tradename "Onpattro"—commercial product. EX2025. Patisiran received regulatory approval in the U.S. and Europe and has been designated by the FDA as a "first-in-class" drug. EX2024. The therapeutic potential of genetic therapy has been appreciated for over 25 years, but effectively delivering nucleic acids to target cells without eliciting vehicle-related toxicity prevented realization of this potential. *E.g.*, EX2016, 38, 42; EX2018, 11. By 2008, the industry-wide failure to identify a solution to the delivery problem resulted in waning confidence. EX2019, 2, 10; EX2018, 11; EX2023, 291-292.

The nucleic acid-lipid particle formulations of the '069 patent met a long-felt need for compositions that could safely and effectively deliver nucleic acids to patient target cells. The combination of effectiveness and low toxicity that characterizes the claimed compositions surprised many in the field, and finally solved the delivery problem that hindered the field for decades.

The petition is a poorly conceived challenge, relying on erroneous legal analysis in each of the under-developed obviousness (Grounds 1-3) and anticipation (Ground 1 and 3) challenges.

Petitioner's obviousness challenges are based ***solely*** on the theory that alleged overlapping ranges create a presumption or *prima facie* case of obviousness under the legal framework in *In re Peterson*, 315 F.3d 1325 (Fed. Cir. 2003) and *E.I. duPont de Nemours & Co. v. Synvina C.V.* 904 F.3d 996 (Fed. Cir. 2018). *E.g.*, Pet. 31-32, 33, 39, 40, 49, 54, 56, 58, 59; Paper 8, Decision on Institution ("DI"), 25-27, 36-37. Petitioner's theory fails for numerous reasons.

First, Petitioner has failed to set forth evidence of prior art ranges that overlap with the claimed ranges, and therefore, the prior art and lack of evidence on which it relies cannot be the basis for establishing a *prima facie* case of obviousness. *Peterson* and *duPont* are inapposite here where the proposition is that "[a] prima facie case of obviousness typically exists ***when the ranges of a claimed composition overlap with ranges disclosed in the prior art***." *Peterson*, 315 F.3d at 1329 (emphasis added). Neither case dictates nor even loosely implies the existence of such presumption when the prior art fails to actually disclose ***any*** ranges overlapping with those claimed. For example, the prior art presented by Petitioner fails to disclose ***any*** concentration range for a phospholipid component—much less one that overlaps with the claimed invention. As such, Petitioner has failed to provide any evidence supporting its arguments of *prima facie* obviousness under the framework of *Peterson* and *duPont*. To hold Petitioner's lack of evidence sufficient to show a *prima facie* case of obviousness

would require a series of unwarranted and unsupported assumptions—a marked departure from the framework of these cases. This is particularly pertinent here as Petitioner's arguments rest solely on its putative "*prima facie*" case, as though that alone meets its ultimate burden of proof. This reliance underscores the deficiencies in the Petition, including not only its failure to address the claimed subject matter as a whole (*i.e.*, the claimed particle formulation), as mandated by statute, but also the lack of any meaningful discussion of motivation to combine or reasonable expectation of success.

Second, regardless of whether the framework of *duPont* and *Peterson* even applies as a threshold matter, Petitioner's obviousness challenges still lack motivation to combine with reasonable expectation of success. As the Federal Circuit has explained, every obviousness challenge requires motivation to combine with an expectation of success. *In re Stepan Co.*, 868 F.3d 1342, 1346 n.1 (Fed. Cir. 2017). The Federal Circuit has explained that even if *prima facie* obviousness is established, it is overcome with a showing that routine optimization does not apply. *duPont*, 904 F.3d at 1006 ("disclosure of very broad ranges may not invite routine optimization")(citing *Genetics Inst., LLC v. Novartis Vaccines & Diagnostics, Inc.*, 655 F.3d 1291, 1306 (Fed. Cir. 2011). The framework of *duPont* and *Peterson* is no exception and is predicated specifically on a routine optimization rationale.

Here, routine optimization is not applicable—rebutting any argument of *prima facie* obviousness. This issue was previously litigated in *Moderna, Inc. et al. v. Arbutus Biopharma Corp.*, IPR2018-00739 ("the '739 IPR") in the context of the same person of ordinary skill in the art ("POSITA"), at the same relevant time, viewing the same specification as the challenged '069 patent. *E.g.*, POPR, 23-29; EX2033, 36:5-13. Here, like in the '739 IPR, Petitioner and its expert expressly embrace the complexity of the technology, emphasize unpredictability, and disavow the notion that arriving at the claimed subject matter would have been a matter of simple optimization. *E.g.*, EX2033, 42:7-10 ("If the range is immense, there would be undue experimentation, I believe, to find a combination or a range that behaved in a desirable light."), 60:5-16 (ranges narrower than those in the cited art are "immense" and "would require undue experimentation, not simple optimization."), 19:25-20:15. Accordingly, routine optimization (and by extension, the framework of *Peterson* and *duPont*) is not a viable rationale in view of the broad ranges cited in the art, as well as expert testimony and extensive literature indicating that developing nucleic acid lipid at the time simply was not considered a routine matter of optimizing variables.

Third, even if Petitioner is credited with some showing of obviousness, unexpected results overcome any such presumption. Extensive experimental testing demonstrates the claimed nucleic acid-lipid particles are surprisingly non-

toxic, non-immunogenic, and more potent and efficacious than prior art compositions. These unexpected results are different in kind and are supported by data from both the '069 patent and post-filing publications that test many different formulations, with many different combinations of lipid components, gene targets, nucleic acid payloads, and methods of production. The petition materials provide no meaningful analysis of the full scope of experimental data presented in the '069 patent and ignore the post-filing publications entirely.

Fourth, additional objective indicia further rebut any case of obviousness. Indeed, the uncontested record demonstrates a long-felt need, failure of others, skepticism in the industry, and commercial success—each of which supports the non-obviousness of the claimed invention.

In Ground 2, Petitioner relies on Lin and Ahmad for the conjured notion that it would be obvious "to increase the cationic lipid to the 50%-65% range in order to potentially increase the transfection." Pet. 50. Lin and Ahmad are irrelevant for Petitioner's purposes. Lin and Ahmad are directed to lipoplexes—a fundamentally different class of particles that are expressly differentiated by the '069 patent from the claimed "nucleic acid-lipid particle." EX1001, 2:12-18, 3:3-10. Even Petitioner's own expert testified that lipoplexes are outside the scope of the challenged claims. EX2001, 122:1-24. Further, Petitioner never once articulates a motivation as to why a POSITA would increase the concentration of cationic

lipids. All Petitioner offers is a series of assertions that such modification "could" increase transfection efficiency. *See* Pet. 49 ("***may*** increase..."), 50 ("...***potentially*** increase..."), ("...***could*** impact..."). Such assertions have never been sufficient to demonstrate obviousness. Notwithstanding Petitioner's misrepresentation of Lin and Ahmad, Ground 2 inherits all the defects of Ground 1 and thus also fails for those same reasons.

Finally, Petitioner's half-baked anticipation challenges are dead on arrival. The anticipation theory relies on the conclusory assertion that the prior art "disclosures are sufficiently specific to anticipate the claimed range." Pet. 33. Missing from Petitioner's analysis is any explanation as to how prior art disclosures can be "sufficiently specific" to anticipate the claimed phospholipid range, when, in fact, the prior art does not disclose ***any*** phospholipid range.[1] As to the other lipid components, the cited ranges at best partially overlap with the claimed range. Missing again is any explanation as to how the disclosures are "sufficiently specific" to establish anticipation.

[1] Even the Board's Institution Decision acknowledges that the cited references lack express disclosure of a phospholipid range and require a series of assumptions. DI, 23-25, 36-37

For these reasons, and those explained in further detail herein, Petitioner fails to meet its burden of demonstrating the unpatentability of the claims, and each Ground under the Petition should be rejected.

II. OVERVIEW OF THE '069 PATENT AND THE PRIOR ART

The '069 patent is directed to the surprising discovery that nucleic acid-lipid particle formulations with high levels of cationic lipids and low levels of conjugated lipids exhibit favorable *in vivo* transfection efficiencies as well as "improved tolerability of the formulations in vivo, resulting in a significant increase in the therapeutic index [a measure of dosage relative to toxic effect] as compared to nucleic acid-lipid particle compositions previously described." EX1001, 5:55-58; *id.*, 5:58-6:3; 11:26-32 (defining the inventive formulations as "extremely useful for systemic applications"); *see also* EX2031, ¶¶25-28. The '069 patent claims nucleic acid-lipid particle formulations with high levels of cationic lipids (50–65 mol%) and low levels of conjugated lipids (0.5–2 mol%)—as well as specific levels of cholesterol/derivative (30-40 mol%) and phospholipid (4-10 mol%).

Prior art taught against the invention claimed by the '069 patent. Largely ignored by the petition, prior art at the time of invention (including ***all*** references cited by Petitioner) instructed that formulations with a high level of cationic lipid were toxic and poorly tolerated *in vivo* and had little to no *in vivo* transfection

efficiency. *E.g.*, EX1003, ¶6; EX1006, 3315; EX2009, 30:34-41; EX2031, ¶¶80-88. Moreover, the prior art instructs that the level of cationic lipid should be minimized, as high levels were deemed unsuitable for *in vivo* transfection. EX1007, 745. Additionally, where conjugated lipids were utilized, the art instructed much higher levels as compared to those claimed. EX1009, 5; EX2031, ¶¶77-78.

Yet, contrary to these teachings, the claimed formulations uniformly withstood rigorous *in vivo* tests that established stability following systemic (*in vivo*) administration, suitability for mammals with no considerable toxicity, and transfection efficiencies superior to conventional formulations. *E.g.*, EX2031, ¶¶89-112.

III. PROCEDURAL HISTORY

Petitioner challenges the '069 patent in the instant proceeding and has previously challenged another patent from this patent family, U.S. Patent No. 9,364,435 ("the '435 patent"), a continuation of the '069 patent, in the '739 IPR. The independent claim in the '069 patent contains a similar limitation to a range of phospholipids as claim 7 of the '435 patent, and, is in fact, narrower. The Board issued a Final Written Decision in the '739 IPR ('739 IPR, Paper 51, "FWD") on September 11, 2019, after institution of the instant proceeding, in which it was determined that claim 7 was patentable. FWD, 51.

IV. CLAIM CONSTRUCTION—NUCLEIC ACID-LIPID PARTICLE

No claim construction is necessary in order to determine that the Petition fails. *Vivid Techs., Inc. v. American Science & Eng'g, Inc.*, 200 F.3d 795, 803 (Fed. Cir. 1999)("only those terms need be construed that are in controversy, and only to the extent necessary to resolve the controversy."). That being said, Petitioner's proffered construction of "nucleic acid-lipid particle" is incorrect.

Claim terms are not construed in the abstract but are construed as to how they would be understood by a POSITA when read in light of the specification and the prosecution history. *Fenner Investments, Ltd. V. Cellco Partnership*, 778 F.3d 1320, 1323 (Fed. Cir. 2015). As such, the term "nucleic acid-lipid particle" should be construed as necessarily including a nucleic acid encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation. This is consistent with the disclosure of the specification, wherein "nucleic acids, when present in the lipid particles of the present invention, are resistant in aqueous solution to degradation with a nuclease." EX1001, 11:42-55; *see also id.*, 11:10-12; EX2009, 4:15-19; EX2031, ¶¶32-33.

Moreover, during prosecution of the underlying application of the '069 patent, applicants specifically touted encapsulation of the nucleic acid. EX1016, 38; EX2031, ¶34. Encapsulation was also argued extensively in the '739 IPR. Thus, prosecution history reinforces the teaching of the specification that the

claimed nucleic acid-lipid particles necessarily require encapsulation of the nucleic acid. *Microsoft Corp. v. Proxyconn, Inc.*, 789 F.3d 1292, 1298 (Fed. Cir. 2015); *Aylus Networks, Inc. v. Apple Inc.*, 856 F.3d 1353, 1361 (Fed. Cir. 2017).

Petitioner does not offer its own claim construction analysis, but merely adopts the Board's preliminary interpretation, notably determined under a different claim construction standard, from the institution decision in the '739 IPR. That construction, however, is unduly broad as it relies on an incomplete reading of the specification and would encompass an empty lipid particle. The portion of the specification relied upon by the Board in its construction of "nucleic acid-lipid particle" is directed at the term "lipid particle," and not "nucleic acid-lipid particle" as required by the claims. '739 IPR, Paper 15, 10-11. That is, as disclosed in the '069 specification, a "lipid particle" "*may* [include a nucleic acid] encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation." EX1001, 11:4–12. A "nucleic acid-lipid particle," however, ***does*** include a nucleic acid encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation.[2] EX1001, 11:22-32, 11:51-55; EX2031, ¶¶35-37.

[2] During his deposition in the '739 IPR, Dr. Janoff repeatedly testified that the claimed particle should be defined as a SNALP. EX2001, 118:19-119:4, 119:9-17, 120:5-6, 121:14-25. Petitioner has never explained the contradiction between its

V. OBVIOUSNESS IN VIEW OF OVERLAPPING RANGES FAILS (GROUNDS 1 & 3)

Petitioner's obviousness challenge rises and falls on its mere alleged presumption of obviousness based only on the theory of overlapping ranges. *E.g.*, Pet., 31-32, 54; DI, 25-26, 37 (citing *Peterson* and *duPont*). This obviousness theory is both legally and factually wrong.

The Federal Circuit has explained that overlapping ranges, without evidence to the contrary, may invoke a presumption of obviousness when "routine optimization" is applicable. Routine optimization, however, is not applicable when broad ranges need to be assessed. *Peterson*, 315 F.3d at 1330 n.1 ("[Overlapping] ranges that are ***not especially broad*** invite ***routine experimentation*** to discover optimum values, rather than require nonobvious invention") (emphasis added); *duPont,* 904 F.3d at 1006. The legal paradigms of "overlapping ranges" and "routine experimentation" do not apply here. *Genetics*, 655 F.3d at 1306 ("Simply put, the typical desire of scientists to find an optimum value within a narrow disclosed range … does not apply to the facts in this case.").

First, there can be no presumption of obviousness in view of overlapping ranges when the cited art ***simply does not disclose an overlapping range*** for a

---

expert's testimony in the '739 IPR and its conclusory statement that the Board's construction in the '739 IPR "is appropriate." EX2003, ¶13.

claimed component. Neither *Peterson* nor *duPont* supports a presumption of obviousness based on the hindsight-driven and selective picking and choosing of disclosures to contrive a range, as Petitioner attempts to do.

Second, "routine optimization" is not a viable rationale for arriving at the claimed subject matter. The testimony of both experts, contemporaneous literature, and industry recognition all illustrate that developing lipid particle formulations for drug delivery was far from a simple or routine matter of optimizing variables.

Furthermore, regardless of the obviousness theory, the petition fails to establish any motivation to combine and a reasonable expectation of success in doing so. It is clear that these requirements to prove a case of obviousness have not been met.

A. No Affirmative Teaching of an Overlapping Phospholipid Range Defeats Petitioner's Obviousness Theory Based on Overlapping Ranges

Under the relevant caselaw, a presumption of obviousness may apply when the prior art ***actually*** discloses overlapping ranges that are not especially broad. *Peterson*, 315 F.3d at 1329-30. When, as here, the prior art fails to disclose any range for a claimed component (let alone ranges that overlap), there can be no presumption of obviousness.

The petition cites to *Peterson* and *duPont*, though neither case supports a presumption of obviousness when ranges are not disclosed but are instead contrived. In both those cases, the court found a *prima facie* case of obviousness based on very close prior art ***expressly*** disclosing component ranges that overlapped with ranges claimed. *Peterson*, 315 F.3d at 1329-32; *duPont,* 904 F.3d at 1011-13. In neither instance did the court find a presumption of obviousness by making a series of assumptions or inferences to arrive at a range not affirmatively disclosed. *Peterson*, 315 F.3d at 1329-32; *duPont,* 904 F.3d at 1011-13.

In contrast to *Peterson* and *duPont*, independent claim 1 of the '069 patent recites four components at different concentrations, including a range for phospholipid "***from 4 mol % to 10 mol % of the total lipid.***" Petitioner claims the cited prior art references explicitly disclose a 0-19% or a 0-19.5% phospholipid range, but it is indisputable that ***none*** of these references disclose any such range, nor ***any*** range for phospholipids specifically. *E.g.*, EX1008, ¶118 (citing EX1003, ¶¶89, 91; EX1004, ¶¶152, 159) *compare with* FWD, 31-32, 35-37. The Board in its Institution Decision did not dispute that the cited refences lacked affirmative disclosure of a phospholipid range, but instead instituted on the basis that a phospholipid range could be contrived through "reasonable inferences." DI, 23, 36; EX2031, ¶¶38-39.

Petitioner cites no authority that the presumption of obviousness under *Peterson* and *duPont* would allow a "reasonable inference" to be satisfied by selective, hindsight-driven picking and choosing, and especially not when dealing with the broad ranges at issue. *Peterson*, 315 F.3d at 1330 n.1; *duPont*, F.3d at 1011 n.15 (in distinguishing *Genetics*, noting that the "case [in *duPont*] presents 'not especially broad' ranges of temperature and pressure."). Put simply, Petitioner attempts to shoehorn the present facts into a legal framework that simply does not apply and Petitioner has not shown the claims are obvious.

i. Ground 1 – The '196 PCT and the '189 Publication do not disclose the recited phospholipid concentration range.

Neither the '196 PCT nor the '189 publication provide affirmative disclosure of any phospholipid range, let alone a phospholipid range that overlaps with the claimed range. *See* FWD, 31-32, 35-37. Petitioner relies on the disclosure of a range for non-cationic lipid from 20% to about 85% of the total lipid present in the particle. EX1008, ¶118 (citing EX1003, ¶¶89, 91; EX1004, ¶¶152, 159). The '196 PCT disclosure is not limited to phospholipids; rather, phospholipids are merely an example of a noncationic lipid component. EX2031, ¶¶40-41.

Similarly, '189 publication states that the non-cationic lipid "typically comprises from about 5 mol % to about 90 mol %, from about 10 mol % to about 85 mol %, from about 20 mol % to about 80 mol %, from about 30 mol% to about 70 mol %, from about 40 mol % to about 60 mol % or about 48 mol% of the total

lipid present in the particle," also failing to expressly teach a phospholipid range, or a range close to the claimed 4-10% range. EX1004, ¶152; EX2031, ¶42.

Petitioner's reliance on the '618 patent does not cure the above deficiencies. It is also irrelevant in term of Petitioner's overlapping ranges theory for obviousness. The '618 patent discloses a nucleic acid lipid complex with 56% cationic lipid, 14% phospholipid, and 30% cholesterol. Pet. 38 (citing EX1017, 34:54-35:23). The disclosure of 14% phospholipid does not result in any "reasonable inference" of a phospholipid range of 4-10%—it is indisputably outside of the claimed range and there is no teaching to lower the percent phospholipid in this formulation. EX2031, ¶43. Furthermore, claim 1 of the '069 has a clear limitation including a conjugated lipid that would make up 0.5-2 mol% of the formulation, not similarly present in the '618 patent. To believe Petitioner's theory in light of these apparent holes would require impermissible hindsight. *In re Arkley*, 455 F.2d 586, 587-88 (C.C.P.A. 1972); *In re Ruschig*, 379 F.2d 990, 995 (C.C.P.A. 1967)) ("Working backward from [the invention], that is by hindsight, it is all very clear what route one would travel through the forest of the specification to arrive at it."); *Orexo AB v. Actavis Elizabeth LLC*, 903 F.3d 1265, 1271 (Fed. Cir. 2018) ("It is inappropriate to use the template provided by the inventor, to render the inventor's contribution obvious.")

Petitioner's assumptions further illustrate impermissible hindsight. Petitioner sets the amount of cationic lipid at 60%, with the only provided reason being that it is at the high end of the disclosed range. Pet 39. Notably, Dr. Janoff does not provide a reason for its selection. This unexplained, unsupported assumption inappropriately serves as the linchpin for the remainder of Petitioner's analysis. EX2031, ¶¶44-50.

Accordingly, as the '196 PCT and '189 publication fail to disclose any range for phospholipid, much less an overlapping range, Ground 1 necessarily fails.

ii. Ground 3 – The '554 Publication also does not disclose or suggest the recited phospholipid concentration range.

As with Ground 1, the petition (Pet. 58) alleges "explicit disclosure of encompassing ranges" for the phospholipid concentration range recited in the challenged claims. The '554 publication, however, does not discuss concentration ranges for phospholipids. *See* Pet. 57-58 (failing to identify any teaching in the asserted prior art regarding phospholipid concentrations); *see also* FWD, 35-37. As already discussed, there can be no presumption of obviousness under *Peterson* and *duPont* when there is no overlapping range in the cited reference, such as here, where a range is contrived through a series of assumptions. Certainly, there is no presumption of obviousness when those assumptions are illogical, unsupported by the references, and clearly driven by improper hindsight.

Petitioner relies on the disclosure of a range for non-cationic lipid from about 20% to about 85% of the total lipid present in the particle. Pet. 57. Like the '196 PCT and the '189 publication, the disclosure of the '554 publication is not limited to phospholipids; rather, phospholipids are merely an example of a noncationic lipid component that may be used. Specifically, the non-cationic lipid may be a neutral uncharged, zwitterionic, or anionic lipids that are capable of producing a stable complex. EX1008, ¶157 (citing EX1005, ¶¶313, 315, 455). In addition, the disclosed non-cationic lipid ranges are not close to the claimed 4-10% range. EX2031, ¶¶51-52.

Petitioner's reliance on the L106 formulation of Table 4 as including cholesterol at 30% does not cure the above deficiencies. Pet. 57. The L106 formulation contains 67% DMOBA (cationic lipid), 30% cholesterol, and 3% 2KPEG-Cholesterol, but does not contain any phospholipid. EX1005, Table 4. Petitioner does not explain why a POSITA would look to the L106 formulation in formulating particles containing a phospholipid. In addition, both cationic and conjugated lipid amounts are outside the claimed ranges, and Petitioner does not explain the relevance of such a formulation to the claimed particles. EX2031, ¶53.

The Board in its Institution Decision also cites to L054, which "includes the cationic lipid DMOBA, cholesterol, the phospholipid DSPC, and the [] PEG-n-DMG in a molar ratio of 50/20/28/2." DI, 36 (citing EX1005, Table 4). Again, a

POSITA would not understand a single formulation as defining a range for the lipid components making up that formulation. Neither the Board or Dr. Janoff explain how a single formulation having 28% phospholipid suggests a phospholipid range of 0-20%. Moreover, the percentage of phospholipid is 28%, well above the claimed range of 4-10%. EX2031, ¶54.

Similar to Ground 1, Petitioner attempts to contrive a range for the phospholipid from the '554 publication, however its analysis is based on unwarranted and unreasonable assumptions. Petitioner sets the amount of cationic lipid at 60%, Pet. 39, that choice then inexplicably serves as the linchpin for the remainder of Petitioner's analysis. EX2031, ¶¶55-56.

Moreover, although the petition identifies a range for the conjugated lipid, Dr. Janoff again fails to account for the amount of conjugated lipid in his declaration. The range of conjugated lipid identified by the petition, however, is at the lower end of the range identified by the '554 publication. Accommodating 19% phospholipid is only mathematically possible if assuming the highest possible cationic lipid (60%) together with the lowest possible cholesterol (20%) and lowest possible conjugated lipid (1%). There is no explanation for any of these choices which appear driven by hindsight. Moreover, selecting the highest possible cationic lipid (60%) together with the lowest possible number in the ranges for

conjugated lipid disclosed by the '554 publication would have been viewed as counterintuitive. EX2031, ¶¶57-58.

Accordingly, as the '554 publication fail to disclose any range for phospholipid, much less an overlapping range, this challenge also necessarily fails on this basis alone.

B. Formulating Nucleic Acid-Lipid Particles Was Not a Matter of Routine Optimization

The legal paradigm based on overlapping ranges is not applicable here as evidenced by the lack of disclosure in the cited references of lipid ranges overlapping with the claimed ranges. *See* Section V.A. Even if considered further, obviousness under *Peterson* and *duPont* is a theory premised on the notion that routine optimization—not undue experimentation—is all that is required to arrive at the claimed subject matter. It is undisputed, however, that at the time of invention, formulating nucleic acid-lipid particles was ***not*** a matter of routine optimization. In other words, routine optimization simply does not apply here and presents another basis why the poorly conceived obviousness challenges in the petition fail.

The undisputed fact that developing nucleic acid-lipid particles at the time was not a simple optimization matter was laid bare in the '739 IPR. EX2006, 403: 22-25 ("Q. In the 2008 timeframe, was developing nucleic acid-lipid particles considered a routine matter of optimizing variables? A. No."); EX2004, ¶58 ("The

effects of making changes to the proportion of other components in the lipid particle would be unpredictable..."), ¶60 ("Making safe and effective nucleic acid-lipid particle formulations was not simply a matter of 'varying the proportion' of cationic lipid in prior art formulations …"); *see also* EX2004, ¶¶57-59, 136; EX2005, 31:22-23 ("Change solvent, change additives, change lots of different variables"), 41:4-6 ("plenty of places to go wrong"), 178:17-18, 180:6-8; EX2006, 404:11-18 ("As I stated multiple times in my deposition, these are multicomponent systems and varying one component at a time was not a viable strategy."). Dr. Janoff repeatedly emphasized the complexity of the field of art at the time. EX2001, 144:18-145:1 ("We're in deep waters, and what you think are simple questions belie — and I don't mean to be pejorative — belie an ignorance of the field that you're questioning me in"), 57:19 ("it's a very technical area"), 58:22-59:1 ("we're in deep water here talking about very technical issues), 61:9-11 ("You're asking me a very, very, very technical question..."), 63:5-11 ("we're in technical deep waters"), 68:12 ("we're in deep technical territory here"); *see also* EX2003, ¶25 (discussing "the complicated nature of what affects transfection efficiencies").[3] Petitioner should not be allowed to switch tact and argue that it

[3] The Institution Decision distinguished between evidence pertinent to the '739 IPR and the present case. DI, 26. However, certain specific cited evidence,

was obvious to arrive at the claimed ranges given the lack of any overlapping prior art disclosure, ample evidence teaching against certain claimed ranges, and their admissions on the complexity of the subject matter. EX2031, ¶¶59-60.

Dr. Thompson explains that, as illustrated throughout the literature at the time, developing lipid carrier particles for nucleic acids was by no means considered a simple or routine matter of optimizing variables. EX2031, ¶61. Those in the field at the time had been struggling for years to find active formulations that were not toxic. Moreover, such lipid particles are multi-components systems. The interaction of the various components was not well understood at the time and there was little guidance available in the art. EX2031, ¶¶60, 76-81.

Dr. Janoff's declaration in this case includes only a single conclusory sentence regarding determining an "optimal proportion" of cationic lipid. EX1008, ¶112; *see also* 37 C.F.R. § 42.65(a). Dr. Janoff cites nothing to support this

---

pertinent to the state of the art generally, and the issue of routine experimentation specifically, cannot be ignored here given the relation of the two patents. The '435 patent and '069 patent share the same specification, the same effective filing date and have been addressed from the same POSITA perspective. EX2033, 35:13-36:15; *compare* EX1008, ¶¶29-32 *with* EX2028, ¶¶29-32.

incorrect and conclusory statement. In fact, the remainder of his testimony unambiguously supports precisely the opposite conclusion. EX2031, ¶¶61-63.

For example, Dr. Janoff emphasizes complexity in the technology and describes lipid ranges ***narrower*** than those in the cited art as "immense." EX1008, ¶74; EX2028, ¶¶73, 112. Dr. Janoff further testified that, in view of such "immense" lipid ranges, "a POSITA would have no way of knowing if [a] lipid combinaton at any given proportion would have resulted in formulations of superior therapeutic index of other formulations." EX1008, ¶74; EX2028, ¶73; *see also* EX2033, 42:3-42:10; EX2031, ¶62.

During cross-examination, Dr. Janoff repeatedly testified that narrower lipid ranges than what is found in the art would be considered "immense" at the time and require "undue experimentation." EX2033, 41:21-42:6 ("There is no way -- there is no way a person of ordinary skill in the art would know what specific proportions might give results that are desired."), 42:7-10 ("If the range is immense, there would be undue experimentation I believe to find a combination or a range that behaved in a desirable light."), 60:5-60:16 ("Immense is big. And by immense maybe I can help a little bit more. By immense, I mean that in order to come up, in order for a person of ordinary skill in the art to find utility because of the immenseness of the range, this would require undue experimentation, not simple optimization"); EX2031, ¶64.

The prior art cited in the petition is consistent with the testimony of both experts and corroborates that forming functioning lipid particles at the time was far from routine, but instead was a function of multiple parameters whose interactions were poorly understood, with limited guidance existing. EX2031, ¶¶65-66. For example, Ahmad emphasizes the lack of mechanistic understanding of lipid-based delivery systems at the time "due to the large number of parameters involved" and observes the lack of empirical investigation ("few investigations to date include a complete examination of lipid performance as a function of lipid-bilayer composition and lipid/DNA charge ratio (ρ_{chg})." *Id.* (references omitted); *see also* EX2003, ¶25; EX1009, 8 ("[It is] essential for us to identify the critical parameters limiting gene delivery in the current systems.").

The evidence of record also demonstrates the recognition in the industry that developing lipid particle formulations for drug delivery was not a simple or routine matter, instead, the field struggled for years to identify effective solutions. EX2031, ¶¶65-66. For example, a MIT immunologist noted that "physical delivery of the [siRNA] to diseased cells is extremely challenging." EX2016, 38; *see also* EX2017, 7248 ("The intrinsic complexity of any such gene delivery vehicle can be expected to present continued challenges ..."). Phillip Sharp, who shared the Nobel Prize in Physiology or Medicine for his earlier work on RNA splicing stated that "The major hurdle right now [for RNAi therapeutics] is

delivery, delivery, delivery." EX2018, 11; *see also* EX2020, 7 ("What's interesting about what we do is that the drug isn't the problem. It's the delivery of it."); EX2019, 2; EX2016, 42; EX2020, 1. Once the first lipid particle-based drug patisiran, was approved for use in humans, the development was hailed in the field with express discussion of the difficulties in overcoming the technical hurdles associated with effective delivery. EX2023, 291-292 ("[Delivery] proved to be a substantially harder problem than we anticipated..."), ("All of those tear-your-hair-out days were worth it to get to today").

In fact, Petitioner has never established that formulating nucleic acid-lipid particles as claimed would have been a matter of routine optimization (or any other obviousness rationale). Petitioner and its expert instead embrace the complexity of formulating nucleic acid-lipid particles, repeatedly arguing unpredictability in adjusting lipid proportions. The obviousness challenges fail for at least this reason alone. *E.g.*, *Stepan*, 868 F.3d at 1346 (rejecting obviousness in view of overlapping ranges because "[m]issing from the Board's analysis is an explanation as to *why* it would have been routine optimization to arrive at the claimed invention.") (emphasis in original).

Accordingly, "routine optimization" is not a viable rationale for arriving at the claimed subject matter. EX2031, ¶67; *see also* EX2007, 14-17.

C. The Broad Ranges of the Prior Art do not Support Routine Optimization

The breadth of the ranges for other lipid components disclosed by the prior art relied upon in the petition further underscore that the presumption of *Peterson* does not apply under the facts of this case. That is, obviousness under *Peterson* and *duPont* does not apply when the disclosed range is so broad so as to encompass a large amount of distinct compositions. *duPont*, 904 F.3d at 1006 ("we have reasoned that disclosure of very broad ranges may not invite routine optimization."); *Genetics*, 655 F.3d at 1306 (explaining it is "the typical desire of scientists to find an optimum value [is] within a ***narrow disclosed*** range," not ranges that are unduly broad) (emphasis added).

The lipid ranges in the cited references are far broader than the ranges that Dr. Janoff describes as "immense" and requiring "undue experimentation, not simple optimization." For example, the '196 PCT teaches that "[t]he cationic lipid typically comprises from about 2% to about 60% of the total lipid present in said particle (EX1003, ¶88), and the '554 publication teaches that the cationic lipid component can comprise from about 2% to about 60% of the total lipid (EX1005, ¶313). Those ranges are much broader than the claimed range of 50 mol% to 65 mol% recited in the challenged claims. EX2031, ¶¶68-69.

For the conjugated lipid, the range for the more expansive "bilayer stabilizing component" genus that is disclosed by the '196 PCT is "from about

0.5% to about 50% of the total lipid present in the particle" (EX1003, ¶93), and the broadest range disclosed by the '554 publication is "from about 1% to about 20%" (EX1005, ¶313). EX2031, ¶70. Again, those ranges are much broader than the claimed range of 0.5mol% to 2mol% of the total lipid.

And as discussed above in Sections V.A.i and V.A.ii, the prior art does not even disclose a range for the phospholipid. Importantly, other than characterizing the ranges as "overlapping," Petitioner does not explain how the claimed ranges would be obtained by "optimization" and "routine experimentation." *See Stepan*, 868 F.3d at 1346 n.1; EX2033, 145:12-22 (Dr. Janoff testifying that a *prima facie* case of obviousness based on overlapping ranges is a legal construct, not a technical analysis).

Not only are the ranges disclosed by the prior art broad, the teachings of prior art encompass an extremely large number of compositions. Specifically, the prior art is not limited to a formulation requiring a phospholipid, but instead disclose a large number of lipid components that may be used, one of which may be a phospholipid. EX2033, 52:9-18 (Dr. Janoff testifying that "a noncationic lipid could be cholesterol, it could be a zwitterionic phospholipid or could be an anionic lipid. The only requirement is that it's not cationic. So, note immediately here, we are talking about an immense number of lipid types."). For example, the '554 publication provides a broad definition for a neutral lipid, stating that it can be

"any of a variety of neutral uncharged, zwitterionic or anionic lipids capable of producing a stable complex," including phospholipid related materials, cholesterol, non-phosphorus containing lipids, and PEG-based polymers. EX1005, ¶455; EX2031, ¶¶71-72.

Rather, Petitioner fails to address the claims as a whole, only addressing the individual lipid components (*see* Section V.D.i) and failing to account for the unpredictability in formulating nucleic acid-lipid particles (*see* Section V.B), as well as the interactions between the components (*see* Section V.D.i). EX2031, ¶73.

D. Petitioner Does not Explain Selecting the Claimed Composition from the Prior Art Ranges

Regardless of whether the *Peterson* presumption applies here (it does not), Petitioner must provide articulated reasoning with some rational underpinning to support a conclusion of obviousness. *KSR Int'l Co. v. Teleflex Inc.,* 550 U.S. 398, 418 (2007). The petition does not identify a reason to select the claimed composition from the prior art references, nor does it address why there would be a reasonable expectation of success of arriving at the claimed compositions. *Intelligent BioSystems, Inc. v. Illumina Cambridge Ltd.*, 821 F.3d 1359, 1367 (Fed. Cir. 2016). Thus, again, the petition fails to demonstrate the unpatentability of the claims. EX2031, ¶74.

i. Claim as a Whole/Interaction of Components

The petition separately parses the claimed amounts of cationic lipids, conjugated lipids, and non-cationic lipids from the references, without regard to one another. That is, the petition separately addresses the limitations of the claims, alleging that each limitation to the specific components of the nucleic acid-lipid particle in isolation are "prima facie obvious." *See, e.g.,* Pet. 33 ("this limitation is *prima facie* obvious"), 39 (same), 40 (same). Missing from the obviousness grounds is a showing that the challenged claims as a whole are obvious. *In re Kahn*, 441 F.3d 977, 986 (Fed. Cir. 2006) ("[M]ere identification in the prior art of each element is insufficient to defeat the patentability of the combined subject matter as a whole."); *In re NTP, Inc.*, 654 F.3d 1279, 1299 (Fed. Cir. 2011); *see also* FWD, 35-37. Indeed, as the statute itself states: "if the differences between the subject matter sought to be patented and the prior art are such that the ***subject matter as a whole*** would have been obvious." 35 U.S.C. §103(a) (2012) (emphasis added); *see generally Graham v. John Deere Co.*, 383 U.S. 1, 17-18 (1966); *TriVascular, Inc. v. Samuels*, 812 F.3d 1056, 1066 (Fed. Cir. 2016).

The petition thus fails to address the fact that the concentrations of different lipid components are highly interdependent. *See* EX2004, ¶¶57-59. At best, the petition attempts to show that some prior art formulations had high concentrations

of cationic lipid. However, these also use a high concentration of conjugated lipid, or are not nucleic acid-lipid particles. *See also* Section IX; EX2031, ¶¶74-79.

ii. Cationic Lipids Were Known to be Toxic

The claimed invention is a nucleic acid-lipid particle comprised of relatively high levels of cationic lipids and low levels of conjugated lipids. This combination was counterintuitive to the then-existing state of the art, as cationic lipids were known to be cytotoxic, systemically toxic, to elicit an adverse complement-mediated immune response, and to cause particle aggregation that resulted in rapid clearance. *E.g.*, EX1007, 745 ("Minimizing the amount of cationic lipid is desirable … fewer, more highly charged molecules should mean a smaller metabolic effort..."); EX1009, 5 ("the cationic lipid contributes significantly to the toxicity observed."); EX2016, 42 ("I wouldn't want anyone injecting cationic lipids into my bloodstream."). The prior art taught that the cationic lipid component of lipid particles should be minimized, regardless of whether used for *in vitro* or *in vivo* purposes. EX2031, ¶¶80-88.

In fact, Petitioner has published extensively about toxicity concerns due specifically to ionizable cationic lipids, including DLinDMA. For instance, one of Petitioner's recent publications states the following:

> Ionizable cationic lipids, such as, but not limited to, DLinDMA, Dlin-KC2-DMA, and Dlin-MC3-DMA, have been shown to accumulate in

> plasma and tissues over time and may be a potential source of toxicity.

EX2037, 21:10-12; EX2031, ¶86. Petitioner own publications explicitly attribute toxicity of ionizable cationic lipids (including DLinDMA) not to charge at physiologic pH — which by its own admission is neutral— but to accumulation and immunogenicity. These unrebutted facts are relevant because they 1) further undermine any motivation/expectation of success at the time; and 2) would have taught away from the claimed compositions.

Moreover, compositions with low levels of conjugated lipid (*i.e.*, 0.5 mol% to 2 mol%) would have been expected to result in unstable particles that aggregate and fail to effectively transfect cells. Conjugated lipids were used to shield the cationic lipids from interacting with negatively charged serum proteins and thereby diminish the adverse effects. Thus, when employing an increased amount of cationic lipid, a POSITA would have had every reason to correspondingly ***increase*** the amount of conjugated lipid. EX2004, ¶¶25-35. The inventors of the '069 patent did just the opposite. EX2031, ¶¶80-88.

Making non-toxic and effective nucleic acid-lipid particle formulations was thus not simply a matter of "varying the proportion" of cationic lipid in prior art formulations. *E.g.*, Pet. 33; Sections V.A. & V.B. As discussed in Sections VII.A and VII.B, the field was hindered by the lack of effective and safe nucleic acid delivery vehicles. That the field struggled for 20 years to find such a delivery

vehicle speaks to the difficulty of the task. Had the solution been a matter of simply optimizing the cationic lipid proportion, it would not have taken such an enormous investment of money and time. EX2004, ¶60; EX2031, ¶¶80-88.

VI. UNEXPECTED RESULTS FURTHER REBUT ANY *PRIMA FACIE* OBVIOUSNESS

To the extent any *prima facie* case of obviousness was ever established, it is rebutted by uncontroverted evidence that developing nucleic acid-lipid particles as claimed was ***not*** a matter of routine optimization of lipid variables. The Federal Circuit has explained in *Peterson* and elsewhere, one may also overcome a *prima facie* case of obviousness "by showing that the claimed range achieves unexpected results." *Peterson*, 315 F.3d at 1330-1331. Any such *prima facie* case here is even further overcome by the extensive experimental data in the '069 patent and post-filing publications showing unexpected results.[4]

[4] As both Petitioner and Dr. Janoff were aware of Patent Owner's evidence of secondary considerations, which were extensively developed throughout the proceeding of the '739 IPR, but did not address any in the Petition or Dr. Janoff's declaration. They should not be permitted to challenge this evidence in Reply here, as it should have been addressed in the petition in the first instance. *E.g.*, *Praxair Distribution, Inc. v. Mallinckrodt Hospital Products*, IPR2016-00777, Paper 10, 9.

The extensive scope of the experimental testing conducted—and ignored by the petition—included many different formulations, with many different combinations of different lipid components, gene targets, nucleic acid payloads and methods of production. *See* EX2008 (summary of exemplary formulations tested and within the scope of the '069 patent claims); EX2007, 18-20. Such testing is more than sufficient to rebut any *prima facie* case of obviousness. *See Peterson*, 315 F.3d at 1331 (unexpected results not commensurate where only two data points were tested, and only one data point produced unexpected results); *duPont*, 904 F.3d at 996 (only a single data point was tested); *In re Greenfield*, 571 F.2d 1185, 1189 (Fed. Cir. 1978) (testing only one species in a large genus).

The prior art instructs that high-level cationic lipid compositions were expected to have poor efficacy and increased cytotoxicity and immunogenicity relative to low-level cationic lipid formulations. *See* EX1006, 3315; EX1007, 745; EX1009, 5; EX2009, 30:34-41. As Dr. Thompson explains, the expectation for the claimed nucleic acid-lipid particle compositions would have been toxic compositions poorly suited for systemic use and little, if any, efficacy. EX2004, ¶¶66-67. Contrary to these expectations, the claimed formulations are well-tolerated and efficacious at far lower dosages than prior art compositions. EX2004, ¶68; *e.g.* EX1001, 74:1-4, Figure 3. These results are "an unexpected difference in kind that supports nonobviousness." *Allergan, Inc. v. Sandoz Inc.*,

796 F.3d 1293, 1306 (Fed. Cir. 2015). Petitioner ignores much of the experimental data presented in the '069 patent, offers no meaningful critique to the data that is addressed, and fails to address that the claimed nucleic acid-lipid particle compositions are substantially non-toxic and non-immunogenic. *See also* EX2004, ¶¶69-71; EX2031, ¶¶89-93.

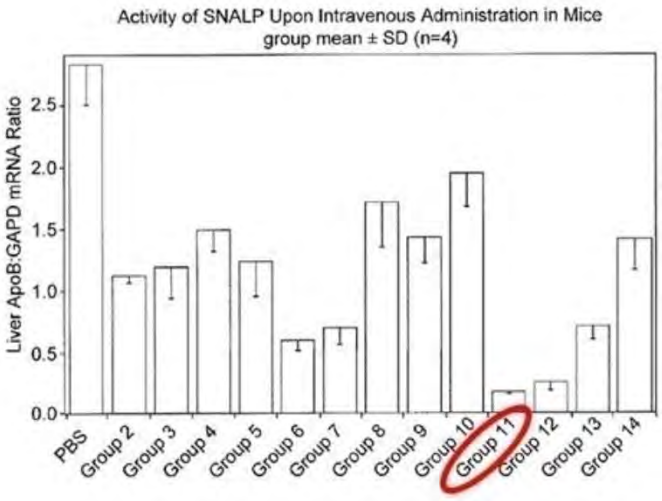
A. The '069 Patent Reports Extensive Testing of Numerous Formulations within the Claimed Range

Patent Owner found that the claimed formulations surprisingly impart increased activity of the encapsulated nucleic acid and improved tolerability of the formulations *in vivo*, resulting in a significant increase in the therapeutic index. EX1016, 38-39, 68-69. Moreover, the claimed formulations are stable in circulation and are substantially non-toxic when administered to mammals. The '069 patent specification provides experimental data for numerous formulations within the scope of claim 1, supporting the unexpected tolerability and efficacy of the claimed compositions. EX2031, ¶¶89-95; *see also* EX2008.

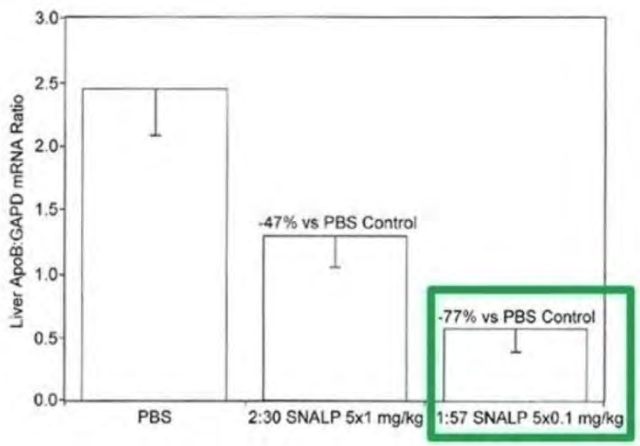
Example 2 of the '069 patent demonstrates that Eg5 siRNA formulations formulated as 1:57 SNALP are potent inhibitors of cell growth *in vitro*. EX1001, 68:51-70:22. Sample 9 (a 1:57 SNALP) was among the most potent inhibitors of tumor cell growth at all siRNA concentrations tested. *Id.*, 70:19-22. As Dr. Janoff admits, the 1:57 SNALP outperforms the 2:40 admitted prior art at low siRNA amounts (EX1008, ¶83), demonstrating the potency of the 1:57 SNALP. And as

Dr. Janoff testified, potency is a factor when discussing toxicity. EX2033, 68:11-16, 69:7-71:1 (relating potency to effectiveness); EX2029, 116 (defining efficacy), 263 (defining potency); EX2031, ¶¶96-98.

Additionally, Example 3 in the '069 patent specification demonstrates that the 1:57 formulations were substantially more effective at silencing the expression of a target gene as compared to all other nucleic acid-lipid particle formulations tested. *See, e.g.*, EX1001, 72:21-24 ("FIG. 2 shows that the 1:57 SNALP formulation … was ***the most potent*** at reducing ApoB expression in vivo (see, Group 11)") (emphasis added); *see also id*., Table 4 (showing Group 11 as 1.4% conjugated lipid (PEG); 57.1% cationic lipid (DLinDMA); 7.4% phospholipid (DPPC); 34.3% cholesterol). Figure 2 (annotated) is shown. EX2031, ¶¶99-101.

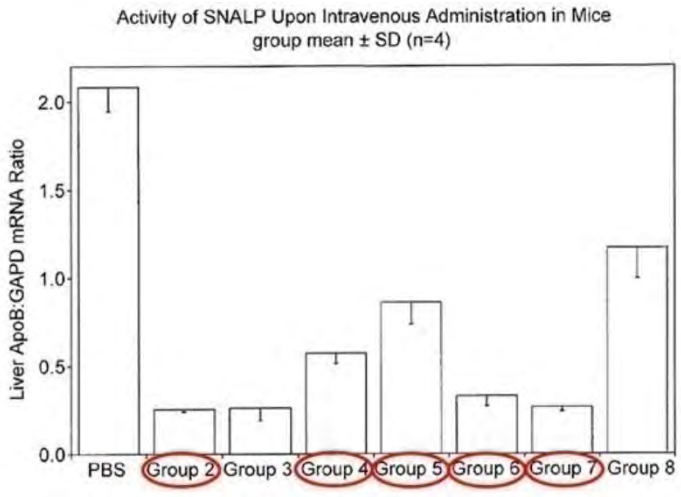


As Dr. Thompson explains, Example 4 demonstrates that 1:57 formulations were ***10 times more efficacious*** as compared to a nucleic acid-lipid particle formulation previously described ("2:30 SNALP")



in mediating target gene silencing *in vivo* ***at a 10-fold lower dose***. EX1001, 73:64-67; EX1016, 39. Figure 3 (annotated) is shown. As explained during *ex parte* prosecution, the 2:30 SNALP formulation contains the greatest amount of cationic lipid of all the SNALP formulations prepared and tested in the *MacLachlan et al.* reference that was cited by the Examiner. EX1016, 39; EX2031, ¶¶101-103.

Example 5 describes testing of five additional "1:57" formulations within the scope of claim 1. EX1001, 74:1-53, Table 6 (see Groups 2 and 4-7), Figure 4. Those formulations included combinations



of different conjugated lipids (PEG_{2000} and PEG_{5000}), cationic lipids (DLinDMA and DODMA), phospholipids (DPPC and DPPE), and cholesterol/derivative (cholesterol and cholestanol). As disclosed in Example 5 and illustrated in Fig. 4,

each of those formulations demonstrated potent silencing activity *in vivo*. EX2031, ¶¶104-106.

As Dr. Thompson explains, Examples 7 and 8 describe testing of tolerability and efficacy using "1:57" SNALPs prepared by different manufacturing processes. EX1001, 75:41-80:45. The tested SNALPs were well-tolerated and efficacious in mediating target gene silencing *in vivo*. *E.g., id.*, 77:61-66 ("There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations."), 80:5-8 ("There was also no obvious change in animal appearance/behavior at any of the dosages tested … there were no obvious changes in platelet count"); EX2031, ¶¶107-111.

The examples presented in the '069 patent demonstrate that nucleic acid-lipid particles were efficacious in silencing multiple different gene targets *in vivo* and discloses data demonstrating nucleic acid-lipid particle compositions across the claimed range are unexpectedly well-tolerated and efficacious. EX2007, 20-22. As indicated above, Examples 3-8 demonstrate potent silencing of ApoB expression *in vivo*. Examples 9-11 further demonstrate *in vivo* silencing of polo-like kinase 1 (PLK-1) expression using different 1:57 SNALP formulations. EX1001, 80:46-86:19. EX2031, ¶¶99-111.

The claimed particles have not only been compared to prior 2:30 and 2:40 formulations, but, as discussed below, post filing-date particles have been

compared to other formulations having conjugated lipid levels above the claimed range. As discussed below in Section VI, particles encompassed by claim 1 were superior in silencing gene expression of Factor VII *in vivo* relative to formulations having conjugated lipid levels above the claimed range. EX2014, Figure 12. Thus, although ignored by Petitioner, other post-filing date evidence reinforces the unexpected properties discussed in the disclosure of the '069 patent. EX2031, ¶¶99-112.

B. Post-Filing Publications Provide Testing Data for a Broad Range of Lipids and Cargo Molecules

As Dr. Thompson explains, Publications following the '069 patent (including Petitioner's own publications) tested dozens more formulations within the scope of claim 1, finding those formulations efficacious and well-tolerated.[5] EX2031, ¶¶113-21; EX2008. The testing reported in post-filing publications (including those by Petitioner) cover different nucleic acid payloads (*e.g.*, siRNA and mRNA), many different cationic lipids, numerous gene targets, various *in vivo* animal models, and humans. EX2031, ¶113.

---

[5] *Genetics*, 655 F.3d at 1307 ("[W]e have held that evidence of unexpected results may be used to rebut a case of *prima facie* obviousness even if that evidence was obtained after the patent's filing or issue date….")

U.S. Patent No. 8,236,943 ("the '943 patent," EX2010) discloses testing of several formulations within the scope of claim 1. For instance, the '943 patent evaluated several 1:57 formulations comprising 1.4% PEG2000-cDMA, 57.1% cationic lipid, 7.1% DPPC, and 34.3% cholesterol. EX2010, 150:15-47; *see also id.,* 151:Table 1 (describing the siRNA cargo). Examples 11 and 13 disclose such formulations comprising five different cationic lipids (*i.e.*, DLin-K-C2 DMA, DLenDMA, γ-DLenDMA, γ-DLen-C2K-DMA, and DLen-C2K-DMA), which were tested for their capacity to silence the ApoB gene in animals following intravenous injection. *Id*., 150:59-151:55, 153:25-55, Figures 4, 7. Each of these formulations demonstrated potent gene silencing activity *in vivo*. EX2031, ¶114.

U.S. Publication No. 2013/0116307 ("the '307 publication," EX2011) discloses testing of additional formulations within the scope of claim 1, including 1:57 formulations comprising 1.4% PEG2000-cDMA, 57.1% cationic lipid, 7.1% DPPC, and 34.3% cholesterol. EX2011, ¶421; *see also id*., Table 1 (describing the siRNA cargo). Such formulations included seven different cationic lipids (*i.e.*, DLin-MC3-DMA, LenMC3, CP-LenMC3, CP-γ-DLen-C2K-DMA, CP-DLen-C2K-DMA, γ-Len-MC3, CP-γ-Len-MC3) that were tested for their capacity to silence gene expression in mice following intravenous injection. *Id*., Examples 17 & 18, ¶¶430-439, Figures 4, 5. As illustrated in Figures 4 and 5, each of these nine

formulations were efficacious in silencing target gene activity *in vivo*. EX2031, ¶115.

Semple (EX2021) discloses testing of formulations within the scope of claim 1 in multiple different *in vivo* animal models. For instance, Semple tested a 1:57 formulation comprising 1.4% PEG2000-cDMA, 57.1% DLin-KC2-DMA, 7.1% DPPC, and 34.3% cholesterol. EX2021, 177 ("Preparation of KC2-SNALP"); *see also id*., ("siRNA synthesis") (describing the TTR siRNA cargo). The formulation was tested for its capacity to silence the TTR gene in mice, rats, and non-human primates following a single intravenous injection. EX2021, 175, 178 ("In vivo nonhuman primate experiments"), Figure 3. As reported, the 1:57 formulation "was well-tolerated in both rodent and nonhuman primates and exhibited in vivo activity at siRNA doses as low as 0.01 mg/kg in rodents, as well as silencing of a therapeutically significant gene (TTR) in nonhuman primates." *Id*., 175; *see also id*., Table 2; EX2022, Supplementary Table 4. The formulations were both efficacious and well-tolerated. EX2021, 175 ("toxicological analysis indicated that the treatment was well tolerated at the dose levels tested, with no treatment-related changes in animal appearance or behavior."). EX2031, ¶116.

U.S. Publication No. 2017/0307608 to Bettencourt ("the '608 publication," EX2012) discloses testing of the commercial product, Onpattro™ (*i.e.*, patisiran). Patisiran includes 50% DLin-MC3-DMA, 1.5% PEG-cDMG, 10% DSPC, 38.5%

cholesterol. EX2012, ¶46, Table 1; *see also id*., ¶43 (describing the TTR siRNA cargo). Testing demonstrated that patisiran is both efficacious and well-tolerated in human subjects. EX2012, ¶103. Patisiran effectively silenced expression of its target—the TTR gene—providing clinical benefit to patients. *Id*., ¶¶121, 132; EX2031, ¶¶117-18.

International Publication No. WO2010/088537 to Akinc ("the '537 publication," EX2014) discloses testing of even more formulations within the scope of claim 1. EX2014, 112, 120, 109 (Table 3) (describing the FVII siRNA cargo). Example 16 discloses 24 formulations, three of which (Groups 23-25) are within the scope of claim 1. *Id*., 120. As illustrated in Fig. 12 below (annotated), Groups 23-25 were superior in silencing gene expression of Factor VII ("FVII") in vivo relative to formulations having conjugated lipid levels above the claimed range. *Id*., Figure 12; EX2031, ¶119.

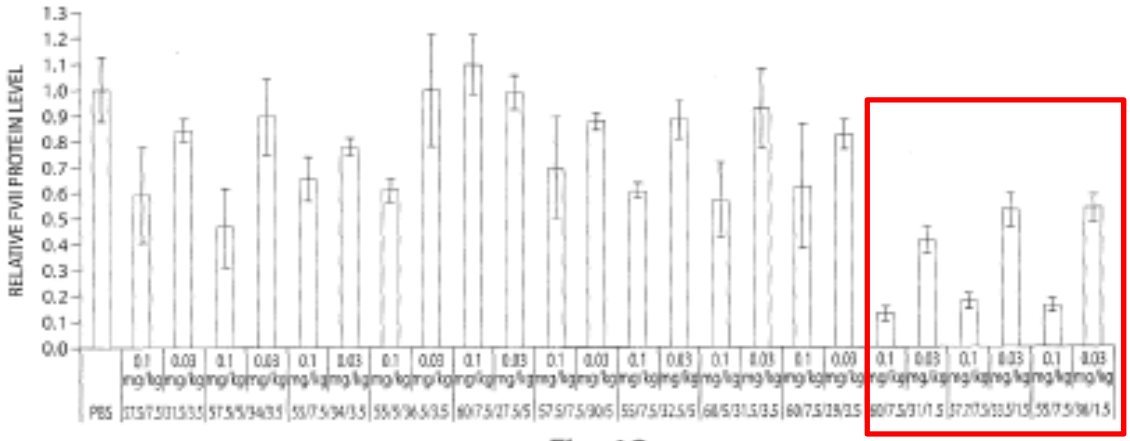


Fig. 12

Remarkably, the petition and Dr. Janoff ignore that Petitioner has published extensively that the claimed formulations are efficacious and well-tolerated for various nucleic acid payloads, including mRNA. *See* EX2002, 70:18-73:4, 75:25-77:7 (confirming his opinions ignored Petitioner's publications); EX2026; EX2015; EX2027. For example, Sedic tested mRNA therapeutic payloads in the LNPs using the same "off-the-shelf" formulation as patisiran (*i.e.*, 50% DLin-MC3-DMA, 1.5% PEG-cDMG, 10% DSPC, 38.5% cholesterol). EX2026, 2 ("850-nucleotide messenger RNA"), 3; EX2012, ¶46. Consistent with the various other reports in the literature, Petitioner reported that the LNPs were efficacious and well-tolerated. EX2026, Abstract ("Overall, these combined studies indicate that LNP-formulated modified mRNA can be administered by intravenous infusion in 2 toxicologically relevant test species and generate supratherapeutic levels of protein (hEPO) in vivo.").[6]

In sum, various post-filing publications, including Petitioner's own publications, which were not addressed in the petition, have reported testing of numerous formulations within the scope of claim 1. This includes testing of many

[6] EX2019, 4 ("[O]nce a delivery technology is found suitable for knocking down genes in a given cell/tissue type, any gene can be targeted."); *see also* EX2006, 404:20-406:7.

different cationic lipids, numerous gene targets, and various *in vivo* animal models. Such publications provide additional evidence that the claimed formulations are surprisingly efficacious and remarkably non-toxic. *See also* EX2007, 23-27; EX2031, ¶¶120-21.

C. Petitioner Fails to Present Any Meaningful Critique to Patent Owner's Evidence Supporting Patentability

The burden is never on Patent Owner to demonstrate the patentability of the claims—it is up to Petitioner to demonstrate the unpatentability of the claims. *E.g. In re Magnum Oil Tools Int'l, Ltd.*, 829 F.3d 1364, 1375 (Fed. Cir. 2016). Instead, Petitioner provides an incomplete and flawed analysis of the data, and its critique falls far short of carrying its burden of demonstrating the unpatentability of the claims. *E.g.*, EX2033, 104:8-9 (Dr. Janoff testifying "I have no reason not to believe the data."). As discussed above, Patent Owner has presented extensive unrebutted evidence showing that the claimed particles demonstrate unexpected results, as well as extensive unrebutted evidence as to other secondary considerations such as skepticism and commercial success (*see* Section VII), all of which support the patentability of the claims. Not only has that evidence been presented in the instant proceeding, it was made of record in the '739 IPR before the instant petition was filed, and again, other than for Examples 2-5 of the instant disclosure, this evidence has never been rebutted. To be clear, Petitioner's critique of Examples 2-5 is inapt.

Turning first to Example 2, Dr. Janoff concedes that the 1:57 formulation (Sample 9) was highly potent in inhibiting cell viability, as well as outperforms Sample 10, which comprises a phospholipid component (13.3%) falling outside the scope of the challenged claims. EX1008, ¶83. Thus, Dr. Janoff has no meaningful criticism of this example. Furthermore, as to Example 3, Dr. Janoff offers only his erroneous speculation that the 1:57 SNALP (Group 11) is not statistically significantly efficacious over Group 12, containing 40.4% cationic lipid. EX1008, ¶85. Dr. Thomson's review of Figure relied upon by Dr. Janoff in making that statement, however, determined that the error bars in fact did not overlap. EX2031, ¶100. Dr. Thompson's review is also consistent with the express disclosure of the specification that the 1:57 composition was the most potent (EX1001, 72:21-24). Notably, Dr. Janoff failed to address this opinion, thus it remains unrebutted. *See* EX1008, ¶85.

As to Example 4 (EX1001, 73:64-67), Dr. Janoff in fact concedes that the testing established that the 1:57 formulation outperformed the 2:30 SNALP by showing more than 10 times the efficacy but attacks the data on the basis that the 2:30 formulation was formulated with the phospholipid DSPC, while the 1:57 formulation was formulated with the phospholipid DPPC. EX1008, ¶86. That criticism, however, is belied by his testimony in the '680 IPR, in which he stated that the'069 patent discloses that DSPC may be substituted for DPPC. EX2030,

¶85 ("A POSITA would understand from these disclosures that DSPC could be substituted for DPPC in the identified formulations."). Finally, Dr. Janoff's only critique of Example 5 is that the preferred proportion for one cationic lipid, DLinDMA, "does not necessarily apply" to all cationic lipids. EX1008, ¶116. But this again falls far short of Petitioner's burden demonstrating the unpatentability of the claims.

Petitioner also asserts that other formulations contain cationic lipids over 50%, citing Lin and Ahmad. As discussed in Section IX, Lin and Ahmad are ***not*** drawn to nucleic acid-lipid particles, but are drawn to lipoplexes, which are a different type of particle compared to a "nucleic acid-lipid particle," excluded from the scope of the challenged claims, and now recognized in the literature as unsuitable for use *in vivo*. In addition, as discussed in Section VI, particles encompassed by claim 1 were superior in silencing gene expression of Factor VII *in vivo* relative to formulations having conjugated lipid levels above the claimed range. EX2014, Figure 12; EX2031, ¶¶152-162.

VII. ADDITIONAL OBJECTIVE INDICIA OF NON-OBVIOUSNESS FURTHER REBUT ANY PRIMA FACIE OBVIOUSNESS[7]

Patent Owner also demonstrated long felt need, failure of others, skepticism

[7] All of the above objective indicia were previously presented in the '739 IPR. '739 IPR, Paper 24, 53-61. Despite being placed on notice of this issue, Petitioner has

in the industry, and commercial success. *E.g.*, '739 IPR, Paper 24, 21-28, 53-61; EX2007, 34; *Apple Inc. v. Samsung Elecs. Co.*, 839 F.3d 1034, 1052 (Fed. Cir. 2016) (en banc).

A. Long-Felt Need – the delivery problem was not solved for over 20 years

"Evidence of a long-felt but unresolved need" weighs in favor of non-obviousness. *Apple*, 839 F.3d at 1056. The therapeutic potential of RNAi has been appreciated for over 25 years. However, effectively delivering RNA to target cells without eliciting vehicle-related toxicity prevented realization of this potential. *See, e.g.,* EX2016, 38, 42; EX2018, 11; EX2004, ¶¶170-71. The mid-2000's saw dramatic growth and investment in RNAi-based therapeutics. Yet, despite $2.5-3.5 billion in investment, no solution for the delivery problem had been found. EX2019, 1; EX2004, ¶¶171-73; EX2031, ¶¶122-30.

By 2008, the industry-wide failure to identify a solution to the delivery challenge resulted in waning confidence that RNAi could deliver on its therapeutic promise. EX2019, 2, 10. Much of the delivery technologies identified around this

---

not made any attempt to rebut or even address the evidence. Petitioner's failure to address the evidence of objective indicia at the petition stage, as required by *Graham*, requires a conclusion that Petitioner has failed to demonstrate the unpatentability of the claims by a preponderance of the evidence.

time are inferior to the claimed invention. EX2019, 10 ("Roche's Factor VII patent application (WO 2010/055041) features Alnylam's 'lipidoid' technology for the rodent studies, but then switched to [Patent Owner's] SNALP liposomes for the nonhuman primate part of the patent application."); *see also* EX2004, ¶174; EX2031, ¶126.

Prior to the first publication of the '435 patent disclosure, Alan Sachs, leader of RNA Therapeutics at Merck, identified delivery as the challenge to successfully developing RNAi drugs.

> What's interesting about what we do is that the drug isn't the problem. **It's the delivery of it.**

EX2020, 7 (emphasis added). As explanation for the lack of viable delivery chemistries, Dr. Sachs elaborated on the nature of the challenge.

> If it were so easy [with targeted therapeutics], one would have to describe why so few examples exist. The same is true in the RNAi delivery process. You can write down the steps. You can write down what you think will happen. But then you have to put it in a 50-nanometer particle that's safe and potent to deliver.

EX2020, 4; *see also* EX2004, ¶¶175-176. The long-felt need for a siRNA delivery vehicle and the difficulty in finding a solution is further exemplified by the 500 person-years and $200M that Patent Owner invested into SNALP technology. EX2019, 8; *see also* EX2004, ¶177. Prior to the serum-stable nucleic acid-lipid

particles disclosed in the '069 patent, there were no proven solutions to the delivery problem that had long plagued the industry. EX2004, ¶178; EX2031, ¶¶123-30. The over 20-year long-felt need in the industry supports a finding of nonobviousness.

B. Failure of Others – those in the art failed to formulate nucleic acid-lipid particles suitable for systemic delivery

"Evidence that others tried but failed to develop a claimed invention may carry significant weight in an obviousness inquiry." *Eurand, Inc. v. Mylan Pharms., Inc.*, 676 F.3d 1063, 1081 (Fed. Cir. 2012); *see also Boehringer Ingelheim Vetmedica, Inc. v. Schering-Plough Corp.*, 320 F.3d 1339, 1354 (Fed. Cir. 2003). The obviousness grounds in the petition demonstrate that there is long-term evidence of a failure of others to achieve the claimed invention. The petition argues that there is a general motivation to obtain efficacious nucleic acid-lipid particles formulated or suitable for systemic delivery. Yet, no prior art publication teaches achievement of the serum-stable nucleic acid-lipid particles of the invention. Indeed, due to toxicity and other concerns, the cited references would have led a POSITA to formulate particles having low cationic lipid and relatively higher conjugated lipid to shield the particles from toxicities. *E.g.*, EX2004, ¶¶25-35, 62, 174; EX2031, ¶¶123-30.

Those in the art failed to achieve nucleic acid-lipid particles suitable for systemic delivery for decades, and instead suffered through 25 years of industry-

wide failure to identify a solution to the delivery problem. The failure of others in the industry supports the non-obviousness of the '069 patent. *See* EX2004, ¶174.

C. Skepticism – those in the art questioned the safety of the SNALP as a suitable delivery platform

"Doubt or disbelief by skilled artisans regarding the likely success of a combination or solution weighs against the notion that one would combine elements in references to achieve the claimed invention." *WBIP, LLC v. Kohler Co.*, 829 F.3d 1317, 1335 (Fed. Cir. 2016). Whether the skepticism is before or after the invention matters not. *Transocean Offshore Deepwater Drilling, Inc. v. Maersk Drilling USA, Inc.*, 699 F.3d 1340, 1352-53 (Fed. Cir. 2012) (skepticism even after invention is probative of non-obviousness).

The art widely believed that cationic lipid content should be minimized to avoid cytotoxicity, aggregation, and unwanted interactions with non-targeted cells. *See* EX2004, ¶¶31, 32, 179. For example, Dr. Zamore of Alnylam, acknowledged in 2003 that various cationic lipid formulations could successfully deliver siRNA to cells *in vitro* but stated that "I wouldn't want anyone injecting cationic lipids into my bloodstream." EX2016, 42; *see* EX2004, ¶180; EX2031, ¶¶131-32.

Dr. Sachs of Merck also expressed skepticism, in 2010, as to the safety of the SNALP platform for delivery of siRNA.

> First are lipid-based delivery systems. At the time of our acquisition of Sirna, they had successfully shown lipid-based delivery to the liver.

> Initially, it was through a collaboration with what is now called [Patent Owner]. That was really the leading standard for the area. Several [applications to begin clinical trials] have been filed with the FDA. We spent a lot of internal research money and time on novel lipids. ***The liability of that platform is absolutely its safety***.

EX2020, 5 (emphasis added); *see* EX2004, ¶181.

These facts rebut the hindsight-driven analysis and assertions of the petition that the nucleic acid-lipid particle compositions of the invention would have been obvious to a POSITA in April 2008. *See* EX2004, ¶179-81; EX2031, ¶133.

D. Commercial Success – the claimed nucleic acid-lipid particle is the first FDA approved siRNA drug

Independent claim 1 of the '069 patent encompasses the first nucleic acid-lipid particle approved by the FDA for gene therapy. The '069 patent is now listed in the Orange Book as protecting the patisiran—tradename "Onpattro"—commercial product. EX2025. Patisiran was developed by Alnylam Pharmaceuticals under license from Patent Owner. EX2004, ¶¶191-193; EX2031, ¶¶134-36.

Patisiran has received tremendous recognition in the field and gained regulatory approval in the U.S. and Europe and has been designated by the FDA as a "first-in-class" drug. EX2024. According to James Cardia, head of business development at RXi Pharmaceuticals, which is developing RNAi treatments: "This approval is key for the RNAi field" and "is transformational." EX2023, 291; *see*

also EX2004, ¶190. The literature explicitly credits the nucleic acid-particle delivery technology for the success of patisiran. EX2023, 291-292 (crediting the groundbreaking nature of patisiran to its delivery vehicle); EX2002, 77:8-86:23; EX2031, ¶¶134-36. *PPC Broadband, Inc. v. Corning Optical Communs. RF, LLC*, 815 F.3d 734, 747 (Fed. Cir. 2016) (explaining that there is a presumption of nexus when the commercial product falls within the scope of the claimed invention) (citing *Ecolochem, Inc. v. S. Cal. Edison Co.*, 227 F.3d 1361, 1378 (Fed. Cir. 2000)).

VIII. ANTICIPATION IN VIEW OF OVERLAPPING RANGES FAILS (GROUNDS 1 AND 3)

The petition also fails to demonstrate anticipation of the claimed ranges.

A. No Affirmative Teaching of a Phospholipid Range Defeats Petitioner's Anticipation Theory

It is axiomatic that to anticipate, ***every element and limitation*** of the claimed invention must be found in a ***single*** prior art reference, arranged as in the claim. *Karsten Mfg. Corp. v. Cleveland Golf Co.*, 242 F.3d 1376, 1383 (Fed. Cir. 2001). As explained above in Section V.A, the prior art does not provide a phospholipid range, much less the range reported by Petitioner in its Tables. *E.g.* Pet. 39. As the prior art fails to disclose every element of the claim, Petitioner's anticipation theories in both Grounds 1 and 3 fail on this basis alone.

B. Ranges in the Art are Not Sufficiently Specific to Anticipate the Claimed Ranges

Petitioner cites to various ranges in the '196 PCT and the '189 and'554 publications in addressing the remaining claimed ranges of the cationic lipid, cholesterol, and conjugated lipid. To the extent that such citations are intended to advance an anticipation theory, that theory also fails. EX2004, ¶¶124-25. Initially, Dr. Janoff considers the claimed phospholipid range of 0.5 to 2 mol% to be broad and immense (EX2033, 60:25-61:24), which directly contradicts his declaration that the ranges of the prior art are sufficiently specific to anticipate the claimed ranges.

Anticipation requires that a reference ***clearly and unequivocally*** disclose the claimed invention without any need for picking, choosing, and combining various disclosures not directly related to each other in the cited reference. *Net MoneyIN, Inc. v. Verisign, Inc.*, 545 F.3d 1359, 1371 (Fed. Cir. 2008); *Arkley*, 455 F.2d at 587. For example, in *Atofina*, although the prior art fully encompassed the claimed range, the Federal Circuit explained that "[g]iven the considerable difference between the claimed range and the range in the prior art, no reasonable fact finder could conclude that the prior art describes the claimed range with sufficient specificity to anticipate th[e] limitation." *Atofina v. Great Lakes Chem. Corp.*, 441 F.3d 991, 999 (Fed. Cir. 2006); *see also* EX2004, ¶¶130-32. The fact pattern and holding of *Atofina* is applicable to Petitioner's assertions of anticipation here.

a. Ground I—Neither the '196 PCT nor the '189 Publication Anticipate the Remaining Claimed Ranges

Claim 1 recites a cationic lipid range of 50mol% to 85mol%, a cholesterol or derivative thereof of 30mol% to 40mol%, a phospholipid range of 4-10% and a conjugated lipid of 0.5 mol% to 2mol%. EX2031, ¶137.

For the cationic lipid, Petitioner relies on the disclosure of the '196 PCT and the '189 publication that "[t]he cationic lipid typically comprises from about 2% to about 60% of the total lipid present in said particles … [i]n other preferred embodiments, the cationic lipid comprises from about 40% to about 50% of the of the total lipid present in said particle." Pet. 32-33 (quoting EX1003, ¶88; EX1004, ¶152). Petitioner relies also on the '618 patent as disclosing a formulation of 56% cationic lipid, 14% phospholipid, and 30% cholesterol, as well as other formulations containing over 50% cationic lipid. Pet. 33 (citing EX1017, 34:54-35:23). Petitioner asserts that "[g]iven the breadth of the claimed range, these disclosures are sufficiently specific to anticipate the claimed range." Pet. 33 (citing EX1008, ¶112); EX2031, ¶¶138-40.

Petitioner does not explain, however, how a range of about 2% to about 60% cationic lipid is sufficiently specific to anticipate the claimed cationic lipid range of 50 mol% to 65 mol%. As in *Atofina*, the claimed range overlaps, but does not fall within the disclosed range. Moreover, a range of 2% to 60% is much broader than the claimed range of 50 mol% to 65 mol%. EX2031, ¶140.

As stated in Section V.A.i, Petitioner's reliance on the '618 patent is ineffectual. The particle containing 56% cationic lipid does not contain any conjugated lipid, and the amount of phospholipid (14%) is outside of the claimed range of 4mol% to 10mol%. EX1017, 35:17. Other formulations containing higher amounts of phospholipid are missing other components, such as the phospholipid and conjugated lipids. *Id.*, 35:20. Such formulations clearly do not anticipate the claims. Petitioner wholly fails to demonstrate how the formulations of the '618 patent relate to its anticipation analysis. EX2031, ¶¶141-42.

As for cholesterol, Petitioner asserts that the prior art teaches that, if present, the cholesterol comprises from about 20 % to about 45%. Pet. 38 (citing EX1003, ¶91; EX1004, ¶152). Petitioner also relies on the '618 patent for disclosing a formulation of 56% cationic lipid, 14% phospholipid, and 30% cholesterol. Pet. 38 (citing EX1017, 34:54-35:23). Again, although the disclosed range of about 20% to about 45% encompasses the claimed range of 30 mol% to 40 mol%, the presence of cholesterol is optional, and the about 20% to about 45% range of the '196 PCT and the '189 publication of an optional component is not sufficiently specific to anticipate. EX2031, ¶143.

Finally, Petitioner relies on the disclosure of a bilayer stabilizing component of about 0.5% to about 25% of the total lipid, asserting that disclosure is sufficiently specific to anticipate. Pet. 40 (citing EX1003, ¶¶92-93; EX1004, ¶152;

EX1008, ¶120). As an initial threshold, a bilayer stabilizing components is not sufficiently specific to a conjugated lipid. Further, the disclosed range is much broader than the claimed range of 0.5 mol% to 2 mol%. Thus, the disclosure of about 0.5% to about 25% is not sufficiently specific to be anticipatory. EX2031, ¶¶144-45.

b. Ground 3—The '554 Publication is Not Anticipatory

For the cationic component limitation, Petitioner relies on the prior art ranges of "about 2% to about 60%" and "about 40% to about 50%." Pet. 55 (citing EX1005, ¶116). Neither range is representative of the cationic lipid range of 50mol% to 65mol% required by claim 1, and a POSITA would not consider the ranges in the '554 publication as describing the claimed range with sufficient specificity. EX2004, ¶¶130-32. Petitioner further relies on the L054, L097, and L109 formulations of Table 4, which include 50% cationic lipid, as well as L060, L061, L098-103, L114, L116, and L117 as containing 52% cationic lipid. Pet. 55-56 (citing EX1008, ¶155). Petitioner does not explain, however, how the formulations containing those amounts of cationic lipids relate to the claimed particles. For example, L054, contains 50% DMOBA (cationic lipid), 20% DSPC (phospholipid), 28% cholesterol, and 2% PEG-n-DMG. EX1005, Table 4. The L054 formulation is not anticipatory as both the phospholipid and cholesterol fall

outside of the ranges for those components required by the claims. EX2031, ¶¶146-49.

As for cholesterol, Petitioner relies on the '554 publication, which teaches that cholesterol may comprises 20% of 45% of the total lipid. Pet. 57 (citing EX1005, ¶313). And as discussed above (Section V.A.ii), Petitioner relies on L106 as an example of a formulation having 30% cholesterol. Pet. 57. L106 contains 67% DMOBA (cationic lipid), 30% cholesterol, and 3% 2KPEG-cholesterol, but no phospholipid, and thus cannot anticipate claim 1. EX1005, Table 4. Petitioner fails to explain the relevance of the L106 formulation to the'554 publication, much less the claimed particles. EX2031, ¶150.

For the conjugated lipid, Petitioner notes that the '554 publication teaches that a PEG-conjugate comprises about 1% to 20% of the total lipid, which it asserts is sufficiently specific to anticipate the claimed range. Pet. 58-59 (citing EX1005, ¶118; EX1008, ¶159). But again, the claimed range and the prior art range are considerably different. As in *Atofina*, this small overlap does not describe the entire claimed range with sufficient specificity to anticipate the limitation. EX1007 ¶¶148, 150, 151; *see also* EX2004, ¶¶130-32; EX2031, ¶151.

IX. GROUND 2 FAILS

Petitioner contends that claims 1-22 of the '069 patent are obvious in view of "patent owner's prior disclosures" in light of Lin and/or Ahmad. The petition,

however, only addresses the cationic lipid limitations of claims 1 and 8. Pet. 49-53; EX1008, ¶¶145-149.

At best, Ground 2 is little more than an attempt to backfill Ground 1 by adding "Lin and/or Ahmad." Thus, Ground 2 inherits all the defects of Ground 1. *See* Sections V, VIII. For example, Ground 2 fails at least because neither the '196 PCT nor the '189 publication discloses phospholipid from 4mol% to 10mol%, as recited in claim 1. *See also* Sections VI-VII (unexpected results and objective indicia further support nonobviousness).

As a threshold matter, "lipoplexes" as in Lin and Ahmad are a fundamentally different type of particle compared to a "nucleic acid-lipid particle," excluded from the scope of the challenged claims, and are expressly differentiated both in the challenged patent and the cited art. *E.g.*, EX2004, ¶¶89, 95-99; EX2031, ¶¶152-54; Sections I-II.

Moreover, Petitioner's argument that increasing cationic lipid "could" increase transfection efficiency is insufficient to establish obviousness. *See* Pet. 49 ("***may*** increase..."), 50 ("...***potentially*** increase..."), *and* EX1008, ¶¶146-149 (parroting the same language). It is well established, however, that such assertions of what one "might" do or what "could" be done have never been sufficient in establishing obviousness. *InTouch Techs. Inc. v. VGo Communs., Inc.*, 751 F.3d 1327,1351-52 (Fed. Cir. 2014) (obviousness analysis failed for stating "that one of

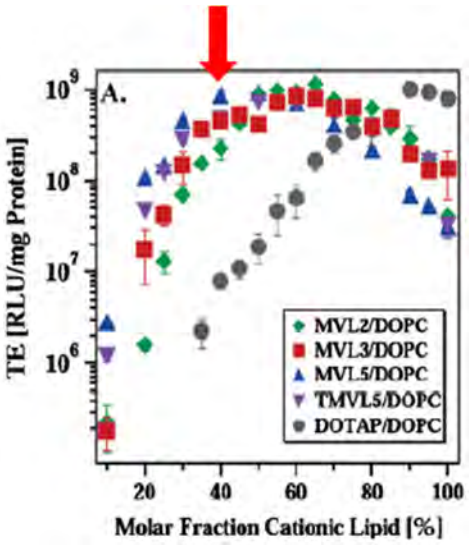
ordinary skill in the art *could* combine these references, not that they *would* have been motivated to do so.") (original emphasis); *PersonalWeb Techs., LLC v. Apple, Inc.*, 848 F.3d 987, 993-4 (Fed. Cir. 2017) (same); *Belden Inc. v. Berk-Tek LLC*, 805 F.3d 1064, 1073 (Fed. Cir. 2015) (same).

Ground 2 also fails because it provides only unsupported and conclusory remarks regarding motivation to combine and expectation of success. *See* EX2004, ¶94; EX2031, ¶152.

A. Lin and Ahmad Do Not Supply the Missing Motivation for Ground 2

The petition fails to establish that one would have been motivated to combine the disclosures of the '196 PCT or the '189 publication with those of Lin/Ahmad, or that there would have been any reasonable expectation of success in doing so. As a threshold matter, Lin and Ahmad are directed to "lipoplexes"—a fundamentally different type of particle compared to a "nucleic acid-lipid particle" and outside the scope of the claimed invention. *E.g.*, EX2004, ¶¶95-100 ("Lipoplexes are ***not*** nucleic acid-lipid particles."); EX2001, 122:1-24 (Dr. Janoff identifying lipoplexes as outside the scope of the challenged claims); EX2009, 2:26-40, 2:52-65 (identifying lipoplexes as structurally and functionally distinct); EX1001, 2:12-18, 3:3-10 (differentiating nucleic acid-lipid particles from lipoplexes); EX2031, ¶¶153-57.

Despite being placed on notice of the above evidence in the '739 IPR, Petitioner does not explain why one would have looked to this fundamentally different technology to modify a SNALP of the cited '196 PCT (or '189 publication) or that they would have had a reasonable expectation of success in doing so. *See* EX2003, ¶25 (acknowledging Lin and Ahmad as lipoplexes); *see also e.g.* Sections II, V.B (establishing the complicated nature of the technology.).



Although Lin/Ahmad tested different lipoplex formulations to compare saturation dynamics relative to control, neither reference instructs the use of any particular cationic lipid concentration in a formulation. In fact, Ahmad's experiment shows reaching saturation around 40 mol%, after which point the rate of enhanced metabolic burden far exceeds any further minimal TE gain. EX1007, Fig. 3A (annotated – shown), 739 ("...at intermediate σ_M, TE exhibits saturated behavior..."), 745 ("This means that much more cationic lipid is required to achieve optimal TE at large lipid/DNA charge ratios."), ("Minimizing the amount of cationic lipid is desirable to reduce cost as well as potential toxic effects of the cationic lipid."); *see also* EX2007, 31-34; EX2031, ¶¶158-6.

B. Petitioner Ignores Content of Lin and Ahmad that Undermines its Obviousness Assertions

According to Petitioner, a cationic lipid variable that may impact transfection efficacy the use of cationic lipids that result in a net neutral charge at physiological pH. Pet. 10. Petitioner's own publications attribute toxicity to bioaccumulation and immunogenicity—not net charge. EX2031, ¶161. Ahmad expressly advocates for multivalent cationic lipids so as to maintain charge with a *lower amount* of molecules, identifying bioaccumulation and metabolic burden as toxicity mechanisms. EX1007, 745.

Although it is not Patent Owner's burden to prove no motivation or reasonable expectation of success, that is the only reasonable conclusion in view of the evidence. Both Lin and Ahmad expressly acknowledge the complicated nature of what affects transfection efficiencies. EX1006, 3315; EX1007, 740. And Dr. Thompson explained during the '739 IPR and explains here that neither Lin nor Ahmad would reasonably be viewed as advocating cationic lipid above 50 mol% in a formulation. EX2004, ¶¶100, 101; EX2031, ¶162. Rather, the central point of Lin and Ahmad was to ***reduce*** cationic lipid (and the corresponding metabolic burden/toxicity) through use of multivalent lipids (MVLs)—that is, lipids that have more positive charge per individual molecule. EX2006, 409:3-19 ("the bottom line message of Lin and Ahmad is how to reduce cationic lipid concentration in a formulation..."), 251:3-22, 252:11-19. Petitioner's expert

acknowledges that Ahmad advocates the use of MVLs as a means for reducing the ***amount*** of cationic lipid. EX2002, 31:7-39:9.

Thus, the teaching in Lin and Ahmad actually undermine Petitioner's obviousness assertion. EX2031, ¶162.

X. THE DEPENDENT CLAIMS ARE NEITHER OBVIOUS NOR ANTICIPATED

Claims 2-22 are not obvious for the same reasons identified above with respect to claim 1 for each of the grounds of challenge. Additional discussion for some of claims 2-20 is provided below which illustrates further reasons why each of the grounds of challenge to these claims fail. EX2031, ¶163.

A. Claim 8

Claim 8 specifies that the cationic lipid comprises from 52 mol% to 62 mol% of the total lipid present in the particle. Given the breadth of the disclosed cationic lipid ranges (*e.g.*, Section V.C), the prior art does not anticipate this narrower range. In addition, those broad ranges do not invite routine experimentation, and as Petitioner provides no reason as to why the prior art suggests this narrower range, much less discussing a reasonable expectation of success, the petition fails to demonstrate that claim 8 is anticipated or rendered obvious by the prior art. EX2031, ¶164.

B. Claim 14

Claim 14 is drawn to the nucleic acid-lipid particle of claim 10, wherein the nucleic acid-lipid particle comprises about 57.1 mol% cationic lipid, about 7.1 mol% phospholipid, about 34.3 mol% cholesterol or a derivative thereof, and about 1.4 mol% PEG-lipid conjugate.

Petitioner asserts that as construed by the examiner, comprising about encompasses ±10, 20, or 30 mol% of each lipid component, and thus this claim is unpatentable for the same reasons set forth as to claim 1. Pet. 45 (citing EX1008, ¶134). Petitioner does not address claim construction using a *Phillips* standard. In addition, the unexpected results reported in the specification, as well as the other objective indicia of nonobviousness, are directly on point, as this claim is drawn to a 1:57 particle. As Petitioner fails to provide a reason to arrive at the claimed lipid amounts, or a reasonable expectation of success of achieving the claimed particle, Petitioner's challenge necessarily fails. EX2031, ¶¶165-66.

C. Claim 15

Claim 15 that the conjugated lipid comprises from 1 mol% to 2 mol% of the total lipid present in the particle. As discussed for claim 8, the breadth of the disclosed ranges of the conjugated lipid do not anticipate the claimed range, nor does the breadth invite routine optimization. *E.g.*, Section V.C. Given no reason to arrive at the claimed range, nor any discussion of a reasonable expectation of

success, the petition fails to demonstrate that claim 15 is anticipated or rendered obvious by the prior art. EX2031, ¶167.

D. Claim 16

Claim 16 is drawn to the nucleic acid-lipid particle of claim 1, wherein the nucleic acid in the nucleic acid-lipid particle is not substantially degraded after incubation of the particle in serum at 37°C for 30 minutes. No explanation is given for why a POSITA would have had a reasonable expectation of success generating serum stable nucleic acid-lipid particles having a high level of cationic lipid and a low level of conjugated lipid. *E.g.*, FWD, 33-34. Moreover, Petitioner is merely engaging in claim mapping, and does not relate serum stability to the teachings of the lipid components. And even assuming *arguendo* it may have been obvious to ***test*** for serum stability, the ability to test does not provide a motivation or reasonable expectation of success of achieving such particles. EX2031, ¶168.

E. Claim 17

Claim 17 is drawn to the nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle. No explanation is given for why a POSITA would have had a reasonable expectation of success generating fully encapsulated nucleic acid-lipid particles having a high level of cationic lipid and a low level of conjugated lipid. FWD, 33-34. Moreover, as noted in a patent in which Dr. Janoff is listed as an author, encapsulation may not

be inferred from the composition or production method, but need be determined by testing. EX2009, 4:15-19; EX2031, ¶¶169-71.

F. Claim 18

Claim 18 is drawn to the nucleic acid-lipid particle of claim 1, wherein the nucleic acid-lipid particle has a lipid:nucleic acid mass ratio of from about 5 to about 15. The petition (Pet. 47) asserts a ratio of 12.5 to 100, which is much broader than the claimed range, and thus the disclosed range does not support anticipation, nor does it invite routine experimentation to arrive at the claimed range. EX2031, ¶172; *e.g.*, Section V.C.

G. Claim 20

Claim 20 is drawn to the nucleic acid-lipid particle of claim 1, wherein the phospholipid comprises from 5 mol% to 9 mol% of the total lipid present in the particle." As discussed in Section V.A, the prior art does not provide a specific phospholipid range. And as discussed for claim 8, the breadth of the disclosed ranges of the neutral lipid do not anticipate the claimed range, nor does the breadth invite routine optimization. *E.g.*, Section V.C. Given no reason to arrive at the claimed range, nor any discussion of a reasonable expectation of success, the petition fails to demonstrate that claim 20 is anticipated or rendered obvious by the prior art. EX2031, ¶173.

H. Claim 21

Claim 21 is drawn to the nucleic acid-lipid particle of claim 1, wherein the cholesterol or derivative thereof comprises from 32 mol% to 36 mol% of the total lipid present in the particle. As discussed for claim 8, the breadth of the disclosed ranges of cholesterol do not anticipate the claimed range, nor does the breadth invite routine optimization. *E.g.*, Section V.C. Given no reason to arrive at the claimed range, nor any discussion of a reasonable expectation of success, the petition fails to demonstrate that claim 21 is anticipated or rendered obvious by the prior art. EX2031, ¶174.

I. Claim 22

Claim 22 is drawn to a "pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier." The petition does not explain why a POSITA would have had a reasonable expectation of success generating a pharmaceutical composition of nucleic acid-lipid particles that have a high level of cationic lipid and a low level of conjugated lipid. A POSITA would have expected the claimed particles to be prone to aggregation and unstable due to a high cationic lipid and low conjugated lipid. EX2004, ¶92; EX2031, ¶175.

XI. CONCLUSION

For the reasons set forth above, the petition fails to meet its burden of establishing the unpatentability of the claims, and Patent Owner respectfully submits that the challenged claims should be found to be not unpatentable.

Respectfully submitted,

Dated: November 13, 2019

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
Reg. No. 52,182

XII. CERTIFICATE OF COMPLIANCE

Pursuant to 37 C.F.R. §42.24(d), the undersigned certifies that this paper contains no more than 14,000 words, not including the portions of the paper exempted by §42.24(b). According to the word-processing system used to prepare this paper, the paper contains 13,879 words.

Respectfully submitted,

Dated: November 13, 2019

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
Reg. No. 52,182

XIII. APPENDIX – LIST OF EXHIBITS

Exhibit No.	Description
2001	Deposition Transcript of Andrew S. Janoff in IPR2018-00739, December 4, 2018
2002	Deposition Transcript of Andrew S. Janoff in IPR2018-00739, April 5, 2019
2003	Reply Declaration of Dr. Andrew S. Janoff in IPR2018-00739, December 4, 2018
2004	Declaration of David H. Thompson, Ph.D. in IPR2018-00739
2005	Deposition Transcript of David H. Thompson in IPR2018-00739, February 4, 2019 (Volume I)
2006	Deposition Transcript of David H. Thompson in IPR2018-00739, February 5, 2019 (Volume II)
2007	Patent Owner's Sur-Reply in IPR2018-00739
2008	Listing of Example Formulations Falling Within the Scope of the '069 Patent Claims
2009	U.S. Patent No. 7,491,409
2010	U.S. Patent No. 8,236,943
2011	U.S. Publication No. 2013/0116307
2012	U.S. Publication No 2017/0307608
2013	U.S. Patent No. 9,404,127
2014	International Publication No. WO 2010/088537
2015	International Publication No. WO 2013/090648

2016	Charles W. Schmidt, *Therapeutic Interference: Small RNA Molecules Act as Blockers of Disease Metabolism* AM. CHEM. SOC'Y 37 (2003)
2017	C. Russell Middaugh & Joshua D. Ramsey, *Analysis of Cationic-Lipid-Plasmid-DNA Complexes*, ANALYTICAL CHEMISTRY 7240 (2007)
2018	Erika Check, *RNA to the Rescue?* 425 NATURE 10 (2003)
2019	Dirk Hausseker, *The Business of RNAi Therapeutics in 2012*, 2 AM. SOC'Y OF GENE & CELL THERAPY (2012)
2020	Luke Timmerman, Merck's Alan Sachs, on RNAi's Big Challenge: Delivery, Delivery, Delivery, XCONOMY (Jan. 21, 2010), https://xconomy.com/national/2010/01/21/mercks-alan-sachs-on-rnais-big-challenge-delivery-delivery-delivery/
2021	Sean C. Semple, et al., *Rational Design of Cationic Lipids for siRNA Delivery*, 28 NATURE BIOTECH. 172 (2010)
2022	Supplementary Figures to Sean C. Semple, et al., *Rational Design of Cationic Lipids for siRNA Delivery*, 28 NATURE BIOTECH. 172 (2010)
2023	Heidi Ledford, *Gene-Silencing Drug Approved: US Government Okays First RNA-Interference Drug — After a 20-Year Wait* 560 NATURE 291 (2018)
2024	*FDA Approves First-of-its Kind Targeted RNA-based Therapy to Treat a Rare Disease*, FOOD AND DRUG ADMIN. (2018), https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm616518.htm
2025	Orange Book: Approved Drug Products with Therapeutic Equivalence Evaluations, *Patent and Exclusivity for: N210922*, FOOD AND DRUG ADMIN. *available at* https://www.accessdata.fda.gov/scripts/cder/ob/patent_info.cfm?Product_No=001&Appl_No=210922&Appl_type=N (last visited Dec. 19, 2018)

2026	Maja Sedic et al., *Safety Evaluation of Lipid Nanoparticle-Formulated Modified mRNA in the Sprague-Dawley Rat and Cynomolgus Monkey*, VETERINARY PATHOLOGY (2017)
2027	Kapil Bahl et al., *Preclinical and Clinical Demonstration of Immunogenicity by mRNA Vaccines against H10N8 and H7N9 Influenza Viruses*, MOLECULAR THERAPY (2017)
2028	Declaration of Andrew S. Janoff in IPR2018-00739
2029	DICTIONARY OF PHARMACY (2004)
2030	Declaration of Andrew S. Janoff in IPR2018-00680
2031	Declaration of David H. Thompson, Ph.D.
2032	Curriculum Vitae of David H. Thompson, Ph.D.
2033	Deposition Transcript of Andrew S. Janoff, October 25, 2019
2034	Doxil Label – FDA (Revised May, 2007), https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/050718s029lbl.pdf
2035	Ian MacLachlan & Pieter Cullis, *Diffusible-PEG-Lipid Stabilized Pasmid Lipid Particles,* 53 ADVANCES IN GENETICS 157 (2005)
2036	Sean C. Semple et al., *Immunogenicity and Rapid Blood Clearance of Liposomes Containing Polyethylene Glycol-Lipid Conjugates and Nucleic Acid,* 312 THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS 1020 (2005)
2037	International Publication No. WO 2017/223135
2038	*New Medicine for Hereditary Rare Disease,* EUROPEAN MED. AGENCY (2018), https://www.ema.europa.eu/en/news/new medicine-hereditary-rare-disease
2039	*Arbutus' LNP Licensee Alnylam Announces FDA Approval of ONPATTRO™ (patisiran), for the Treatment of ATTR Amyloidosis,* ARBUTUS BIOPHARMA (2018), https://investor.arbutusbio.com/news-releases/news-release-details/arbutus-lnp licensee-alnylam-announces-fda-approval-onpattrotm

CERTIFICATE OF SERVICE

This is to certify that I caused to be served a true and correct copies of the foregoing Patent Owner's Response Pursuant to 37 C.F.R. § 42.107 and corresponding Exhibits 2028 through 2039 on this 13th day of November 2019, on the Petitioner at the correspondence address of the Petitioner as follows:

Michael Fleming
C. Maclain Wells
IRELL & MANELLA LLP
mfleming@irell.com
mwells@irell.com
ModernaIPR@irell.com

Respectfully submitted,

Dated: November 13, 2019

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
Reg. No. 52,182

JOINT APPENDIX 66

Paper No. ___
Filed: March 31, 2020

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

MODERNA THERAPEUTICS, INC.,
Petitioner,

v.

ARBUTUS BIOPHARMA CORPORATION,
Patent Owner.

Case IPR2019-00554
Patent No. 8,058,069

PATENT OWNER'S SUR-REPLY

TABLE OF CONTENTS

Page

I. INTRODUCTION

The Reply is largely an untimely attempt to cure deficiencies identified in the Patent Owner Response (POR). Consolidated Trial Practice Guide, 73 ("Petitioner may not submit new evidence in reply that it could have presented earlier, e.g. to make out a prima facie case of unpatentability."s); *Intelligent Bio-Sys., Inc. v. Illumina Cambridge Ltd.*, 821 F.3d 1359, 1369 (Fed. Cir. 2016) ("Unlike district court litigation… the expedited nature of IPRs bring with it an obligation for petitioners to make their case in their petition to institute.").

Much of the Reply relies on attacking arguments Patent Owner ("PO") never made, mischaracterizing the deposition testimony of PO's expert, and blatantly ignoring detrimental testimony from Petitioner's first expert. Beyond that, the Reply newly attempts to overstate the importance of irrelevant parameters (e.g., N/P ratio), fabricate "trends," and falsely argue non-toxic cationic lipids. These arguments not only lack any supporting evidence, but are contradicted by the references of record, including Petitioner's own publications.

First, Petitioner's anticipation charge is unaddressed in the Reply, and now appears abandoned.

As to Petitioner's obviousness assertions, PO's Response ("POR") (*e.g.*, 2-4, 11-31) laid out in detail how Petitioner failed to substantiate the "routine optimization" rationale at the heart of the cited *Peterson* and *du Pont* cases—*i.e.*,

the only obviousness theory identified in the petition and instituted by the Board under *SAS*. *E.g.*, Pet. 31-33, 38-40, 54, 56-59; Decision on Institution ("DI"), 24-27, 35-37. In fact, up until the Reply, Petitioner and its expert, Dr. Janoff, agreed on the inapplicability of routine optimization. The petition materials, presumably concerned by extensive experimental testing reported in the '069 patent, embraced the complexity of the technology and argued wild unpredictability. During cross-examination, Petitioner's expert witness repeatedly testified the prior art lipid ranges are "immense" and "would require undue experimentation, not simple optimization." EX2033, 60:5-16; 42:7-10; 19:25-20:15; POR 4, 19-27. As explained in the POR, Dr. Janoff was correct in this regard, undermining the ill-conceived obviousness case in the petition.

With the deficiencies in the petition case laid bare, Petitioner belatedly attempts to cure them—ignoring the evidence and testimony of its own witness entirely and now asserting "routine optimization."[1] Even if this untimely argument is entertained, it can be rejected on the merits for at least the reasons set forth

[1] Even in Reply, Petitioner continues its erratic oscillation on this point. While now arguing routine optimization, Petitioner returns to embracing complexity and unpredictability when attacking the extensive experimental testing. *E.g.,* Reply 24-25; *compare* EX2006, 405:5-12.

below. The evidence is overwhelming — achieving the nucleic acid-lipid particles of the '069 patent was *not* a matter of routine optimization.

To the extent any *prima facie* case of obviousness was established by identification of overlapping lipid ranges in the art, that case is rebutted by the extensive experimental data in the '069 patent and numerous post-filing publications showing unexpected results, including Petitioner's own publications. As corroborated in the literature (and unrebutted by Petitioner), high-level cationic lipid formulations (*e.g.*, 50-65% cationic lipid) would have been expected to have relatively poor *in vivo* activity and elicit increased toxicity and immunogenicity compared to lower-level cationic lipid formulations. EX1006, 3315; EX1007, 745; EX1009, E96; EX2009, 30:34-41.

PO, however, found that the claimed formulations surprisingly impart increased activity of the nucleic acid payload and improved tolerability of the formulations *in vivo*, resulting in a significant increase in the therapeutic index. POR, 31-42. Moreover, the claimed formulations are stable in circulation and are substantially non-toxic when administered to mammals. These surprising results are different in kind, and the Reply fails to demonstrate otherwise.

As such, when all the evidence of record is weighed and considered, Petitioner fails to meet its burden of demonstrating the unpatentability of the

claims by a preponderance of the evidence, and the patentability of the claims should be affirmed.

II. CLAIM CONSTRUCTION

No claim construction is necessary in order to determine that the Petition fails. The petition (23) offered no claim construction analysis, just the conclusory assertion that the claims should be construed as in the DI in IPR2018-00739 under a different claim construction standard—that is, "a particle that comprises a nucleic acid and lipids, in which the nucleic acid *may be* encapsulated in the lipid portion of the particle." *See also* EX1008, ¶88 (same). It ignored the specification and the prosecution history (including in IPR2018-00739) entirely, which is particularly pertinent under the different *Phillips* standard. The petition materials failed to satisfy the basic requirements of petition content. *See* 37 C.F.R. §42.104 (requiring analysis of how the claims are to be construed); 35 U.S.C. §312(a)(3).

To the extent that the Board is inclined to reach claim construction, Petitioner's proffered construction of ***"nucleic acid lipid particle***" is not only inconsistent with the specification, but it is unreasonably broad at least in that it encompasses empty particles. POR, 10. Dr. Anchordoquy concedes as much, testifying the term "nucleic acid lipid particle" could not encompass an empty particle because such a reading is excluded by the language of the claim itself.

EX1020, ¶30. Indeed, the claimed term is not "lipid particle" but "nucleic acid lipid particle." POR, 10; EX2031, ¶¶32-33.

Moreover, Petitioner's construction is inconsistent with the prosecution history, which includes the record in IPR2018-00739. *See* Patent Owner Preliminary Response ("POPR"), 15-17; POR, 9-10; *see also* IPR2018-00739, Paper 12, 16-17 (same); IPR2018-00739, Paper 24, 11-13 (same). *Kakun Pharm. Co. v. Iancu*, No. 18-2232, slip. op. at (Fed. Cir. March 23, 2020) (noting that statements by applicant to induce patent grant are particularly helpful); *Aylus Networks, Inc. v. Apple Inc.*, 856 F.3d 1353, 1358 (Fed. Cir. 2017) (holding that statements made during an IPR can be relied on to support prosecution disclaimer). During prosecution, applicants specifically discussed encapsulation as a feature of the claimed particles. EX1016, 38; EX2031, ¶34; POR, 9-10. And as discussed above, encapsulation was extensively argued during IPR2018-00739, with PO expressly arguing that the claims require encapsulation. POR, 9-10. That history clearly reflects the inventors understanding the claims to require encapsulation of the nucleic acid and cannot be ignored.

Even if the Board were to consider Petitioner's argument in Reply after review of the intrinsic record, it would not find in Petitioner's favor. Petitioner's new argument in reply is inconsistent with Dr. Janoff's testimony, which also contradicts testimony from Petitioner's second witness, Dr. Anchordoquy. Dr.

Janoff repeatedly testified that the same term "nucleic acid lipid particle", in view of the same specification as the '069 patent, should be limited to SNALP, a definition narrower than any construction previously or currently proffered. EX2001, 118:19-119:4, 119:9-17, 120:5-6, 121:14-25. Dr. Janoff additionally testified that lipoplex particles were fundamentally different than those described in the current specification, and outside the scope of the patent. EX2001, 122:1-24. In this regard, his testimony is consistent with the evidence of record. EX1001, 2:8-18, 2:50-54 (contrasting lipoplexes); EX2031, ¶¶152-58; EX1025, 13:15-22; EX2009, 2:54-67 (contrasting liposomes and lipoplexes); *see also* POR, 5, 44. Instead of addressing Dr. Janoff's testimony and the evidence he cited, Petitioner ignores the evidence and advances, through Dr. Anchordoquy, arguments that rely heavily on lipoplexes. To the extent the new argument in Reply is considered, it can be rejected as contradicted by the evidence of record.

III. DR. ANCHORDOQUY IS NOT A QUALIFIED EXPERT

Dr. Anchordoquy is not a qualified expert in the relevant field and his testimony should be given little, if any, weight. Dr. Anchordoquy is a zoologist, not a lipid chemist with formal training in the subject matter at hand. EX1020, ¶9.

Clearly concerned by this new witness' thin resume, Petitioner staged unprompted and rehearsed questioning during redirect during Dr. Anchordoquy's recent deposition. EX2043, 77:4-79:25. But that testimony is hardly reassuring.

See e.g., EX2043, 77:22-23 (describing his zoology training as broadly encompassing "everything related to animals"); 78:6-14 (testifying he was not in the offered biophysics program);78:13-21; 79:10-11 (identifying other individuals and lab equipment unrelated to the technology at hand in this proceeding). His prepared remarks during redirect may shed further light on his lack of qualifications but offer no reassurance his declaration testimony should be afforded any meaningful weight.

Moreover, Dr. Anchordoquy fails to meet Petitioner's own definition of the ordinary artisan. EX1020, ¶25; DI, 11-12 (discussing the level of ordinary skill in the art). Dr. Anchordoquy cites, as support for his expertise in the field, U.S. Patent No. 7,914,714. EX1020, ¶14. That patent, however, is neither the same nor similar to the technology at issue. Dr. Anchordoquy himself admitted that he has no experience with SNALPs. EX2043, 70:13-14; *see also id.*, 9:24-25 (noting he is an expert in lyophilization). Indeed, it does not seem that Dr. Anchordoquy has any relevant patents or publications at all.

In attempt to distract from the short-comings of its own expert, Petitioner spuriously attacks Dr. Thompson as lacking experience with cationic lipids because his current research involves developing polymer lipid carrier particles. Reply, 2-3. This illogical argument is akin to attacking a climate scientist as ignorant of greenhouse gases on the basis she is developing technology to reduce

them. Dr. Thompson is currently developing lipid carriers using polymers as a replacement of the more toxic cationic lipids, which he routinely uses as benchmarks in his research. EX1025, 8:19-9:10. Unlike Petitioner's expert, Dr. Thompson has published extensively and has decades of experience with lipid particles (including cationic lipids) and their *in vivo* delivery. EX1025, 7:19-25:6.

The Reply (2-3) attacks Dr. Thompson for citing a 2014 patent (EX2012) in discussing the formulation patisiran (trade name, Onpattro), widely reported in the literature and by the manufacturer (and described by Dr. Janoff) as having "50% cationic lipid, 38.5% cholesterol, 10% DSPC and 1.5% PEG." EX2003, ¶33. But, the evidence of record is clear, and each of Dr. Thompson (EX2031, ¶¶117-118, 134-136), Dr. Janoff (EX2003, ¶33), and Dr. Anchordoquy (EX2043, 71:19-24, 72:24-73:3) each agree that the challenged claims encompass patisiran.

IV. PETITIONER ABANDONS ANTICIPATION

The petition (5) asserted that claims 1-22 were anticipated by or obvious over the '196 PCT, the '189 publication, or the '554 publication. The POR (50-55) provided extensive argument as to why the petition failed to demonstrate anticipation of the claimed ranges. The Reply offers no rebuttal evidence or argument, thus PO maintains Petitioner has not shown anticipation of claims 1-22.

V. THE PETITION FAILS TO DEMONSTRATE THE OBVIOUSNESSS OF THE CLAIMS

As discussed in the POR, the Petition did not address whether the POSITA would consider formulating nucleic acid-lipid particles a matter of routine optimization, and the unrefuted evidence of record demonstrated that it was not. POR, 19-24. In addition, the Petition never explained how the broad ranges for lipid components disclosed by the prior art were sufficient under the caselaw as being the conditions required by the claims. POR, 25-27. Accordingly, the petition failed to meet its burden of demonstrating the obviousness of the claims under *Peterson* and *du Pont* by a preponderance of the evidence.

VI. PETITIONER'S NEWLY ADVANCED OBVIOUSNESS THEORIES ALSO FAIL

The Reply now attempts to back-fill the identified holes in its petition case,[2] belatedly arguing motivation and a reasonable expectation of success of achieving the claimed particles through routine experimentation. Such arguments should not be presented for the first time on reply and should be disregarded. However, as explained below, even if considered, they fall far short of establishing obviousness by a preponderance of the evidence.

---

[2] Petitioner also improperly attempted such gap-filling in contravention of proper scope for re-direct during Dr. Janoff's deposition. EX2033, 147:18-166:9.

In addition, Petitioner argues mere possibilities in asserting what "could" or can" be done. *E.g.*, Reply, 4 ("structures existed at the time of the '069 patent that *can* meet the claim limitations"), 13 ("POSITA would have understood … *potential* cationic lipid concentration ranges"), 22 ("could embrace"), 28 ("could be attributable"). Such assertions have never been sufficient to support obviousness. *PersonalWeb Techs., LLC v. Apple, Inc.*, 848 F.3d 987, 944-95 (Fed. Cir. 2017) ("reasoning...that [references] *could* be combined...is not enough: it does not imply a motivation").

At best, the allegations of unpatentability in the petition amount to little more than notice pleading. Such challenges, therefore, are insufficient and do not meet the statutory requirement of "particularity," and cannot be remedied on reply.

A. An Overlapping Phospholipid Range is NOT Disclosed

Petitioner's obviousness case asserted a presumption of obviousness based on alleged disclosure of overlapping ranges in the cited prior art where no such phospholipid range is disclosed. *E.g.*, POR, 12-19. Petitioner (Pet., 39, 58) contrived a phospholipid range of 0-19% or 0-19.5% through a series of assumptions. That was acknowledged by the Board in the DI, crediting a phospholipid range on the basis one could be manufactured through "reasonable inferences." DI, 23, 36.

The POR (12-19), however, explains the disconnect between the proffered legal theory (*i.e.*, presumption of obviousness under *Peterson* and *du Pont*) and the facts (*i.e.*, content of the prior art). Neither of those cases, or any other identified overlapping range case, supports an overlapping range presumption where there is no overlapping range. Petitioner disputes none of this in reply.

The Reply (5) does not dispute the cited art fails to expressly disclose a phospholipid range, it simply asserts that fact "is irrelevant." Not so. Petitioner cites to *IXI IP, LLC v. Samsung Elecs. Co., LTD.*, 903 F.3d 1257, 1264-1265 (Fed. Cir. 2018); it is inapposite. *IXI* is not an overlapping range case at all, and certainly does not support a presumption of obviousness under *Peterson* and *du Pont* by making a series of inferences to arrive at a range not affirmatively disclosed. Petitioner's attempt to shoehorn the present facts into an inapplicable legal framework must be rejected.

The Reply (5-6) also does not contest that its contrived phospholipid range of 0-19% or 0-19.5% is *not* at all reasonable. POR (14-19). Instead, Petitioner (Reply 5-6) abandons its original argument in favor of arguing a much broader phospholipid range of 5-90mol%. This too must be inferred from a more generic discussion of "non-cationic/neutral lipid"., The Reply falsely claiming Dr. Thompson agreed during deposition. Dr. Thompson expressly rejected it. *E.g.*, EX1025, 169:9-13 ("That's not the way I read this….").

Furthermore, Reply (5) also attempts to pivot to a new reference—Protiva's '910 publication (EX1015) that was cited by the Examiner during *ex parte* prosecution. Prosecution counsel's representation of the Examiner's Office Action rejection (which was overcome) is hardly an admission or evidence probative of the perspective of a POSITA. If Petitioner wanted to rely on the '910 publication as a ground reference it should have done so in its petition. *See also* 35 U.S.C. §325(d).

Accordingly, Petitioner's challenge based on a presumption of obviousness in view of overlapping ranges at least fails due to the lack of identified overlapping ranges.

B. Petitioner's Belated Assertions of Routine Optimization

As discussed in the POR (19-24), obviousness based on routine optimization was never substantiated in the petition materials. In fact, until the Reply, it was undisputed that developing nucleic acid-lipid particles was *not* a simple matter of routine optimization. That is supported by the scientific literature, testimony of Dr. Thompson, and testimony of Petitioner's first expert, Dr. Janoff. *E.g.*, EX2006, 403:22-25 ("Q. In the 2008 timeframe, was developing nucleic acid-lipid particles considered a routine matter of optimizing variables? A. No."); EX2001, 144:18-145:1; *see also* EX2014, Fig. 12 (demonstrating that even small changes in the amount of conjugated lipid concentration can impact the efficacy of the particles).

Moreover, Dr. Janoff repeatedly testified that he considered lipid ranges even narrower than those in the prior art to be immense. *E.g.*, EX1008, ¶74; EX2028, ¶¶73, 112; EX2033, 42:7-10 ("[i]f the range is immense, there would be undue experimentation I believe to find a combination or a range that behaved in a desirable light."). The petition (*e.g.*, 36) is littered with arguments of complexity and unpredictability, acknowledging that "even minor variations in lipid percentages appeared to impact efficacy."

The Reply ignores the extensive evidence to the contrary, its own prior arguments, and testimony of its previous expert, and attempts a 180º turn. To the extent the new routine optimization argument is considered, it fails on the merits for at least the reasons set forth below.

1. Petitioner Invalid Assumption of Four Lipid Component Systems

The Reply (6-7), without explanation or analysis, erroneously presumes a four-lipid component starting point. This assumption acts as a lynchpin for its new optimization argument. This reasoning is wholly circular (assuming a four-component system to arrive at the conclusion of a four-component system) is driven by nothing but impermissible hindsight, not any logical underpinnings to an obviousness assertion. *In re Kahn*, 441 F.3d 977, 988 (2006). Moreover, this hindsight is contradicted by the very references cited by Petitioner, none of which identify the four lipid components claimed as being required in a formulation.

Lin, Ahmad, and the '618 and '613 patents disclose two-component lipid particles, lacking both cholesterol and conjugated lipid. EX1006, 3308 (describing particles comprising cationic and phospholipid); EX007, 740-41 (same); EX1017, Fig. 2 (same); EX1012, 1:54-57, 1:66-67 (same). Bennett and the '618 and '505 patents disclose three-component lipid particles which lack conjugated lipid. EX1010, 51 (Figures 1 & 2) (describing particles comprising cationic, phospholipid, and cholesterol); EX1017, 34:65-35:25 (same); EX1013, 5:21-24 (same).

The '554, '196, and '189 publications describe phospholipid, cholesterol, and conjugated lipid as optional components. EX1005, ¶¶12, 120 (conjugated lipid optional), ¶¶97-115 (cholesterol optional), ¶92 (neutral lipid is optionally a phospholipid); EX1004, ¶150 (conjugated lipid optional), ¶152 (cholesterol optional), ¶79 (non-cationic lipid is optionally a phospholipid); *see also* EX1025, 205:7-16 (possible to have particles without phospholipid, cholesterol, or conjugated lipid). The '196 publication describes phospholipid and cholesterol are optional components. EX1003, ¶89 (describing that cholesterol and phospholipids are optional), ¶216 (exemplifying three-component particle). Furthermore, most of the formulations disclosed in the '554 publication are not four-component lipid particles — most lack a phospholipid entirely. EX1005, Table IV. Other

formulations of the '554 publication lack both phospholipid and cholesterol (*e.g.*, L100) and yet others are five-component systems (*e.g.*, L086, L104). *Id*.

Moreover, some lipid particle delivery platforms lack cationic lipid entirely. For example, Dr. Thompson explains that his research developing polymer-based particles as one of the efforts to "advance the field [] beyond cationic lipid particles." EX1025, 7:12-18.

Petitioner's optimization argument can be rejected at least for being based on this faulty premise.

2. Petitioner's Spurious Assertion of a "Trend" Toward Increased Cationic Lipid

Another cornerstone of the belated Reply (13-14) optimization argument is the false assertion of a "trend" in the prior art towards higher cationic lipid concentrations. There was no such trend in the art.

As Dr. Anchordoquy confirmed, there was no scientific analysis here—rather, Petitioner's counsel conjured this "trend" by cherry-picking only ***three*** data points from a very limited time frame—2003 to late 2004. EX2043, 52:9-16 (confirming he did not construct the "trend"), 61:20-22 (confirming only 3 datapoints), 61:13-25 (confirming no statistical fit or regression analysis). As Dr. Anchordoquy conceded, there were "hundreds, if not thousands" publications existing before the time of invention (filed in 2008). EX2043, 29:25-30:4, 30:25-31:7 (more than 30 patent publications by PO by 2008).

Curiously, the Reply (14) points to PO's '910 publication (citing Example 12, Figure 23) as somehow confirming this fabricated trend when it does just the opposite. Dr. Anchordoquy conceded during cross-examination the '910 publication expressly identifies the 30mol% cationic lipid concentration (not the 40mol% that serves as the basis of Petitioner's optimization argument) as best performing in this in vitro screen. EX2043, 12:18-20, 16:6-11, EX1015 ¶335 ("SNALP comprising 30% DLinDMA was more effective in reducing luciferase expression in the Neuro2A cells than SNALP comprising DODAC or DODMA were."). Dr. Anchordoquy further acknowledged that all subsequent *in vivo* testing (EX1015 ¶¶337-354) was limited to formulations having 30% or less cationic lipid, which admittedly illustrates the lack of interest in higher cationic lipid concentrations. EX2043, 16:6-11; 17:24-19:24; EX1025, 184:4-186:14. Finally, Dr. Anchordoquy conceded that plotting even one of the datapoints from the '910 publication (none were plotted in the reply materials) would destroy the fabricated "trend." EX2043, 59:7-61:7.

3. Petitioner's New N/P Ratio Argument is an Irrelevant Distraction

The Reply (11-13, 16-17) argues that a newly introduced parameter, N/P ratio, would somehow drive a POSITA's optimization efforts to arrive at the claimed composition. *E.g.*, EX1020, ¶¶72-75, EX2043, 33:17-35:15. Even if

considered, there is no explanation how this admittedly irrelevant parameter would render challenged claims obvious.

As an initial matter, argument based on N/P ratio should be given little weight due to the fact that neither Petitioner nor Dr. Anchordoquy provide any of their underlying calculations. 37 C.F.R. §42.65. This is especially important given Dr. Anchordoquy conceded during cross-examination, that there are a number of variables involved, some of which he did not know, requiring him to make certain (unexplained) assumptions. EX2043, 36:8-40:17, 44:20-45:16. He further conceded he actually made "approximations" rather than any precise calculation. *Id*., 44:24-45:13. As a result, both PO and the Board are denied to ability to critically evaluate the accuracy of Dr. Anchordoquy's assumptions.

Beyond that, Petitioner's assertion that an N/P ratio of 6 was recognized as "optimized," while a lower ratio (*e.g.*, "approximately 3") is suboptimal, lacks a shred of supporting evidence. Reply, 11-12. The evidence of record states the contrary. For example, the Reply ignores the fact that the N/P ratio for the optimized commercial product, patisiran, is 3.4 (EX2041). When probed during deposition, Dr. Anchordoquy conceded that he had calculated the N/P ratio but chose to exclude it from his declaration. EX2043, 65:13-66:2; 66:19-67:13. Such information is clearly probative as to the lack of credibility to this argument and should not have been withheld.

The Reply (17) points to Lin and Ahmad but similarly fails to inform the Board that these references describe maintaining a constant N/P ratio of 2.8—*i.e.*, contrary to the new optimization argument. *See, e.g.*, (Lin) EX1006, 3314; (Ahmad) EX1007, 743. The '196 and '189 publications identify its most narrow preferred charge ratio expansively—anywhere from 2:1 to 6:1. EX1003, ¶126; EX1004, ¶197 (same).

Finally, as Dr. Anchordoquy admitted during deposition, the N/P ratio is ultimately irrelevant to the claimed composition, as N/P ratio is neither recited nor dependent on any of the concentrations of the lipid component concentrations that are recited. That is, Dr. Anchordoquy conceded that the amount of conjugated lipid, cholesterol, and conjugated lipid all have no impact on the N/P ratio. EX2043, 35:16-36:2. In fact, Dr. Anchordoquy conceded that the N/P ratio is ultimately irrelevant to the concentration of the cationic lipid.

> Q. (By Mr. Rosato) Okay. It is possible for two different lipid particle formulations to have different cationic lipid concentrations but have the same N-to-P ratio?
>
> A. Yeah.

EX2043, 40:18-22.

Accordingly, N/P ratio argument is an irrelevant distraction, unsupported by any credible evidence.

4. The Conjugated Lipid Range was Not Obvious

The Reply (18-19) newly offers a motivation for adding a conjugated lipid, but as pointed out in the POR (3, 27) such arguments were never made in the petition, neither was necessary corresponding argument demonstration a reasonable expectation of success of arriving at the claimed range.

Even if considered, Petitioner's reply arguments are still unavailing. The Reply ignores the fact that, even if this optional component were included, much higher amounts of conjugated lipid were considered optimal at the time of invention. That was especially pertinent if the amount of cationic lipid was hypothetically increased. As Dr. Thompson has testified, both in his declaration (EX2031, ¶¶48-49) and his deposition (EX1025, 57:1-7, 62:3-6, 132:9-133:23, 175:3-13), amounts such as 5-10% were more typically used. See also, the newly cited MacLachlan chapter (EX1024, 258), citing Judge (EX2042) as providing evaluation of preferred conjugated lipid concentration. EX2042, 335 (describing 10% conjugated lipid); EX2043, 31:15-32:16.

Neither the petition nor the Reply provide any meaningful rebuttal to PO's evidence and argument that higher cationic lipid together with lowering conjugated lipid would have been counterintuitive at the time. Petitioner's entire analysis reduces to the legally deficient argument that one "could" have included conjugated lipid.

5. The Claimed Cholesterol Range Was Not Obvious

The Reply (20) newly argues motivation to include cholesterol on the basis of adding rigidity to the particle, but never explains why this necessitates cholesterol in a formation at all, let alone the specific concentrations claimed.

The cited art all describe cholesterol as an optional component. EX1003, ¶91 ("If present, the cholesterol….."); EX1004, ¶152 (same); EX1005, ¶98. The particles of Lin (EX1006) and Ahmad (EX1007) contain no cholesterol at all. *See also* EX1025, 205:12-14. The petition and Reply never provide any evidence based analysis of why a POSITA would have included cholesterol at the claimed concentration. *E.g.*, EX1020, ¶106 (Dr. Anchordoquy failing to cite evidence for the proposition that the "claimed range of 30-48mol% is squarely within the generally acceptable ranges in the field."). At best, Petitioner's argument amount to no more than a POSTA "could" have included cholesterol.

6. Petitioner fails to establish the claimed phospholipid concentration was obvious

The Reply (21) argument of including a phospholipid as a "bilayer stabilizing component" is untimely and insufficient.

The cited art provides a broad list of optional bilayer stabilizing components far more expansive than just a phospholipid. EX1004, ¶88. Petitioner does not explain why one would specifically select a phospholipid as opposed to any other compound. Moreover, Petitioner advances the same argument presented for

cholesterol with only slightly different verbiage, but does not explain why *both* phospholipid and cholesterol would be added for particle rigidity/stability. The prior art cited by Petitioner discusses phospholipid as optional. EX1003, ¶89; EX1004, ¶79; EX1005, ¶455; *see also* EX1025, 205:7-9 (phospholipid not necessary). Again, Petitioner's arguments distill down to little more than a phospholipid "could" be present.

Finally, the independent claim at issue here has a similar limitation to a range of phospholipids as claim 7 of the '435 patent. The Board issued a Final Written Decision ("FWD") in IPR2018-00739 on September 11, 2019, after institution of the instant proceeding, in which it was determined that claim 7 was not shown to be obvious. FWD, 51. And as was discussed in the POPR (4), both the instant petition and the petition in IPR2018-00739 asserts essentially the same grounds over the same refences.

VII. EXPERIMENTAL TESTING FURTHER CONFIRMS THE PATENTABILITY OF THE CLAIMS

Incredibly, while first disregarding its own claims of unpredictability and undue experimentation to now belatedly argue routine optimization, the Reply (24-25) inexplicably reverts back to unpredictability to argue the test data is not commensurate with the challenged claim scope. Petitioner's oscillation on this point is dizzying. If the art is as complex and unpredictable as asserted in the

petition, by Dr. Janoff, and now here at Reply, there is no *prima facie* case remaining.

If the testing data is reached, it is far more than "a single data point" as the Reply (25) falsely states. The POR (33-42) explains in detail testing of various different cationic, conjugated and phospholipids, as well as different cholesterols, and various different formulation methods tested against various gene targets, which were found surprisingly well-tolerated, potently efficacious *in vivo*. *E.g.*, EX2008. The evidence summarized there identifies at least fifty-five different formulations in this regard—not "a single data point" as Petitioner incredibly states.

As to Example 2, the Reply (26) mischaracterizes the potency of the 1:57 SNALP (Sample 9 in Fig. 1) as unfounded argument by PO. But the reference itself expressly states as much. *E.g.*, EX1001, 70:19-22 (identifying the 1:57 SNALP (Sample 9) as "among the most potent"). In fact, Dr. Janoff agreed, testifying that the 1:57 formulation (Sample 9) was the most potent at low siRNA. EX1008, ¶83; POR, 33-34; EX2033, 68:11-16, 69:7-71:1.

The Reply (26) argument on Example 3 fails at least for relying on the new and irrelevant N/P ratio parameter. Moreover, Petitioner's argument of insubstantial difference between Sample 11 (1:57 formulation) and Sample 12 (one of the tested 2:40 formulations) is at odds with the express disclosure of the '069

patent (EX1001, 72:20-23), which states "that the 1:57 SNALP...was the most potent at reducing ApoB expression in vivo (*see* Group 11)." Unfounded attorney argument does not displace the express disclosure of the reference.

Finally, as to Example 4, Petitioner does not dispute that the tested claimed formulation was "10 times as efficacious…at a 10-fold lower dose" compared to 2:30 formulation. *See* EX1001, 73:63-66. Instead, Petitioner (Reply, 26) argues this should be disregarded on the unsubstantiated basis that the 2:40 formulation represented the closest prior art. This argument is unsupported and contrary to the evidence, and was further rejected by Dr. Thompson during deposition. EX1025, 179:9-181:3, 181:7-183:9. Moreover, as noted above, the '910 publication confirms prior focus was actually on formulations having 30mol% cationic lipid or less. EX2043, 16:6-19:24. Regardless of Petitioner's unfounded assertions of what is "closer," the striking data and superior performance reported in Example 4 would not be ignored by a POSITA.

The Reply (27) attacks arguments that PO never made. PO never argued that unexpected results may be demonstrated by any apparent efficacy. As correctly argued in the POR (32-33), Petitioner's proffered standard of only "superior" efficacy qualifying as unexpected is not the law. Here, extensive testing data demonstrates well-tolerated and high in vivo potency of formulations as claimed. Petitioner fails to present any meaningful rebuttal on that point.

As to the extensive post-filing date publications further illustrating surprising tolerance and efficacy (POR, 37-42), Petitioner's only response (Reply, 27) is to cherry pick a single formulation, from one experiment in one reference and falsely assert inoperability. EX2006, 411:14-412:25. The extensive testing reported in the '069 patent and other publications is more than sufficient to rebut any *prima facie* case of obviousness, and the Reply fails to demonstrate otherwise.

VIII. BELATED ATTACKS ON OBJECTIVE INDICIA ARE UNFOUNDED AND UNAVAILING

As to long-felt need, the Reply (28) speculates that the investments discussed in the prior art "could" be attributable to SNALPs other than PO's. But Dr. Janoff previously confirmed he conducted no meaningful evaluation, he simply didn't know the facts. EX2002, 82:20-84:7. Similarly, for the evidence that Roche adopted Patent Owner's SNALP technology for non-human primate studies, Petitioner offers only unfounded speculation. *Id.*, 85:9-86:4. Speculation is neither evidence nor meaningful rebuttal. *Rambus Inc. v. Rea*, 731 F.3d 1248, 1257 (Fed. Cir. 2013) (finding no substantial evidence where Board's conclusion of no nexus rested solely on conjecture).

The Reply (28) also argues as to failure of others and skepticism that ionizable cationic lipids were known to be substantially nontoxic. This false narrative was extensively addressed in PO's sur-reply in IPR2018-00739. Paper 34, 27-31. Petitioner's own publications criticize ionizable cationic lipids,

including DLinDMA, as toxic. EX2037, 21:10-12 (noting that ionizable cationic lipids have been shown to accumulate in plasma and tissues over time are thus a potential source of toxicity); EX2031, ¶86; *see also* POR, 29-30. In addition, the record is replete with evidence illustrating a concern with the toxicity of cationic lipids, without differentiating between types of cationic lipids. *E.g.*, EX1007, 745 ("Minimizing the amount of cationic lipid is desirable....fewer, more highly charged molecules should mean a smaller metabolic effort..."); EX1009, 5 ("the cationic lipid contributes significantly to the toxicity observed."); EX2016, 42 ("I wouldn't want anyone injecting cationic lipids into my bloodstream.").

The Reply (28-29) now falsely argues patisiran does not use the claimed ranges. This is another remarkable and unexplained departure from the testimony of Dr. Janoff who, like Dr. Thompson, characterized patisiran as "50% cationic lipid, 38.5% cholesterol, 10% DSPC, and 1.5% PEG." EX2003, ¶33. Petitioner also ignores the fact that the manufacturer of patisiran lists it in the orange book as being covered by the '069 patent. EX2025.

Even if the average nominal concentrations purportedly calculated (but not shown) are considered, patisiran would still fall within the scope of the claims because, at a minimum, a large portion of a population of particles would meet the claimed lipid concentrations. Dr. Anchordoquy conceded this would be true due to reasonably expected variation of lipid concentration in a particle population.

EX2043, 71:19-24, 72:8-73:3 (confirming variation of 45-55mol% cationic lipid); *see also* 73:4-76:15; EX2040 (Petitioner previously arguing variation in a particle population cannot be ignored).

The Reply (28-29) also tries to downplay commercial success by newly arguing that the success of patisiran is due to the specific DLinKC2DMA cationic lipid. This is yet another concocted litigation argument and unexplained departure Petitioner's previous argument. In IPR2018-00739, Petitioner asserted that patisiran's success was due to the siRNA "drug," not the formulation. Presumably Petitioner now changes tack because PO identified scientific literature expressly credited PO's delivery formulation for patisiran's success. EX2023. Regardless, the specifically identified cationic lipid is still a "cationic lipid" as claimed.

IX. CONCLUSION

For at least the reasons set forth in the POR and above, the challenged claims should be found ***not unpatentable.***

Respectfully submitted,

Date: March 31, 2020

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
Reg. No. 52,182

CERTIFICATE OF COMPLIANCE

Pursuant to § 42.24(d), the undersigned certifies that this paper contains no more than 5,600 words, not including the portions of the paper exempted by § 42.24(b). According to the word-processing system used to prepare this paper, the paper contains 5,428 words.

Respectfully,

Dated: March 31, 2020

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
Reg. No. 52,182

X. APPENDIX – LIST OF EXHIBITS

Exhibit No.	Description
2001	Deposition Transcript of Andrew S. Janoff in IPR2018-00739, December 4, 2018
2002	Deposition Transcript of Andrew S. Janoff in IPR2018-00739, April 5, 2019
2003	Reply Declaration of Dr. Andrew S. Janoff in IPR2018-00739, December 4, 2018
2004	Declaration of David H. Thompson, Ph.D. in IPR2018-00739
2005	Deposition Transcript of David H. Thompson in IPR2018-00739, February 4, 2019 (Volume I)
2006	Deposition Transcript of David H. Thompson in IPR2018-00739, February 5, 2019 (Volume II)
2007	Patent Owner's Sur-Reply in IPR2018-00739
2008	Listing of Example Formulations Falling Within the Scope of the '069 Patent Claims
2009	U.S. Patent No. 7,491,409
2010	U.S. Patent No. 8,236,943
2011	U.S. Publication No. 2013/0116307
2012	U.S. Publication No 2017/0307608
2013	U.S. Patent No. 9,404,127
2014	International Publication No. WO 2010/088537
2015	International Publication No. WO 2013/090648

2016	Charles W. Schmidt, *Therapeutic Interference: Small RNA Molecules Act as Blockers of Disease Metabolism* AM. CHEM. SOC'Y 37 (2003)
2017	C. Russell Middaugh & Joshua D. Ramsey, *Analysis of Cationic-Lipid-Plasmid-DNA Complexes*, ANALYTICAL CHEMISTRY 7240 (2007)
2018	Erika Check, *RNA to the Rescue?* 425 NATURE 10 (2003)
2019	Dirk Hausseker, *The Business of RNAi Therapeutics in 2012*, 2 AM. SOC'Y OF GENE & CELL THERAPY (2012)
2020	Luke Timmerman, Merck's Alan Sachs, on RNAi's Big Challenge: Delivery, Delivery, Delivery, XCONOMY (Jan. 21, 2010), https://xconomy.com/national/2010/01/21/mercks-alan-sachs-on-rnais-big-challenge-delivery-delivery-delivery/
2021	Sean C. Semple, et al., *Rational Design of Cationic Lipids for siRNA Delivery*, 28 NATURE BIOTECH. 172 (2010)
2022	Supplementary Figures to Sean C. Semple, et al., *Rational Design of Cationic Lipids for siRNA Delivery*, 28 NATURE BIOTECH. 172 (2010)
2023	Heidi Ledford, *Gene-Silencing Drug Approved: US Government Okays First RNA-Interference Drug — After a 20-Year Wait* 560 NATURE 291 (2018)
2024	*FDA Approves First-of-its Kind Targeted RNA-based Therapy to Treat a Rare Disease*, FOOD AND DRUG ADMIN. (2018), https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm616518.htm
2025	Orange Book: Approved Drug Products with Therapeutic Equivalence Evaluations, *Patent and Exclusivity for: N210922*, FOOD AND DRUG ADMIN. *available at* https://www.accessdata.fda.gov/scripts/cder/ob/patent_info.cfm?Product_No=001&Appl_No=210922&Appl_type=N (last visited Dec. 19, 2018)

2026	Maja Sedic et al., *Safety Evaluation of Lipid Nanoparticle-Formulated Modified mRNA in the Sprague-Dawley Rat and Cynomolgus Monkey*, VETERINARY PATHOLOGY (2017)
2027	Kapil Bahl et al., *Preclinical and Clinical Demonstration of Immunogenicity by mRNA Vaccines against H10N8 and H7N9 Influenza Viruses*, MOLECULAR THERAPY (2017)
2028	Declaration of Andrew S. Janoff in IPR2018-00739
2029	DICTIONARY OF PHARMACY (2004)
2030	Declaration of Andrew S. Janoff in IPR2018-00680
2031	Declaration of David H. Thompson, Ph.D.
2032	Curriculum Vitae of David H. Thompson, Ph.D.
2033	Deposition Transcript of Andrew S. Janoff, October 25, 2019
2034	Doxil Label – FDA (Revised May, 2007), https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/050718s029lbl.pdf
2035	Ian MacLachlan & Pieter Cullis, *Diffusible-PEG-Lipid Stabilized Pasmid Lipid Particles,* 53 ADVANCES IN GENETICS 157 (2005)
2036	Sean C. Semple et al., *Immunogenicity and Rapid Blood Clearance of Liposomes Containing Polyethylene Glycol-Lipid Conjugates and Nucleic Acid,* 312 THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS 1020 (2005)
2037	International Publication No. WO 2017/223135
2038	*New Medicine for Hereditary Rare Disease,* EUROPEAN MED. AGENCY (2018), https://www.ema.europa.eu/en/news/new medicine-hereditary-rare-disease
2039	*Arbutus' LNP Licensee Alnylam Announces FDA Approval of ONPATTRO™ (patisiran), for the Treatment of ATTR Amyloidosis,* ARBUTUS BIOPHARMA (2018), https://investor.arbutusbio.com/news-releases/news-release-details/arbutus-lnp licensee-alnylam-announces-fda-approval-onpattrotm
2040	"Patent Owner: One Way Particle Variation," Slide 27 from Petitioner's Demonstrative Exhibits, Case Nos. IPR2018-00739, -00680, June 6, 2019

2041	Patisiran N/P Ratio
2042	Adam Judge et al., *Hypersensitivity and Loss of Disease Site Targeting Caused by Antibody Responses to PEGylated Liposomes*, 13 MOLECULAR THERAPY (February 2006)
2043	Deposition Transcript of Thomas J. Anchordoquy, Ph.D., March 24, 2020

CERTIFICATE OF SERVICE

This is to certify that I caused to be served a true and correct copies of the foregoing Patent Owner's Sur-Reply and Exhibits 2040-2043, on this 31st day of March, 2019, on the Petitioner at the correspondence address of the Petitioner as follows:

Michael Fleming
C. Maclain Wells
IRELL & MANELLA LLP
mfleming@irell.com
mwells@irell.com
ModernaIPR@irell.com

Respectfully submitted,

Date: March 31, 2020

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
Reg. No. 52,182

JOINT APPENDIX 67

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

MODERNA THERAPEUTICS, INC.,
Petitioner,

v.

PROTIVA BIOTHERAPEUTICS, INC.,
Patent Owner.

Case IPR2018-00739
Patent No. 9,364,435

DECLARATION OF DAVID H. THOMPSON, PH. D.

TABLE OF CONTENTS

I, David H. Thompson, declare as follows:

I. QUALIFICATIONS

1. I am a Professor of Chemistry at Purdue University and Director of the Medicinal Chemistry Group in the Purdue Center for Cancer Research. My primary research interests include development of transiently-stable carrier systems for drug and nucleic acid delivery.

2. I received my Ph.D. in Organic Chemistry from Colorado State University in 1984. I also hold a Bachelor of the Arts in Biology and a Bachelor of Science in Chemistry from the University of Missouri, Columbia.

3. I have been a visiting professor at numerous institutions including, Chulalongkorn University, Department of Pharmaceutics; Technical University of Denmark, Department of Micro & Nanotechnology; Japan Advanced Institute of Science & Technology, Department of Biomaterials; Osaka University, Department of Applied Chemistry; University of Florida, Department of Pharmaceutics; and University of British Columbia, Department of Biochemistry.

4. I am listed as a co-inventor on 7 United States patents. I have also published more than 140 peer reviewed scientific papers.

5. I have studied, taught, practiced, and conducted research involving the formulation, use, characterization, and delivery of lipid particles. I have expertise with the delivery of therapeutic agents using lipid particles.

6. A copy of my Curriculum Vitae, attached as EX2010, contains further details on my education, experience, publications, and other qualifications to render an expert opinion in this matter.

II. SCOPE OF WORK

7. I understand that a petition was filed with the United States Patent and Trademark Office for inter partes review of U.S. Patent No. 9,364,435 ("the '435 patent," EX1001).

8. I further understand that the Patent Trial and Appeal Board ("PTAB" or the "Board") has decided to institute inter partes review of claims 1-20 of the '435 patent under 35 U.S.C. §§ 102 and 103 based on the disclosures of WO2005/007196 ("the '196 PCT," EX1002), US 2006/134189 ("the '189 PCT," EX1003), Lin, et al, "Three-Dimensional Imaging of Lipid Gene-Carriers: Membrane Charge Density Controls Universal Transfection Behavior in Lamellar Cationic Liposome-DNA Complexes," ("Lin," EX1005), Ahmad, et al, "New multivalent cationic lipids reveal bell curve for transfection efficiency versus membrane charge density: lipid–DNA complexes for gene delivery," ("Ahmad," EX1006), and US 2006/0240554 ("the '554 publication," EX1004).

9. I have been specifically asked to provide my expert opinions on the patentability of the claims of the '435 patent in view of the asserted grounds in the petition. In connection with this analysis, I have reviewed the '435 patent and the

prior art cited against the patentability of claims 1-20. I have also reviewed and considered the petition, Dr. Janoff's declaration and deposition transcript, and the Board's Decision on Institution of Inter Partes Review, and may cite these documents in this declaration.

10. I am being compensated at a rate of $600 per hour for my work in this matter. I am also being reimbursed for reasonable and customary expenses associated with my work in this investigation. My compensation is not contingent on the outcome of this matter or the specifics of my testimony.

III. LEGAL STANDARDS

11. I have been advised that a claimed invention is not patentable under an anticipation theory (35 U.S.C. § 102) if all claim elements are found in a single prior art reference. I further understand that anticipation is about prior invention and therefore the single prior art reference must be found to disclose all elements of the claimed invention arranged as in the claim. I also understand that picking, choosing, and combining various embodiments disclosed within a single reference is not proper under an anticipation theory.

12. I understand that differences between the prior art reference and a claimed invention, however slight, invoke the question of obviousness, not anticipation.

13. I have been advised that a claimed invention is not patentable under 35 U.S.C. § 103 if it is obvious. A patent claim is unpatentable if the claimed invention would have been obvious to a person of ordinary skill in the field at the time the claimed invention was made. This means that even if all of the requirements of the claim cannot be found in a single prior art reference that would anticipate the claim, a person of ordinary skill in the relevant field who knew about all this prior art would have come up with the claimed invention.

14. I have further been advised that the ultimate conclusion of whether a claim is obvious should be based upon several factual determinations. That is, a determination of obviousness requires inquiries into: (1) the level of ordinary skill in the field; (2) the scope and content of the prior art; (3) what difference, if any, existed between the claimed invention and the prior art; and (4) any objective indicia of nonobviousness.

15. I have been advised that, in determining the level of ordinary skill in the field that someone would have had at the time the claimed invention was made, I should consider: (1) the levels of education and experience of persons working in the field; (2) the types of problems encountered in the field; and (3) the sophistication of the technology.

16. I have been advised that a patent claim composed of several elements is not proved obvious merely by demonstrating that each of its elements was

independently known in the prior art. In evaluating whether such a claim would have been obvious, I may consider whether there is a reason that would have prompted a person of ordinary skill in the field to combine the elements or concepts from the prior art in the same way as in the claimed invention.

17. I have also been advised, however, that I must be careful not to determine obviousness using the benefit of hindsight; many true inventions might seem obvious after the fact. I should put myself in the position of a person of ordinary skill in the field at the time the claimed invention was made and I should not consider what is known today or what is learned from the teaching of the patent.

18. Finally, I have been advised that any obviousness rationale for modifying or combining prior art must include a showing that a person of ordinary skill would have had a reasonable expectation of success.

19. With regard to objective indicia of nonobviousness, I have been advised that any objective evidence may be considered as an indication that the claimed invention would not have been obvious at the time the claimed invention was made. I understand that the purpose of objective indicia is to prevent a hindsight analysis of the obviousness of the claims.

20. I have been advised that there are several factors that may be considered as objective indicia. These factors include the long-felt need, skepticism, unexpected results and commercial success of the invention.

21. I have been further advised that in order for objective indicia to be significant, there must be a sufficient nexus between the claimed invention and the evidence of objective indicia. I understand that this nexus serves to provide a link between the merits of the claimed invention and the evidence of objective indicia provided.

IV. LEVEL OF ORDINARY SKILL IN THE ART

22. I have been advised that, in determining the level of ordinary skill in the art that someone would have had at the time the claimed invention was made, I should consider: (1) the levels of education and experience of persons working in the field; (2) the types of problems encountered in the field; and (3) the sophistication of the technology. I have been advised that an invention must be evaluated not through the eyes of the inventor, who may have been of exceptional skill, but as by one of ordinary skill in the art.

23. I understand that Dr. Janoff defined a person of ordinary skill as one that "would have specific experience with lipid particle formation and use in the context of delivering therapeutic payloads, and would have a Ph.D., an M.D., or similar advanced degree in an allied field (*e.g.*, biophysics, microbiology,

biochemistry) or an equivalent combination of education and experience." EX1007 ¶31. I understand this was adopted by the Board. Paper 15 at 5-7.

24. In my opinion, the level of ordinary skill defined by Dr. Janoff and the Board is inappropriate. With regard to the "specific experience," Dr. Janoff states that the "level of skill is representative of the inventors on the '435 patent and authors/inventors of prior art cited herein." EX1007 ¶31. The inventors of the '435 patent, however, are artisans of exceptional skill in the subject matter of the '435 patent. Thus, in my view, Dr. Janoff has not simply applied a slightly higher level of skill in the art in setting forth his opinions in his declaration, but has assumed a much higher level of skill than that of a person of ordinary skill in the art.

V. BACKGROUND

25. An objective of genetic therapy at the time, and to this day, is the development of drugs — that is, nucleic acids — to treat systemic diseases such as cancer, inflammation, virus infection, and cardiovascular disease. While genetic therapy holds the promise of highly specific targeting of disease pathways, it was known that this promise would only be realized through the development of appropriate delivery vehicles. Delivery is critical because a therapeutic agent is useless if it does not reach its target. This is particularly true with nucleic acids — large, negatively charged molecules — that cannot simply be given to a patient systemically (*e.g.*, intravenously) and allowed to passively enter cells, as would be

the case with many small molecule drugs. Therapeutic nucleic acids require an effective delivery vehicle, which historically has proved to present a considerable technical obstacle. *See, e.g.*, EX2016 ("You can write down the steps. You can write down what you think will happen. But then you have to put it in a 50-nanometer particle that's safe and potent to deliver."); EX2014 at 11 ("The major hurdle right now is delivery, delivery, delivery," says Sharp), ("Khvorova believes that the medical benefits of RNAi will be huge if the delivery issues can be resolved.").

26. The first generation of nucleic acid delivery systems that were developed included cationic liposome nucleic acid complexes (also known as liposomes). *See* EX1002 ¶8 (defining "cationic liposome complex" as lipoplex); EX2007, 2:27-28 (same). Lipoplexes were found to be unsuitable for many applications, particularly systemic uses, due in large part to the toxic nature of the cationic lipids. *See, e.g.*, EX1008 at 5.

27. The toxicity of cationic lipids is observed both at the systemic level and at the cellular level. Cationic lipids cause clustering of membrane glycoproteins on cell surfaces, thereby disrupting normal cellular protein trafficking and receptor recycling, and thus are cytotoxic to cells themselves. Toxicity also occurs at the organ level, as these lipids are often not readily biodegradable, such that they accumulate to cytotoxic concentrations in the liver

and spleen. Cationic lipids also have immunostimulatory capacity and have been associated with immunogenic and inflammatory responses. The presence of cationic lipids also results in these complexes being rapidly cleared from the body, further limiting their therapeutic utility. Furthermore, it was understood that the cationic lipid component can cause aggregation of lipid particles.

28. These technical obstacles of toxicity, immunogenicity, and aggregation due to use of cationic lipids in a delivery vehicle for nucleic acids was well known in 2008 and thus those in the field at the time sought to minimize the cationic lipid component of a lipid delivery vehicle. This is evidenced in the references cited in the petition.

29. For example, Ahmad teaches that the cationic lipid component should be minimized to reduce cytotoxicity and metabolic effort associated with elimination of cationic lipids.

> Minimizing the amount of cationic lipid is desirable to reduce cost as well as potential toxic effects of the cationic lipid. In addition, achieving a given σ_M with fewer, more highly charged molecules should mean a ***smaller metabolic effort for the elimination of the lipids*** from the cell.

EX1006 at 7.

30. Gao discusses toxicity caused by the cationic lipid component.

> Detailed toxicological studies … revealed that the cationic lipid contributes significantly to the toxicity observed. Similar toxic effects

> are also noticeable in systemic gene delivery via the tail vein with other types of cationic lipids. Symptoms include acute pulmonary hypotension, induction of inflammatory cytokines, tissue infiltration of neutrophils in lungs, decrease in white cell counts, and in some cases tissue injury in liver and spleen. In humans, various degrees of adverse inflammatory reactions, including flulike symptoms with fever and airway inflammation, ….

EX1008 at 5.

31. Gao further discloses that the cationic lipid component caused unwanted interactions with serum proteins, including complement.

> Another factor related to the severity of transfection-related side effects is complement activation and adsorption of serum proteins onto their surface, which in turn act as opsonins to trigger the uptake of opsonized particles by macrophages and other immune cells. Various strategies have been considered to deal with the toxic responses.

EX1008 at 5-6; *see also* Table 1.

32. Gao also describes that the cationic lipid component of lipoplexes caused unwanted interactions with non-target cells.

> Once administered in vivo, lipoplexes tend to interact with negatively charged blood components and form large aggregates that could be absorbed onto the surface of circulating red blood cells, trapped in a thick mucus layer, or embolized in microvasculatures, preventing them from reaching the intended target cells in the distal location.

EX1008 at 5.

33. Additionally, it was appreciated that the cationic lipid component of lipoplexes caused aggregation of lipid particles. EX1008 at 9 ("[T]he polycations in either lipoplexes or polyplexes have the intrinsic property of causing significant aggregation in biological matrices full of negatively charged molecules …."); *see also* EX1004 ¶136.

34. Therefore, it was well established at the time of filing of the patent that cationic lipids used in a delivery vehicle for nucleic acids were toxic, immunogenic, and caused aggregation. As Dr. Zamore of Alnylam stated, "I wouldn't want anyone injecting cationic lipids into my bloodstream." EX2011 at 42.

35. At the filing date of the patent, the aim of those working in the field sought lipid particles that were substantially non-toxic and therefore suitable for systemic applications. However, at that time, the development of nucleic acid-lipid particles that were suitable for systemic applications had not been achieved. Furthermore, it was widely understood at that time that in order to design nucleic acid-lipid particles suitable for systemic use the amount of cationic lipid in the formulation should be kept as low as possible, because of concerns over the known toxic effects of cationic lipids.

VI. CLAIM CONSTRUCTION

36. The petition materials provided an unreasonably broad construction of "nucleic acid-lipid particle," stating that it should be construed as "a composition of lipids and a nucleic acid for delivering a nucleic acid to a target site on interest." Pet. 24.

37. I have been apprised that the Board, in its Institution Decision, rejected the construction in the petition and offered a different one. That is, the Board construed "nucleic acid-lipid particle" as "a particle that comprises a nucleic acid and lipids, in which the nucleic acid may be encapsulated in the lipid portion of the particle." Paper 15 at 10-11 (citing EX1001, 11:14–22).

38. In my opinion, both constructions of "nucleic acid-lipid particle" in the petition materials and in the Institution Decision are incorrect and unreasonably broad at least to the extent they encompass lipid particles lacking any encapsulated nucleic acid. The petition materials and the Board focused on a different term — *i.e.*, the term "lipid particle" — and only incompletely address the corresponding discussion in the '435 patent specification. But the claimed term is not "lipid particle," the claimed term is "nucleic acid-lipid particle."

39. A "nucleic acid-lipid particle" expressly includes a nucleic acid. According to the '435 patent, "nucleic acids, when present in the lipid particles of the present invention, are resistant in aqueous solution to degradation with a

nuclease." EX1001, 11:51-54. The '435 patent describes nucleic acid encapsulation in the lipid particle as conferring resistance to such enzymatic degradation. EX1001, 11:20-22 ("[T]he active agent or therapeutic agent may be encapsulated in the lipid, thereby protecting the agent from enzymatic degradation."); *see also* EX2007 4:15-19; 22:40-47; 23:1-3; 23:27-29; 26:35-37 (describing resistance to nuclease enzymatic degradation as indicating nucleic encapsulation in the liposomes).

40. A "lipid particle" "*may* [include a nucleic acid] encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation." EX1001, 11:14–22. A "nucleic acid-lipid particle," however, *does* include a nucleic acid encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation. EX1001, 11:23-31, 11:51-54.

41. I understand that, during cross-examination, Dr. Janoff testified multiple times that the lipid particles as claimed are defined as SNALPs. *See, e.g.*, EX2028, 118:18-119:4, 119:9-17, 120:5-6, 121:14-25. Dr. Janoff cited to a provision of U.S. Patent No. 9,404,127 ("the '127 patent," EX2029) at 5:15-22 that is identically recited in the '435 patent. *Compare* EX2029, 5:15-22 *with* EX1001, 19:19-26.

42. Dr. Janoff is correct in that the specification repeatedly identifies SNALPs as the invention of the patent for delivering a nucleic acid payload. *See*

e.g., EX1001, 3:9-13 ("The present invention provides novel, serum-stable lipid particles …."), 47:23-24 ("[T]he lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP)…"), 3:32-37, 14:20-25.

43. In my opinion, a fair and reasonable reading of the '435 patent specification supports Dr. Janoff's position in that there is no meaningful distinction between the '435 patent specification's descriptions of a "lipid particle" containing a nucleic acid (*i.e.*, a nucleic acid-lipid particle) and particle characteristics that confer serum stability. *Compare* EX1001, 11:14-22, 11:51-54 ("[N]ucleic acids, when present in the lipid particles of the invention, are resistant in aqueous solution to degradation with a nuclease.") *with* 13:32-37 ("'Serum-stable' in relation to nucleic acid-lipid particles such as SNALP means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA").

44. In my opinion, a narrow focus on a linguistic difference between a nucleic acid-lipid particle and the term "SNALP" is misguided and risks overlooking pertinent disclosure and context provided in the '435 patent. The '435 patent specification states that "nucleic acids, when present in the lipid particles of the invention, are resistant in aqueous solution to degradation with a nuclease." *Id.*, 11:51-54. Such physical properties of the particles providing nuclease degradation resistance, or encapsulation of the nucleic acid, are also as described in the '435

patent specification as conferring the identified serum stability. *Id*., 13:32-37. This is certainly true if the claimed particles are SNALP, as supported not only by the specification of the '435 but as affirmed by petitioner's expert Dr. Janoff.

45. I disagree with the Board's analysis presented at pages 9-10 of the Institution Decision at least for the reasons explained above. As explained above, there is no meaningful distinction between a nucleic acid-lipid particle and a SNALP in the context of the '435 patent. None of the provisions of the '435 patent specification identified by the Board indicate otherwise. Paper 15 at 9-10. The Board's discussion of whether particles are "limited to in vivo use," is confusing and loses sight of both the context of the '435 patent specification and a reasonable perspective of a person of ordinary skill in the art. For example, the Board cites to an example of SNALP being tested for transfection activity in vitro as indicating the same composition is not a SNALP. Paper 15 at 10 (citing Example 2 at 69:6–70:52 ("Eg5 siRNA Formulated as 1:57 SNALP are Potent Inhibitors of Cell Growth In Vitro")). The composition in Example 2 is expressly described as "1:57 SNALP." A person of ordinary skill in the art would understand that composition can be both 1) formulated such that the nucleic acid is encapsulated in the lipid particle, rendering the composition extremely useful for systemic applications; and 2) tested for in vitro transfection activity. It is not uncommon for compositions to be assessed for in vitro transfection activity and then subject to testing in vivo.

46. Regardless of whether the Board construes "nucleic acid-lipid particle" as a SNALP as indicated by Dr. Janoff; as a lipid particle with an encapsulated nucleic acid (thereby protecting it from enzymatic degradation); or under the broad construction presented in the Institution Decision, the petition materials fail to establish the unpatentability of claims 1-20.

VII. GROUND 1 – CLAIMS 1-20 ARE NOT OBVIOUS IN VIEW OF PATENT OWNER'S DISCLOSURES IN THE '196 PCT AND '189 PUBLICATION

47. It is my opinion that the petition fails to demonstrate that claims 1-20 of the '435 patent are obvious in view of the '196 PCT and the '189 publication. Since the petition materials provide no meaningful discussion of the '189 publication, the below arguments focus on lack of obviousness in view of the '196 PCT.

48. Although I understand that it is not Patent Owner's burden to prove, it is my opinion that claims 1-20 of the '435 patent are not obvious.

A. Claim 1

49. First, the petition materials fail to address all the lipid components of the claimed nucleic acid-lipid particle composition. Second, the petition materials fail to address the combination of the lipid components of the claimed nucleic acid-lipid particle composition. Third, the petition materials fail to explain why a person of ordinary skill in the art would have wanted to combine the individual lipid

components disclosed in the '196 PCT. Fourth, the petition materials fail to address that a skilled artisan would have had no reasonable expectation of success that the claimed nucleic acid-lipid particle composition would be well-tolerated and efficacious. Finally, much of the evidence of unexpected results within the patent is disregarded, and what is considered is mischaracterized in view of the prior art.

50. As an initial observation, the assertion of obviousness in the petition materials is based on alleged overlapping ranges between the '196 PCT and the challenged claims. But the petition materials fail to identify ranges that overlap for each of the claim components.

51. For example, claim 1 recites "a conjugated lipid that inhibits aggregation of particles." Rather than identifying disclosure in the '196 PCT that is specific for a conjugated lipid range, the petition materials cite to a range provided for "a bilayer stabilizing component." Pet. 39; EX1007 ¶117.

52. The '196 PCT makes clear that a "bilayer stabilizing component" is not the same as a "conjugated lipid that inhibits aggregation of particles." *See, e.g.*, EX1002 ¶92 ("Suitable BSCs include, but are not limited to, polyamide oligomers, peptides, proteins, detergents, lipid-derivatives, PEG-lipids, …"). Bilayer stabilizing components include a broad class of structurally and chemically diverse molecules. Numerous bilayer stabilizing components (*e.g.*, polyamide oligomers,

peptides, proteins, detergents, and lipid-derivatives) would not be considered a conjugated lipid by a person of ordinary skill in the art.

53. While the '196 PCT lists a general range for the bilayer stabilizing component category, a person of ordinary skill in the art would not have interpreted the stated range (*e.g.*, 0.5% to 25%) as being applicable to each listed bilayer stabilizing component example. For example, a skilled artisan would have appreciated that if the bilayer stabilizing component were a detergent, 25% would have been an unreasonably high level. This is because at this concentration of detergent the lipids would be solubilized and no longer form a lipid particle.

54. The '196 PCT discloses seven nucleic acid-lipid particle compositions — all of which have either 7.5 or 15 mol % cationic lipid and 10 mol % conjugated lipid. EX1002 ¶¶216, 223, 228, 232.

55. Therefore, the petition materials fail to identify in '196 PCT "a conjugated lipid that inhibits aggregation of particles" as required by claim 1.

56. In my opinion, as explained in further detail below, there would have been no good reason why a person of ordinary skill in the art would combine the different range disclosures for different lipid components from the '196 PCT so as to arrive at the claimed nucleic acid-lipid particle. Nor would one would reasonably expect such formulations to work. The petition materials fail to demonstrate otherwise.

57. Those in the field at the time recognized that the properties of any lipid particle are conferred not by the amount of any individual component but by the interaction of the combined components as a whole. Furthermore, if the skilled person were to vary one component (by taking the amount of that component specified for a particular formulation), it would then be necessary to decide which of the other components would need to be varied in order to accommodate the change in proportions of the overall composition.

58. The effects of making changes to the proportion of other components in the lipid particle would be unpredictable. Such changes, even if apparently minor in nature, would have little assurance of producing a functional lipid particle suitable for systemic use. The idea of simply "cherry-picking" specific amounts of individual components from different formulations, or the different ranges in the '196 PCT, when designing lipid particles is therefore something which would have made no technical sense to the skilled person.

59. Dr. Janoff states that "determining the optimal proportion of cationic lipid for a given lipid combination would be a simple matter of varying the proportion using prior art methodologies." EX1007 ¶110. I disagree. As explained above, the properties of a formulation are not conferred by the amount of one single component. Properties such as safety and efficacy are conferred by the combination of components in the entire formulation.

60. Moreover, Dr. Janoff's stated reason disregards the state of the art at the time of the invention. Making safe and effective nucleic acid-lipid particle formulations was not simply a matter of "varying the proportion" of cationic lipid in prior art formulations. As discussed above, the field of genetic medicine was hindered by the lack of effective and safe nucleic acid delivery vehicles. That the field struggled for 20 years to find such a delivery vehicle speaks to the difficulty of the task. *See generally* EX2015. Had the solution been a matter of simply optimizing the cationic lipid proportion, it would not have taken such an enormous investment of money and time.

61. As discussed elsewhere herein, the high cationic lipid levels claimed would have been disfavored in view of well-established toxicity concerns. Moreover, inclusion of a conjugated lipid in a formulation with high cationic lipid would have been expected to occur at much higher levels than claimed.

62. Conjugated lipid had been incorporated into lipid particles to help shield positive charge and reduce nonspecific interactions with blood components, leading to enhanced systemic clearance. Lipid particle compositions at the time typically used much higher levels of conjugated lipid than is claimed by the '435 patent, such as 10% PEG (*i.e.*, 5- to 20-times more than the claimed formulations). For example, Doxil, the first FDA approved liposome formulation contained 5% PEG-conjugated lipid. EX2034. Likewise lipid particles for the delivery of nucleic

acids commonly used 10% PEG. EX2032 at 174; EX2033 at 1021; EX1002 ¶¶216, 223, 228, 232.

63. The petition materials fail to provide a reasonable expectation of success for the claimed nucleic acid-lipid particle in view of the '196 PCT and the state of the art at the date of the patent filing. In my opinion, a person of ordinary skill in the art would not have expected a nucleic acid-lipid particle composition with a high level of cationic lipid and a low level of conjugated lipid to be safe and effective.

64. As discussed above, the prior art taught that the cationic lipid component of lipid particles should be minimized, regardless of whether used for in vitro or in vivo purposes. For example, it was appreciated that cationic lipids are directly cytotoxic. Cationic lipids, in addition, elicit unwanted immune reactions (*e.g.*, inflammation), off-target cellular interactions (*e.g.*, blood cells), and aggregation. Furthermore compositions with low levels of conjugated lipid (*i.e.*, 0.5 mol % to 2 mol %) would have been expected to result in unstable particles that aggregate and fail to effectively transfect cells. Hence the claimed nucleic acid-lipid particle would have been expected to be cytotoxic and ineffective.

65. Accordingly, claim 1 is not obvious in view of the '196 PCT at least because there would have been no expectation of success that the claimed nucleic acid-lipid particle would be safe and effective.

B. Unexpected Results

66. I reviewed the experimental data in the '435 patent. In my opinion, the results of the experimental data would have been quite surprising to a POSITA. As explained in further detail below, those in the art at the time would have expected formulations as claimed to result in significantly lower activity and higher levels of toxicity compared to what is reported in the experimental results.

67. The prior art expressly instructs that high-level cationic lipid formulations were expected to have poor *in vivo* activity and display increased toxicity and immunogenicity relative to low-level cationic lipid formulations. *See* EX1005 at 3315; EX1006 at 745; EX1008 at E96; EX2007, 30:34:41 (Issued patent naming Dr. Janoff as an inventor and explaining that "[t]here may be a limit to the use of cationic lipoplexes [in vivo] because of their toxicity."). As such, the expectation for the claimed formulations would have been toxic formulations unsuitable for systemic use and little, if any, efficacy.

68. Contrary to these expectations, the claimed formulations are well-tolerated and possess favorable *in vivo* efficacy at far lower dosages than prior art formulations. Data for numerous formulations within the scope of claim 1 are found in the '435 patent and support the unexpected degree of tolerability and efficacy. *See also* Section XI.

69. The petition materials present an incomplete and flawed analysis of the data within the '435 patent. First, the petition materials fail to consider that, unexpectedly, the claimed formulations are not toxic nor do they cause unwanted immune responses. Second, although the petition materials purport to address the data supporting unexpected efficacy, these data are analyzed without consideration of what a person of ordinary skill in the art would have expected.

70. Dr. Janoff says "the ***sole basis*** for alleged novelty of the '435 patent claims is that a nucleic acid-lipid particle comprising component lipids in the claimed proportions achieves ***unexpected efficacy*** making the claims patentably distinct from the prior art." EX1007 ¶75 (emphases added). Not so. The '435 patent also discloses that the claimed formulations "are substantially non-toxic to mammals such as humans." EX1001, 6:2-5; *see also* EX1001, 6:26-30, 11:51, 14:40-42, 23:2-3, 47:9-18.

71. For example, the '435 patent used various measurements (*e.g.*, body weight, appearance and behavior, and platelet count) to assess the toxicity of the claimed compositions following systemic administration to mice. Unexpectedly, no significant toxicity was detected by any measure.

72. Body weight was monitored after systemic administration of a 1:57 formulation. EX1001, 79:33-37. A sharp decline was considered indicative of toxicity and would have been the expected result. However, "there was very little

effect on body weight 24 hours" even at the highest dosages. EX1001, 79:33-34; *see also id*., 79:34-36 ("The maximum weight loss of 3.6±0.7% was observed at the highest drug dose of 17 mg/kg."), Figure 8. Moreover, the '435 patent explains that "[t]here was also no obvious change in animal appearance/behavior at any of the dosages tested." EX1001, 79:36-37.

73. Platelet count was also measured after systemic administration of a 1:57 nucleic acid-lipid particle composition. An increase or decrease in platelet count is indicative of toxicity and would have been the expected result. *See* EX1001, 79:38-41. However, "the mean platelet volume did not change in SNALP-treated groups." EX1001, 79:44; *see also* Figure 9.

74. No significant toxicity was observed even after multiple systemic doses of the 1:57 nucleic acid-lipid particle composition. Mice were dosed intravenously multiple times per week for a total of 5 weeks. EX1001, 81:20-82:25. Similar to the single dose of 1:57, no treatment-related toxicity was observed. EX1001, 82:30-36 ("The treatment regimen was well tolerated with no apparent signs of treatment-related toxicity."), ("[T]reatment with 1:57 SNALP-formulated PLK1424 caused a significant increase in the survival of Hep3B tumor-bearing mice. This in vivo anti-tumor effect was observed in the absence of any apparent toxicity or immune stimulation.").

75. For example, the claimed nucleic acid-lipid particle compositions effectively induced silencing of the targeted gene (*i.e.*, ApoB) when administered systemically to mice. Table 4 lists the nucleic acid-lipid particle compositions tested as part of Example 3.

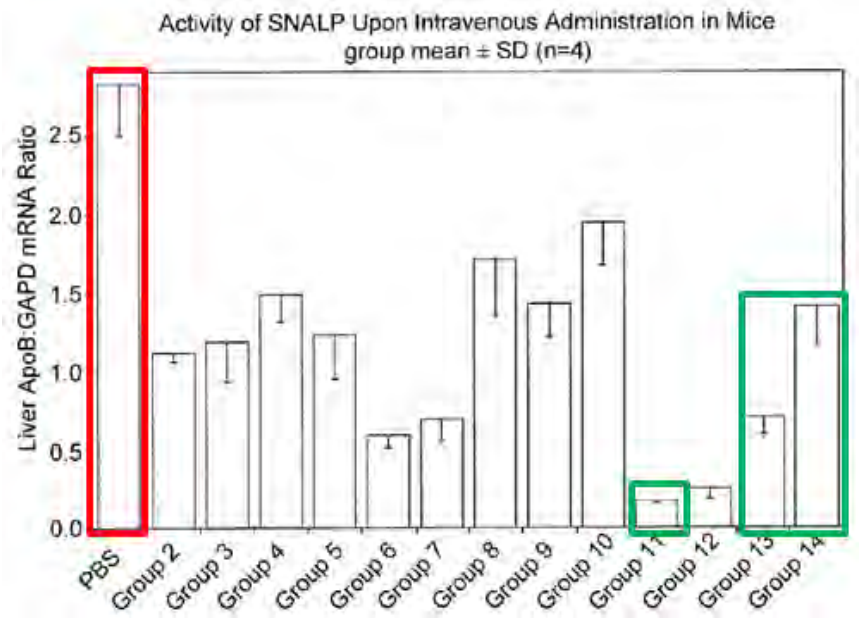
TABLE 4

Characteristics of the SNALP formulations used in this study.

			Finished Product Characterization		
Group	Formulation Composition Lipid Name & Mole %	Lipid/Drug Ratio	Size (nm)	Poly-dispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2 | 40 | 10 | 48	12.4	59	0.15	93
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 2.2 | 44.4 | 53.3	10.7	55	0.17	91
4	PEG(2000)-C-DMA | DLinDMA | DOPC | Cholesterol 2 | 40 | 10 | 48	12.5	59	0.16	92
5	PEG(2000)-C-DMA | DLinDMA | DMPC | Cholesterol 2 | 40 | 10 | 48	12.2	56	0.11	92
6	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.8 | 36.4 | 18.2 | 43.6	13.8	66	0.16	93
7	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 2 | 40 | 10 | 48	12.4	56	0.12	92
8	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 27.0 | 6.8 | 64.9	16.5	60	0.10	93
9	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.3 | 25.3 | 12.7 | 60.8	18.1	74	0.13	92
10	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 2.5 | 25.0 | 12.5 | 60.0	19.2	60	0.13	93
11	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.4 | 34.3	17.8	79	0.09	94
12	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.0 | 40.4 | 10.1 | 48.5	23.6	72	0.11	93
13	PEG(2000)-C-DMA | DLinDMA | DPPC 2 | 70 | 28	8.7	73	0.09	87
14	PEG(2000)-C-DMA | DLinDMA | DPPC 1.6 | 54.7 | 43.8	11.3	65	0.11	87

EX1001, Table 4. Amongst those tested were nucleic acid-lipid particle compositions within the scope (*i.e.*, Groups 11, 13, and 14) and outside the scope of claim 1. The expectation for nucleic acid-lipid particle compositions of Groups 11, 13, and 14 would have been little, if any, gene silencing. That is, based on what was known in the art at the time, Groups 11, 13, and 14 would have been expected

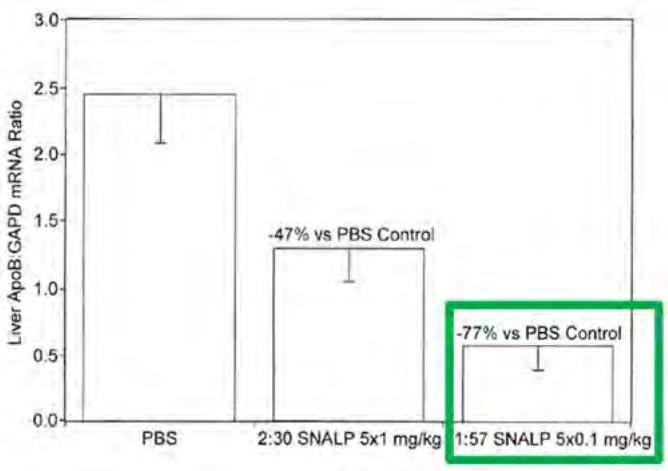
to yield reductions in gene expression similar to the "untreated" control group (*i.e.*, PBS). However, each of the nucleic acid-lipid particle compositions within the scope of claim 1 (Groups 11, 13, and 14) showed significant gene silencing relative to the control group (*i.e.*, PBS). EX1001, FIG 2. Even more surprisingly, these nucleic acid-lipid particle compositions yielded gene silencing levels which were at least comparable and in many cases superior to prior art compositions that have a much lower cationic lipid level (*e.g.*, 25 mol % to 40 mol % cationic lipid). EX1001, FIG 2 (red and green highlighting added).



For instance, the 1:57 nucleic acid-lipid particle composition, "was substantially more effective at silencing the expression of a target gene as compared to prior art nucleic acid-lipid particles ('2:40 SNALP')." EX1001, 6:10-14. Furthermore, the

1:57 nucleic acid-lipid particle composition "was the most potent at reducing" gene expression in vivo. EX1001, 72:25-27.

76. The 1:57 nucleic acid-lipid particle composition was also compared to the prior art 2:30 composition. Surprisingly, the 1:57 nucleic acid-lipid particle composition was "more than 10 times as efficacious as the 2:30 SNALP" composition in silencing gene expression in mouse liver when administered systemically. EX1001, Figure 3 (reproduced below). Remarkably, the 1:57 nucleic acid-lipid particle composition achieved these results at "a 10-fold lower dose" than the 2:30 composition. That is, even at a 10-fold lower dose, the claimed nucleic acid-lipid particle composition produced more gene silencing *in vivo* than the prior art composition.



EX1001, FIG 3 (green highlighting added).

77. Dr. Janoff states that "[a]t most, [Example 4] established that the 1:57 SNALP comprised of the specific species of lipid components and nucleic acid to

lipid ratio disclosed, dosed as disclosed, outperformed the 2:30 SNALP comprised of the lipid species disclosed and dosed as disclosed." EX1007 ¶85. Dr. Janoff further states that "given the disclosures in the '435 patent, a POSITA would not expect all alternative data points falling within the recited numeric range to perform like the 1:57 SNALP." EX1007 ¶112. These comments disregard the breadth of formulations tested. As discussed below, Examples 5 and 6 describe testing of additional formulations within the scope of claim 1 which also yield considerable levels of gene silencing in vivo. EX1001, 74:5-58. I discuss those results in further detail below.

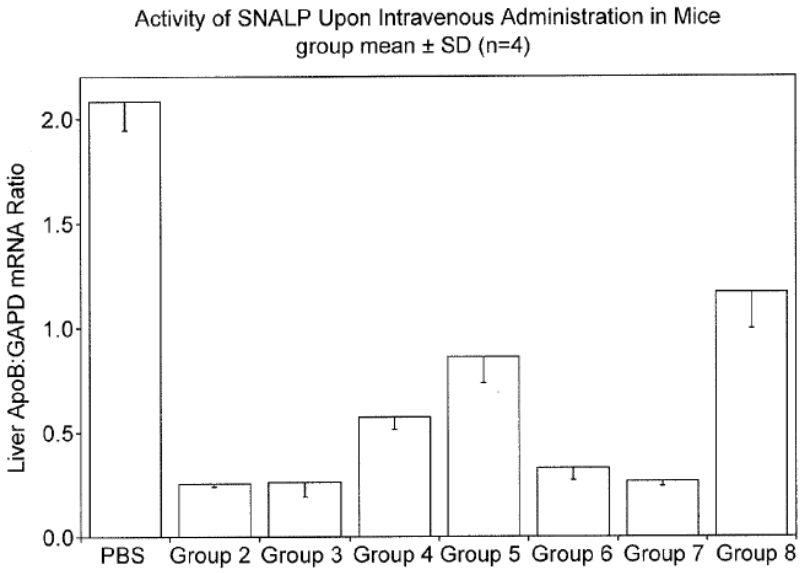
78. Example 5 discloses the testing of seven formulations within the scope of claim 1. EX1001, 74:11-58, Table 6.

TABLE 6

Characteristics of the SNALP formulations used in this study.

Group	Formulation Composition Lipid Name & Mole %	Lipid/ Drug Ratio	Finished Product Characterization: Size (nm)	Poly-dispersity	% Encapsulation
2	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	76	0.06	89
3	PEG(2000)-C-DMA | DLinDMA | Cholesterol 1.5 | 61.5 | 36.9	8.1	76	0.04	86
4	PEG(2000)-C-DMA | DODMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.0	72	0.05	95
5	PEG(5000)-C-DMA | DLinDMA | DPPC | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	9.6	52	0.16	89
6	PEG(2000)-C-DMA | DLinDMA | DPPC | Cholestanol 1.4 | 57.1 | 7.1 | 34.3	8.9	68	0.10	94
7	PEG(2000)-C-DMA | DLinDMA | DPPE | Cholesterol 1.4 | 57.1 | 7.1 | 34.3	8.9	72	0.07	95
8	PEG(2000)-C-DMA | DLinDMA | DPPC 1.8 | 70.2 | 28.1	8.6	74	0.13	86

EX1001, Table 6. Figure 4 displays the level of gene silencing following systemic administration of each of the seven formulations as compared to the performance of a control (*i.e.*, PBS). A person of ordinary skill in the art would have expected these formulations to be ineffective — that is, produce levels of gene silencing similar to that of the control. EX1001, FIG. 4. Contrary to expectations, these formulations produce significant reductions in gene expression.



EX1001, FIG 4.

79. Dr. Janoff states "Example 5 in the '435 patent shows variation of the cationic lipid apparently impacts efficacy" and concludes that "[a] POSITA would understand these results to suggest that a preferred proportion for one cationic lipid (*e.g.*, DLinDMA) does not necessarily apply to all other cationic lipids (*e.g.*, DODMA)." While there is some variation in the efficiency of formulations depending on which cationic lipid is used, (*compare* Group 4 (DODMA) *with*

Group 2 (DLinDMA)), this does not take away from the conclusion that both DODMA and DLinDMA are unexpectedly efficacious.

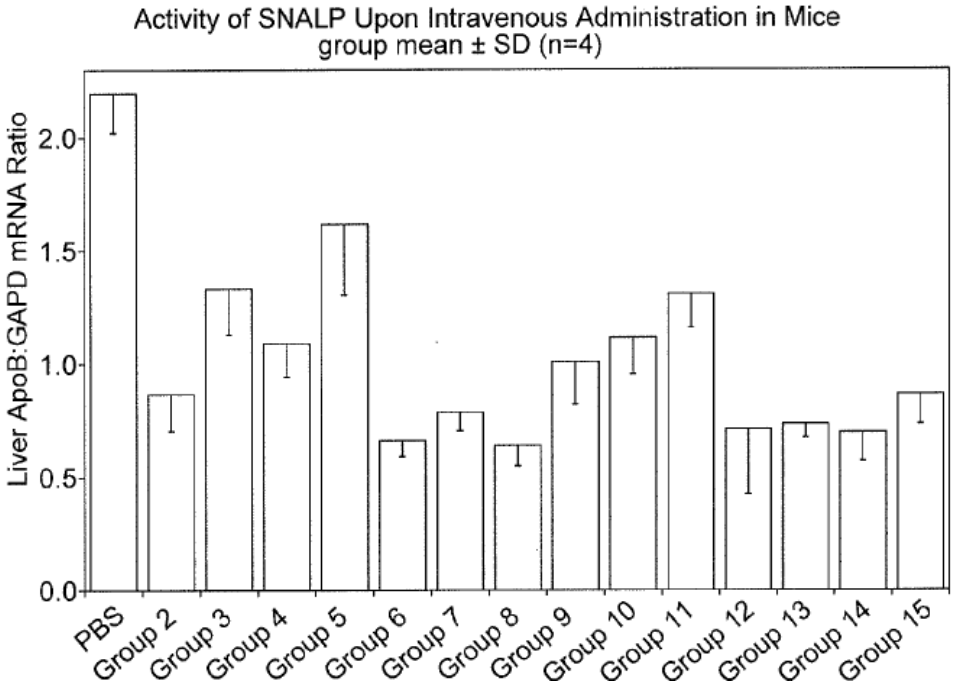
80. Example 6 discloses the testing of 14 formulations within the scope of claim1 (*i.e.*, 54 mol % to 68 mol % cationic lipid). EX1001, 74:11-58, Table 7.

TABLE 7

Characteristics of the SNALP formulations used in this study.

	Formulation Composition, Mole %		Lipid/	Finished Product Characterization		
Group	PEG(2000)-C-DMA | DLinDMA | Cholesterol		Drug Ratio	Size (nm)	Poly-dispersity	% Encapsulation
2	1.5 | 61.5 | 36.9		6.1	80	0.07	92
3	1.4 | 54.8 | 43.8		6.6	74	0.05	89
4	2.0 | 61.2 | 36.7		6.2	71	0.11	91
5	1.8 | 54.5 | 43.6		6.7	67	0.09	91
6	1.3 | 68.1 | 30.6		7.4	91	0.06	89
7	1.2 | 61.8 | 37.1		8.0	87	0.10	90
8	1.7 | 67.8 | 30.5		7.6	81	0.07	91
9	1.4 | 56.3 | 42.3		8.6	75	0.11	92
10	1.9 | 61.3 | 36.8		8.2	72	0.10	91
11	1.8 | 56.1 | 42.1		8.8	70	0.10	90
12	1.3 | 66.7 | 32.0		9.5	89	0.09	89
13	1.2 | 61.7 | 37.0		10.0	87	0.10	91
14	1.7 | 66.4 | 31.9		9.6	82	0.11	90
15	1.5 | 61.5 | 36.9		10.1	79	0.10	91

EX1001, Table 7. Figure 5 depicts the level of gene silencing activity achieved with the 14 formulations and compares this activity to the expected result — the performance of the PBS control.



EX1001, FIG 5. Surprisingly, each of the nucleic acid-lipid particle compositions demonstrated significant levels of gene silencing. The '435 patent notes "that the 1:62 SNALP formulation was one of the most potent inhibitors of ApoB expression at two different lipid:drug ratios (*i.e.*, 6.1 & 10.1) among the phospholipid-free SNALP formulations tested (see Groups 2 & 15)." EX1001, 75:45-48.

81. In sum, the '435 patent discloses data demonstrating nucleic acid-lipid particle compositions across the claimed range are unexpectedly well-tolerated and efficacious.

C. Claims 2-20

82. As an initial matter, the analysis of the dependent claims in the petition and Dr. Janoff's declaration is often difficult to follow. For instance,

Ground 1 alleges obviousness of claims 1-20, but many of the dependent claims include language that appears to invoke an unspecified anticipation theory. *See, e.g.*, EX1007 ¶124 ("Given the breadth of the claimed range for the phospholipid, these disclosures are sufficiently specific to disclose the claimed range. In addition, given the explicit disclosure of encompassing ranges, this limitation is *prima facie* obvious."). Additionally, most of the claims simply map disclosure without any explanation as to its significance. *See, e.g.*, EX1007 ¶124. Finally, the dependency of claims is largely ignored therefore it is challenging to determine what theory of unpatentability is actually being advanced. *See, e.g.*, EX1007 ¶125 (discussing claim 8 which depends from claims 1 and 5). I have done my best to respond to what seems to be the theory of unpatentability for each of the dependent claims.

83. It is my opinion that claims 2-20 are not obvious for the same reasons identified above with respect to claim 1. Additional discussion for some of claims 2-20 is provided below.

Claim 4. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from 50 mol % to 65 mol % of the total lipid present in the particle.

84. Dr. Janoff merely refers to his discussion of the cationic lipid level recited and claim 1 and concludes:

> Given the breadth of the claimed range, the disclosures above are sufficiently specific to disclose the claimed range. Not only does the disclosed broader range substantially overlap with the claimed range,

> a preferred embodiment in the reference recites a narrower range that also partially overlaps. In addition, given the explicit disclosure of overlapping ranges, this limitation is *prima facie* obvious.

EX1007 ¶121. I do not know what range Dr. Janoff asserts belongs to a "preferred embodiment." Moreover, claim 4 recites a narrower range of cationic lipid than claim 1 — a range that is well represented by the tested range of cationic lipid and, in particular, a nucleic acid-lipid particle composition with 57 mol % cationic lipid was found unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 4 is even stronger than it is for claim 1.

Claim 5. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.

85. Neither the petition nor Dr. Janoff points to disclosure of nucleic acid-lipid particles with a mixture of phospholipid and cholesterol. Instead, reliance is placed on long lists of non-cationic lipids including phospholipids, sterols, and non-phosphorous containing lipids and generic ranges for non-cationic lipid and cholesterol. EX1007 ¶122 (citing EX1002 ¶¶89, 91). These paragraphs do not disclose nucleic acid-lipid particle compositions that contain a mixture of phospholipid and cholesterol. To the extent it is argued that a skilled artisan might select both lipid components for inclusion in a nucleic acid-lipid particle, I

understand that an obviousness theory requires a reason to make such a combination and a reasonable expectation of success — both of which are absent.

86. Moreover, claim 5 requires a mixture of phospholipid and cholesterol such as that found in 1:57 nucleic acid-lipid particle compositions which are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 5 is even stronger than it is for claim 1.

Claim 6. The nucleic acid-lipid particle of claim 5, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

87. Dr. Janoff relies on a long list of non-cationic lipids for disclosure of DPPC and DSPC. EX1007 ¶123 (citing EX1002 ¶¶89, 128). It is unclear how the cited disclosure relates to the disclosure cited in the context of claims 1 and 5, from which claim 6 depends. To the extent that Dr. Janoff is arguing that a skilled artisan might select DPPC or DSPC from a long list of non-cationic lipids and incorporate one or both into a nucleic acid-lipid particle composition that includes cholesterol, I understand that such a theory requires a reason to make the formulation and a reasonable expectation of success — both of which are absent.

Claim 7. The nucleic acid-lipid particle of claim 5, wherein the phospholipid comprises from 3 mol % to 15 mol % of the total lipid present in the particle.

88. As an initial matter, the ’196 PCT does not disclose a range for a phospholipid component, much less a range for a phospholipid component in a

nucleic acid-lipid particle composition that includes cholesterol. Instead, Dr. Janoff arrives at the limitation through arithmetical manipulations of ranges of non-cationic lipid and cholesterol two different patent documents. EX1007 ¶124. Specifically, Dr. Janoff selects the 20% to 85% range of non-cationic lipid from the '196 PCT and subtracts from this range the 20% to 45 % range of cholesterol disclosed in the '618 patent. A person of ordinary skill in the art would not consider these calculations to amount to disclosure of a range of phospholipid. There is no basis to conclude that the '196 PCT provides an "explicit disclosure" of a range of 0% to 20% phospholipid. EX1007 ¶124. Moreover, the 1:57 nucleic acid-lipid particle composition is encompassed by claim 7 and is unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 7 is even stronger than it is for claim 1.

Claim 8. The nucleic acid-lipid particle of claim 5, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle.

89. As an initial matter, the '196 PCT does not disclose a range for cholesterol in a formulation that includes phospholipid. Dr. Janoff relies on disclosure in the '196 PCT of a range of 20 mol % to 45 mol % cholesterol. However, this is insufficient to make claim 8, which depends from claims 1 and 5, obvious. Dr. Janoff also relies on a lipoplex composition in the '618 patent that contains 30% cholesterol. A person of ordinary skill in the art would not look to

lipoplex compositions for guidance in making nucleic acid-lipid particle compositions. Moreover, the 1:57 nucleic acid-lipid particle composition is encompassed by claim 8 and is unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 8 is even stronger than it is for claim 1.

Claim 12. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from 1 mol % to 2 mol % of the total lipid present in the particle.

90. Neither the petition nor Dr. Janoff provides any reason to use the range of conjugated lipid of claim 12 in a nucleic acid-lipid particle composition with the claimed ranges of cationic and non-cationic lipid components. *See* EX1007 ¶¶119, 120. Any discussion of reasonable expectation of success is similarly missing. Moreover, claim 12 recites a narrower range of cationic lipid than claim 1 — a range that is well represented by the tested range of cationic lipid and, in particular, nucleic acid-lipid particle compositions with 57 mol % cationic lipid are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 12 is even stronger than it is for claim 1.

Claim 13. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

91. The petition and Dr. Janoff rely solely on one paragraph of the ’196 PCT, which states “[i]n some embodiments, the siRNA molecule is fully encapsulated within the lipid bilayer of the nucleic acid-lipid particle such that the

nucleic acid in the nucleic acid-lipid particle is resistant in aqueous solution to degradation by a nuclease." EX1002 ¶11. Dr. Janoff provides no explanation for why a person of ordinary skill in the art would have had a reasonable expectation of success generating fully encapsulated nucleic acid-lipid particles that have a high level of cationic lipid and a low level of conjugated lipid. Among other issues, a person of ordinary skill in the art would have expected the claimed nucleic acid-lipid particle to be unstable and thus incapable of encapsulating nucleic acids.

Claim 14. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.

92. Dr. Janoff provides no explanation for why a person of ordinary skill in the art would have had a reasonable expectation of success generating a pharmaceutical composition of nucleic acid-lipid particles that have a high level of cationic lipid and a low level of conjugated lipid. As discussed above, a person of ordinary skill in the art would have expected the claimed particles to be prone to aggregation and to be unstable due to the combination of a high level of cationic lipid and a low level of conjugated lipid. Consequently, a person of ordinary skill in the art would not have expected to successfully generate a pharmaceutical composition.

Claim 16. A method for the in vivo delivery of a nucleic acid, the method comprising: administering to a mammalian subject a nucleic acid-lipid particle of claim 1.

Claim 17. A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising: administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 1.

Claim 18. The method of claim 17, wherein the disease or disorder is a viral infection.

Claim 19. The method of claim 17, wherein the disease or disorder is a liver disease or disorder.

Claim 20. The method of claim 17, wherein the disease or disorder is cancer.

93. Dr. Janoff does not include any discussion of reasonable expectation of success. However, the '196 PCT teaches that nucleic acid-lipid particles for systemic use should have cationic lipid in the range of 5 mol % to 15 mol % — that is, much lower than the claimed range. As I discuss in detail above, a skilled artisan would have expected the claimed nucleic acid-lipid particles to be too toxic for in vivo administration. Thus, a person of ordinary skill in the art would not have had a reasonable expectation of success of obtaining nucleic acid-lipid particles suitable for administration to mammalian subjects.

VIII. GROUND 2 – CLAIMS 1-20 ARE NOT OBVIOUS IN VIEW OF PATENT OWNER'S PRIOR DISCLOSURES IN LIGHT OF LIN AND/OR AHMAD

94. The petition alleges that Lin and Ahmad provide additional support, over "patent owner's prior disclosures." Although Ground 2 alleges that claims 1-20 are obvious, the petition materials only address the cationic lipid limitations of claims 1 and 4. Ground 2 lacks a plausible explanation as to why a person of

ordinary skill in the art would want to combine disclosure regarding "lipoplexes" with nucleic acid-lipid particle technology as described in the '196 PCT. Moreover, the statement in the petition materials that a skilled artisan would have had a reasonable expectation of success is conclusory, nonsensical, and appears to be based on a misapprehension of the prior art.

95. The petition materials state that "the Lin and Ahmad systems tested helper lipids and cationic lipids to create carrier particles for nucleic acids, *i.e.*, 'nucleic acid-lipid particles,' the same general carrier particles described in the Patent Owner's prior disclosures." Pet. 50. Lin and Ahmad are directed to lipoplexes. EX1002 ¶8 (defining "cationic liposome complex" as lipoplex); EX2007, 2:27-28 (same). Lipoplexes are ***not*** nucleic acid-lipid particles.

96. Lipoplexes are distinct from the claimed nucleic acid-lipid particles. Lipoplexes and nucleic acid-lipid particles have different lipid compositions. *See, e.g.*, EX1002, ¶85 (explaining that nucleic acid-lipid particles "comprise a nucleic acid …, a cationic lipid, a noncationic lipid and a bilayer stabilizing component"); EX1005 at 2 (describing lipoplexes that comprise DNA and cationic and neutral lipids); EX1006 at 2-3 (same); EX1002 ¶¶6, 8 (contrasting lipoplexes and nucleic acid-lipid particle).

97. Lipoplexes and nucleic acid-lipid particles have different structures.

> For example, liposomal bilayers form around encapsulated nucleic acids, thereby protecting the nucleic acids from degradation by environmental nucleases; lipoplexes, by contrast, do not encapsulate nucleic acids, and hence, cannot completely sequester them away from environmental nucleases. Moreover, liposomes can encapsulate, in their aqueous compartments, other bioactive agents in addition to nucleic acids; lipoplexes, by contrast, cannot because they do not encapsulate aqueous volume.

EX2007, 2:54-62; *see also* EX2028, 122:1-24 (acknowledging distinction between lipoplexes and nucleic acid-lipid particles).

98. Lipoplexes are also functionally distinct and were known to be unsuitable for many applications. EX2007, 2:26-40 (explaining that "lipoplexes suffer from several major drawbacks when used in gene therapy, including low stability, high cytotoxicity, non-biodegradability, poor condensation and protection of DNA, serum sensitivity, large size and lack of tissue specificity."); EX1002, ¶6; EX1005 at 3315; EX1008 at 5-6.

99. The '196 PCT explicitly distinguishes lipoplexes from the nucleic acid-lipid particles described therein. *Compare* EX1002, ¶6 ("Cationic liposome complexes, however, are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects.") *with id.*, ¶2 ("[T]he present invention is directed to using a small interfering RNA (siRNA)

encapsulated in a serum-stable lipid particle having a small diameter suitable for systemic delivery.").

100. Dr. Janoff asserts that a person of ordinary skill in the art "a POSITA would have found it obvious to use the insights of Lin regarding increasing the cationic mole fraction of nucleic acid lipid particles to increase transfection efficiency and the disclosures of the Patent Owner's prior disclosures regarding nucleic acid-lipid particles with a cationic lipid proportion greater than 50%." EX1007 ¶142. I disagree. Because of the differences between lipoplexes and the claimed nucleic acid-lipid particles, a person of ordinary skill in the art would not have found teachings regarding lipoplexes to be relevant for the development of nucleic acid-lipid particle compositions.

101. Moreover, the petition materials falsely assert that "[a] POSITA would understand the testing of Ahmad to support the proposition that for certain formulations, cationic lipids can increase transfection efficiency when they are incorporated above 50 mol%." Pet. 49; EX1007 ¶139. But Ahmad discloses that transfection efficiencies for most lipoplexes are insensitive to cationic lipid increases over the range of 40 mol % to 80 mol % cationic lipid content. EX1006, Figure 3A. Similarly, Lin discloses that transfection efficiency for lipoplexes varies dramatically depending on which cationic lipid and non-cationic lipid are used. For example, the transfection efficiency of DOSPA/DOPC lipoplexes is

insensitive over the range of 20 mol % to 100 mol % cationic lipid, EX1005, Figure 4A, and DOTAP/DOPE lipoplexes are insensitive to cationic lipid increases over the entire tested range (0 mol % to 70 mol %), EX1005 Figure 4D. If anything, Lin supports the unpredictability of lipid particle chemistries. Consequently, there is no teaching or suggestion in Lin or Ahmad to increase the cationic lipid component above 50% is beneficial.

102. Lin and Ahmad further provide that the cationic lipid component of lipid particles should be minimized, regardless of whether used for in vitro or in vivo purposes. For example, Ahmad specifically teaches that the cationic lipid component should be minimized.

> A relatively low lipid/DNA charge ratio, therefore, can be considered optimal since it allows for achievement of maximum TE with the least amount of cationic lipid. This is due to the unexpected increase of σ_M* with ρchg. ***Minimizing the amount of cationic lipid is desirable to reduce cost as well as potential toxic effects of the cationic lipid***. In addition, achieving a given σ_M with fewer, more highly charged molecules should mean a smaller metabolic effort for the elimination of the lipids from the cell.

EX1006 at 7 (emphasis added). This teaching is unequivocal and would not have been ignored by a person of ordinary skill in the art.

103. The Board in its institution decision stated that:

> We do recognize that Ahmad is concerned with the toxicity of cationic lipids, but Ahmad noted that "with the amounts of cationic lipid employed in our in vitro experiments, we find no toxic effects on the cells as judged by cell morphology and the amount of total cellular protein." Ex. 1006, 746. Because claim 1 is not limited to in vivo use of the claimed nucleic acid-lipid particle, the statement in Ahmad to which Patent Owner directs us concerning minimizing the amount of cationic lipid to avoid cost and toxicity, is not necessarily persuasive that Ahmad does not encourage increased amounts of cationic lipid in certain circumstances.

Paper 15 at 31-32. I disagree. Ahmad states without qualification that cationic lipid should be minimized to avoid toxicity. EX1006 at 7, 9. Moreover, Ahmad does not qualify the conclusion that cationic lipid should be minimized even though no toxicity was observed in a single particular set of her experiments.

104. Furthermore, the toxicity assay is not described in sufficient detail to meaningfully assess the results, and in any event, that in a single set of experiments "toxic effects" were not observed does not take away from Ahmad's unequivocal teaching or the teaching of other prior art references consistent with Ahmad. E.g., EX1008 at 5. Among other things, persons of ordinary skill in the art appreciated that in vitro toxicity is dependent on how and when it is measured. For example, if toxicity is measured too long after treatment with a toxic formulation, growth of the surviving cells will give the false impression that the formulation is not toxic. This is because surviving cells will continue to grow and, in time, replace those

cells killed by the toxic formulation. A person of ordinary skill in the art would not ignore the problem of cytotoxicity based on a single unspecified observation to the contrary.

105. In my opinion, as explained in further detail below, there would have been no good reason why a person of ordinary skill in the art would combine the different range disclosures for different lipid components from the '196 PCT so as to arrive at the claimed nucleic acid-lipid particle. Nor would one reasonably expect such formulations to work. The petition materials fail to demonstrate otherwise.

106. The petition materials state that "given the success of generating nucleic acid-lipid particles with a cationic lipid proportion greater than 50% as described in the Patent Owner's prior disclosures, a POSITA would have appreciated a reasonable expectation of doing so." EX1007 ¶141. However, this statement is confusing to me because neither the '196 PCT nor the '189 publication disclose nucleic acid-lipid particles with cationic lipid proportion greater than 50%. EX1002 ¶¶ 216, 223, 228, 232 (disclosing formulations of 7.5 mol % and 15 mol % cationic lipid); EX1003 ¶¶289, 291-293, 295, 303, 311, 319, 327, 335, 343, 351, 361, 369, 377, 385 (disclosing formulations of 30 mol % and 40 mol % cationic lipid).

107. Moreover, if anything, Lin and Ahmad strengthen the conclusion I arrived at in Ground 1 that a person of ordinary skill in the art would not have a reasonable expectation of success. For example, Ahmad expressly states that cationic lipid should be minimize to avoid toxicity and cost. EX1006 at 7.

IX. GROUND 3 – CLAIMS 1-20 ARE NEITHER ANTICIPATED BY NOR OBVIOUS IN VIEW OF THE '554 PUBLICATION

108. It is my opinion that the petition fails to demonstrate that claims 1-20 of the '435 patent are unpatenable in view of the '554 publication.

A. Claim 1

1. Anticipation by L054

109. The '554 publication does not disclose the claimed nucleic acid-lipid particle composition, as claimed. First, L054 formulation of Table 4 is a lipid mixture for making particles, not a particle. Second, the petition fails to establish that L054-derived lipid particles would qualify as a nucleic-acid lipid particle as claimed, as there is no evidence that the L054-derived lipid particles encapsulate nucleic acid. Separately, the petition fails to establish that a L054-derived lipid particle of the '554 publication is suitable for systemic delivery as required by claim 1.

110. The challenged claims are directed to a nucleic acid-lipid particle. The L054 lipid formulation identified in Table 4 of the '554 publication is a listing of lipid components or mixture of lipids used to form particles, not a nucleic acid-

lipid particle as claimed. The petition materials confuse the composition of the input formulation (*i.e.*, lipids of Table 4) with something different—*i.e.*, the output formulation (*i.e.* lipid particles). There is no disclosure in the '554 publication regarding the composition of particles generated using the L054 formulation. This is an important distinction because a person of ordinary skill in the art would not assume that the composition of a lipid particle will be exactly the same as the composition of the lipid formulation utilized as a mixture of lipids to make the particles. Testing of the finished particle composition is necessary to account for variations in the molar fractions of the lipid components in the starting lipid formulation compared to the molar fractions of the lipid components in the resulting particles. EX2012 at 7242 ("Perhaps surprisingly, however, the composition of CLDCs is not usually determined at all—rather, the composition is simply assumed to be defined by the identity and amounts of the components originally introduced. In the future, however, as gene therapy agents approach pharmaceutical reality, more rigorous criteria likely will be required."); EX2013 (FDA guidance) at 3 (recommending labeling with the "amount of each lipid component used in the formulation based on the ***final form*** of the product" and "[a]n expression of the molar ratio of each individual lipid to the drug substance is also recommended for each individual lipid in the ***finished formulation***), 8 (recommending reporting of "characteristics or attributes specific to the liposome

formulation" including "[l]ipid content (***to demonstrate consistency with the intended formulation***).").

111. Experimental data presented in the '435 patent illustrates that the input and output formulations are not identical. For example, the '435 patent discloses that the lipid to drug ratio (*i.e.*, lipid to nucleic acid ratio) calculated from the input components is not identical to that of the finished product. *See, e.g.*, EX1001, 79:50-80:9 (reporting different input and final lipid to drug ratios for SNALP formulations). I understand that Dr. Janoff agrees with this assessment. *See* EX2028, 155:2-25 (indicating that there could be a difference between input and output lipid-to-drug ratio).

112. The '554 publication further evidences that the input molar ratio of lipids is not identical to that of the finished product. For example, the '554 publication discloses that lipid particles made from the lipid mixtures in Table IV (*e.g.*, L054) are "characterized in term of particle size, Zeta potential, alcohol content, total lipid content, nucleic acid encapsulated, and total nucleic acid concentration." EX1004 ¶634. That is, lipid and nucleic acid content and Zeta potential (*i.e.*, a measure of surface charge which relates to the amount of cationic lipid present in the particle) must be empirically determined and is not identical to the starting materials used to make the particles. *See* generally EX2013.

113. The methods disclosed in the '554 publication, including the identified detergent dialysis methods, would be expected to skew the molar ratio of lipids in the finished particles relative to the starting materials. For example, cholesterol-based detergents (*e.g.*, BIGCHAP and deoxy-BIGCHAP) are biased toward extracting cholesterol. *See* EX1004 ¶165 (listing BIGCHAP and deoxy-BIGCHAP as examples of suitable detergents for use with the detergent dialysis method of particle formation). The predictable result of using cholesterol-based detergents is less cholesterol in the finished particles than in the starting materials. When less cholesterol is incorporated into nanoparticles, the molar ratio of the remaining components (*i.e.*, cationic lipid, phospholipid, and conjugated lipid) is increased. I understand that Dr. Janoff agrees that cholesterol in the starting lipid mixture may not be quantitatively incorporated into finished particles. EX2028, 157:12-158:16 (explaining how failure to recover cholesterol in a particle would change the amount of the remaining components).

114. A similar situation would apply to organic solvent-based methods. That is, differences in solubility in organic solvent amongst the lipid components of the mixture will result in differential incorporation of components into nanoparticles.

115. In the L054 example, while the lipid formulation is listed as 50/20/28/2, the molar fractions of the same lipids in the resulting particle would be

expected be different and presumably outside the scope of the challenged claims. The L054 lipid mixture has cationic lipid content and conjugated lipid content that are at the edge of the claimed ranges. Because L054 has cationic lipid content and conjugated lipid content on the edge of the claimed range, even small differences in incorporation of components will result in lipid particles that are outside the claimed range of cationic lipid content and conjugated lipid content. For example, if cholesterol is not quantitatively incorporated, see above, particles derived from the L054 lipid mixture would have more than 2 mol % conjugated lipid.

116. Accordingly, the ’554 publication fails to disclose a lipid particle composition produced by the L054 lipid mixture that is within the scope of claim 1.

117. The petition materials fail to establish that the L054-derived nanoparticles encapsulate nucleic acid, as is required by claim 1. As discussed above, the term “nucleic acid-lipid particles” excludes particles that do not encapsulate nucleic acid. The encapsulation state of the nucleic acids in nanoparticles made with the L054 lipid mixture is unknown.

118. To the extent that the ’554 publication discusses structure, it suggests that only serum-stable lamellar embodiments encapsulate nucleic acids.

> In one embodiment, the present invention provides a serum-stable formulated molecular composition … in which the biologically active molecule is encapsulated in a lipid bilayer and is protected from

> degradation (for example, where the composition adopts a lamellar structure).

EX1004 ¶136; *see also* ¶316. As discussed above, the L054 embodiment has not been tested for serum stability and whether it adopts a lamellar structure that encapsulates the nucleic acid is similarly unknown.

119. Encapsulation of nucleic acids cannot be assumed based on the composition and formulation method. I understand that Dr. Janoff agrees with me.

> However, neither of [the prior art] preparations were dialyzed against high salt buffers subsequent to liposome formation, the reported amounts of encapsulated DNA actually may include a significant percentage of unencapsulated DNA. Since [the prior art] liposomal formulations were not exposed to DNAase degradation to determine the percentage of DNA actually sequestered in the liposomes, the high reported amounts probably do not reflect actually encapsulated DNA.

EX2007, 4:11-19. The ’554 publication does not disclose dialyzing nanoparticles made from the L054 lipid mixture against high salt buffers or exposing the preparation to DNAse degradation. Moreover, the ’554 publication does not disclose methods for determining encapsulation of nucleic acids.

120. Moreover, as indicated above, Dr. Janoff opined that the lipid particles of the ’435 patent are SNALP. That is, the inventive lipid particles are serum stable nucleic acid-lipid particle that are “extremely useful for systemic

applications" and "can exhibit extended circulation lifetimes following intravenous (i.v.) injection." EX1001, 11:36-38.

121. However, the petition and Dr. Janoff fail to address whether L054-derived nanoparticles are formulated for systemic administration. The '554 publication distinguishes between embodiments formulated for *in vitro* use and those formulated for *in vivo* use. *See, e.g.*, EX1004 ¶¶136, 462. Furthermore, the '554 publication stresses that serum-stability is a critical property of *in vivo* formulations. *See, e.g.*, EX1004 ¶¶14, 15, 158. However, L054 was only tested in vitro. *See* EX1004 ¶395 ("FIG. 16 shows a non-limiting example of ***in vitro efficacy*** of siNA nanoparticles …") (emphasis added). Specifically, the L054 formulation was not evaluated for serum stability — a property identified as critical for embodiments formulated for systemic (*in vivo*) use. *See* EX1004 ¶¶158 (providing a serum stability test "for determining whether a formulated molecular composition will be effective for delivery of a biologically active molecule into a biological system, …"), ¶592 (Example 7 Evaluation of Serum Stability of Formulated siNA Compositions). Furthermore, while another exemplary formulation (outside the scope of the '435 claims) was tested *in vivo*, the L054 formulation was not. EX1004 ¶596.

122. Furthermore, a person of ordinary skill in the art would have expected particles derived from L054 to be too toxic for systemic use. Such a skilled artisan

would have expected DMOBA, the cationic lipid used in the L054 lipid mixture (see Table IV), to be toxic. A person of ordinary skill in the art would have appreciated that the dimethylamino group on the aryl ring is a good leaving group upon protonation and, as such, has the potential to alkylate cysteines and lysines on cellular proteins. *See* EX1004, Table IV (DMOBA structure). Additionally, DMOBA would have been expected to accumulate in liver and spleen because the arylether groups make elimination through mammalian detoxification pathways more difficult. The resulting accumulation and protein modifications would result in organ toxicity rendering lipid particles using DMOBA inappropriate for systemic use.

123. Accordingly, the petition materials fail to identify a nucleic acid-lipid particle within the scope of claim 1.

2. Anticipation by ranges

124. The petition and Dr. Janoff assert that claim 1 is anticipated by ranges disclosed in the ’554 publication However, the petition and Dr. Janoff fail to demonstrate that a person of ordinary skill in the art would have understood the separate disclosures of ranges for the various components to represent a single formulation. Moreover, I understand that anticipation by ranges requires a factual inquiry into whether the ranges are disclosed with “sufficient specificity.” Such an inquiry is wholly absent from the petition and Dr. Janoff’s declaration.

125. To the extent that these ranges are discussed, there is no explanation in the '554 publication or Dr. Janoff's declaration to indicate how the disclosed ranges for the three different components would represent or be combined into a single embodiment.

126. For example, Dr. Janoff points to three paragraphs in the '554 publication for disclosure of ranges of lipid components. *See* EX1007 ¶¶146, 150, 151. These paragraphs are reproduced in relevant part below.

> ***In one embodiment***, the cationic lipid component …of a composition of invention comprises from about 2% to about 60%, from about 5% to about 45%, from about 5% to about 15%, or from about 40% to about 50% of the total lipid present in the formulation.

EX1004 ¶116 (emphasis added).

> ***In one embodiment***, the neutral lipid component of a composition of the invention comprises from about 5% to about 90%, or from about 20% to about 85% of the total lipid present in the formulation.

EX1004 ¶313 (emphasis added).

> ***In one embodiment***, the PEG conjugate … of a composition of the invention comprises from about 1% to about 20%, or from about 4% to about 15% of the total lipid present in the formulation.

EX1004 ¶118 (emphasis added).

127. These paragraphs state that "***in one embodiment***" the cationic lipid component can span four different ranges. Further, "***in one embodiment***" the neutral lipid component can span two different ranges. Still further, "***in one***

embodiment" the PEG conjugate can span two different ranges. There is no explanation in the '554 publication or Dr. Janoff's declaration to indicate how these eight different ranges for three different components would represent or be combined into a single embodiment. Moreover, as with L054-derived particles, the petition and Dr. Janoff fail to address whether any of the embodiments relied upon are suitable for systemic administration.

128. Also absent is sufficient demonstration that ranges or examples relied upon in '554 publication disclose the claimed range for "a conjugated lipid that inhibits aggregation of particles" (*i.e.*, claim element 1(d)). The petition relies on paragraph ¶504 of the '554 publication to support that it is "desirable to include other components" that serve to prevent aggregation. Pet. 55. The petition materials, however, fail to demonstrate or explain which, if any, specific embodiments in the '554 publication includes conjugated lipid such that aggregation of particles is inhibited as claimed.

129. Moreover, as with L054-derived particles, the petition and Dr. Janoff fail to address whether any of the embodiments relied upon are suitable for systemic administration.

130. Furthermore, the petition and Dr. Janoff fail to demonstrate that a person of ordinary skill in the art would have understood the separate disclosures of ranges for the various components to represent a single formulation. For

example, relevant to such an inquiry is an analysis as to the size of the prior art ranges and the differences between the prior art ranges and the claimed ranges. No such analysis was provided by Dr. Janoff — he merely asserts "[g]iven the breadth of the claimed range, these disclosures are sufficiently specific to anticipate the claimed range." EX1007 ¶¶148, 150, 151.

131. Based on my review of the size of the prior art ranges and the extent of the differences between the prior art ranges and the claimed ranges, it is my opinion that there are considerable differences between the prior art and claimed ranges. The ranges disclosed by the '554 publication which Dr. Janoff cites are very broad, and thus the '554 publication fails to provide the required sufficient specificity to anticipate the lipid component ranges of claim 1.

132. Therefore, in my opinion, the lipid component ranges of the '554 publication do not anticipate the lipid component ranges of the claimed nucleic acid-lipid particle composition at least because the ranges disclosed by the '554 publication which Dr. Janoff cites are very broad, the '554 publication and Dr. Janoff fail to indicate how these broad ranges arrive at the specific ranges recited in claim 1, and a person of ordinary skill in the art would not have understood the separate disclosures of ranges for the various components to represent a single formulation.

3. Obvious over ranges

133. The claimed nucleic acid-lipid particle composition is not obvious in view the lipid component ranges of the '554 publication. First, the '554 publication fails to disclose the combination of the lipid components of the claimed nucleic acid-liposome particle. Second, a skilled artisan would have had no reason to combine individualized lipid components in the '554 publication to arrive at the claimed nucleic acid-lipid particle. Third, the petition materials fail to address that a skilled artisan would have had no reasonable expectation of success that the claimed nucleic acid-lipid particle composition would be well-tolerated and efficacious. Finally, unexpected results further demonstrate the nonobvious nature of the claimed nucleic acid-lipid particle composition.

134. To the extent that the petition and Dr. Janoff argue that the claims are obvious over the disclosed ranges of lipids in the '554 publication, this argument fails for the same reasons as discussed in Ground 1. That is, the petition materials rely on generic disclosure of ranges for individual lipid components that may be used in lipid particle compositions. But a person of ordinary skill in the art knew that the properties of lipid particle compositions (*e.g.*, cytotoxicity and efficacy) derive from the entire composition (*i.e.*, cationic, non-cationic, and conjugated lipids), rather than the individual lipid components. Given the interdependent nature of the components of the claimed nucleic acid-lipid particle composition,

the petition's per-limitation approach to addressing the levels of cationic, non-cationic, and conjugated lipids is inadequate.

135. Moreover, "a conjugated lipid that inhibits aggregation of particles" is required by the challenged claims (*i.e.*, claim element 1(d)). The petition materials, however, rely on paragraph ¶504 of the '554 publication for the proposition that it is "desirable to include other components" that serve to prevent particle aggregation. EX1007 ¶151. The petition materials, however, fail to demonstrate or explain which, if any, specific embodiments in the '554 publication includes a conjugated lipid such that aggregation of particles is inhibited as claimed.

136. Lastly, it is my understanding that a claim is only obvious if a person of ordinary skill in the art would have had some motivation to modify the cited reference, and a reasonable expectation of success in doing so. Dr. Janoff does not discuss these aspects. However, it is my opinion that there would not have been any motivation to modify the cited disclosures of the '554 publication. As already discussed, the state of the art was unequivocal that a high level of cationic lipid should be avoided, and that much higher levels of conjugated lipids should be used. To the extent that Dr. Janoff is suggesting that a person of ordinary skill in the art could simply vary the concentrations of each component until they arrived at the claimed invention, I disagree. The field of art is unpredictable. Furthermore,

there was no guidance whatsoever that would suggest to a skilled artisan to increase the cationic lipid levels while decreasing the conjugate lipid levels.

137. It is also my opinion that there would not have been any reasonable expectation of success. As discussed, a person of ordinary skill in the art would not have expected lipid particle formulations that departed so drastically from the instructions in the prior art to successfully exhibit any efficacy (*e.g.*, gene silencing). The expected result of using the claimed lipid particle formulations would have been little to no efficacy, accompanied by a host of unwanted side effects — *e.g.*, toxicity, *in vivo* aggregation, and immunogenicity

B. Claims 2-20

138. According to the petition, claims 1-20 are being challenged under anticipation and obviousness theories. However, only a handful of claims unambiguously identify both an anticipation and obvious theory.

Claim	Theory
2, 3, 5, 6, 9, 13-20	From these disclosures, a POSITA would appreciate that the claim limitation is expressly disclosed. EX1007 ¶¶153, 154, 156, 157, 161, 165-172.
4	[G]iven the explicit disclosure of overlapping ranges, this limitation is *prima facie* obvious. EX1007 ¶155.

Claim	Theory
7	Given the breadth of the claimed range for the phospholipid, these disclosures are sufficiently specific to anticipate the claimed range. EX1007 ¶158. This limitation is *prima facie* obvious. EX1007 ¶159.
8	Given the breadth of the claimed range, these disclosures are sufficiently specific to anticipate the claimed range. Moreover, given the explicit disclosure of an encompassing range, this limitation is *prima facie* obvious. EX1007 ¶160.
10	Because one of the listed species of PEG-lipid conjugates is disclosed, this element is anticipated. EX1007 ¶162.
11	This limitation would have been obvious in view of the '554 publication in light of the knowledge of a POSITA. EX1007 ¶163.
12	For the reasons stated above, the '554 publication discloses this range with sufficient specificity to anticipate. In the alternative, this range is *prima facie* obvious given the overlapping range in the '554 publication. EX1007 ¶164.

139. Additionally, most of the claims simply map disclosure without any explanation as to its significance. *See, e.g.*, EX1007 ¶160. Finally, the dependency of claims is largely ignored therefore it is challenging to determine what theory of unpatentability is actually being advanced. *See, e.g.*, EX1007 ¶160 (discussing

claim 8 which depends from claims 1 and 5). I have done my best to respond to what seems to be the theory of unpatentability for each of the dependent claims.

Claim 4. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from 50 mol % to 65 mol % of the total lipid present in the particle.

140. Dr. Janoff presents no independent argument for claim 4, but rather refers back to his discussion of claim 1. For the same reasons as claim 1, the petition and Dr. Janoff fail to demonstrate the unpatentability of claim 4. Moreover, claim 4 recites a narrower range of cationic lipid than claim 1 — a range that is well represented by the tested range of cationic lipid and, in particular, nucleic acid-lipid particle compositions with 57 mol % cationic lipid which are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 4 is even stronger than it is for claim 1.

141. Accordingly, the ’554 publication neither anticipates nor makes obvious claim 4.

Claim 5. The nucleic acid-lipid particle of claim 1, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof.

142. Dr. Janoff appears to suggest that claim 5 is anticipated by the L054 formulation. EX1007 ¶156 (citing EX1004 Table IV). As I discussed above, L054 does not anticipate claim 1 and therefore cannot anticipate claim 5.

143. To the extent that Dr. Janoff is asserting an anticipation theory based on disclosure in paragraphs 85 and 455, he provides no guidance for why a person of ordinary skill in the art would select a mixture of cholesterol and phospholipid from long lists of non-cationic lipids which include phospholipids, nonphosphorous containing lipids, and sterols.

144. To the extent that Dr. Janoff is presenting an obviousness theory, I understand that to require a reason to combine these disclosures and a reasonable expectation of success — both of which are absent. Moreover, claim 5 requires a mixture of phospholipid and cholesterol such as that found in 1:57 formulations which are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 5 is even stronger than it is for claim 1.

145. Accordingly, the '554 publication neither anticipates nor makes obvious claim 5.

Claim 6. The nucleic acid-lipid particle of claim 5, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

146. Dr. Janoff states that "a POSITA would appreciate that the claim limitation is expressly disclosed" in paragraph 85 of the '554 publication.

> Suitable neutral lipids include those comprising ***any of a variety of neutral uncharged, zwitterionic or anionic lipids*** capable of producing a stable complex. They are preferably neutral, although

> they can alternatively be positively or negatively charged. … [list of neutral lipids].

EX1004 ¶85. To the extent that Dr. Janoff is expressing an anticipation theory, I understand that picking and choosing claim elements from a prior art disclosure is not proper. To the extent that he is arguing that the claim is obvious, I understand that such a theory requires a reason to combine and reasonable expectation of success — both of which are absent.

147. Accordingly, the '554 publication neither anticipates nor makes obvious claim 6.

Claim 7. The nucleic acid-lipid particle of claim 5, wherein the phospholipid comprises from 3 mol % to 15 mol % of the total lipid present in the particle.

148. Dr. Janoff appears to be alleging that claim 7 is anticipated or obvious over paragraph 455, which is a long list of neutral lipid components and generic ranges for neutral lipids and cholesterol disclosed in paragraphs 117-119 of the '554 publication. Dr. Janoff arrives at the "disclosure" of 3 mol % to 15 mol % phospholipid through a series of unexplained assumptions and mathematical manipulations. Dr. Janoff selects the 20% to 85% range of neutral lipid and the 20% to 45 % range of cholesterol from the paragraphs below.

> In one embodiment, the neutral lipid component of a composition of the invention comprises from about 5% to about 90%, or from about 20% to about 85% of the total lipid present in the formulation.

> In one embodiment, the PEG conjugate (*i.e.*, PEG-DAG, PEG-cholesterol, PEG-DMB) of a composition of the invention comprises from about 1% to about 20%, or from about 4% to about 15% of the total lipid present in the formulation.
>
> In one embodiment, the cholesterol component of a composition of the invention comprises from about 10% to about 60%, or from about 20% to about 45% of the total lipid present in the formulation.

EX1004 ¶¶117-119. Dr. Janoff then concludes:

> When cholesterol is present, the range for a phospholipid is thus 0-40%. Not only does the disclosed range encompass the claimed range, when combined with a cationic lipid proportion in the 60% range and cholesterol in the 20-40% range, the range for the phospholipid is decreased to 0%-20%. Given the breadth of the claimed range for the phospholipid, these disclosures are sufficiently specific to anticipate the claimed range.

EX1007 ¶158. I do not understand this argument. There is just no basis to conclude that the '554 publication "discloses" a range of 0% to 40% or 0% to 20% phospholipid.

149. Moreover, 1:57 nucleic acid-lipid particle compositions are encompassed by claim 7 and are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 7 is even stronger than it is for claim 1.

150. Accordingly, the '554 publication neither anticipates nor makes obvious claim 7.

Claim 8. The nucleic acid-lipid particle of claim 5, wherein the cholesterol or derivative thereof comprises from 30 mol % to 40 mol % of the total lipid present in the particle.

151. As an initial matter, claim 8 depends from claims 1 and 5. One of the anticipation theories presented in claim 5 was based on the L054 lipid mixture, which has 28 mol % cholesterol and therefore cannot be used to make particles with 30 mol % to 40 mol % cholesterol as recited in claim 8. The other anticipation theory in claim 5 was based on disclosure in paragraphs 85 and 455 which recite long lists of non-cationic lipids. I do not understand the relevance of paragraph 119, which recites ranges of cholesterol, to an anticipation theory. Among other reasons, this paragraph does not address the phospholipid content required by claim 5.

152. Dr. Janoff also argues that paragraph 119 represents "an encompassing range" and therefore the claim is prima facie obvious. But again, claim 8 depends from claim 5 which requires a mixture of phospholipid and cholesterol. This "encompassing range" for cholesterol does not make any mention of phospholipid. Furthermore, a reason to combine these disclosures and reasonable expectation of success are absent from Dr. Janoff's analysis.

153. Dr. Janoff also argues that "the '554 publication also includes various specific formulations which include cholesterol at a 30% proportion. Id., Table 4 (*e.g.*, L106)." First, L106 is a lipid mixture and not a nucleic acid-lipid particle.

Second, even if particles were made from L106, these particles would not meet the limitations of claim 8 which depends from claims 1 and 5. L106 has 3% conjugated lipid, which is well above the range of claim 1 (*i.e.*, 0.5% to 2%). Further L106 does not "comprise[] a mixture of a phospholipid and cholesterol or a derivative thereof" as required by claim 5. L106, as with the other examples from the '554 publication with 30% cholesterol, do not meet the limitations of claim 8.

154. Moreover, 1:57 nucleic acid-lipid particle compositions are encompassed by claim 8 and are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 8 is even stronger than it is for claim 1.

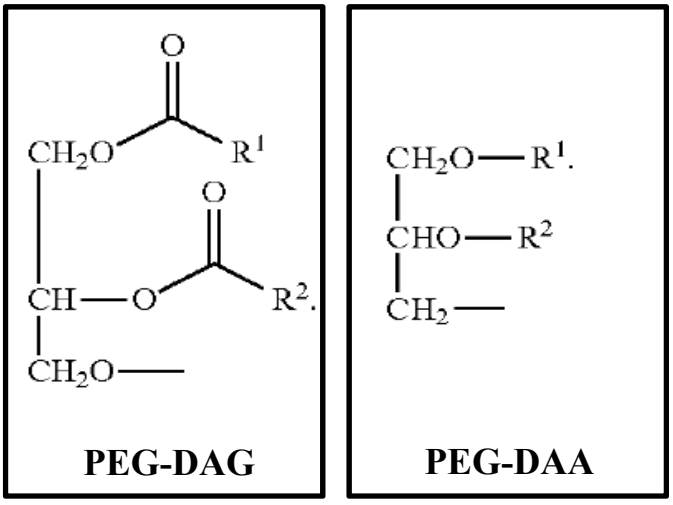
155. Accordingly, the '554 publication neither anticipates nor makes obvious claim 8.

Claim 11. The nucleic acid-lipid particle of claim 10, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

156. Dr. Janoff admits, that the '554 publication does not disclose a PEG-dialkyloxypropyl (PEG-DAA) conjugates as required in claim 11 and thus the '554 publication does not anticipate claim 11.

157. Dr. Janoff alleges that "[a] POSITA would have been aware that PEG-dialkyloxypropyl (PEG-DAA) conjugates could be used in lieu of PEG-diacylglycerol (PEG-DAG) conjugates." EX1007 ¶163. Dr. Janoff points to

EX1014 as the "'910 publication" and states that it discloses PEG-DAA conjugates. I do not know what EX1014 is, but it is not the '910 publication. Regardless, Dr. Janoff's argument is based on the false assumption that PEG-DAG and PEG-DAA conjugates are interchangeable. This is not the case. PEG-DAG conjugates have an ester moiety linking the acyl chains (R^1 and R^2 in the figure) to the backbone whereas PEG-DAA conjugates have an ether moiety linking the acyl chains to the backbone. *See* EX1001, 53:53-54:14 (inset). As compared to PEG-DAA conjugates, PEG-DAG conjugates are more hydrophilic which directly impacts the physical properties of the particles. Moreover, PEG-DAG conjugates are more easily metabolized by cells. A skilled artisan would not consider PEG-DAG and PEG-DAA conjugates to be equivalent or interchangeable.



158. Accordingly, the '554 publication neither anticipates nor makes obvious claim 11.

Claim 12. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises from 1 mol % to 2 mol % of the total lipid present in the particle.

159. Dr. Janoff presents no independent argument for claim 12, but rather refers back to his discussion of claim 1. For the same reasons as claim 1, the petition and Dr. Janoff fail to demonstrate the unpatentability of claim 12. Moreover, claim 12 recites a narrower range of cationic lipid than claim 1 — a range that is well represented by the tested range of cationic lipid and, in particular, nucleic acid-lipid particle composition with 57 mol % cationic lipid are unexpectedly well-tolerated and efficacious. *See* Section VIII.B. Thus, the nexus of the unexpected results and claim 12 is even stronger than it is for claim 1.

Claim 13. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

160. Dr. Janoff alleges that "a POSITA would understand that full encapsulation requires only an excess of cationic lipid with regard to the nucleic acid for electrostatic interaction." EX1007 ¶165. I disagree. Encapsulation is only determined by testing and cannot be inferred from the composition or production method. I understand that Dr. Janoff's shares my views. As he disclosed in one of his own patents:

> Additionally, [two prior art references] report encapsulation of 1-4 micrograms per micromole of spermine-condensed SV40 plasmid DNA in liposomes. However, neither of their preparations were dialyzed against high salt buffers subsequent to liposome formation,

> the reported amounts of encapsulated DNA actually may include a significant percentage of unencapsulated DNA. Since these liposomal formulations were not exposed to DNAase degradation to determine the percentage of DNA actually sequestered in the liposomes, the high reported amounts probably do not reflect actually encapsulated DNA.

EX2007 (Janoff) 4:15-19. Dr. Janoff's statements about full encapsulation are remarkably similar to how the term is defined in the '435 patent.

> The term "fully encapsulated" indicates that the active agent or therapeutic agent in the lipid particle is not significantly degraded after exposure to serum or a nuclease or protease assay that would significantly degrade free DNA, RNA, or protein. In a fully encapsulated system, preferably less than about 25% of the active agent or therapeutic agent in the particle is degraded in a treatment that would normally degrade 100% of free active agent or therapeutic agent, more preferably less than about 10%, and most preferably less than about 5% of the active agent or therapeutic agent in the particle is degraded.

EX1001, 23:5-15. That is, full encapsulation requires a determination that the nucleic acid is protected from nuclease degradation.

161. Dr. Janoff does not point to a nuclease degradation assay in the '554 publication — because there is no such disclosure. Instead he alleges that encapsulation finds support in paragraph 11.

> The encapsulation of anionic compounds using cationic lipids is essentially quantitative due to electrostatic interaction. In addition, it

> is believed that the cationic lipids interact with the negatively charged cell membranes initiating cellular membrane transport (Akhtar et al., 1992, Trends Cell Bio., 2, 139; Xu et al., 1996, Biochemistry 35, 5616).

EX1004 ¶11. This paragraph is describing prior art formulations and not embodiments of the '554 publication. Additionally, paragraph 11 is describing prior art cationic liposome-DNA complexes (*i.e.*, lipoplexes). I understand that Dr. Janoff asserts that lipoplexes do not encapsulate nucleic acids. EX2007, 2:54-59. ("[L]iposomal bilayers form around encapsulated nucleic acids, thereby protecting the nucleic acids from degradation by environmental nucleases; lipoplexes, by contrast, do not encapsulate nucleic acids, and hence, cannot completely sequester them away from environmental nucleases."). I do not understand why Dr. Janoff suggests paragraph 11 is relevant to the limitation of claim 13.

162. Accordingly, the '554 publication neither anticipates nor makes obvious claim 13.

> **Claim 14. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.**

163. With respect to claim 14, Dr. Janoff points to disclosure of a pharmaceutical carrier and summarily concludes that this limitation is "expressly disclosed." EX1007 ¶162. As I discussed above, claim 1 is not anticipated under any theory nor are any claims depending from claim 1.

164. To the extent that Dr. Janoff is presenting an obviousness theory, I understand that it requires a reason to combine and reasonable expectation of success — both of which are absent.

> **Claim 16. A method for the in vivo delivery of a nucleic acid, the method comprising: administering to a mammalian subject a nucleic acid-lipid particle of claim 1.**
>
> **Claim 17. A method for treating a disease or disorder in a mammalian subject in need thereof, the method comprising: administering to the mammalian subject a therapeutically effective amount of a nucleic acid-lipid particle of claim 1.**
>
> **Claim 18. The method of claim 17, wherein the disease or disorder is a viral infection.**
>
> **Claim 19. The method of claim 17, wherein the disease or disorder is a liver disease or disorder.**
>
> **Claim 20. The method of claim 17, wherein the disease or disorder is cancer.**

165. As I discussed above, the ’554 publication neither anticipates nor makes obvious the nucleic acid-lipid particles of claim 1. Therefore, any method that claims the in vivo delivery of the nucleic acid-lipid particles of claim 1 is similarly not anticipated and not obvious over the ’554 publication.

166. A person of ordinary skill in the art would not find it obvious to administer the nanoparticles of the ’554 publication to mammalian subjects in vivo. This is because a skilled artisan would have expected the disclosed nanoparticles to be too toxic for in vivo administration. More particularly, a skilled artisan would have expected DMOBA and DMLBA (see Table IV) to be toxic. A

person of ordinary skill in the art would have appreciated that the dimethylamino group on the aryl ring is a good leaving group upon protonation and, as such, has potential to alkylate cysteines and lysines on cellular proteins. Additionally, DMOBA and DMLBA would have been expected to accumulate in liver and spleen because the arylether groups make elimination through mammalian detoxification pathways more difficult. The resulting accumulation and protein modifications would result in organ toxicity rendering lipid particles using DMOBA and DMLBA inappropriate for systemic use.

167. In sum, a person of ordinary skill in the art would not have been motivated to administer the nanoparticles of the ’554 publication to mammalian subjects. And, consequently, claims 16-20 are not obvious.

X. OBJECTIVE INDICIA OF NONOBVIOUSNESS

168. The nucleic acid-lipid particle formulations of the ’435 patent solved a long-felt need for compositions that could safely and effectively deliver nucleic acids to target cells of patients. Skilled artisans were skeptical that compositions having high levels of cationic lipid (*i.e.*, 50 mol % to 85 mol %) and low levels of conjugated lipid (*i.e.*, 0.5 mol % to 2 mol %) would be effective and well-tolerated when administered in vivo. The combination of effectiveness and low toxicity that characterizes the claimed compositions surprised many in the field, including me. Finally, the unique properties of the claimed nucleic acid-lipid particle

formulations solved the delivery problem that hindered the field of siRNA drugs. Onpattro™, a first in class siRNA drug was recently approved for use in the United States and Europe and is a nucleic acid-lipid particle composition within the scope of claim 1.

169. My opinion that the challenged claims of the '435 patent are not obvious in view of the prior art, as set forth above, is not dependent on objective indicia of nonobviousness. However, such objective indicia further support the conclusion that the challenged claims would not have been obvious to a person of ordinary skill in the art at the time of the invention.

A. Long-felt need

170. In 1998, it was discovered that double-stranded RNA molecules could mediate a sequence-specific destruction of mRNA in the model organism, *C. elegans*. EX2011 (ACS) at 41. This phenomenon was dubbed RNA interference or RNAi. A few years later, RNAi was shown to work in mammalian cells but only if the size of the double-stranded RNA was limited to 21-23 nucleotides. The biomedical potential of targeted destruction mammalian mRNAs was immediately appreciated. Fire and Mello were awarded the Nobel Prize in Physiology in 2006 for their discovery of RNAi. EX2011 at 41.

171. RNAi can be used to selectively disable mRNAs in mammalian cells and thereby silence expression of a protein (*e.g.*, an aberrant protein causing

disease). However, a major challenge in bringing RNAi to the clinic was delivery of the small interfering ("siRNA") to cells. EX2011 at 38 ("[P]hysical delivery of the drugs to diseased cells is extremely challenging."). In 2003, for example, Dr. Phillip Sharp, Nobel Laureate and co-founder of Alnylam Pharmaceuticals, was asked about the challenges that lie ahead for RNAi drugs he answered "Delivery, delivery, delivery." EX2014 at 11. Dr. Phillip Zamore, co-Founder of Alnylam Pharmaceuticals, shared Dr. Sharp's concern about delivery. EX2011 at 42.

172. Delivery of RNAi to cells in vivo was widely regarded as a major challenge in the field. For instance, Dr. Anastasia Khvorova, Director of Biology at Dharmacon, acknowledged that the delivery systems developed prior to 2003 for antisense-based nucleic acid drugs were not promising for delivery of RNA drugs.

> Khvorova believes that the medical benefits of RNAi will be huge if the delivery issues can be resolved. "But we've looked at a lot of the delivery methods that have been used for antisense, and so far I haven't been impressed," she says.

EX2014 at 11.

173. The mid-2000's (*i.e.*, 2005-2008) saw dramatic growth and investment in RNAi-based therapeutics. A number of large pharmaceutical companies entered the RNAi space through acquisition of biotechnology companies that owned the intellectual property rights to various siRNA molecules, such as Merck's acquisition of Sirna Therapeutics. Yet, despite $2.5-3.5 billion in

investment, no solution for the delivery problem had been found. EX2015 at 1. In fact, all four RNAi drugs in clinical trials at this time were naked RNA — that is, the siRNA was administered without a delivery vehicle. None of those four RNAi drugs in clinical trial were ultimately successful.

174. By 2008, the industry-wide failure to identify a solution to the delivery challenge resulted in waning confidence that RNAi could deliver on its therapeutic promise. The large pharmaceutical companies that had entered the RNAi space just a few years prior, began searching for delivery solutions by acquiring nascent delivery technologies and developing in-house delivery programs. EX2015 at 2 ("Big Pharma quickly realized the mistake of putting IP before enablement as they scrambled to scout for delivery technologies and found the majority of them not to live up to their claims."), 10 ("Big Pharma may also have relied too much on assurances by pure-play companies that delivery technologies were more mature than they really were."). Much of the delivery technologies identified around this time were inferior to Patent Owner's SNALP technology. EX2015 at 10 ("It is interesting for example that Roche's Factor VII patent application (WO 2010/055041) features Alnylam's 'lipidoid' technology for the rodent studies, but then switched to Tekmira's SNALP liposomes for the nonhuman primate part of the patent application.").

175. Prior to the first publication of the '435 patent disclosure, Alan Sachs, leader of RNA Therapeutics at Merck, identified delivery as the challenge to successfully developing RNAi drugs.

> I have no doubt that RNAi, if it hasn't already, will absolutely demonstrate efficacy. It's an incredible drug. What's interesting about what we do is that the drug isn't the problem. It's the delivery of it.

EX2016 at 7.

176. Dr. Sachs indicated that while there were a large number of delivery technologies available in mid-2009, few had been tested systemically in a relevant animal model.

> We have a graph we've disclosed which represents the number of opportunities we have looked at to do exactly what you describe, which is collaborate, particularly in the delivery space to advance this field. We are fully funded to do that, not just the evaluation, but the actual work. And what's really disappointing is that when you look at that graph, which is current as of mid-2009, there were 250-260 interesting opportunities, and there are really only two or three which have data that's valuable—meaning they have data from non-human primates.

EX2016 at 4. As explanation for the lack of viable delivery chemistries, Dr. Sachs elaborated on the nature of the challenge.

> There's a lot of hype, and there's a lot of ideas. But it's not a straightforward problem. Injecting something in the bloodstream, leading to something appearing in the cytoplasm in the RNA-silencing

> complex, there are a lot of black boxes between those two steps. People who are entering the field start with a white paper. It's much like people who started on targeted therapeutics years ago started with a white paper. If it were so easy, one would have to describe why so few examples exist. The same is true in the RNAi delivery process. You can write down the steps. You can write down what you think will happen. But then you have to put it in a 50-nanometer particle that's safe and potent to deliver.

Id.

177. The long-felt need for a siRNA delivery vehicle and the difficulty in finding a solution is further exemplified by the effort and money that Patent Owner invested into SNALP technology.

> [I]t took Tekmira 500 person-years and over US$200M to turn a single technology, SNALP, into the prolific drug development engine it is today.

EX2015 at 8.

178. Prior to the nucleic acid-lipid particles disclosed in the '435 patent, there were no proven solutions to the delivery problem.

B. Skepticism

179. It was widely believed that cationic lipid content should be minimized to avoid cytotoxicity, aggregation, and unwanted interactions with the immune system and non-target cells.

180. For example, Dr. Zamore of Alnylam, acknowledged that various cationic lipid formulations could successfully deliver siRNA to cells in vitro but stated that "I wouldn't want anyone injecting cationic lipids into my bloodstream." EX2011 at 42.

181. Dr. Sachs of Merck also expressed skepticism as to the safety of the SNALP platform for delivery of siRNA.

> First are lipid-based delivery systems. At the time of our acquisition of Sirna, they had successfully shown lipid-based delivery to the liver. Initially, it was through a collaboration with what is now called [Vancouver, BC-based] Tekmira. That was really the leading standard for the area. Several [applications to begin clinical trials] have been filed with the FDA. We spent a lot of internal research money and time on novel lipids. ***The liability of that platform is absolutely its safety***.

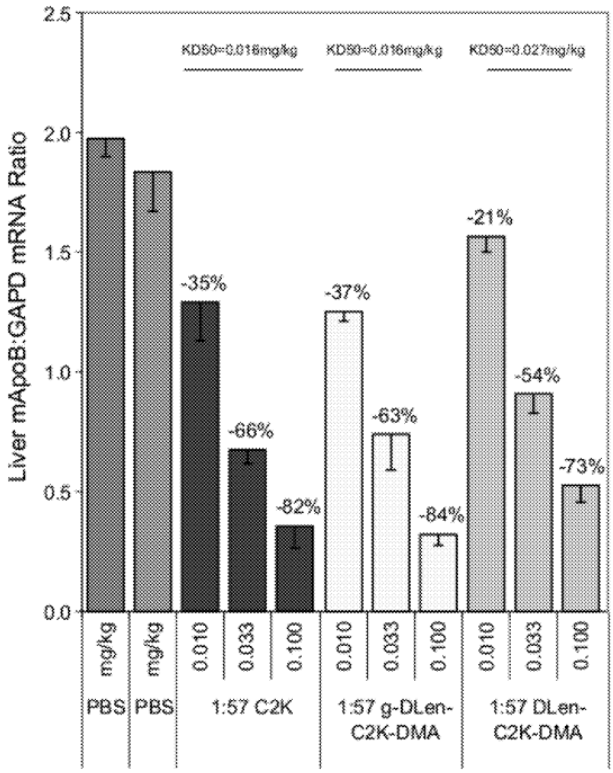
EX2016 at 5.

C. Unexpected Results

182. It is surprising and entirely unexpected that the first FDA approved RNAi drug to meet the long-felt need for a delivery vehicle for RNAi drugs was one that does not minimize the cationic lipid component. Even more surprisingly the claimed formulations have a low level of conjugated lipid.

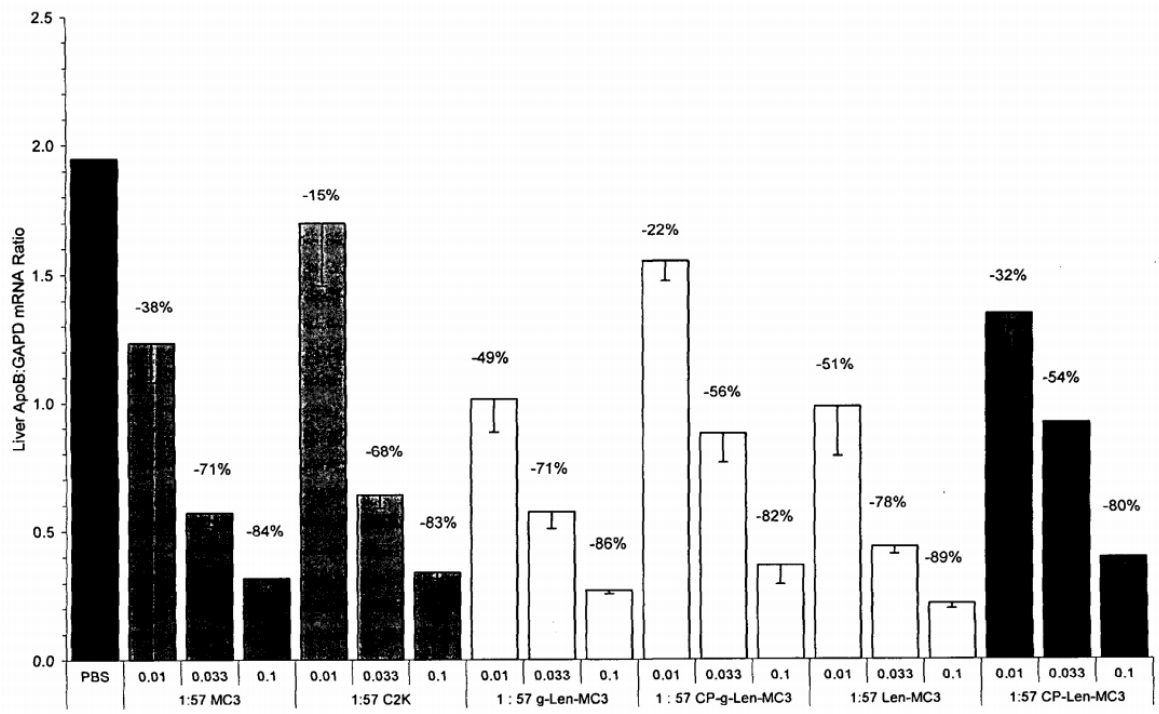
183. Patent Owner's patents and publications that were published after the filing date of the '435 patent provide data for additional formulations within the scope of claim 1.

184. For example, in U.S. Patent No. 8,236,943 (the "'943 patent," EX2017) tested several 1:57 formulations. Specifically, the '943 patent tested 1:57 formulations that used C2K, g-DLen-C2K-DMA, or DLen-C2K-DMA as the cationic lipid. Figure 7 depicts levels of gene silencing obtained after systemic administration of 0.010, 0.033, or 0.100 mg/kg of the various nucleic acid-lipid particle formulations. EX2017, 153:45-47.



EX2017, Figure 7. Each formulation exhibited gene silencing activity far superior to that of the control PBS group. Further, the "SNALP formulation containing g-DLen-C2K-DMA displayed similar ApoB silencing activity at all three doses" and "the SNALP formulation containing DLen-C2K-DMA displayed considerable potency in silencing ApoB mRNA expression." EX2017, 153:45-55.

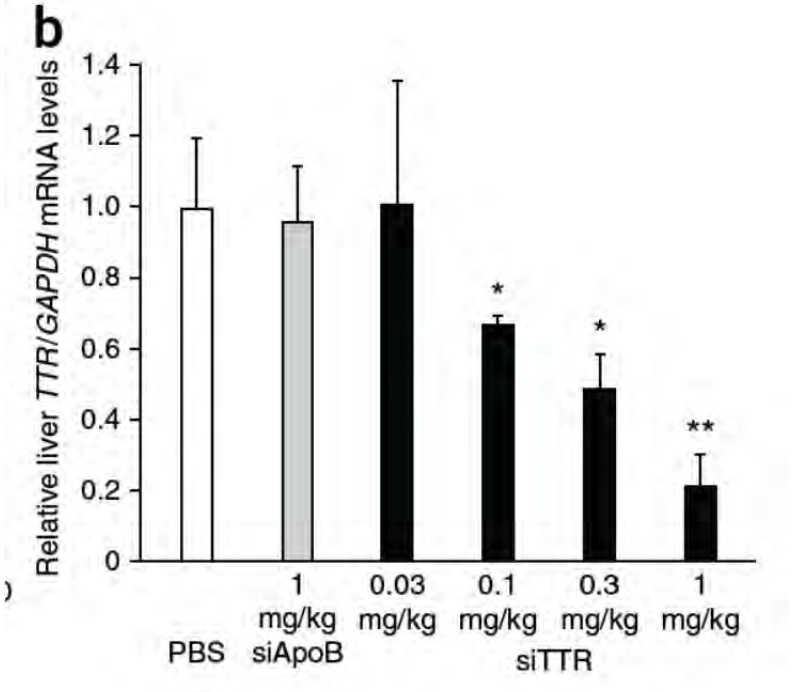
185. U.S. Publication No. 2013/0116307 ("'307 publication," EX2018) also tested 1:57 formulations. Specifically, the '307 publication tested 1:57 formulations that used MC3, C2K, g-Len-MC3, CP-g-Len-MC3, Len-MC3, and CP-Len-MC3 as the cationic lipid.



EX2018, Figure 5. Figure 5 depicts levels of gene silencing obtained after systemic administration of 0.010, 0.033, or 0.100 mg/kg of the various nucleic acid-lipid

particle formulations. EX2018 ¶439. Each formulation exhibited gene silencing activity far superior to that of the control PBS group. Further, the '307 publication explains that "a SNALP formulation containing either CP-γ-LenMC3 or CP-LenMC3 displayed similar ApoB silencing activity compared to a SNALP formulation containing the C2K benchmark cationic lipid at all three doses." EX2018 ¶439.

186. Yet more 1:57 formulations were disclosed in Semple et al., *Rational Design of Cationic Lipids for siRNA Delivery*, 28 Nature Biotechnology 172-178 (2010) ("Semple," EX2021). Specifically, Semple tested 1:57 formulations that used KC2 as the cationic lipid. EX2021 at 177. Semple measured gene silencing following systemic administration in non-human primates. EX2021 at 174.



EX2021, Figure 3b. Semple explains that "[a] clear dose response was obtained with an apparent ED_{50} of ~0.3 mg/kg." EX2021 at 175. Further, "toxicological analysis indicated that the treatment was well tolerated at the dose levels tested, with no treatment-related changes in animal appearance or behavior." EX2021 at 175; *see also* id. ("Clinical signs were observed daily and body weights, serum chemistry and hematology parameters were measured 72 h after dosing. KC2-SNALP was very well tolerated at the high dose levels examined (relative to the observed ED_{50} dose) with no dose-dependent, clinically significant changes in key serum chemistry or hematology parameters."); EX2022 [Supplementary Materials Doc], Table 4.

187. U.S. Publication No. 2017/0307608 to Bettencourt ("'608 publication," EX2019) discloses testing of a 1:50 formulation. The '608 publication is directed to the commercial product, Onpattro™ (*i.e.*, patisiran). The patisiran formulation is disclosed in Table 1. EX2019 ¶46, Table 1. Converting to mol % yields the following:

	DLin-MC3-DMA	**PEG-c-DMG**	**DSPC**	**Cholesterol**
mg	12.7	1.5	3.1	5.9
mol %	50	1.5	10	38.5

That is, patisiran is encompassed by claim 1.

188. The ’608 publication discloses the testing of patisiran in human subjects. As with the testing of 1:57 formulations in mice and non-human primates, patisiran was well tolerated in humans. EX2019 ¶103 (“The use of patisiran did not result in any significant changes in hematologic, liver, or renal measurements or in thyroid function, and there were no drug-related serious adverse events or any study-drug discontinuations because of adverse events.”). Patisiran effectively silenced expression of its target — the TTR protein. EX2019 ¶121. (“[T]reatment of patients with FAP with patisiran led to robust, dose-dependent, and statistically significant knockdown of serum TTR protein levels.”). The disclosed study found clinical benefit to treatment of patients with patisiran. EX2019 ¶132.

189. A person of ordinary skill in the art would understand that the unexpected results of the ’435 patent are not limited to formulations comprising specific ratios of components or comprising a specific type of cationic lipid. Specifically, between the ’435 patent and subsequent publications, nucleic acid-lipid particle formulations with cationic lipid in the range of 50 mol % to 70 mol % and conjugated lipid in the range of 1.2 mol % to 2 mol % were tested and found to be efficacious and well tolerated. Additionally, nucleic acid-lipid particle formulations with eight different cationic lipids were tested and found to be efficacious and well tolerated. Finally, nucleic acid-lipid particle formulations within the scope of the claims were found efficacious and well tolerated in non-

human primates and humans. These data span nearly the entire claimed ranges of cationic and conjugated lipid and are entirely unexpected.

D. Commercial Success

190. The nucleic acid-lipid particles claimed by the ’435 patent have achieved tremendous commercial success. Patisiran — tradename “Onpattro” — is a first in class siRNA drug. *See, e.g.*, EX2023 (Patisiran Nature News)at 291 (“US regulators have approved the first therapy based on RNA interference (RNAi), a technique that can be used to silence specific genes linked to disease.”), (“‘This approval is key for the RNAi field,’ says James Cardia, head of business development at RXi Pharmaceuticals in Marlborough, Massachusetts, which is developing RNAi treatments. ‘This is transformational.’”); EX2024 (FDA Release) (“FDA approves first-of-its kind targeted RNA-based therapy to treat a rare disease.”); EX2025 (EMA Release) (“The European Medicines Agency's (EMA) Committee for Medicinal Products for Human Use (CHMP) has recommended granting a marketing authorisation for Onpattro (patisiran), …”). . Patisiran received regulatory approval in Europe on July 28, 2018 and the United States on August 10, 2018. *Id*.

191. Patisiran was developed by Alnylam Pharmaceuticals under license from Arbutus. Specifically, Alnylam licenses the nucleic acid-lipid particle technology claimed in the ’435 patent from Arbutus. EX2026 (Arbutus press

release). Under the license, Alnylam owes Arbutus royalties on the patisiran product. *Id*.

192. Patisiran is encompassed by claim 1 of the '435 patent. EX2019, Table 1.

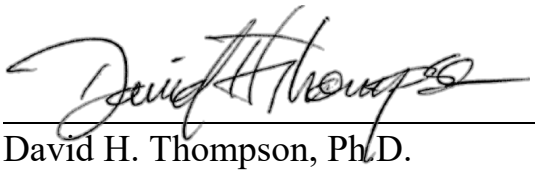
	Nucleic acid	**Cationic lipid**	**Non-cationic lipid**		**Conjugated lipid**
	siRNA-TTR	**DLin-MC3-DMA**	**Cholesterol**	**DSPC**	**PEG-c-DMG**
mg	2.0	12.7	5.9	3.1	1.5
mol %		50	38.5	10	1.5

193. The '435 patent is one of the patents that encompasses the patisiran commercial product. EX2027.

XI. CONCLUDING STATEMENTS

194. In signing this declaration, I understand that the declaration will be filed as evidence in a contested case before the Patent Trial and Appeal Board of the United States Patent and Trademark Office. I acknowledge that I may be subject to cross-examination in this case and that cross-examination will take place within the United States. If cross-examination is required of me, I will appear for cross-examination within the United States during the time allotted for cross-examination.

195. I declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Dated: December 21, 2018 By: 

David H. Thompson, Ph.D.

JOINT APPENDIX 68

Paper No. ___
Filed: December 21, 2018

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

MODERNA THERAPEUTICS, INC.,
Petitioner,

v.

PROTIVA BIOTHERAPEUTICS, INC.,
Patent Owner.

Case IPR2018-00739
Patent No. 9,364,435

PATENT OWNER'S RESPONSE PURSUANT TO 37 C.F.R. § 42.120

TABLE OF CONTENTS

I. STATEMENT OF PRECISE RELIEF REQUESTED

Moderna Therapeutics, Inc. ("Petitioner") filed a petition for *inter partes* review of claims 1-20 of U.S. Patent No. 9,364,435 (the "'435 patent," EX1001). The Board issued its decision instituting trial (Paper 15) on all grounds set forth in the petition. Protiva Biotherapeutics, Inc. ("Patent Owner") hereby requests that the Board now issue a final written decision confirming that claims 1-20 are not unpatentable.

II. INTRODUCTION

The nucleic acid-lipid particles claimed by the '435 patent have achieved tremendous recognition in the field of genetic therapy. The '435 patent is now listed in FDA's Orange Book as protecting the patisiran—tradename "Onpattro"—commercial product. EX2027. Patisiran received regulatory approval in the U.S. and Europe and has been designated by the FDA as a "first-in-class" drug. EX2024.

The therapeutic potential of genetic therapy has been appreciated for over 25 years, but effectively delivering nucleic acids to target cells without eliciting vehicle-related toxicity has prevented realization of this potential. *See e.g.*, EX2011 at 38, 42; EX2014 at 11. By 2008, the industry-wide failure to identify a solution to the delivery problem resulted in waning confidence. EX2015 at 2, 10. Dr. Phillip Sharp, Nobel Laureate and co-founder of Alnylam Pharmaceuticals, was asked about the challenges that lie ahead for RNAi drugs he answered "Delivery, delivery,

delivery." EX2014 at 11; *see also* EX2016, Title of Article ("Merck's Alan Sachs, on RNAi's Big Challenge: Delivery, Delivery, Delivery"); EX2011 at 42 ("Delivery, delivery, delivery.").

The nucleic acid-lipid particle formulations of the '435 patent solved a long-felt need for compositions that could safely and effectively deliver nucleic acids to target cells of patients. Skilled artisans were skeptical that compositions having high levels of cationic lipid (i.e., 50 mol % to 85 mol %) and low levels of conjugated lipid (i.e., 0.5 mol % to 2 mol %) would be effective, let alone well-tolerated when administered *in vivo*. The combination of effectiveness and low toxicity that characterizes the claimed compositions surprised many in the field, and finally solved the delivery problem that hindered the field for decades.

Given the innovation protected by the '435 patent, the petition is a poorly conceived challenge. It seeks troubling shortcuts rather than providing any *bona fide* obviousness analysis addressing motivation to combine and reasonable expectation of success in view of the state of the art at the time. In numerous instances, the petition fails to coherently identify the specific invalidity theories on which its challenge is based.

The obviousness challenges of Grounds 1 and 3 argue for a *prima facie* case of obviousness on a per-limitation basis for what it contends are overlapping ranges of individual claim elements. Petitioner then rests on its putative "*prima facie*" case

as though that alone meets its ultimate burden of proof. But Petitioner fails to address the claimed subject matter as a whole (i.e., the claimed particle formulation), as mandated by statute, instead repeatedly arguing only that each "limitation is prima facie obvious." Analysis of whether there would have been a motivation to combine or any reasonable expectation of success is wholly absent from the petition.

Moreover, the petition provides no meaningful analysis of the full scope of experimental data presented in the '435 patent itself. Instead, Petitioner incompletely addresses a subset, never explains what would be expected/unexpected, and operates on the erroneous assumption that comprehensive superiority relative to the prior art is necessary. Patent Owner's expert, Dr. David Thompson, explains that the experimental results reported in the '435 patent (as well as numerous post-filing publications) demonstrate that the claimed formulations are surprisingly well-tolerated and efficacious at far lower dosages than prior art compositions.

Ground 3 does not work any better as an anticipation argument. While the challenged claims are directed to a nucleic acid-lipid particle, the petition relies on disclosure in the reference of a lipid mixture used to make particles. As confirmed by Petitioner's own expert, the petition has wrongly assumed that the lipid mixture in a starting solution would have the same molar ratio of components as the resulting

particle. *See e.g.*, 2028, 155:2-25; 156:1-158:16; *see also* EX2012 at 7242; EX2013 at 3.

Ground 2 is an undeveloped attempt to backfill Ground 1. The petition incompletely addresses the challenged claims (addressing only claim element "1(b)" and claim 4), ignores the claimed subject matter as a whole, and lacks explanation as to whether there would be any reasonable expectation of success. The later point is particularly pertinent in this instance, as Petitioner cites to the references of "Lin and/or Ahmad"—both of which are addressing "lipoplex" formulations, a fundamentally different type of lipid delivery system. Petitioner's own expert has characterized lipoplexes as fundamentally distinct, both during cross-examination and in his previous publications. EX2028, 122:18-24; EX2007, 2:27-40.

For these reasons, and those explained in further detail herein, the petition challenges should be rejected.

III. DR. JANOFF'S DECLARATION IS ENTITLED NO WEIGHT

The Declaration of Dr. Janoff submitted with the petition (EX1007) should be accorded no weight for at least the reasons set forth below.

First, Dr. Janoff's declaration merely adopts the attorney arguments set forth in the petition and should be weighted accordingly. The direct testimony itself characterizes the declaration as such, where Dr. Janoff repeatedly states his opinions "are based on the petition." EX1007, ¶27 ("My opinion[s] expressed in this

declaration are based on the Petition ...."), ¶¶5-7. During cross-examination, Dr. Janoff confirmed that his direct testimony was based on the petition. EX2028, 93:10-11 ("There is a petition. I based my opinions on the petition"), 91:18-92:20, 92:21-93:11, 26:12-27:5.[1]

Attorney argument is not elevated to testimonial evidence simply by virtue of being parroted by a witness. *E.g. InfoBionic, Inc. v. Braemar Manufacturing*, LLC, IPR2015-01704, Paper 11; *Elbit Sys. of Am., LLC v. Thales Visionix, Inc.*, 881 F.3d 1354, 1359 (Fed. Cir. 2018)("'Attorney argument is not evidence'")(quoting *Icon Health & Fitness, Inc. v. Strava, Inc.*, 849F.3d 1034, 1043 (Fed. Cir. 2017)); *Estee Lauder Inc. v. L'Oreal, S.A.*, 129 F.3d 588, 595 (Fed. Cir. 1997).

Second, conduct by both the witness and counsel during cross-examination of Dr. Janoff should give the Board considerable pause in crediting any direct testimony of the witness. Petitioner's counsel, for instance, interjected to accuse the questioning lawyer of misstating the witness' testimony over 20 times. *E.g.*, EX2028, 32:11-2, 35:1-2, 43:13-14, 49:11-12, 119:5-17, 148:18-149:5, 170:11-12. Petitioner's counsel repeatedly interrupted questioning by instructing the witness to review certain documents prior to answering the question pending. *E.g.*, *id.*, 42:7-17,

[1] Dr. Janoff demonstrated a general unfamiliarity with the petition materials throughout the deposition. *E.g.* EX2028, 31:9-32:25 (unable to recall using the term "lipid particle"); 90:3-91:8 (seemingly unable to recognize the petition).

50:23-51:1, 101:4-15, 151:8-9. Improper witness coaching was discussed multiple times. *E.g.*, *id*., 41:9-42:17, 48:13-25, 101:15, 138:20-139:25. Questions were objected to as allegedly "vague" more than 40 times. *E.g.*, *id*., 28:5, 32:11, 35:9, 36:22, 45:19, 57:1, 58:4, 76:15, 85:11, 104:17, 126:22-23, 138:12-18, 146:6-18, 157:18-19; Trial Practice Guide, 77 Fed. Reg. 78755, 48772 (Aug. 14, 2012) (identifying "Objection, vague" as an improper objection).

Such conduct was disruptive and prejudicial as it often prompted recalcitrance from the witness and, in some instances, refusal to answer straightforward questions about terms or statements in Dr. Janoff's own documents. For example, taking cue from the defending lawyer, Dr. Janoff repeatedly protested about questions being "vague" or lacking context. EX2028, 35:7-19, 45:5-22, 29:19-30:11 ("it's a vague, vague, vague question."), 56:21-57:25, 62:17-65:17 (refusing to answer questions about experience working with liposomes at "The Liposome Company, Inc." as too vague to understand).

Dr. Janoff refused to clarify terms and statements made in his declaration. *Id*., 33:6-14 ("lipid particle" is too vague to understand without context); *cf*. EX1007¶¶32, 75; EX2028, 34:7-35:25 ("therapeutic payload" is too vague), 36:1-37:5; *cf*. EX1007, ¶32; EX2028, 86:6-87:1 (when asked "what a PEG is, the abbreviation P-E-G," testifying that "a peg is something that I could hammer into a piece of wood.").

Dr. Janoff refused to answer simple questions regarding statements in his own patent (U.S. Patent No. 7,491,409 – "the '409 patent," EX2007). *E.g.*, EX2028, 138:12-139-23 (refusing to answer whether he is familiar with the concept of nucleic acids being encapsulated in a liposome); *cf.* EX2007, 3:1-2 ("the concept of encapsulating bioactive agents in liposomes is not new"); EX2028, 136:5-138:11; 145:15-147:17; *see generally id.*, 133:9-151:20.

The impropriety of the conduct was not lost on the witness. EX2028, 36:24-25 ("I'm not trying to be rebarbative here."), 57:11-13, 123:19-20, 144:19-20, 143:23-145:5 (testifying that questions demonstrate "ignorance" on the part of Patent Owner's lawyer).

Such conduct is particularly prejudicial in the context of the present proceeding, as Patent Owner has repeatedly raised issues as to the lack of clarity in the petition materials. *E.g.*, POPR at 5, 9-10, 24, 26; EX2008, 7:9-9:8. Accordingly, giving no weight to Dr. Janoff's direct testimony is appropriate under the present circumstances where the petitioner and the witness have frustrated the discovery process and declined to provide clarity when asked. Furthermore, given the extraordinary actions to frustrate the discovery process and preclude a fair examination of the witness in the present case, any disputed issue of material fact should be resolved in favor of Patent Owner. *See e.g.*, 37 C.F.R. §42.12(a)(5) and (b)(1); Office Trial Practice Guide at 48772; *California Institute of Technology v.*

Enzo Life Sciences, Inc., Patent Interference No. 105,496, Paper 117, 5-6 (March 30, 2010) (giving no weight to the expert's direct testimony and resolving issues of fact in favor of the non-offending party an appropriate remedy for violating cross-examination guidelines).

IV. STATE OF THE ART

An objective of genetic therapy was the development of drugs — that is, nucleic acids — to treat systemic diseases such as cancer, inflammation, virus infection, and cardiovascular disease. Delivery of nucleic acids has been particularly challenging because they are large, negatively charged molecules that cannot simply be given to a patient systemically (e.g., intravenously) and allowed to passively enter cells, as would be the case with many small molecule drugs. Therapeutic nucleic acids require an effective delivery vehicle, which has proved to present considerable technical obstacles. *See, e.g.*, EX2014 ("The major hurdle right now is delivery, delivery, delivery," said Sharp in 2003); EX2014 at 11 (stating in 2003, "Khvorova believes that the medical benefits of RNAi will be huge if the delivery issues can be resolved."); *see also* EX2009 ¶25.

The first generation of nucleic acid delivery systems that were developed included cationic liposome nucleic acid complexes (also known as liposomes). *See* EX1002 ¶8; EX2007, 2:27-40, 2:57-67. Lipoplexes were found to be unsuitable for

many applications, particularly systemic uses, due in large part to the toxic nature of the cationic lipids. *See, e.g.*, EX1008 at 5; *see also* EX2009 ¶26.

The toxicity of cationic lipids occurs at the cellular and organ levels, as these lipids are often not readily biodegradable and accumulate to cytotoxic concentrations in the liver and spleen. Cationic lipids also have immunostimulatory capacity and are associated with immunogenic and inflammatory responses. The obstacles to using cationic lipids as a nucleic acid delivery vehicle posed by, e.g., toxicity, immunogenicity, and aggregation, were well known prior to 2008. As one industry executive stated, "I wouldn't want anyone injecting cationic lipids into my bloodstream." EX1004 ¶136; EX1006 at 7; EX1008 at 5-6, 9; EX2011 at 42; *see also* EX2009 ¶¶28-35.

V. PERSON OF ORDINARY SKILL IN THE ART

It is well-settled that an "invention must be evaluated not through the eyes of the inventor, who may have been of exceptional skill, but as by one of 'ordinary skill.'" *Interconnect Planning Corp. v. Feil*, 774 F.2d 1132, 1138 (Fed. Cir. 1985).

The level of skill of the ordinary artisan upon which the petition materials are based is incorrect for a number of reasons. First, the petition equates the level of skill of the ordinary artisan with the level of skill of the inventors of the '435 patent. This is indicative of impermissible hindsight. EX1007, ¶31; EX2028, 44:9-12 (putting level of ordinary skill as that of the inventors of the patent). Thus, the petition has

improperly assumed a much higher level of skill than that of a person of ordinary skill in the art ("POSITA"). *See also* EX2009, ¶¶22-24.

Second, Petitioner's definition of a POSITA is ultimately indeterminable. The petition defines a POSITA as someone that "would have specific experience with lipid particle formation and use in the context of delivering therapeutic payloads." Pet. 5-6; EX1007, ¶31. During cross-examination, Dr. Janoff was unable (or unwilling) to clarify what this means, repeatedly indicating that Petitioner's own definition is "too vague" to understand. EX2028, 33:6-11, 34:7-35:25, 38:20-39:9; *see also id*., 36:1-37:5; *see generally* EX2028, 27:6-43:8.

Petitioner's definition of a POSITA, at a minimum, taints the obviousness arguments in the petition as using an incorrect and erroneous perspective. Obviousness must be assessed from the perspective of "a person having ordinary skill in the art" at the time the invention was made. 35 U.S.C. §103(a) (2012); *see generally Graham v. John Deere Co.*, 383 U.S. 1, 17-18 (1966). Because the petition sets the level much higher, to that of the inventors, Petitioner has failed to conduct an appropriate analysis. It is axiomatic that Petitioner has not met its burden of demonstrating obviousness where the threshold question of how a person of skill is defined—a fundamental factual basis for any corresponding analysis—is, by its expert's own admission, indeterminable. *Id*. at 17; *see also* EX2009 ¶¶23-24.

VI. CLAIM CONSTRUCTION

A claim subject to *inter partes* review receives the broadest reasonable construction or interpretation in light of the specification of the patent in which it appears ("BRI"). *See* 37 C.F.R. § 42.100(b). However, the Board may not construe a term "so broadly that its constructions are unreasonable under general claim construction principles." *Microsoft Corp. v. Proxyconn, Inc.*, 789 F.3d 1292, 1298 (Fed. Cir. 2015).

The petition was based on an overbroad construction of "nucleic acid-lipid particle." Pet. 24. The Board rejected Petitioner's construction and offered one of its own — that is, "nucleic acid-lipid particle" is "a particle that comprises a nucleic acid and lipids, in which the nucleic acid may be encapsulated in the lipid portion of the particle." Paper 15 at 10-11 (citing EX1001, 11:14–22). According to Dr. Thompson, both of these constructions are unreasonably broad, at least to the extent they encompass lipid particles lacking any encapsulated nucleic acid. *See also* EX2009 ¶¶39-40.

A "nucleic acid-lipid particle" expressly includes a nucleic acid. According to the '435 patent, "nucleic acids, when present in the lipid particles of the present invention, are resistant in aqueous solution to degradation with a nuclease." EX1001, 11:51-54. The '435 patent describes nucleic acid encapsulation in the lipid particle as conferring resistance to such enzymatic degradation. EX1001, 11:20-22;

see also EX2007, 4:15-19; 22:40-47; 23:1-3; 23:27-29; 26:35-37. A "lipid particle" "*may* [include a nucleic acid] encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation." EX1001, 11:14–22. A "nucleic acid-lipid particle," however, *does* include a nucleic acid encapsulated in the lipid portion of the particle, thereby protecting it from enzymatic degradation. EX1001, 11:23-31, 11:51-54; *see also* EX2009 ¶39.

Petitioner's expert testified multiple times that the lipid particles as claimed are defined as SNALPs. *See, e.g.*, EX2028, 118:18-119:4, 119:9-17, 120:5-6, 121:14-25. He cited to a provision of U.S. Patent No. 9,404,127 ("the '127 patent," EX2029) at 5:15-22 that is identically recited in the '435 patent. *Compare* EX2029, 5:15-22 *with* EX1001, 19:19-26. Indeed, the '435 patent specification repeatedly identifies SNALPs as the invention for delivering a nucleic acid payload. *See e.g.*, EX1001, 3:9-13 ("The present invention provides novel, serum-stable lipid particles …."), 47:23-24 ("[T]he lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP)…"), 3:32-37, 14:20-25; *see also* EX2009 ¶¶41-42.

According to Dr. Thompson, a fair and reasonable reading of the '435 patent specification supports Dr. Janoff's position in that there is no meaningful distinction between the '435 patent specification's descriptions of a "lipid particle" containing a nucleic acid (*i.e.*, a nucleic acid-lipid particle) and particle characteristics that confer

serum stability. *Compare* EX1001, 11:14-22, 11:51-54 *with* 13:32-37. ("'Serum-stable' in relation to nucleic acid-lipid particles such as SNALP means that the particle is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA"); *see also* EX2009 ¶43.

It is Dr. Thompson's opinion that a narrow focus on a linguistic difference between a nucleic acid-lipid particle and the term "SNALP" is misguided and risks overlooking pertinent disclosure and context provided in the '435 patent. For example, the '435 patent specification states that "nucleic acids, ***when present in the lipid particles of the invention***, are resistant in aqueous solution to degradation with a nuclease." *Id*., 11:51-54 (emphasis added). Such physical properties of the particles providing nuclease degradation resistance, or encapsulation of the nucleic acid, are also as described in the '435 patent specification as conferring the identified serum stability. *Id*., 13:32-37. This is certainly true if the claimed particles are SNALP, as supported not only by the specification of the '435 but as affirmed by petitioner's expert. EX2009 ¶44.

Regardless of whether the Board construes "nucleic acid-lipid particle" as a SNALP as indicated by Petitioner's expert; as a lipid particle with an encapsulated nucleic acid (thereby protecting it from enzymatic degradation); or under the broad construction presented in the Institution Decision, the petition fails to establish the unpatentability of claims 1-20. EX2009 ¶¶45-46.

VII. RESPONSE TO GROUND 1

The claimed invention is a nucleic acid-lipid particle comprised of relatively high levels of cationic lipids and low levels of conjugated lipids. This combination was counter-intuitive to the then-existing state of the art, as cationic lipids were known to be cytotoxic, systemically toxic, to elicit an adverse complement-mediated immune response, and to cause particle aggregation that resulted in rapid clearance. Conjugated lipids were used to shield the cationic lipids from interacting with negatively charged serum proteins and thereby diminish the adverse effects. Thus, when employing an increased amount of cationic lipid, a POSITA would have had every reason to correspondingly ***increase*** the amount of conjugated lipid. EX2009, ¶¶25-35. The inventors of the '435 patent did just the opposite.

A. The petition fails to address the claims as a whole

The petition separately parses the claimed amounts of cationic lipids, conjugated lipids, and non-cationic lipids from the references without regard to one another. The petition even states that individual limitations are allegedly "prima facie obvious." *See, e.g.,* Pet. 34 ("this limitation is prima facie obvious"), 39 (same), 40 (same). Missing from Ground 1 is a showing that the challenged claims as a whole are obvious. *In re Kahn*, 441 F.3d 977, 986 (Fed. Cir. 2006) ("[M]ere identification in the prior art of each element is insufficient to defeat the patentability of the combined subject matter as a whole."); *In re NTP, Inc.*, 654 F.3d 1279, 1299

(Fed. Cir. 2011). Indeed, as the statute itself states: "if the differences between the subject matter sought to be patented and the prior art are such that the ***subject matter as a whole*** would have been obvious." 35 U.S.C. § 103(a) (2012) (emphasis added); *see generally Graham*, 383 U.S. at 17-18; *TriVascular, Inc. v. Samuels*, 812 F.3d 1056, 1066 (Fed. Cir. 2016).

The petition fails to address the fact that the concentrations of different lipid components are highly interdependent. *See* EX2009, ¶¶57-59. At best, the petition attempts to show that some prior art formulations had high concentrations of cationic lipid. However, these also use a high concentration of conjugated lipid, or are not nucleic acid-lipid particles. *See also* Section VII.C.,D..

B. The prior art does not teach each and every claim element

The disclosure and ranges relied upon in the petition do not actually overlap with what is claimed. Based on this, the petition fails to show that the prior art taught each and every claim element, and for this reason alone, Ground 1 fails. *E.g.*, *In re Royka*, 490 F.2d 981, 983-85 (CCPA 1974); *see also* EX2009, ¶¶50-55.

1. The petition fails to identify a range for conjugated lipid

Claim 1 recites in part, "a conjugated lipid that inhibits aggregation of particles comprising from 0.5 mol % to 2 mol % of the total lipid present in the particle." The petition represents that the '196 PCT teaches conjugated lipids at concentrations of 0.5% to 25%. Pet. 39. Rather than identifying disclosure in the

'196 PCT disclosing a conjugated lipid range, the petition cites to a range provided for "a bilayer stabilizing component." Pet. 39[2]; EX1007 ¶117; *see also* EX2009, ¶¶51-54.

The '196 PCT makes clear that a "bilayer stabilizing component" is not the same as a "conjugated lipid that inhibits aggregation of particles." *See* EX1002 ¶92. Bilayer stabilizing components include a broad class of structurally and chemically diverse molecules. As Dr. Thompson explains, numerous bilayer stabilizing components (e.g., polyamide oligomers, peptides, proteins, detergents, and lipid-derivatives) would not be considered a conjugated lipid. EX2009, ¶¶52-53.

While the '196 PCT lists a general range for the bilayer stabilizing component category, according to Dr. Thompson, a POSITA would not have interpreted the stated range (e.g., 0.5% to 25%) as being applicable to each listed bilayer stabilizing component example. For example, a POSITA would have appreciated that if the bilayer stabilizing component were a detergent, 25% would have been an unreasonably high level. At this concentration of detergent, the lipids would be solubilized and would no longer be in particle form. EX2009, ¶¶52-53.

[2] The petition seemingly acknowledges the lack of disclosure by arriving at the "teaching" in the '196 PCT through splicing together isolated phrases from two paragraphs. *Compare* Pet. 39 *with* EX1002 ¶¶92-93.

Moreover, while the '196 PCT discloses exemplary compositions with conjugated lipid, all have much higher levels of conjugated lipid than is claimed. EX1002 ¶¶216, 223, 228, 232 (disclosing nucleic acid-lipid particle compositions with 10 mol % conjugated lipid); *see also* EX2009, ¶¶54-55.

Accordingly, the petition fails to identify in '196 PCT "a conjugated lipid that inhibits aggregation of particles" as required by claim 1. EX2009, ¶55.

2. The petition's reliance on broad ranges and non-grounds references is misplaced

Petitioner fails to demonstrate that the recited concentration of cationic lipid is taught by the asserted prior art. Specifically, claim 1 recites, "a cationic lipid comprising from 50 mol % to 85 mol % of the total lipid present in the particle."

Petitioner cited the '196 PCT's disclosure of "about 2% to about 60%" and "about 40% to about 50%" for the cationic lipid component. Pet. 33 (citing EX1002 ¶88). The '196 PCT, however, emphasizes that "for systemic delivery, the cationic lipid may comprise from about 5% to about 15% of the total lipid." EX1002, ¶88. This range does not overlap with the recited range in claim 1, and was not addressed.

The petition also cites to the '618 patent, a non-Grounds document, solely for the contention that prior art taught formulations "containing over 50% cationic lipid." *Id*. This contention highlights the petition's legally-deficient approach of identifying individual components without regard to the claimed invention as a whole. The '618 patent does not disclose any compositions that include a conjugated

lipid. *Id*. According to Dr. Thompson, a POSITA would not find the cited formulation of the '618 instructive for the development of the claimed invention. EX2009, ¶89.

C. The petition fails to articulate a reason to combine the prior art disclosures

As explained by Dr. Thompson, there would have been no reason for a POSITA to combine the different range disclosures parsed for different lipid components from the '196 PCT so as to arrive at the claimed nucleic acid-lipid particle. The petition fails to demonstrate otherwise. EX2009, ¶18.

Those in the field at the time recognized that the properties of any lipid particle are conferred not by the amount of any individual component but by the interaction of the combined components as a whole. More specifically, Dr. Thompson explained that if a POSITA were to vary one component it would then be necessary to decide which of the other components would need to be varied in order to accommodate the change in proportions of the overall composition. EX2009, ¶¶57-58; *see also* Section VII.A.

The effects of making changes to the proportion of other components in the lipid particle would be unpredictable. Such changes, even if apparently minor in nature, would not be expected to produce a functional lipid particle suitable for systemic use. According to Dr. Thompson, the idea of simply "cherry-picking" specific amounts of individual components from different formulations, or the

different ranges in the '196 PCT, when designing lipid particles is therefore something which would have made no technical sense to a POSITA. EX2009, ¶58.

The petition states that "determining the optimal proportion of cationic lipid for a given lipid combination would be a simple matter of varying the proportion using prior art methodologies." EX1007 ¶110. This is wrong. As Dr. Thompson explains, the properties of a formulation are not conferred by the amount of one single component. Properties such as lack of toxicity and efficacy are conferred by the combination of components in the entire formulation. EX2009, ¶59.

Moreover, the petition's stated reason disregards the state of the art at the time of the invention. Making non-toxic and effective nucleic acid-lipid particle formulations was not simply a matter of "varying the proportion" of cationic lipid in prior art formulations. As discussed above, the field was hindered by the lack of effective and safe nucleic acid delivery vehicles. That the field struggled for 20 years to find such a delivery vehicle speaks to the difficulty of the task. Had the solution been a matter of simply optimizing the cationic lipid proportion, it would not have taken such an enormous investment of money and time. EX2009, ¶60.

As discussed elsewhere herein, the high cationic lipid levels claimed would have been disfavored in view of well-established toxicity concerns. Moreover, Dr. Thompson explains that inclusion of a conjugated lipid in a formulation with high cationic lipid would have been expected to occur at much higher levels than claimed.

For example, conjugated lipid had been incorporated into lipid particles to help shield positive charge and reduce nonspecific interactions with blood components. Lipid particle compositions at the time typically used much higher levels of conjugated lipid than is claimed by the '435 patent, such as 10% PEG (i.e., 5- to 20-times more than the claimed formulations). For example, Doxil, the first FDA approved liposome formulation contained 5% PEG-conjugated lipid. EX2034. Likewise lipid particles for the delivery of nucleic acids commonly used 10% PEG. EX2032 at 174; EX2033 at 1021; EX1002 ¶¶216, 223, 228, 232; *see also* EX2009, ¶¶61-62.

Accordingly, a POSITA would not have been motivated based on the '196 PCT to make the claimed composition.

D. The petition fails to show any reasonable expectation of success

The petition lacks any showing of an expectation of success in making the proposed modifications to arrive at the claimed nucleic acid-lipid particle composition. For this reason alone, the petition fails to demonstrate obviousness of the challenged claims. *In re Stepan Co.*, 868 F.3d 1342, 1346 n.1 (Fed. Cir. 2017) ("Whether a rejection is based on combining disclosures from multiple references, combining multiple embodiments from a single reference, or selecting from large lists of elements in a single reference, there must be a motivation to make the combination and a reasonable expectation that such a combination would be

successful, otherwise a skilled artisan would not arrive at the claimed combination.").

According to Dr. Thompson, a POSITA would not have expected a nucleic acid-lipid particle composition with a high level of cationic lipid and a low level of conjugated lipid to be non-toxic and effective. This is because, as discussed above, the prior art taught that the cationic lipid component of lipid particles should be minimized, regardless of whether used for *in vitro* or *in vivo* purposes. For example, it was appreciated that cationic lipids are directly cytotoxic. Cationic lipids, in addition, elicit unwanted immune reactions (e.g., inflammation), off-target cellular interactions (e.g., blood cells), and aggregation. Furthermore compositions with low levels of conjugated lipid (i.e., 0.5 mol % to 2 mol %) would have been expected to result in unstable particles that aggregate and fail to effectively transfect cells. Hence the claimed nucleic acid-lipid particle would have been expected to be cytotoxic and ineffective. *See* Section VII.A; EX2009, ¶¶63-65.

Accordingly, claim 1 is not obvious in view of the '196 PCT at least because there was no expectation of success that the claimed nucleic acid-lipid particle would be non-toxic and effective. EX2009, ¶¶63-65.

E. The petition mischaracterizes and fails to address the full scope of unexpected results

Relying on repeated citations to *Peterson*, Petitioner erroneously claims that its "*prima facie*" showing shifts the burden to Patent Owner to demonstrate

unexpected results. Pet. 34. Petitioner then argues that the data in the '435 patent is "insufficient" as rebuttal because not all tested compositions were superior. *Id*. But Petitioner applies the wrong legal standard and, moreover, fails to justify its (unstated) assumption that claimed formulations were expected to work as well as those in the prior art.

A *prima facie* case of obviousness may be rebutted by showing a POSITA would have found the properties of the claimed invention "surprising or unexpected." *In re Geisler*, 116 F.3d 1465, 1469 (Fed. Cir. 1997). It is unnecessary to demonstrate that the claimed compositions are ***superior*** to those of the prior art. *Ryco, Inc. v. Ag-Bag Corp.*, 857 F.2d 1418, 1424 (Fed. Cir. 1988) ("Nothing in the patent statute requires that an invention be superior to the prior art to be patentable."). Rebuttal does not require a showing of superiority to the prior art.

Petitioner operates on the assumption that superiority of the claimed range relative to the prior art is necessary and never explains what would be "unexpected" from the perspective of a POSITA at the time. But as discussed above (*see* Section IV), the prior art expressly instructs that high-level cationic lipid compositions were expected to have poor efficacy and increased cytotoxicity and immunogenicity relative to low-level cationic lipid formulations. *See* EX1005 at 3315; EX1006 at 745; EX1008 at E96; EX2007, 30:34:41. As Dr. Thompson explains, the expectation for the claimed nucleic acid-lipid particle compositions would have been toxic

compositions unsuitable for systemic use and little, if any, efficacy. EX2009, ¶¶66-67.

Contrary to these expectations, the claimed formulations are well-tolerated and efficacious at far lower dosages than prior art compositions. EX2009, ¶68; *e.g.* EX1001, 74:1-4, Figure 3. These results are "an unexpected difference in kind that supports nonobviousness." *Allergan, Inc. v. Sandoz Inc.*, 796 F.3d 1293, 1306 (Fed. Cir. 2015).

The petition states that "[t]he ***sole basis*** for alleged novelty of the '435 patent claims is that a nucleic acid-lipid particle comprising component lipids in the claimed proportions achieves unexpected efficacy." Pet. 14 (emphasis added). Not so. The '435 patent emphasizes that the claimed formulations "are substantially non-toxic to mammals such as humans." EX1001, 6:2-5; see also EX1001, 6:26-30, 11:51, 14:40-42, 23:2-3, 47:9-18. Petitioner fails to address that the claimed nucleic acid-lipid particle compositions are substantially non-toxic and non-immunogenic. *See also* EX2009, ¶¶69-71.

Nucleic acid-lipid particle compositions of claim 1 are well-tolerated at dosages sufficient to produce a therapeutic effect. EX2009, ¶68. For example, animals administered a single systemic dose of a 1:57 composition displayed no outward signs of toxicity as measured by body weight, appearance/behavior, and platelet count. EX1001, 79:33-41, Figures 8, 9. Additional experiments

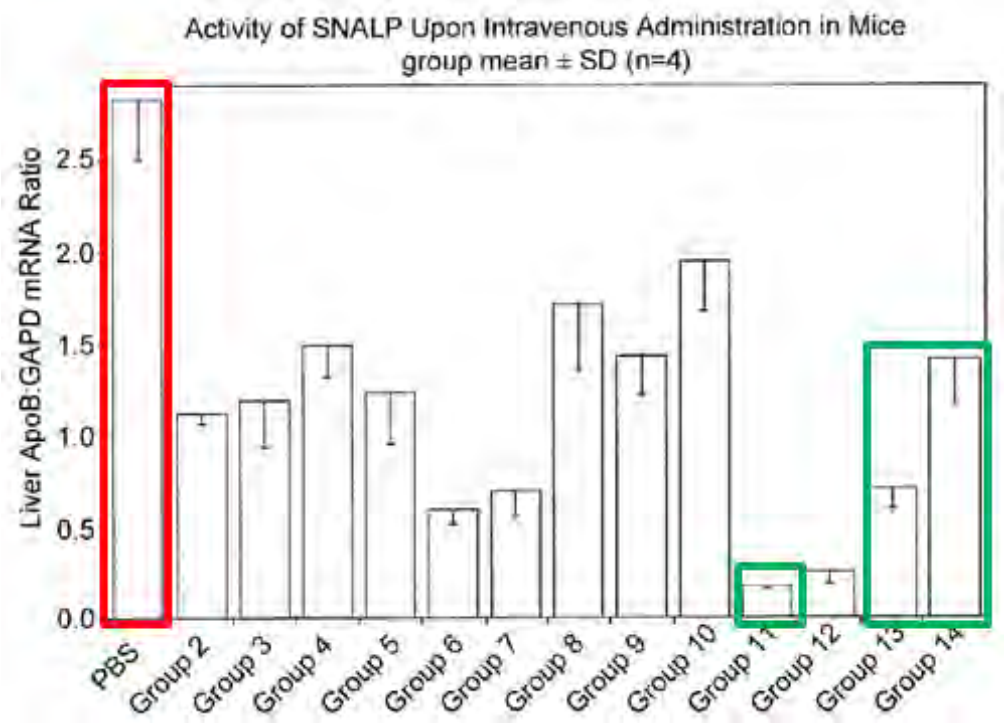
demonstrated that multiple systemic doses of a 1:57 composition produced a therapeutic effect, but no treatment related toxicity and no immune stimulation. EX1001, 81:20-82:25, 82:30-36; *see also* EX2009, ¶¶71-74.

According to Dr. Thompson, a POSITA would have expected that administering lipid particles with a high level of cationic lipid (i.e., 57 mol %) and low shielding (i.e., 1.4 mol % conjugated lipid) would have been toxic. Surprisingly, these particles are substantially non-toxic and do not cause unwanted immune reactions. *E.g.* EX2009, ¶67.

As discussed, a POSITA would have expected little to no efficacy (e.g., gene silencing) following systemic administration of the claimed nucleic acid-lipid particle compositions. However, compositions across the scope of claim 1 were tested and all induce gene silencing to levels similar to, and in some cases far superior to, prior art formulations with much lower cationic lipid levels. EX2009, ¶67.

For example, the ’435 patent tested nucleic acid-lipid particle compositions of claim 1 (i.e., Groups 11, 13, and 14) and prior art compositions. The expectation for compositions of claim 1 would have been little, if any, gene silencing. That is, these formulations would have been expected to yield reductions in gene expression similar to the PBS control group. Unexpectedly, each of these formulations showed significant gene silencing when compared to the control group. EX1001, FIG 2.

Even more surprisingly, according to Dr. Thompson, these formulations induced gene silencing levels which were at least comparable and in many cases superior to prior art formulations that have a much lower cationic lipid level (e.g., 25 mol % to 40 mol % cationic lipid). *See* EX1001, Table 4; *see also* EX2009, ¶75.



EX1001, FIG 2 (red and green highlighting added). For instance, the 1:57 composition (i.e., group 11), "was substantially more effective at silencing the expression of a target gene as compared to prior art nucleic acid-lipid particles ('2:40 SNALP')" (i.e., Groups 2, 4-8). EX1001, 6:10-14, Figure 2. Furthermore, the 1:57 formulation "was the most potent at reducing" gene expression *in vivo*. EX1001, 72:25-27; *see also* EX2009, ¶75.

A 1:57 composition was further compared to a prior art 2:30 composition in Example 4. Surprisingly, a 1:57 composition was "more than 10 times as efficacious as the 2:30 SNALP" composition in silencing gene expression *in vivo*. EX1001, Figure 3. Remarkably, the 1:57 formulation achieved these results at "***a 10-fold lower dose***" than the conventional 2:30 SNALP formulation. EX1001, 6:10 (emphasis added); *see also* EX2009, ¶76.

Petitioner states that "[a]t most, [Example 4] established that the 1:57 SNALP comprised of the specific species of lipid components and nucleic acid to lipid ratio disclosed, dosed as disclosed, outperformed the 2:30 SNALP comprised of the lipid species disclosed and dosed as disclosed." Pet. 22. "[A] POSITA would not expect all alternative data points falling within the recited numeric range to perform like the 1:57 SNALP." Pet. 36. These remarks ignore the breadth of compositions tested. *See also* EX2009, ¶77.

For instance, Examples 5 and 6 describe testing of additional compositions of claim 1 which also yield considerable levels of gene silencing following systemic administration. EX1001, 74:5-58. Example 5 discloses the testing of seven compositions of claim 1 (e.g., 1:57, 1:62 and 2:70). EX1001, 74:11-58, Table 6, Figure 4. Each composition within Example 5 induced significant levels of gene silencing. Example 6 discloses the testing of 14 compositions of claim 1 (i.e., 54 mol % to 68 mol % cationic lipid). EX1001, 74:11-58, Table 7. Each composition within

Example 6 induced significant levels of gene silencing. Moreover, the '435 patent notes "that the 1:62 SNALP formulation was one of the most potent inhibitors of ApoB expression at two different lipid:drug ratios (i.e., 6.1 & 10.1) among the phospholipid-free SNALP formulations tested (see Groups 2 & 15)." EX1001, 75:45-48; *see also* EX2009, ¶¶78-81.

According to Dr. Thompson, a POSITA would have expected these formulations to be ineffective — that is, produce levels of gene silencing similar to that of the control. EX1001, FIG. 4. Surprisingly, these formulations exhibited significant *in vivo* gene silencing activity. *See also* EX2009, ¶¶78-81.

The petition does not address the breadth of compositions tested (i.e., cationic lipid level ranging from 54 mol % to 70 mol %), rather it argues that the data can be dismissed because "variation of the cationic lipid apparently impacts efficacy" and that a POSITA "would understand these results to suggest that a preferred proportion for one cationic lipid (e.g., DLinDMA) does not necessarily apply to all other cationic lipids (e.g., DODMA)." Pet. 37-38. As explained by Dr. Thompson, while there is some variation in the efficiency of formulations depending on which cationic lipid is used, this does not take away from the conclusion that both DODMA and DLinDMA are unexpectedly efficacious. EX2009, ¶79. Moreover, as will be discussed in Section X, several additional cationic lipids were tested after the filing of the '435 patent and provide further support for the conclusion that the

compositions across the claimed ranges are surprisingly efficacious and well-tolerated.

F. The petition fails to address prior art teachings contrary to the proffered obviousness theory

At the time the '435 patent was filed, a POSITA understood that lipid particles containing cationic lipids could be used to deliver nucleic acids to cells *in vitro*, however such lipid particles were too toxic for *in vivo* applications. EX2011 at 42 ("I wouldn't want anyone injecting cationic lipids into my bloodstream."); EX2009, ¶¶25-35. The petition does not even mention toxicity, much less provide a reason to disregard the plain teachings of the prior art.

In determining whether a POSITA would have been motivated to make a particular modification or combination, "the full field of the invention must be considered." *In re Dow Chem. Co.*, 837 F.2d 469, 473 (Fed. Cir. 1988). A reference is said to "teach away" if a POSITA upon reading the reference "would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the applicant." *Monarch Knitting Mach. Corp. v. Sulzer Morat GmbH*, 139 F.3d 877, 885 (Fed. Cir. 1998). "[R]eferences that teach away cannot serve to create a prima facie case of obviousness." *McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1354.

As discussed above, the prior art unequivocally taught away from using a level of cationic lipid such as those claimed in the '435 patent. For example, it was

known that "cationic lipid contributes significantly to the toxicity observed" in prior art lipid particles. EX1008 at E96. It was also appreciated that cationic lipid interacts with serum proteins which leads to aggregation and rapid clearance from the body. EX1008 at 9. That is, cationic lipid was known to make lipid particles toxic and ineffective when administered systemically. Consequently, the prior art expressly taught minimization of the cationic lipid component. *E.g.*, EX1006 at 745 ("Minimizing the amount of cationic lipid is desirable"). Consistent with the state of the art, the '196 PCT teaches that the cationic lipid component should be minimized. EX1002 ¶88 ("[F]or systemic delivery, the cationic lipid may comprise ***about 5% to about 15%*** of the total lipid present in said particle.") (emphasis added); *see also* Sections IV, VII.C.; EX2009, ¶¶25-35, 66-68.

Accordingly, the prior art taught away from the proposed modifications, and a POSITA would not have found the claimed invention obvious in view of the '196 PCT and the '189 publication. *See* EX2009, ¶¶47-49.

G. Dependent Claims 2-20

Dependent claims 2-20 are not rendered obvious at least for the reasons discussed above, and by virtue of their dependency from claim 1—which the petition fails to demonstrate as unpatentable. Furthermore, claims 4, 5, 7, and 12 recite additional limitations that provide for an even more coextensive nexus with the unexpected results disclosed in the '435 patent. *See* EX2009, ¶¶82-86, 88, 90.

The challenges to claims 2-20 are also not presented with the requisite degree of particularity. 35 U.S.C. § 312(a)(3); 37 C.F.R. § 42.104(b). The petition cites to disclosure in the '196 PCT, and patents incorporated by reference into the '196 PCT, but fails to account for any dependencies of the challenged claims — these challenges appear to be nothing more than a claim mapping exercise. *See 10X Genomics, Inc. v. Bio-Rad Lab. Inc.*, IPR2018-01206, Paper 7 at 15 ("Petitioner's alternatively and inconsistently defined combination of elements is not sufficient to allow for a reasoned analysis"). Furthermore, while the petition indicates that claims 1-20 are being challenged as obvious, *see* Pet. 32, the discussion for some invokes an unexplained anticipation theory. *See, e.g.* Pet. 41 (claim 4), 42-43 (claim 7); *see also* EX2009, ¶¶82-84.

Additional discussion for some of claims 2-20 is provided below.

1. Claim 5

The petition relies on long lists of non-cationic lipids including phospholipids, sterols, and non-phosphorous containing lipids and generic ranges for non-cationic lipid and cholesterol. EX1007 ¶122 (citing EX1002 ¶¶89, 91). These paragraphs do not disclose nucleic acid-lipid particle compositions that contain a mixture of phospholipid and cholesterol. To the extent it is argued that a POSITA might select phospholipid and cholesterol for inclusion in a nucleic

acid-lipid particle, the petition does not provide a motivation, nor any reasonable expectation of success. EX2009 ¶¶85-86.

2. Claim 6

The challenge to claim 6 is nothing more than a bare claim mapping that pays no regard to the dependencies of the claim. To the extent that it is argued that a POSITA might incorporate DPPC or DSPC into a nucleic acid-lipid particle composition that includes cholesterol, the petition provides neither a motivation nor any reasonable expectation of success. EX2009 ¶87.

3. Claim 7

The '196 PCT does not disclose a range for a phospholipid component, much less one in a nucleic acid-lipid particle that includes cholesterol. Instead, the petition uses arithmetical manipulations of ranges of non-cationic lipid and cholesterol in two different patent documents. EX1007 ¶124. A POSITA would not consider these calculations to disclose a range of phospholipid in the '196 PCT. EX2009 ¶88.

4. Claim 8

The challenge to claim 8 is a bare claim mapping. The '196 PCT does not disclose a range for cholesterol in a formulation that includes phospholipid, as required by claim 5, from which claim 8 depends. The relevance of a composition in the '618 patent that does not contain conjugated lipid, as required by claim 1, is unexplained. EX2009 ¶89.

5. Claim 13

No explanation is given for why a POSITA would have had a reasonable expectation of success generating fully encapsulated nucleic acid-lipid particles having a high level of cationic lipid and a low level of conjugated lipid. As explained by Dr. Thompson, a POSITA would have expected the claimed nucleic acid-lipid particle to be unstable and incapable of encapsulating nucleic acids. EX2009 ¶91.

6. Claim 14

The petition does not explain why a POSITA would have had a reasonable expectation of success generating a pharmaceutical composition of nucleic acid-lipid particles that have a high level of cationic lipid and a low level of conjugated lipid. A person of ordinary skill in the art would have expected the claimed particles to be prone to aggregation and unstable due to a high cationic lipid and low conjugated lipid. EX2009, ¶92.

7. Claims 16-20

Whether there was a reasonable expectation of success for these claims is not discussed. The '196 PCT teaches nucleic acid-lipid particles for systemic use at a much lower cationic lipid range, 5 mol % to 15 mol %. A PHOSITA would have expected the claimed nucleic acid-lipid particles to be too toxic for *in vivo* administration, and thus not suitable for administration to mammalian subjects. EX2009, ¶93. Additionally, as will be discussed in detail below, objective indicia of nonobviousness further support the patentability of claims 1-20. Section X.

VIII. RESPONSE TO GROUND 2

Petitioner contends that claims 1-20 of the '435 patent are obvious in view of "patent owner's prior disclosures" in light of Lin and/or Ahmad. It is unclear what exactly constitutes "patent owner's prior disclosures," or even, precisely, what claims are being challenged. For example, although Ground 2 alleges that claims 1-20 are obvious, the petition only addresses the cationic lipid limitations of claims 1 and 4. Pet. 48-51; EX1007 ¶¶138-142.

At best, Ground 2 is little more than an attempt to backfill Ground 1 by adding "Lin and/or Ahmad." Thus, Ground 2 inherits all the defects discussed above with respect to Ground 1. Ground 2 is further deficient because the petition misrepresents the disclosures of Lin and Ahmad, and provides only unsupported and conclusory remarks regarding motivation to combine and expectation of success. *See* EX2009 ¶94.

A. The petition fails to assess the full scope and content of Lin and Ahmad

Petitioner cites to Lin and Ahmad solely to address the claimed range of cationic lipid in claims 1 and 4. Pet. 49. However, Ground 2 fails because the petition presents a misleading and incomplete assessment of the prior art, and never addresses the prior art's express instruction to minimize the level of cationic lipid. *See also* EX2009 ¶¶94-102.

Lin and Ahmad are directed to lipoplexes — fundamentally different lipid particles than the claimed nucleic acid-lipid particles. The '196 PCT, among other publications including a patent to Dr. Janoff, explicitly distinguishes lipoplexes from the claimed nucleic acid-lipid particle. EX1002 ¶8 (defining "cationic liposome complex" as lipoplex); EX2007, 2:27-28 (same); *compare* EX1002, ¶6 ("Cationic liposome complexes, however, are large, poorly defined systems that are not suited for systemic applications and can elicit considerable toxic side effects.") *with id*., ¶2 ("[T]he present invention is directed to using a small interfering RNA (siRNA) encapsulated in a serum-stable lipid particle having a small diameter suitable for systemic delivery."); *compare* EX2007, 2:26-40 (patent to Janoff explaining that "lipoplexes suffer from several major drawbacks when used in gene therapy, including low stability, high cytotoxicity, non-biodegradability, poor condensation and protection of DNA, serum sensitivity, large size and lack of tissue specificity.") *with id*., 2:52-67 (explaining that liposomes, unlike lipoplexes, can encapsulate nucleic acids and are not cytotoxic); EX2028, 122:1-24 (explaining that the inventors did not define the term SNALP as inclusive of lipoplex); *see also* EX2009, ¶¶94-99.

The petition brushes aside the distinctions between lipolexes and nucleic acid-lipid particles by stating that "the Lin and Ahmad systems tested helper lipids and cationic lipids to create carrier particles for nucleic acids, i.e., 'nucleic acid-lipid

particles,' the same general carrier particles described in the Patent Owner's prior disclosures." Pet. 50. Not so. As explained by Dr. Thompson, and confirmed by Dr. Janoff, a POSITA would have recognized that the lipoplexes disclosed by Lin and Ahmad are distinct from the claimed nucleic acid-lipid particles. EX2007, 2:26-40. Among other differences, lipoplexes have different compositions than nucleic acid-lipid particles. *See e.g.,* EX1005 at 2 (describing lipoplexes that comprise DNA and cationic and neutral lipids); EX1006 at 2-3 (same); EX1002 ¶¶6, 8 (contrasting lipoplex and nucleic acid-lipid particle compositions); *see also* EX2009, ¶¶100-101.

Given the fundamental differences between lipoplexes and the claimed nucleic acid-lipid particles, a POSITA would not have found teachings regarding lipoplexes to be instructive for the development of the claimed invention. EX2009, ¶100.

As explained by Dr. Thompson, even if considered relevant, neither reference teaches or suggests increasing the cationic lipid content above 50 mol %. For example, the transfection efficiency of DOSPA/DOPC lipoplexes is insensitive over the range of 20 mol % to 100 mol % cationic lipid and DOTAP/DOPE lipoplexes are insensitive to cationic lipid increases over the entire tested range (0 mol % to 70 mol %). EX1005, FIG. 4A, 4D. Similarly, Ahmad discloses that transfection efficiencies for most lipoplexes are insensitive to cationic lipid increases over the range of 40 mol % to 80 mol % cationic lipid content. EX1006, Figure 3A. Consequently, there

is no teaching or suggestion in Lin or Ahmad to increase the cationic lipid component above 50%. *See* EX2009 ¶101.

Moreover, Ahmad expressly teaches that cationic lipid content should be minimized. EX1006 at 7 ("Minimizing the amount of cationic lipid is desirable to reduce cost as well as potential toxic effects of the cationic lipid."). In the Institution Decision, the Board commented that this teaching from Ahmad is not necessarily persuasive that Ahmad does not encourage increased amounts of cationic lipid in certain circumstances." Paper 15 at 31-32. Dr. Thompson respectfully disagrees and notes that Ahmad's statement of minimizing the amount of cationic lipid is without qualification and is consistent with the literature at the time. *See* Section IV. A POSITA would recognize that the aversion for high levels of cationic lipid is well documented in the literature, and would not dismiss the problems of cytotoxicity associated with cationic lipids as limited only to particular circumstances. *See* EX2009, ¶102-4.

B. The petition fails to demonstrate motivation to combine

With respect to a motivation to combine Lin and Ahmad with "Patent Owner's prior disclosures," the petition offers only conclusory and inaccurate statements. *See* EX2009 ¶105.

The petition states that a POSITA "would have been aware that the lipid proportions used could impact transfection efficiency." Pet. 50. The petition in

essence, suggests that the prior art provided an invitation to conduct further experimentation and research, but such invitation does not amount to a motivation to combine. *Eurand, Inc. v. Mylan Pharms., Inc.*, 676 F.3d 1063, 1072 (Fed. Cir. 2012) ("Evidence of obviousness, …, is insufficient unless it indicates that the possible options skilled artisans would have encountered were 'finite,' 'small,' or 'easily traversed,' and that skilled artisans would have had a reason to select the route that produced the claimed invention."); *see also* EX2009, ¶¶106-7. Furthermore, a POSITA would have also been aware that lipid proportions affect, *inter alia*, stability, aggregation, toxicity, and immune stimulation in addition to transfection efficiency. EX2009, ¶¶25-35. The invention is not simply a nucleic acid-lipid particle that is capable of transfecting cells, but one which avoids the known and well-documented problems associated with cationic lipid-containing particles. Petitioner fails to indicate how—or even which—"lipid proportions" should be manipulated to impact transfection efficiency. As explained above, the properties of nucleic acid-lipid particles do not depend on any single component. *See id*., ¶¶57-59

Accordingly, any motivation to combine expressed in the petition is based on a misguided understanding of the art. *See* EX2009 ¶¶105-7.

C. The petition fails to show any reasonable expectation of success

The petition states that "given the success of generating nucleic acid-lipid particles with a cationic lipid proportion greater than 50% … a POSITA would have

appreciated a reasonable expectation of doing so." Pet. 50-51. Petitioner does not identify what disclosures it relies upon, or where such "given" success can be located in the references. First, Lin and Ahmad do not teach or suggest generating nucleic acid-lipid particles at all. Second, none of the nucleic acid-lipid particle compositions disclosed in the '196 PCT and the '189 publication have cationic lipid content greater than 50 mol %. EX1002 ¶¶ 216, 223, 228, 232 (disclosing formulations of 7.5 mol % and 15 mol % cationic lipid); EX1003 ¶¶289, 291-293, 295, 303, 311, 319, 327, 335, 343, 351, 361, 369, 377, 385 (disclosing compositions of 30 mol % and 40 mol % cationic lipid). Accordingly, the petition's basis for reasonable expectation of success is unsupported in the prior art. *See* EX2009 ¶107.

Petitioner's stated expectation of success becomes little more than circular and conclusory reasoning. Petitioner assumes that success is a "given," and then uses this assumption as a basis for concluding that success would be expected. The combination of references of Ground 2 does not support the requisite expectation of success.

Claims 1-20 are not obvious over "Patent Owner's prior disclosures" in view of Lin and/or Ahmad for the reasons stated above. Additionally, as will be discussed in detail below, objective indicia of nonobviousness further support the patentability of claims 1-20. *See* Section X.

IX. RESPONSE TO GROUND 3

The petition impermissibly conflates alternate theories of anticipation and obviousness into a singular discussion such that it is impossible to discern which of the various portions of the '554 publication cited to are meant to support which alternate theory. *See Wasica Fin. GmbH v. Cont'l Auto. Sys.*, 853 F.3d 1272, 1286 (Fed. Cir. 2017) (affirming patentability determination where "[Petitioner] offered only a conclusory and sweeping allegation that 'to the extent that any of the variances in claim scope are not necessarily shown in the above [anticipation analysis], such variances would have been obvious to a [POSITA].'"). However, regardless of which theory is being asserted, the '554 publication neither anticipates nor renders obvious the claimed invention. *See also* EX2009 ¶108.

A. The prior art does not disclose each and every element of the challenged claims

Petitioner appears to advance both anticipation by the L054 lipid mixture, and anticipation by ranges of components of lipid particles. Both theories fall far short of what the law requires.

1. L054 is not a nucleic acid-lipid particle

The petition asserts that claim 1 is anticipated by the L054 lipid mixture. Pet. 52. A claim is anticipated only if each and every limitation recited in the challenged claim is found in a single prior art reference. *Net MoneyIN, Inc. v. VeriSign, Inc.*, 545 F.3d 1359, 1371 (Fed. Cir. 2008). Claim 1 recites a nucleic acid-lipid

particle with specific concentration ranges of a cationic lipid (50 mol% to 85 mol%), non-cationic lipid (13 mol% to 49.5 mol%), conjugated lipid (0.5 mol% to 2 mol%) in a nucleic acid-lipid particle. The L054 lipid mixture is not a particle and fails to meet at least these limitations. *See* EX2009 ¶109-10.

L054 is not a lipid particle, but rather a mixture, i.e., the lipid solution that may be used to make particles. The petition wrongly assumes that the solution and any resulting particle would have the same molar ratio of components. Quite the contrary, the input formulation (i.e., nucleic acid and lipid mixture) and the output formulation (i.e. lipid particles) are not identical. EX2028, 155:2-25, 156:1-157:11 (explaining that input versus final lipid-to-drug ratio may be different), 157:15-158:16 (explaining that, for instance, cholesterol may not be recovered in the lipid particle). Even the '435 patent explains that the lipid-to-drug ratio (i.e., lipid to nucleic acid ratio) calculated from the input components is not identical to that of the finished product. *See, e.g.,* EX1001, 79:50-80:9 (reporting different input and final lipid to drug ratios for SNALP formulations); *see also* EX2009 ¶109-10.

It was widely documented in the art that a finished lipid particle must be tested to determine its composition. EX2012 at 7242; *see also* EX1004 ¶634. It was known that the method of lipid particle formation effects the incorporation of lipids and nucleic acids into finished particles. EX1004 ¶¶165, 463; *see also* EX2009, ¶109-14.

The significance of the difference between the starting lipid ratio and that of the final lipid particle is one the reasons why FDA Guidance specifies identifying the lipid ratio of the finished formulation. EX2013 at 3 (recommending labeling with the "amount of each lipid component used in the formulation based on the ***final form*** of the product" and "[a]n expression of the molar ratio of each individual lipid to the drug substance is also recommended for each individual lipid in the ***finished formulation***), 8 (recommending reporting of "characteristics or attributes specific to the liposome formulation" including "[l]ipid content (***to demonstrate consistency with the intended formulation***)."); *see also* EX2009, ¶109-15.

The '554 publication is entirely silent as to the composition of the particle formed from the L054 mixture. The L054 lipid mixture cannot meet each and every limitation recited in independent claim 1. EX2028, 157:15-158:16; *see also* EX2009 ¶¶115-16.

Claim 1 also recites "[a] nucleic acid-lipid particle." As discussed above, the term "nucleic acid-lipid particle" excludes particles that do not encapsulate nucleic acid. The encapsulation state of the nucleic acids in lipid particles made with the L054 lipid mixture is entirely unaddressed. Encapsulation of nucleic acids cannot be assumed based on the composition and formulation method. *See* EX2007, 4:11-19; *see also* EX2009 ¶117-18.

Moreover, the '554 publication does not define "encapsulate," disclose encapsulation level for any nanoparticle composition, nor disclose an assay for determining whether or not a nucleic acid is encapsulated in a lipid particle. Petitioner's expert testified that context is needed in order to know what is meant by the term "encapsulate" — that is, it has more than one meaning. *See, e.g.*, EX2028, 137:16-138:16, 146:22-147:1 ("I don't know what we really mean by encapsulated DNA, and I don't know what the method is, …."), 147:18-22 ("Q. You don't know what encapsulation means? A. It's a fungible term. It means different things to different people in different contexts."). Petitioner's expert has also asserted, in the context of one of his own patents, that testing is needed to determine encapsulation. EX2007, 4:11-19; *see also* EX2009 ¶¶118-19.

In contrast to the '554 publication, the '435 patent provides a definition, an assay for encapsulation, and reports encapsulation values. *See, e.g.*, EX1001, 22:55-62 ("In preferred embodiments, a SNALP comprising a nucleic acid such as an interfering RNA (e.g., siRNA) is fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In certain instances, the nucleic acid in the SNALP is not substantially degraded after exposure of the particle to a nuclease at 37°C. for at least about 20, 30, 45, or 60 minutes."), Tables 2-6. Petitioner's expert agrees that the '435 patent provides the necessary context for "encapsulation." EX2028, 198:4-17, 199:5-9 ("Q. So it's encapsulated in

some way? You're just giving a specific way? A. Encapsulated in the lipid portion."). That is, a nucleic acid is encapsulated when it is inside the lipid particle and protected from nuclease degradation. *See also* EX2009 ¶¶117-19.

Separately, both experts agree that there is no meaningful difference between the inventive lipid particles of the '435 patent and SNALP. *Id*., ¶120. That is, the inventive lipid particles are serum-stable nucleic acid-lipid particles that are "extremely useful for systemic applications" which "can exhibit extended circulation lifetimes following intravenous (i.v.) injection." EX1001, 11:36-38. The '554 publication distinguishes between embodiments formulated for *in vitro* use and those formulated for *in vivo* use. *See, e.g.*, EX1004 ¶¶136, 462. Having emphasized this distinction, the '554 publication then proceeded to only test L054 *in vitro*. *See Id*., ¶395. Nor were L054-derived particles evaluated for serum stability. *See Id.*, ¶¶158, 592, 596. A POSITA would have expected the L054-dervied lipid particles to be too toxic and inappropriate for systemic use based on the structure of the cationic lipid (i.e. DMOBA). EX2009, ¶¶121-22.

In sum, an anticipation theory based on L054 is deficient in many aspects, including not expressly or inherently meeting each of the recited claim limitations. *See* EX2009 ¶123.

2. The prior art ranges are not sufficiently specific

Petitioner also cites to various ranges in the '554 publication. To the extent that such citations are intended to advance an anticipation theory, such theory also fails. *See* EX2009 ¶¶124-25.

Anticipation requires that a reference clearly and unequivocally disclose the claimed invention without any need for picking, choosing, and combining various disclosures not directly related to each other in the cited reference. *In re Arkley*, 455 F.2d 586, 587-88 (CCPA 1972); *see also Net MoneyIN,* 545 F.3d at 1371. Here, Petitioner fails to demonstrate that the disparate ranges of various components in the '554 publication represent a single lipid particle formulation. The Petition cites to three different paragraphs in the '554 publication for the ranges of the different lipid components. Pet. 53-56 (citing to EX1004 ¶¶116, 118, 313). Yet, each of these paragraphs states that the disclosed ranges are applicable to one embodiment. There is no indication that those embodiments are to be combined or are related to one another. These paragraphs state that "***in one embodiment***" the cationic lipid component can span four different ranges. Further, "***in one embodiment***" the neutral lipid component can span two different ranges. Still further, "***in one embodiment***" the PEG conjugate can span two different ranges. No explanation is provided as to how these eight different ranges for three different components might be combined. *See* EX2009, ¶¶125-29.

Contrary to Petitioner's representation, considerable differences exist between the prior art ranges and the claimed ranges. S*ee also Atofina v. Great Lakes Chem. Corp*., 441 F.3d 991, 999 (Fed. Cir. 2006) ("Considerable difference" between the claimed range and the prior art weighed against a finding of sufficient specificity). In *Atofina*, the claim at issue required that a "process be conducted in the presence of 0.1 to 5 moles of oxygen per 100 moles of methylene chloride, at a temperature of between 330 and 450 [degrees] C." With respect to the oxygen ratio, the prior art disclosed a range of "0.001 to 1.0 percent" *Id*. at 1000. However, the Federal Circuit reasoned that the prior art range "overlaps but does not fall within the range of ratios claimed" and that "the disclosure of a 0.001 to 1.0 percent range in [the prior art] does not constitute a specific disclosure of 0.1 percent to 5.0 percent." *Id*. "No reasonable fact finder could determine that this overlap describes the entire claimed range with sufficient specificity to anticipate this limitation of the claim." *Id*. With respect to the temperature range, the prior art disclosed a much broader range of 100 to 500 °C. Even though the prior art fully encompassed the claimed range, the Federal Circuit explained that "[g]iven the considerable difference between the claimed range and the range in the prior art, no reasonable fact finder could conclude that the prior art describes the claimed range with sufficient specificity to anticipate th[e] limitation." *Id*. at 999; *see also* EX2009, ¶¶130-32.

The fact pattern and holding of *Atofina* is applicable here. Claim 1 recites a cationic lipid range of 50 mol % to 85 mol %. For this limitation, Petitioner relies on the prior art ranges of "about 2% to about 60%" and "about 40% to about 50%." Pet. 53 (citing EX1004 ¶116). Neither range is representative of the cationic lipid range of claim 1. Further, a POSITA would not consider the ranges in the '554 publication as describing the claimed range with sufficient specificity. EX2009, ¶¶130-32.

Similarly, independent claim 1 recites a conjugated lipid range of 0.5 mol % to 2 mol%. Here, Petitioner relies on the disclosed range of "about 1% to about 20%." Again, the claimed range and the prior art range are considerably different. The prior art broadly suggests any concentration from 1% up to about 20%. In contrast, the claimed range is limited to 0.5 mol% to 2 mol%. As in *Atofina*, the small overlap does not describe the entire claimed range with sufficient specificity to anticipate the limitation. EX1007 ¶¶148, 150, 151; *see also* EX2009, ¶¶130-32.

B. The challenged claims are not obvious over the '554 publication

With respect to an obviousness theory in Ground 3, this fails for many of the same reasons discussed in Ground 1: 1) It considers each component in a piecemeal fashion without addressing the claims as a whole, 2) fails to articulate any reason to combine the prior art disclosures, and 3) does not articulate any reasonable expectation of success in making the proposed modifications. *See* EX2009 ¶¶133-34.

As discussed in responding to Ground 1, the Petitioner fails to address the claims as a whole. *Supra,* Section VII. The claimed invention is a nucleic acid-lipid particle that simultaneously has both a high level of cationic lipids ***and*** a low level of conjugated lipids. Petitioner does not provide any showing that the '554 publication would have taught or suggested the use of nucleic acid-lipid particles with high levels of cationic lipids and low levels of conjugated lipids. EX2009 ¶135. Given the highly interdependent nature of the components claimed, Petitioner's per-limitation approach to addressing the concentrations of cationic lipid, non-cationic lipid, and conjugated lipid is inadequate to address the claimed invention as a whole. *See id.*, ¶¶57-59.

A conclusion of obviousness requires articulating reason to combine or modify the prior art — Petitioner provides none. *See* EX2009, ¶¶136-37.

The petition also injects a single sentence "obvious to try" rationale at page 56. Under "obvious to try" the petition must at least analyze "the characteristics of the science or technology, its state of advance, the nature of the known choices, the specificity or generality of the prior art, and the predictability of results in the area of interest." *Abbott Labs. V. Sandoz, Inc.*, 544 F.3d 1341, 1352 (Fed. Cir. 2008). Petitioner does not address any of those factors.

The requisite discussion of whether there was a reasonable expectation of success is also nonexistent in Ground 3. Petitioner never once addresses whether a

POSITA would have reasonably expected any modification to be successful. *See* EX2009, ¶¶136-37.

C. Dependent Claims 2-20

Dependent claims 2-20 are neither anticipated nor rendered obvious at least for the reasons discussed above, and by virtue of their dependency from claim 1—which the petition fails to demonstrate as unpatentable. Furthermore, claims 4, 5, 7, and 12 recite additional limitations that provide for an even more coextensive nexus with the unexpected results disclosed in the '435 patent. *See* EX2009 ¶¶140-45, 148-50, 159. The challenges to claims 2-20 are also not presented with the requisite degree of particularity. 35 U.S.C. § 312(a)(3); 37 C.F.R. § 42.104(b); *see also* POPR, 19-22. The petition cites to disclosure in the '554 publication, but often fail to indicate how the cited disclosure relates to the previous discussion of claim 1 or whether the assertion is one of anticipation or obviousness. For a vast majority of the claims, it is entirely unclear what the specific basis of challenge is being advanced. For example, the petition does not state whether claims 2, 3, 5, 6, 9, and 13-20 are being challenged as allegedly anticipated or obvious. EX2009 ¶¶138-39.

Additional discussion for some of claims 2-20 is provided below.

1. Claim 5

The L054 mixture does not anticipate claim 1 and therefore cannot anticipate claim 5. Paragraphs 85 and 455 do not disclose nucleic acid-lipid particle

compositions that contain a mixture of phospholipid and cholesterol. To the extent it is argued that a POSITA might select phospholipid and cholesterol for inclusion in a nucleic acid-lipid particle, the petition does not provide a reason to do so, nor any reasonable expectation of success. EX2009 ¶¶142-45.

2. Claim 6

Picking and choosing claim elements from a long list of neutral lipids in paragraph 85, randomly combined with other unspecified components, is not sufficient under an anticipation theory. To the extent it is argued that paragraph 85 makes obvious claim 6, the petition does not provide a reason to select DPPC and DSPC from this long list for incorporation into a lipid particle, nor a reasonable expectation of success. EX2009 ¶¶146-47.

3. Claim 7

If anticipation is being argued, the challenge fails because the '554 publication does not disclose any ranges for phospholipids. If obviousness is being argued, it fails because it is based on a series of unexplained assumptions (e.g., "when cholesterol is present," "when combined with a cationic lipid proportion in the 60% range") and calculations using unrelated disclosures. EX2009 ¶¶148-50.

4. Claim 8

Claim 8 depends from claims 1 and 5. If anticipation is argued there is no relationship between the disclosure mapped for claim 8 to that mapped for claims 1 and 5. If obviousness is argued, this challenge fails because claim 8 depends from

claim 5 which requires a mixture of phospholipid and cholesterol. This "encompassing range" for cholesterol does not make any mention of phospholipid and thus is missing a claim element. Additionally, neither a reason to combine these disclosures nor a reasonable expectation of success are addressed.

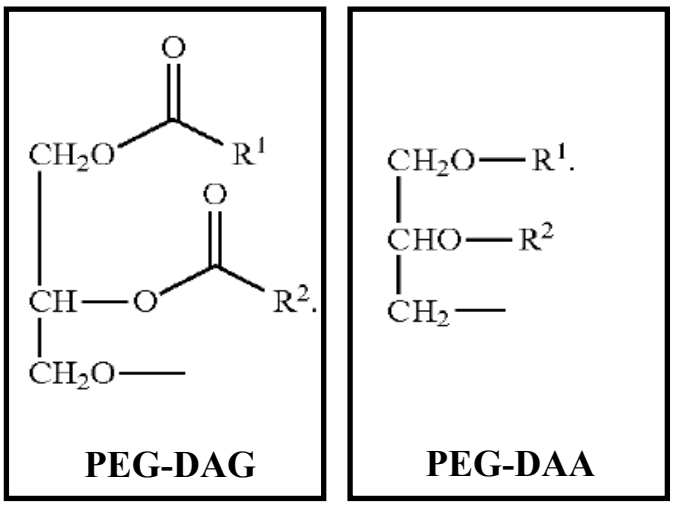
The petition refers to a formulation which includes cholesterol at a 30% proportion, citing Table 4 (e.g., L106)." EX1007 ¶160. First, L106 is simply a lipid mixture—not a particle. Second, if particles were made from L106, they would not meet the limitations of claim 8. L106 has 3% conjugated lipid, which is well above the range of claim 1 (i.e., 0.5% to 2%). Further L106 does not "comprise[] a mixture of a phospholipid and cholesterol or a derivative thereof" as required by claim 5. L106, as with the other examples from the '554 publication with 30% cholesterol, does not meet the limitations of claim 8. EX2009 ¶¶151-55.

5. Claim 11

The '554 publication does not disclose PEG-dialkyloxypropyl (PEG-DAA) conjugates as required in claim 11, as admitted in the petition, and thus does not anticipate claim 11. *See* EX2009 ¶156.

The petition alleges that "PEG-dialkyloxypropyl (PEG-DAA) conjugates could be used in lieu of PEG-diacylglycerol (PEG-DAG) conjugates" as disclosed in Patent Owner's "'910 publication." Pet. 61 (citing EX1014). EX1014 is not the '910 publication. Regardless, this argument is based on the false assumption that

PEG-DAG and PEG-DAA conjugates are interchangeable. PEG-DAG conjugates have an ester moiety linking the acyl chains (R^1 and R^2 in the figure below) to the backbone whereas PEG-DAA conjugates have an ether moiety linking the acyl chains to the backbone. See EX1001, 53:53-54:14. As compared to PEG-DAA conjugates, PEG-DAG conjugates are more hydrophilic which directly impacts the physical properties of the particles. Moreover, PEG-DAG conjugates are more easily metabolized by cells. A POSITA would not consider PEG-DAG and PEG-DAA conjugates to be equivalent or interchangeable and would not have reason to substitute them as alleged in the petition. EX2009 ¶¶156-58.



6. Claim 13

The petition alleges that "a POSITA would understand that full encapsulation requires only an excess of cationic lipid with regard to the nucleic acid for electrostatic interaction." EX1007 ¶165. But both experts agree that encapsulation

cannot be inferred from the composition or production method and must be determined by testing. EX2007, 4:15-19; EX2009 ¶¶160-62.

7. Claims 14, 16-20

If anticipation is argued, these challenges fail because there is no relationship between the disclosure mapped for claims 16-20 to the disclosure asserted in claim 1. EX2009 ¶¶163-67.

If asserting obviousness, a POSITA would not find it obvious to administer the nanoparticles of the '554 publication to mammalian subjects *in vivo*. This is because, *inter alia*, a POSITA would have expected the disclosed nanoparticles to be too toxic for *in vivo* administration. As explained by Dr. Thompson, a POSITA would have expected DMOBA and DMLBA (see Table IV) to be too toxic for *in vivo* administration, including organ toxicity. Lipid particles using DMOBA and DMLBA were considered inappropriate for systemic use. Consequently, claims 16-20 are not obvious. EX2009 ¶¶163-67.

Claims 1-20 are not anticipated and not obvious over the '554 publication for the reasons stated above. Additionally, as will be discussed in detail below, objective indicia of nonobviousness further support the patentability of claims 1-20. *See* Section X.

X. OBJECTIVE INDICIA OF NONOBVIOUSNESS

Objective indicia of non-obviousness "guard against slipping into the use of hindsight, and the temptation to read into the prior art the teachings of the invention in issue." *Graham*, 383 US at 17-18, 36. They may include the existence of a long-felt but unsatisfied need for the invention, failure of others, unexpected results, and others. *Id*.; *Apple Inc. v. Samsung Elecs. Co.*, 839 F.3d 1034, 1052 (Fed. Cir. 2016) (en banc). Objective evidence of non-obviousness "may often be the most probative and cogent evidence in the record." *Id*. (quoting *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1538-39 (Fed. Cir. 1983)). "Objective indicia of nonobviousness must be considered in every case" where presented and "it is error to reach a conclusion of obviousness until all [Graham] factors [including objective indicia] are considered." *Id*. at 1048.

There is a presumption of nexus for objective considerations when a patentee shows that the asserted objective evidence is tied to a specific product and that product "is the invention disclosed and claimed in the patent." *WBIP, LLC v. Kohler Co.*, 829 F.3d 1317, 1339 (Fed. Cir. 2016)(quoting *J.T. Eaton & Co. v. Atl. Paste & Glue Co.*, 106 F.3d 1563, 1571 (Fed. Cir. 1997)). In the present case, the '435 patent is listed in the Orange Book for patisiran and independent claim 1 and several dependent claims cover the product. This nexus supports the objective evidence discussed.

The nucleic acid-lipid particles claimed by the '435 patent have achieved tremendous recognition in the field as embodied by a first-in-class siRNA drug. EX2024 ("FDA approves first-of-its kind targeted RNA-based therapy to treat a rare disease."). The drug, patisiran — tradename "Onpattro" — is encompassed by claim 1. EX2019, Table 1. The '435 patent is listed in FDA's Orange Book as protecting patisiran. EX2027. Patisiran received regulatory approval in the United States, and was recommended for approval in Europe. EX2024; EX2025; *see also* EX2009 ¶168.

Patisiran is the first therapy based on RNA interference (RNAi), to silence specific genes linked to disease. *See, e.g.,* EX2023 at 291. FDA approval was hailed in the industry: "This approval is key for the RNAi field," stated James Cardia, head of business development at RXi Pharmaceuticals, which is developing RNAi treatments. "This is transformational." *See* EX2009 ¶190.

The nucleic acid-lipid particle formulations of the '435 patent solved a long-felt need for compositions that could safely and effectively deliver nucleic acids to target cells of patients. Skilled artisans were skeptical that compositions having high levels of cationic lipid (i.e., 50 mol % to 85 mol %) and low levels of conjugated lipid (i.e., 0.5 mol % to 2 mol %) would be effective and well-tolerated when administered *in vivo*. The combination of effectiveness and low toxicity that

characterizes the claimed compositions surprised many in the field, and finally solved the delivery problem that hindered the field of siRNA drugs. *See id*., ¶168-69.

A. Long-felt need

"Evidence of a long-felt but unresolved need can weigh in favor of the non-obviousness of an invention because it is reasonable to infer the need would not have persisted had the solution been obvious." *Apple,* 839 F.3d at 1056.

The therapeutic potential of RNAi has been appreciated for over 25 years, however effectively delivering short-interfering RNA to target cells without eliciting vehicle-related toxicity has prevented realization of this potential. *See, e.g.,* EX2011 at 38, 42; EX2014 at 11; *see* EX2009 ¶¶170-71.

The mid-2000's saw dramatic growth and investment in RNAi-based therapeutics. Yet, despite $2.5-3.5 billion in investment, no solution for the delivery problem had been found. EX2015 at 1; EX2009 ¶¶171-73.

By 2008, the industry-wide failure to identify a solution to the delivery challenge resulted in waning confidence that RNAi could deliver on its therapeutic promise. EX2015 at 2, 10. Much of the delivery technologies identified around this time are inferior to Patent Owner's claimed technology. EX2015 at 10 ("Roche's Factor VII patent application (WO 2010/055041) features Alnylam's 'lipidoid' technology for the rodent studies, but then switched to [Patent Owner's] SNALP

liposomes for the nonhuman primate part of the patent application."). *See* EX2009 ¶174.

Prior to the first publication of the '435 patent disclosure, Alan Sachs, leader of RNA Therapeutics at Merck, identified delivery as the challenge to successfully developing RNAi drugs.

> What's interesting about what we do is that the drug isn't the problem.
>
> ***It's the delivery of it***.

EX2016. As explanation for the lack of viable delivery chemistries, Dr. Sachs elaborated on the nature of the challenge. *See* EX2009 ¶175.

> If it were so easy [with targeted therapeutics], one would have to describe why so few examples exist. The same is true in the RNAi delivery process. You can write down the steps. You can write down what you think will happen. But then you have to put it in a 50-nanometer particle that's safe and potent to deliver.

EX2016 at 4; *see also* EX2009 ¶176.

The long-felt need for a siRNA delivery vehicle and the difficulty in finding a solution is further exemplified by the 500 person-years and $200M that Patent Owner invested into SNALP technology. EX2015 at 8; *see also* EX2009 ¶177.

Prior to the serum-stable nucleic acid-lipid particles disclosed in the '435 patent, there were no proven solutions to the delivery problem that had long plagued the industry. EX2009 ¶178.

B. Failure of Others

"Evidence that others tried but failed to develop a claimed invention may carry significant weight in an obviousness inquiry." *Eurand*, 676 F.3d at 1081. "[L]ittle better evidence negating an expectation of success than actual reports of failure." *Boehringer Ingelheim Vetmedica, Inc. v. Schering-Plough Corp.*, 320 F.3d 1339, 1354 (Fed. Cir. 2003).

The obviousness grounds in the petition demonstrate that there is long-term evidence of a failure of others to achieve the claimed invention. The petition is based on the argument that there is a general motivation to obtain efficacious nucleic acid-lipid particles formulated or suitable for systemic delivery, yet the fact remains that, despite any general motivation, no prior art publication teaches achievement of the serum-stable nucleic acid-lipid particles of the invention. Indeed, due to toxicity and other concerns the cited references would have led a POSITA to formulate particles having low cationic lipid and relatively higher conjugated lipid to shield the particles from toxicities. *E.g.* EX2009 ¶¶25-35, 62, 174.

Despite the motivation alleged in the petition, those in the art failed to achieve a nucleic acid-lipid particles suitable for systemic delivery for decades, and instead suffered through 25 years of industry-wide failure to identify a solution to the delivery problem. The failure of others in the industry supports the non-obviousness of the '435 patent. *See* EX2009 ¶174.

C. Skepticism

"Doubt or disbelief by skilled artisans regarding the likely success of a combination or solution weighs against the notion that one would combine elements in references to achieve the claimed invention." *WBIP, LLC v. Kohler Co.*, 829 F.3d 1317, 1335 (Fed. Cir. 2016). Whether the skepticism is before or after the invention matters not. *See, e.g.*, *Transocean Offshore Deepwater Drilling, Inc. v. Maersk Drilling USA, Inc.*, 699 F.3d 1340, 1353 (Fed. Cir. 2012) (skepticism even after invention is probative of non-obviousness).

So it was here. The art widely believed that cationic lipid content should be minimized to avoid cytotoxicity, aggregation, and unwanted interactions with the immune system and non-targeted cells. *See* EX2009 ¶¶31, 32, 179.

For example, Dr. Zamore of Alnylam, acknowledged in 2003 that various cationic lipid formulations could successfully deliver siRNA to cells *in vitro* but stated that "I wouldn't want anyone injecting cationic lipids into my bloodstream." EX2011 at 42; *see* EX2009 ¶180.

Dr. Sachs of Merck also expressed skepticism, in 2010, as to the safety of the SNALP platform for delivery of siRNA.

> First are lipid-based delivery systems. At the time of our acquisition of Sirna, they had successfully shown lipid-based delivery to the liver. Initially, it was through a collaboration with what is now called [Patent Owner]. That was really the leading standard for the area. Several

> [applications to begin clinical trials] have been filed with the FDA. We spent a lot of internal research money and time on novel lipids. ***The liability of that platform is absolutely its safety***.

EX2016; *see* EX2009 ¶181.

These facts rebut the hindsight-driven analysis and assertions of the petition that the nucleic acid-lipid particle compositions of the invention would have been obvious to a POSITA in April 2008. *See* EX2009 ¶179-81.

D. Unexpected Results

Completely absent from the petition is any discussion of the real-world facts that the nucleic acid-lipid particle composition for systemic administration was met with skepticism before it was employed in clinical trials, and its results found surprising after the data were released and FDA approval granted. *See* EX2028, 54:21-55:4 (Dr. Janoff is unfamiliar with Onpattro™ (patisiran)). Indeed, it was surprising and entirely unexpected that the first FDA approved RNAi drug to meet the long-felt need for a delivery vehicle for RNAi drugs was one that does not minimize the cationic lipid component. Even more surprisingly the claimed formulation has a low level of conjugated lipid. EX2009 ¶¶182.

Patent Owner's patents and publications published after the filing date of the '435 patent provide data regarding additional compositions of claim 1. For example, in U.S. Patent No. 8,236,943 (EX2017) and U.S. Publication No. 2013/0116307 (EX2018), eight different cationic lipids were tested within 1:57 formulations.

EX2017, Figure 7; EX2018, Figure 5. Each formulation exhibited gene silencing activity far superior to that of the control PBS group. EX2009 ¶¶183-85.

Yet other 1:57 formulations were disclosed in Semple et al., *Rational Design of Cationic Lipids for siRNA Delivery*, 28 Nature Biotechnology 172-178 (2010) ("Semple," EX2021). Specifically, Semple tested 1:57 formulations that used KC2 as the cationic lipid. EX2021 at 177. Importantly, Semple measured gene silencing following systemic administration in non-human primates. EX2021 at 174. Semple found the formulations to be well-tolerated and effective. EX2021 at 175; EX2022, Table 4; *see also* EX2009 ¶186.

U.S. Publication No. 2017/0307608 to Bettencourt ("'608 publication," EX2019) discloses testing a 1:50 formulation. The '608 publication is directed to the commercial product, Onpattro™ (i.e., patisiran). The patisiran formulation is disclosed in Table 1. EX2019 ¶46, Table 1, and converting from milligrams to mol % confirms the 1:50 formulation and that patisiran is encompassed by claim 1. *See* EX2009 ¶187.

The '608 publication discloses the testing of patisiran in human subjects. As with the testing of 1:57 formulations in other animals, patisiran was well tolerated in humans. EX2019 ¶103. Patisiran effectively silenced expression of its target — the TTR protein. EX2019 ¶121. The disclosed study found clinical benefit to treatment of patients with patisiran. EX2019 ¶132; *see* EX2009 ¶188.

As explained by Dr. Thompson, a POSITA would understand that the surprising and unexpected results of the '435 patent as exemplified above are not limited to formulations comprising specific ratios of components or comprising a specific type of cationic lipid. Specifically, between the '435 patent and subsequent publications, nucleic acid-lipid particle compositions of claim 1 were tested and found efficacious and well tolerated. Nucleic acid-lipid particle formulations with eight different cationic lipids were tested and found efficacious and well tolerated. Finally, nucleic acid-lipid particle formulations within the scope of the claims were found efficacious and well tolerated in non-human primates and humans. These data span nearly the entire claimed ranges of cationic and conjugated lipid and are surprising and entirely unexpected in view of the state of the art as of April 2008. *See* EX2009 ¶189.

E. Commercial Success

Patisiran was developed by Alnylam Pharmaceuticals under license from Patent Owner. Specifically, Alnylam licenses the nucleic acid-lipid particle technology claimed in the '435 patent from Patent Owner. EX2026. Under the license, Alnylam owes Patent Owner royalties on the patisiran product. *Id.*; *see also* EX2009 ¶¶191-93.

XI. THE PETITION DOES NOT COMPORT WITH STATUTORY REQUIREMENTS

The statute governing *inter partes* review requires that any ground for unpatentability be presented "with particularity." 53 USC §312(b); *see also* 37 CFR §42.104(b). Moreover, in *SAS*, the Supreme Court noted that §311(a) of the statute does not contemplate that the Director may "depart from the petition and institute a *different* inter partes review of his design." *SAS Inst., Inc. v. Iancu*, 138 S. Ct. 1348, 1356 (2018).

All three grounds presented in the petition include various alternate legal theories and/or different permutations or combinations of the "Patent Owner's prior disclosures." In some instances, the stated grounds are so convoluted that it is unclear what basis of review Petitioner is requesting. Ground 3 is particularly egregious. Ground 3 is presented as alternate legal theories of anticipation or obviousness, none of which are sufficiently explained, and no single theory comprehensively addresses the entire set. The petition renders it impossible to institute a trial in compliance with *SAS*.

While raised earlier, these issues were not addressed in the Institution Decision. See POPR at 19-22; EX2008 at X. In fact, the Institution Decision compounded these issues when it rejected the petition's overbroad construction of "nucleic acid-lipid particle," Paper 15 at X, thereby instituting trial of the Board's own design. *SAS*, 138 S. Ct. at 1356.

XII. CONCLUSION

For at least the reasons set forth above, petitioner has failed to meet its burden and the challenged claims should be found *not unpatentable*.

Respectfully submitted,

Date: December 21, 2018

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
Reg. No. 52,182

CERTIFICATE OF COMPLIANCE

Pursuant to §42.24(d), the undersigned certifies that this paper contains no more than 14,000 words, not including the portions of the paper exempted by §42.24(b). According to the word-processing system used to prepare this paper, the paper contains 13,826 words.

Respectfully submitted,

Date: December 21, 2018

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
Reg. No. 52,182

XIII. APPENDIX

EXHIBIT NO.	DESCRIPTION
2001	Declaration of Edward R. Reines in Support of Patent Owner's Motion for *Pro Hac Vice* Admission
2002	*In re Reines*, No. 14-MA004 (14-4) (Fed. Cir. Nov. 5, 2014)
2003	Personal Statement of Edward R. Reines
2004	Tam P. et al., *Stabilized Plasmid-Lipid Particles for Systemic Gene Therapy* 7 GENE THERAPY 1867-1874 (2000)
2005	Huang L. et al., *Liposomal Gene Delivery: A Complex Package* 15 NATURE BIOTECHNOLOGY 620-621 (1997)
2006	Pak C.C., Erukulla R.K., Ahl, P.L., Janoff, A.S. and Meers, P., *Elastase-Activated Liposomal Delivery to Nucleated Cells*. 1419 BIOCHIM. BIOPHY. ACTA 111-126 (1999)
2007	U.S. Patent No. 7,491,409
2008	Transcript of October 2, 2018 Conference Call
2009	Declaration of David H. Thompson, Ph.D.
2010	*Curriculum Vitae* of David H. Thompson, Ph.D.
2011	Charles W. Schmidt, *Therapeutic Interference: Small RNA Molecules Act as Blockers of Disease Metabolism* AM. CHEM. SOC'Y 37 (2003)

2012	C. Russell Middaugh & Joshua D. Ramsey, *Analysis of Cationic-Lipid-Plasmid-DNA Complexes*, ANALYTICAL CHEMISTRY 7240 (2007)
2013	*Liposome Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation, Guidance for Industry*, FOOD AND DRUG ADMINISTRATION (2018)
2014	Erika Check, *RNA to the Rescue?* 425 NATURE 10 (2003)
2015	Dirk Hausseker, *The Business of RNAi Therapeutics in 2012* 2 AM. SOC'Y OF GENE & CELL THERAPY (2012)
2016	Luke Timmerman, *Merck's Alan Sachs, on RNAi's Big Challenge: Delivery, Delivery, Delivery*, XCONOMY (Jan. 21, 2010), https://xconomy.com/national/2010/01/21/mercks-alan-sachs-on-rnais-big-challenge-delivery-delivery-delivery/
2017	U.S. Patent No. 8,236,943
2018	U.S. Publication No. 2013/0116307
2019	U.S. Publication No 2017/0307608
2020	Combined Declaration for Patent Application and Power of Attorney in U.S. Patent Application No. 90/914,615.
2021	Sean C. Semple, et al., *Rational Design of Cationic Lipids for siRNA Delivery*, 28 NATURE BIOTECH. 172 (2010)
2022	Supplementary Figures to Sean C. Semple, et al., *Rational Design of Cationic Lipids for siRNA Delivery*, 28 NATURE BIOTECH. 172 (2010)
2023	Heidi Ledford, *Gene-Silencing Drug Approved: US Government Okays First RNA-Interference Drug — After a 20-Year Wait* 560 NATURE 291 (2018)

2024	*FDA Approves First-of-its Kind Targeted RNA-based Therapy to Treat a Rare Disease*, FOOD AND DRUG ADMIN. (2018), https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm616518.htm
2025	*New Medicine for Hereditary Rare Disease,* EUROPEAN MED. AGENCY (2018), https://www.ema.europa.eu/en/news/new-medicine-hereditary-rare-disease
2026	*Arbutus' LNP Licensee Alnylam Announces FDA Approval of ONPATTRO™ (patisiran), for the Treatment of ATTR Amyloidosis*, ARBUTUS BIOPHARMA (2018), https://investor.arbutusbio.com/news-releases/news-release-details/arbutus-lnp-licensee-alnylam-announces-fda-approval-onpattrotm
2027	Orange Book: Approved Drug Products with Therapeutic Equivalence Evaluations, *Patent and Exclusivity for: N210922*, FOOD AND DRUG ADMIN. *available at* https://www.accessdata.fda.gov/scripts/cder/ob/patent_info.cfm?Product_No=001&Appl_No=210922&Appl_type=N (last visited Dec. 19, 2018)
2028	Deposition Transcript of Andrew S. Janoff, December 4, 2018
2029	U.S. Patent No. 9,404,127
2030	Intentionally Left Blank
2031	Intentionally Left Blank
2032	Ian MacLachlan & Pieter Cullis, *Diffusible-PEG-Lipid Stabilized Pasmid Lipid Particles*, 53 ADVANCES IN GENETICS 157 (2005)
2033	Sean C. Semple et al., *Immunogenicity and Rapid Blood Clearance of Liposomes Containing Polyethylene Glycol-Lipid Conjugates and Nucleic Acid*, 312 THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS 1020 (2005)
2034	Doxil Label – FDA (Revised May, 2007), https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/050718s029lbl.pdf

2035-2039	Intentionally Left Blank
2040	Declaration of David H. Thompson, Ph.D. in Support of Patent Owner's Contingent Motion to Amend
2041	U.S. Provisional Patent Application Number 61/045,228
2042	U.S. Patent Application Number 12/424,367
2043	U.S. Patent Application Number 13/253,917
2044	U.S. Patent Application Number 13/928,309
2045	U.S. Patent Application Number 14/462,441

CERTIFICATE OF SERVICE

I certify that the foregoing Patent Owner's Response Pursuant to 37 C.F.R. § 42.120 and Exhibits 2009-2045 were served on this 21st day of December, 2018, on the Petitioner at the following electronic service addresses:

Michael Fleming
C. Maclain Wells
IRELL & MANELLA LLP
mfleming@irell.com
mwells@irell.com
ModernaIPR@irell.com

Respectfully submitted,

Date: December 21, 2018

/ Michael T. Rosato /
Michael T. Rosato, Lead Counsel
Reg. No. 52,182