JOINT APPENDIX 01

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July 13, 2022

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PATENT NUMBER: *8,058,069*
ISSUE DATE: *November 15, 2011*

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

Rodney Glover
Certifying Officer

US008058069B2

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

(10) **Patent No.: US 8,058,069 B2**
(45) **Date of Patent: Nov. 15, 2011**

(54) **LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY**

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(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 0 days.

(21) Appl. No.: **12/424,367**

(22) Filed: **Apr. 15, 2009**

(65) **Prior Publication Data**

US 2010/0130588 A1 May 27, 2010

Related U.S. Application Data

(60) Provisional application No. 61/045,228, filed on Apr. 15, 2008.

(51) **Int. Cl.**
C07H 21/04 (2006.01)
C12N 15/88 (2006.01)

(52) **U.S. Cl.** **435/458**; 536/24.5

(58) **Field of Classification Search** 536/24.5; 435/458

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,394,448	A	7/1983	Szoka, Jr. et al.	
4,438,052	A	3/1984	Weder et al.	
4,515,736	A	5/1985	Deamer	
4,598,051	A	7/1986	Papahadjopoulos et al.	
4,897,355	A	1/1990	Eppstein et al.	
5,013,556	A	5/1991	Woodle et al.	
5,171,678	A	12/1992	Behr et al.	
5,208,036	A	5/1993	Eppstein et al.	
5,225,212	A	7/1993	Martin et al.	
5,264,618	A	11/1993	Felgner et al.	
5,279,833	A	1/1994	Rose	
5,283,185	A	2/1994	Epand et al.	
5,320,906	A	6/1994	Eley et al.	
5,545,412	A	8/1996	Eppstein et al.	
5,578,475	A	11/1996	Jessee et al.	
5,641,662	A	6/1997	Debs et al.	
5,656,743	A	8/1997	Busch et al.	
5,703,055	A	12/1997	Felgner et al.	
5,705,385	A	1/1998	Bally et al.	
5,820,873	A	10/1998	Choi et al.	
5,976,567	A	11/1999	Wheeler et al.	
5,981,501	A	11/1999	Wheeler et al.	
6,534,484	B1	3/2003	Wheeler et al.	
6,586,410	B1	7/2003	Wheeler et al.	
6,649,780	B1	11/2003	Eibl et al.	
6,815,432	B2	11/2004	Wheeler et al.	
6,858,224	B2	2/2005	Wheeler et al.	
7,422,902	B1	9/2008	Wheeler et al.	
7,799,565	B2 *	9/2010	MacLachlan et al.	435/458
7,807,815	B2 *	10/2010	MacLachlan et al.	536/24.5
7,838,658	B2 *	11/2010	MacLachlan et al.	536/24.5
2001/0048940	A1	12/2001	Tousignant et al.	
2003/0077829	A1	4/2003	MacLachlan	
2003/0143732	A1	7/2003	Fosnaugh et al.	
2004/0063654	A1	4/2004	Davis et al.	
2004/0142892	A1	7/2004	Finn et al.	
2004/0253723	A1	12/2004	Tachas et al.	
2004/0259247	A1	12/2004	Tuschl et al.	
2005/0064595	A1	3/2005	MacLachlan et al.	
2005/0118253	A1	6/2005	MacLachlan et al.	
2006/0008910	A1	1/2006	MacLachlan et al.	
2007/0042031	A1	2/2007	MacLachlan et al.	
2009/0291131	A1 *	11/2009	Maclachlan et al.	424/450

FOREIGN PATENT DOCUMENTS

WO	WO 91/16024	A1	10/1991
WO	WO 93/05162	A1	3/1993
WO	WO 93/12240	A1	6/1993
WO	WO 93/12756	A2	7/1993
WO	WO 93/24640	A2	12/1993
WO	WO 93/25673	A1	12/1993
WO	WO 95/02698	A1	1/1995
WO	WO 95/18863	A1	7/1995
WO	WO 95/35301	A1	12/1995
WO	WO 96/02655	A1	2/1996
WO	WO 96/10390	A1	4/1996
WO	WO 96/40964	A2	12/1996
WO	WO 96/41873	A1	12/1996
WO	WO 01/05374	A1	1/2001
WO	WO 02/034236	A2	5/2002
WO	WO 02/087541	A1	11/2002
WO	WO 03/097805	A2	11/2003
WO	WO 2004/065546	A2	8/2004
WO	WO 2004/110499	A1	12/2004

(Continued)

OTHER PUBLICATIONS

Arpicco, S., et al., "Preparation and Characterization of Novel Cationic Lipids Developed for Gene Transfection," Proceed. Int'l Symp. Control. Rel. Bioact. Mater. (Controlled Release Society, Inc.), 1999, vol. 26, pp. 759-760.

Arpicco, S., et al., "Synthesis, characterization and transfection activity of new saturated and unsaturated cationic lipids," IL Farmaco, 2004, vol. 59, pp. 869-878.

Ballas, N., et al., "Liposomes bearing a quatemary ammonium detergent as an efficient vehicle for functional transfer of TMV-RNA into plant protoplasts," Biochimica et Biophysica Acta, 1988, vol. 939, pp. 8-18.

(Continued)

Primary Examiner — Brian Whiteman
(74) *Attorney, Agent, or Firm* — Kilpatrick Townsend & Stockton LLP

(57) **ABSTRACT**

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.

22 Claims, 24 Drawing Sheets

FOREIGN PATENT DOCUMENTS

WO	WO 2005/007196	A2	1/2005
WO	WO 2005/026372	A1	3/2005
WO	WO 2005/120152	A2	12/2005
WO	WO 2009/086558	A1	7/2009
WO	WO 2009/111658	A2	9/2009
WO	WO 2010/042877	A1	4/2010
WO	WO 2010/048228	A2	4/2010
WO	WO 2010/088537	A2	8/2010
WO	WO 2010/105209	A1	9/2010

OTHER PUBLICATIONS

Barinaga, M., "Step Taken Toward Improved Vectors for Gene Transfer," Science, 1994, vol. 266, p. 1326.

Bass, *Nature*, 2001, 411: 428-9.

Beale, G., et al., "Gene Silencing Nucleic Acids Designed by Scanning Arrays: Anti-EGFR Activity of siRNA, Ribozyme and DNA Enzymes Targeting a Single Hybridization-accessible Region using the Same Delivery System," Journal of Drug Targeting, 2003, vol. 11, No. 7, pp. 449-456.

Behr, J.-P., "Synthetic Gene-Transfer Vectors," Acc. Chem. Res., 1993, vol. 26, pp. 274-278.

Brigham, K., et al., "Rapid Communication: In vivo Transfection of Murine Lungs with a Functioning Prokaryotic Gene Using a Liposome Vehicle," The American Journal of the Medical Sciences, vol. 298, No. 4, pp. 278-281, 1989.

Brummelkamp, et al., "A System for Stable Expression of Short Interfering RNAs in Mammalian Cells," Science, 2002, V. 296. pp. 550-553.

Cevc, G., "How Membrane Chain-Melting Phase-Transition Temperature is Affected by the Lipid Chain Asymmetry and Degree of Unsaturation: An Effective Chain-Length Model," Biochemistry, 1991, vol. 30, pp. 7186-7193.

Cortesi, R., et al., "Effect of cationic liposome composition on in vitro cytotoxicity and protective effect on carried DNA," International Journal of Pharmaceutics, 1996, vol. 139, pp. 69-78.

Crystal, R., "Transfer of Genes to Humans: Early Lessons and Obstacles to Success," Science, 1995, vol. 270, pp. 404-410.

Culver K., "The First Human Gene Therapy Experiment," Gene Therapy: A Handbook for Physicians, 1994, pp. 33-40.

Duzgunes, N., "Membrane Fusion," Subcellular Biochemistry, 1985, vol. 11, pp. 195-286.

Dwarki, V.J., et al., "Cationic Liposome-Mediated RNA Transfection," Methods in Enzymology, 1993, vol. 217, pp. 644-654.

Enoch, H., et al., "Formation and properties of 1000-Å-diameter, single-bilayer phospholipid vesicles," Proc. Natl. Acad. Sci. USA, 1979, vol. 76, No. 1, pp. 145-149.

Felgner, J., et al., "Cationic Lipid-Mediated Transfection in Mammalian Cells: 'Lipofection,'" J. Tiss. Cult. Meth., 1993, vol. 15, pp. 63-68.

Felgner, J., et al., "Enhanced Gene Delivery and Mechanism Studies with a Novel Series of Cationic Lipid Formulations," The Journal of Biological Chemistry, 1994, vol. 269, No. 4, pp. 2550-2561.

Felgner, P., et al., "Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure," Proc. Natl. Acad. Sci. USA, 1987, vol. 84, pp. 7413-7417.

Felgner, P.L., et al., "Cationic Liposome Mediated Transfection," Proc. West. Pharmacol. Soc., 1989, vol. 32, pp. 115-121.

Gao, X., et al., "A Novel Cationic Liposome Reagent for Efficient Transfection of Mammalian Cells," Biochem. Biophys. Res. Comm., 1991, vol. 179, No. 1, pp. 280-285.

Gershon, H., et al., "Mode of Formation and Structural Feature of DNA-Cationic Liposome Complexes Used for Transfection," Biochemistry, 1993, vol. 32, pp. 7143-7151.

Guy-Caffey, J., et al., "Novel Polyaminolipids Enhance the Cellular Uptake of Oligonucleotides," The Journal of Biological Chemistry, 1995, vol. 270, No. 52, pp. 31391-31396.

Hawley-Nelson, P., et al., "LipofectAmine™ Reagent: A New, Higher Efficiency Polycationic Liposome Transfection Reagent," Focus, 1993, vol. 15, No. 3, pp. 73-80.

Hyde, S., et al., "Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy," Nature, 1993, vol. 362, pp. 250-255.

Jiang, L., et al., "Comparison of protein precipitation methods for sample preparation prior to proteomic analysis," Journal of Chromatography A, 2004, vol. 1023, pp. 317-320.

Juliano, R., et al., "The Effect of Particle Size and Charge on the Clearance Rates of Liposomes and Liposome Encapsulated Drugs," Biochem. Biophys. Res. Commun., 1975, vol. 63, No. 3, pp. 651-658.

Keough, K., "Influence of chain unsaturation and chain position on thermotropism and intermolecular interactions in membranes," Biochem. Soc. Transactions, 1990, vol. 18, No. 5, pp. 835-837.

Legendre, J.-Y. et al., "Delivery of Plasmid DNA into Mammalian Cell Lines Using pH- Sensitive Liposomes: Comparison with Cationic Liposomes," Pharm. Res., 1992, vol. 9, No. 10, pp. 1235-1242.

Leventis, R., et al., "Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles," Biochem. Biophys. Acta, 1990, vol. 1023, pp. 124-132.

Liu, et al., "Cationic Liposome-mediated Intravenous Gene Delivery", J. Biol. Chem., 1995, V. 270, pp. 24864-24870.

Marshall, E., "Gene Therapy's Growing Pains," Science, 1995, vol. 269, pp. 1050-1055.

Orkin, S., et al., NIH Report, Report and Recommendations of the Panel to Assess the NIH Investment in Research on Gene Therapy, 1995.

Paul, C., et al., "Effective expression of small interfering RNA in human cells," Nature Biotech., 2002, vol. 20, pp. 505-508.

Puyal, C., et al., "A new cationic liposome encapsulating genetic material: A potential delivery system for polynucleotides," Eur. J. Biochem., 1995, vol. 228, pp. 697-703.

Sorensen, et al., "Gene Silencing by Systemic Delivery of Synthetic siRNAs in Adult Mice", J. Biol. Chem., 2003, V. 327, pp. 761-766.

Spagnou, S., et al., "Lipidic Carriers of siRNA: Differences in the Formulation, Cellular Uptake, and Delivery with Plasmid DNA," Biochemistry, 2004, vol. 43, pp. 13348-13356.

Stamatatos, L., et al., "Interactions of Cationic Lipid Vesicles with Negatively Charged Phospholipid Vesicles and Biological Membranes," Biochemistry, 1988, vol. 27, pp. 3917-3925.

Szoka, F., et al., "Comparative Properties and Methods of Preparation of Lipid Vesicles (Liposomes)," Ann. Rev. Biophys. Bioeng., 1980, vol. 9, pp. 467-508.

Szoka, F., et al., "Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation," Proc. Natl. Acad. Sci. USA, 1978, vol. 75, No. 9, pp. 4194-4198.

Templeton, "Cationic Liposome-mediated Gene Delivery In vivo", Bioscience Reports, 2002, vol. 22, No. 2, pp. 283-295.

Van Der Woude, I., et al., "Parameters influencing the introduction of plasmid DNA into cells by the use of synthetic amphiphiles as a carrier system," Biochimica et Biophysica Acta, 1995, vol. 1240, pp. 34-40.

Wheeler, et al., "Stabilized Plasmid-lipid Particles: Constructions and Characterization," Gene Therapy, V. 6, pp. 271-281, 1999.

Wilson, R., et al., "Counterion-Induced Condensation of Deoxyribonucleic Acid," A Light-Scattering Study, Biochemistry, 1979, vol. 18, No. 11, pp. 2192-2196.

Woodle, M.C., et al., "Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes," Biochimica et Biophysica Acta, 1992, vol. 1105, pp. 193-200.

Zhu, N., et al., "Systemic Gene Expression After Intravenous DNA Delivery into Adult Mice," Science, 1993, vol. 261, pp. 209-211.

Elbashir, et al.; Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells; Nature; May 2001; pp. 494-498; vol. 411.

* cited by examiner

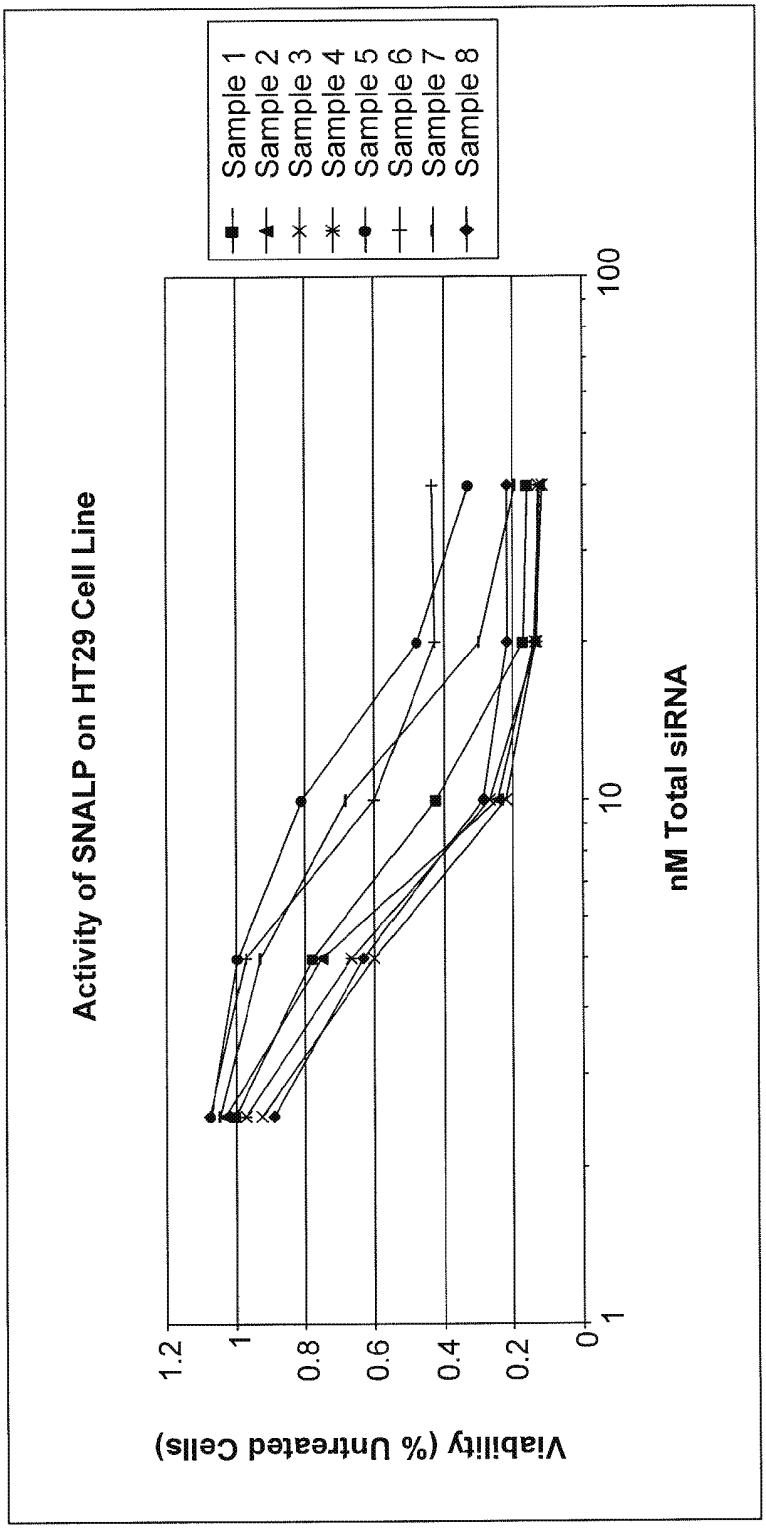

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
nM Total siRNA
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100

FIG. 1A

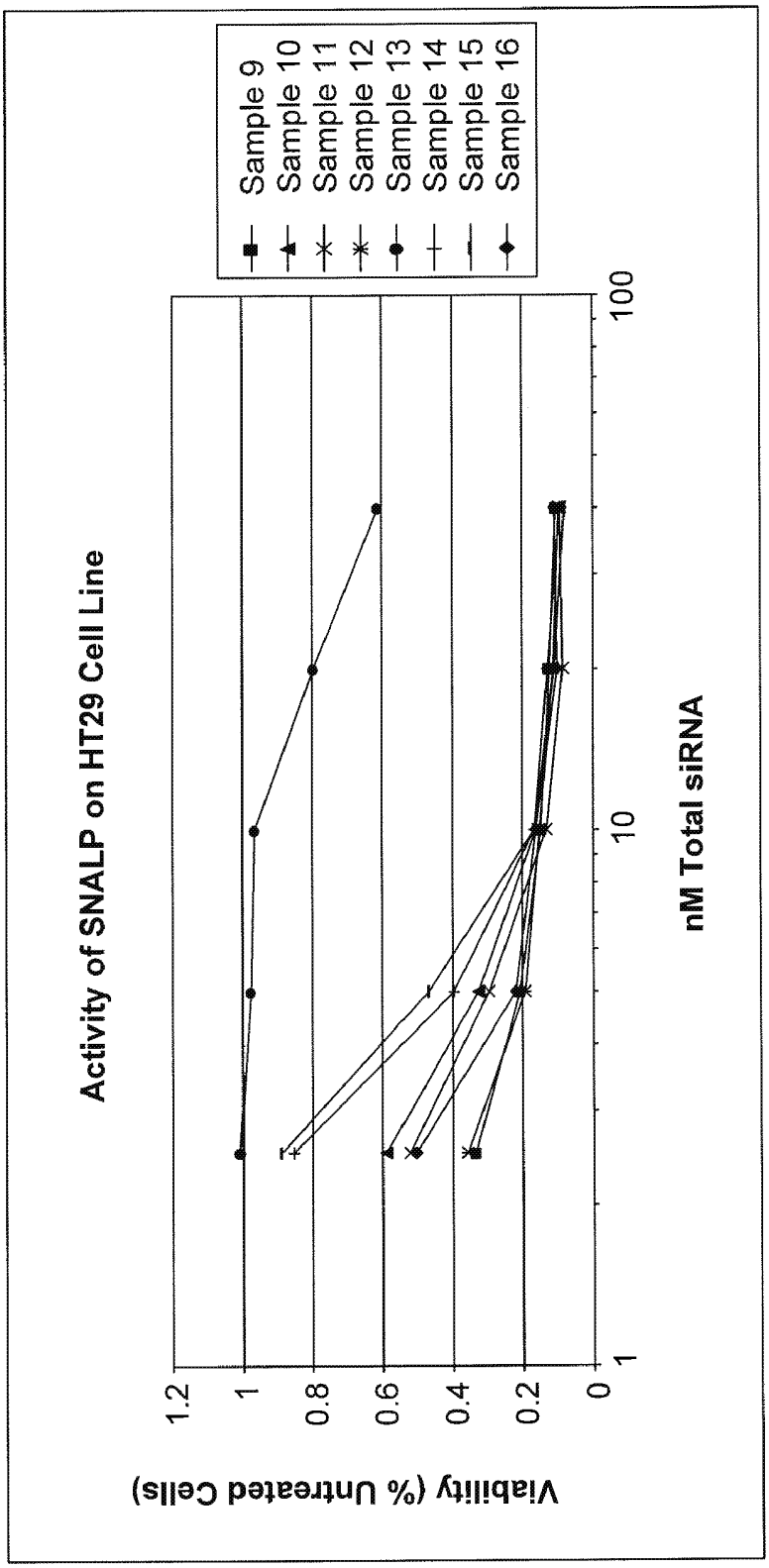

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 9
Sample 10
Sample 11
Sample 12
Sample 13
Sample 14
Sample 15
Sample 16

FIG. 1B

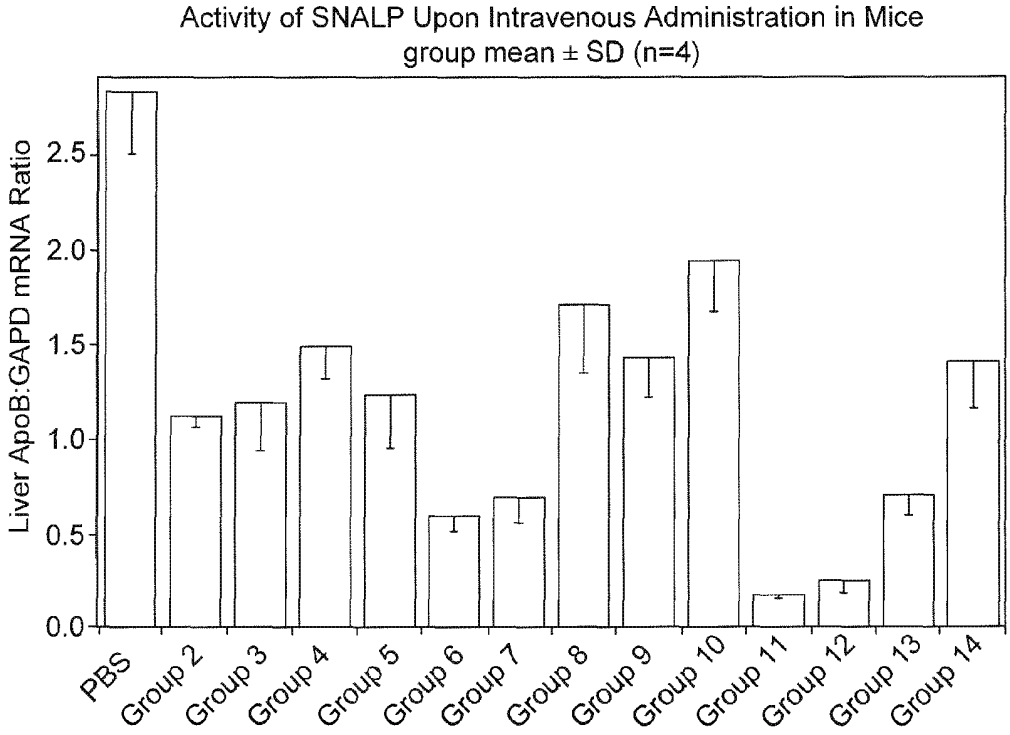

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.5
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

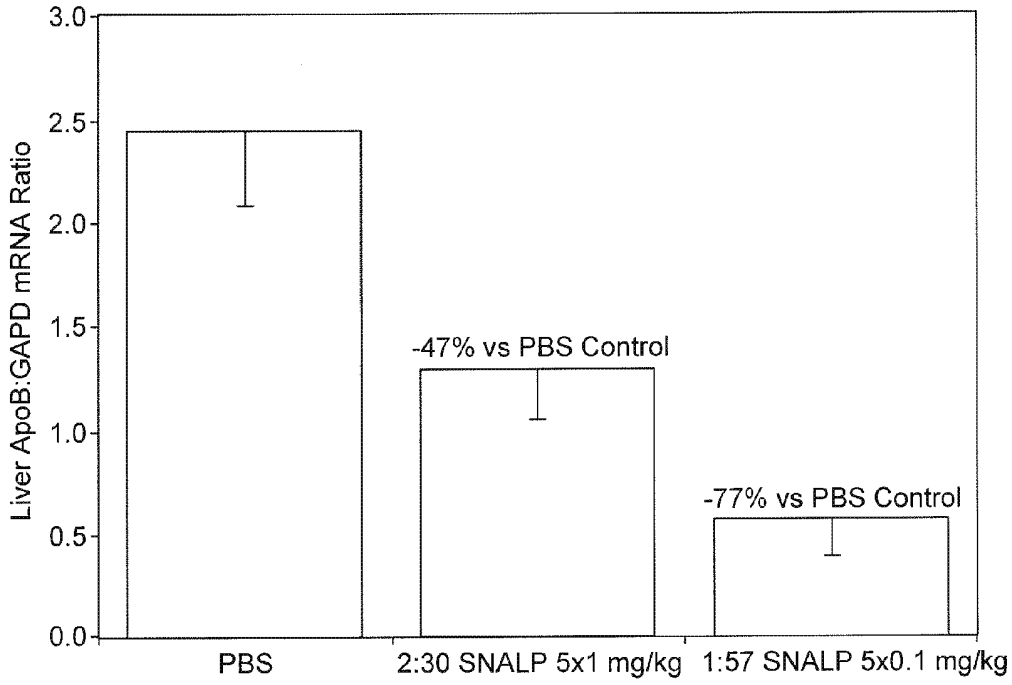

Liver ApoB:GAPD mRNA Ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5x1 mg/kg
1:57 SNALP 5x0.1 mg/kg

FIG. 3

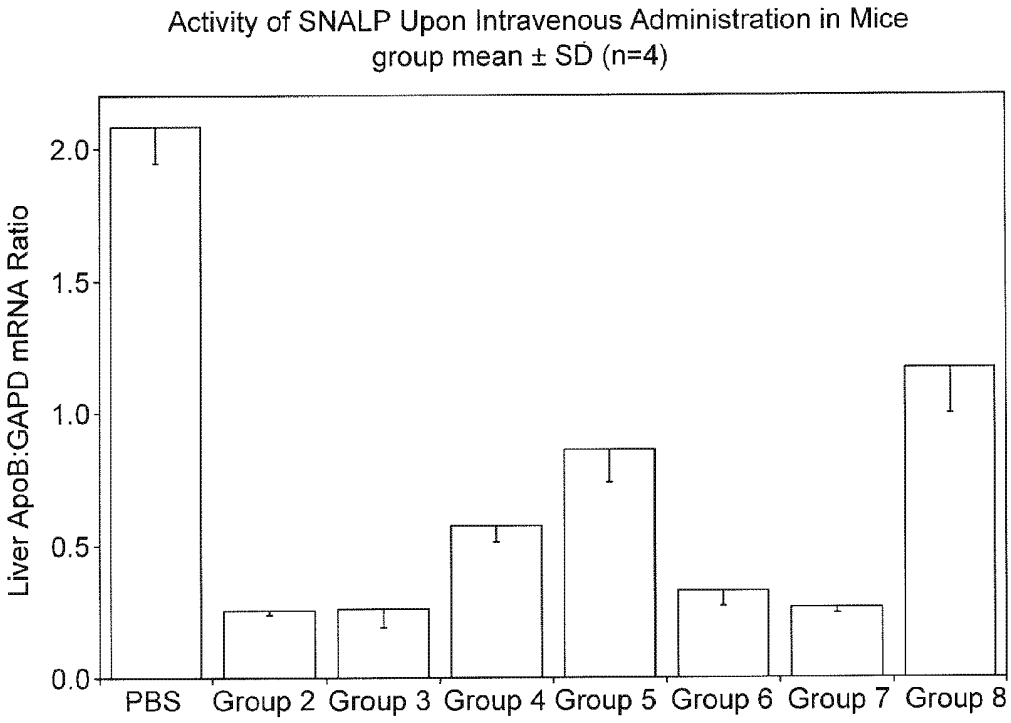

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

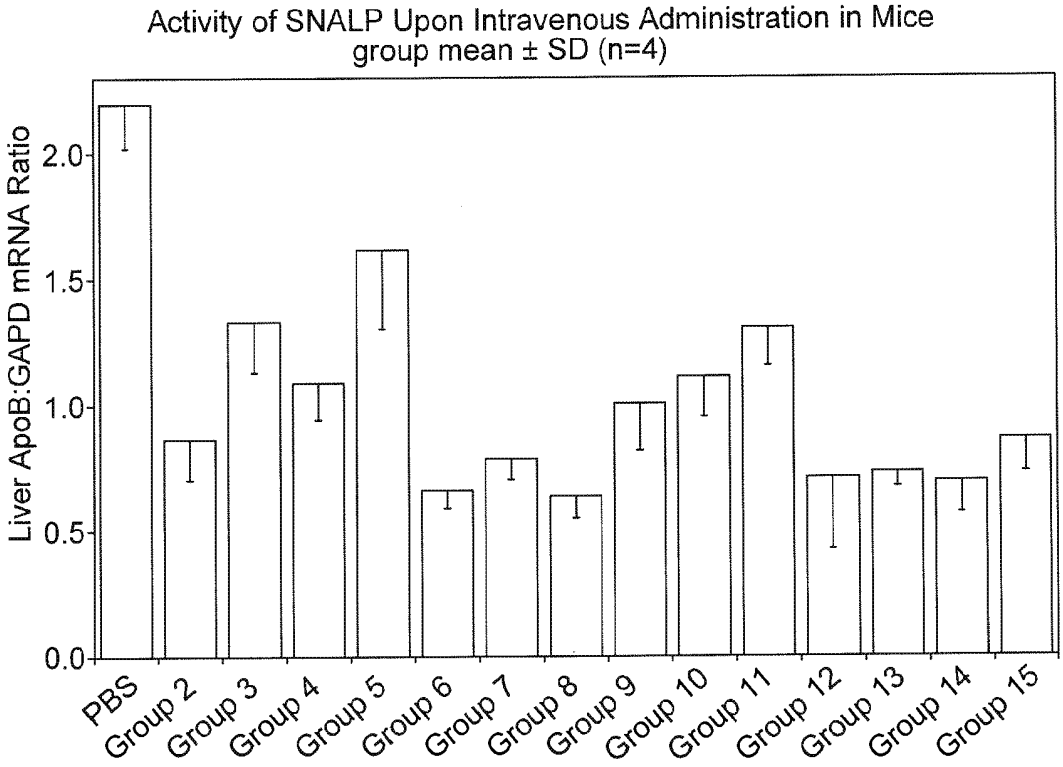

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

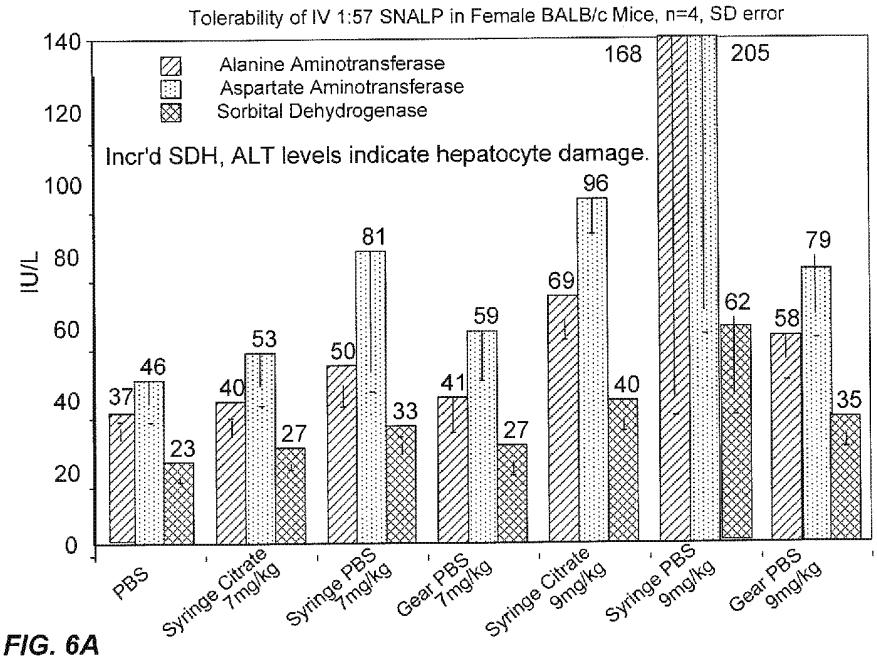

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
0
20
40
60
80
100
120
140
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
168
205
62
58
79
35
PBS
Syringe 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

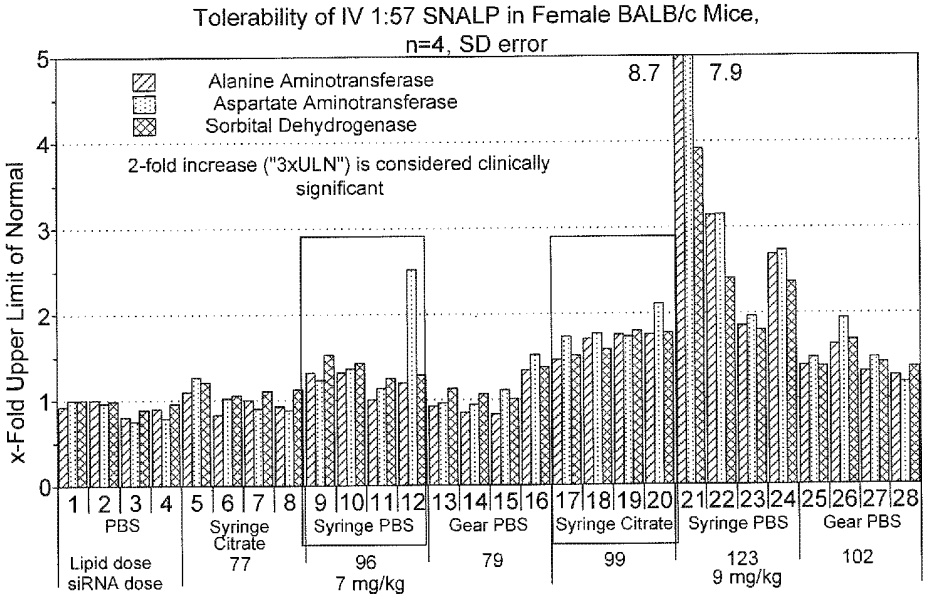

Tolerability of IV 1:57 SNALP in Female BALB/c Mice,
n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
1
2
3
4
5
8.7
7.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 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

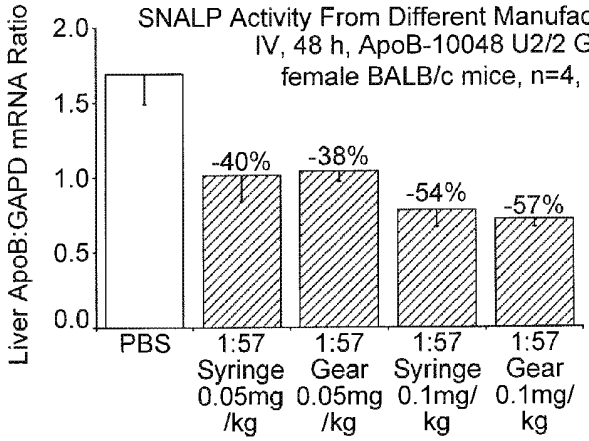


FIG. 7B

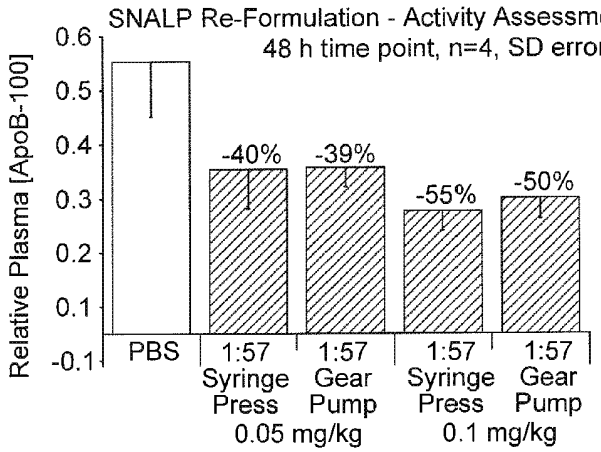
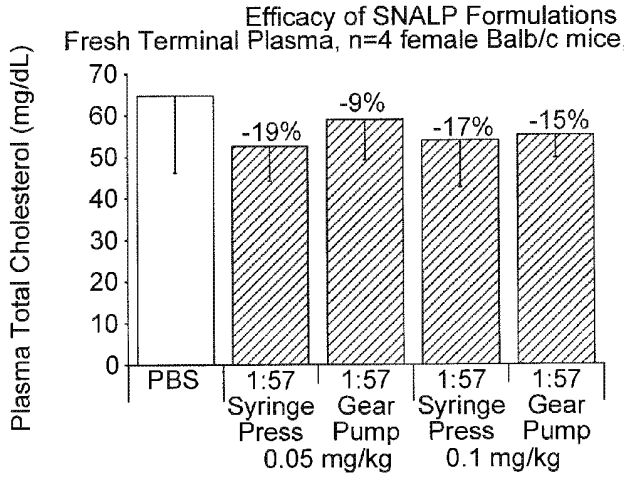


FIG. 7C



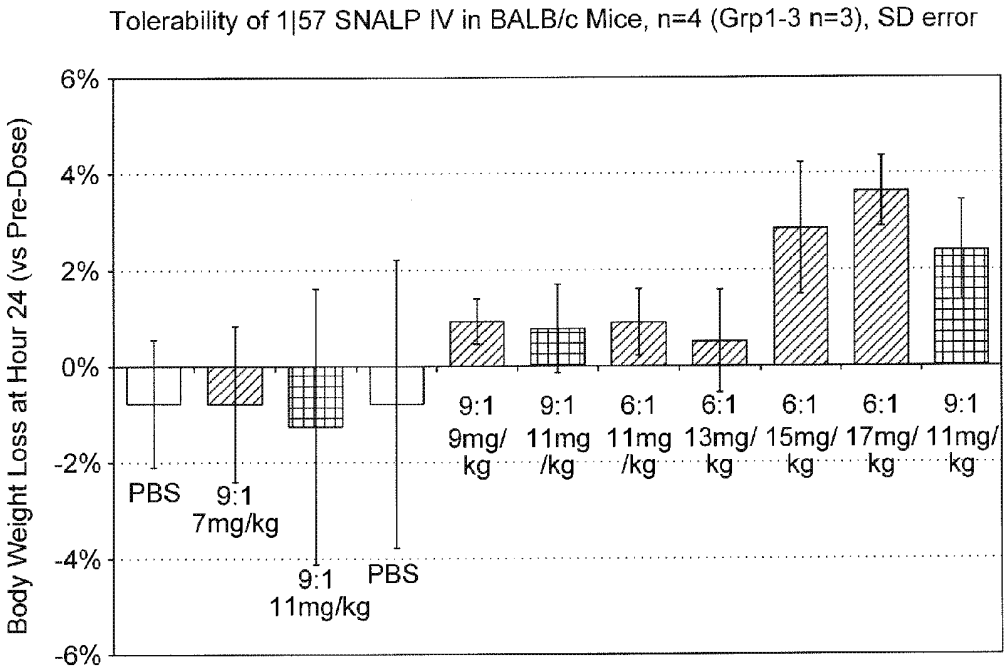

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

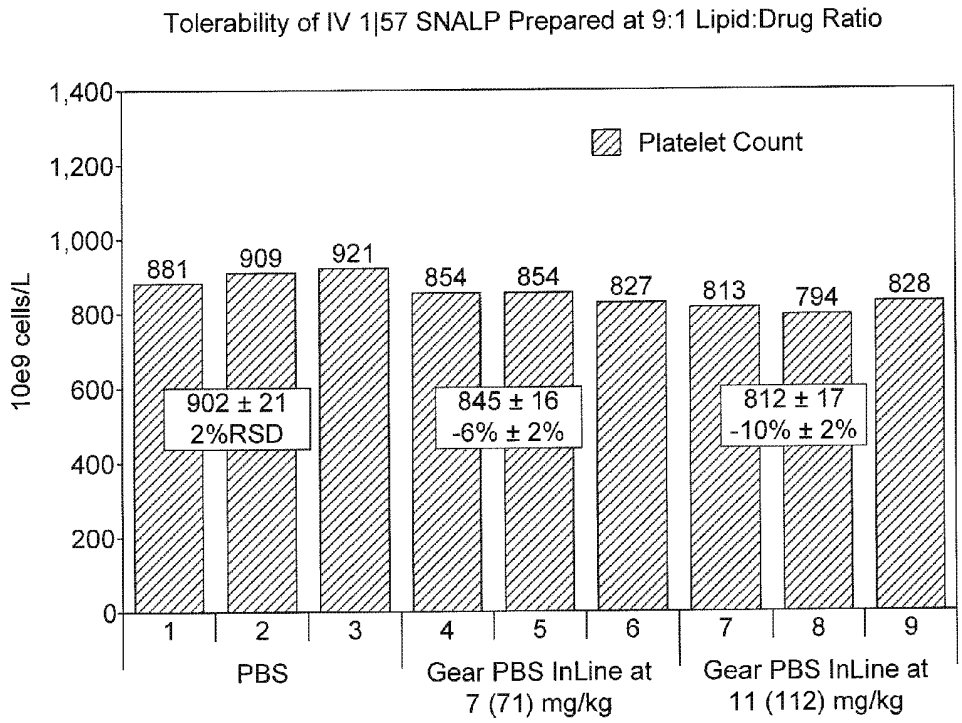

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
Platelet Count
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at
7 (71) mg/kg
Gear PBS InLine at
11 (112) mg/kg

FIG. 9

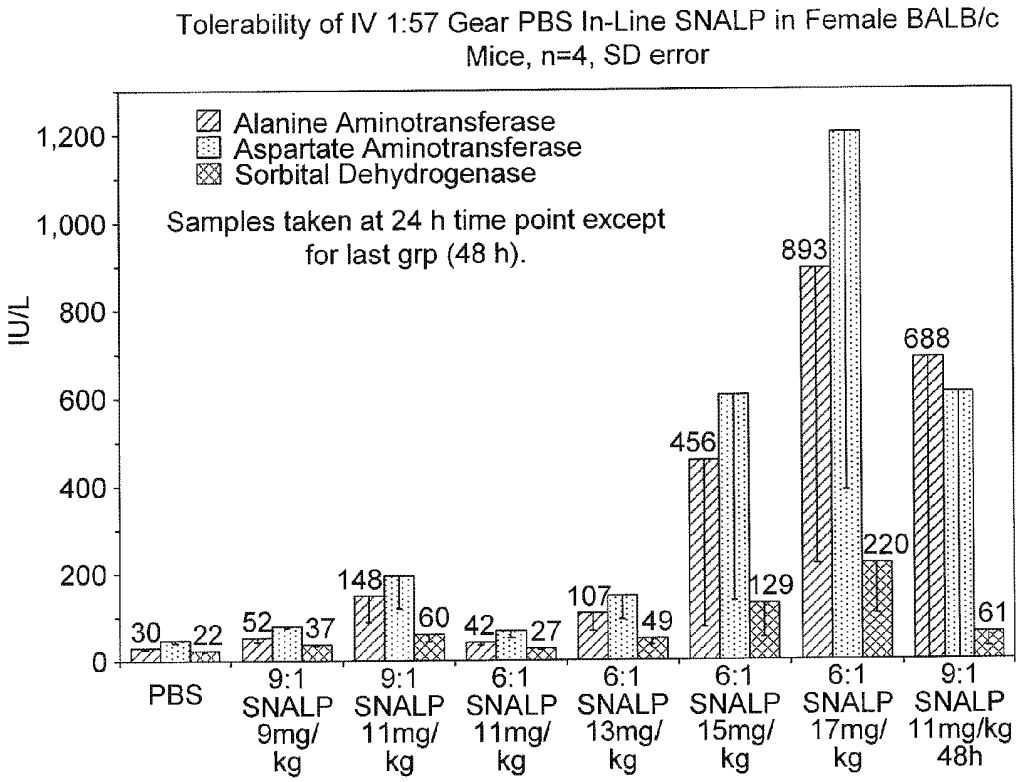

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
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

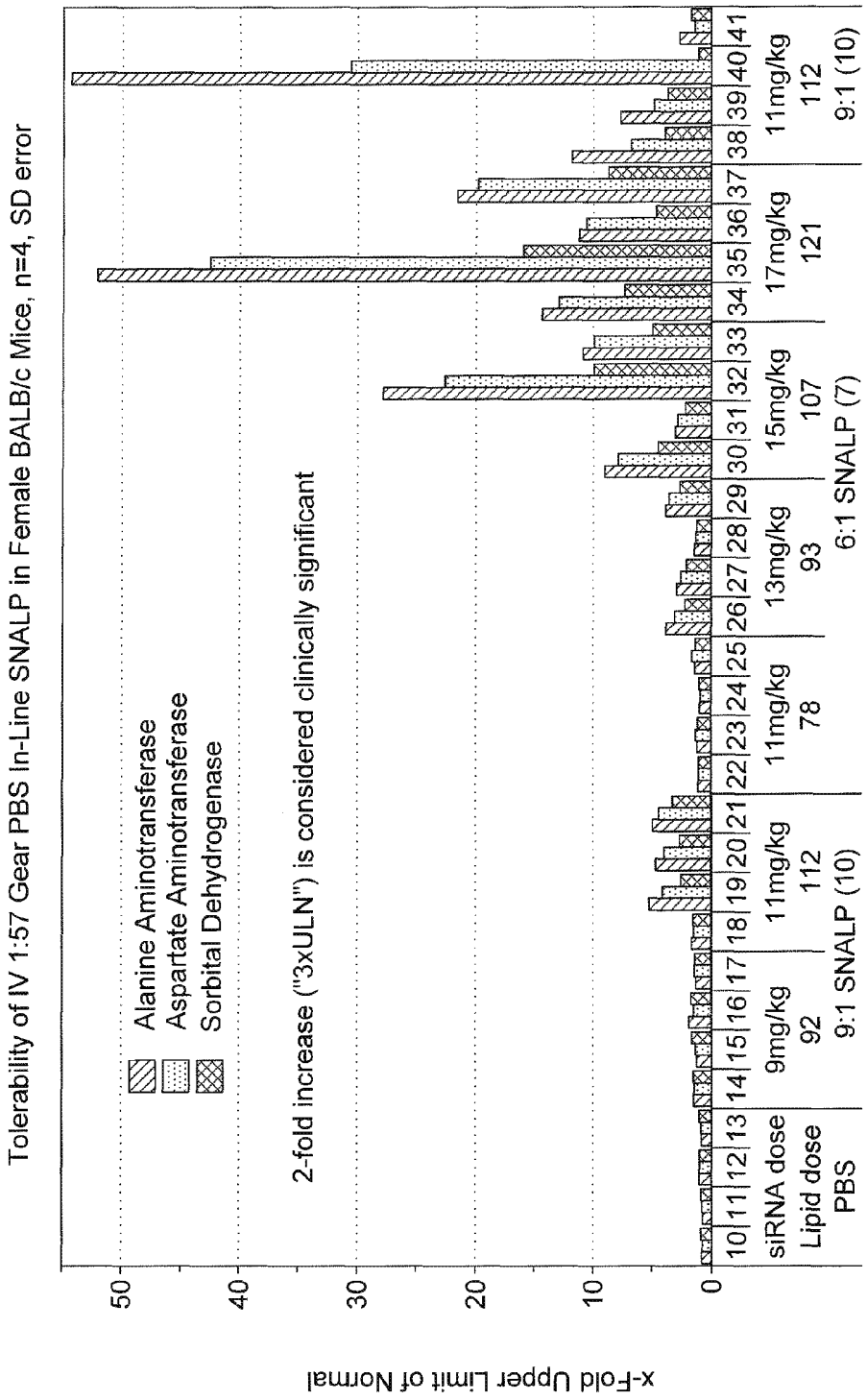

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
10
20
30
40
50
10 11 12 13 14 15 16 17 18 19 20 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

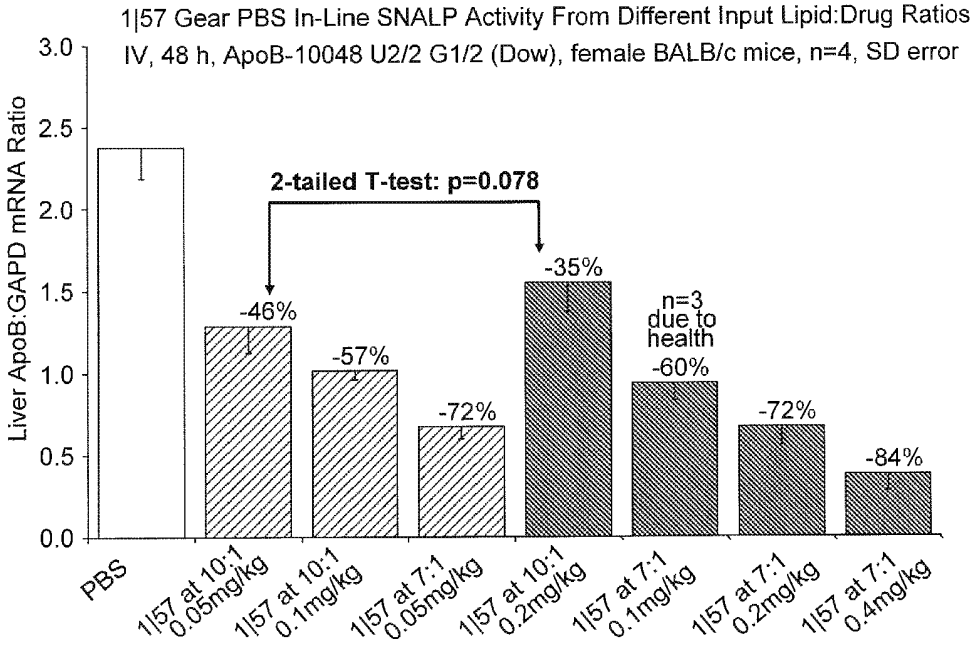
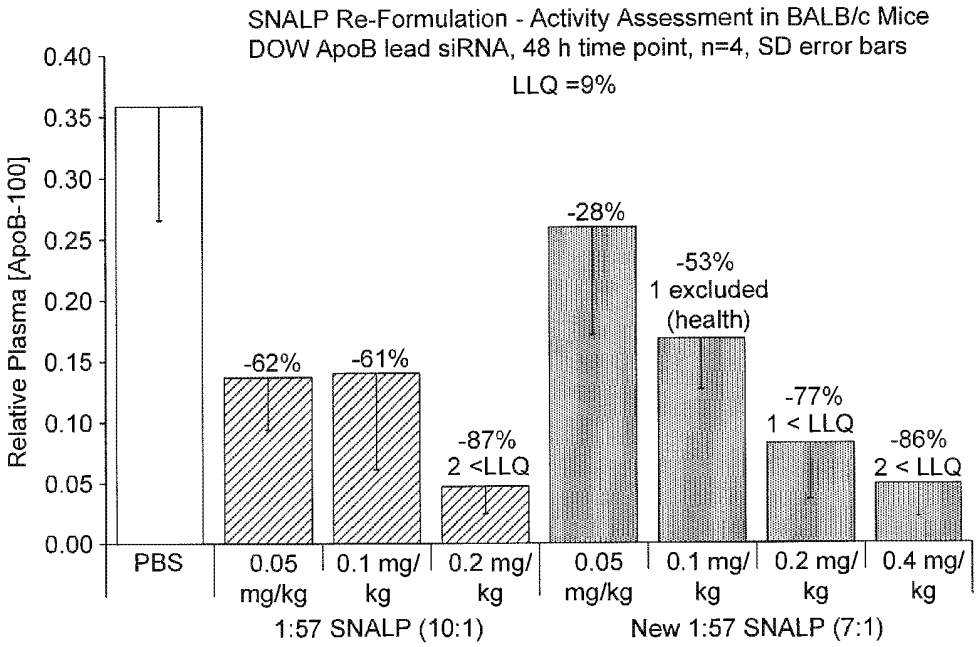


FIG. 11B



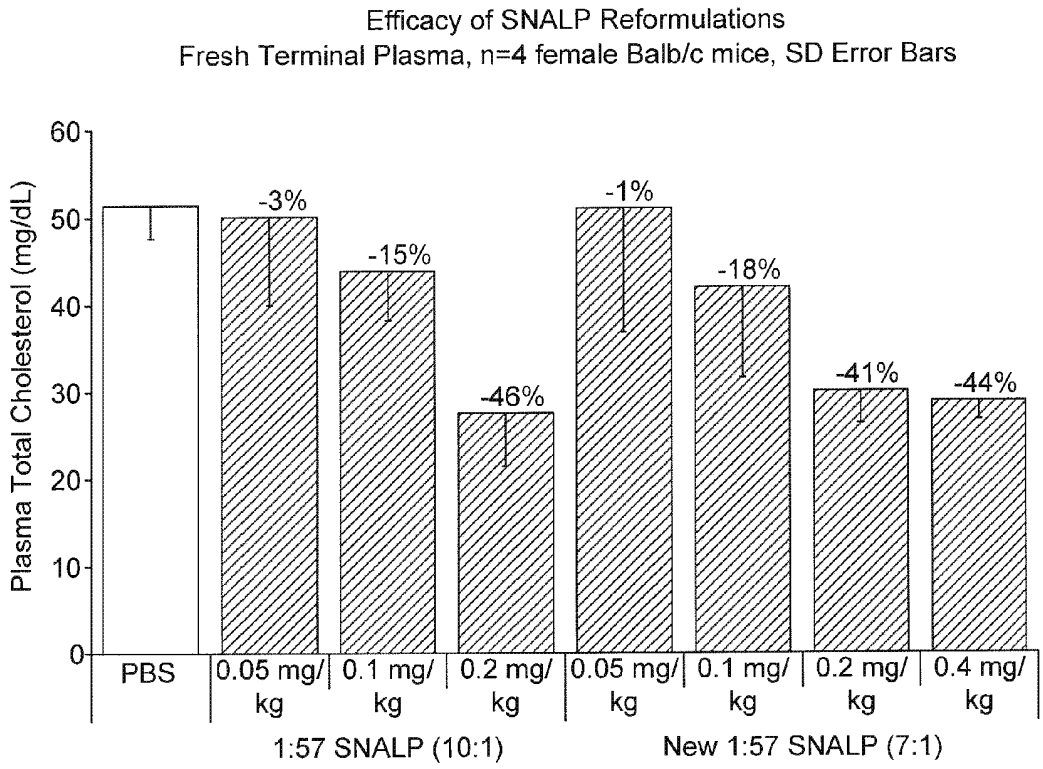

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 12

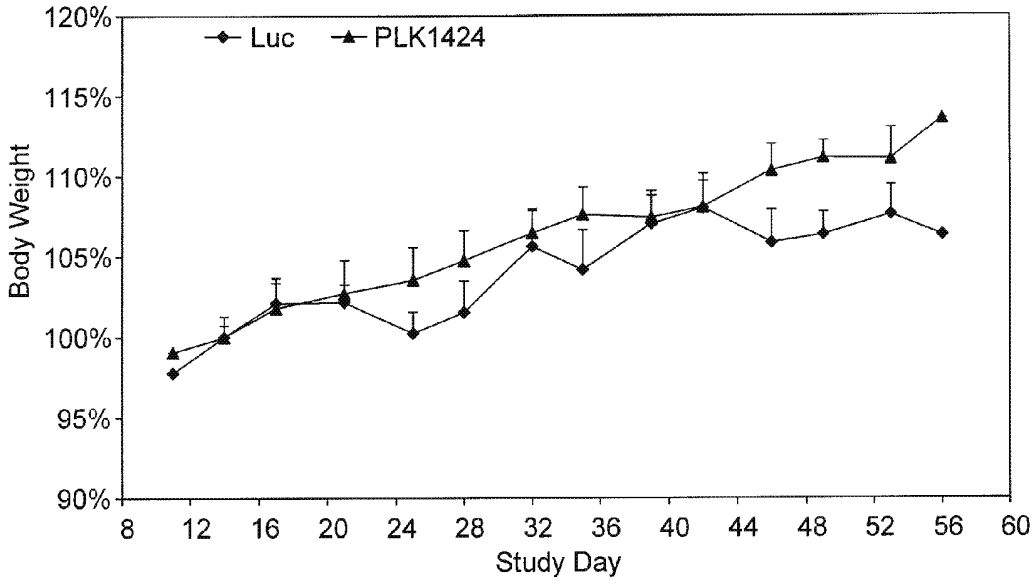

120%
115%
110%
105%
100%
95%
90%
Body Weight
Luc
PLK1424
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

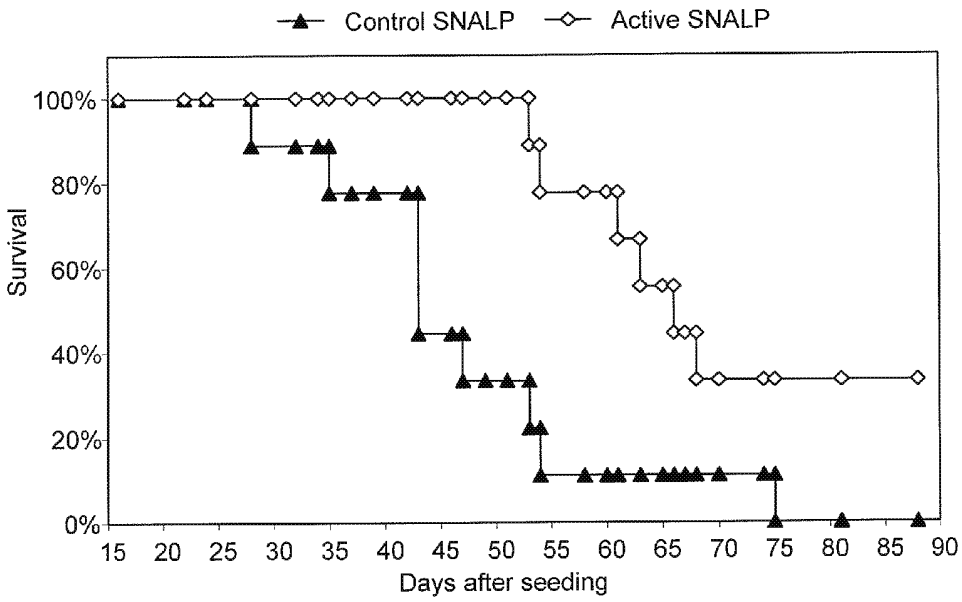

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Days after seeding

FIG. 14

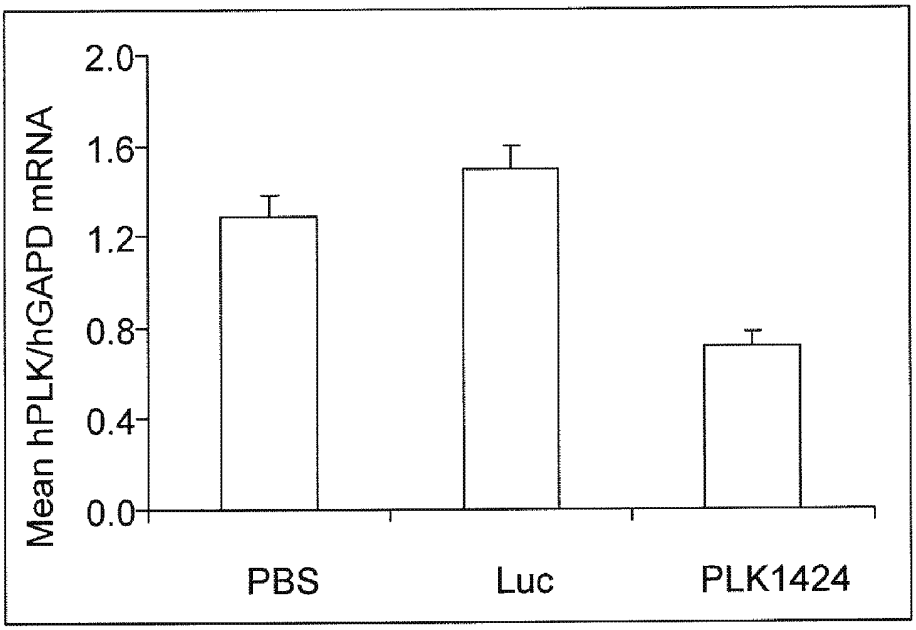


FIG. 15

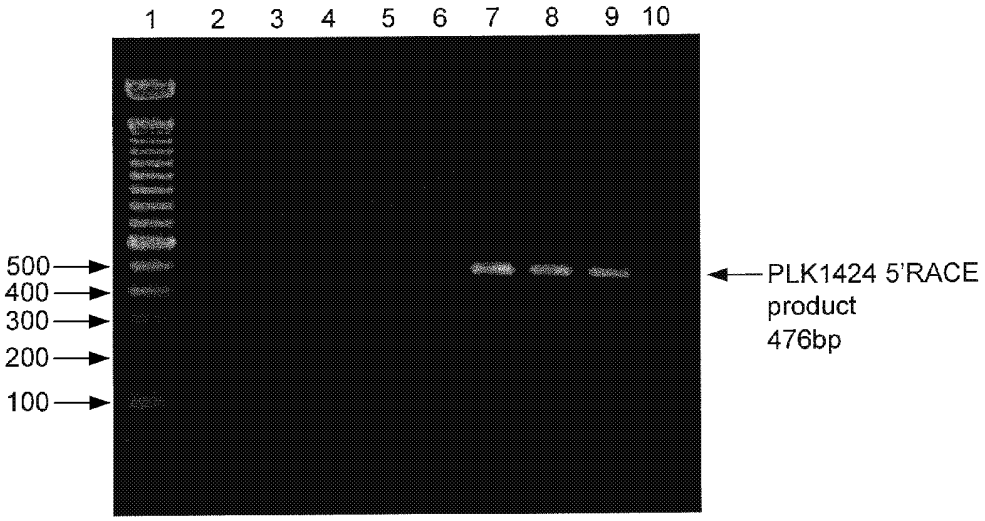
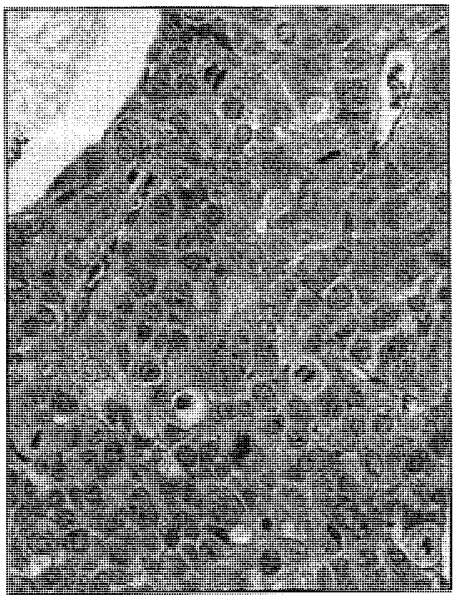
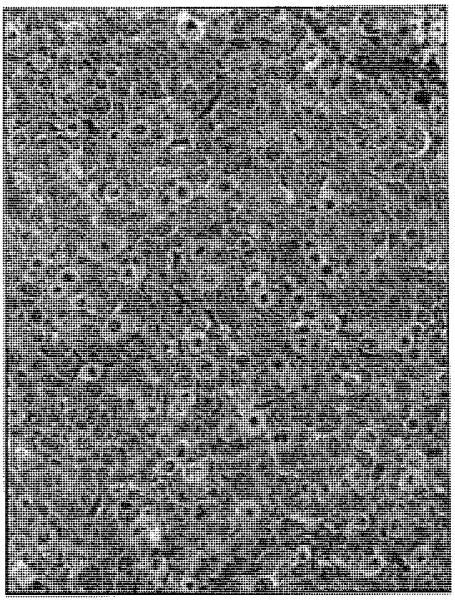

1 2 3 4 5 6 7 8 9 10
500
400
300
200
100
PLK1424 5'RACE
product
476bp

FIG. 16

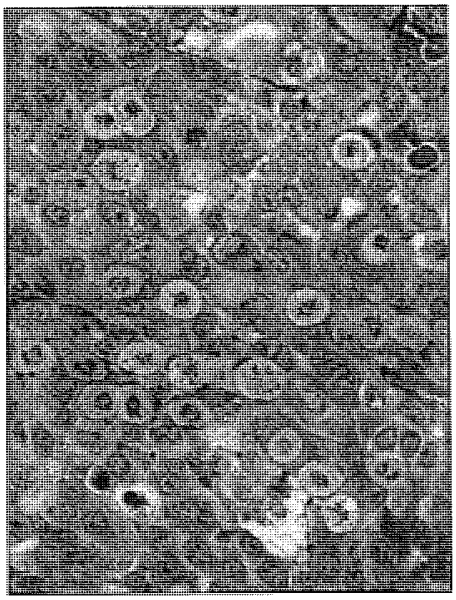
x200 mag



x400 mag



x200 mag



x400 mag

FIG. 17

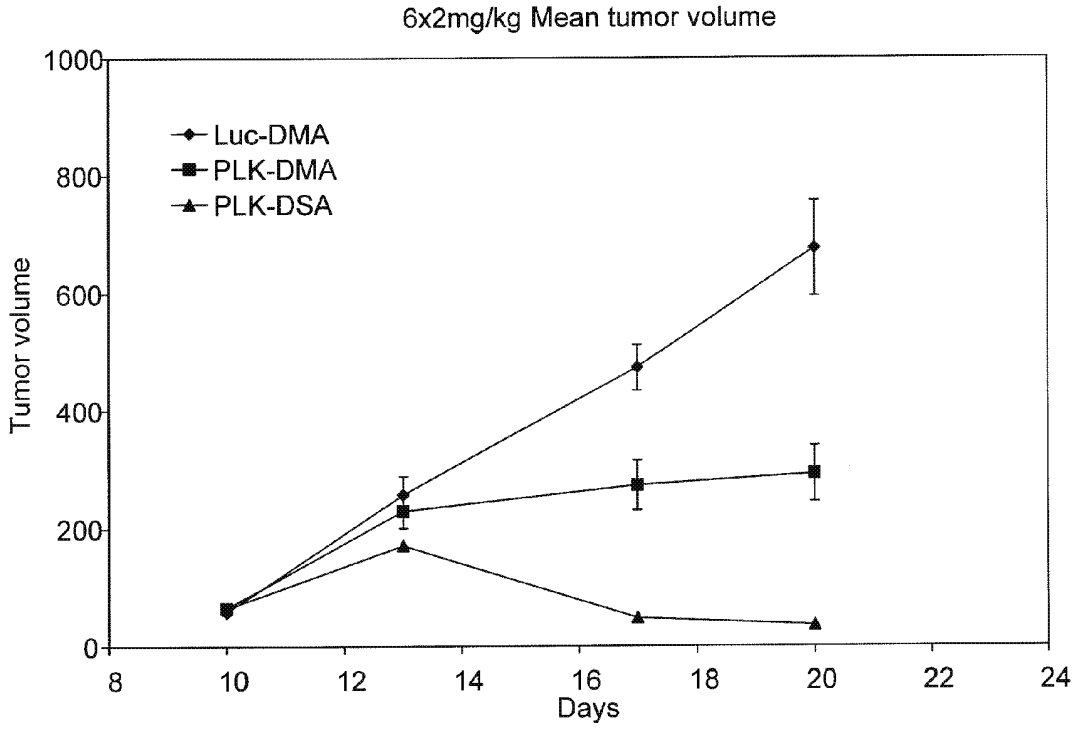

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

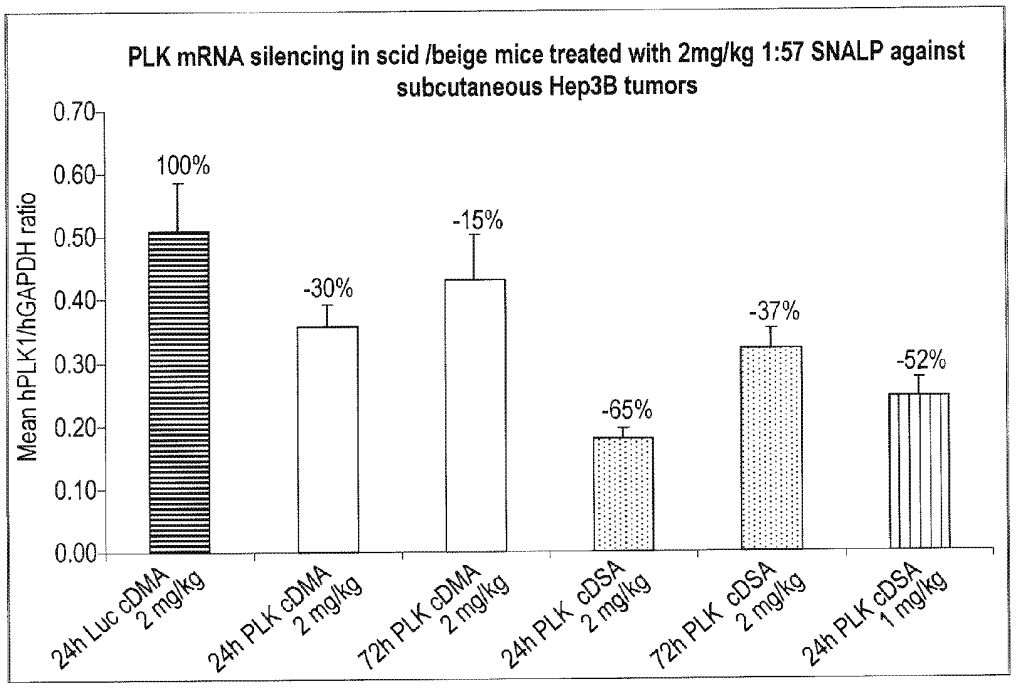

PLK mRNA silencing in scid /beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

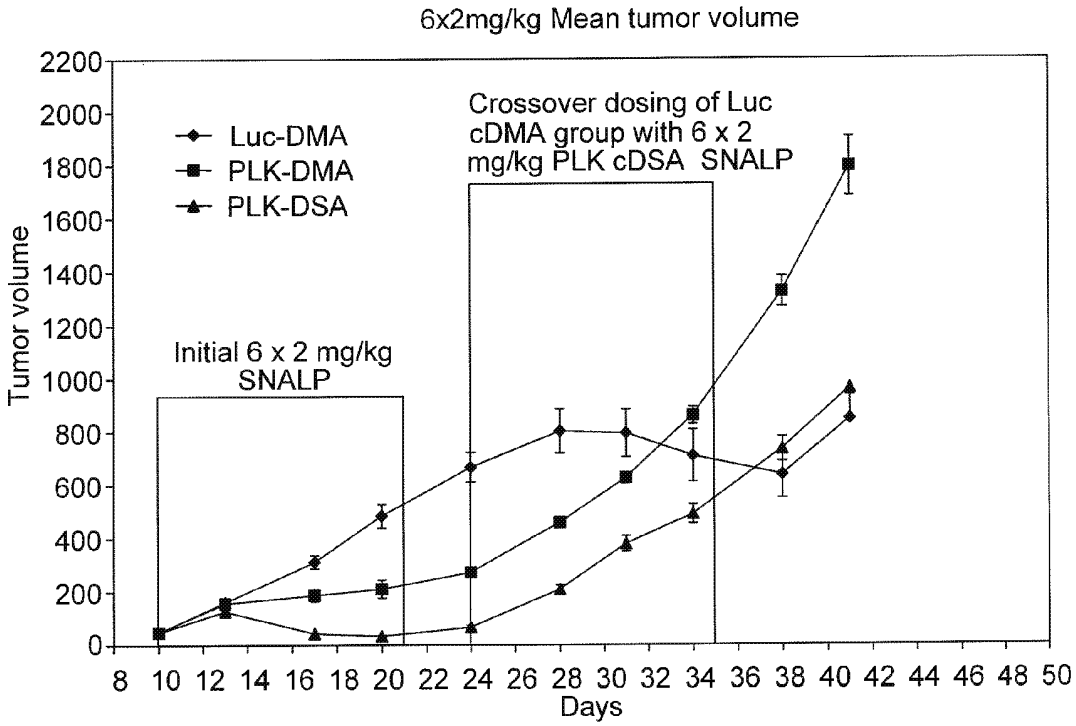

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
Initial 6 x 2 mg/kg SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

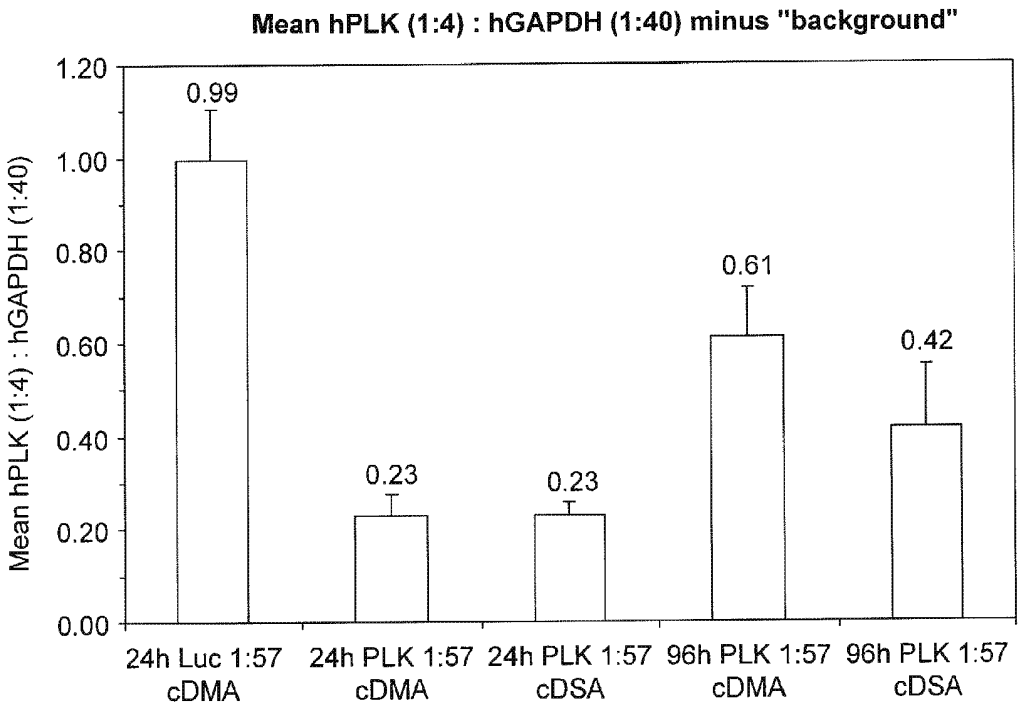

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

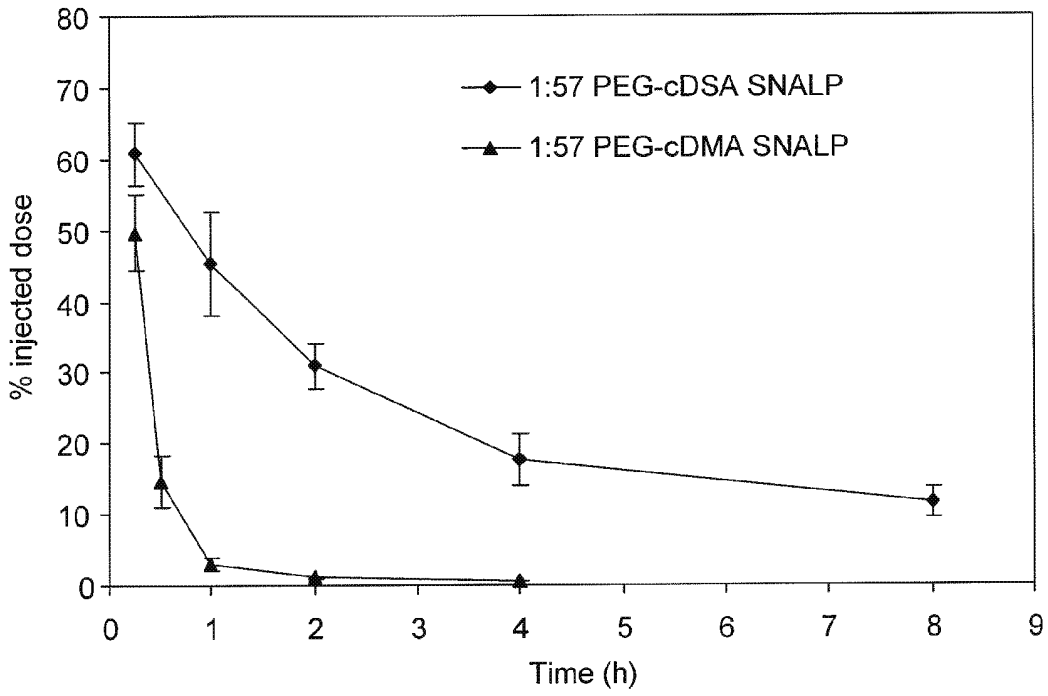

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCES TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Application No. 61/045,228, filed Apr. 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

Not applicable.

REFERENCE TO A "SEQUENCE LISTING"

Not applicable.

BACKGROUND OF THE INVENTION

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.

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.

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.

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. Pat. No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Publication No. 20030073640.

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)).

Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Pat. 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.

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.

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.

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

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).

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.

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.

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).

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.

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.

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.

The present invention also provides pharmaceutical compositions comprising a lipid particle described herein (e.g., SNALP) and a pharmaceutically acceptable carrier.

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).

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).

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).

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

FIG. **1**A (Samples 1-8) and FIG. **1**B (Samples 9-16) illustrate data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

FIG. **2** illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **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.

FIG. **4** illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **5** illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **6**A (expressed as IU/L) and FIG. **6**B (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.

FIG. **7**A (expressed as liver ApoB:GAPD mRNA ratio), FIG. **7**B (expressed as relative plasma ApoB-100 concentration), and FIG. **7**C (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.

FIG. **8** illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

FIG. **9** illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

FIG. **10**A (expressed as IU/L) and FIG. **10**B (expressed as x-Fold Upper Limit of Normal) illustrate data demonstrating that clinically significant liver enzyme elevations (3×ULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

FIG. **11**A (expressed as liver ApoB:GAPD mRNA ratio) and FIG. **11**B (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.

FIG. **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).

FIG. **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.

FIG. **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.

FIG. **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.

FIG. **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.

FIG. **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).

FIG. **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.

FIG. **19** illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

FIG. **20** illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

FIG. **21** illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

FIG. **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

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, FIG. **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, FIG. **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").

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.

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.

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

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

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.

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.

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).

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.

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.

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.

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.

"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.

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.

Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5×SSC, and 1% SDS, incubating at 42° C., or, 5×SSC, 1% SDS, incubating at 65° C., with wash in 0.2×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).

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 1×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.

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.

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.

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, Wis.), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology*, Ausubel et al., eds. (1995 supplement)).

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/).

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.

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.

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.

"Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

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.

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.

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.

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.

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).

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. Pat. 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.

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.

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.

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.

The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

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, palmitoyloleoylphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

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. Pat. 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.

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.

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.

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.

"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.

"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.

"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.

"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.

The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

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

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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-dimethyl-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.

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 Oct. 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 Dec. 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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-sn3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed Dec. 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-w-methyl-poly(ethylene glycol) (2 KPEG-DMG). The synthesis of 2 KPEG-DMG is described in U.S. Pat. No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

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.

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.

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.

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, Calif.). "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.

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.

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).

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).

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.

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.

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-IDSA), 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.

The present invention also provides a pharmaceutical composition comprising a lipid particle (e.g., SNALP) described herein and a pharmaceutically acceptable carrier.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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

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.

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

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.

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.

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.

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.

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.

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

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).

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.

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.

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.

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

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).

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.

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.

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.

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.

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.

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. Pat. 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. Pat. 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. Pat. 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.

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, N.J.); human IL-6 and TNF-α (eBioscience; San Diego, Calif.); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences; San Diego, Calif.)).

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

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).

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.

To form a long dsRNA, for synthetic RNAs, the complement is also transcribed in vitro and hybridized to form a dsRNA. If a naturally occurring 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.

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. Pat. 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.

Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules of the inven-

tion 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, Calif.). 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.

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

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.

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.

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. Pat. 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.

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.

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 l'-position.

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. Pat. 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'-β-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

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.

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)).

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. Pat. 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.

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.

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 Hepatitis 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. Pat. No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed Mar. 20, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

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, 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 Jan. 26, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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 Ser. 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 Ser. No. 12/343,342, filed Dec. 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 Apr. 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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 Ser. No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

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.

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. Pat. No. 6,174,861), angiostatin (see, e.g., U U.S. Pat. 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.

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)).

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)).

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

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.

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 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.

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.

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

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.

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.

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.

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.

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.

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.

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

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.

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. Pat. 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); Penis et al., *Brain Res Mol Brain Res.,* 15; 57:310-20 (1998); and U.S. Pat. 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. Pat. 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.

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

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.

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.

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. Pat. 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. Pat. 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.

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.

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. Pat. 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

Nucleic acids associated with lipid particles 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).

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.

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 immuno-

stimulatory 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 Dec. 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Pat. 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

In certain embodiments, the active agent associated with the lipid particles 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 therapeutic 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.

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., gefltinib (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.

Examples of conventional hormonal therapeutic agents include, without limitation, steroids (e.g., dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

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.).

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.

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.

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.

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, immunovir, 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

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.

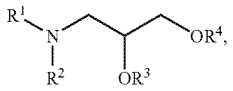
In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (e.g., siRNA, aiRNA, and/or miRNA), a cationic lipid (e.g., a cationic lipid of Formulas I, II, and/or III), a non-cationic lipid (e.g., cholesterol alone 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. Pat. 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

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.

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-dimethyl-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. Pat. 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, N.Y., 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, Wis., USA).

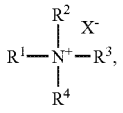
Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.



(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).

Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.

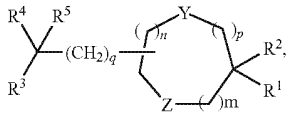


(II)

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.

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, optionally substituted 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.

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).

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.

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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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

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.

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. Pat. 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, 2 KPEG-DMG, and a mixture thereof.

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. Pat. 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, monomethoxypolyethyleneglycolacetic acid (MePEG-CH_2COOH) is particularly useful for preparing PEG-lipid conjugates including, e.g., PEG-DAA conjugates.

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.

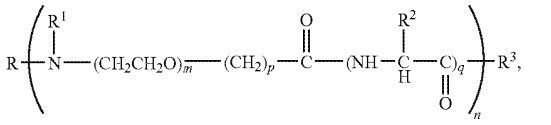
In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, e.g., non-ester containing 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.

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.

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).

The term "ATTR" or "polyamide" refers to, without limitation, compounds described in U.S. Pat. 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:

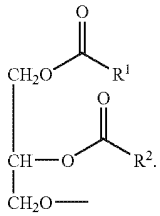
(IV)



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.

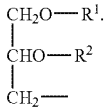
The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups 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)



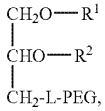
The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:

(VI)



In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:

(VII)

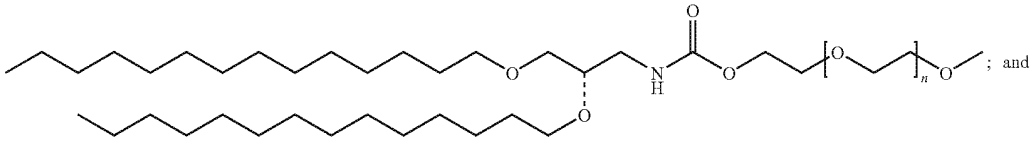


wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated 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.

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.

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).

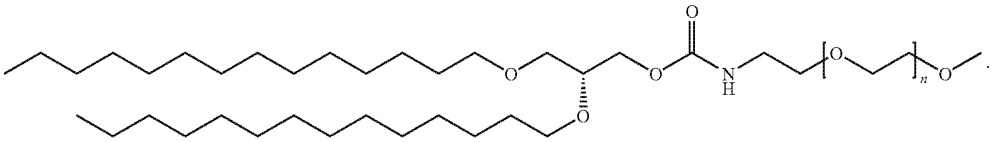
In particular embodiments, the PEG-lipid conjugate is selected from:



; and

(PEG-C-DMA)

-continued



(PEG-C-DOMG)

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).

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.

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.

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. Pat. 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.

Suitable CPLs include compounds of Formula VIII:

A-W—Y (VIII),

wherein A, W, and Y are as described below.

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.

"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.

"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.

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.

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. Pat. 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.

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.

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.

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.

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

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.

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.

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.

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.

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.

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.

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 deliv-

ers 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.

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.

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.

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.

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. Pat. 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.

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.

In some embodiments, the nucleic acids in the SNALP are precondensed as described in, e.g., U.S. patent application Ser. No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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, Wis., 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.

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.

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), (10:1), 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.

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 preformed 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. Pat. 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

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.

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).

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

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.

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.

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.

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.

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.

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

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.

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. Pat. 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. Pat. 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.

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.

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. Pat. Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 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. Pat. No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

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.

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.

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. Pat. 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.

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.

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.

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.

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.

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.

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

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.

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.

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.

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

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.

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).

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

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

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

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.

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).

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. Pat. No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis et al. eds.) Academic Press Inc. San Diego, Calif. (1990); Arnheim & Levinson (Oct. 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.

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 polynucleotides, 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 polynucleotides 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.

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

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

siRNA: All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, Colo.). The siRNAs were desalted and annealed using standard procedures.

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, Ala.); 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

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'-C**U**GAAGACC**U**GAAGACAA**U**dTdT-3'	1	6/42 = 14.3%	6/38 = 15.8%
	3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUUA-5'	2		

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.

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	Formulation Composition, Mole % PEG(2000)-C-DMA | DLinDMA |	Lipid/Drug	Finished Product Characterization		
No.	DPPC | Cholesterol	Ratio	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
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

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, Wis.), a resazurin dye that is reduced by metabolically active cells to the fluorogenic 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.

FIG. **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, FIG. **1**B, Sample 9).

Example 3

ApoB siRNA Formulated as 1:57 SNALP have Potent Silencing Activity In Vivo

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'-AGU**G**UCA**U**CACAC**U**GAAUACC-3'	3	7/42 = 16.7%	7/38 = 18.4%
		3'-GU**U**CACAGUAGU**G**UG**A**C**U**UAU-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.

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).

(GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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).

TABLE 4

Characteristics of the SNALP formulations used in this study.

	Formulation Composition	Lipid/Drug	Finished Product Characterization		
Group	Lipid Name & Mole %	Ratio	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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase

Example 4

ApoB siRNA Formulated as 1:57 SNALP have Potent Silencing Activity In Vivo

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

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.

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.

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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.

	Formulation Composition	Lipid/Drug	Finished Product Characterization		
Group	Lipid Name & Mole %	Ratio	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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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 | DLinDMA | 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
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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy*, 13:494 (2006).

FIG. **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

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

Animal Model: Female BALB/c mice, 5 wks old, n=4 per group/cage.

siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

Tolerability:

Group	Formulation	IV Injection siRNA mg/kg	IV Injection 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	IV Injection 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:

Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

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.

Formulation Summary:

	1:57 (9:1) + DOW siRNA	Particle Size Zavg (nm)	Poly	% Encap	Final L:D (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

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.

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 16,000×g & 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.

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 16,000×g (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.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

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

There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. FIG. **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.

FIG. **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

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

Animal Model: Female BALB/c mice, 7 wks old.

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:

Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

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.

Formulation Summary:

		Particle Size			
	1|57 SNALP Gear PBS In-Line	Zavg (nm)	Poly	% Encap	Final L:D (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

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.

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).

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.

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 16,000×g & 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.

Groups 12-19: Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16,000×g (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.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

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:

FIG. **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.

FIG. **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.

FIG. **10** shows that clinically significant liver enzyme elevations (3×ULN) 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:

FIG. **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.

FIG. **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:

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 FIG. **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

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'	5	6/38 = 15.8%
	3'-NNUC**U**AGUGGGAG**G**AAUUUAU-5'	6	
PLK1424 U4/G	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3'	5	7/38 = 18.4%
	3'-NNUCUA**G**UG**G**AG**G**AAUUUAU-5'	7	

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

20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay
A B	20 to seed	I.H. 1.5×10^6 Hep3B	Luc 1:57 PLK 1424 1:57	9 9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10×2 mg/kg	When moribund	Survival Body Weights

Test Articles

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 (28 mM lipid)
B	PLK1424 U4/GU SNALP 1:57 (28 mM lipid)
	PLK1424 U4/G SNALP 1:57 (28 mM 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 30 G (⅜") 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

FIG. **13** shows the mean body weights of mice during therapeutic dosing of PLK1424 SNALP in the Hep3B intra-

hepatic (I.H.) tumor model. The treatment regimen was well tolerated with no apparent signs of treatment-related toxicity.

FIG. **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

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.

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

20 SCID/beige mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	Sacrifice	Assay
A	20 to	I.H.	PBS	6	1 × 2 mg/kg	24 h after	Tumor QG
B	seed	1 × 10^6	Luc 1:57	7	Day 20	treatment	Tumor RACE-PCR
C		Hep3B	PLK 1424 1:57	7			Histopathology

Test Articles

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. 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 30 G (⅜") 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

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.

FIG. **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.

FIG. **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.

FIG. **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

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

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.

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 6×2 mg/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.

FIG. **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.

FIG. **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 FIG. **18**.

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 FIG. **20**, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

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.

FIG. **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.

FIG. **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.

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.

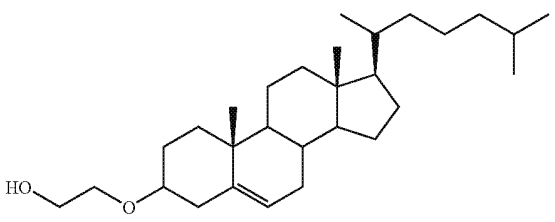
Example 12

Synthesis of Cholesteryl-2'-Hydroxyethyl Ether

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 (2×50 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%).

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 (3×100 ml). The organic phases were combined, washed with water (2×150 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%).

The structures of the cholesterol derivatives cholesteryl-2'-hydroxyethyl ether and cholesteryl-4'-hydroxybutyl ether are as follows:



Cholesteryl-2'-hydroxyethyl ether

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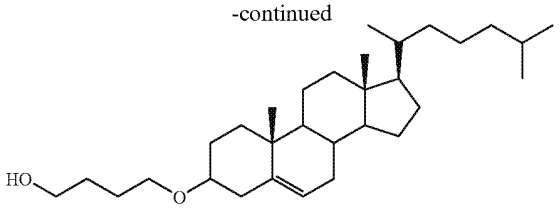

Cholesteryl-4'-hydroxybutyl ether

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.

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What is claimed is:

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 a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the phospholipid comprises from 4 mol % to 10 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.

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 **2**, wherein said siRNA is about 19 to about 25 base pairs in length.

7. The nucleic acid-lipid particle of claim **2**, wherein said siRNA comprises 3' overhangs.

8. The nucleic acid-lipid particle of claim **1**, wherein the cationic lipid comprises from 52 mol % to 62 mol % of the total lipid present in the particle.

9. The nucleic acid-lipid particle of claim **1**, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

10. The nucleic acid-lipid particle of claim **1**, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

11. The nucleic acid-lipid particle of claim **10**, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

12. The nucleic acid-lipid particle of claim **11**, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

13. The nucleic acid-lipid particle of claim **12**, wherein the PEG has an average molecular weight of about 2,000 daltons.

14. 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.

15. 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.

16. 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.

17. The nucleic acid-lipid particle of claim **1**, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

18. 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.

19. 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.

20. 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.

21. 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.

22. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim **1** and a pharmaceutically acceptable carrier.

* * * * *

JOINT APPENDIX 02

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United States Patent and Trademark Office

July 13, 2022

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

PATENT NUMBER: *8,492,359*
ISSUE DATE: *July 23, 2013*

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

Rodney Glover
Certifying Officer

US008492359B2

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

(10) **Patent No.: US 8,492,359 B2**
(45) **Date of Patent: *Jul. 23, 2013**

(54) **LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY**

(75) Inventors: **Edward Yaworski**, Maple Ridge (CA); **Kieu Lam**, Surrey (CA); **Lloyd Jeffs**, Delta (CA); **Lorne Palmer**, Vancouver (CA); **Ian MacLachlan**, Mission (CA)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 59 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **13/253,917**

(22) Filed: **Oct. 5, 2011**

(65) **Prior Publication Data**

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Related U.S. Application Data

(63) Continuation of application No. 12/424,367, filed on Apr. 15, 2009, now Pat. No. 8,058,069.

(60) Provisional application No. 61/045,228, filed on Apr. 15, 2008.

(51) **Int. Cl.**
C12N 15/11 (2006.01)

(52) **U.S. Cl.**
USPC ..... **514/44 A**

(58) **Field of Classification Search**
USPC ..... 514/44 A
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,394,448	A	7/1983	Szoka, Jr. et al.
4,438,052	A	3/1984	Weder et al.
4,515,736	A	5/1985	Deamer
4,598,051	A	7/1986	Papahadjopoulos et al.
4,897,355	A	1/1990	Eppstein et al.
5,013,556	A	5/1991	Woodle et al.
5,171,678	A	12/1992	Behr et al.
5,208,036	A	5/1993	Eppstein et al.
5,225,212	A	7/1993	Martin et al.
5,264,618	A	11/1993	Felgner et al.
5,279,833	A	1/1994	Rose
5,283,185	A	2/1994	Epand et al.
5,320,906	A	6/1994	Eley et al.
5,334,761	A	8/1994	Gebeyehu et al.
5,545,412	A	8/1996	Eppstein et al.
5,578,475	A	11/1996	Jessee
5,627,159	A	5/1997	Shih et al.
5,641,662	A	6/1997	Debs et al.
5,656,743	A	8/1997	Busch et al.
5,674,908	A	10/1997	Haces et al.
5,703,055	A	12/1997	Felgner et al.
5,705,385	A	1/1998	Bally et al.
5,736,392	A	4/1998	Hawley-Nelson et al.
5,820,873	A	10/1998	Choi et al.
5,877,220	A	3/1999	Schwartz et al.
5,885,613	A	3/1999	Holland et al.
5,958,901	A	9/1999	Dwyer et al.
5,976,567	A	11/1999	Wheeler et al.
5,981,501	A	11/1999	Wheeler et al.
6,020,202	A	2/2000	Jessee
6,020,526	A	2/2000	Schwartz et al.
6,034,135	A	3/2000	Schwartz et al.
6,051,429	A	4/2000	Hawley-Nelson et al.
6,075,012	A	6/2000	Gebeyehu et al.
6,165,501	A	12/2000	Tirosh et al.
6,172,049	B1	1/2001	Dwyer et al.
6,251,939	B1	6/2001	Schwartz et al.
6,284,267	B1	9/2001	Aneja
6,287,591	B1	9/2001	Semple et al.
6,339,173	B1	1/2002	Schwartz et al.
6,376,248	B1	4/2002	Hawley-Nelson et al.
6,534,484	B1	3/2003	Wheeler et al.
6,586,410	B1	7/2003	Wheeler et al.
6,638,529	B2	10/2003	Schwartz et al.
6,649,780	B1	11/2003	Eibl et al.
6,671,393	B2	12/2003	Hays et al.
6,696,424	B1	2/2004	Wheeler
6,815,432	B2	11/2004	Wheeler et al.
6,858,224	B2	2/2005	Wheeler et al.

(Continued)

FOREIGN PATENT DOCUMENTS

CA	2309727	A1	4/1999
CA	2271582	A1	11/1999

(Continued)

OTHER PUBLICATIONS

Arpicco, S., et al., "Preparation and Characterization of Novel Cationic Lipids Developed for Gene Transfection," Proceed. Int'l Symp. Control. Rel. Bioact. Mater. (Controlled Release Society, Inc.), 1999, vol. 26, pp. 759-760.

Arpicco, S., et al., "Synthesis, characterization and transfection activity of new saturated and unsaturated cationic lipids," IL Farmaco, 2004, vol. 59, pp. 869-878.

Ballas, N., et al., "Liposomes bearing a quaternary ammonium detergent as an efficient vehicle for functional transfer of TMV-RNA into plant protoplasts," Biochimica et Biophysica Acta, 1988, vol. 939, pp. 8-18.

Barinaga, M., "Step Taken Toward Improved Vectors for Gene Transfer," Science, 1994, vol. 266, p. 1326.

Bass, "The Short Answer," *Nature*, 2001, 411: 428-9.

Beale, G., et al., "Gene Silencing Nucleic Acids Designed by Scanning Arrays: Anti-EGFR Activity of siRNA, Ribozyme and DNA Enzymes Targeting a Single Hybridization-accessible Region using the Same Delivery System," Journal of Drug Targeting, 2003, vol. 11, No. 7, pp. 449-456.

(Continued)

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(74) *Attorney, Agent, or Firm* — Kilpatrick Townsend & Stockton LLP

(57) **ABSTRACT**

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.

21 Claims, 24 Drawing Sheets

U.S. PATENT DOCUMENTS

7,166,745	B1	1/2007	Chu et al.	
7,422,902	B1	9/2008	Wheeler et al.	
7,479,573	B2	1/2009	Chu et al.	
7,601,872	B2	10/2009	Chu et al.	
7,687,070	B2	3/2010	Gebeyehu et al.	
7,745,651	B2	6/2010	Heyes et al.	
7,799,565	B2	9/2010	MacLachlan et al.	
7,803,397	B2	9/2010	Heyes et al.	
7,807,815	B2	10/2010	MacLachlan et al.	
7,838,658	B2	11/2010	MacLachlan et al.	
7,901,708	B2	3/2011	MacLachlan et al.	
7,915,450	B2	3/2011	Chu et al.	
7,982,027	B2	7/2011	MacLachlan et al.	
8,058,068	B2	11/2011	Hawley-Nelson et al.	
8,058,069	B2 *	11/2011	Yaworski et al.	435/458
8,158,827	B2	4/2012	Chu et al.	
8,227,443	B2	7/2012	MacLachlan et al.	
2001/0048940	A1	12/2001	Tousignant et al.	
2003/0069173	A1	4/2003	Hawley-Nelson et al.	
2003/0077829	A1	4/2003	MacLachlan	
2003/0143732	A1	7/2003	Fosnaugh et al.	
2004/0063654	A1	4/2004	Davis et al.	
2004/0142892	A1	7/2004	Finn et al.	
2004/0253723	A1	12/2004	Tachas et al.	
2004/0259247	A1	12/2004	Tuschl et al.	
2005/0064595	A1	3/2005	MacLachlan et al.	
2005/0118253	A1	6/2005	MacLachlan et al.	
2005/0260757	A1	11/2005	Gebeyehu et al.	
2006/0008910	A1	1/2006	MacLachlan et al.	
2006/0147514	A1	7/2006	Gebeyehu et al.	
2006/0228406	A1	10/2006	Chiou et al.	
2007/0042031	A1	2/2007	MacLachlan et al.	
2007/0202598	A1	8/2007	Chu et al.	
2007/0202600	A1	8/2007	Chu et al.	
2009/0143583	A1	6/2009	Chu et al.	
2009/0291131	A1	11/2009	MacLachlan et al.	
2010/0159593	A1	6/2010	Chu et al.	
2012/0136073	A1	5/2012	Yang et al.	
2012/0238747	A1	9/2012	Chu et al.	

FOREIGN PATENT DOCUMENTS

CA	2330741	A1	11/1999
CA	2397016	A1	7/2001
JP	03-126211		5/1991
JP	05-202085		8/1993
JP	06-080560		3/1994
WO	WO 91/16024	A1	10/1991
WO	WO 93/05162	A1	3/1993
WO	WO 93/12240	A1	6/1993
WO	WO 93/12756	A2	7/1993
WO	WO 93/24640	A2	12/1993
WO	WO 93/25673	A1	12/1993
WO	WO 95/02698	A1	1/1995
WO	WO 95/18863	A1	7/1995
WO	WO 95/35301	A1	12/1995
WO	WO 96/02655	A1	2/1996
WO	WO 96/10390	A1	4/1996
WO	WO 96/41873	A1	12/1996
WO	WO 98/51285	A2	11/1998
WO	WO 00/03683	A2	1/2000
WO	WO 00/15820	A1	3/2000
WO	WO 00/62813	A2	10/2000
WO	WO 01/05374	A1	1/2001
WO	WO 01/05873	A1	1/2001
WO	WO 02/34236	A2	5/2002
WO	WO 02/087541	A1	11/2002
WO	WO 03/097805	A2	11/2003
WO	WO 2004/065546	A2	8/2004
WO	WO 2004/110499	A1	12/2004
WO	WO 2005/007196	A2	1/2005
WO	WO 2005/026372	A1	3/2005
WO	WO 2005/035764	A1	4/2005
WO	WO 2005/120152	A2	12/2005
WO	WO 2006/002538	A1	1/2006
WO	WO 2006/053430	A1	5/2006
WO	WO 2007/056861	A1	5/2007
WO	WO 2010/048228	A2	4/2010
WO	WO 2010/088537	A2	8/2010
WO	WO 2010/105209	A1	9/2010

OTHER PUBLICATIONS

Behr, J.-P., "Synthetic Gene-Transfer Vectors," Acc. Chem. Res., 1993, vol. 26, pp. 274-278.

Brigham, K., et al., "Rapid Communication: In vivo Transfection of Murine Lungs with a Functioning Prokaryotic Gene Using a Liposome Vehicle," The American Journal of the Medical Sciences, vol. 298, No. 4, pp. 278-281, 1989.

Brummelkamp, et al., "A System for Stable Expression of Short Interfering RNAs in Mammalian Cells," Science, 2002, V. 296. pp. 550-553.

Cevc, G., "How Membrane Chain-Melting Phase-Transition Temperature is Affected by the Lipid Chain Asymmetry and Degree of Unsaturation: An Effective Chain-Length Model," Biochemistry, 1991, vol. 30, pp. 7186-7193.

Chonn et al., "Recent advances in liposomal drug-delivery systems," Current Opinion in Biotechnology, 1995, vol. 6, pp. 698-708.

Cortesi, R., et al., "Effect of cationic liposome composition on in vitro cytotoxicity and protective effect on carried DNA," International Journal of Pharmaceutics, 1996, vol. 139, pp. 69-78.

Crystal, R., "Transfer of Genes to Humans: Early Lessons and Obstacles to Success," Science, 1995, vol. 270, pp. 404-410.

Culver K., "The First Human Gene Therapy Experiment," Gene Therapy: A Handbook for Physicians, 1994, pp. 33-40.

Duzgunes, N., "Membrane Fusion," Subcellular Biochemistry, 1985, vol. 11, pp. 195-286.

Dwarki, V.J., et al., "Cationic Liposome-Mediated RNA Transfection," Methods in Enzymology, 1993, vol. 217, pp. 644-654.

Elbashir, et a., "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells," Nature, May 2001, pp. 494-498, vol. 411.

Enoch, H., et al., "Formation and properties of 1000-Å-diameter, single-bilayer phospholipid vesicles," Proc. Natl. Acad. Sci. USA, 1979, vol. 76, No. 1, pp. 145-149.

Felgner, J., et al., "Cationic Lipid-Mediated Transfection in Mammalian Cells: 'Lipofection,'" J. Tiss. Cult. Meth., 1993, vol. 15, pp. 63-68.

Felgner, J., et al., "Enhanced Gene Delivery and Mechanism Studies with a Novel Series of Cationic Lipid Formulations," The Journal of Biological Chemistry, 1994, vol. 269, No. 4, pp. 2550-2561.

Felgner, P., et al., "Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure," Proc. Natl. Acad. Sci. USA, 1987, vol. 84, pp. 7413-7417.

Felgner, P.L., et al., "Cationic Liposome Mediated Transfection," Proc. West. Pharmacol. Soc., 1989, vol. 32, pp. 115-121.

Gao, X., et al., "A Novel Cationic Liposome Reagent for Efficient Transfection of Mammalian Cells," Biochem. Biophys. Res. Comm., 1991, vol. 179, No. 1, pp. 280-285.

Gershon, H., et al., "Mode of Formation and Structural Feature of DNA-Cationic Liposome Complexes Used for Transfection," Biochemistry, 1993, vol. 32, pp. 7143-7151.

Global Newswire, retrieved from http://globalnewswire.com on Feb. 27, 2013, Tekmira sues Alnylam Pharmaceuticals for repeated misuse of tradescrets and confidential information, Mar. 16, 2011, pp. 1-3.

Guy-Caffey, J., et al., "Novel Polyaminolipids Enhance the Cellular Uptake of Oligonucleotides," The Journal of Biological Chemistry, 1995, vol. 270, No. 52, pp. 31391-31396.

Hawley-Nelson, P., et al., "LipofectAmine™ Reagent: A New, Higher Efficiency Polycationic Liposome Transfection Reagent," Focus, 1993, vol. 15, No. 3, pp. 73-80.

Heyes et al., "Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids," Journal of Controlled Release, 2005, vol. 107, pp. 276-287.

Heyes et al., "Synthesis of novel cationic lipids: effect of structural modification on the efficiency of gene transfer," J. Med. Chem., 2002, vol. 45, pp. 99-114.

Hyde, S., et al., "Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy," Nature, 1993, vol. 362, pp. 250-255.

Jiang, L., et al., "Comparison of protein precipitation methods for sample preparation prior to proteomic analysis," Journal of Chromatography A, 2004, vol. 1023, pp. 317-320.
Juliano, R., et al., "The Effect of Particle Size and Charge on the Clearance Rates of Liposomes and Liposome Encapsulated Drugs," Biochem. Biophys. Res. Commun., 1975, vol. 63, No. 3, pp. 651-658.
Keough, K., "Influence of chain unsaturation and chain position on thermotropism and intermolecular interactions in membranes," Biochem. Soc. Transactions, 1990, vol. 18, No. 5, pp. 835-837.
Lawrence et al. "The formation, characterization and stability of non-ionic surfactant vesicles," S.T.P. Pharma Sciences, 1996, vol. 6, No. 1, pp. 49-60.
Lawrence et al., "Synthesis and aggregation properties of dialkyl polyoxyethylene glycerol ethers," Chemistry and Physics of Lipids, 1996, 82(2):89-100.
Legendre, J.-Y. et al., "Delivery of Plasmid DNA into Mammalian Cell Lines Using pH-Sensitive Liposomes: Comparison with Cationic Liposomes," Pharm. Res., 1992, vol. 9, No. 10, pp. 1235-1242.
Leventis, R., et al., "Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles," Biochem. Biophys. Acta, 1990, vol. 1023, pp. 124-132.
Liu, et al., "Cationic Liposome-mediated Intravenous Gene Delivery", J. Biol. Chem., 1995, V. 270, pp. 24864-24870.
Marshall, E., "Gene Therapy's Growing Pains," Science, 1995, vol. 269, pp. 1050-1055.
Murahashi et al., "Synthesis and evaluation of neoglycolipid for liposome modification," Biol. Pharm. Bull., 1997, 20(6):704-707.
Orkin, S., et al., NIH Report, Report and Recommendations of the Panel to Assess the NIH Investment in Research on Gene Therapy, 1995.
Parr et al., Factors influencing the retention and chemical stability of polly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles, Biochimica et Biophysica Acta, 1994, 1195:21-30.
Paul, C., et al., "Effective expression of small interfering RNA in human cells," Nature Biotech., 2002, vol. 20, pp. 505-508.
Puyal, C., et al., "A new cationic liposome encapsulating genetic material: A potential delivery system for polynucleotides," Eur. J. Biochem., 1995, vol. 228, pp. 697-703.
Sawada et al., "Microemulsions in supercritical CO_2 utilizing the polyethyleneglycol dialkylglycerol and their use for the solubilization of hydrophiles," Dyes and Pigments, 2005, pp. 64-74, vol. 65.
Shin, et al. "Acid-triggered release via dePEGylation of DOPE liposomes containing acid-labile vinyl ether PEG-lipids," Journal of Controlled Release, 2003, vol. 91, pp. 187-200.
Song et al., "Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes," Biochimica et Biophysica Acta, 2002, 1558:1-13.
Sorensen, et al., "Gene Silencing by Systemic Delivery of Synthetic siRNAs in Adult Mice", J. Biol. Chem., 2003, V. 327, pp. 761-766.
Spagnou, S., et al., "Lipidic Carriers of siRNA: Differences in the Formulation, Cellular Uptake, and Delivery with Plasmid DNA," Biochemistry, 2004, vol. 43, pp. 13348-13356.
Stamatatos, L., et al., "Interactions of Cationic Lipid Vesicles with Negatively Charged Phospholipid Vesicles and Biological Membranes," Biochemistry, 1988, vol. 27, pp. 3917-3925.
Szoka, F., et al., "Comparative Properties and Methods of Preparation of Lipid Vesicles (Liposomes)," Ann. Rev. Biophys. Bioeng., 1980, vol. 9, pp. 467-508.
Szoka, F., et al., "Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation," Proc. Natl. Acad. Sci. USA, 1978, vol. 75, No. 9, pp. 4194-4198.
Templeton, "Cationic Liposome-mediated Gene Delivery in vivo", Bioscience Reports, 2002, vol. 22, No. 2, pp. 283-295.
Van Der Woude, I., et al., "Parameters influencing the introduction of plasmid DNA into cells by the use of synthetic amphiphiles as a carrier system," Biochimica et Biophysica Acta, 1995, vol. 1240, pp. 34-40.
Wheeler, et al., "Stabilized Plasmid-lipid Particles: Constructions and Characterization," Gene Therapy, V. 6, pp. 271-281, 1999.
Wilson, R., et al., "Counterion-Induced Condensation of Deoxyribonucleic Acid," A Light-Scattering Study, Biochemistry, 1979, vol. 18, No. 11, pp. 2192-2196.
Woodle, M.C., et al., "Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes," Biochimica et Biophysica Acta, 1992, vol. 1105, pp. 193-200.
Zhu, N., et al., "Systemic Gene Expression After Intravenous DNA Delivery into Adult Mice," Science, 1993, vol. 261, pp. 209-211.

* cited by examiner

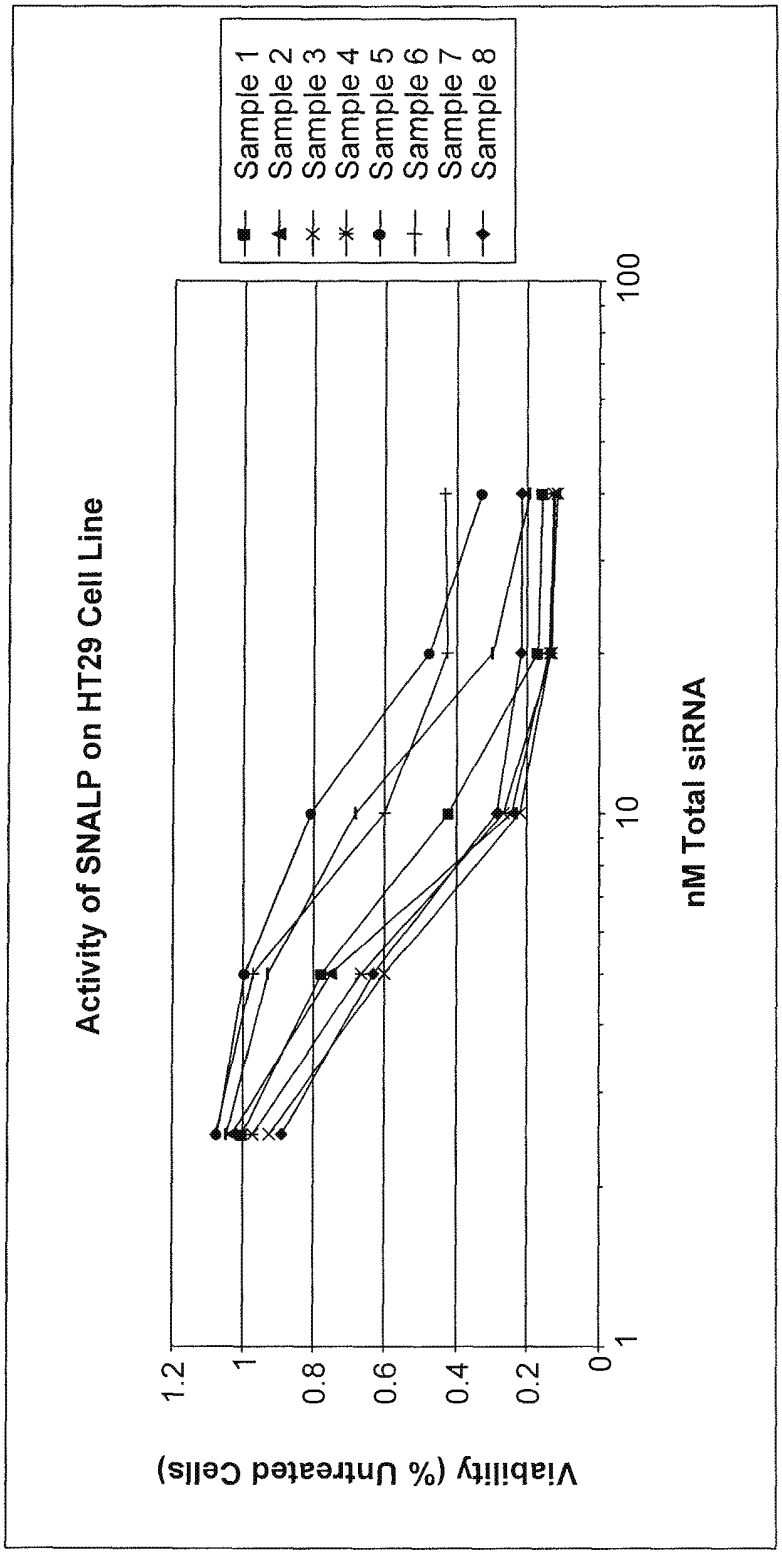

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8

FIG. 1A

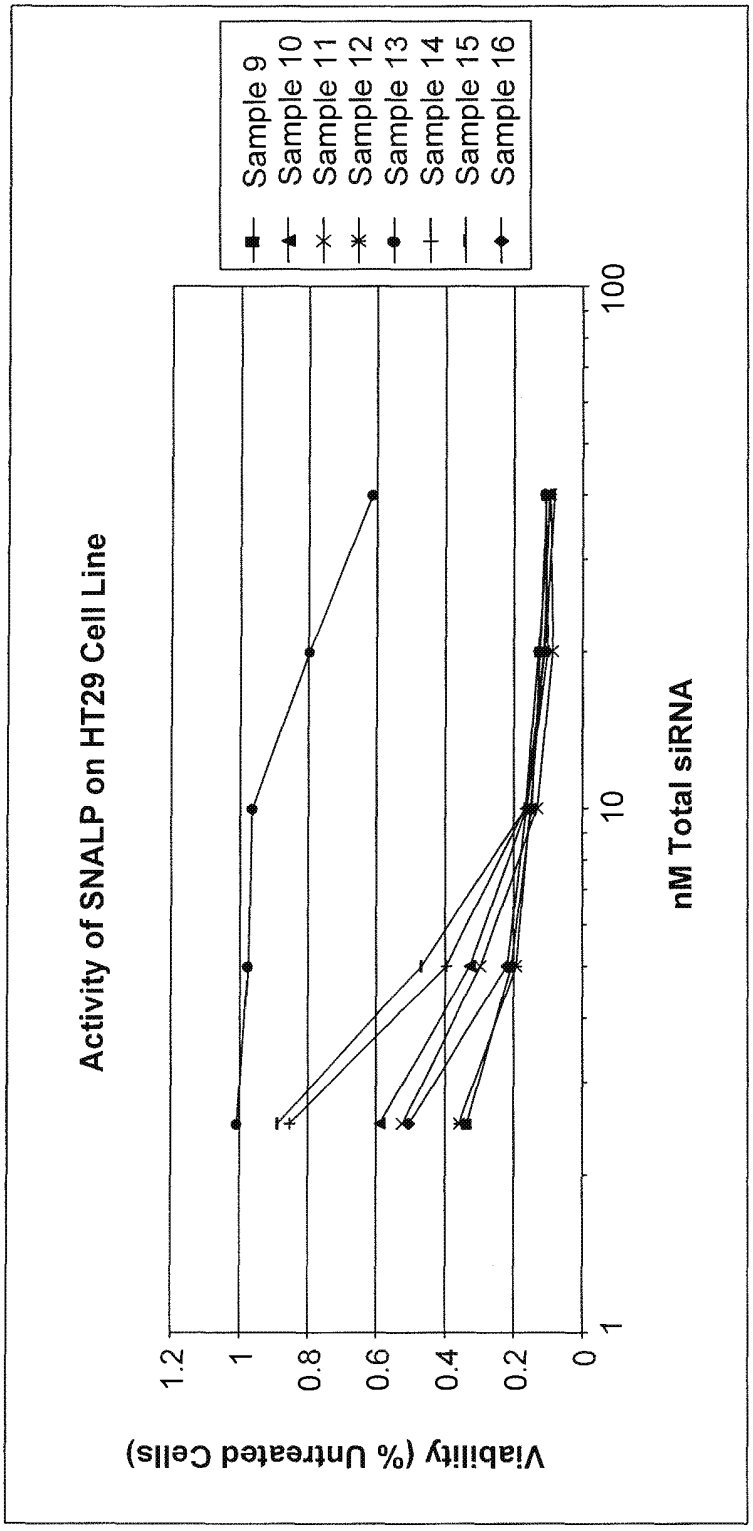

Sample 9
Sample 10
Sample 11
Sample 12
Sample 13
Sample 14
Sample 15
Sample 16
Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA

FIG. 1B

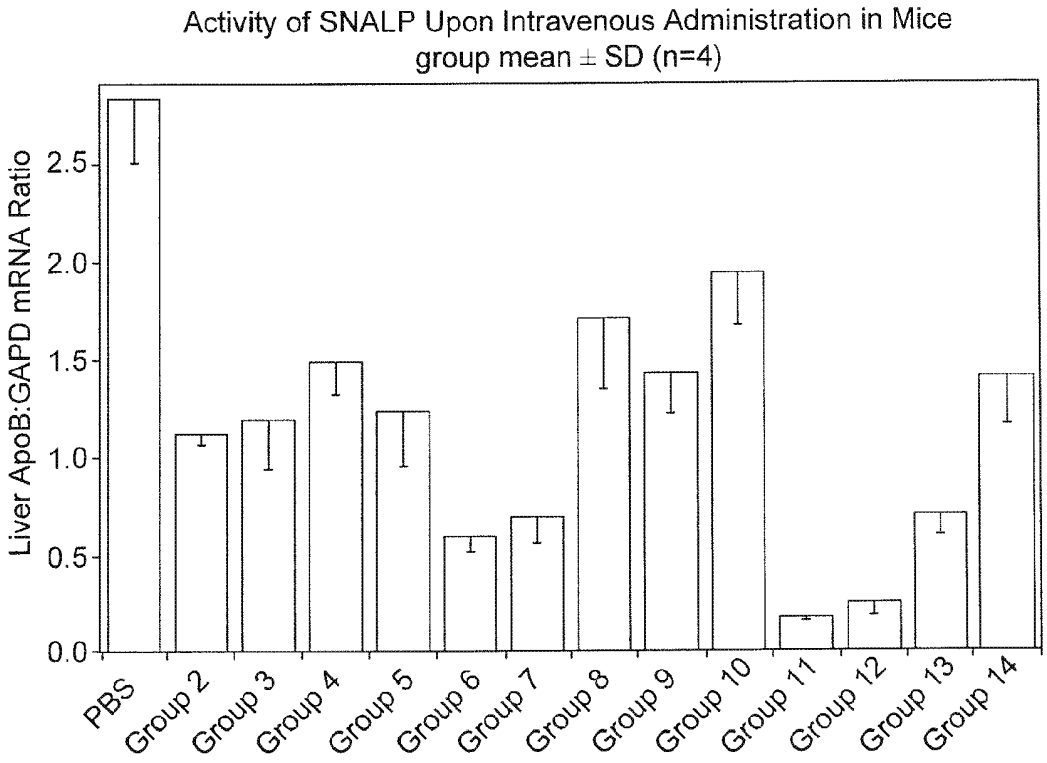

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.5
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

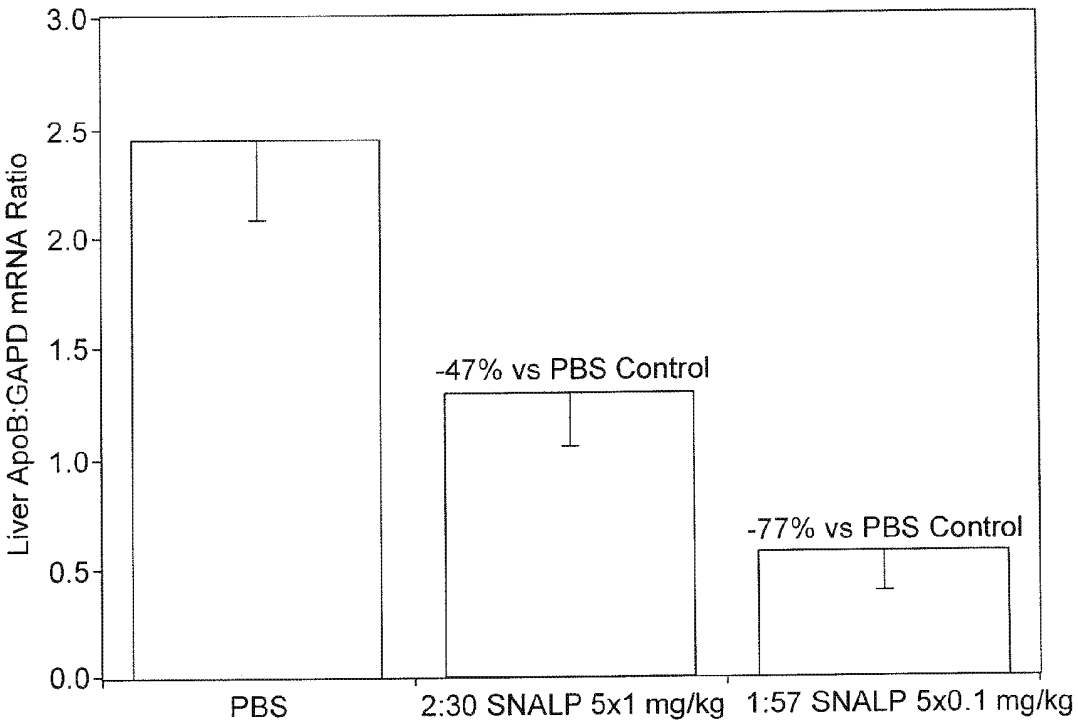

Liver ApoB:GAPD mRNA Ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5x1 mg/kg
1:57 SNALP 5x0.1 mg/kg

FIG. 3

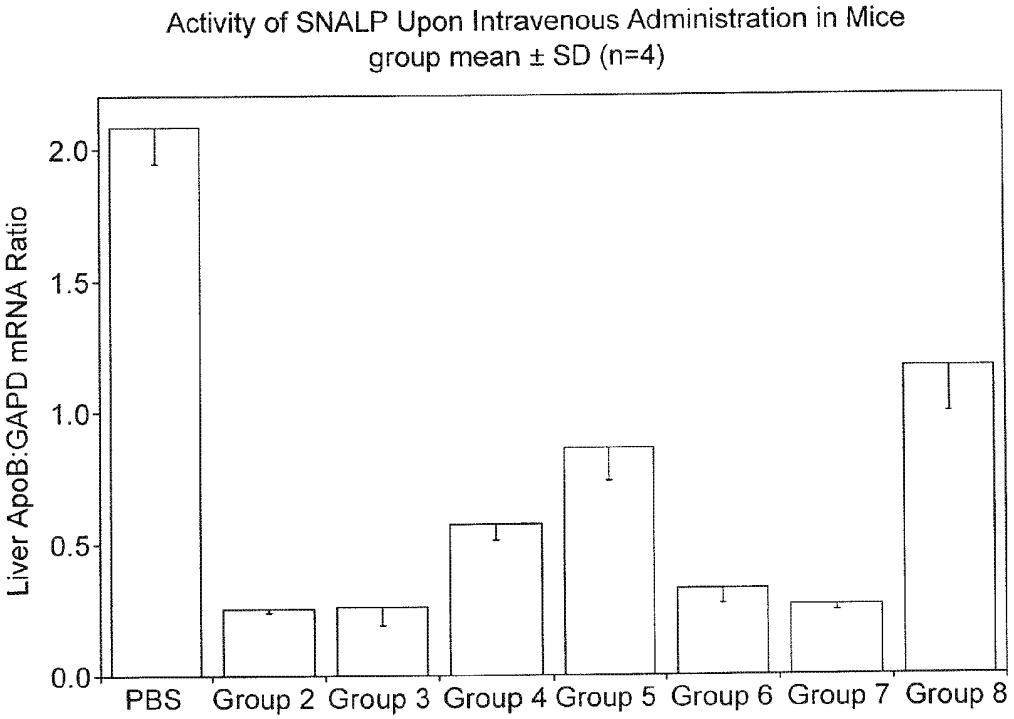

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

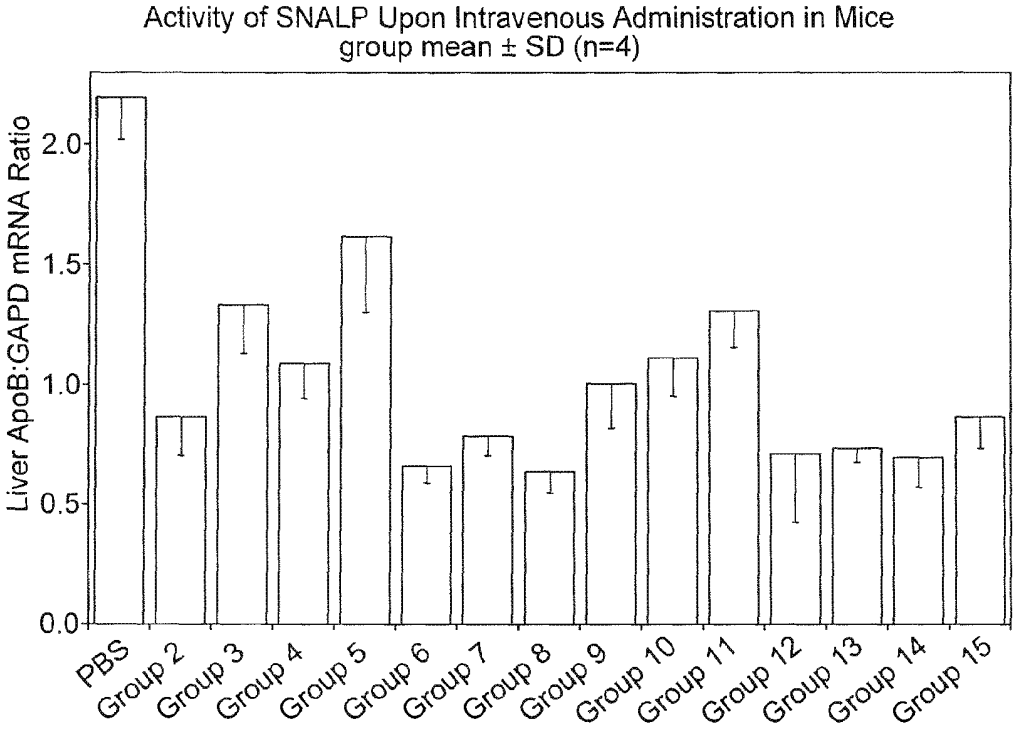

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

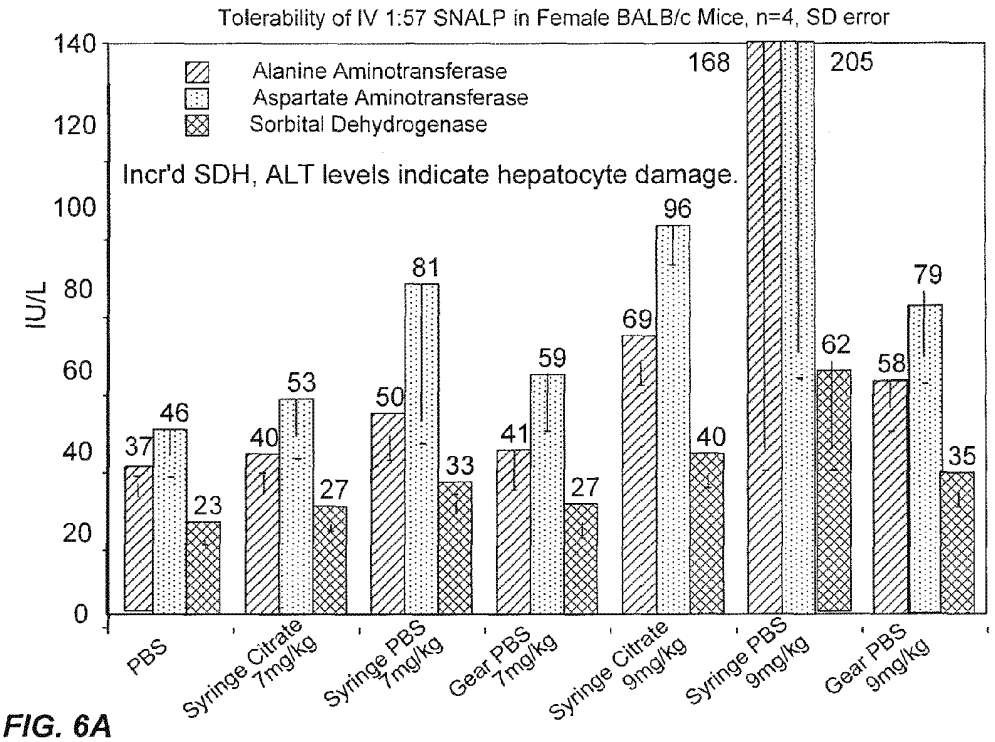

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
140
120
100
80
60
40
20
0
168
205
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
62
58
79
35
PBS
Syringe Citrate 7mg/kg
Syringe PBS 7mg/kg
Gear PBS 7mg/kg
Syringe Citrate 9mg/kg
Syringe PBS 9mg/kg
Gear PBS 9mg/kg

FIG. 6A

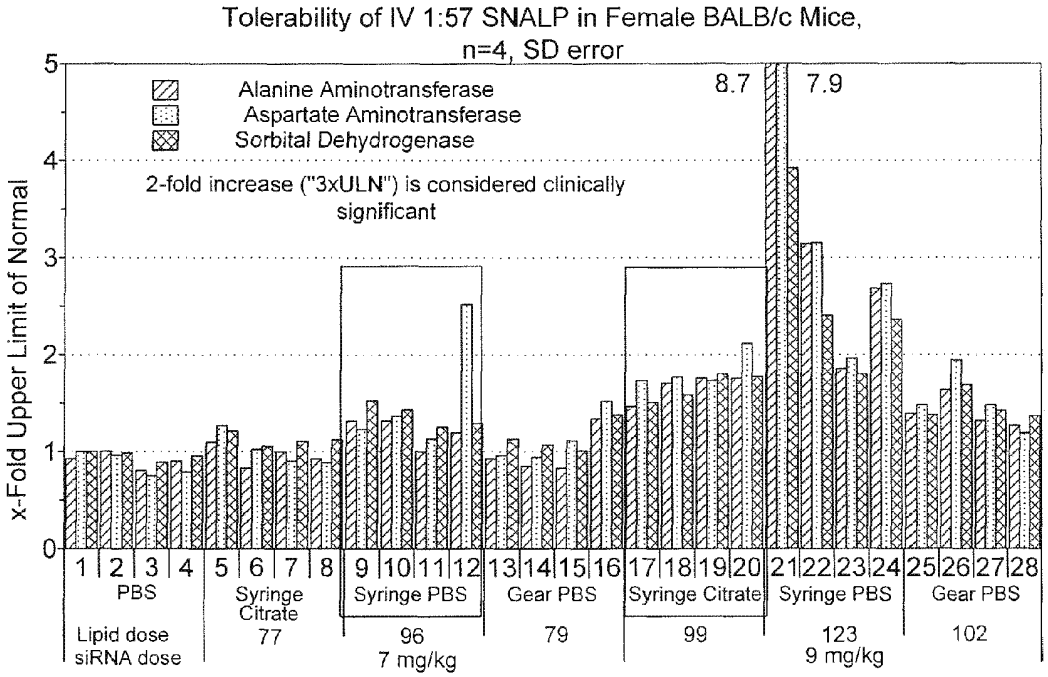

Tolerability of IV 1:57 SNALP in Female BALB/c Mice,
n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
5
4
3
2
1
0
8.7
7.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 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

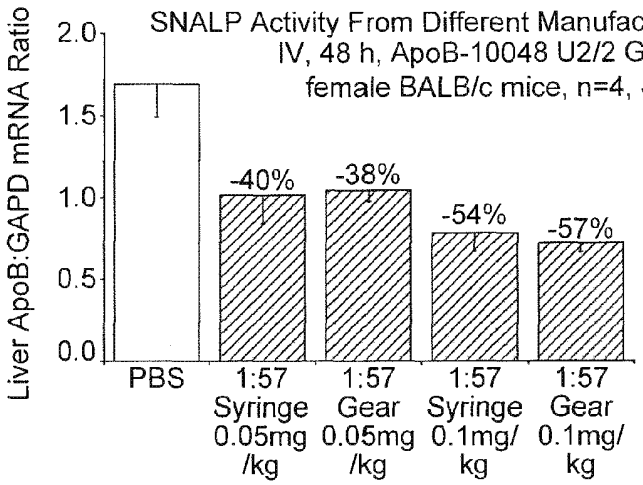


FIG. 7B

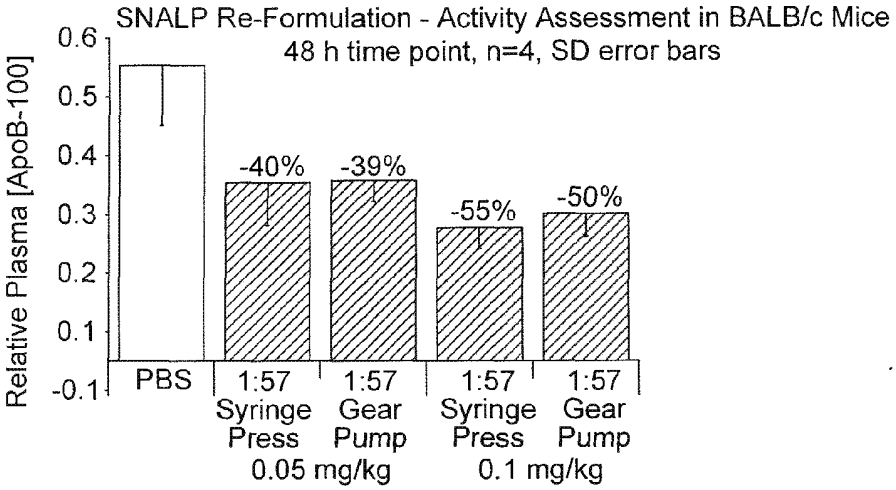
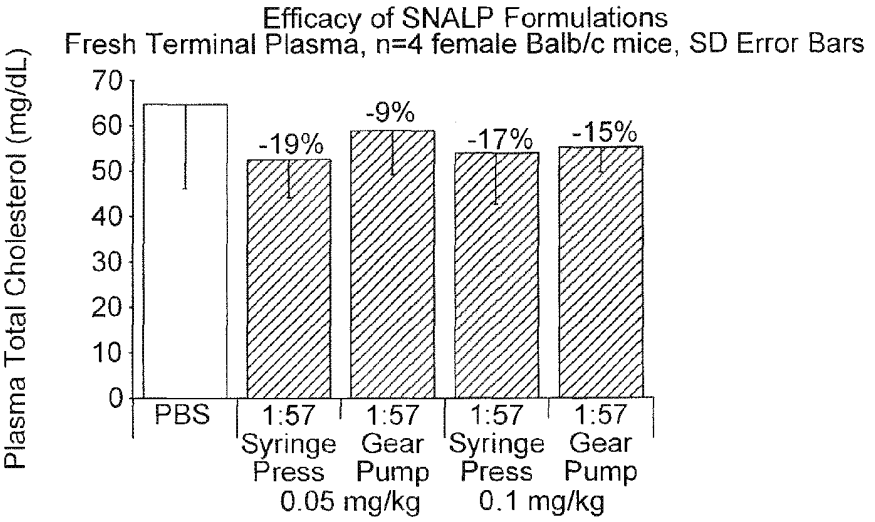


FIG. 7C



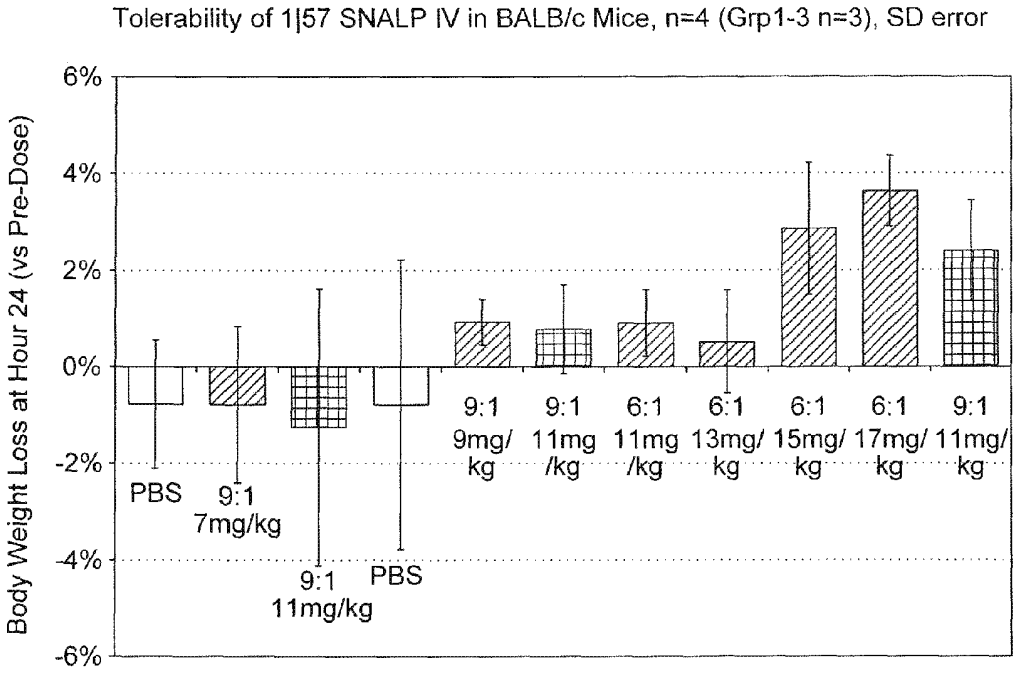

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

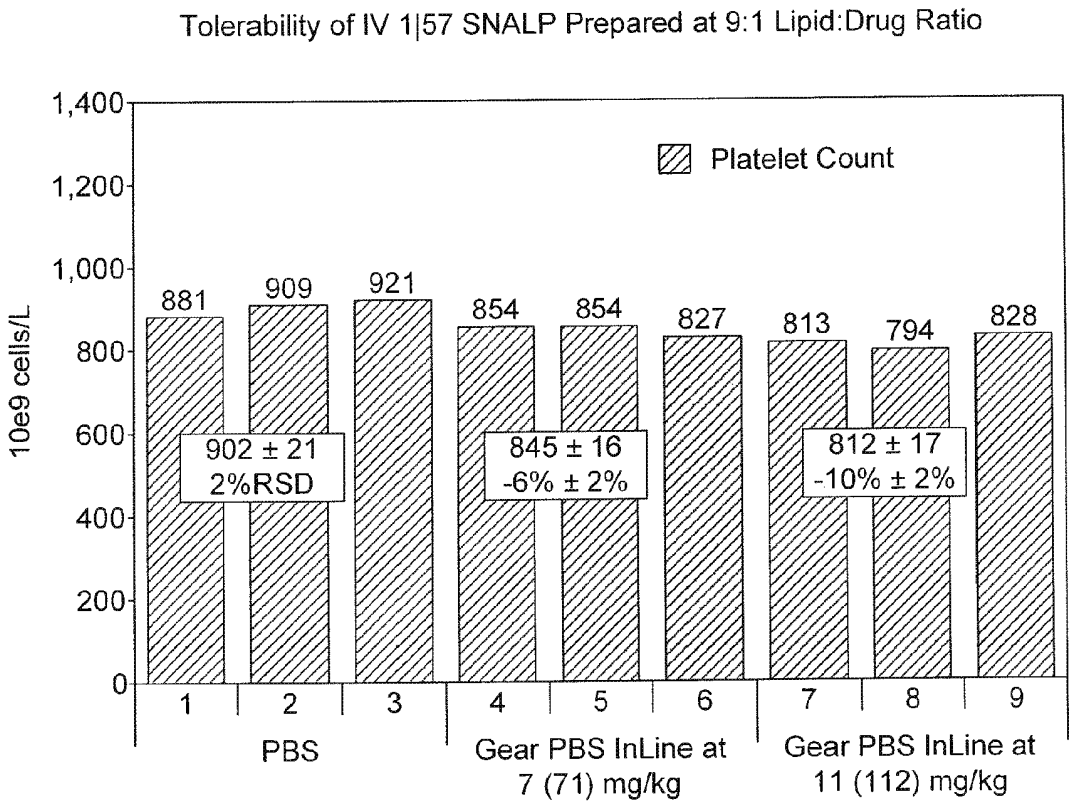

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
Platelet Count
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at
7 (71) mg/kg
Gear PBS InLine at
11 (112) mg/kg

FIG. 9

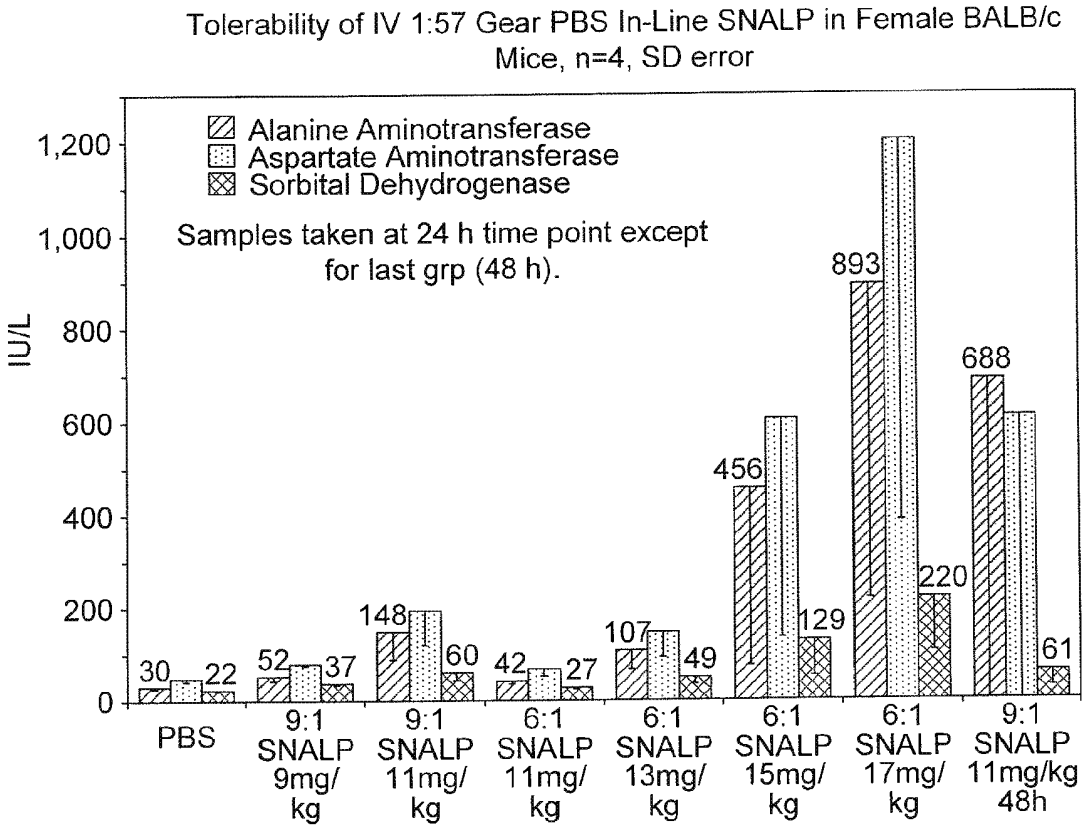

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
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

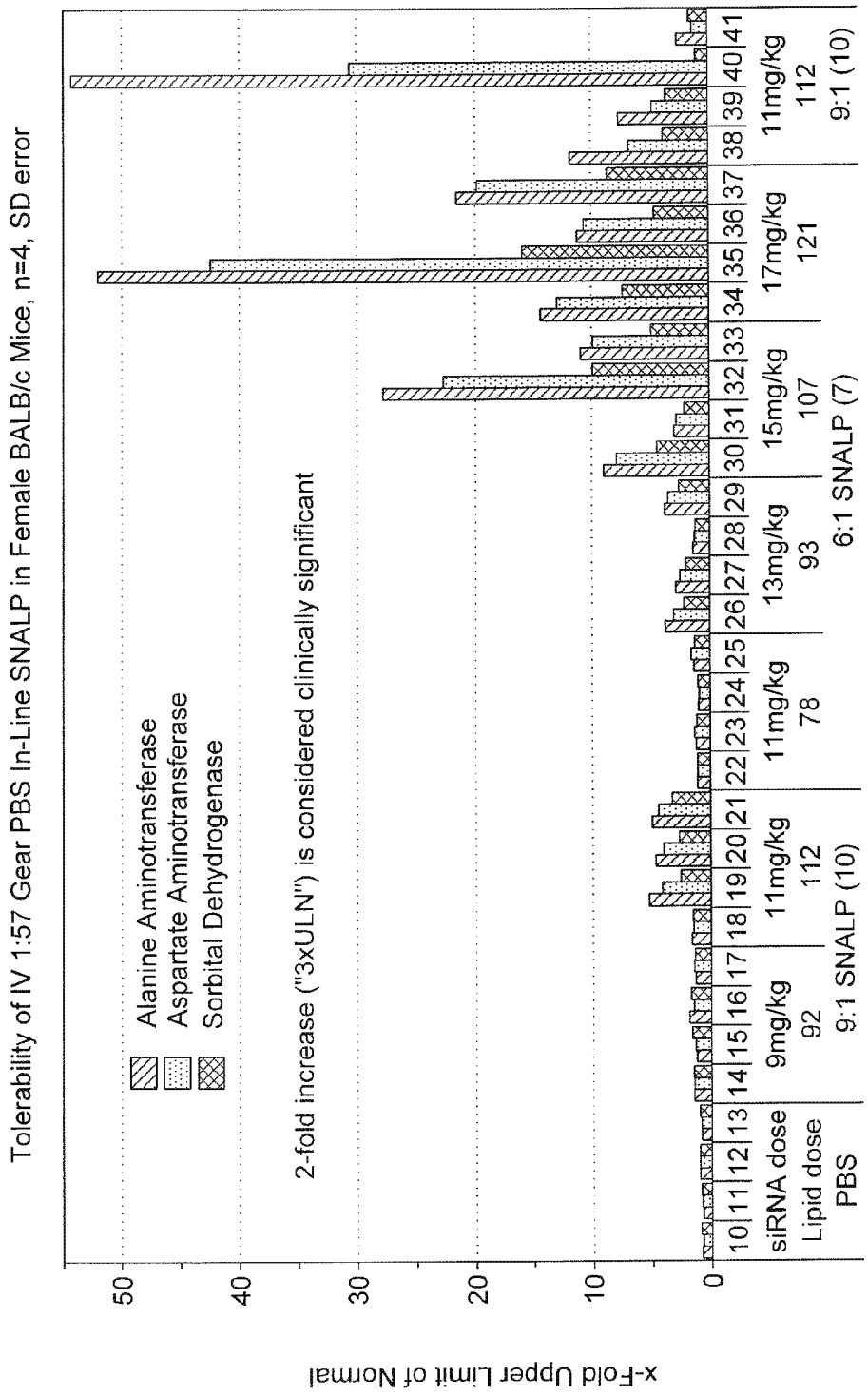

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
10
20
30
40
50
10 11 12 13 14 15 16 17 18 19 20 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

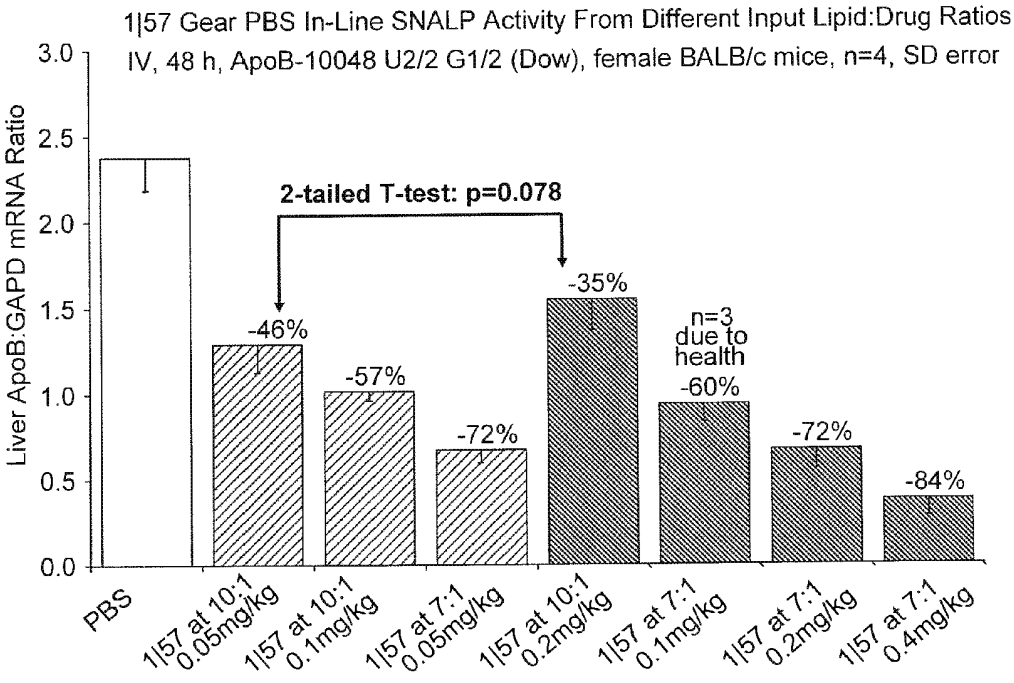
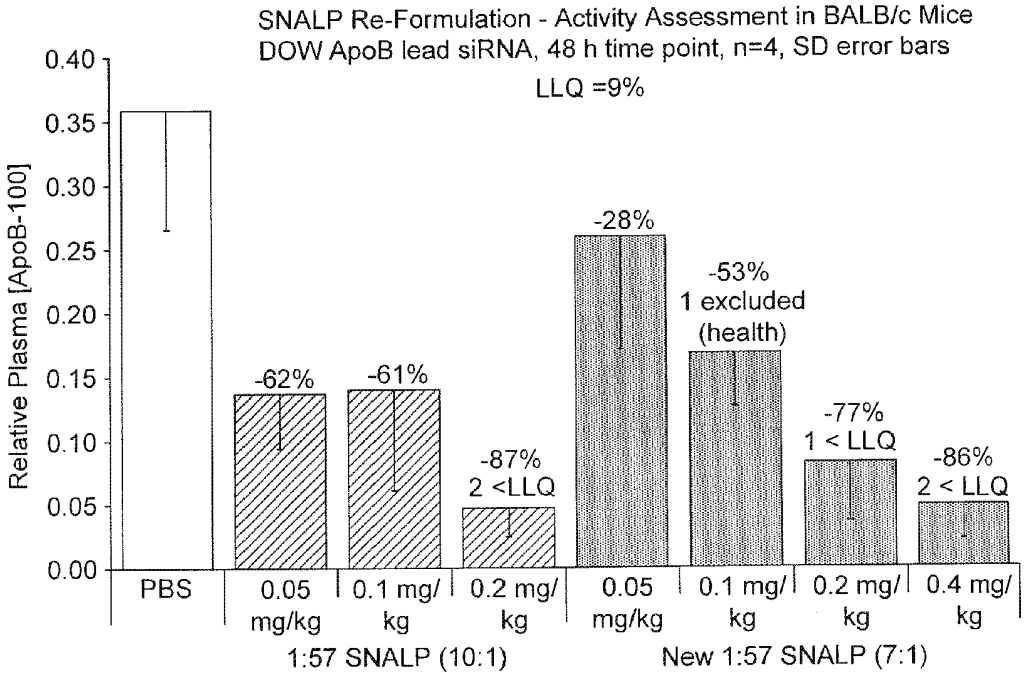


FIG. 11B



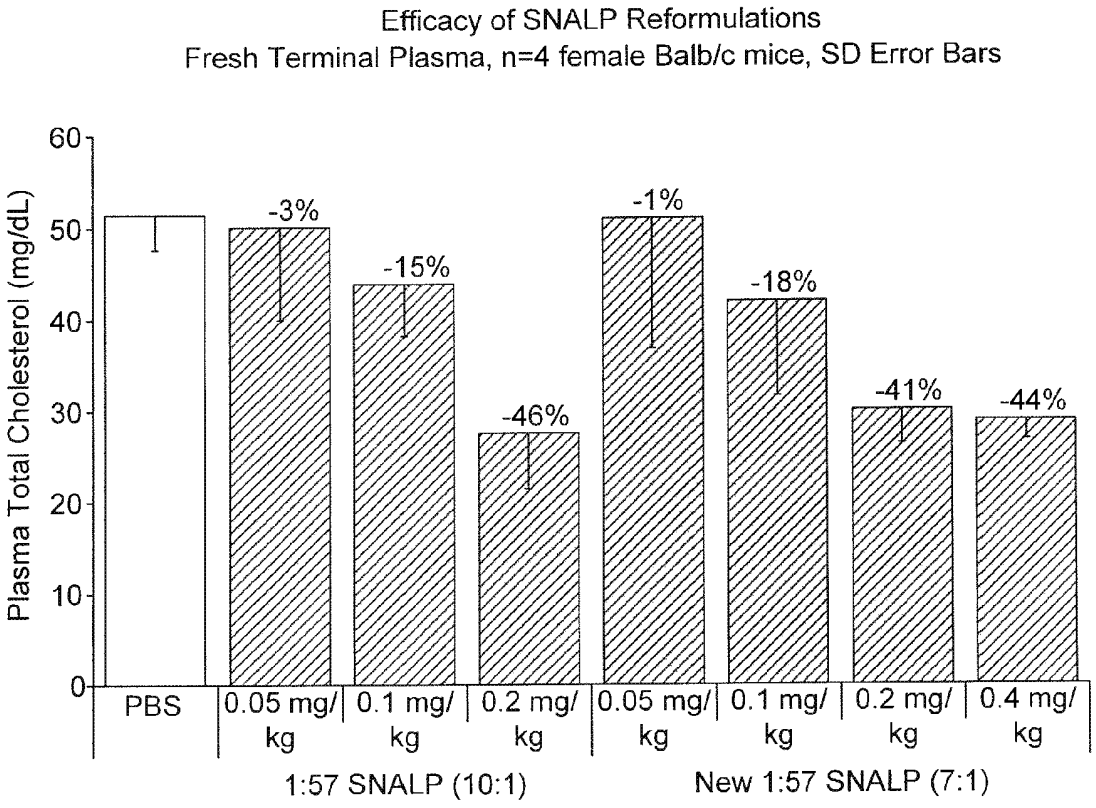

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 12

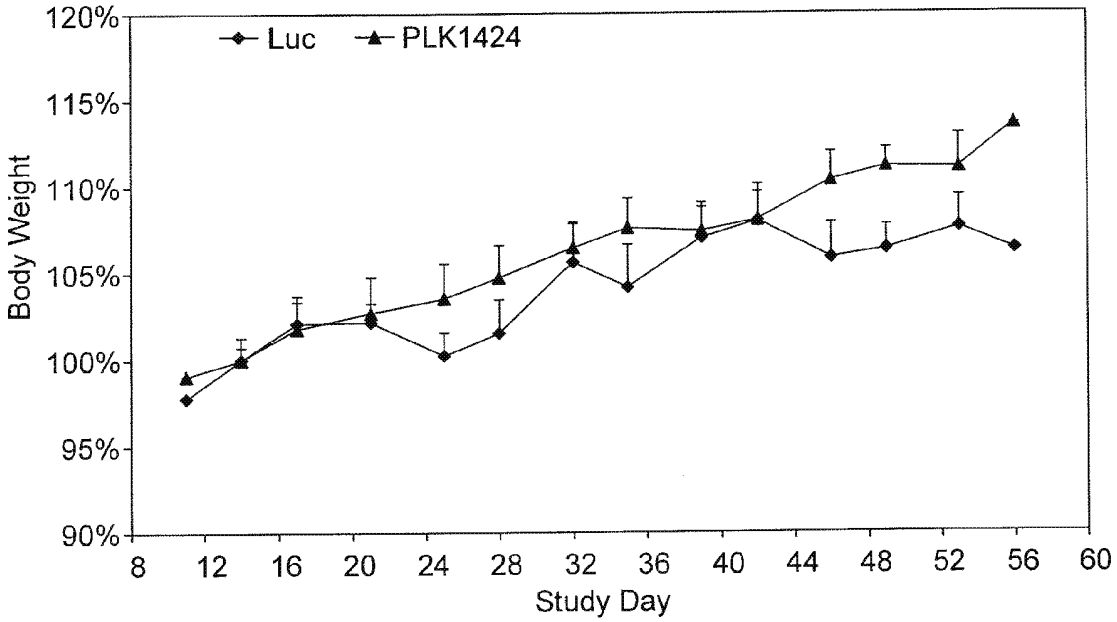

Luc
PLK1424
Body Weight
120%
115%
110%
105%
100%
95%
90%
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

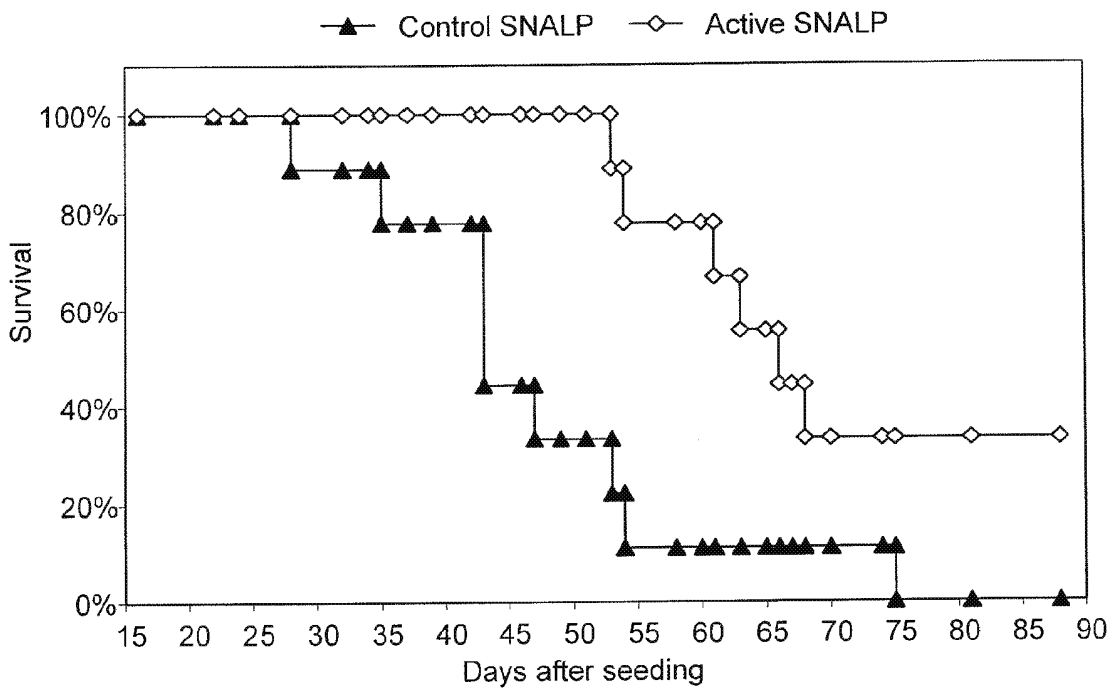

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Days after seeding

FIG. 14

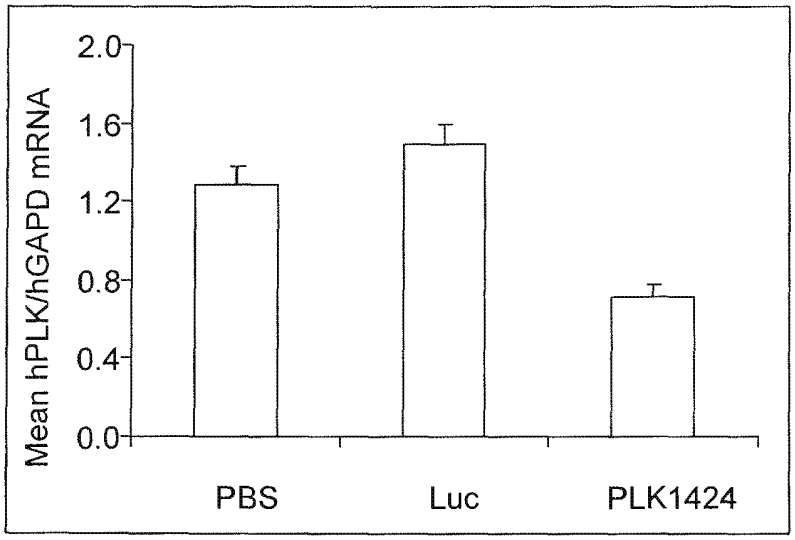


FIG. 15

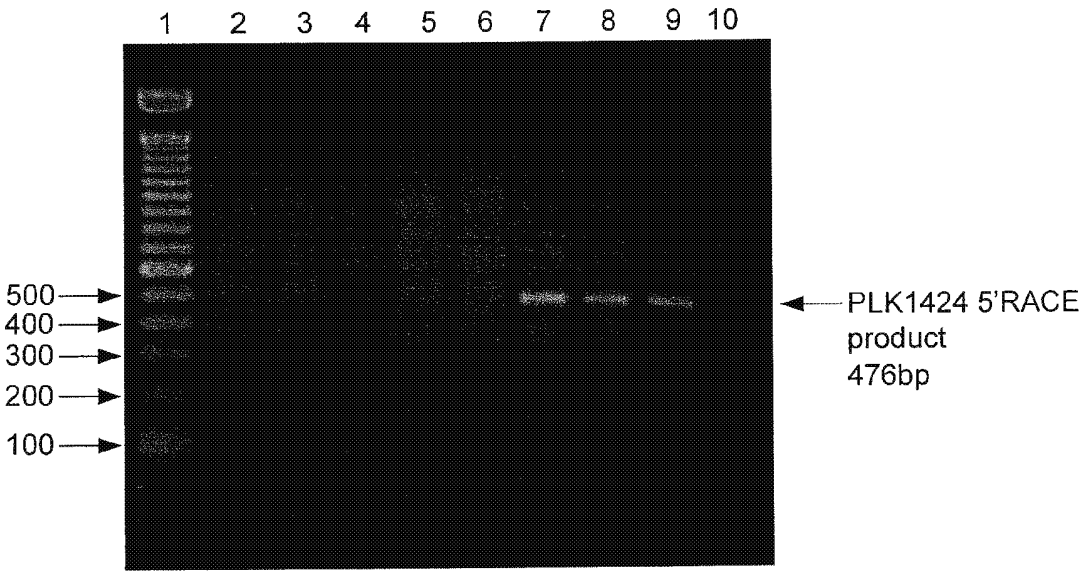
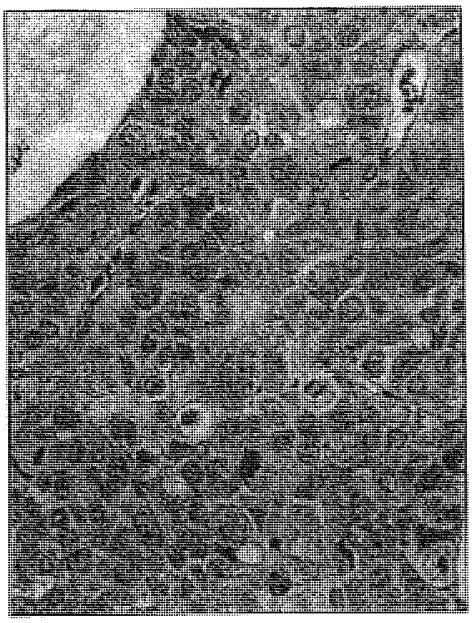
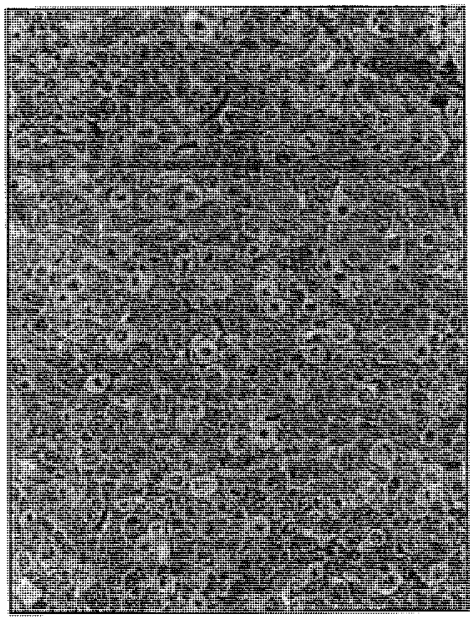

1 2 3 4 5 6 7 8 9 10
500
400
300
200
100
PLK1424 5'RACE
product
476bp

FIG. 16

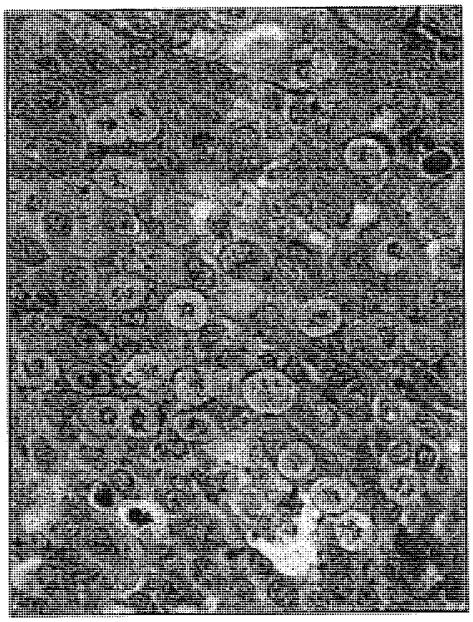
x200 mag



x400 mag



x200 mag



x400 mag

FIG. 17

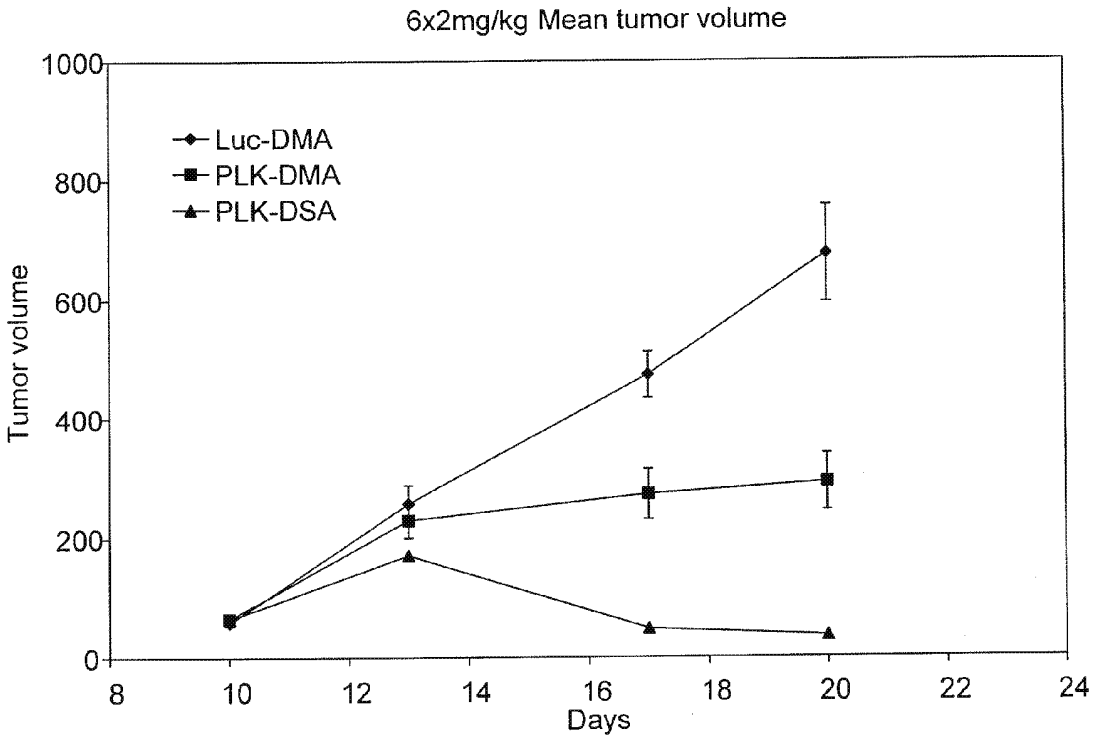

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

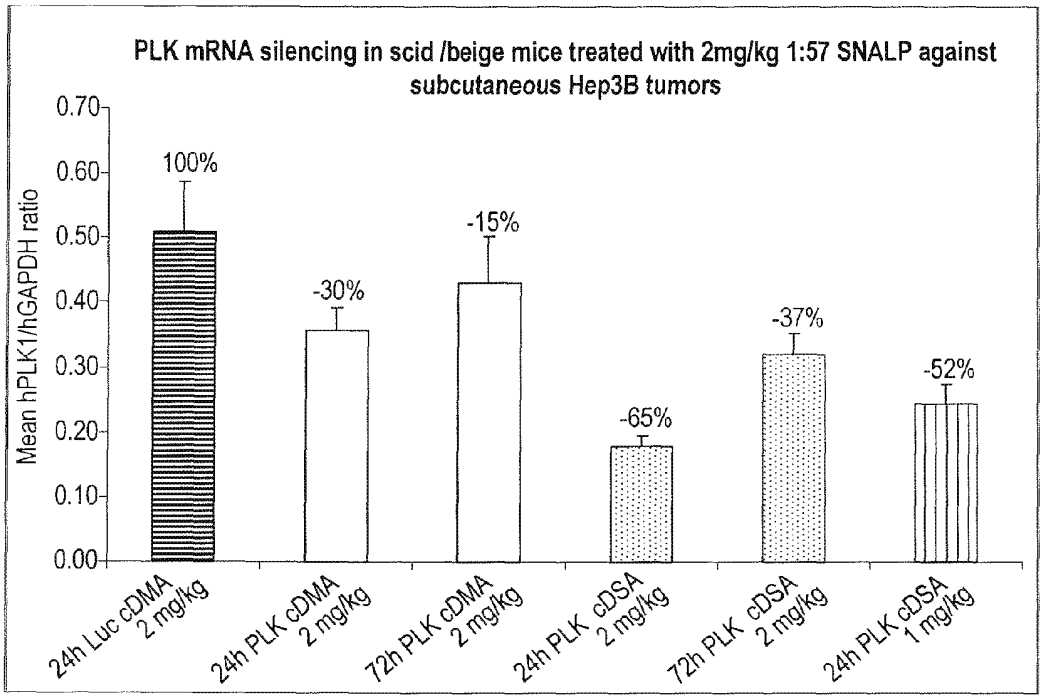

PLK mRNA silencing in scid /beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

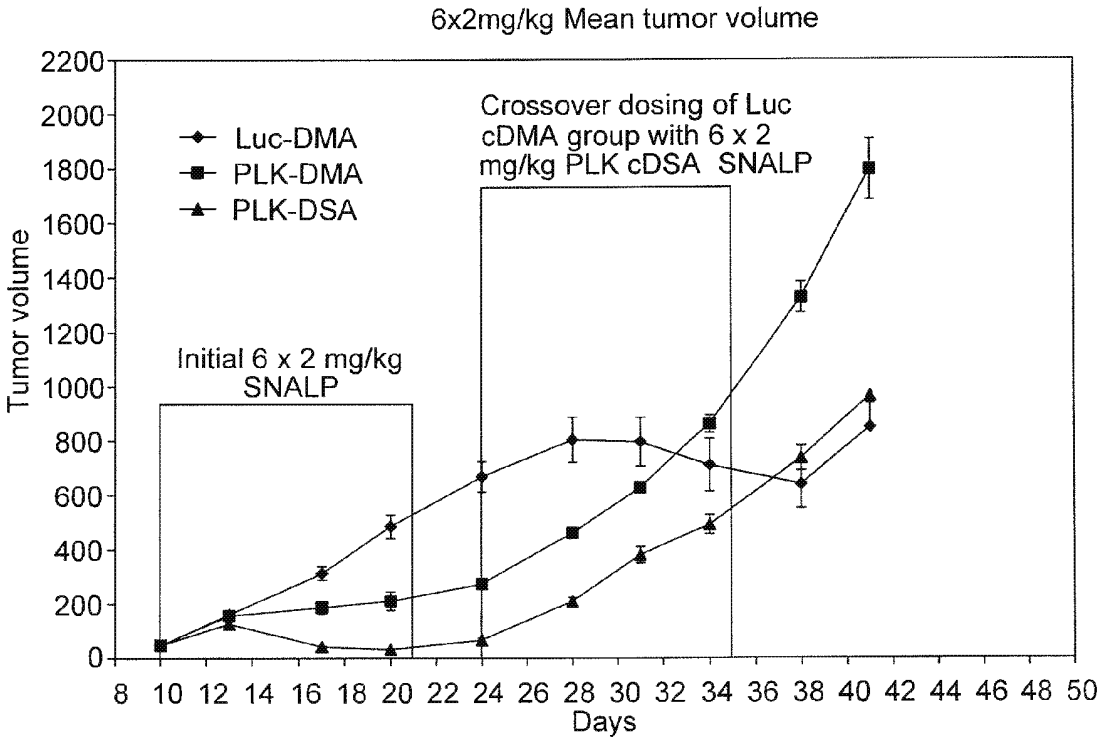

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Initial 6 x 2 mg/kg SNALP
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

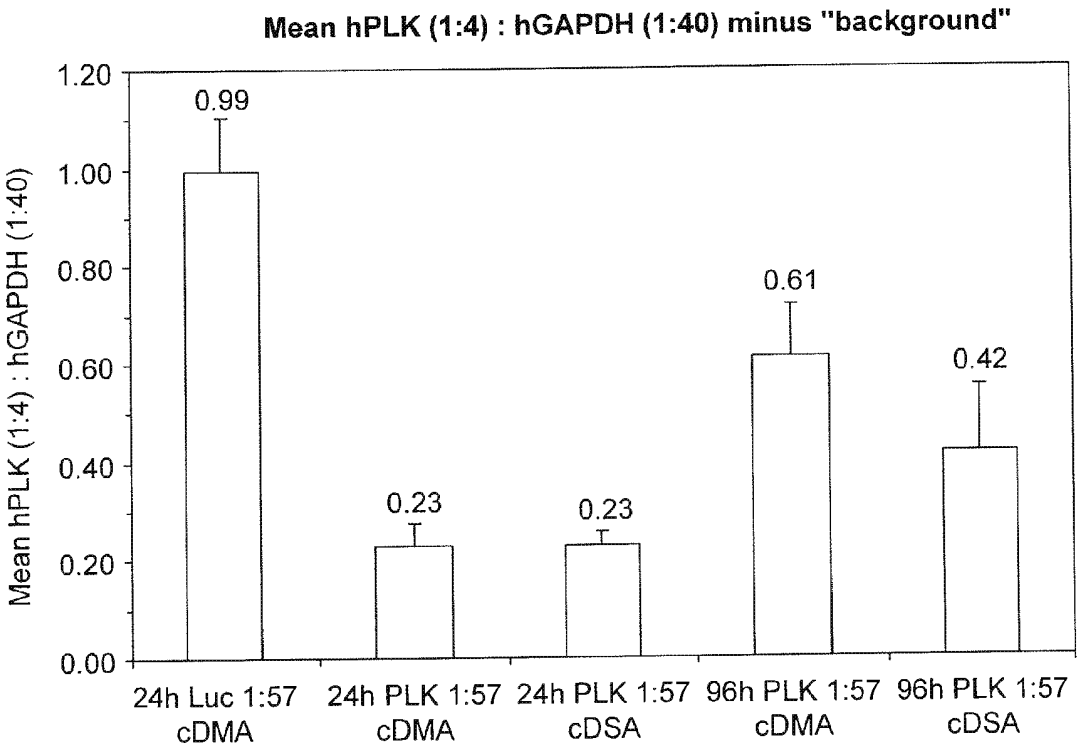

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

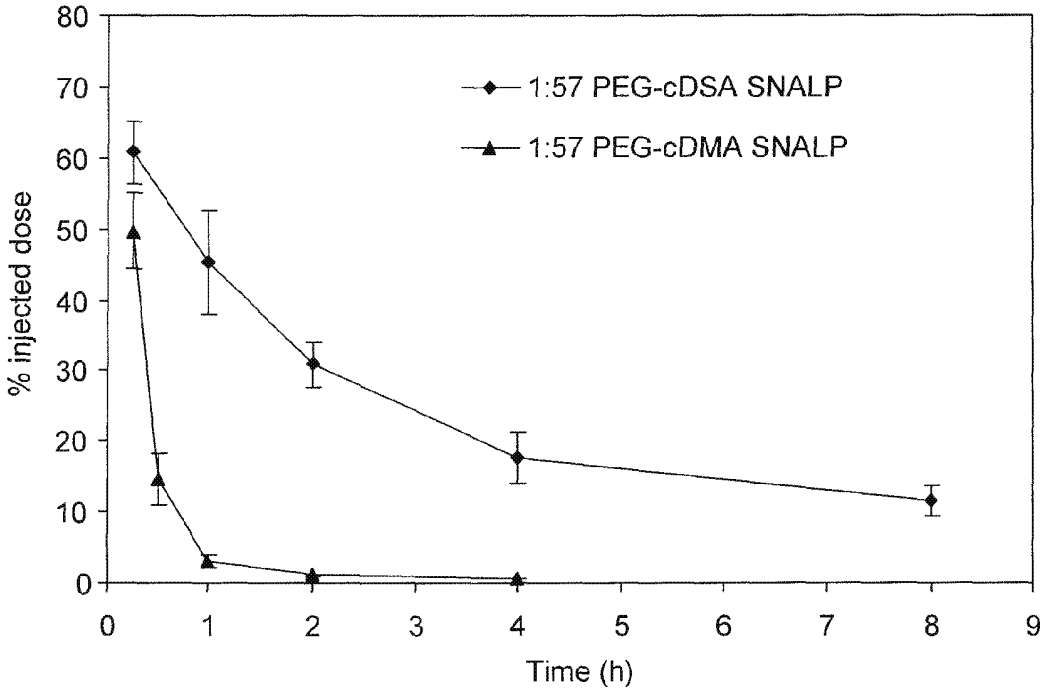

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCES TO RELATED APPLICATIONS

The present application is a continuation of U.S. application Ser. No. 12/424,367 filed Apr. 15, 2009, now U.S. Pat. No. 8,058,069, which application claims priority to U.S. Provisional Application No. 61/045,228, filed Apr. 15, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

Not applicable.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

The Sequence Listing written in file-77-2.TXT, created on Mar. 13, 2012, 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

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.

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.

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.

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. Pat. No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Publication No. 20030073640.

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)).

Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Pat. 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.

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.

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.

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

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).

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.

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.

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).

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.

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.

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.

The present invention also provides pharmaceutical compositions comprising a lipid particle described herein (e.g., SNALP) and a pharmaceutically acceptable carrier.

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).

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).

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).

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

FIG. **1**A (Samples 1-8) and FIG. **1**B (Samples 9-16 illustrate data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

FIG. **2** illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **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.

FIG. **4** illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **5** illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **6**A (expressed as IU/L) and FIG. **6**B (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.

FIG. **7**A (expressed as liver ApoB:GAPD mRNA ratio), FIG. **7**B (expressed as relative plasma ApoB-100 concentration), and FIG. **7**C (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.

FIG. **8** illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

FIG. **9** illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

FIG. **10**A (expressed as IU/L) and FIG. **10**B (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.

FIG. **11**A (expressed as liver ApoB:GAPD mRNA ratio) and FIG. **11**B (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.

FIG. **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).

FIG. **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.

FIG. **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.

FIG. **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.

FIG. **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.

FIG. **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).

FIG. **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.

FIG. **19** illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

FIG. **20** illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

FIG. **21** illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

FIG. **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

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, FIG. **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, FIG. **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").

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.

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.

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

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

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.

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.

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).

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.

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.

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.

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.

"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.

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.

Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5×SSC, and 1% SDS, incubating at 42° C., or, 5×SSC, 1% SDS, incubating at 65° C., with wash in 0.2×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 dena-

turation 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).

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 1×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.

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.

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.

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, Wis.), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology*, Ausubel et al., eds. (1995 supplement)).

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 (www.ncbi.nlm.nih.gov/).

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.

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.

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.

"Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

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.

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.

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.

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.

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).

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. Pat. 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.

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.

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.

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.

The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

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, palmitoyloleoylphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

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 Publi-

cation Nos. 20060083780 and 20060240554; U.S. Pat. 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.

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.

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.

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.

"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.

"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.

"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.

"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.

The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

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

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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-dimethyl-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.

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 Oct. 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 Dec. 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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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 embodi-

ments, 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.

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.

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.

Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-sn3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed Dec. 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) (2 KPEG-DMG). The synthesis of 2 KPEG-DMG is described in U.S. Pat. No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

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.

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.

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.

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, Calif.). "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.

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.

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).

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).

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.

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.

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.

The present invention also provides a pharmaceutical composition comprising a lipid particle (e.g., SNALP) described herein and a pharmaceutically acceptable carrier.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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

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.

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

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, oligonucleotides 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.

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.

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.

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.

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.

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

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 Nykanen et al., *Cell,* 107:309 (2001)), or may lack overhangs (i.e., have blunt ends).

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.

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.

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.

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

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).

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.

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., 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.

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.

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 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.

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.

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. Pat. 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. Pat. 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. Pat. 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.

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, N.J.); human IL-6 and TNF-α (eBioscience; San Diego, Calif.); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences; San Diego, Calif.)).

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

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).

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.

To form a long dsRNA, for synthetic RNAs, the complement is also transcribed in vitro and hybridized to form a dsRNA. If a naturally occurring 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.

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.

Pat. 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.

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, Calif.). 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.

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

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.

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.

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. Pat. 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.

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.

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.

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. Pat. 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

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.

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)).

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. Pat. 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.

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.

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. Pat. No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed Mar. 20, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

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, 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 Jan. 26, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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); COPS signalosome subunits such as CSN1, CSN2, CSN3, CSN4, CSN5 (JAB1; Genbank Accession No. NM_006837); CSN6, CSN7A, CSN7B, and CSN8; ubiquitin ligases such as COPT (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 Ser. 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 Ser. No. 12/343,342, filed Dec. 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 Apr. 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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 Ser. No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

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.

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. Pat. No. 6,174,861), angiostatin (see, e.g., U.S. Pat. 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.

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, TFG-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)).

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)).

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

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.

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 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.

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.

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

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.

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.

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.

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.

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.

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.

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

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.

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. Pat. 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); Penis et al., *Brain Res Mol Brain Res.,* 15; 57:310-20 (1998); and U.S. Pat. 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. Pat. 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.

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

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 a, *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.

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.

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. Pat. No. 5,631,359. An example of the hepatitis 8 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. Pat. 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.

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.

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. Pat. 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

Nucleic acids associated with lipid particles 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).

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.

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 Dec. 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Pat. 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

In certain embodiments, the active agent associated with the lipid particles 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 therapeutic 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.

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 (Iresse), 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.

Examples of conventional hormonal therapeutic agents include, without limitation, steroids (e.g., dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

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.).

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.

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.

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.

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, immunovir, 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

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.

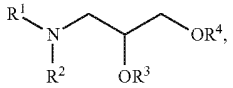
In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (e.g., siRNA, aiRNA, and/or miRNA), a cationic lipid (e.g., a cationic lipid of Formulas I, II, and/or III), a non-cationic lipid (e.g., cholesterol alone 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. Pat. 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

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.

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-dimethyl-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. Pat. 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, N.Y., 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, Wis., USA).

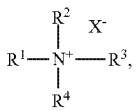
Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.



(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).

Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.

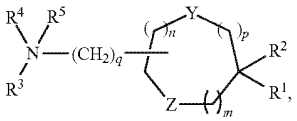


(II)

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.

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, optionally substituted 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.

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).

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.

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

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.

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.

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.

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.

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 stearate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

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.

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.

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.

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.

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

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.

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. Pat. 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, 2 KPEG-DMG, and a mixture thereof.

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. Pat. 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.

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.

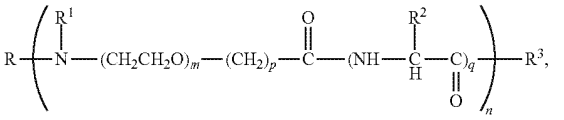
In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, e.g., non-ester containing 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.

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.

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).

The term "ATTA" or "polyamide" refers to, without limitation, compounds described in U.S. Pat. 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)

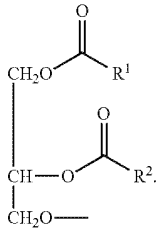


wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R' is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R^1 and the nitrogen to which they are bound form an azido moiety; R^2 is a member of the group selected from hydrogen, optionally 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.

The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R' and

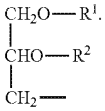
R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C_{16}), 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)



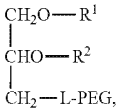
The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:

(VI)



In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:

(VII)

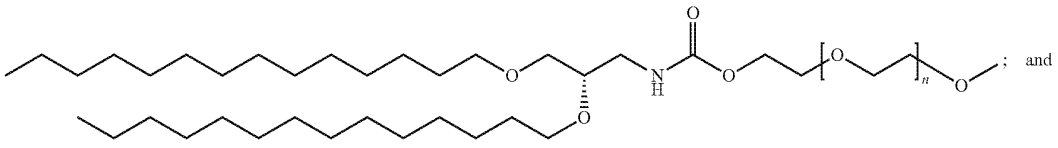


wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated 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.

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.

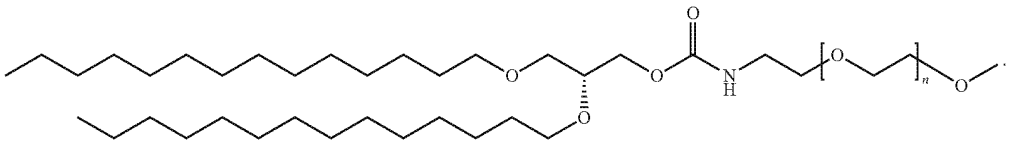
In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide 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).

In particular embodiments, the PEG-lipid conjugate is selected from:



; and

(PEG-C-DMA)



(PEG-C-DOMG)

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).

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.

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.

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. Pat. 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.

Suitable CPLs include compounds of Formula VIII:

A-W-Y (VIII),

wherein A, W, and Y are as described below.

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.

"W" is a polymer or an oligomer such as a hydrophilic polymer or oligomer. Preferably, the hydrophilic polymer is a biocompatible 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.

"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.

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.

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. Pat. 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.

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.

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.

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.

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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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. Pat. 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.

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.

In some embodiments, the nucleic acids in the SNALP are precondensed as described in, e.g., U.S. patent application Ser. No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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, Wis., 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.

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.

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), (10:1), 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.

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 preformed 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. Pat. 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

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.

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).

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

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.

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.

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.

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.

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.

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

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.

For in vivo administration, administration can be in any manner known in the art, e.g., by injection, oral administration, inhalation (e.g., intranasal 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. Pat. 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. Pat. 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.

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.

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. Pat. Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 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. Pat. No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

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.

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.

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. Pat. 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.

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.

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.

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.

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.

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.

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 kilo-

gram 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

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.

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.

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.

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

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.

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).

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

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

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 ^3H, ^{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

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.

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).

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. Pat. No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis et al. eds.) Academic Press Inc. San Diego, Calif. (1990); Arnheim & Levinson (Oct. 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.

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 polynucleotides, 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 polynucleotides 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.

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

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

siRNA:

All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, Colo.). The siRNAs were desalted and annealed using standard procedures.

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-[(-Methoxy-poly(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, Ala.); 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 % t 5 mol %, and the amount of lipid conjugate will be 1.5 mol % f 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

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.

geting 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, Wis.), a resazurin dye that is reduced by metabolically active cells to the fluorogenic 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.

FIG. **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, FIG. **1**B, Sample 9).

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'	1	6/42 = 14.3%	6/38 = 15.8%
	3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUUA-5'	2		

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.

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).

Example 3

ApoB siRNA Formulated as 1:57 SNALP have Potent Silencing Activity In Vivo

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

TABLE 2

Characteristics of the SNALP formulations used in this study.

	Formulation Composition, Mole %		Finished Product Characterization		
Sample No.	PEG(2000)-C-DMA|DLinDMA|DPPC|Cholesterol	Lipid/Drug Ratio	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
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

Silencing of Eg5 by siRNA transfection causes mitotic arrest and apoptosis in mammalian cells. Cell viability following transfection with SNALP containing an siRNA tar-

density lipoproteins (LDL). Mutations in ApoB are associated with hypercholesterolemia. ApoB occurs in the plasma in 2 main forms, ApoB48 and ApoB 100, which are synthe-

sized 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'-AGU**G**UCA**U**CACAC**U**GAAUACC-3'	3	7/42 = 16.7%	7/38 = 18.4%
		3'-GU**U**CACAGUA**G**U**G**UGAC**U**UAU-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.

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).

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Pa-

TABLE 4

Characteristics of the SNALP formulations used in this study.

	Formulation Composition	Lipid/Drug	Finished Product Characterization		
Group	Lipid Name & Mole %	Ratio	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

nomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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

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.

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.

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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.

	Formulation Composition	Lipid/Drug	Finished Product Characterization		
Group	Lipid Name & Mole %	Ratio	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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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|DLinDMA|Cholesterol	Lipid/ Drug Ratio	Finished Product Characterization Size (nm)	Poly-dispersity	% Encap-sulation
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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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

Animal Model: Female BALB/c mice, 5 wks old, n=4 per group/cage.

siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

Tolerability:

Group	Formulation	IV Injection siRNA mg/kg	IV Injection 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	IV Injection 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:

Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

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.

Formulation Summary:

	1:57 (9:1) + DOW siRNA	Particle Size Zavg (nm)	Poly	% Encap	Final L:D (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

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.

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 16,000×g & 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.

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 16,000×g (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.

Termination:

Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

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

There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. FIG. **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.

FIG. **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

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

Animal Model: Female BALB/c mice, 7 wks old.

siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

CBC/Diff:

Group	# Mice	Test Article	IV Dosage Encap. siRNA	IV Dosage 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	IV Dosage 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	IV Dosage 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:

Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

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.

Formulation Summary:

	1\57 SNALP Gear PBS	Particle Size			Final L:D
	In-Line	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

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.

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).

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.

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 16,000×g & 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.

Groups 12-19:

Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16,000×g (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.

Termination:

Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

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:

FIG. **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.

FIG. **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.

FIG. **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:

FIG. **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.

FIG. **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:

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 FIG. **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

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'-AGAUCACCCUCCUUAAAUANN-3' 3'-NNUCUAGUGGGAGGAAUUUAU-5'	5 6	6/38 = 15.8%
PLK1424 U4/G	5'-AGAUCACCCUCCUUAAAUANN-3' 3'-NNUCUAGUGGGAGGAAUUUAU-5'	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 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

20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay
A B	20 to seed	I.H. 1.5×10^6 Hep3B	Luc 1:57 PLK 1424 1:57	9 9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10 × 2 mg/kg	When moribund	Survival Body Weights

Test Articles

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 (28 mM lipid)
B	PLK1424 U4/GU SNALP 1:57 (28 mM lipid)
	PLK1424 U4/G SNALP 1:57 (28 mM 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 (⅜") 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.

-continued

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

FIG. **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.

FIG. **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

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.

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

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. 1×10^6 Hep3B	PBS	6	1×2 mg/kg Day 20	24 h after treatment	Tumor QG
B			Luc 1:57	7			Tumor RACE-PCR Histopathology
C			PLK 1424 1:57	7			

Test Articles

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. 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 (⅜") 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

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.

FIG. **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.

FIG. **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.

FIG. **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

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

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.

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 6×2 mg/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.

FIG. **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.

FIG. **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 FIG. **18**.

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 FIG. **20**, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

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.

FIG. **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.

FIG. **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.

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

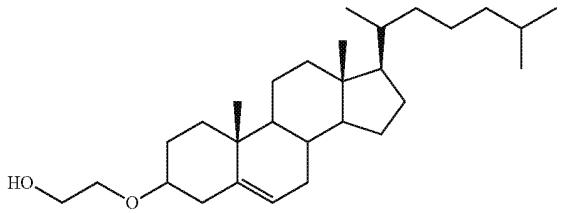
Step 1:

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 (2×50 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%).

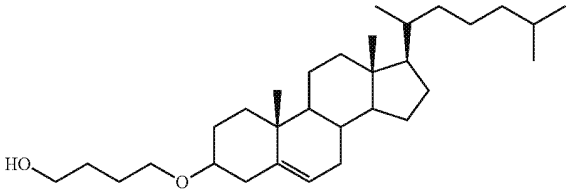
Step 2:

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 (3×100 ml). The organic phases were combined, washed with water (2×150 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%).

The structures of the cholesterol derivatives cholesteryl-2'-hydroxyethyl ether and cholesteryl-4'-hydroxybutyl ether are as follows:



Cholesteryl-2′-hydroxyethyl ether



Cholesteryl-4′-hydroxyethyl ether

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.

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What is claimed is:

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 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.

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 at least one modified nucleotide.

4. The nucleic acid-lipid particle of claim **2**, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

5. The nucleic acid-lipid particle of claim **2**, wherein the siRNA is about 19 to about 25 base pairs in length.

6. The nucleic acid-lipid particle of claim **2**, wherein the siRNA comprises 3' overhangs.

7. The nucleic acid-lipid particle of claim **1**, wherein the cationic lipid comprises from 50 mol % to 60 mol % of the total lipid present in the particle.

8. The nucleic acid-lipid particle of claim **1**, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

9. The nucleic acid-lipid particle of claim **1**, wherein the phospholipid comprises from 4 mol % to 15 mol % of the total lipid present in the particle.

10. The nucleic acid-lipid particle of claim **1**, wherein the phospholipid comprises from 4 mol % to 12 mol % of the total lipid present in the particle.

11. The nucleic acid-lipid particle of claim **1**, wherein the phospholipid comprises from 5 mol % to 12 mol % of the total lipid present in the particle.

12. The nucleic acid-lipid particle of claim **1**, wherein the phospholipid comprises from 6 mol % to 12 mol % of the total lipid present in the particle.

13. The nucleic acid-lipid particle of claim **1**, wherein the cholesterol or derivative thereof comprises from 30 mol % to 35 mol % of the total lipid present in the particle.

14. The nucleic acid-lipid particle of claim **1**, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

15. The nucleic acid-lipid particle of claim **14**, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

16. The nucleic acid-lipid particle of claim **15**, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

17. The nucleic acid-lipid particle of claim **16**, wherein the PEG has an average molecular weight of about 2,000 daltons.

18. 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.

19. The nucleic acid-lipid particle of claim **14**, 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.

20. The nucleic acid-lipid particle of claim **1**, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

21. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim **1** and a pharmaceutically acceptable carrier.

* * * * *

JOINT APPENDIX 03

8270078

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United States Patent and Trademark Office

July 13, 2022

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

PATENT NUMBER: *8,822,668*
ISSUE DATE: *September 2, 2014*

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

W. Montgomery
Wanda Montgomery
Certifying Officer

US008822668B2

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

(10) **Patent No.: US 8,822,668 B2**
(45) **Date of Patent: *Sep. 2, 2014**

(54) **LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY**

(71) Applicant: **Protiva Biotherapeutics, Inc.**, Burnaby (CA)

(72) Inventors: **Edward Yaworski**, Maple Ridge (CA); **Kieu Lam**, Surrey (CA); **Lloyd Jeffs**, Delta (CA); **Lorne Palmer**, Vancouver (CA); **Ian MacLachlan**, Mission (CA)

(73) Assignee: **Protiva Biotherapeutics, Inc.**, Burnaby BC (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.: **13/928,309**

(22) Filed: **Jun. 26, 2013**

(65) **Prior Publication Data**

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Related U.S. Application Data

(63) Continuation of application No. 13/253,917, filed on Oct. 5, 2011, now Pat. No. 8,492,359, which is a continuation of application No. 12/424,367, filed on Apr. 15, 2009, now Pat. No. 8,058,069.

(60) Provisional application No. 61/045,228, filed on Apr. 15, 2008.

(51) **Int. Cl.**
C07H 21/04 (2006.01)

(52) **U.S. Cl.**
USPC **536/24.5**

(58) **Field of Classification Search**
USPC 536/24.5
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,394,448	A	7/1983	Szoka, Jr. et al.
4,438,052	A	3/1984	Weder et al.
4,515,736	A	5/1985	Deamer
4,598,051	A	7/1986	Papahadjopoulos et al.
4,897,355	A	1/1990	Eppstein et al.
5,013,556	A	5/1991	Woodle et al.
5,171,678	A	12/1992	Behr et al.
5,208,036	A	5/1993	Eppstein et al.
5,225,212	A	7/1993	Martin et al.
5,264,618	A	11/1993	Felgner et al.
5,279,833	A	1/1994	Rose
5,283,185	A	2/1994	Epand et al.
5,320,906	A	6/1994	Eley et al.
5,334,761	A	8/1994	Gebeyehu et al.
5,545,412	A	8/1996	Eppstein et al.
5,578,475	A	11/1996	Jessee et al.
5,627,159	A	5/1997	Shih et al.
5,641,662	A	6/1997	Debs et al.
5,656,743	A	8/1997	Busch et al.
5,674,908	A	10/1997	Haces et al.
5,703,055	A	12/1997	Felgner et al.
5,705,385	A	1/1998	Bally et al.
5,736,392	A	4/1998	Hawley-Nelson et al.
5,820,873	A	10/1998	Choi et al.
5,877,220	A	3/1999	Schwartz et al.
5,885,613	A	3/1999	Holland et al.
5,958,901	A	9/1999	Dwyer et al.
5,976,567	A	11/1999	Wheeler et al.
5,981,501	A	11/1999	Wheeler et al.
6,020,202	A	2/2000	Jessee
6,020,526	A	2/2000	Schwartz et al.
6,034,135	A	3/2000	Schwartz et al.
6,051,429	A	4/2000	Hawley-Nelson et al.
6,075,012	A	6/2000	Gebeyehu et al.
6,165,501	A	12/2000	Tirosh et al.
6,172,049	B1	1/2001	Dwyer et al.
6,251,939	B1	6/2001	Schwartz et al.
6,284,267	B1	9/2001	Aneja
6,287,591	B1	9/2001	Semple et al.
6,339,173	B1	1/2002	Schwartz et al.
6,376,248	B1	4/2002	Hawley-Nelson et al.
6,534,484	B1	3/2003	Wheeler et al.
6,586,410	B1	7/2003	Wheeler et al.
6,638,529	B2	10/2003	Schwartz et al.

(Continued)

FOREIGN PATENT DOCUMENTS

CA	2309727	A1	4/1999
CA	2271582	A1	11/1999

(Continued)

OTHER PUBLICATIONS

Arpicco, S. et al., "Preparation and Characterization of Novel Cationic Lipids Developed for Gene Transfection," Proceed. Int'l Symp. Control. Rel. Bioact. Mater. (Controlled Release Society, Inc.), 1999, vol. 26, pp. 759-760.

Arpicco, S. et al., "Synthesis, characterization and transfection activity of new saturated and unsaturated cationic lipids," IL Farmaco, 2004, vol. 59, pp. 869-878.

Ballas, N., et al., "Liposomes bearing a quaternary ammonium detergent as an efficient vehicle for functional transfer of TMV-RNA into plant protoplasts," Biochimica et Biophysica Acta, 1988, vol. 939, pp. 8-18.

Barinaga, M., "Step Taken Toward Improved Vectors for Gene Transfer," Science, 1994, vol. 266, p. 1326.

Bass, "The Short Answer," Nature, 2001, 411: 428-9.

(Continued)

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(74) *Attorney, Agent, or Firm* — Kilpatrick Townsend & Stockton LLP

(57) **ABSTRACT**

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.

23 Claims, 24 Drawing Sheets

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,649,780	B1	11/2003	Eibl et al.	
6,671,393	B2	12/2003	Hays et al.	
6,696,424	B1	2/2004	Wheeler	
6,815,432	B2	11/2004	Wheeler et al.	
6,858,224	B2	2/2005	Wheeler et al.	
7,166,745	B1	1/2007	Chu et al.	
7,422,902	B1	9/2008	Wheeler et al.	
7,479,573	B2	1/2009	Chu et al.	
7,601,872	B2	10/2009	Chu et al.	
7,687,070	B2	3/2010	Gebeyehu et al.	
7,745,651	B2	6/2010	Heyes et al.	
7,799,565	B2 *	9/2010	MacLachlan et al.	435/458
7,803,397	B2	9/2010	Heyes et al.	
7,807,815	B2 *	10/2010	MacLachlan et al.	536/24.5
7,838,658	B2 *	11/2010	MacLachlan et al.	536/24.5
7,901,708	B2	3/2011	MacLachlan et al.	
7,915,450	B2	3/2011	Chu et al.	
7,982,027	B2 *	7/2011	MacLachlan et al.	536/24.5
8,058,068	B2	11/2011	Hawley-Nelson et al.	
8,058,069	B2 *	11/2011	Yaworski et al.	435/458
8,101,741	B2 *	1/2012	MacLachlan et al.	536/24.5
8,158,827	B2	4/2012	Chu et al.	
8,188,263	B2 *	5/2012	MacLachlan et al.	536/24.5
8,227,443	B2 *	7/2012	MacLachlan et al.	514/44 A
8,236,943	B2 *	8/2012	Lee et al.	536/24.5
8,283,333	B2 *	10/2012	Yaworski et al.	514/44 A
8,455,455	B1 *	6/2013	Robbins et al.	514/44 A
8,492,359	B2 *	7/2013	Yaworski et al.	514/44 A
8,513,403	B2 *	8/2013	MacLachlan et al.	536/24.5
8,569,256	B2 *	10/2013	Heyes et al.	514/44 A
8,598,333	B2 *	12/2013	MacLachlan et al.	536/24.5
2001/0048940	A1	12/2001	Tousignant et al.	
2003/0069173	A1	4/2003	Hawley-Nelson et al.	
2003/0072794	A1	4/2003	Boulikas	
2003/0077829	A1	4/2003	MacLachlan	
2003/0143732	A1	7/2003	Fosnaugh et al.	
2004/0063654	A1	4/2004	Davis et al.	
2004/0142892	A1	7/2004	Finn et al.	
2004/0253723	A1	12/2004	Tachas et al.	
2004/0259247	A1	12/2004	Tuschl et al.	
2005/0064595	A1	3/2005	MacLachlan et al.	
2005/0118253	A1	6/2005	MacLachlan et al.	
2005/0260757	A1	11/2005	Gebeyehu et al.	
2006/0008910	A1	1/2006	MacLachlan et al.	
2006/0147514	A1	7/2006	Gebeyehu et al.	
2006/0228406	A1	10/2006	Chiou et al.	
2007/0042031	A1	2/2007	MacLachlan et al.	
2007/0202598	A1	8/2007	Chu et al.	
2007/0202600	A1	8/2007	Chu et al.	
2009/0143583	A1	6/2009	Chu et al.	
2009/0291131	A1	11/2009	MacLachlan et al.	
2010/0159593	A1	6/2010	Chu et al.	
2012/0136073	A1	5/2012	Yang et al.	
2012/0238747	A1	9/2012	Chu et al.	

FOREIGN PATENT DOCUMENTS

CA	2330741	A1	11/1999
CA	2397016	A1	7/2001
CA	2513623		8/2004
JP	03-126211		5/1991
JP	05-202085		8/1993
JP	06-080560		3/1994
WO	91/16024	A1	10/1991
WO	93/05162	A1	3/1993
WO	93/12240	A1	6/1993
WO	93/12756	A2	7/1993
WO	93/24640	A2	12/1993
WO	93/25673	A1	12/1993
WO	95/02698	A1	1/1995
WO	95/18863	A1	7/1995
WO	95/35301	A1	12/1995
WO	96/02655	A1	2/1996
WO	96/10390	A1	4/1996
WO	96/40964	A2	12/1996
WO	96/41873	A1	12/1996
WO	98/51285	A2	11/1998
WO	00/03683	A2	1/2000
WO	00/15820	A1	3/2000
WO	00/62813	A2	10/2000
WO	01/05374	A1	1/2001
WO	01/05873	A1	1/2001
WO	01/93836		12/2001
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WO	03/097805	A2	11/2003
WO	2004/065546	A2	8/2004
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WO	2005/026372	A1	3/2005
WO	2005/035764	A1	4/2005
WO	2005/120152	A2	12/2005
WO	2006/002538	A1	1/2006
WO	2006/053430	A1	5/2006
WO	2007/056861	A1	5/2007
WO	2009/086558	A1	7/2009
WO	2009/111658	A2	9/2009
WO	2010/042877	A1	4/2010
WO	2010/048228	A2	4/2010
WO	2010/088537	A2	8/2010
WO	2010/105209	A1	9/2010

OTHER PUBLICATIONS

Beale, G. et al., "Gene Silencing Nucleic Acids Designed by Scanning Arrays: Anti-EGFR Activity of siRNA, Ribozyme and DNA Enzymes Targeting a Single Hybridization-accessible Region using the Same Delivery System," Journal of Drug Targeting, 2003, vol. 11, No. 7, pp. 449-456.

Behr, J.-P., "Synthetic Gene-Transfer Vectors," Acc. Chem. Res., 1993, vol. 26, pp. 274-278.

Brigham, K. et al., "Rapid Communication: In vivo Transfection of Murine Lungs with a Functioning Prokaryotic Gene Using a Liposome Vehicle," The American Journal of the Medical Sciences, vol. 298, No. 4, pp. 278-281, 1989.

Brummelkamp et al., "A System for Stable Expression of Short Interfering RNAs in Mammalian Cells," Science, 2002, V. 296. pp. 550-553.

Cevc, G., "How Membrane Chain-Melting Phase-Transition Temperature is Affected by the Lipid Chain Asymmetry and Degree of Unsaturation: An Effective Chain-Length Model," Biochemistry, 1991, vol. 30, pp. 7186-7193.

Chonn et al., "Recent advances in liposomal drug-delivery systems," Current Opinion in Biotechnology, 1995, vol. 6, pp. 698-708.

Cortesi, R. et al., "Effect of cationic liposome composition on in vitro cytotoxicity and protective effect on carried DNA," International Journal of Pharmaceutics, 1996, vol. 139, pp. 69-78.

Crystal, R., "Transfer of Genes to Humans: Early Lessons and Obstacles to Success," Science, 1995, vol. 270, pp. 404-410.

Culver K., "The First Human Gene Therapy Experiment," Gene Therapy: A Handbook for Physicians, 1994, pp. 33-40.

Duzgunes, N., "Membrane Fusion," Subcellular Biochemistry, 1985, vol. 11, pp. 195-286.

Dwarki, V.J., et al., "Cationic Liposome-Mediated RNA Transfection," Methods in Enzymology, 1993, vol. 217, pp. 644-654.

Elbashir et al., "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells," Nature, May 2001, pp. 494-498, vol. 411.

Enoch, H. et al., "Formation and properties of 1000-Å-diameter, single-bilayer phospholipid vesicles," Proc. Natl. Acad. Sci. USA, 1979, vol. 76, No. 1, pp. 145-149.

Felgner, J. et al., "Cationic Lipid-Mediated Transfection in Mammalian Cells: 'Lipofection,'" J. Tiss. Cult. Meth., 1993, vol. 15, pp. 63-68.

Felgner, J., et al., "Enhanced Gene Delivery and Mechanism Studies with a Novel Series of Cationic Lipid Formulations," The Journal of Biological Chemistry, 1994, vol. 269, No. 4, pp. 2550-2561.

Felgner, P. et al., "Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure," Proc. Natl. Acad. Sci. USA, 1987, vol. 84, pp. 7413-7417.

(56) **References Cited**

OTHER PUBLICATIONS

Felgner, P.L. et al., "Cationic Liposome Mediated Transfection," Proc. West. Pharmacol. Soc., 1989, vol. 32, pp. 115-121.
Gao, X. et al., "A Novel Cationic Liposome Reagent for Efficient Transfection of Mammalian Cells," Biochem. Biophys. Res. Comm., 1991, vol. 179, No. 1, pp. 280-285.
Gershon, H. et al., "Mode of Formation and Structural Feature of DNA-Cationic Liposome Complexes Used for Transfection," Biochemistry, 1993, vol. 32, pp. 7143-7151.
Global Newswire, retrieved from http://globalnewswire.com on Feb. 27, 2013, Tekmira sues Alnylam Pharmaceuticals for repeated misuse of tradesecrets and confidential information, Mar. 16, 2011, pp. 1-3.
Guy-Caffey, J. et al., "Novel Polyaminolipids Enhance the Cellular Uptake of Oligonucleotides," The Journal of Biological Chemistry, 1995, vol. 270, No. 52, pp. 31391-31396.
Hawley-Nelson, P. et al., "LipofectAmine™ Reagent: A New, Higher Efficiency Polycationic Liposome Transfection Reagent," Focus, 1993, vol. 15, No. 3, pp. 73-80.
Heyes et al., "Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids," Journal of Controlled Release, 2005, vol. 107, pp. 276-287.
Heyes et al., "Synthesis of novel cationic lipids: effect of structural modification on the efficiency of gene transfer," J. Med. Chem., 2002, vol. 45, pp. 99-114.
Hyde, S. et al., "Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy," Nature, 1993, vol. 362, pp. 250-255.
Jiang, L. et al., "Comparison of protein precipitation methods for sample preparation prior to proteomic analysis," Journal of Chromatography A, 2004, vol. 1023, pp. 317-320.
JP06080560—English abstract from CAplus 2 pages, 1994.
Juliano, R. et al., "The Effect of Particle Size and Charge on the Clearance Rates of Liposomes and Liposome Encapsulated Drugs," Biochem. Biophys. Res. Commun., 1975, vol. 63, No. 3, pp. 651-658.
Keough, K., "Influence of chain unsaturation and chain position on thermotropism and intermolecular interactions in membranes," Biochem. Soc. Transactions, 1990, vol. 18, No. 5, pp. 835-837.
Krichevsky, A. et al., "RNAi functions in cultured mammalian neurons," PNAS, 99(18):11926-29, 2002.
Lawrence et al., "The formation, characterization and stability of non-ionic surfactant vesicles," S.T.P. Pharma Sciences, 1996, vol. 6, No. 1, pp. 49-60.
Lawrence et al., "Synthesis and aggregation properties of dialkyl polyoxyethylene glycerol ethers," Chemistry and Physics of Lipids, 1996, 82(2):89-100.
Legendre, J.-Y. et al., "Delivery of Plasmid DNA into Mammalian Cell Lines Using pH-Sensitive Liposomes: Comparison with Cationic Liposomes," Pharm. Res., 1992, vol. 9, No. 10, pp. 1235-1242.
Leventis, R. et al., "Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles," Biochem. Biophys. Acta, 1990, vol. 1023, pp. 124-132.
Liu et al., "Cationic Liposome-mediated Intravenous Gene Delivery", J. Biol. Chem., 1995, V. 270, pp. 24864-24870.
Marshall, E., "Gene Therapy's Growing Pains," Science, 1995, vol. 269, pp. 1050-1055.
Murahashi et al., "Synthesis and evaluation of neoglycolipid for liposome modification," Biol. Pharm. Bull., 1997, 20(6):704-707.
Orkin, S. et al., NIH Report, Report and Recommendations of the Panel to Assess the NIH Investment in Research on Gene Therapy, 1995, pp. 1-41.
Parr et al., Factors influencing the retention and chemical stability of polly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles, Biochimica et Biophysica Acta, 1994, 1195:21-30.
Paul, C. et al., "Effective expression of small interfering RNA in human cells," Nature Biotech., 2002, vol. 20, pp. 505-508.
Puyal, C. et al., "A new cationic liposome encapsulating genetic material: A potential delivery system for polynucleotides," Eur. J. Biochem., 1995, vol. 228, pp. 697-703.
Sawada et al., "Microemulsions in supercritical CO_2 utilizing the polyethyleneglycol dialkylglycerol and their use for the solubilization of hydrophiles," Dyes and Pigments, 2005, pp. 64-74, vol. 65.
Shin et al. "Acid-triggered release via dePEGylation of DOPE liposomes containing acid-labile vinyl ether PEG-lipids," Journal of Controlled Release, 2003, vol. 91, pp. 187-200.
Song et al., "Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes," Biochimica et Biophysica Acta, 2002, 1558:1-13.
Sorensen et al., "Gene Silencing by Systemic Delivery of Synthetic siRNAs in Adult Mice", J. Biol. Chem., 2003, V. 327, pp. 761-766.
Spagnou, S. et al., "Lipidic Carriers of siRNA: Differences in the Formulation, Cellular Uptake, and Delivery with Plasmid DNA," Biochemistry, 2004, vol. 43, pp. 13348-13356.
Stamatatos, L. et al., "Interactions of Cationic Lipid Vesicles with Negatively Charged Phospholipid Vesicles and Biological Membranes," Biochemistry, 1988, vol. 27, pp. 3917-3925.
Szoka, F. et al., "Comparative Properties and Methods of Preparation of Lipid Vesicles (Liposomes)," Ann. Rev. Biophys. Bioeng., 1980, vol. 9, pp. 467-508.
Szoka, F. et al., "Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation," Proc. Natl. Acad. Sci. USA, 1978, vol. 75, No. 9, pp. 4194-4198.
Templeton, "Cationic Liposome-mediated Gene Delivery In vivo", Bioscience Reports, 2002, vol. 22, No. 2, pp. 283-295.
Van Der Woude, I. et al., "Parameters influencing the introduction of plasmid DNA into cells by the use of synthetic amphiphiles as a carrier system," Biochimica et Biophysica Acta, 1995, vol. 1240, pp. 34-40.
Wheeler et al., "Stabilized Plasmid-lipid Particles: Constructions and Characterization," Gene Therapy, V. 6, pp. 271-281, 1999.
Wilson, R. et al., "Counterion-Induced Condensation of Deoxyribonucleic Acid, A Light-Scattering Study," Biochemistry, 1979, vol. 18, No. 11, pp. 2192-2196.
Woodle, M.C. et al., "Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes," Biochimica et Biophysica Acta, 1992, vol. 1105, pp. 193-200.
Zhu, N. et al., "Systemic Gene Expression After Intravenous DNA Delivery into Adult Mice," Science, 1993, vol. 261, pp. 209-211.

* cited by examiner

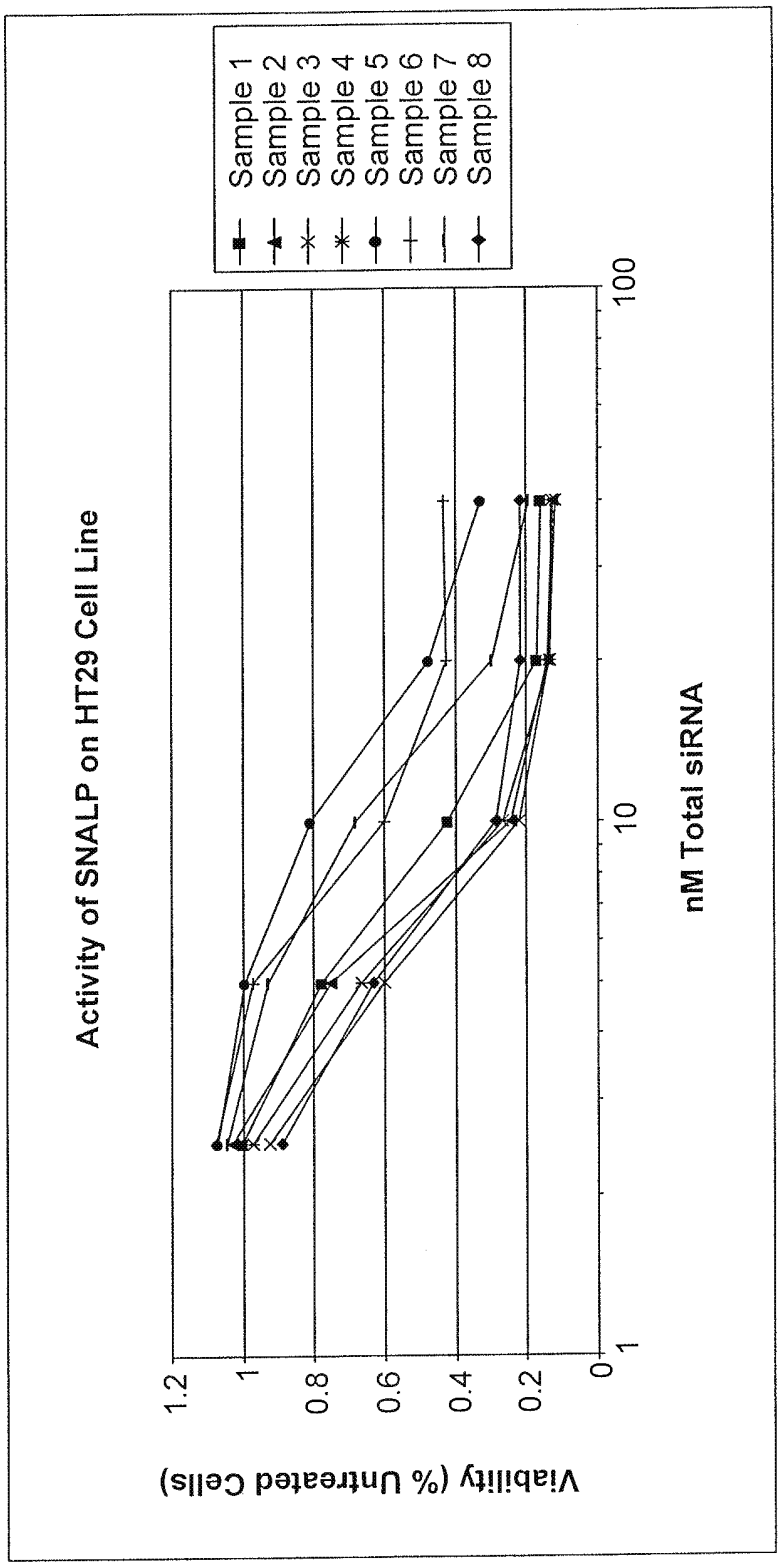

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8

FIG. 1A

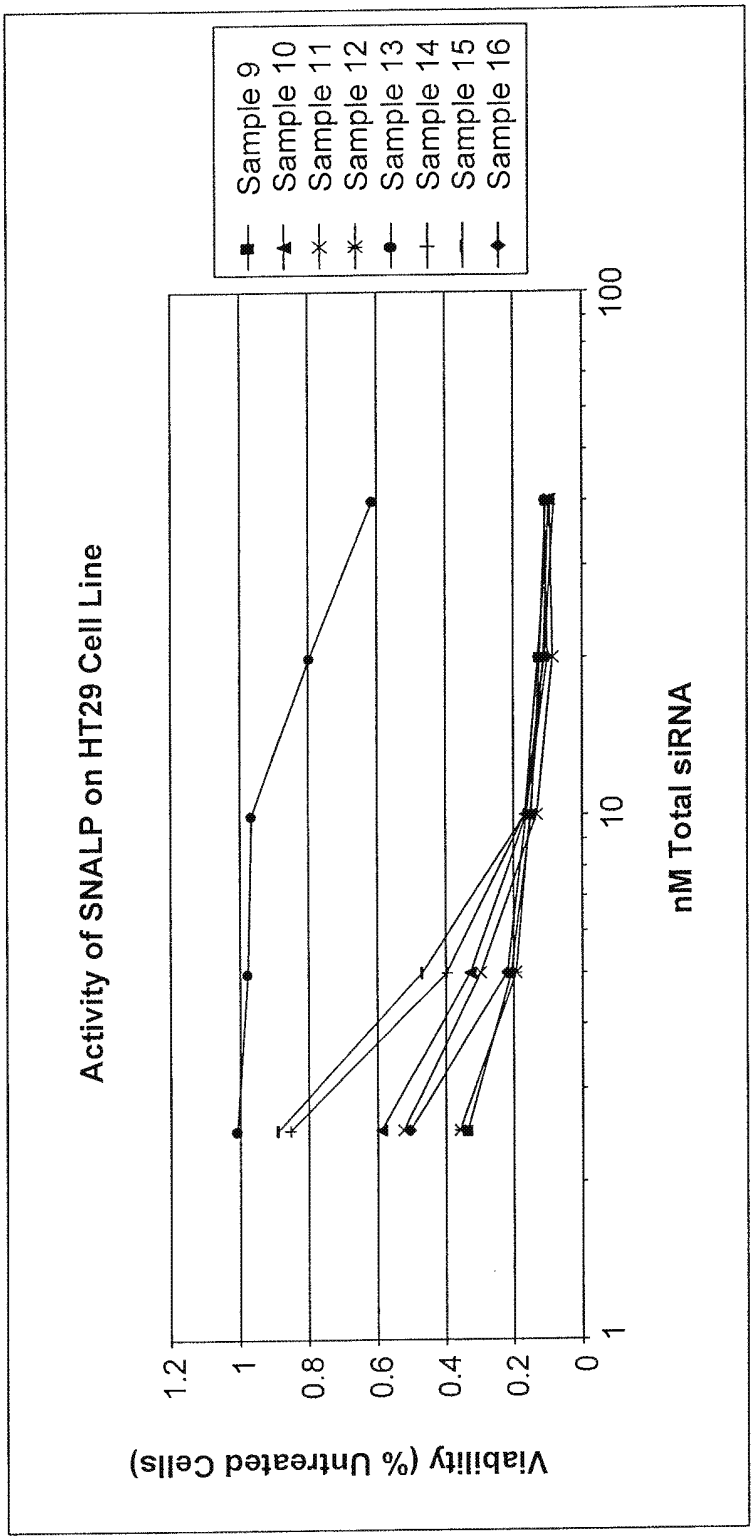

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 9
Sample 10
Sample 11
Sample 12
Sample 13
Sample 14
Sample 15
Sample 16

FIG. 1B

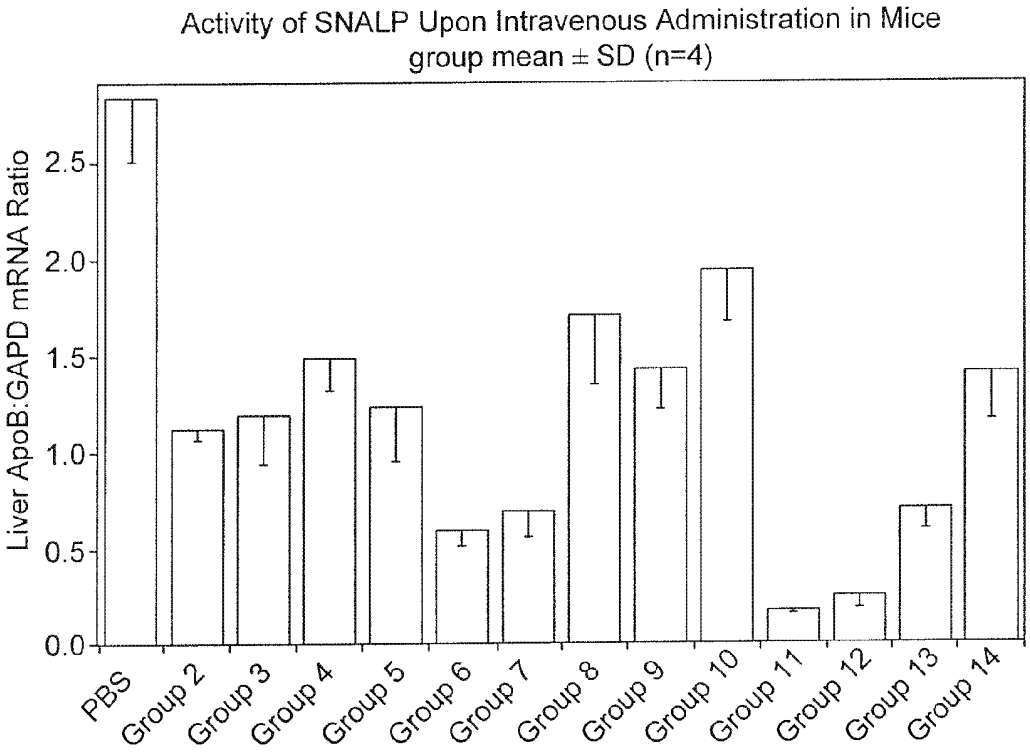

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
2.5
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

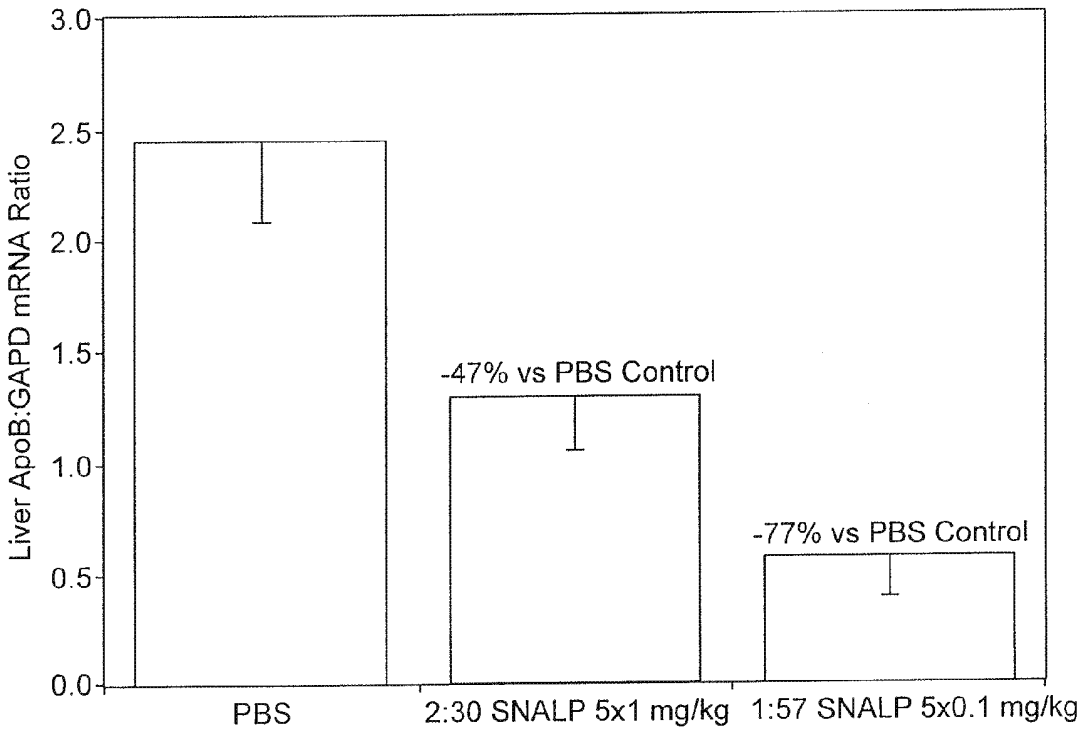

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Liver ApoB:GAPD mRNA Ratio
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5x1 mg/kg
1:57 SNALP 5x0.1 mg/kg

FIG. 3

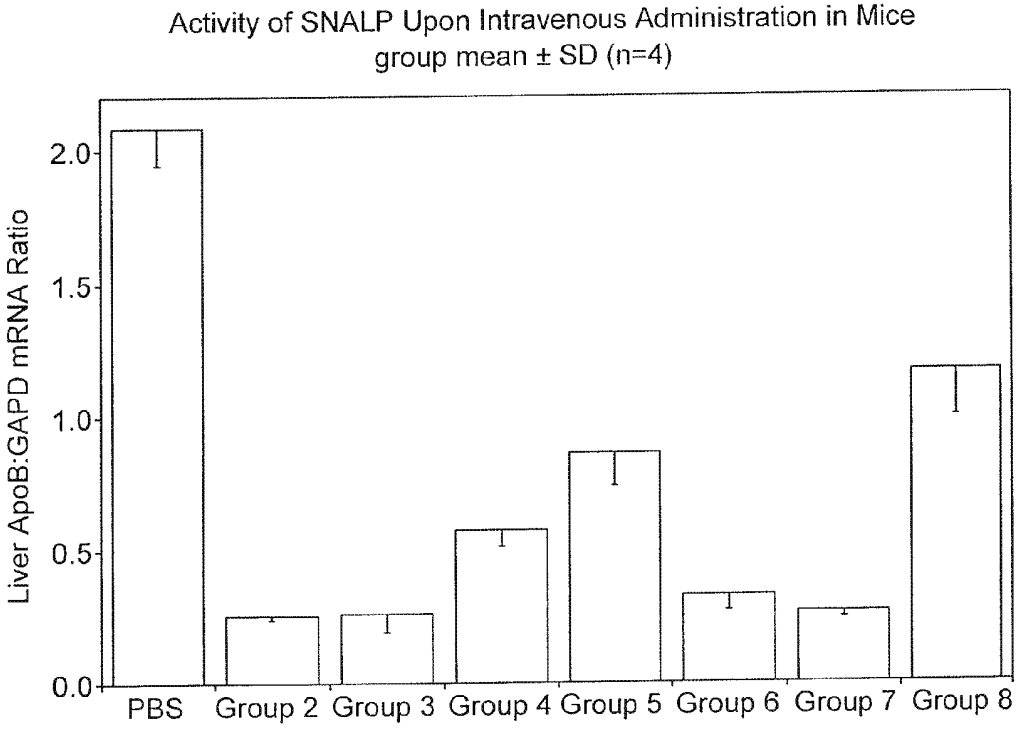

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

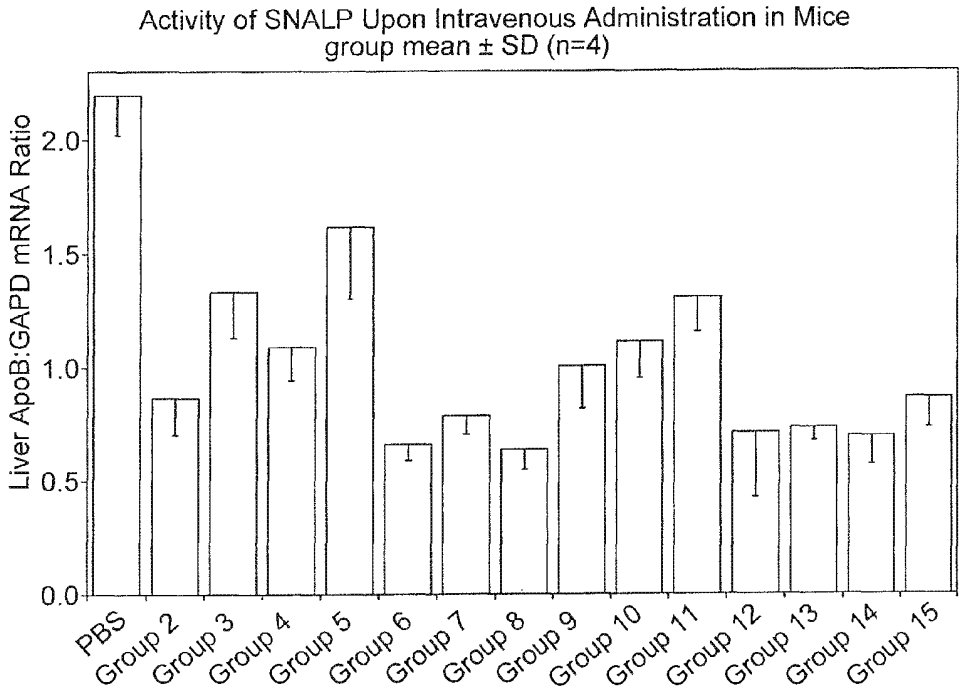

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

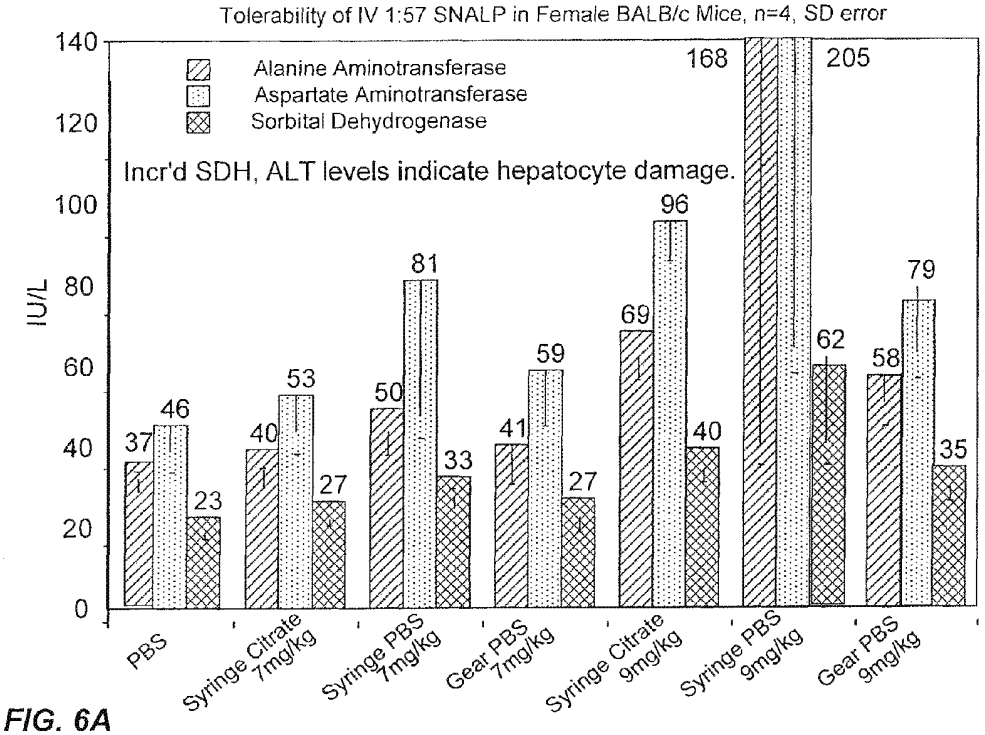

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
0
20
40
60
80
100
120
140
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
168
205
62
58
79
35
PBS
Syringe 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

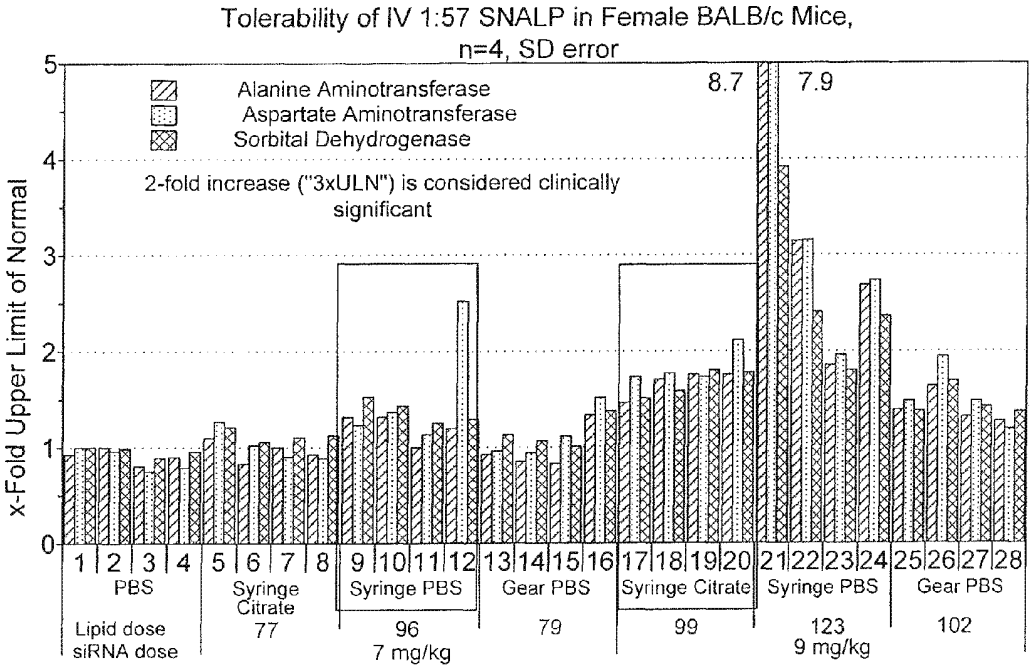

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
1
2
3
4
5
8.7
7.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 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

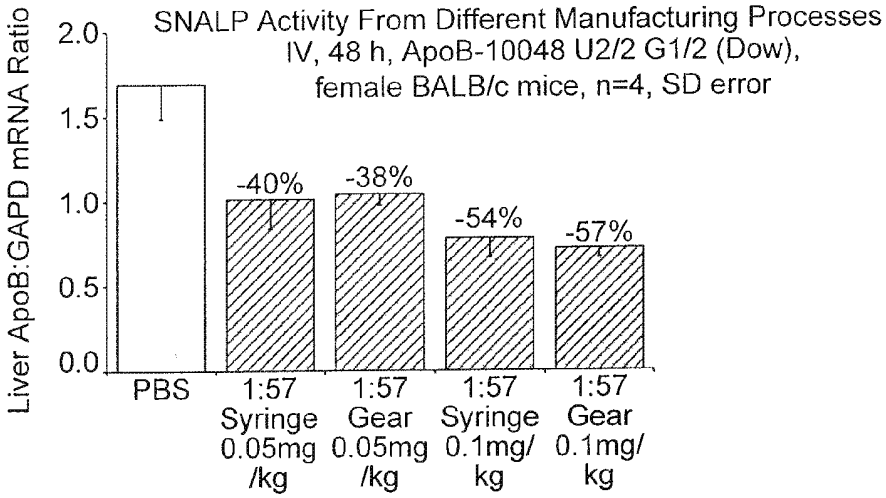


FIG. 7B

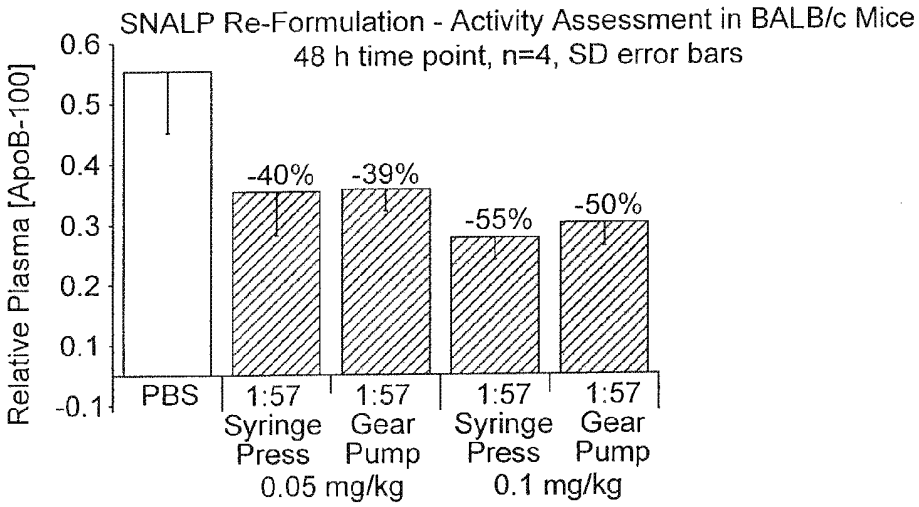
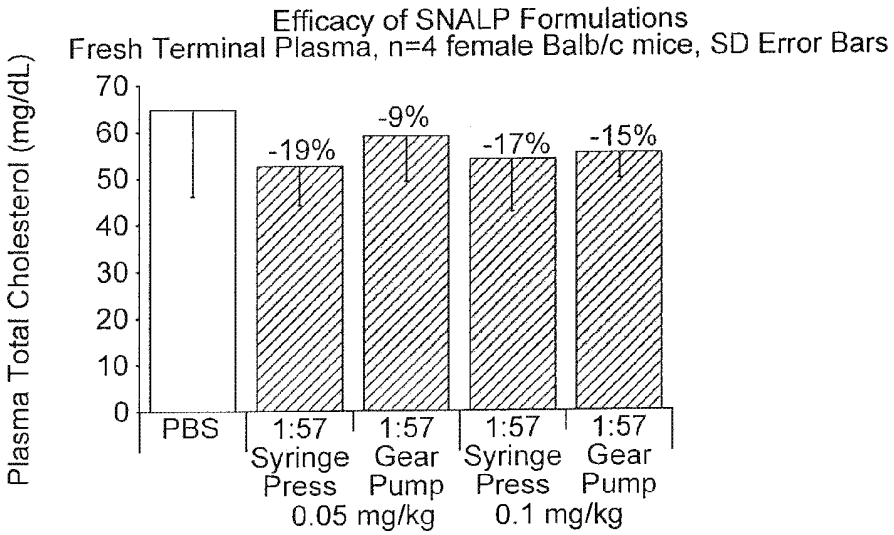


FIG. 7C



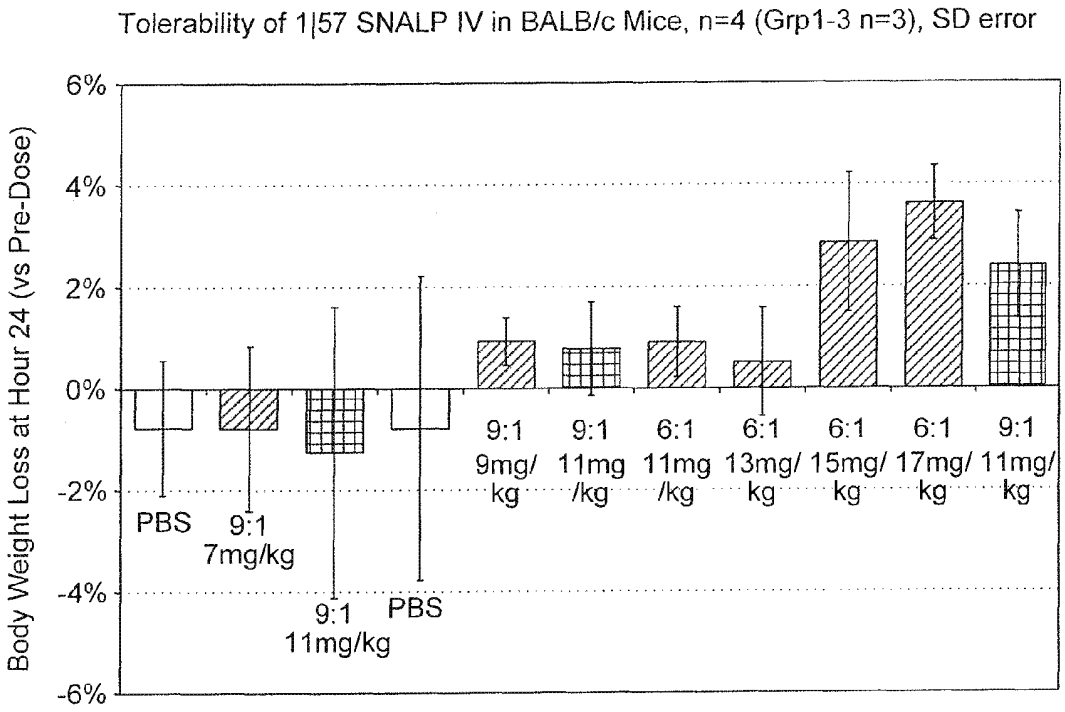

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

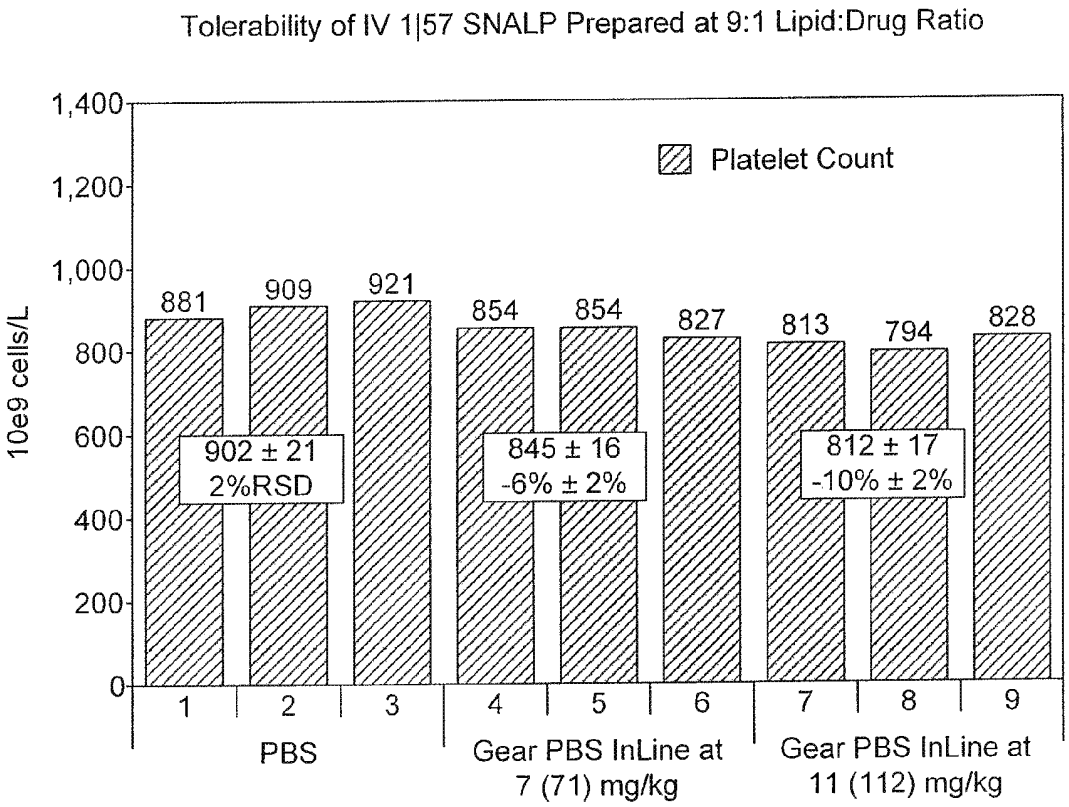

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
Platelet Count
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at
7 (71) mg/kg
Gear PBS InLine at
11 (112) mg/kg

FIG. 9

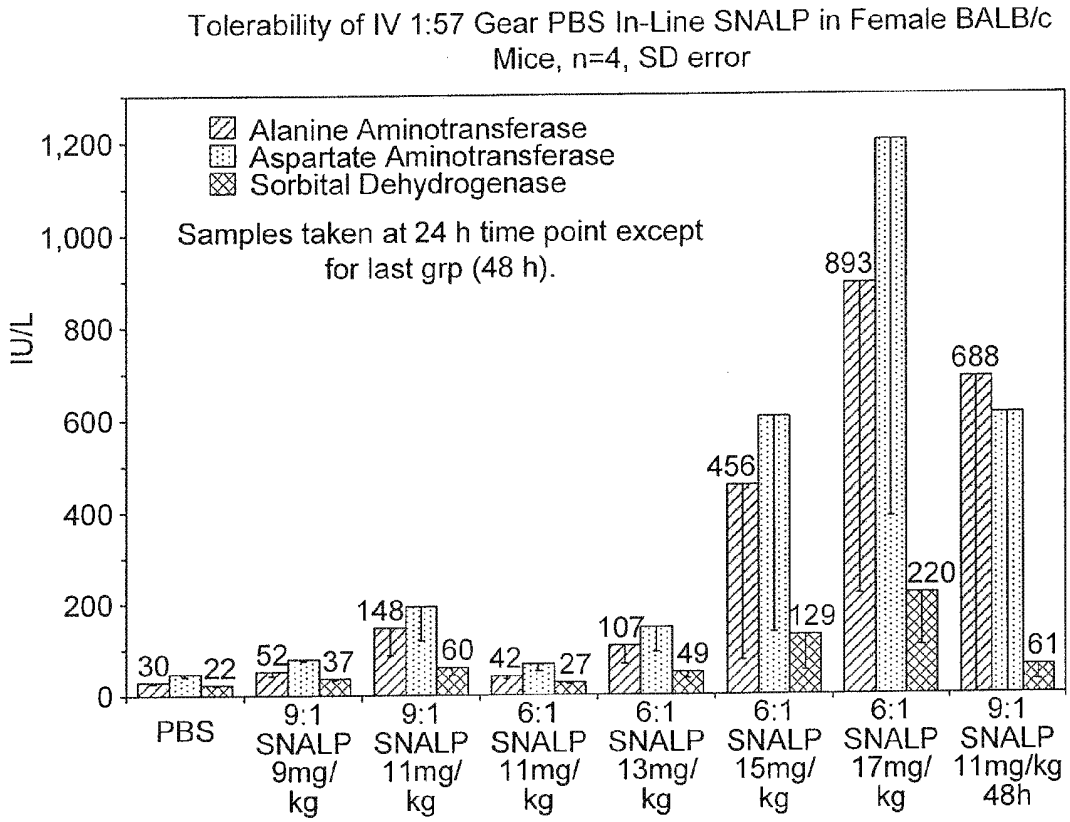

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
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

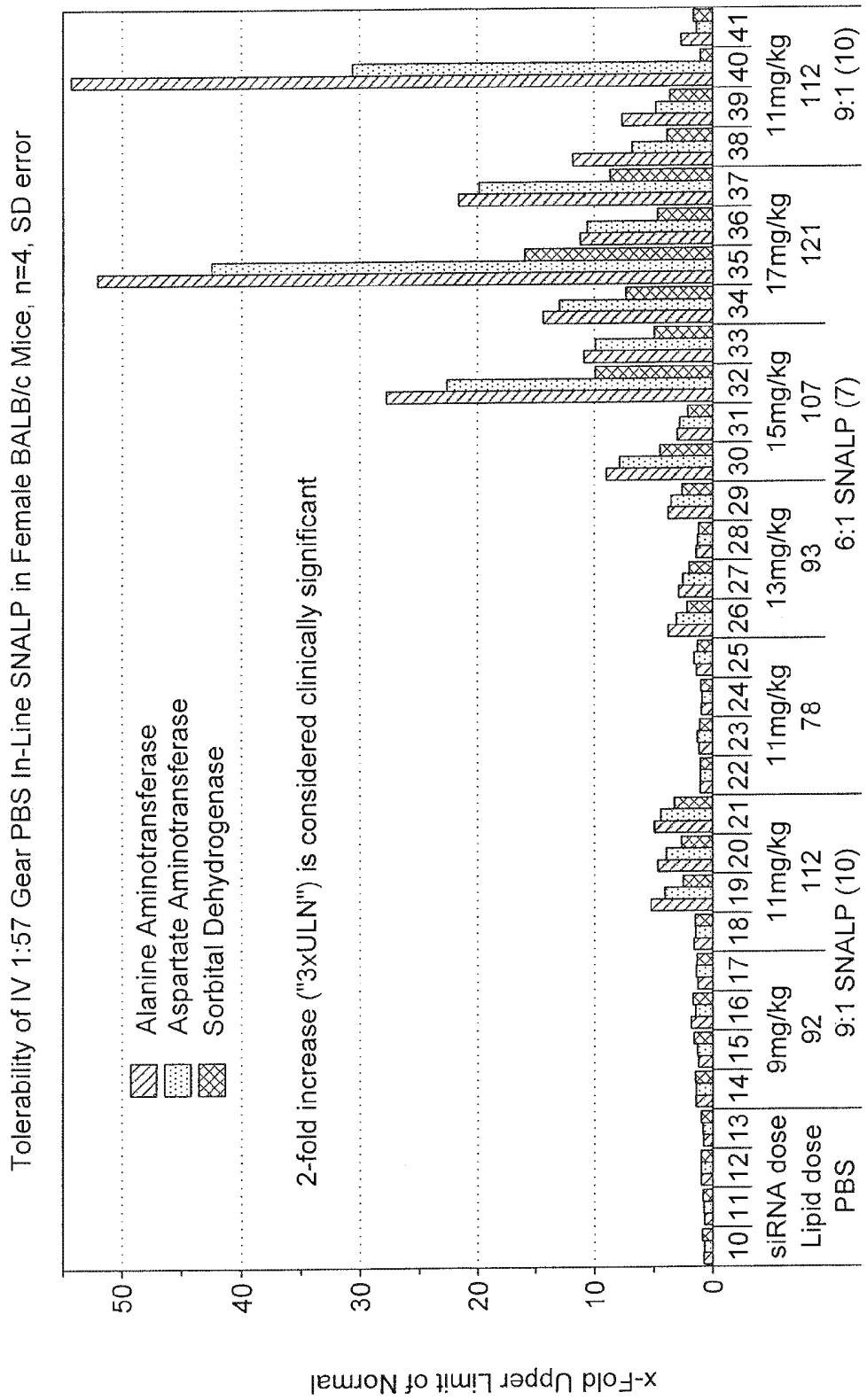

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
10
20
30
40
50
10 11 12 13 14 15 16 17 18 19 20 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

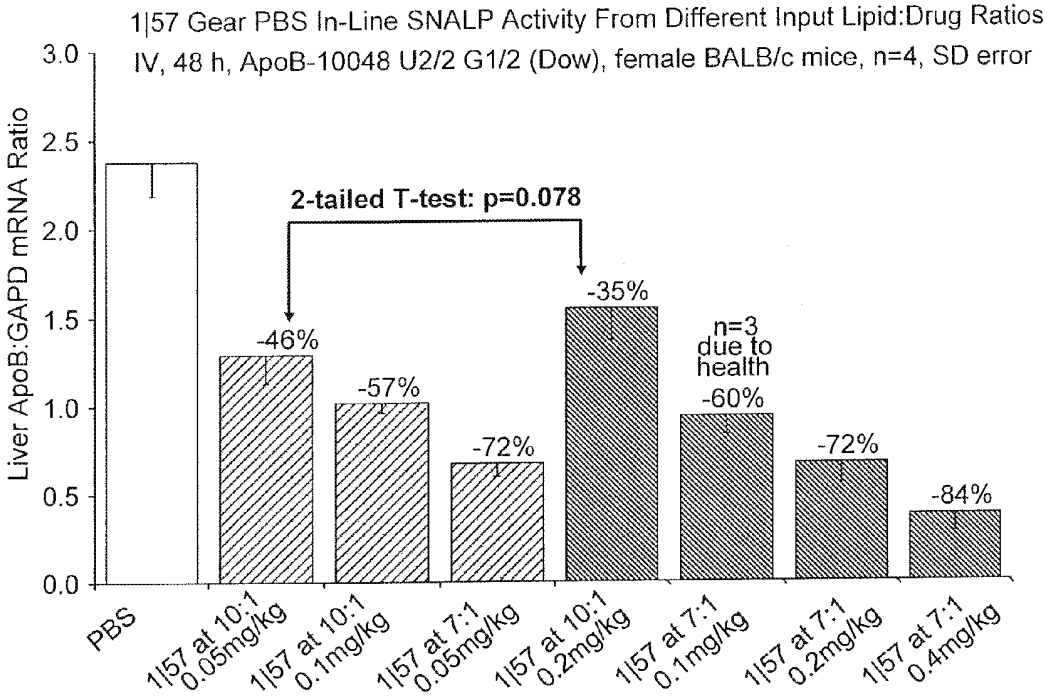
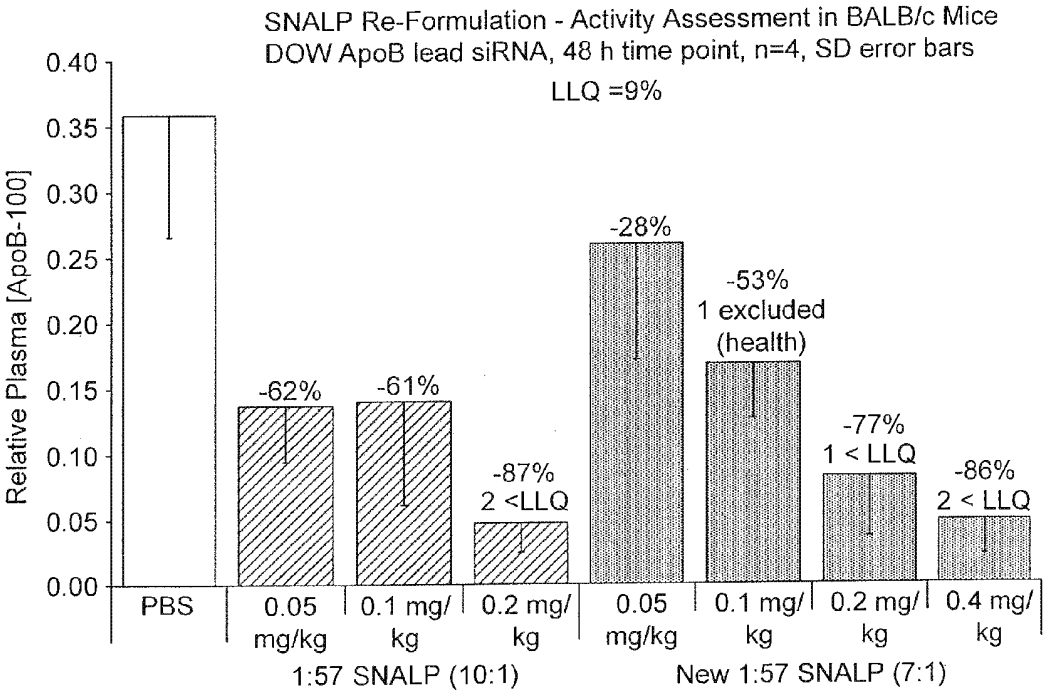


FIG. 11B



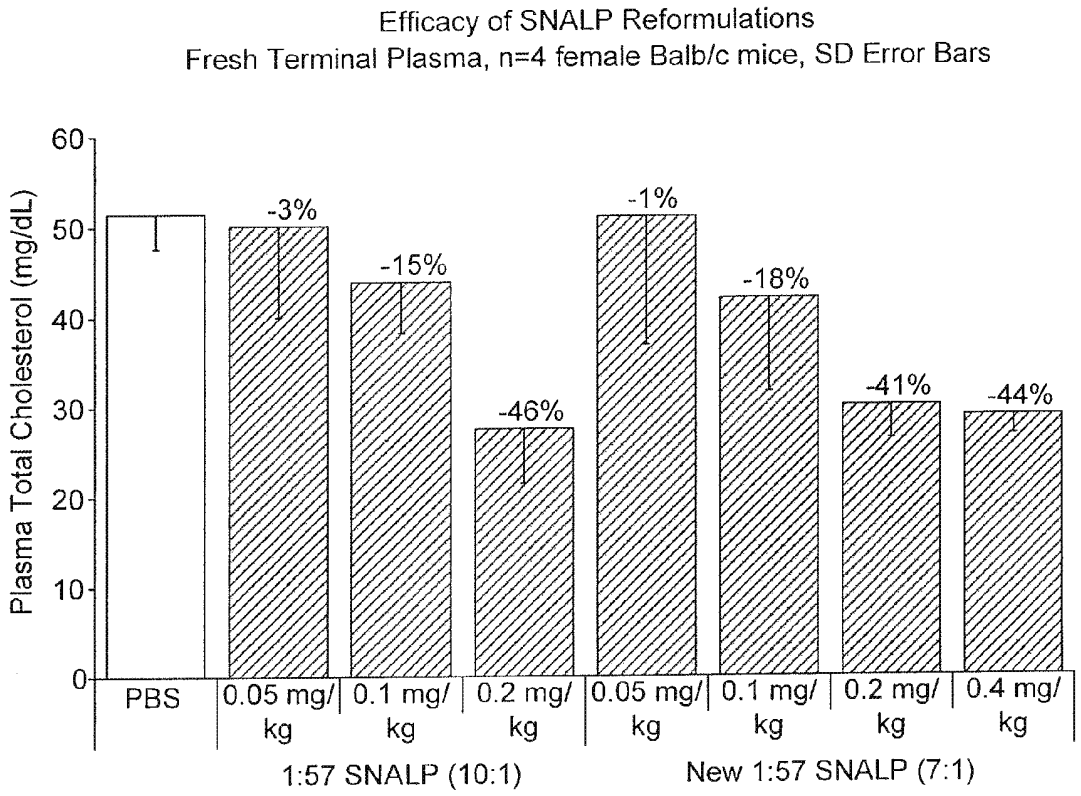

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 12

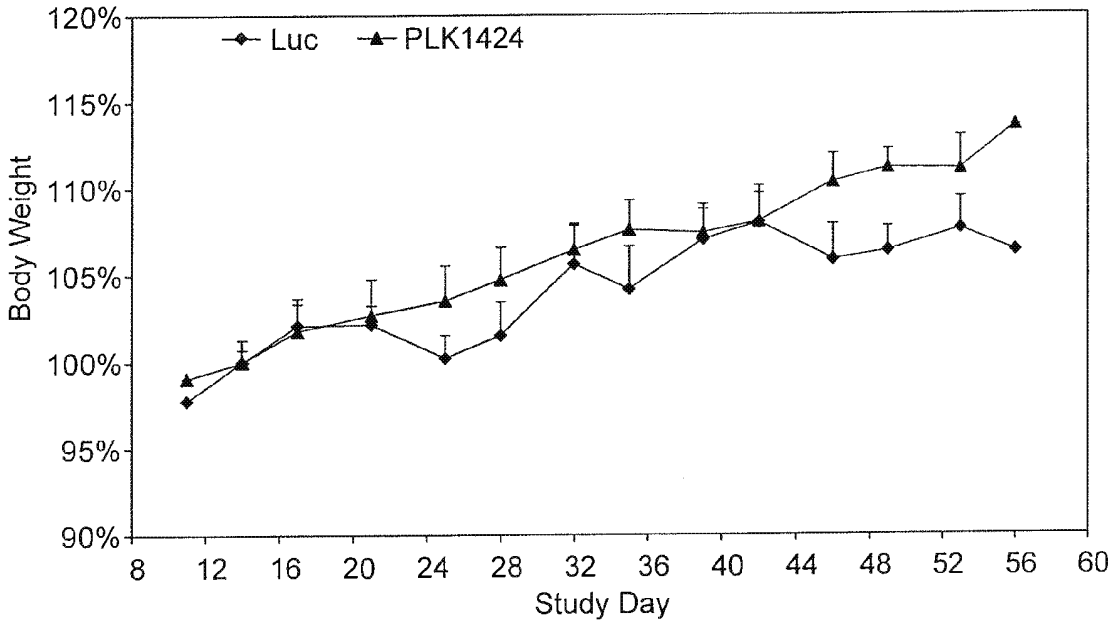

120%
115%
110%
105%
100%
95%
90%
Body Weight
Luc
PLK1424
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

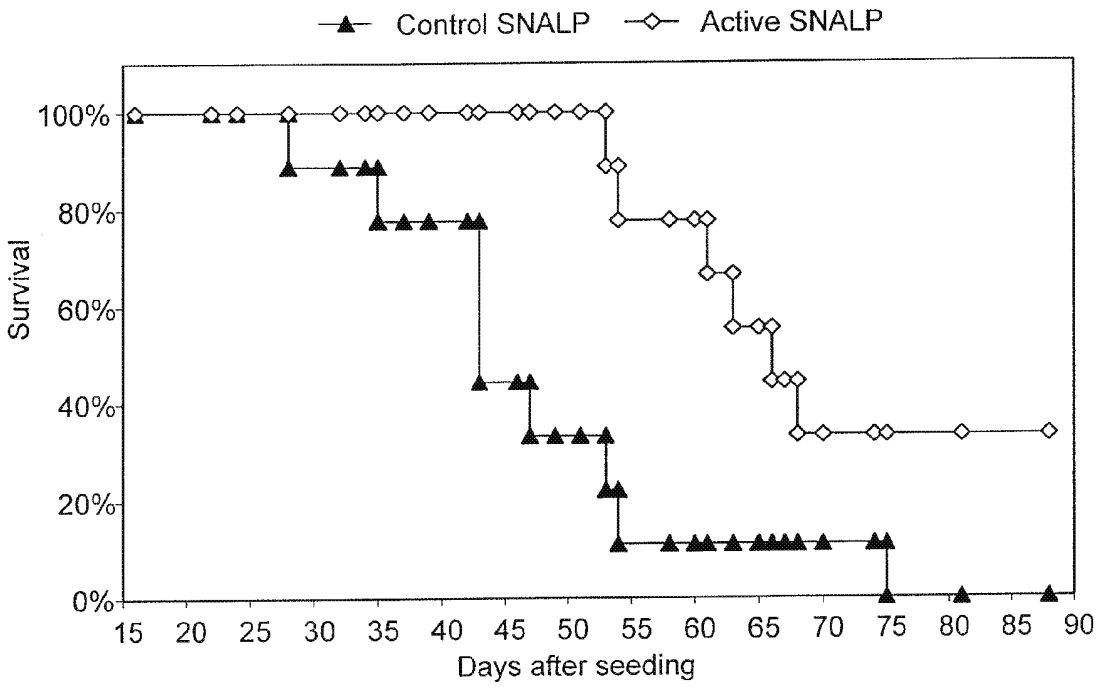

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Days after seeding

FIG. 14

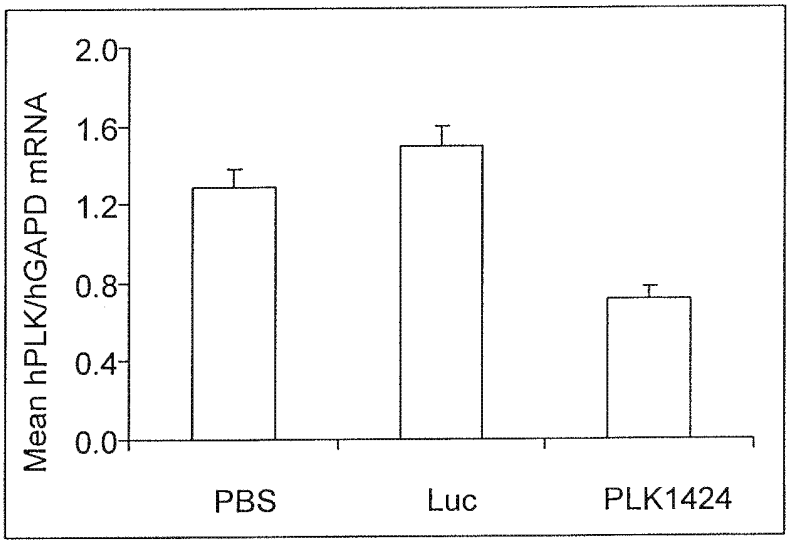


FIG. 15

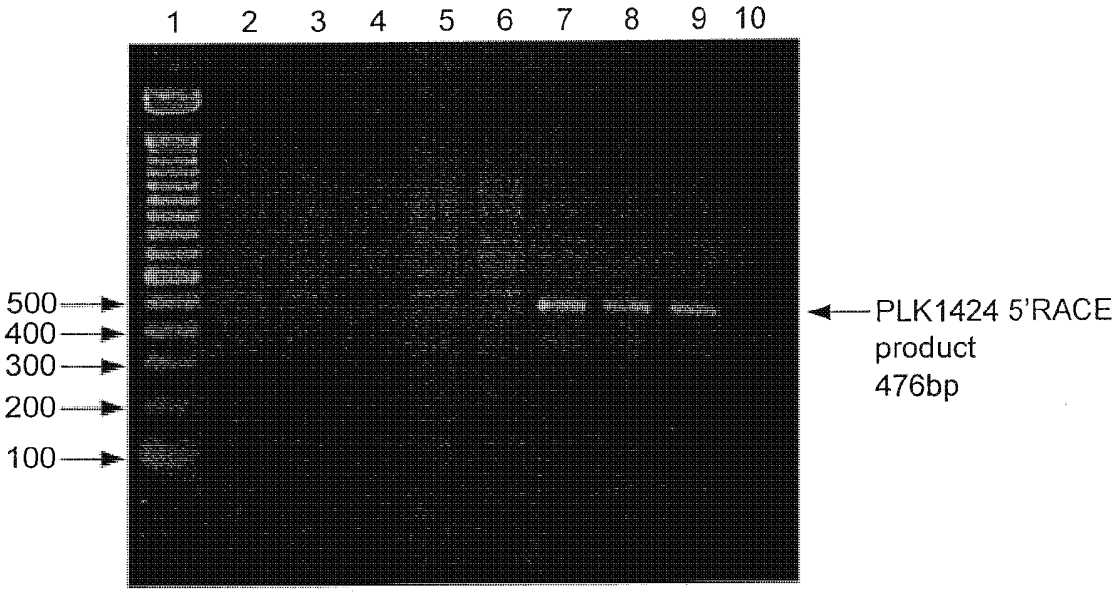
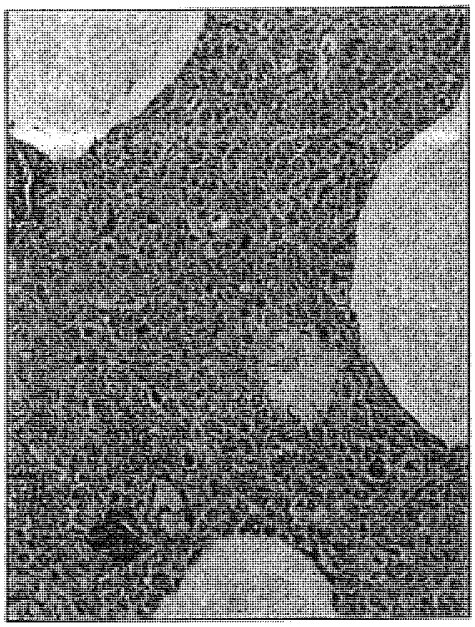
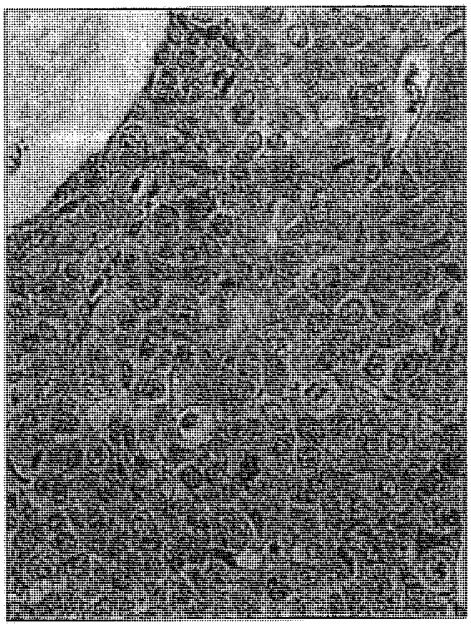

1
2
3
4
5
6
7
8
9
10
500
400
300
200
100
PLK1424 5'RACE
product
476bp

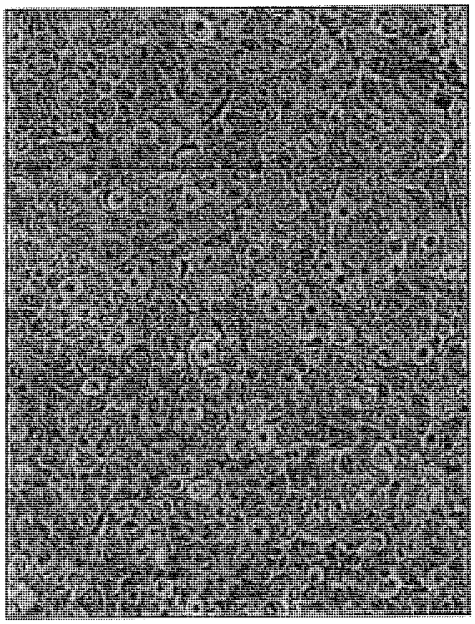
FIG. 16



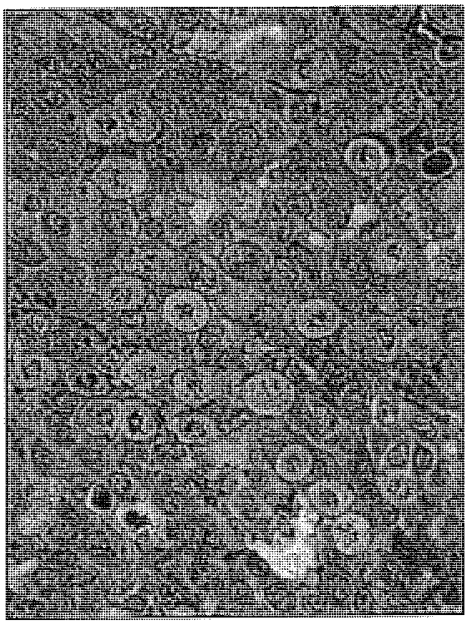
x200 mag



x400 mag



x200 mag



x400 mag

FIG. 17

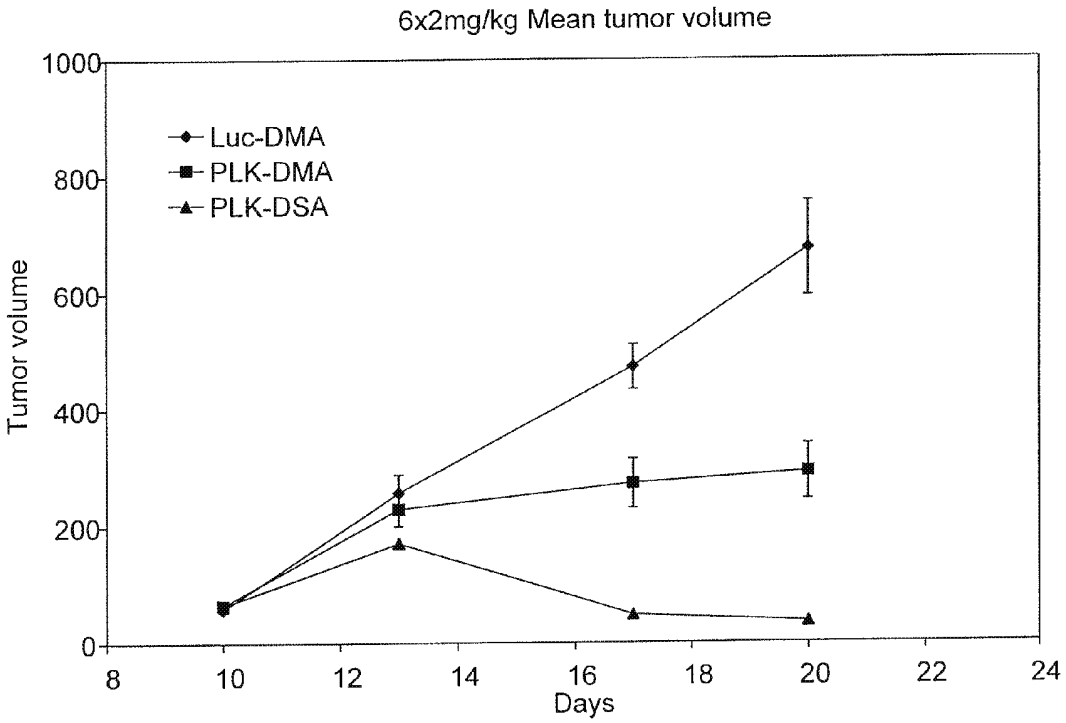

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

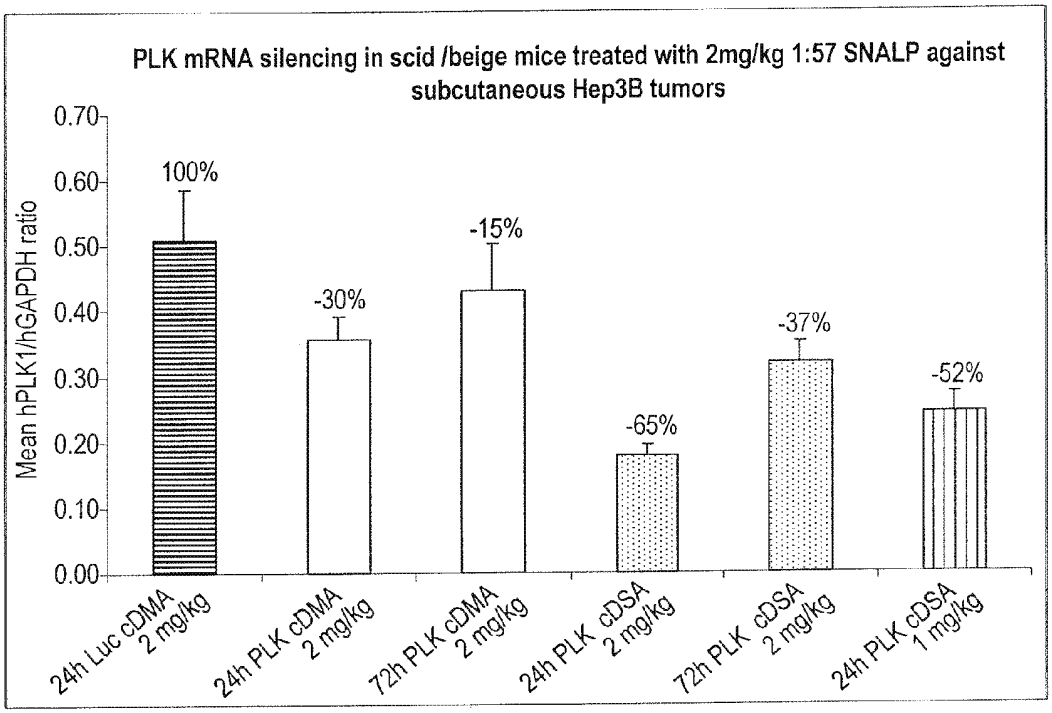

PLK mRNA silencing in scid /beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

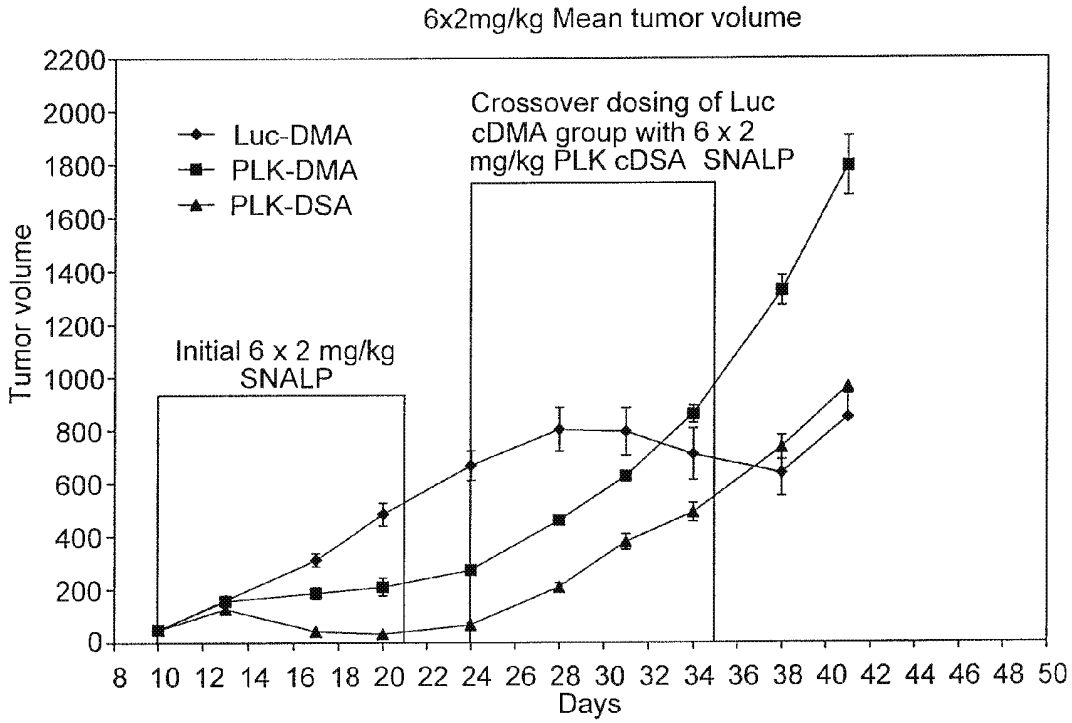

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Initial 6 x 2 mg/kg SNALP
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

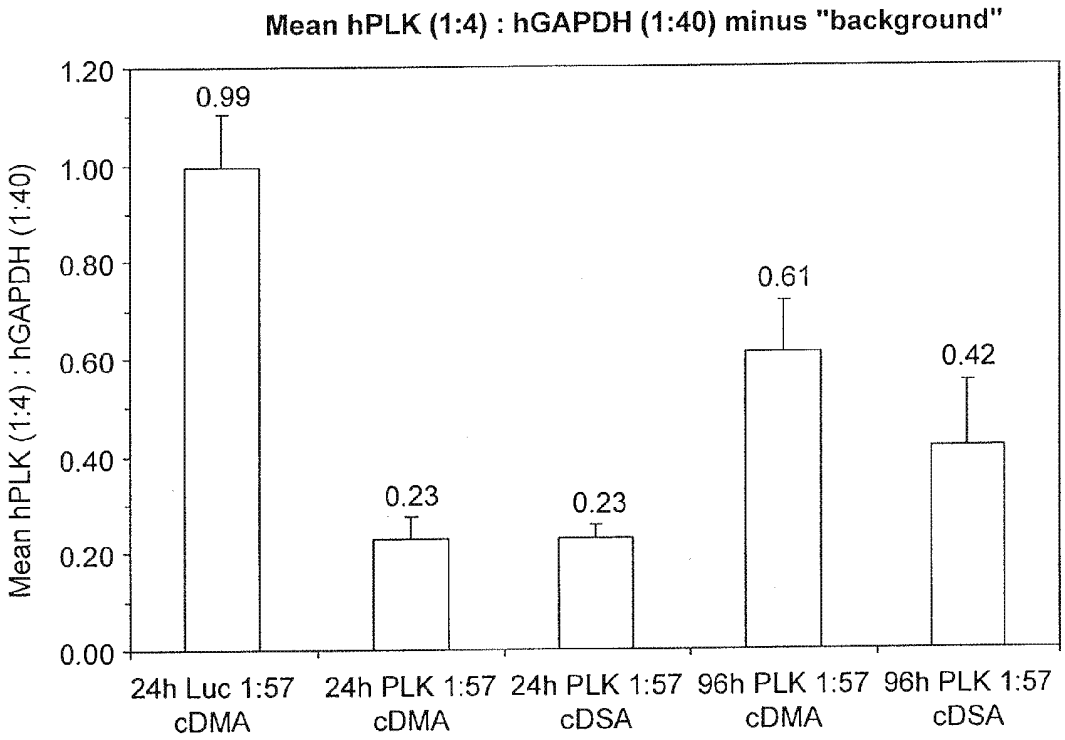

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

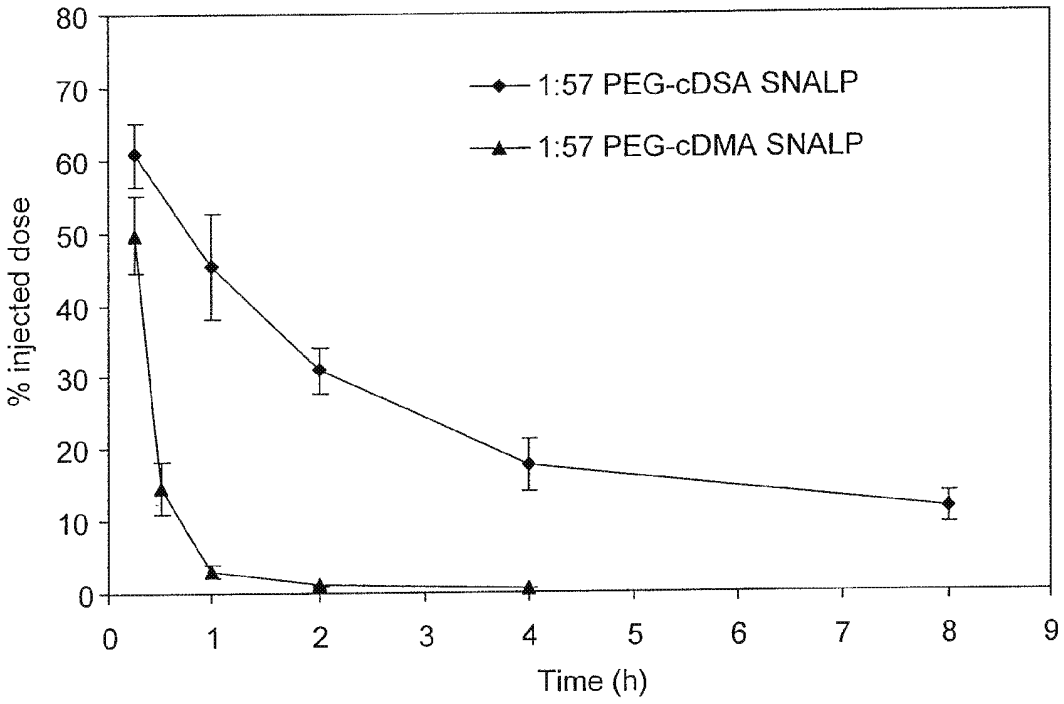

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCES TO RELATED APPLICATIONS

The present application is a continuation of U.S. application Ser. No. 13/253,917, filed Oct. 5, 2011, now U.S. Pat. No. 8,492,359, which application is a continuation of 12/424,367 filed Apr. 15, 2009, now U.S. Pat. No. 8,058,069, which application claims priority to U.S. Provisional Application No. 61/045,228, filed Apr. 15, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

Not applicable.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

The Sequence Listing written in file -77-3.TXT, created on Aug. 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

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.

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.

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.

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. Pat. No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent. Publication No. 20030073640.

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)).

Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Pat. 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.

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.

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.

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

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).

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.

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.

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).

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.

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.

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.

The present invention also provides pharmaceutical compositions comprising a lipid particle described herein (e.g., SNALP) and a pharmaceutically acceptable carrier.

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).

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).

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).

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

FIG. **1**A (Samples 1-8) and FIG. **1**B (Samples 9-16) illustrate data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

FIG. **2** illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **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.

FIG. **4** illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **5** illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **6**A (expressed as IU/L) and FIG. **6**B (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.

FIG. **7**A (expressed as liver ApoB:GAPD mRNA ratio), FIG. **7**B (expressed as relative plasma ApoB-100 concentration), and FIG. **7**C (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.

FIG. **8** illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

FIG. **9** illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

FIG. **10**A (expressed as IU/L) and FIG. **10**B (expressed as x-Fold Upper Limit of Normal) illustrate data demonstrating that clinically significant liver enzyme elevations (3×ULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

FIG. **11**A (expressed as liver ApoB:GAPD mRNA ratio) and FIG. **11**B (expressed as relative plasma ApoB-100 con-

centration) 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.

FIG. **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).

FIG. **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.

FIG. **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.

FIG. **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.

FIG. **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.

FIG. **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).

FIG. **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.

FIG. **19** illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

FIG. **20** illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

FIG. **21** illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

FIG. **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

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, FIG. **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, FIG. **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").

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.

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.

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

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

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.

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.

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).

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.

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.

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.

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.

"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.

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.

Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5×SSC, and 1% SDS, incubating at 42° C., or, 5×SSC, 1% SDS, incubating at 65° C., with wash in 0.2×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).

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 1×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.

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.

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.

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, Wis.), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology*, Ausubel et al., eds. (1995 supplement)).

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/).

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.

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.

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.

"Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

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.

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.

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.

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.

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).

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. Pat. 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.

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.

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.

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.

The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

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, palmitoyloleoylphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

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 Publi-

cation Nos. 20060083780 and 20060240554; U.S. Pat. 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.

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.

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.

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.

"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.

"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.

"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.

"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.

The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

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

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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-dimethyl-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.

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 Oct. 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 Dec. 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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-sn3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed Dec. 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. Pat. No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

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.

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.

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.

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, Calif.). "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.

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.

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).

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).

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.

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.

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 formula-

tions 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.

The present invention also provides a pharmaceutical composition comprising a lipid particle (e.g., SNALP) described herein and a pharmaceutically acceptable carrier.

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, intraarticular, intralesional, intratracheal, subcutaneous, and intradermal. Preferably, the mammalian subject is a human.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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

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.

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

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.

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.

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.

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.

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.

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

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).

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.

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.

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.

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

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).

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.

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.

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.

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.

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.

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. Pat. 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. Pat. 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. Pat. 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.

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, N.J.); human IL-6 and TNF-α (eBioscience; San Diego, Calif.); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences; San Diego, Calif.)).

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

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).

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.

To form a long dsRNA, for synthetic RNAs, the complement is also transcribed in vitro and hybridized to form a dsRNA. If a naturally occurring 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.

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. Pat. 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.

Preferably, siRNA are chemically synthesized. The oligonucleotides that comprise the siRNA molecules of the inven-

tion 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, Calif.). 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.

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

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.

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.

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. Pat. 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.

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.

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.

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. Pat. 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'-β-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

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.

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)).

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. Pat. 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.

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.

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 Hepatitis 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. Pat. No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed Mar. 20, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

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, 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 Jan. 26, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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 (JAB 1; 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 Ser. 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 Ser. No. 12/343,342, filed Dec. 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 Apr. 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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 Ser. No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

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.

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. Pat. No. 6,174,861), angiostatin (see, e.g., U.S. Pat. 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.

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)).

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)).

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

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.

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 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.

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.

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

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.

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.

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.

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.

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.

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.

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

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.

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. Pat. 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. Pat. 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. Pat. 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.

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

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.

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.

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. Pat. 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. Pat. 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.

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.

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. Pat. 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

Nucleic acids associated with lipid particles 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).

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.

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 immuno-

stimulatory 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 Dec. 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Pat. 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

In certain embodiments, the active agent associated with the lipid particles 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 therapeutic 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.

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.

Examples of conventional hormonal therapeutic agents include, without limitation, steroids (e.g., dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

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.).

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.

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.

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.

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, immunovir, idoxuridine, imiquimod, indinavir, inosine, integrase inhibitors, interferon type III (e.g., IFN-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

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.

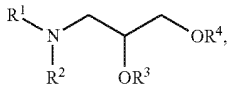
In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (e.g., siRNA, aiRNA, and/or miRNA), a cationic lipid (e.g., a cationic lipid of Formulas I, II, and/or III), a non-cationic lipid (e.g., cholesterol alone 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. Pat. 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

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.

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-dimethyl-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. Pat. 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, N.Y., 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, Wis., USA).

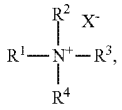
Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.



(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).

Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.

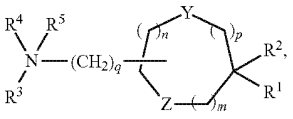


(II)

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.

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, optionally substituted 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.

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).

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.

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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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

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.

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. Pat. 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.

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. Pat. 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.

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.

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.

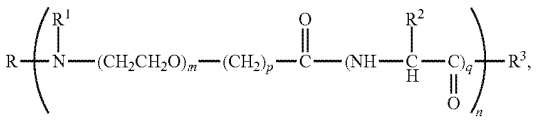
In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, e.g., carbonate (—OC(O)O—), succinoyl, phosphate esters (—O—(O)POH—O—), sulfonate esters, and combinations thereof.

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 con-

taining 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).

The term "ATTA" or "polyamide" refers to, without limitation, compounds described in U.S. Pat. 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:

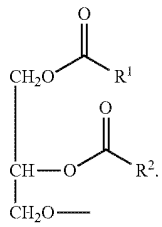
(IV)



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.

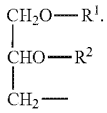
The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups 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)



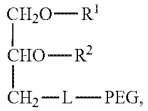
The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:

(VI)



In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:

(VII)

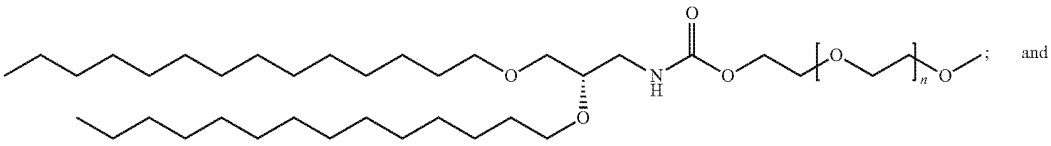


wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated 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.

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.

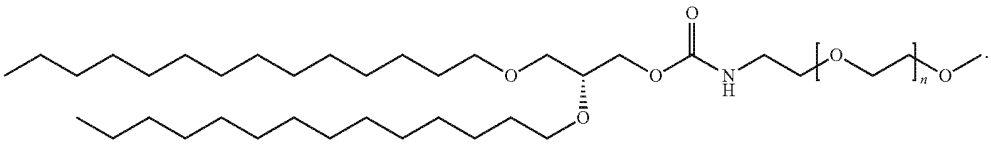
In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide 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).

In particular embodiments, the PEG-lipid conjugate is selected from:



; and

(PEG-C-DMA)



(PEG-C-DOMG)

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).

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.

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.

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. Pat. 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.

Suitable CPLs include compounds of Formula VIII:

A-W-Y (VIII),

wherein A, W, and Y are as described below.

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.

"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.

"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.

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.

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. Pat. 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.

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.

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.

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.

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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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. Pat. 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.

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.

In some embodiments, the nucleic acids in the SNALP are precondensed as described in, e.g., U.S. patent application Ser. No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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, Wis., 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.

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 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.

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), (10:1), 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.

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 preformed 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. Pat. 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

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.

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).

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

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.

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.

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.

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.

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.

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

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.

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. Pat. 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. Pat. 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.

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.

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. Pat. Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 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. Pat. No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

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.

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.

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. Pat. 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.

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.

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.

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.

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.

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.

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

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.

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.

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.

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

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.

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).

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

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

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

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.

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).

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. Pat. No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis et al. eds.) Academic Press Inc. San Diego, Calif. (1990); Arnheim & Levinson (Oct. 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); Lomeli 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.

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 polynucleotides, 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 polynucleotides 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.

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

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

siRNA: All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, Colo.). The siRNAs were desalted and annealed using standard procedures.

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, Ala.); 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

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'-C**U**GAAGACC**U**GAAGACAA**U**dTdT-3'	1	6/42 = 14.3%	6/38 = 15.8%
	3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUUA-5'	2		

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.

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	Formulation Composition, Mole %	Lipid/Drug	Finished Product Characterization		
No.	PEG(2000)-C-DMA|DLInDMA|DPPC|Cholesterol	Ratio	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
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

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, Wis.), a resazurin dye that is reduced by metabolically active cells to the fluorogenic product resorufin. The human colon cancer cell line HT29 was cultured using standard tissue culture techniques. 72 hours after SNALP application, CeilTiter-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.

FIG. **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, FIG. **1**B, Sample 9).

Example 3

ApoB siRNA Formulated as 1:57 SNALP have Potent Silencing Activity In Vivo

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 ApoB 100, 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'-AGU**G**UCA**U**CACAC**U**GAAUACC-3'	3	7/42 = 16.7%	7/38 = 18.4%
		3'-GU**U**CACAGUAGU**G**U**G**AC**U**UAU-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.

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.

	Formulation Composition	Lipid/Drug	Finished Product Characterization		
Group	Lipid Name & Mole %	Ratio	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

TABLE 4-continued

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
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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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

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.

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.

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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

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.

Example 6

ApoB siRNA Formulated as 1:62 SNALP have Potent Silencing Activity In Vivo

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|DLinDMA|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
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

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **4** shows that the 1:57 and 1:62 SNALP formulations had comparable ApoB silencing activity in vivo (see, e.g., Groups 2 & 3).

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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

Animal Model: Female BALB/c mice, 5 wks old, n=4 per group/cage.

siRNA payload: ApoB 10048 U2/2 G1/2 siRNA.

Tolerability:

		IV Injection	
Group	Formulation	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:

		IV Injection	
Group	Formulation	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:

Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

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.

Formulation Summary:

		Particle Size			
	1:57 (9:1) + DOW siRNA	Zavg (nm)	Poly	% Encap	Final L:D (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

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.

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 16,000×g & 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.

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 16,000×g (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.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

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

There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. FIG. **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.

FIG. 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

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

Animal Model: Female BALB/c mice, 7 wks old.

siRNA payload: ApoB 10048 U2/2 G1/2 siRNA.

CBC/Diff:

	#		IV Dosage	
Group	Mice	Test Article	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:

	#		IV Dosage	
Group	Mice	Test Article	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:

	#		IV Dosage	
Group	Mice	Test Article	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:

Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

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.

Formulation Summary:

		Particle Size			
	1|57 SNALP Gear PBS In-Line	Zavg (nm)	Poly	% Encap	Final L:D (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

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.

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).

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.

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 16,000×g & 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.

Groups 12-19: Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16,000×g (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.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

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:

FIG. **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.

FIG. **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.

FIG. **10** shows that clinically significant liver enzyme elevations (3×ULN) 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:

FIG. **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.

FIG. **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:

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 FIG. **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

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'	5	6/38 = 15.8%
	3'-NNUC**U**AGUGGGAG**G**AAUUUAU-5'	6	
PLK1424 U4/G	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3'	5	7/38 = 18.4%
	3'-NNUCUA**G**UG**G**GA**G**GAAUUUAU-5'	7	

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

20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay
A B	20 to seed	I.H. 1.5×10^6 Hep3B	Luc 1:57 PLK 1424 1:57	9 9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10 × 2 mg/kg	When moribund	Survival Body Weights

Test Articles

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 (28 mM lipid)
B	PLK1424 U4/GU SNALP 1:57 (28 mM lipid) PLK1424 U4/G SNALP 1:57 (28 mM 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 30 G (⅜") 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

FIG. **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.

FIG. **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

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.

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

20 SCID/beige mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	Sacrifice	Assay
A B C	20 to seed	I.H. 1×10^6 Hep3B	PBS Luc 1:57 PLK 1424 1:57	6 7 7	1 × 2 mg/kg Day 20	24 h after treatment	Tumor QG Tumor RACE-PCR Histopathology

Test Articles

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. 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 30 G (⅜") 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

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.

FIG. **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.

FIG. **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.

FIG. **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

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

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.

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 6×2 mg/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.

FIG. **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.

FIG. **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 FIG. **18**.

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 FIG. **20**, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

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.

FIG. **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.

FIG. **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.

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.

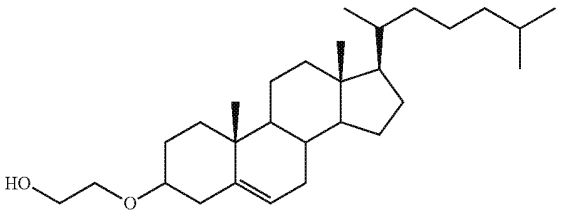
Example 12

Synthesis of Cholesteryl-2'-Hydroxyethyl Ether

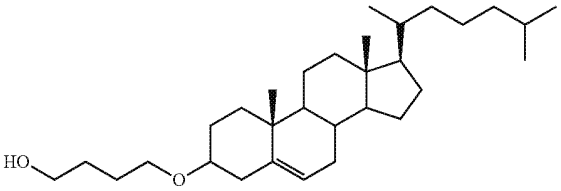
Step 1: A 250 ml round bottom flask containing cholesterol (5.0 g, 12.9 mmol) and a stir bar was sealed and flushed with nitrogen. Toluenesulphonyl chloride (5.0 g, 26.2 mmol) was weighed into a separate 100-mL round bottom flask, also sealed and flushed with nitrogen. Anhydrous pyridine (2×50 ml) was 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%).

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 (3×100 ml). The organic phases were combined, washed with water (2×150 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%).

The structures of the cholesterol derivatives cholesteryl-2'-hydroxyethyl ether and cholesteryl-4'-hydroxybutyl ether are as follows:



Cholesteryl-2′-hydroxyethyl ether



Cholesteryl-4′-hydroxyethyl ether

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.

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What is claimed is:

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 an interfering RNA.

3. The nucleic acid-lipid particle of claim **2**, wherein the interfering RNA comprises a small interfering RNA (siRNA).

4. The nucleic acid-lipid particle of claim **3**, wherein the siRNA comprises at least one modified nucleotide.

5. The nucleic acid-lipid particle of claim **3**, wherein the siRNA comprises at least one 2'-O-methyl (2'OMe) nucleotide.

6. The nucleic acid-lipid particle of claim 3, wherein the siRNA is about 19 to about 25 base pairs in length.

7. The nucleic acid-lipid particle of claim 3, wherein the siRNA comprises 3' overhangs.

8. The nucleic acid-lipid particle of claim 1, wherein the cationic lipid comprises from 50 mol % to 60 mol % of the total lipid present in the particle.

9. The nucleic acid-lipid particle of claim 1, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

10. The nucleic acid-lipid particle of claim 1, wherein the cholesterol or derivative thereof comprises from 30 mol % to 35 mol % of the total lipid present in the particle.

11. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

12. The nucleic acid-lipid particle of claim 11, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

13. The nucleic acid-lipid particle of claim 12, wherein the PEG-DAA conjugate comprises a PEG-dimyristyloxypropyl (PEG-DMA) conjugate, a PEG-distearyloxypropyl (PEG-DSA) conjugate, or a mixture thereof.

14. The nucleic acid-lipid particle of claim 13, wherein the PEG has an average molecular weight of about 2,000 daltons.

15. 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.

16. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

17. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.

18. 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.

19. 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.

20. 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.

21. The method of claim 20, wherein the disease or disorder is a viral infection.

22. The method of claim 20, wherein the disease or disorder is a liver disease or disorder.

23. The method of claim 20, wherein the disease or disorder is cancer.

* * * * *

JOINT APPENDIX 04

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

July 13, 2022

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

PATENT NUMBER: *9,364,435*
ISSUE DATE: *June 14, 2016*

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

Rodney Glover
Certifying Officer

US009364435B2

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

(10) **Patent No.: US 9,364,435 B2**
(45) **Date of Patent: *Jun. 14, 2016**

(54) **LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY**

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(72) Inventors: **Edward Yaworski**, Maple Ridge (CA); **Kieu Lam**, Surrey (CA); **Lloyd Jeffs**, Delta (CA); **Lorne Palmer**, Vancouver (CA); **Ian MacLachlan**, Mission (CA)

(73) Assignee: **PROTIVA BIOTHERAPEUTICS, INC.**, Burnaby, BC (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.: **14/462,441**

(22) Filed: **Aug. 18, 2014**

(65) **Prior Publication Data**

US 2015/0164799 A1 Jun. 18, 2015

Related U.S. Application Data

(63) Continuation of application No. 13/928,309, filed on Jun. 26, 2013, now Pat. No. 8,822,668, which is a continuation of application No. 13/253,917, filed on Oct. 5, 2011, now Pat. No. 8,492,359, which is a continuation of application No. 12/424,367, filed on Apr. 15, 2009, now Pat. No. 8,058,069.

(60) Provisional application No. 61/045,228, filed on Apr. 15, 2008.

(51) **Int. Cl.**

C12N 15/11	(2006.01)
A61K 9/127	(2006.01)
A61K 48/00	(2006.01)
C07H 21/00	(2006.01)
C07J 9/00	(2006.01)
C12N 15/113	(2010.01)

(52) **U.S. Cl.**

CPC ***A61K 9/1272*** (2013.01); ***A61K 9/1271*** (2013.01); ***A61K 48/0025*** (2013.01); ***C07H 21/00*** (2013.01); ***C07J 9/00*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 15/113*** (2013.01); *C12N 2310/14* (2013.01); *C12N 2320/32* (2013.01)

(58) **Field of Classification Search**

CPC . C12N 15/113; C12N 2310/14; A61K 9/1271

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,394,448	A	7/1983	Szoka, Jr. et al.
4,438,052	A	3/1984	Weder et al.
4,515,736	A	5/1985	Deamer
4,598,051	A	7/1986	Papahadjopoulos et al.
4,897,355	A	1/1990	Eppstein et al.
5,013,556	A	5/1991	Woodle et al.
5,171,678	A	12/1992	Behr et al.
5,208,036	A	5/1993	Eppstein et al.
5,225,212	A	7/1993	Martin et al.
5,264,618	A	11/1993	Felgner et al.
5,279,833	A	1/1994	Rose
5,283,185	A	2/1994	Epand et al.
5,320,906	A	6/1994	Eley et al.
5,334,761	A	8/1994	Gebeyehu et al.
5,545,412	A	8/1996	Eppstein et al.
5,578,475	A	11/1996	Jessee
5,627,159	A	5/1997	Shih et al.
5,641,662	A	6/1997	Debs et al.
5,656,743	A	8/1997	Busch et al.
5,674,908	A	10/1997	Haces et al.
5,703,055	A	12/1997	Felgner et al.
5,705,385	A	1/1998	Bally et al.
5,736,392	A	4/1998	Hawley-Nelson et al.
5,820,873	A	10/1998	Choi et al.
5,877,220	A	3/1999	Schwartz et al.
5,885,613	A	3/1999	Holland et al.
5,958,901	A	9/1999	Dwyer et al.
5,976,567	A	11/1999	Wheeler et al.
5,981,501	A	11/1999	Wheeler et al.
6,020,202	A	2/2000	Jessee

(Continued)

FOREIGN PATENT DOCUMENTS

CA	2309727	A1	4/1999
CA	2271582	A1	11/1999

(Continued)

OTHER PUBLICATIONS

Arpicco, S., et al., "Preparation and Characterization of Novel Cationic Lipids Developed for Gene Transfection," Proceed. Int'l Symp. Control. Rel. Bioact. Mater. (Controlled Release Society, Inc.), 1999, vol. 26, pp. 759-760.

Arpicco, S., et al., "Synthesis, characterization and transfection activity of new saturated and unsaturated cationic lipids," IL Farmaco, 2004, vol. 59, pp. 869-878.

Ballas, N., et al., "Liposomes bearing a quaternary ammonium detergent as an efficient vehicle for functional transfer of TMV-RNA into plant protoplasts," Biochimica et Biophysica Acta, 1988, vol. 939, pp. 8-18.

Barinaga, M., "Step Taken Toward Improved Vectors for Gene Transfer," Science, 1994, vol. 266, p. 1326.

Bass, "The Short Answer," Nature, 2001, 411: 428-9.

Beale, G., et al., "Gene Silencing Nucleic Acids Designed by Scanning Arrays: Anti-EGFR Activity of siRNA, Ribozyme and DNA Enzymes Targeting a Single Hybridization-accessible Region using the Same Delivery System," Journal of Drug Targeting, 2003, vol. 11, No. 7, pp. 449-456.

(Continued)

Primary Examiner — Brian Whiteman

(74) *Attorney, Agent, or Firm* — Kilpatrick Townsend & Stockton LLP

(57) **ABSTRACT**

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.

20 Claims, 24 Drawing Sheets

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,020,526	A	2 2000	Schwartz et al.	
6,034,135	A	3 2000	Schwartz et al.	
6,051,429	A	4 2000	Hawley-Nelson et al.	
6,075,012	A	6 2000	Gebeyehu et al.	
6,165,501	A	12 2000	Tirosh et al.	
6,172,049	B1	1 2001	Dwyer et al.	
6,251,939	B1	6 2001	Schwartz et al.	
6,284,267	B1	9 2001	Aneja	
6,287,591	B1	9 2001	Semple et al.	
6,339,173	B1	1 2002	Schwartz et al.	
6,376,248	B1	4 2002	Hawley-Nelson et al.	
6,534,484	B1	3 2003	Wheeler et al.	
6,586,410	B1	7 2003	Wheeler et al.	
6,638,529	B2	10 2003	Schwartz et al.	
6,649,780	B1	11 2003	Eibl et al.	
6,671,393	B2	12 2003	Hays et al.	
6,696,424	B1	2 2004	Wheeler et al.	
6,815,432	B2	11 2004	Wheeler et al.	
6,858,224	B2	2 2005	Wheeler et al.	
7,166,745	B1	1 2007	Chu et al.	
7,422,902	B1	9 2008	Wheeler et al.	
7,479,573	B2	1 2009	Chu et al.	
7,601,872	B2	10 2009	Chu et al.	
7,687,070	B2	3 2010	Gebeyehu et al.	
7,745,651	B2	6 2010	Heyes et al.	
7,799,565	B2	9 2010	MacLachlan et al.	
7,803,397	B2	9 2010	Heyes et al.	
7,807,815	B2	10 2010	MacLachlan et al.	
7,838,658	B2	11 2010	MacLachlan et al.	
7,901,708	B2	3 2011	MacLachlan et al.	
7,915,450	B2	3 2011	Chu et al.	
7,982,027	B2	7 2011	MacLachlan et al.	
8,058,068	B2	11 2011	Hawley-Nelson et al.	
8,058,069	B2	11 2011	Yaworski et al.	
8,101,741	B2	1 2012	MacLachlan et al.	
8,158,827	B2	4 2012	Chu et al.	
8,188,263	B2	5 2012	MacLachlan et al.	
8,227,443	B2	7 2012	MacLachlan et al.	
8,236,943	B2 *	8 2012	Lee et al.	536 24.5
8,283,333	B2	10 2012	Yaworski et al.	
8,455,455	B1	6 2013	Robbins et al.	
8,492,359	B2 *	7 2013	Yaworski et al.	514 44 A
8,513,403	B2	8 2013	MacLachlan et al.	
8,569,256	B2	10 2013	Heyes et al.	
8,598,333	B2	12 2013	MacLachlan et al.	
8,822,668	B2 *	9 2014	Yaworski et al.	536 24.5
9,006,417	B2 *	4 2015	Yaworski et al.	536 24.5
2001 0048940	A1	12 2001	Tousignant et al.	
2003 0069173	A1	4 2003	Hawley-Nelson et al.	
2003 0072794	A1	4 2003	Boulikas	
2003 0077829	A1	4 2003	MacLachlan	
2003 0143732	A1	7 2003	Fosnaugh et al.	
2004 0063654	A1	4 2004	Davis et al.	
2004 0142892	A1	7 2004	Finn et al.	
2004 0253723	A1	12 2004	Tachas et al.	
2004 0259247	A1	12 2004	Tuschi et al.	
2005 0064595	A1	3 2005	MacLachlan et al.	
2005 0118253	A1	6 2005	MacLachlan et al.	
2005 0260757	A1	11 2005	Gebeyehu et al.	
2006 0008910	A1	1 2006	MacLachlan et al.	
2006 0147514	A1	7 2006	Gebeyehu et al.	
2006 0228406	A1	10 2006	Chiou et al.	
2006 0240093	A1	10 2006	MacLachlan et al.	
2007 0042031	A1	2 2007	MacLachlan et al.	
2007 0202598	A1	8 2007	Chu et al.	
2007 0202600	A1	8 2007	Chu et al.	
2009 0143583	A1	6 2009	Chu et al.	
2009 0291131	A1	11 2009	MacLachlan et al.	
2010 0130588	A1	5 2010	Yaworski et al.	
2010 0159593	A1	6 2010	Chu et al.	
2012 0058188	A1	3 2012	MacLachlan et al.	
2012 0136073	A1	5 2012	Yang et al.	
2012 0183581	A1	7 2012	Yaworski et al.	
2012 0238747	A1	9 2012	Chu et al.	
2014 0065228	A1	3 2014	Yaworski et al.	

FOREIGN PATENT DOCUMENTS

CA	2330741	A1	11 1999
CA	2397016	A1	7 2001
CA	2513623		8 2004
JP	03-126211		5 1991
JP	05-202085		8 1993
JP	06-080560		3 1994
JP	2002-525063		8 2002
JP	2003-505401		2 2003
JP	2007-524349		8 2007
WO	91 16024	A1	10 1991
WO	93 05162	A1	3 1993
WO	93 12240	A1	6 1993
WO	93 12756	A2	7 1993
WO	93 24640	A2	12 1993
WO	93 25673	A1	12 1993
WO	95 02698	A1	1 1995
WO	95 18863	A1	7 1995
WO	95 35301	A1	12 1995
WO	96 02655	A1	2 1996
WO	96 10390	A1	4 1996
WO	96 40964	A2	12 1996
WO	96 41873	A1	12 1996
WO	98 51285	A2	11 1998
WO	00 03683	A2	1 2000
WO	00 15820	A1	3 2000
WO	00 62813	A2	10 2000
WO	01 05374	A1	1 2001
WO	01 05873	A1	1 2001
WO	01 75164	A2	10 2001
WO	01 93836		12 2001
WO	02 34236	A2	5 2002
WO	02 087541	A1	11 2002
WO	03 097805	A2	11 2003
WO	2004 065546	A2	8 2004
WO	2004 110499	A1	12 2004
WO	2005 007196	A2	1 2005
WO	2005 026372	A1	3 2005
WO	2005 035764	A1	4 2005
WO	2005 120152	A2	12 2005
WO	2006 002538	A1	1 2006
WO	2006 053430	A1	5 2006
WO	2007 056861	A1	5 2007
WO	2009 086558	A1	7 2009
WO	2009 111658	A2	9 2009
WO	2010 042877	A1	4 2010
WO	2010 048228	A2	4 2010
WO	2010 088537	A2	8 2010
WO	2010 105209	A1	9 2010

OTHER PUBLICATIONS

Behr, J.-P., "Synthetic Gene-Transfer Vectors," Acc. Chem. Res., 1993, vol. 26, pp. 274-278.

Brigham, K., et al., "Rapid Communication: In vivo Transfection of Murine Lungs with a Functioning Prokaryotic Gene Using a Liposome Vehicle," The American Journal of the Medical Sciences, vol. 298, No. 4, pp. 278-281.

Brummelkamp, et al., "A System for Stable Expression of Short Interfering RNAs in Mammalian Cells," Science, 2002, V. 296, pp. 550-553.

Ceve, G., "How Membrane Chain-Melting Phase-Transition Temperature is Affected by the Lipid Chain Asymmetry and Degree of Unsaturation: An Effective Chain-Length Model," Biochemistry, 1991, vol. 30, pp. 7186-7193.

Chonn et al., "Recent advances in liposomal drug-delivery systems," Current Opinion in Biotechnology, 1995, vol. 6, pp. 698-708.

Cortesi, R., et al., "Effect of cationic liposome composition on in vitro cytotoxicity and protective effect on carried DNA," International Journal of Pharmaceutics, 1996, vol. 139, pp. 69-78.

Crystal, R., "Transfer of Genes to Humans: Early Lessons and Obstacles to Success," Science, 1995, vol. 270, pp. 404-410.

Culver K., "The First Human Gene Therapy Experiment," Gene Therapy: A Handbook for Physicians, 1994, pp. 33-40.

Duzgunes, N., "Membrane Fusion," Subcellular Biochemistry, 1985, vol. 11, pp. 195-286.

(56) **References Cited**

OTHER PUBLICATIONS

Dwarki, V.J., et al., "Cationic Liposome-Mediated RNA Transfection," Methods in Enzymology, 1993, vol. 217, pp. 644-654.
Elbashir, et a., "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells," Nature, May 2001, pp. 494-498, vol. 411.
Enoch, H., et al., "Formation and properties of 1000-Å-diameter, single-bilayer phospholipid vesicles," Proc. Natl. Acad. Sci. USA, 1979, vol. 76, No. 1, pp. 145-149.
Felgner, J., et al., "Cationic Lipid-Mediated Transfection in Mammalian Cells: 'Lipofection.'" J. Tiss. Cult. Meth., 1993, vol. 15, pp. 63-68.
Felgner, J., et al., "Enhanced Gene Delivery and Mechanism Studies with a Novel Series of Cationic Lipid Formulations," The Journal of Biological Chemistry, 1994, vol. 269, No. 4, pp. 2550-2561.
Felgner, P., et al., "Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure," Proc. Natl. Acad. Sci. USA, 1987, vol. 84, pp. 7413-7417.
Felgner, P.L., et al., "Cationic Liposome Mediated Transfection," Proc. West. Pharmacol. Soc., 1989, vol. 32, pp. 115-121.
Gao, X., et al., "A Novel Cationic Liposome Reagent for Efficient Transfection of Mammalian Cells," Biochem. Biophys. Res. Comm., 1991, vol. 179, No. 1, pp. 280-285.
Gershon, H., et al., "Mode of Formation and Structural Feature of DNA-Cationic Liposome Complexes Used for Transfection," Biochemistry, 1993, vol. 32, pp. 7143-7151.
Global Newswire, retrieved from http: globalnewswire.com on Feb. 27, 2013, Tekmira sues Alnylam Pharmaceuticals for repeated misuse of tradesecrets and confidential information, Mar. 16, 2011, pp. 1-3.
Guy-Caffey, J., et al., "Novel Polyaminolipids Enhance the Cellular Uptake of Oligonucleotides," The Journal of Biological Chemistry, 1995, vol. 270, No. 52, pp. 31391-31396.
Hawley-Nelson, P., et al., "LipofectAmine™ Reagent: A New, Higher Efficiency Polycationic Liposome Transfection Reagent," Focus, 1993, vol. 15, No. 3, pp. 73-80.
Heyes et al., "Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids," Journal of Controlled Release, 2005, vol. 107, pp. 276-287.
Heyes et al., "Synthesis of novel cationic lipids: effect of structural modification on the efficiency of gene transfer," J. Med. Chem., 2002, vol. 45, pp. 99-114.
Hyde, S., et al., "Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy," Nature, 1993, vol. 362, pp. 250-255.
Jiang, L., et al., "Comparison of protein precipitation methods for sample preparation prior to proteomic analysis," Journal of Chromatography A, 2004, vol. 1023, pp. 317-320.
Juliano, R., et al., "The Effect of Particle Size and Charge on the Clearance Rates of Liposomes and Liposome Encapsulated Drugs," Biochem. Biophys. Res. Commun., 1975, vol. 63, No. 3, pp. 651-658.
Keough, K., "Influence of chain unsaturation and chain position on thermotropism and intermolecular interactions in membranes," Biochem. Soc. Transactions, 1990, vol. 18, No. 5, pp. 835-837.
Krichevsky, A. et al., "RNAi functions in cultured mammalian neurons," PNAS, 99(18):11926-29, 2002.
Lawrence et al. "The formation, characterization and stability of non-ionic surfactant vesicles," S.T.P. Pharma Sciences, 1996, vol. 6, No. 1, pp. 49-60.
Lawrence et al., "Synthesis and aggregation properties of dialkyl polyoxyethylene glycerol ethers," Chemistry and Physics of Lipids, 1996, 82(2):89-100.
Legendre, J.-Y. et al., "Delivery of Plasmid DNA into Mammalian Cell Lines Using pH-Sensitive Liposomes: Comparison with Cationic Liposomes," Pharm. Res., 1992, vol. 9, No. 10, pp. 1235-1242.
Leventis, R., et al., "Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles," Biochem. Biophys. Acta, 1990, vol. 1023, pp. 124-132.
Liu, et al., "Cationic Liposome-mediated Intravenous Gene Delivery", J. Biol. Chem., 1995, V. 270, pp. 24864-24870.
Marshall, E., "Gene Therapy's Growing Pains," Science, 1995, vol. 269, pp. 1050-1055.
Murahashi et al., "Synthesis and evaluation of neoglycolipid for liposome modification," Biol. Pharm. Bull., 1997, 20(6):704-707.
Orkin, S., et al., NIH Report, Report and Recommendations of the Panel to Assess the NIH Investment in Research on Gene Therapy, 1995.
Parr et al., Factors influencing the retention and chemical stability of polly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles, Biochimica et Biophysica Acta, 1994, 1195:21-30.
Paul, C., et al., "Effective expression of small interfering RNA in human cells," Nature Biotech., 2002, vol. 20, pp. 505-508.
Puyal, C., et al., "A new cationic liposome encapsulating genetic material: A potential delivery system for polynucleotides," Eur. J. Biochem., 1995, vol. 228, pp. 697-703.
Sawada et al., "Microemulsions in supercritical CO_2 utilizing the polyethyleneglycol dialkylglycerol and their use for the solubilization of hydrophiles," Dyes and Pigments, 2005, pp. 64-74, vol. 65.
Shin, et al. "Acid-triggered release via dePEGylation of DOPE liposomes containing acid-labile vinyl ether PEG-lipids," Journal of Controlled Release, 2003, vol. 91, pp. 187-200.
Song et al., "Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes," Biochimica et Biophysica Acta, 2002, 1558:1-13.
Sorensen, et al., "Gene Silencing by Systemic Delivery of Synthetic siRNAs in Adult Mice", J. Biol. Chem., 2003, V. 327, pp. 761-766.
Spagnou, S., et al., "Lipidic Carriers of siRNA: Differences in the Formulation, Cellular Uptake, and Delivery with Plasmid DNA," Biochemistry, 2004, vol. 43, pp. 13348-13356.
Stamatatos, L., et al., "Interactions of Cationic Lipid Vesicles with Negatively Charged Phospholipid Vesicles and Biological Membranes," Biochemistry, 1988, vol. 27, pp. 3917-3925.
Szoka, F., et al., "Comparative Properties and Methods of Preparation of Lipid Vesicles (Liposomes)," Ann. Rev. Biophys. Bioeng., 1980, vol. 9, pp. 467-508.
Szoka, F., et al., "Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation," Proc. Natl. Acad. Sci. USA, 1978, vol. 75, No. 9, pp. 4194-4198.
Templeton, "Cationic Liposome-mediated Gene Delivery In vivo", Bioscience Reports, 2002, vol. 22, No. 2, pp. 283-295.
Van Der Woude, I., et al., "Parameters influencing the introduction of plasmid DNA into cells by the use of synthetic amphiphiles as a carrier system," Biochimica et Biophysica Acta, 1995, vol. 1240, pp. 34-40.
Wheeler, et al., "Stabilized Plasmid-lipid Particles: Constructions and Characterization," Gene Therapy, V. 6, pp. 271-281.
Wilson, R., et al., "Counterion-Induced Condensation of Deoxyribonucleic Acid. A Light-Scattering Study," Biochemistry, 1979, vol. 18, No. 11, pp. 2192-2196.
Woodle, M.C., et al., "Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes," Biochimica et Biophysica Acta, 1992, vol. 1105, pp. 193-200.
Zhu, N., et al., "Systemic Gene Expression After Intravenous DNA Delivery into Adult Mice," Science, 1993, vol. 261, pp. 209-211.

* cited by examiner

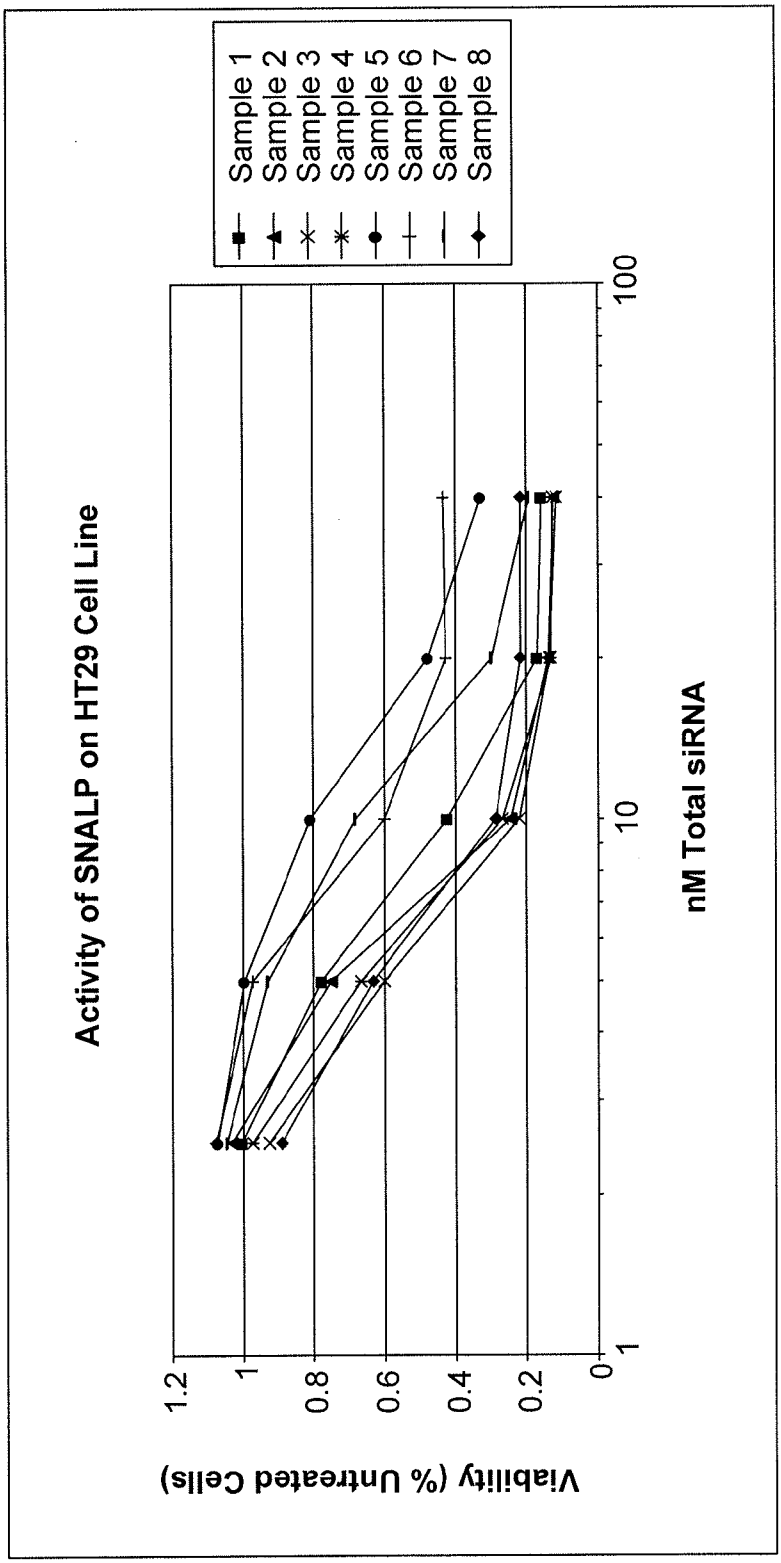

Activity of SNALP on HT29 Cell Line
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA

FIG. 1A

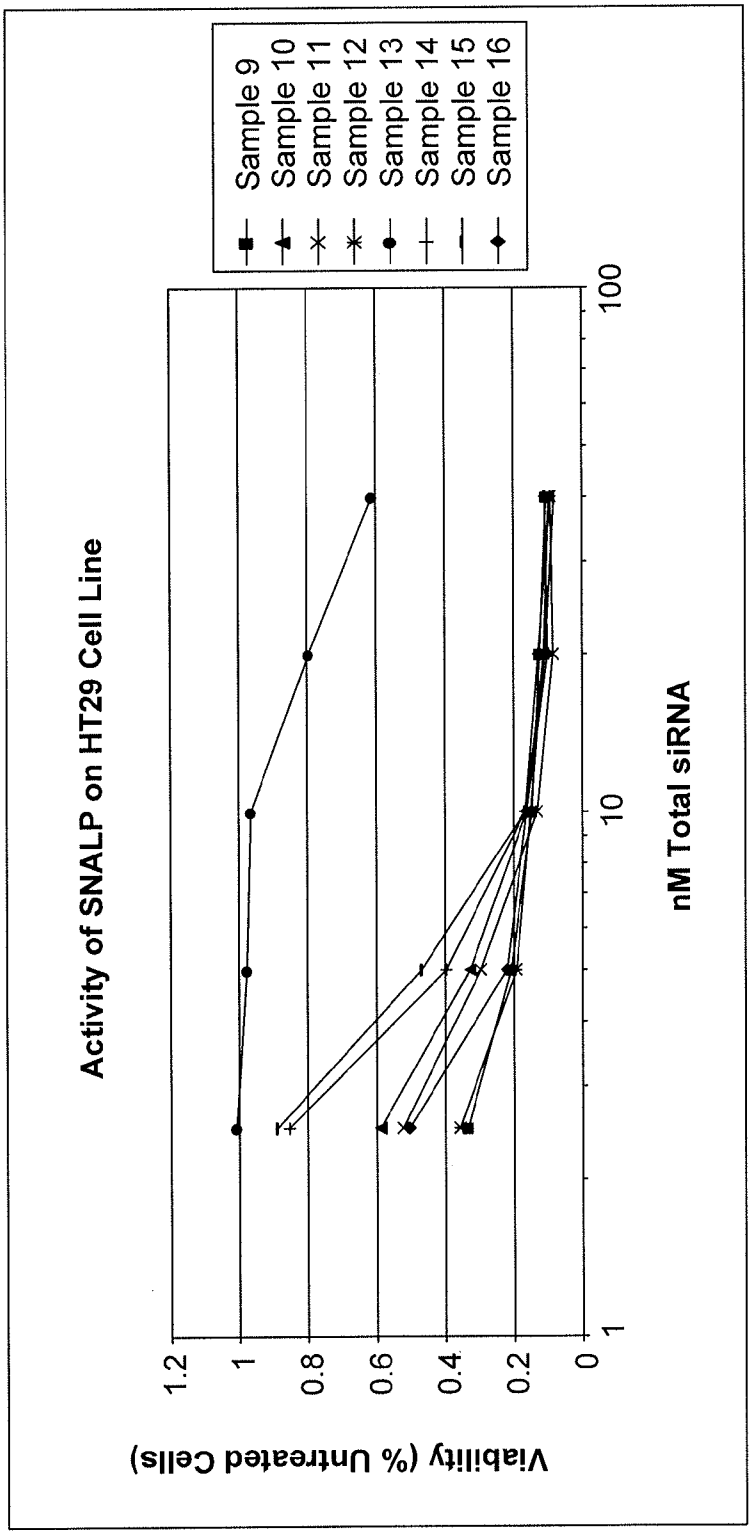

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 9
Sample 10
Sample 11
Sample 12
Sample 13
Sample 14
Sample 15
Sample 16

FIG. 1B

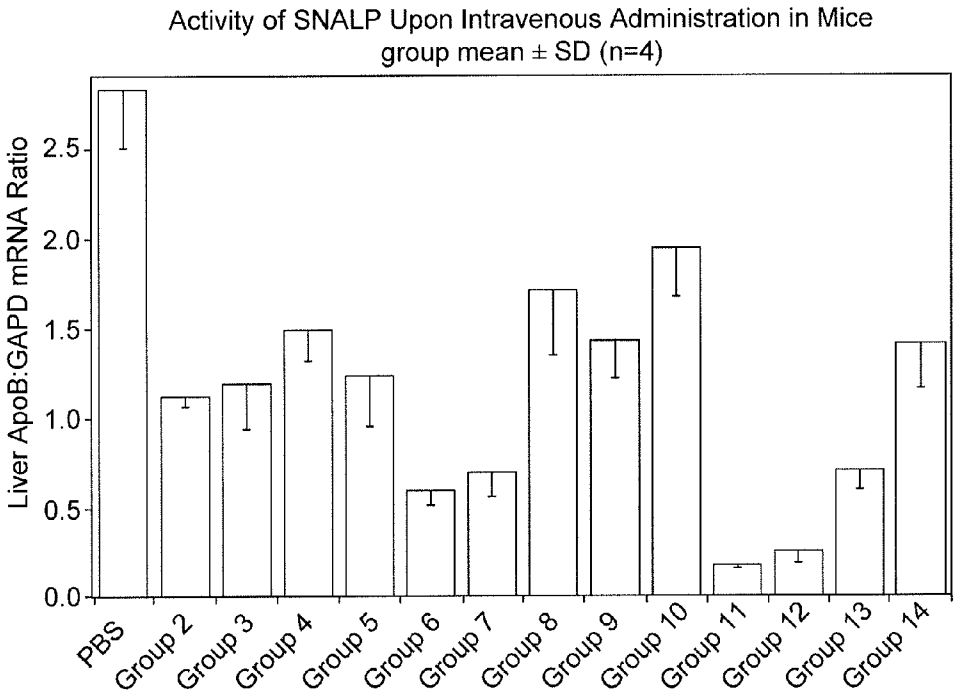

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
2.5
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

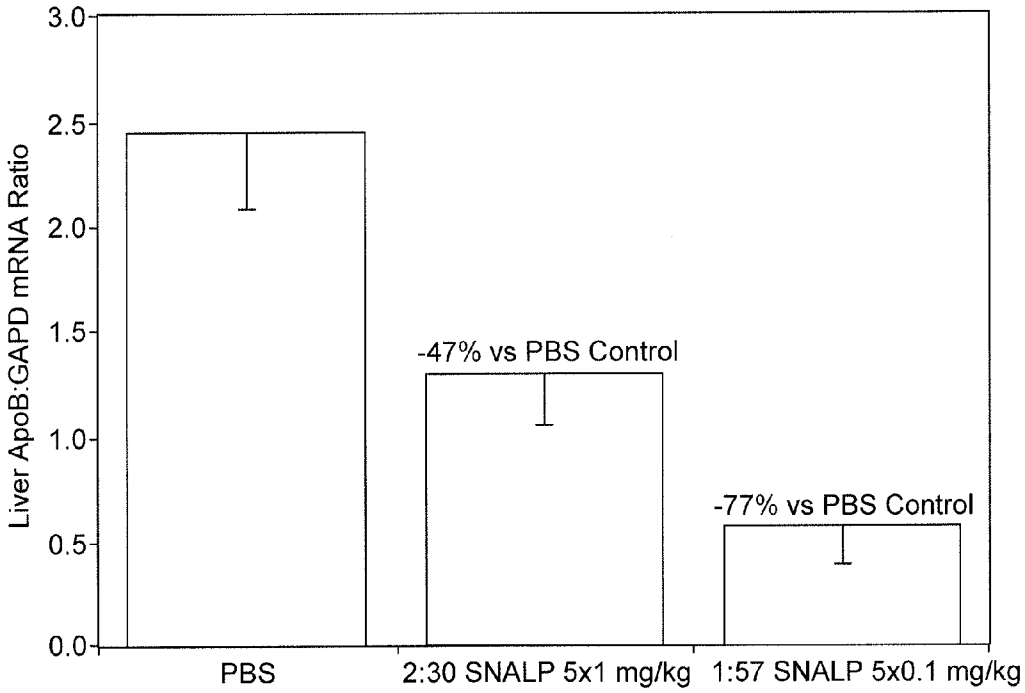

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Liver ApoB:GAPD mRNA Ratio
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5x1 mg/kg
1:57 SNALP 5x0.1 mg/kg

FIG. 3

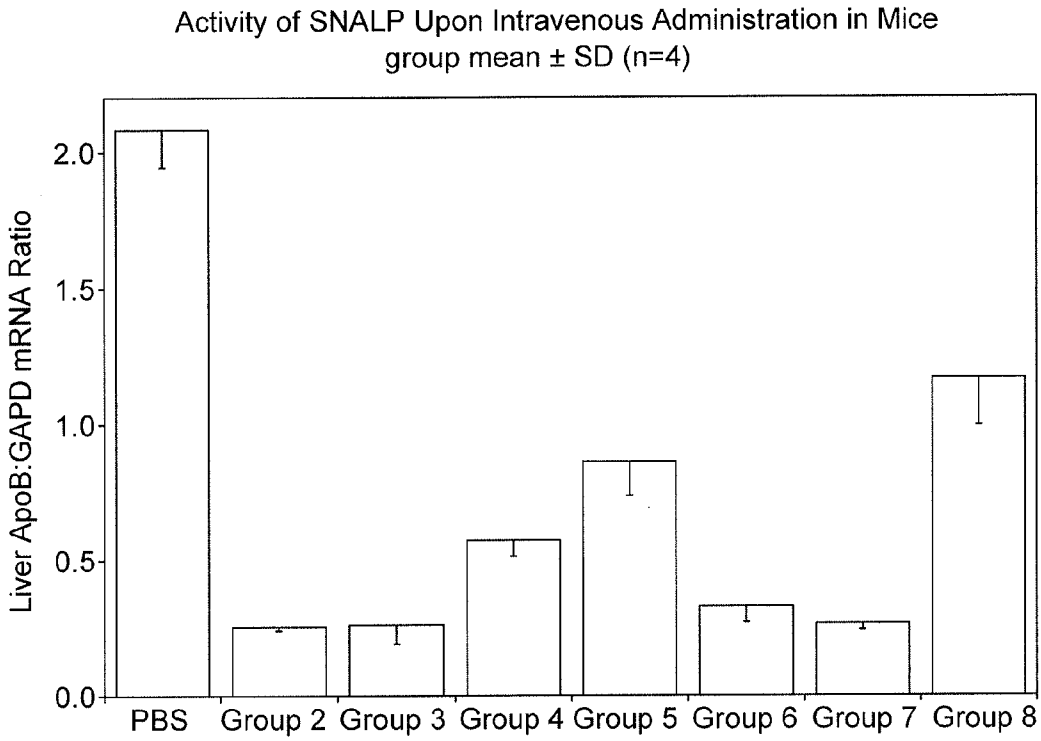

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

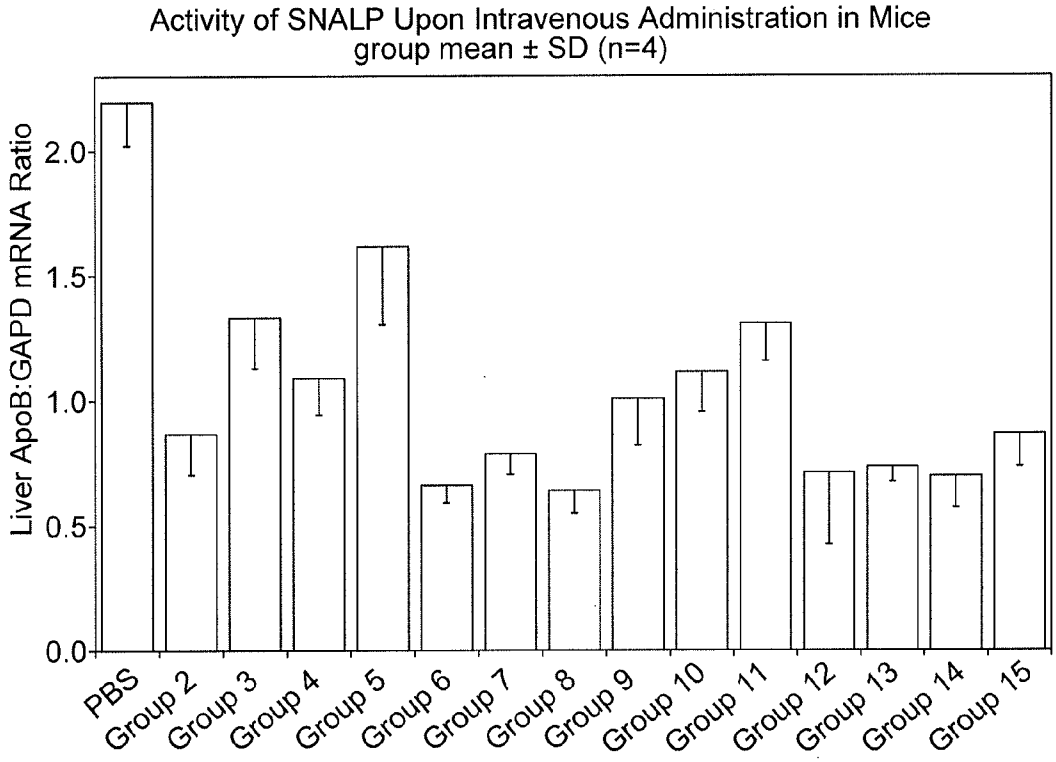

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

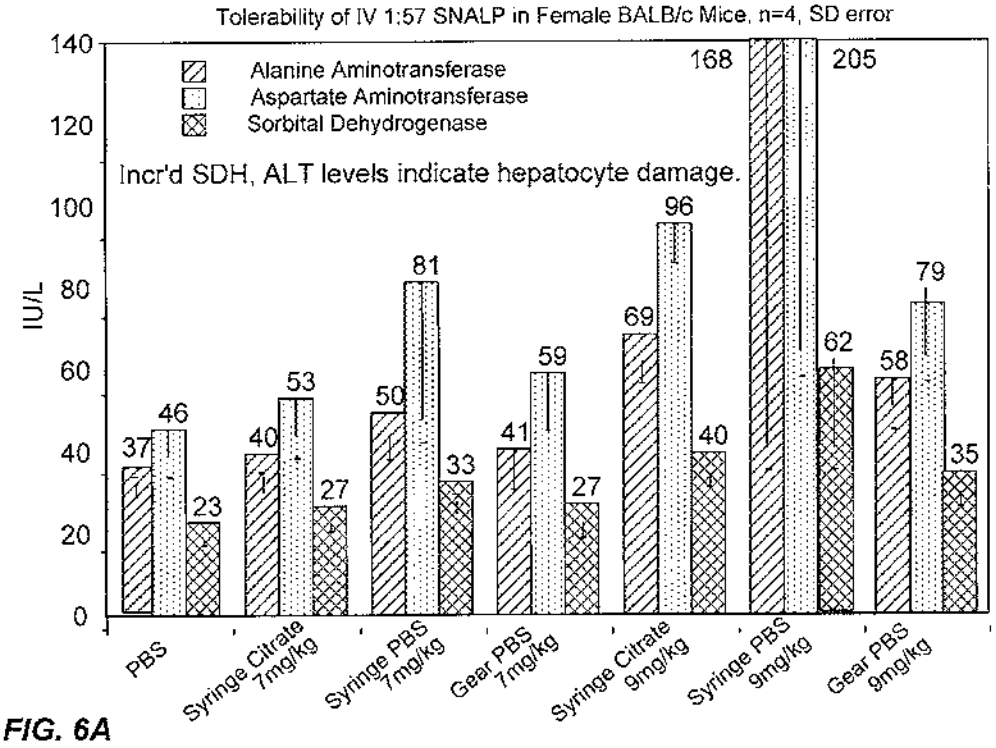

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
0
20
40
60
80
100
120
140
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
168
205
62
58
79
35
PBS
Syringe 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

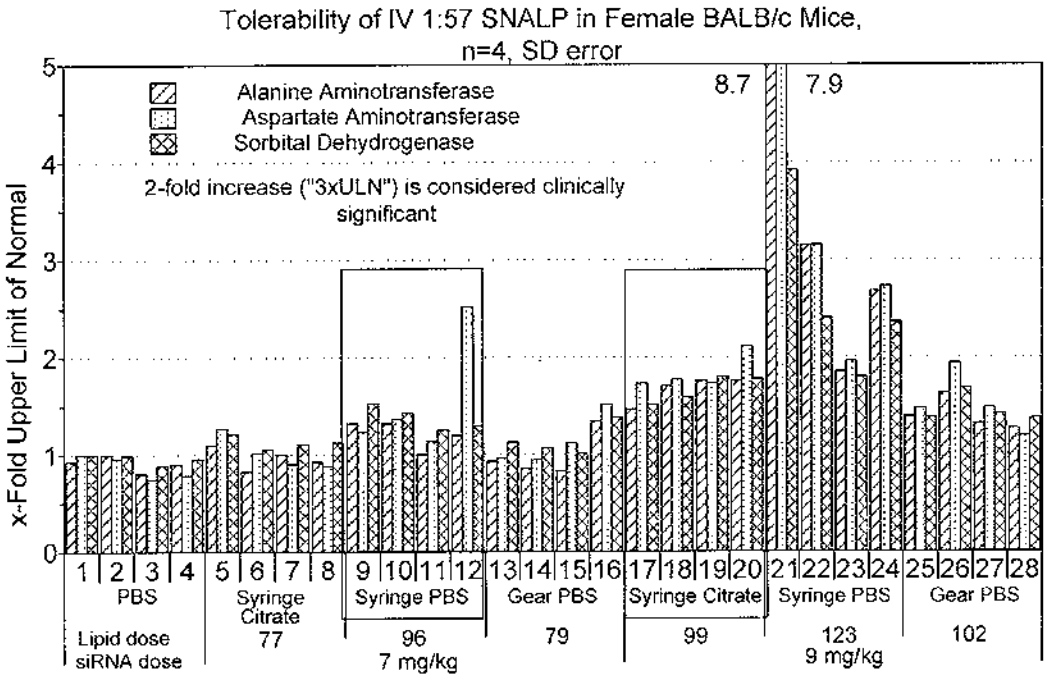

Tolerability of IV 1:57 SNALP in Female BALB/c Mice,
n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
1
2
3
4
5
8.7
7.9
1 2 3 4
PBS
5 6 7 8
Syringe Citrate
9 10 11 12
Syringe PBS
13 14 15 16
Gear PBS
17 18 19 20
Syringe Citrate
21 22 23 24
Syringe PBS
25 26 27 28
Gear PBS
Lipid dose
siRNA dose
77
96
7 mg/kg
79
99
123
9 mg/kg
102

FIG. 6B

FIG. 7A

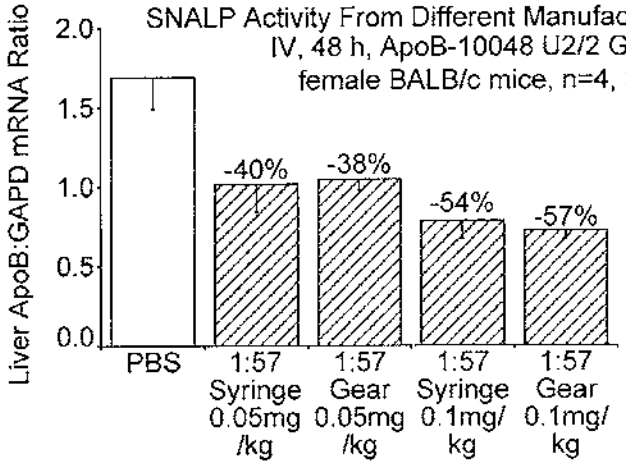


FIG. 7B

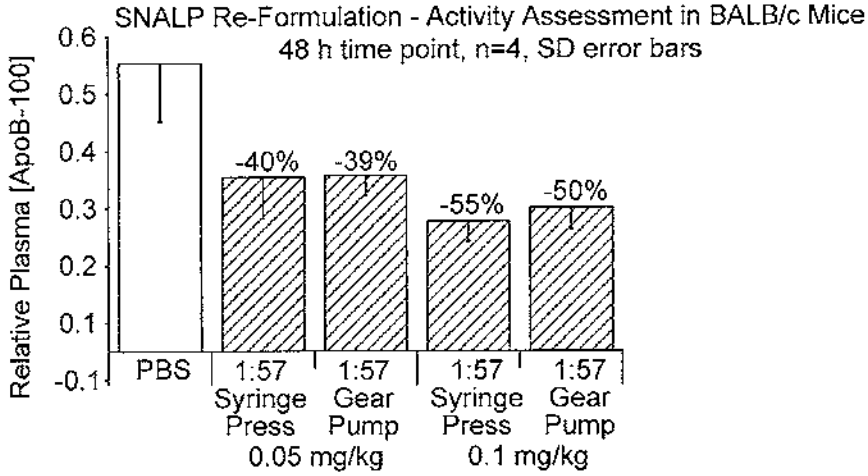
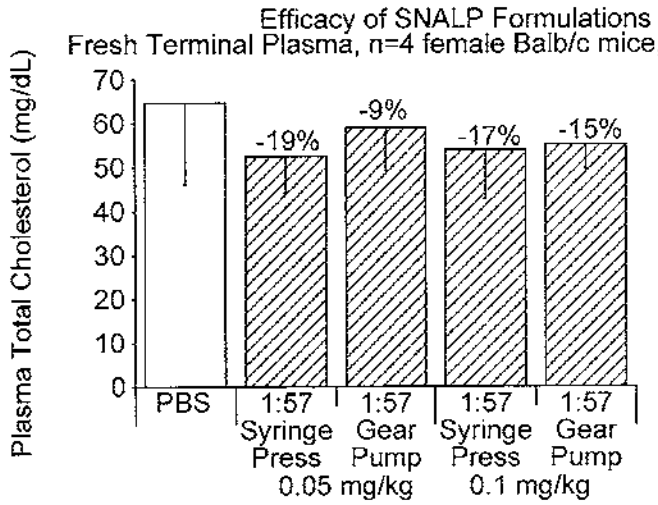


FIG. 7C



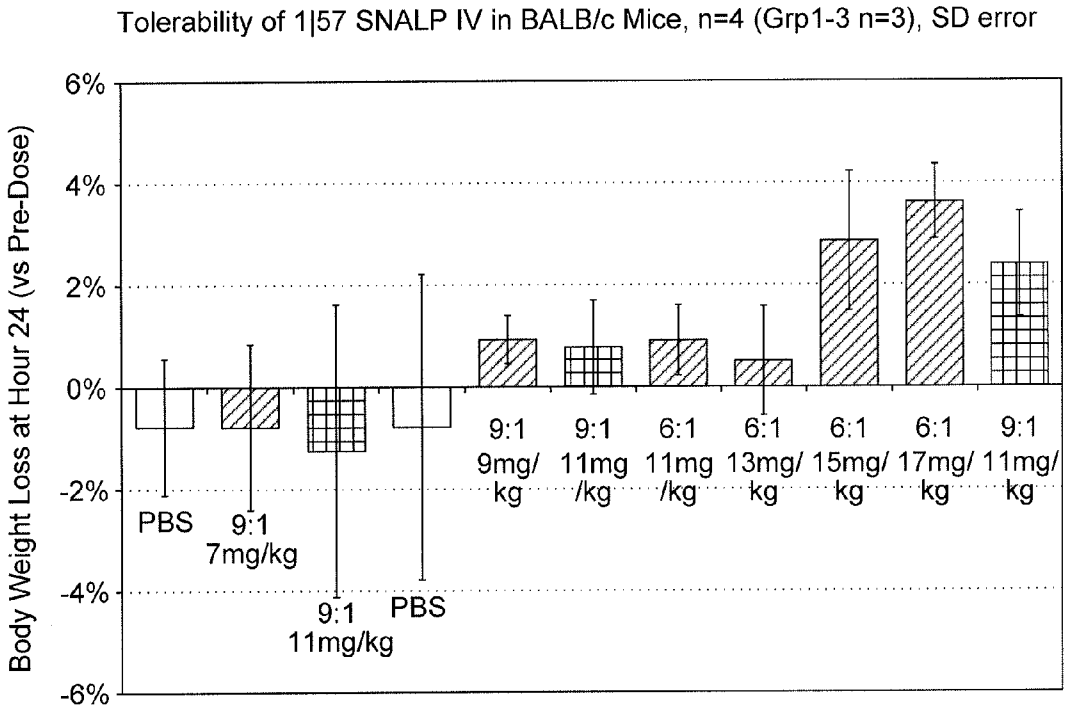

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

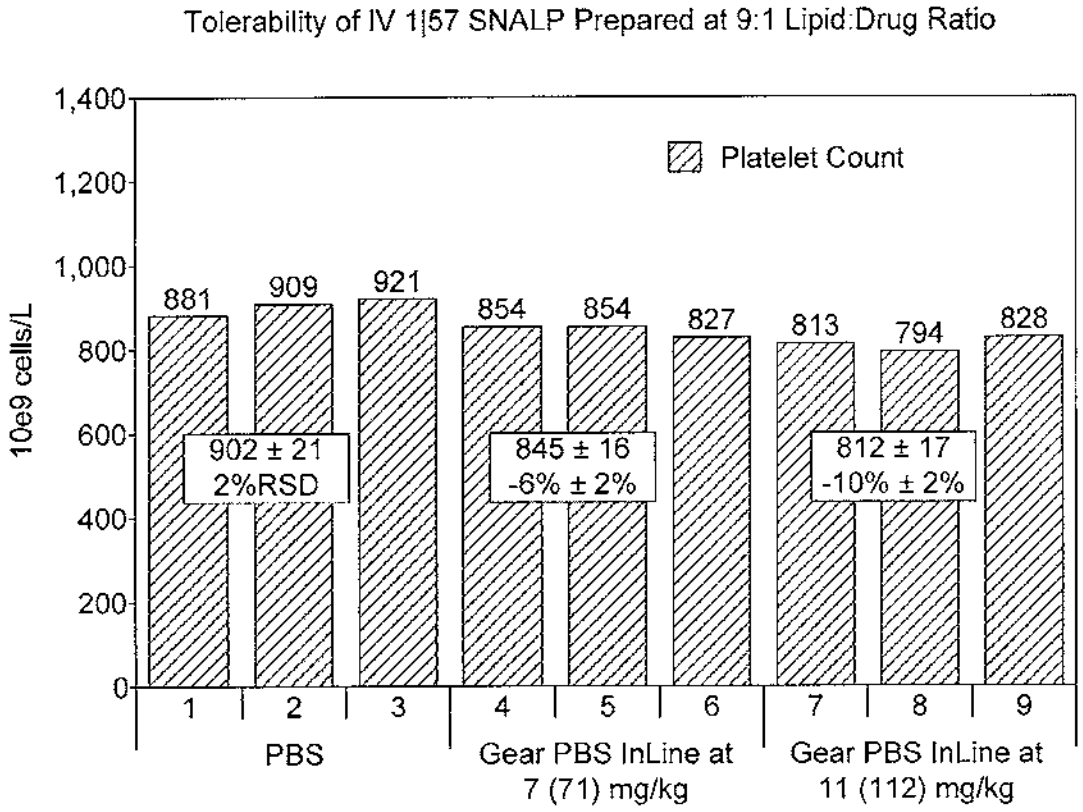

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
Platelet Count
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at
7 (71) mg/kg
Gear PBS InLine at
11 (112) mg/kg

FIG. 9

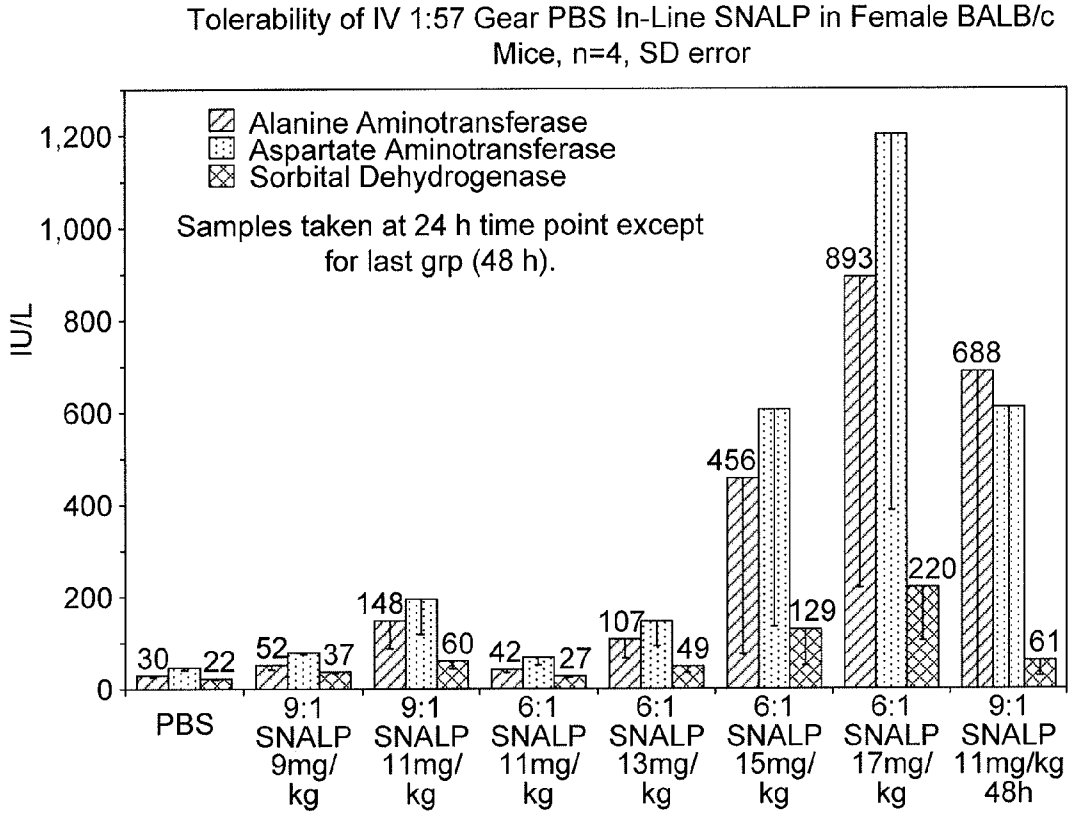

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
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

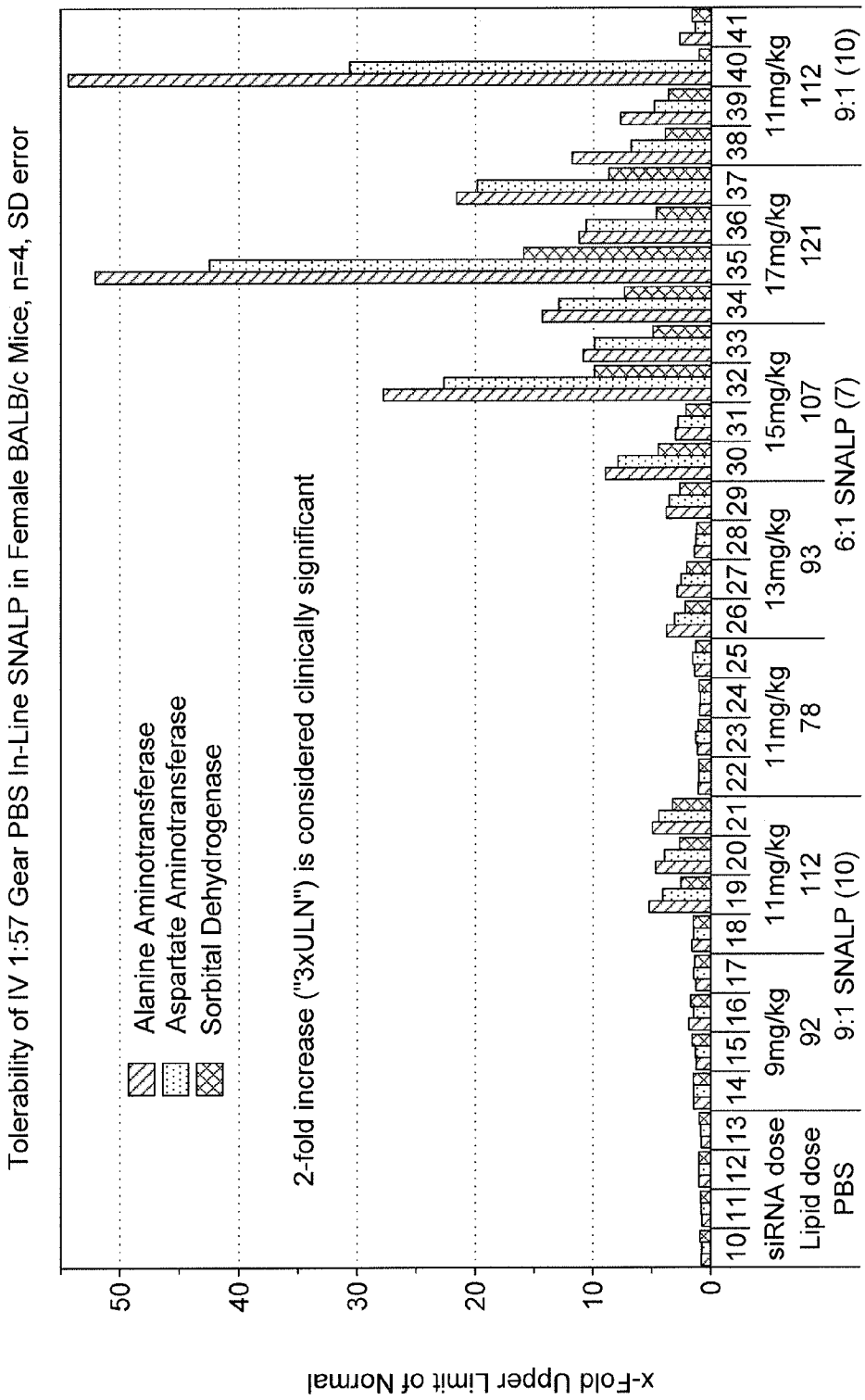

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
10
20
30
40
50
10 11 12 13 14 15 16 17 18 19 20 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

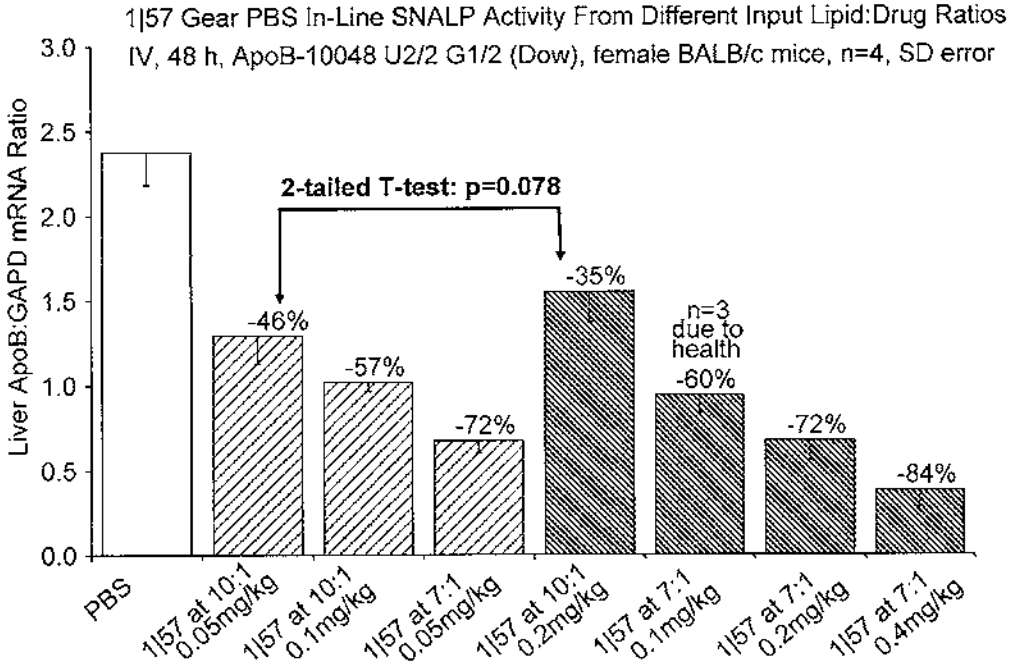
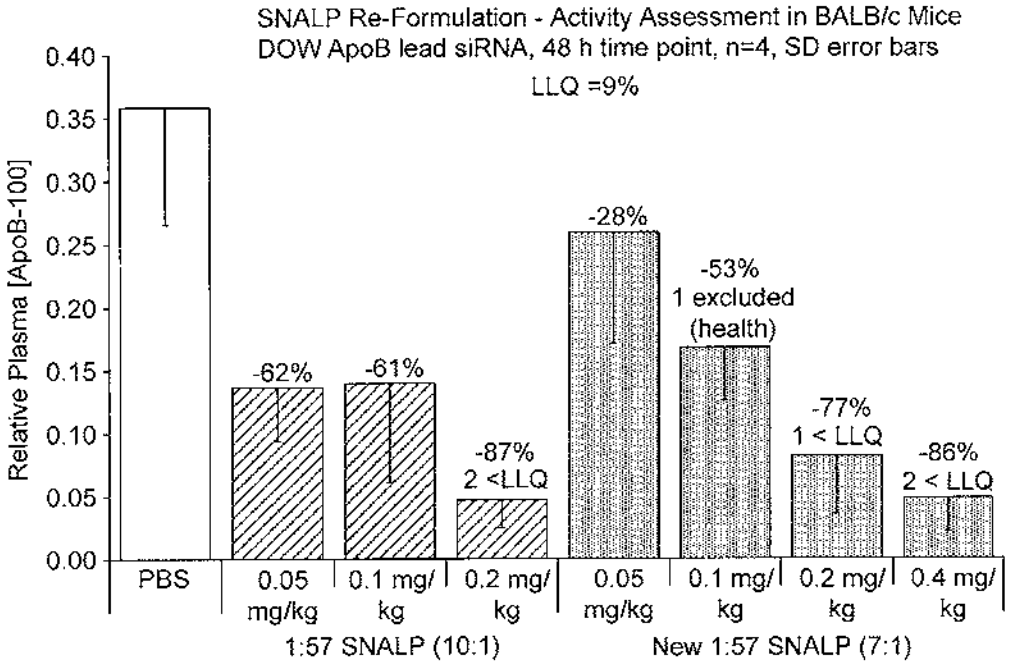


FIG. 11B



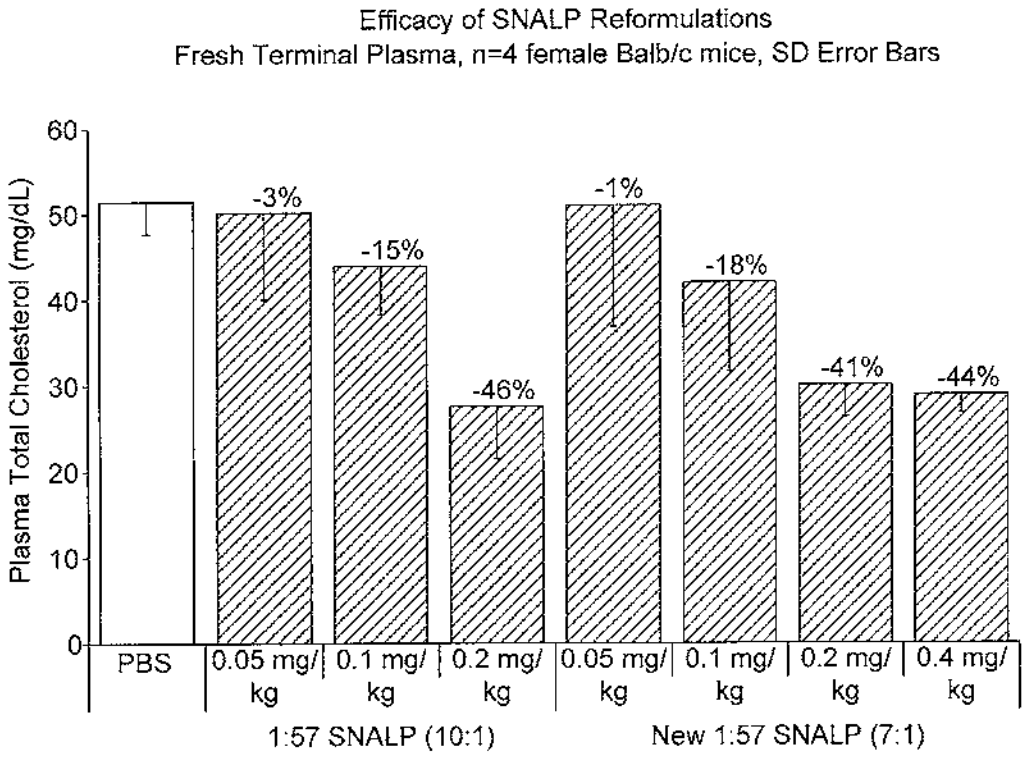

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 12

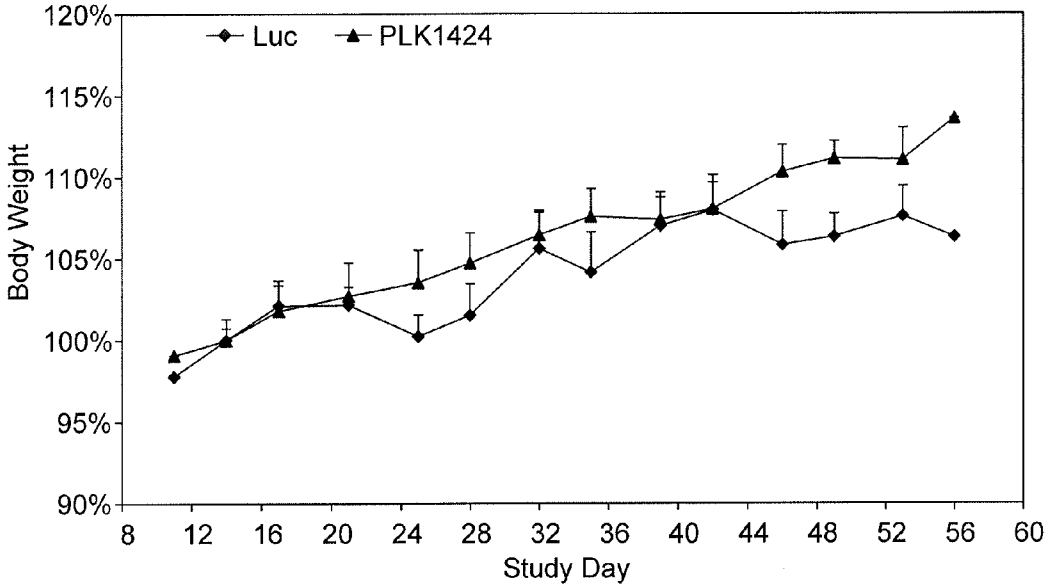

Luc
PLK1424
Body Weight
120%
115%
110%
105%
100%
95%
90%
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

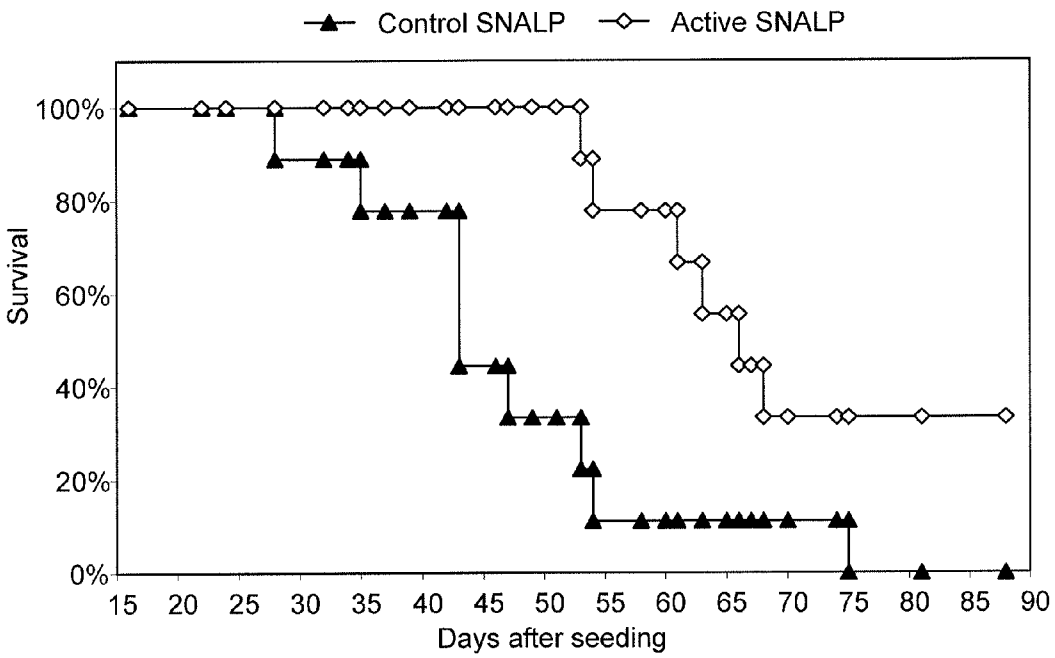

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Days after seeding

FIG. 14

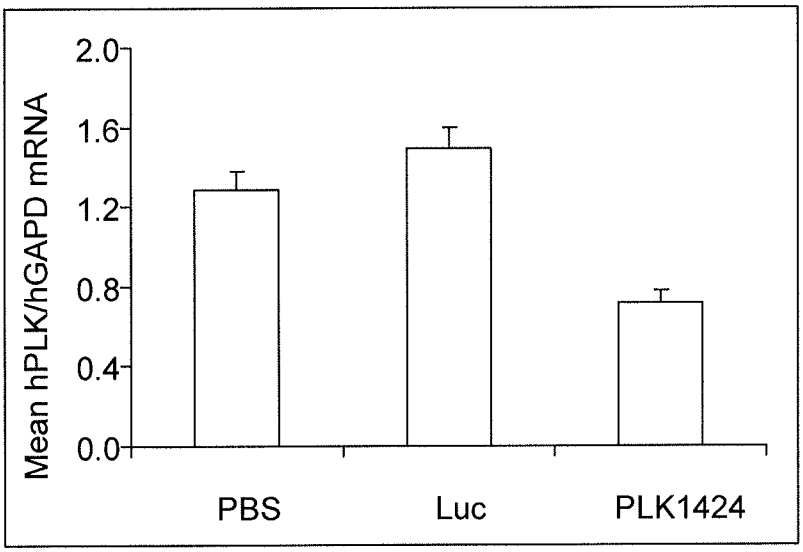


FIG. 15

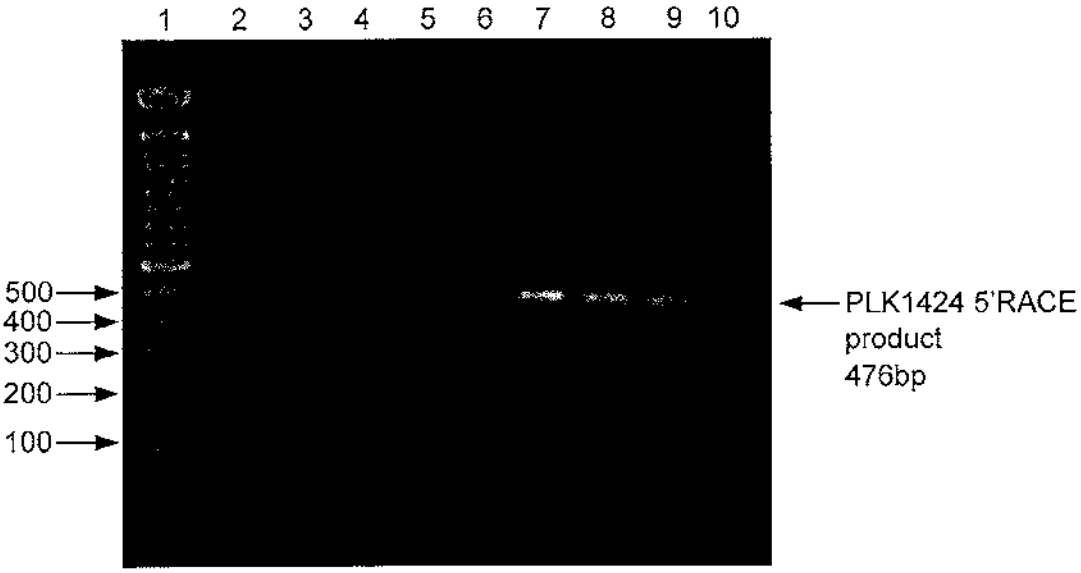
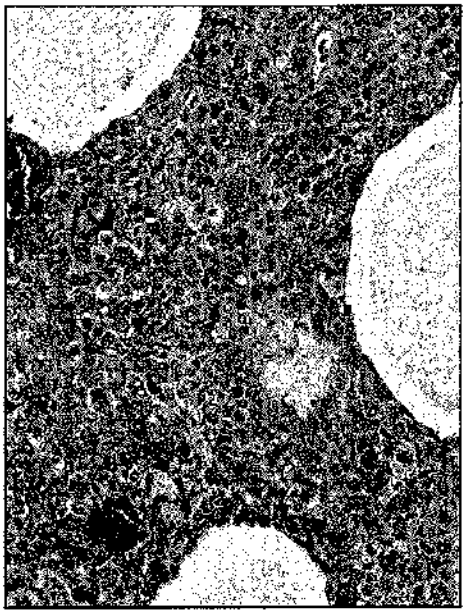
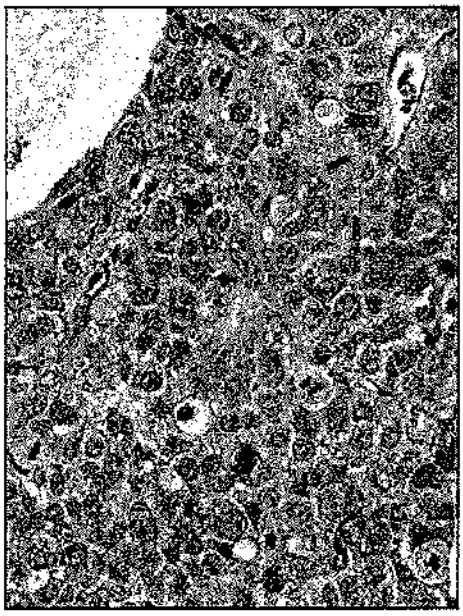

1 2 3 4 5 6 7 8 9 10
500
400
300
200
100
PLK1424 5'RACE
product
476bp

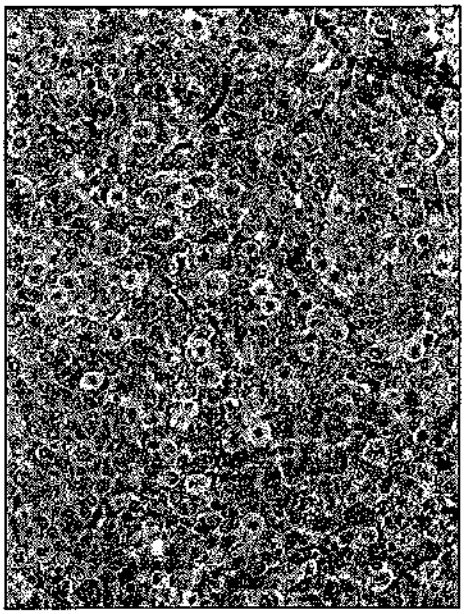
FIG. 16



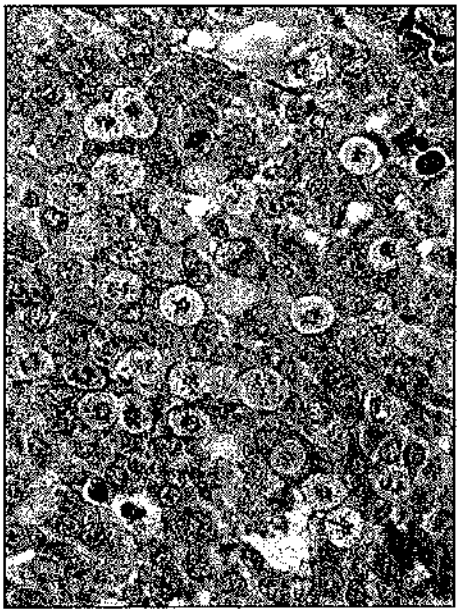
x200 mag



x400 mag



x200 mag



x400 mag

FIG. 17

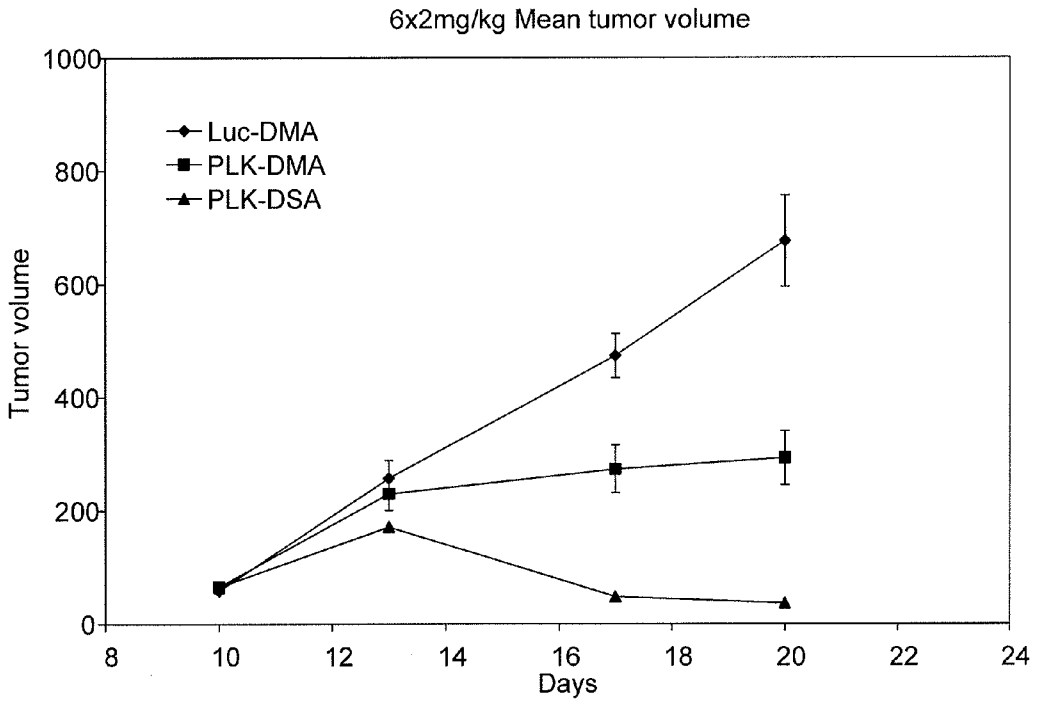

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

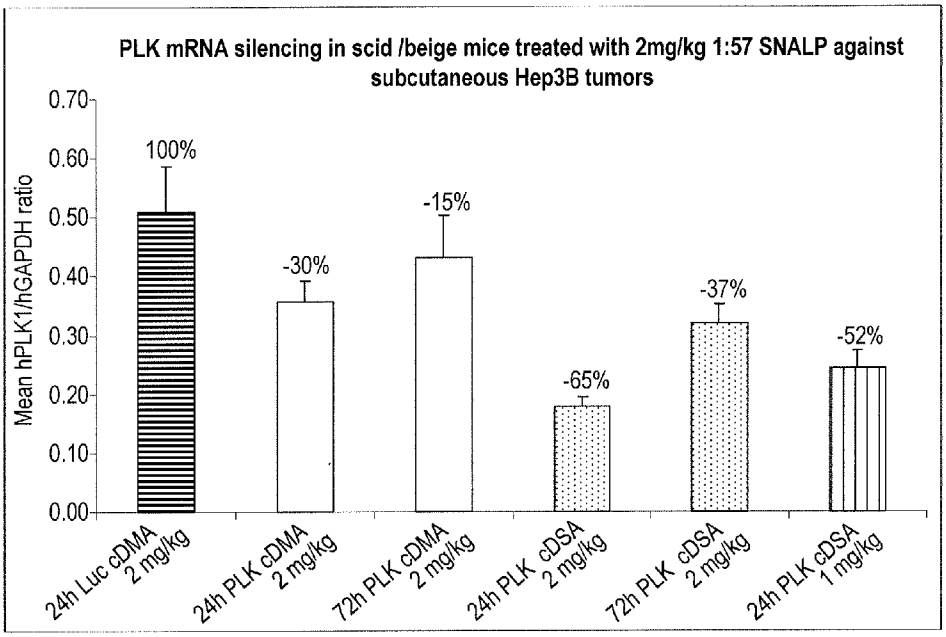

PLK mRNA silencing in scid /beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

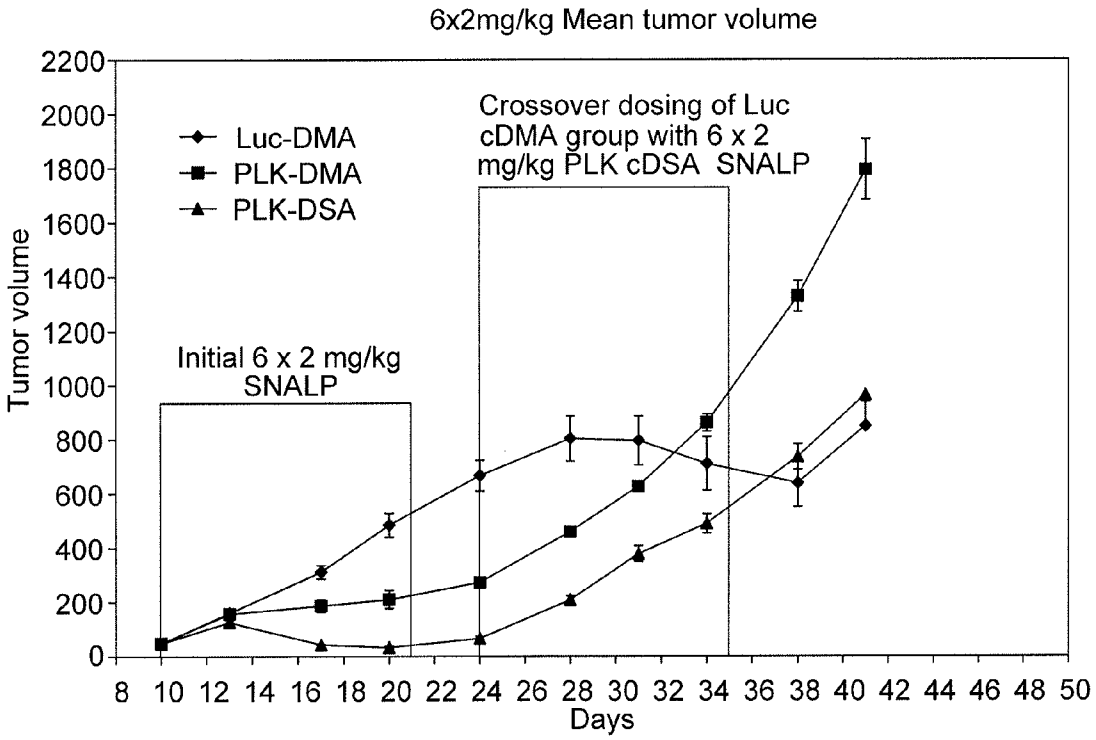

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Initial 6 x 2 mg/kg SNALP
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

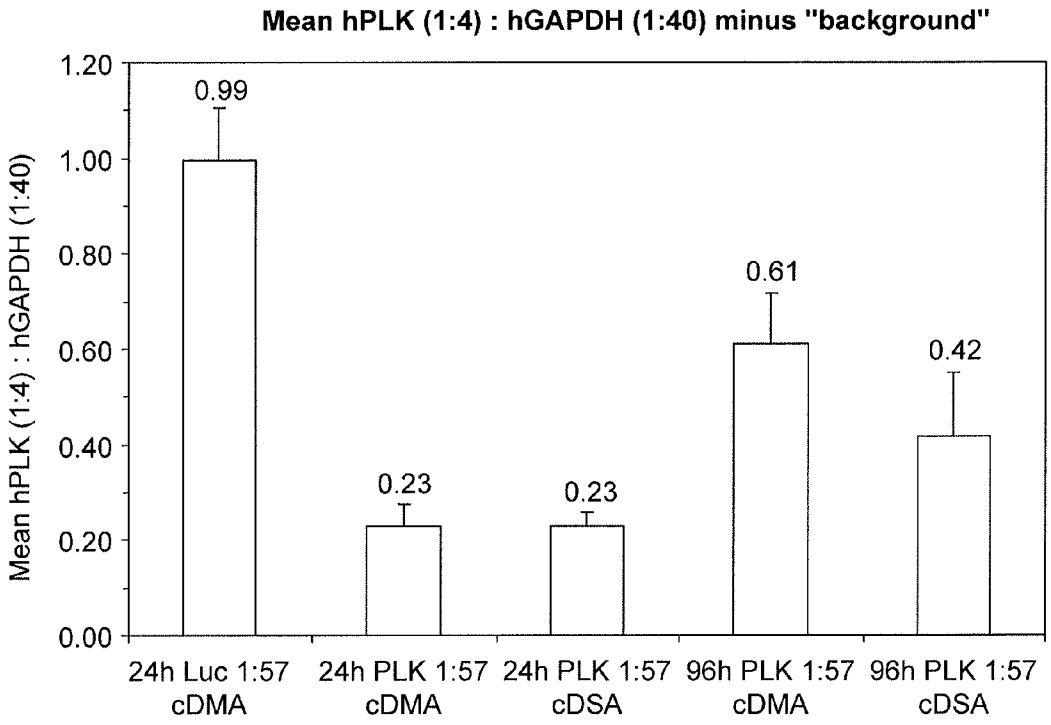

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

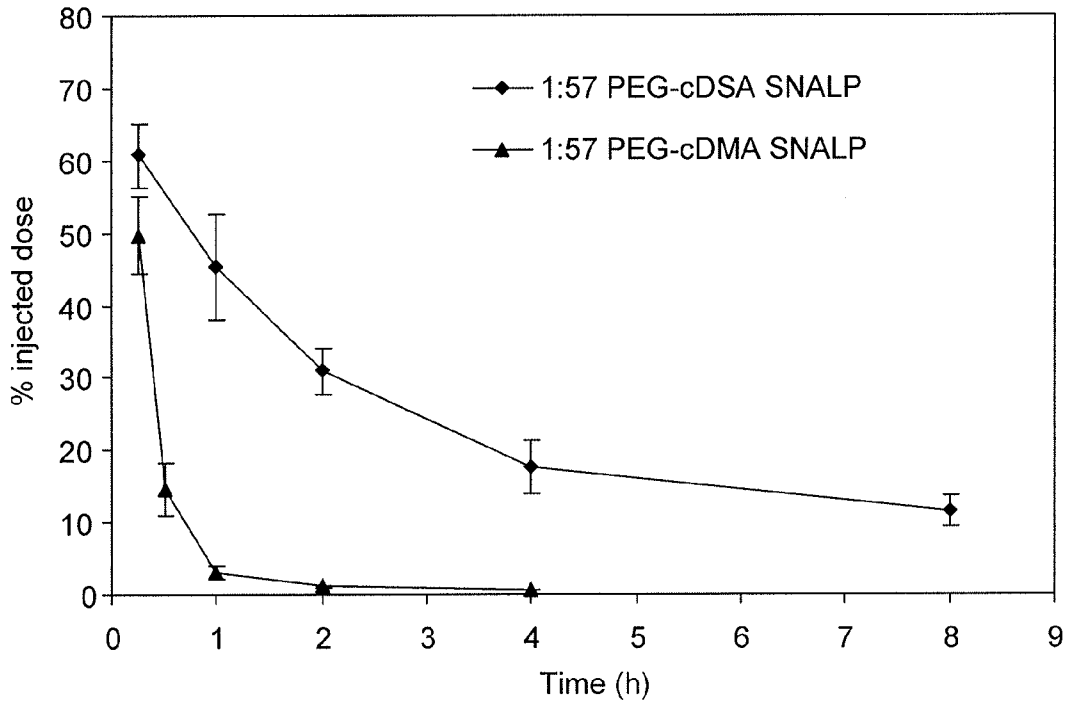

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCES TO RELATED APPLICATIONS

The present application is a continuation of U.S. application Ser. No. 13/928,309, filed Jun. 26, 2013, which application is a continuation of Ser. No. 13/253,917, filed Oct. 5, 2011, now U.S. Pat. No. 8,492,359, which application is a continuation of Ser. No. 12/424,367 filed Apr. 15, 2009, now U.S. Pat. No. 8,058,069, which application claims priority to U.S. Provisional Application No. 61/045,228, filed Apr. 15, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

Not applicable.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED AS AN ASCII TEXT FILE

The Sequence Listing written in file-77-3.TXT, created on Aug. 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

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.

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.

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.

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. Pat. No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Publication No. 20030073640.

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)).

Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Pat. 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.

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.

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.

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

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).

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.

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.

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).

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.

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.

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.

The present invention also provides pharmaceutical compositions comprising a lipid particle described herein (e.g., SNALP) and a pharmaceutically acceptable carrier.

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).

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).

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).

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

FIG. **1**A (Samples 1-8) and FIG. **1**B (Samples 9-16) illustrate data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

FIG. **2** illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **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.

FIG. **4** illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **5** illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **6**A (expressed as IU/L) and FIG. **6**B (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.

FIG. **7**A (expressed as liver ApoB:GAPD mRNA ratio), FIG. **7**B (expressed as relative plasma ApoB-100 concentration), and FIG. **7**C (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.

FIG. **8** illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

FIG. **9** illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

FIG. **10**A (expressed as IU/L) and FIG. **10**B (expressed as x-Fold Upper Limit of Normal) illustrate data demonstrating that clinically significant liver enzyme elevations (3×ULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

FIG. **11**A (expressed as liver ApoB:GAPD mRNA ratio) and FIG. **11**B (expressed as relative plasma ApoB-100 con-

centration) 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.

FIG. **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).

FIG. **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.

FIG. **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.

FIG. **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.

FIG. **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.

FIG. **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).

FIG. **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.

FIG. **19** illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

FIG. **20** illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

FIG. **21** illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

FIG. **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

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, FIG. **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, FIG. **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").

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.

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.

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

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

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.

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.

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).

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.

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.

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.

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 (PBMC's), 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.

"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.

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.

Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5×SSC, and 1% SDS, incubating at 42° C., or, 5×SSC, 1% SDS, incubating at 65° C., with wash in 0.2×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).

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 1×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.

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.

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.

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, Wis.), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology*, Ausubel et al., eds. (1995 supplement)).

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/).

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.

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.

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.

"Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

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.

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.

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.

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.

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).

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. Pat. 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.

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.

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.

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.

The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

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, palmitoyloleoylphosphatidylglycerol (POPG), and other anionic modifying groups joined to neutral lipids.

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 Publi-

cation Nos. 20060083780 and 20060240554; U.S. Pat. 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.

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.

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.

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.

"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.

"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.

"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.

"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.

The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

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

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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 Oct. 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 Dec. 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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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 embodi-

ments, 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.

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.

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.

Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-sn3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed Dec. 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. Pat. No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

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.

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.

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.

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, Calif.). "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.

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.

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).

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).

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.

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.

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.

The present invention also provides a pharmaceutical composition comprising a lipid particle (e.g., SNALP) described herein and a pharmaceutically acceptable carrier.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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

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.

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

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, oligonucleotoides 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.

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.

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.

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.

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.

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

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 Nyilnen et al., *Cell*, 107:309 (2001)), or may lack overhangs (i.e., have blunt ends).

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.

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.

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.

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

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).

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.

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.

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.

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 at 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.

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.

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. Pat. 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. Pat. 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. Pat. 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.

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. N.J.): human IL-6 and TNF-α (eBioscience; San Diego. Calif.); and mouse IL-6, TNF-α, and IFN-γ (BD Biosciences: San Diego, Calif.)).

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

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).

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.

To form a long dsRNA. for synthetic RNAs, the complement is also transcribed in vitro and hybridized to form a dsRNA. If a naturally occurring 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.

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. Pat. 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.

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, Calif.). 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.

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

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.

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.

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. Pat. 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.

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.

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.

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. Pat. 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

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.

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)).

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. Pat. 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.

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.

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 Hepatitis 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. Pat. No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed Mar. 20, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

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 (Genbank 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, 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 Jan. 26, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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 Ser. 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 Ser. No. 12/343,342, filed Dec. 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 Apr. 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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 Ser. No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

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.

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. Pat. No. 6,174,861), angiostatin (see, e.g., U.S. Pat. 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.

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., *FESS Lett.*, 527:274 (2002)).

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)).

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

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.

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.

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.

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

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 at al., *Science*, 294:853-858; Lau et al., *Science*, 294:858-862; and Lee et al., *Science*, 294:862-864.

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.

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.

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.

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.

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.

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

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.

Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein syn-

thesis 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. Pat. 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); Penis et al., *Brain Res Mol Brain Res.*, 15; 57:310-20 (1998); and U.S. Pat. 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. Pat. 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.

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

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.

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.

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. Pat. 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. Pat. 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.

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.

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. Pat. 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

Nucleic acids associated with lipid particles 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).

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.

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 Dec. 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Pat. 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

In certain embodiments, the active agent associated with the lipid particles 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 therapeutic 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.

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.

Examples of conventional hormonal therapeutic agents include, without limitation, steroids (e.g., dexamethasone), finasteride, aromatase inhibitors, tamoxifen, and goserelin as well as other gonadotropin-releasing hormone agonists (GnRH).

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.).

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.

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, litertinoin, 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.

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.

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-ξ, 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, tro-

mantadine, 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

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.

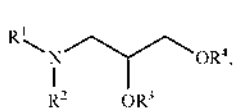
In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (e.g., siRNA, aiRNA, and/or miRNA), a cationic lipid (e.g., a cationic lipid of Formulas I, II, and/or III), a non-cationic lipid (e.g., cholesterol alone 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. Pat. 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

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.

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. Pat. 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, N.Y., 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, Wis., USA).

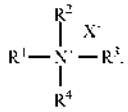
Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.



(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 tetradecrienyl (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).

Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.

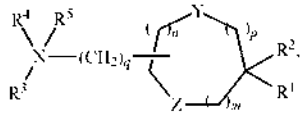


(II)

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 tetradecrienyl (C_{14}) and R^4 is linoleyl (C_{18}). In a preferred embodi-

ment, 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.

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, optionally substituted 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 C1-C6 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.

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-TAPC1), 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).

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.

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

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.

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.

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.

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.

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 sterate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyldimethyl ammonium bromide, ceramide, sphingomyelin, and the like.

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.

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.

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.

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.

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

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.

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. Pat. 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.

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. Pat. 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.

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.

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.

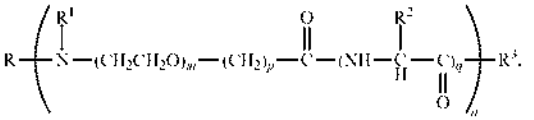
In other embodiments, an ester containing linker moiety is used to couple the PEG to the lipid. Suitable ester containing linker moieties include, e.g., carbonate (—OC(O)O—), succinoyl, phosphate esters (—O—(O)POH—O—), sulfonate esters, and combinations thereof.

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).

The term "ATTA" or "polyamide" refers to, without limitation, compounds described in U.S. Pat. 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:

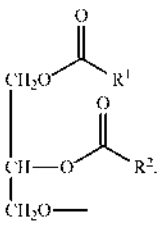
(IV)



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.

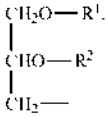
The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups 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)



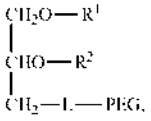
The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:

(VI)



In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:

(VII)

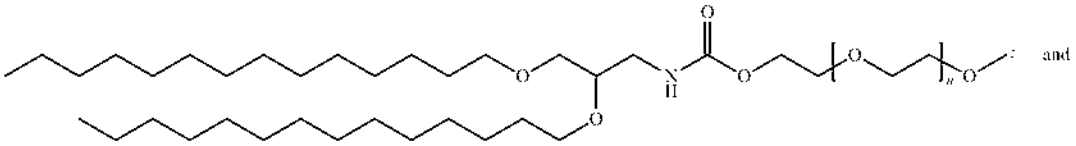


wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated 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.

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.

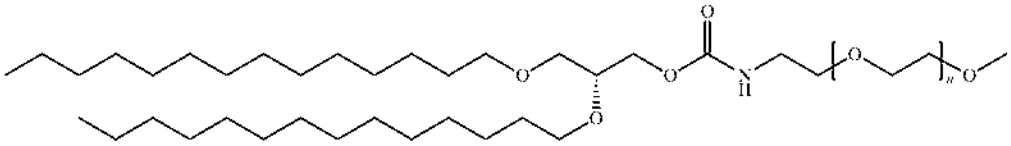
In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide 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).

In particular embodiments, the PEG-lipid conjugate is selected from:



and

(PEG-C-DMA)



(PEG-C-DOMG)

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).

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.

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.

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. Pat. No. 6,852,334 and PCT Publication No. WO 00/62813, disclosures of which are herein incorporated by reference in their entirety for all purposes.

Suitable CPLs include compounds of Formula VIII:

$$A\text{-}W\text{-}Y \qquad \text{(VIII)},$$

wherein A, W, and Y are as described below.

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.

"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.

"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.

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.

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. Pat. 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.

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.

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.

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.

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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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. Pat. 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.

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.

In some embodiments, the nucleic acids in the SNALP are precondensed as described in, e.g., U.S. patent application Ser. No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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, Wis., 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.

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.

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.

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 preformed 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. Pat. 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

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.

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).

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

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.

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.

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.

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.

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.

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

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.

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, 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. Pat. 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. Pat. 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.

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.

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. Pat. Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 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. Pat. No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

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.

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.

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. Pat. 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.

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.

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.

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.

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.

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.

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 dis-

ease 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

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.

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.

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.

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

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.

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).

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

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

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 ^3H, ^{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

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.

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).

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. Pat. No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis et al. eds.) Academic Press Inc. San Diego, Calif. (1990); Arnheim & Levinson (Oct. 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); Lomeli 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.

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 polynucleotides, 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 polyuycleotides 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.

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

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

siRNA: All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, Colo.). The siRNAs were desalted and annealed using standard procedures.

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-phosphocolinel; Avanti Polar Lipids; Alabaster, Ala.); 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 % f 5 mol %, and the amount of lipid conjugate will be 1.5 mol % f 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 % f 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

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'	1	6/42 = 14.3%	6/38 = 15.8%
	3'-dTdTGACUUCUGGACUUCUGUUA-5'	2		

Column 1: "U/U" = 2'OMe uridine modified siRNA duplex

Column 2: 2'OMe modified nucleotides are underlined in both antisense. The siRNA duplex was chemically or enzymatically synthesized. "dT" = deoxy 2' thymine [2'H] nucleotides, 2' deoxy nucleotides, 2' O [2' methoxy ethyl] [MOE] modified, and/or locked nucleic acid [LNA] modified bases. "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.

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.

	Formulation Composition, Mole %					Finished Product Characterization			
			PEG (2000)-CDMA		Lipid				
Sample No.	DLinDMA DPPC		Cholesterol		Drug Ratio	Size (nm)	Poly-dispersity	% 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
8	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
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	81
16	2	40	10	48	24.7	67	0.14	92

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, Wis.), 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.

FIG. 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, FIG. 1B, Sample 9).

Example 3

ApoB siRNA Formulated as 1:57 SNALP have Potent Silencing Activity In Vivo

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	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 XX_123456.
Column 2: The numbers refer to the identification of 2'OMe chemical modifications in each strand.
Column 3: 2'OMe modified nucleotides are underlined in both antistrand. The siRNA duplex can alternatively or additionally comprise 2' deoxy 2' fluoro (2'F) nucleotides, 2' deoxy nucleotides, 5' (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.

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.

			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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics: Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy*, 13:494 (2006).

FIG. **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

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

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.

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.

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy*, 13:494 (2006).

FIG. 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

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.

		Lipid	Finished Product Characterization		
Group	Formulation Composition Lipid Name & Mole %	Drug Ratio	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 DPPC 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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy*, 13:494 (2006).

FIG. 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

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.

	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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy*, 13:494 (2006).

FIG. 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

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

Animal Model: Female BALB/c mice, 5 wks old, n = 4 per group/cage.

siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

Tolerability:

		IV Injection	
Group	Formulation	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:

		IV Injection	
Group	Formulation	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:

Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

Formulation Details:

1. Lipid composition "1157 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.

Formulation Summary:

		Particle Size			
	1:57 (9:1) + DOW siRNA	Zavg (nm)	Poly	% Encap	Final L:D (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

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.

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 16,000×g & 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.

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.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

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

There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. FIG. **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.

FIG. **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

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

Animal Model: Female BALB/c mice, 7 wks old.

siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

CBC/Diff:

			IV Dosage	
Group	# Mice	Test Article	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:

			IV Dosage	
Group	# Mice	Test Article	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:

			IV Dosage	
Group	# Mice	Test Article	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:

Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

Formulation Details:

1. "1157 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.

Formulation Summary:

	1 57 SNALP	Particle Size			Final L:D
	Gear PBS In-Line	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

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.

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).

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.

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 16.000×g & 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.

Groups 12-19: Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16.000×g (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.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

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:

FIG. **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.

FIG. **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.

FIG. **10** shows that clinically significant liver enzyme elevations (3×ULN) 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:

FIG. **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.

FIG. **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:

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 FIG. **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

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'-AGAUCACCCUCCUUAAAUAANN-3' 3'-NNUCUAGUGGGAGGAAUUUAU-5'	5 6	6/38 = 15.8%
PLK1424 U4/G	5'-AGAUCACCCUCCUUAAAUAANN-3' 3'-NNUCUAGUGGGAGGAAUUUAU-5'	5 7	7/38 = 18.4%

[illegible] The number after "PLK" refers to the nucleotide position of the 5' base of the sense strand relative to the start codon (AUG) of the human PLK-1 mRNA sequence NM_005030. [illegible] 2'-O-methyl (2'OMe) nucleotides are indicated in bold and underlined. The siRNA duplex was chemically synthesized with 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, underlined (U) ribonucleotide, or ribonucleotide having complementarity to the target sequence (antisense strand) or the complementary strand (sense strand).

[illegible] In this modified siRNA duplex, 2'OMe nucleotides in the double stranded (ds) region of the siRNA duplex are present in

Experimental Groups

20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay
A B	20 to seed	I.H. 1.5×10^6 Hep3B	Luc 1:57 PLK 1424 1:57	9 9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10×2 mg kg	When moribund	Survival Body Weights

Test Articles

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 (28 mM lipid)
B	PLK1424 U4 GU SNALP 1:57 (28 mM lipid)
	PLK1424 U4 G SNALP 1:57 (28 mM 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.

-continued

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

FIG. 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.

FIG. 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

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.

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

20 SCID/beige mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	Sacrifice	Assay
A	20 to	I.H.	PBS	6	1×2 mg kg	24 h after	Tumor QG
B	seed	1×10^6	Luc 1:57	7	Day 20	treatment	Tumor RACE-PCR
C		Hep3B	PLK 1424 1:57	7			Histopathology

Test Articles

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. 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 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

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.

FIG. **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.

FIG. **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.

FIG. **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

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

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.

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 6×2 mg/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.

FIG. **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.

FIG. **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 FIG. **18**.

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 FIG. **20**, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

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.

FIG. **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.

FIG. **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.

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.

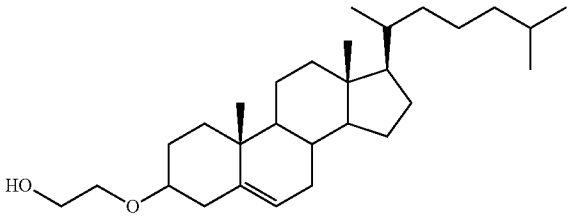
Example 12

Synthesis of Cholesteryl-2'-Hydroxyethyl Ether

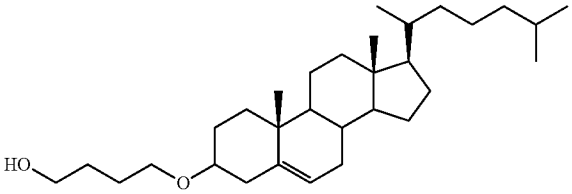
Step 1: A 250 ml round bottom flask containing cholesterol (5.0 g, 12.9 mmol) and a stir bar was sealed and flushed with nitrogen. Toluenesulphonyl chloride (5.0 g, 26.2 mmol) was weighed into a separate 100-mL round bottom flask, also sealed and flushed with nitrogen. Anhydrous pyridine (2×50 ml) was 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%).

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 (3×100 ml). The organic phases were combined, washed with water (2×150 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%).

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

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.

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What is claimed is:

1. 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.

2. The nucleic acid-lipid particle of claim **1**, wherein the nucleic acid comprises an interfering RNA, mRNA, an antisense oligonucleotide, a ribozyme, a plasmid, an immunostimulatory oligonucleotide, or mixtures thereof.

3. The nucleic acid-lipid particle of claim **2**, wherein the interfering RNA comprises a small interfering RNA (siRNA), an asymmetrical interfering RNA (aiRNA), a microRNA (miRNA), or mixtures thereof.

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.

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.

6. The nucleic acid-lipid particle of claim **5**, wherein the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof.

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.

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.

9. The nucleic acid-lipid particle of claim 1, wherein the conjugated lipid that inhibits aggregation of particles comprises a polyethyleneglycol (PEG)-lipid conjugate.

10. The nucleic acid-lipid particle of claim 9, wherein the PEG-lipid conjugate comprises a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG-dialkyloxypropyl (PEG-DAA) conjugate, or a mixture thereof.

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.

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.

13. The nucleic acid-lipid particle of claim 1, wherein the nucleic acid is fully encapsulated in the nucleic acid-lipid particle.

14. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 1 and a pharmaceutically acceptable carrier.

15. 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.

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.

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.

18. The method of claim 17, wherein the disease or disorder is a viral infection.

19. The method of claim 17, wherein the disease or disorder is a liver disease or disorder.

20. The method of claim 17, wherein the disease or disorder is cancer.

* * * * *

JOINT APPENDIX 05

5270078

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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

July 13, 2022

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

PATENT NUMBER: *9,504,651*
ISSUE DATE: *November 29, 2016*

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

W. Montgomery

Wanda Montgomery
Certifying Officer

US009504651B2

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

(10) **Patent No.: US 9,504,651 B2**
(45) **Date of Patent: Nov. 29, 2016**

(54) **LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY**

(71) Applicant: **PROTIVA BIOTHERAPEUTICS, INC.**, Burnaby (CA)

(72) Inventors: **Ian MacLachlan**, Mission (CA); **Lloyd Jeffs**, Burnaby (CA); **Lorne R. Palmer**, Burnaby (CA); **Cory Giesbrecht**, Vancouver (CA)

(73) Assignee: **PROTIVA BIOTHERAPEUTICS, INC.**, Burnaby (CA)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 15 days.

(21) Appl. No.: **14/304,578**

(22) Filed: **Jun. 13, 2014**

(65) **Prior Publication Data**

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Related U.S. Application Data

(60) Continuation of application No. 13/684,066, filed on Nov. 21, 2012, which is a continuation of application No. 12/965,555, filed on Dec. 10, 2010, now Pat. No. 8,329,070, which is a division of application No. 10/611,274, filed on Jun. 30, 2003, now Pat. No. 7,901,708.

(60) Provisional application No. 60/392,887, filed on Jun. 28, 2002.

(51) **Int. Cl.**

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A61K 47/24	(2006.01)
B01J 13/04	(2006.01)
A61K 9/127	(2006.01)
A61K 47/48	(2006.01)
A61K 31/7084	(2006.01)
A61K 31/7088	(2006.01)
A61K 47/44	(2006.01)

(52) **U.S. Cl.**
CPC ***A61K 9/1271*** (2013.01); ***A61K 9/127*** (2013.01); ***A61K 9/1277*** (2013.01); ***A61K 31/7084*** (2013.01); ***A61K 31/7088*** (2013.01); ***A61K 47/10*** (2013.01); ***A61K 47/24*** (2013.01); ***A61K 47/44*** (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,394,448 A 7/1983 Szoka, Jr. et al.
4,438,052 A 3/1984 Weder et al.
(Continued)

FOREIGN PATENT DOCUMENTS

CA 2041075 A1 10/1991
CA 2309727 A1 4/1999
(Continued)

OTHER PUBLICATIONS

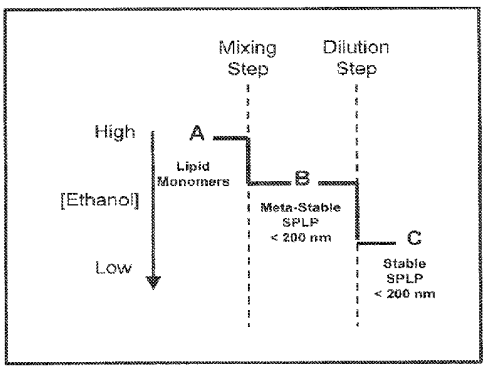
Lasic, D. D. "Novel applications of Liposomes" Tibtech, 1998, v. 16, 307-321.*
(Continued)

Primary Examiner — Mina Haghighatian
Assistant Examiner — Erin Hirt
(74) *Attorney, Agent, or Firm* — Kilpatrick Townsend & Stockton 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.

14 Claims, 15 Drawing Sheets



(56) **References Cited**

U.S. PATENT DOCUMENTS

4,515,736	A		5/1985	Deamer
4,598,051	A		7/1986	Papahadjopoulos et al.
4,687,661	A		8/1987	Kikuchi et al.
4,897,355	A		1/1990	Eppstein et al.
5,013,556	A		5/1991	Woodle et al.
5,171,678	A		12/1992	Behr et al.
5,208,036	A		5/1993	Eppstein et al.
5,225,212	A		7/1993	Martin et al.
5,264,618	A		11/1993	Felgner et al.
5,279,833	A		1/1994	Rose
5,283,185	A		2/1994	Epand et al.
5,320,906	A		6/1994	Eley et al.
5,334,761	A		8/1994	Gebeyehu et al.
5,424,073	A		6/1995	Rahman et al.
5,478,860	A		12/1995	Wheeler et al.
5,545,412	A		8/1996	Eppstein et al.
5,552,157	A		9/1996	Yagi et al.
5,578,475	A		11/1996	Jessee
5,593,622	A	*	1/1997	Yoshioka A61K 9/1271 264/4.3
5,627,159	A		5/1997	Shih et al.
5,641,662	A		6/1997	Debs et al.
5,653,996	A		8/1997	Hsu
5,656,743	A		8/1997	Busch et al.
5,674,908	A		10/1997	Haces et al.
5,703,055	A		12/1997	Felgner et al.
5,705,385	A	*	1/1998	Bally et al. 435/320.1
5,736,392	A		4/1998	Hawley-Nelson et al.
5,820,873	A		10/1998	Choi et al.
5,830,430	A	*	11/1998	Unger et al. 424/1.21
5,877,220	A		3/1999	Schwartz et al.
5,885,613	A		3/1999	Holland et al.
5,958,901	A		9/1999	Dwyer et al.
5,976,567	A		11/1999	Wheeler et al.
5,981,501	A		11/1999	Wheeler et al.
6,007,838	A		12/1999	Alving et al.
6,020,202	A		2/2000	Jessee
6,034,135	A		3/2000	Schwartz et al.
6,051,429	A		4/2000	Hawley-Nelson et al.
6,075,012	A		6/2000	Gebeyehu et al.
6,093,348	A		7/2000	Kowalski et al.
6,165,501	A		12/2000	Tirosh et al.
6,172,049	B1		1/2001	Dwyer et al.
6,251,939	B1		6/2001	Schwartz et al.
6,284,267	B1		9/2001	Aneja
6,287,591	B1		9/2001	Semple et al.
6,339,173	B1		1/2002	Schwartz et al.
6,376,248	B1		4/2002	Hawley-Nelson et al.
6,534,484	B1		3/2003	Wheeler et al.
6,586,410	B1		7/2003	Wheeler et al.
6,596,305	B1		7/2003	Edgerly-Plug
6,638,529	B2		10/2003	Schwartz et al.
6,649,780	B1		11/2003	Eibl et al.
6,671,393	B2		12/2003	Hays et al.
6,696,424	B1		2/2004	Wheeler et al.
6,734,171	B1	*	5/2004	Saravolac A61K 48/0008 424/450
6,815,432	B2		11/2004	Wheeler et al.
6,835,395	B1		12/2004	Semple et al.
6,843,942	B2		1/2005	Katinger et al.
6,858,224	B2		2/2005	Wheeler et al.
7,166,745	B1		1/2007	Chu et al.
7,341,738	B2		3/2008	Semple et al.
7,422,902	B1		9/2008	Wheeler et al.
7,479,573	B2		1/2009	Chu et al.
7,601,872	B2		10/2009	Chu et al.
7,641,915	B2		1/2010	Chen et al.
7,687,070	B2		3/2010	Gebeyehu et al.
7,745,651	B2		6/2010	Heyes et al.
7,799,565	B2	*	9/2010	MacLachlan et al. 435/458
7,803,397	B2		9/2010	Heyes et al.
7,807,815	B2		10/2010	MacLachlan et al.
7,901,708	B2	*	3/2011	MacLachlan A61K 9/127 424/450
7,915,450	B2		3/2011	Chu et al.
7,982,027	B2		7/2011	MacLachlan et al.
8,058,068	B2		11/2011	Hawley-Nelson et al.
8,058,069	B2	*	11/2011	Yaworski et al. 435/458
8,158,827	B2		4/2012	Chu et al.
8,227,443	B2		7/2012	MacLachlan et al.
8,283,333	B2	*	10/2012	Yaworski et al. ..... 514/44 A
8,329,070	B2	*	12/2012	MacLachlan A61K 9/127 264/4.1
8,466,122	B2	*	6/2013	Heyes et al. ..... 514/44 A
8,492,359	B2	*	7/2013	Yaworski et al. ..... 514/44 A
9,005,654	B2		4/2015	MacLachlan et al.
9,006,417	B2		4/2015	Yaworski et al.
2001/0048940	A1		12/2001	Tousignant et al.
2003/0069173	A1		4/2003	Hawley-Nelson et al.
2003/0072794	A1		4/2003	Boulikas
2003/0077829	A1		4/2003	MacLachlan
2003/0083272	A1		5/2003	Wiederholt et al.
2003/0124033	A1		7/2003	Baker et al.
2003/0143732	A1		7/2003	Fosnaugh et al.
2004/0032037	A1		2/2004	Katinger et al.
2004/0037874	A1		2/2004	Hong et al.
2004/0063654	A1		4/2004	Davis et al.
2004/0142025	A1		7/2004	MacLachlan et al.
2004/0142892	A1		7/2004	Finn et al.
2004/0253723	A1		12/2004	Tachas et al.
2004/0259247	A1		12/2004	Tuschi et al.
2005/0064595	A1		3/2005	MacLachlan et al.
2005/0118253	A1		6/2005	MacLachlan et al.
2005/0260757	A1		11/2005	Gebeyehu et al.
2006/0058249	A1		3/2006	Tong et al.
2006/0147514	A1		7/2006	Gebeyehu et al.
2006/0228406	A1		10/2006	Chiou et al.
2007/0042031	A1		2/2007	MacLachlan et al.
2007/0202598	A1		8/2007	Chu et al.
2007/0202600	A1		8/2007	Chu et al.
2008/0200417	A1		8/2008	Semple et al.
2009/0143583	A1		6/2009	Chu et al.
2009/0191259	A1		7/2009	Li et al.
2010/0159593	A1		6/2010	Chu et al.
2011/0216622	A1		9/2011	MacLachlan et al.
2012/0136073	A1		5/2012	Yang et al.
2012/0238747	A1		9/2012	Chu et al.
2013/0303587	A1		11/2013	Yaworski et al.
2014/0044772	A1	*	2/2014	MacLachlan A61K 9/127 424/450
2016/0032320	A1		2/2016	Yaworski et al.

FOREIGN PATENT DOCUMENTS

CA	2271582	A1	11/1999
CA	2330741	A1	11/1999
CA	2397016	A1	7/2001
CA	2457959	A1	12/2002
CA	2427640	A1	5/2003
CA	2473135	A1	6/2003
CA	2490983	A1	1/2004
CA	2491164	A1	1/2004
CA	2513623		8/2004
EP	0 055 576	A1	7/1982
EP	1 013 268	A	6/2000
EP	1 203 614	A1	11/2000
IE	911350		4/1991
JP	03-126211		5/1991
JP	05-202085		8/1993
JP	06-080560		3/1994
JP	02-525063		8/2002
JP	03-505401		2/2003
JP	07-524349		8/2007
WO	89/07929	A1	9/1989
WO	91/16024	A1	10/1991
WO	91/16039	A1	10/1991
WO	93/05162	A1	3/1993
WO	93/09236	A1	5/1993
WO	93/12240	A1	6/1993
WO	93/12756	A2	7/1993
WO	93/24640	A2	12/1993
WO	93/25673	A1	12/1993
WO	95/02698	A1	1/1995
WO	95/18863	A1	7/1995

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

WO	95/35301	A1	12/1995	
WO	96/02655	A1	2/1996	
WO	96/10390	A1	4/1996	
WO	96/40964	A2	12/1996	
WO	96/41873	A1	12/1996	
WO	98/51278	A2 †	11/1998	
WO	98/51285	A2	11/1998	
WO	WO 9851278	A2 *	11/1998	..... A61K 9/00
WO	99/14346	A2	3/1999	
WO	00/03683	A2	1/2000	
WO	00/15820	A1	3/2000	
WO	00/29103	A1	5/2000	
WO	00/62813	A2	10/2000	
WO	01/05373	A1	1/2001	
WO	01/05374	A1	1/2001	
WO	01/05873	A1	1/2001	
WO	01/75164	A2	10/2001	
WO	01/93836	A1	12/2001	
WO	02/34236	A2	5/2002	
WO	02/43699		6/2002	
WO	02/43699	A2	6/2002	
WO	02/085434	A1	10/2002	
WO	02/087541	A1	11/2002	
WO	03/059381	A2	7/2003	
WO	03/097805	A2	11/2003	
WO	2004/002453	A1	1/2004	
WO	2004/065546	A2	8/2004	
WO	2004/110499	A1	12/2004	
WO	2005/007196	A2	1/2005	
WO	2005/026372	A1	3/2005	
WO	2005/120152	A2	12/2005	
WO	2015/011633	A1	1/2015	

OTHER PUBLICATIONS

Meyer, O. et al. "Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides." J. Biol. Chem. 1998, 273, 15621-15627.*

Arpicco, S., et al., "Preparation and Characterization of Novel Cationic Lipids Developed for Gene Transfection," Proceed. Int'l Symp. Control. Rel. Bioact. Mater. (Controlled Release Society, Inc.), 1999, vol. 26, pp. 759-760.

Arpicco, S., et al., "Synthesis, characterization and transfection activity of new saturated and unsaturated cationic lipids," IL Farmaco, 2004, vol. 59, pp. 869-878.

Ballas, N., et al., "Liposomes bearing a quaternary ammonium detergent as an efficient vehicle for functional transfer of TMV-RNA into plant protoplasts," Biochimica et Biophysica Acta, 1988, vol. 939, pp. 8-18.

Barinaga, M., "Step Taken Toward Improved Vectors for Gene Transfer," Science, 1994, vol. 266, p. 1326.

Bass, "The short answer," Nature, 2001, vol. 411, pp. 428-429.

Batzri et al. "Single Bilayer Liposomes Prepared without Sonication," Biochimica et Biophysica acta 1973, vol. 298, pp. 1015-1019.

Beale, G., et al., "Gene Silencing Nucleic Acids Designed by Scanning Arrays: Anti-EGFR Activity of siRNA, Ribozyme and DNA Enzymes Targeting a Single Hybridization-accessible Region using the Same Delivery System," Journal of Drug Targeting, 2003, vol. 11, No. 7, pp. 449-456.

Behr, J.-P., "Synthetic Gene-Transfer Vectors," Acc. Chem. Res., 1993, vol. 26, pp. 274-278.

Brigham, K., et al., "Rapid Communication: In vivo Transfection of Murine Lungs with a Functioning Prokaryotic Gene Using a Liposome Vehicle," The American Journal of the Medical Sciences, vol. 298, No. 4, pp. 278-281, 1989.

Brummelkamp et al., "A System for Stable Expression of Short Interfering RNAs in Mammalian Cells," Science, 2002, vol. 296, pp. 550-553.

Cevc, G., "How Membrane Chain-Melting Phase-Transition Temperature is Affected by the Lipid Chain Asymmetry and Degree of Unsaturation: An Effective Chain-Length Model," Biochemistry, 1991, vol. 30, pp. 7186-7193.

Chonn et al., "Recent advances in liposomal drug-delivery systems," Current Opinion in Biotechnology, 1995, pp. 698-708, vol. 6.

Cortesi, R., et al., "Effect of cationic liposome composition on in vitro cytotoxicity and protective effect on carried DNA," International Journal of Pharmaceutics, 1996, vol. 139, pp. 69-78.

Crystal, R., "Transfer of Genes to Humans: Early Lessons and Obstacles to Success," Science, 1995, vol. 270, pp. 404-410.

Culver K., "The First Human Gene Therapy Experiment," Gene Therapy: A Handbook for Physicians, 1994, pp. 33-40.

Duzgunes, N., "Membrane Fusion," Subcellular Biochemistry, 1985, vol. 11, pp. 195-286.

Dwarki, V.J., et al., "Cationic Liposome-Mediated RNA Transfection," Methods in Enzymology, 1993, vol. 217, pp. 644-654.

Elbashir, S. et al., "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells," Nature, 411(24):494-498 (May 2001).

Enoch, H., et al., "Formation and properties of 1000-Å-diameter, single-bilayer phospholipid vesicles," Proc. Natl. Acad. Sci. USA, 1979, vol. 76, No. 1, pp. 145-149.

Felgner, J., et al., "Cationic Lipid-Mediated Transfection in Mammalian Cells: 'lipofection'," J. Tiss. Cult. Meth., 1993, vol. 15, pp. 63-68.

Felgner, J., et al., "Enhanced Gene Delivery and Mechanism Studies with a Novel Series of Cationic Lipid Formulations," The Journal of Biological Chemistry, 1994, vol. 269, No. 4, pp. 2550-2561.

Felgner, P., et al., "Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure," Proc. Natl. Acad. Sci. USA, 1987, vol. 84, pp. 7413-7417.

Felgner, P.L., et al., "Cationic Liposome Mediated Transfection," Proc. West. Pharmacol. Soc., 1989, vol. 32, pp. 115-121.

Gao, X., et al., "A Novel Cationic Liposome Reagent for Efficient Transfection of Mammalian Cells," Biochem. Biophys. Res. Comm., 1991, vol. 179, No. 1, pp. 280-285.

Georgopapadakou, N. H., ed., "Drug Transport in Antimicrobial and Anticancer Chemotherapy," CRC Press, Marcel Dekker, Inc. (1995).

Gershon, H., et al., "Mode of Formation and Structural Feature of DNA-Cationic Liposome Complexes Used for Transfection," Biochemistry, 1993, vol. 32, pp. 7143-7151.

Guy-Caffey, J., et al., "Novel Polyaminolipids Enhance the Cellular Uptake of Oligonucleotides," The Journal of Biological Chemistry, 1995, vol. 270, No. 52, pp. 31391-31396.

Hawley-Nelson, P., et al., "LipofectAmine™ Reagent: A New, Higher Efficiency Polycationic Liposome Transfection Reagent," Focus, 1993, vol. 15, No. 3, pp. 73-80.

Heyes et al., "Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids," Journal of Controlled Release, 2005, vol. 107, pp. 276-287.

Heyes et al., "Synthesis of novel cationic lipids: effect of structural modification on the efficiency of gene transfer," J. Med. Chem., 2002, vol. 45, pp. 99-114.

Hirota et al., "Simple Mixing Device to Reproducibly Prepare Catonic Lipid-DNA Complexes (Lipoplexes)", BioTechniques, Aug. 1999, vol. 27, No. 2, pp. 286-290.

Hyde, S. et al., "Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy," Nature, 1993, vol. 362, pp. 250-255.

Isele et al., "Large-Scale Production of Liposomes Containing Monomeric Zinc Phthalacyanine by Controlled Dilution of Organic Solvents" Journal of Pharmaceutical Sciences, Nov. 1994, vol. 83, No. 11, pp. 1608-1616.

Jiang, L., et al., "Comparison of protein precipitation methods for sample preparation prior to proteomic analysis," Journal of Chromatography A, 2004, vol. 1023, pp. 317-320.

Juliano, R., et al., "The Effect of Particle Size and Charge on the Clearance Rates of Liposomes and Liposome Encapsulated Drugs," Biochem. Biophys. Res. Commun., 1975, vol. 63, No. 3, pp. 651-658.

Keough, K., "Influence of chain unsaturation and chain position on thermotropism and intermolecular interactions in membranes," Biochem. Soc. Transactions, 1990, vol. 18, No. 5, pp. 835-837.

(56) **References Cited**

OTHER PUBLICATIONS

Krichevsky, A. et al., "RNAi functions in cultured mammalian neurons," PNAS, 99(18):11926-29, 2002.
Lawrence et al. "The formation, characterization and stability of non-ionic surfactant vesicles," S.T.P. Pharma Sciences, 1996, vol. 6, No. 1, pp. 49-60.
Lawrence et al., "Synthesis and aggregation properties of dialkyl polyoxyethylene glycerol ethers," Chemistry and Physics of Lipids, 1996, 82(2):89-100.
Legendre, J.-Y. et al., "Delivery of Plasmid DNA into Mammalian Cell Lines Using pH-Sensitive Liposomes: Comparison with Cationic Liposomes," Pharm. Res., 1992, vol. 9, No. 10, pp. 1235-1242.
Leventis, R. et al., "Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles," Biochem. Biophys. Acta, 1990, vol. 1023, pp. 124-132.
Liu et al., "Cationic liposome-mediated intravenous gene delivery," J. Biol. Chem. 1995, vol. 270, pp. 24864-24870.
MacLachlan, "Liposomal Formulations for Nucleic Acid Delivery," Antisense Drug Technologies, Second Edition; 2007; pp. 237-270.
Marshall, E., "Gene Therapy's Growing Pains," Science, 1995, vol. 269, pp. 1050-1055.
Maurer et al. "Spontaneous Entrapment of Polynucleotides upon Electrostatic Interaction with Ethanol-Destabilized Cationic Liposomes," Biological Journal, May 2001, vol. 80, pp. 2310-2326.
Murahashi et al., "Synthesis and evaluation of neoglycolipid for liposome modification," Biol. Pharm. Bull., 1997, 20(6):704-707.
Orkin, S. et al., NIH Report, Report and Recommendations of the Panel to Assess the NIH Investment in Research on Gene Therapy, 1995.
Parr et al., Factors influencing the retention and chemical stability of polly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles, Biochimica et Biophysica Acta, 1994, 1195:21-30.
Paul, C. et al., "Effective expression of small interfering RNA in human cells," Nature Biotech., 2002, vol. 20, pp. 505-508.
Puyal, C. et al., "A new cationic liposome encapsulating genetic material: A potential delivery system for polynucleotides," Eur. J. Biochem., 1995, vol. 228, pp. 697-703.
Sawada et al., "Microemulsions in supercritical CO2 utilizing the polyethyleneglycol dialkylglycerol and their use for the solubilization of hydrophiles," Dyes and Pigments, 2005, pp. 64-74, vol. 65.
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.
Shin et al. "Acid-triggered release via dePEGylation of DOPE liposomes containing acid-labile vinyl ether PEG-lipids," Journal of Controlled Release, 2003, vol. 91, pp. 187-200.
Song et al., "Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes," Biochimica et Biophysica Acta, 2002, 1558:1-13.
Sorensen et al., "Gene Silencing by Systemic Delivery of Synthetic siRNAs in Adult Mice," J. Mol. Biol., Apr. 4, 2003, vol. 4, pp. 761-766.
Spagnou, S. et al., "Lipidic Carriers of siRNA: Differences in the Formulation, Cellular Uptake, and Delivery with Plasmid DNA," Biochemistry, 2004, vol. 43, pp. 13348-13356.
Stamatatos, L. et al., "Interactions of Cationic Lipid Vesicles with Negatively Charged Phospholipid Vesicles and Biological Membranes," Biochemistry, 1988, vol. 27, pp. 3917-3925.
Szoka, F. et al., "Comparative Properties and Methods of Preparation of Lipid Vesicles (Liposomes)," Ann. Rev. Biophys. Bioeng., 1980, vol. 9, pp. 467-508.
Szoka, F. et al., "Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation," Proc. Natl. Acad. Sci. USA, 1978, vol. 75, No. 9, pp. 4194-4198.
Templeton, "Cationic liposome-mediated intravenous gene delivery in vivo," Bioscience Reports, 2002, vol. 22, No. 2, pp. 283-295.
Van Der Woude, I. et al., "Parameters influencing the introduction of plasmid DNA into cells by the use of synthetic amphiphiles as a carrier system," Biochimica et Biophysica Acta, 1995, vol. 1240, pp. 34-40.
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.
Wheeler et al., "Stabilized plasmid-lipid particles: construction and characterization," Gene Ther., 1999, vol. 6, No. 2, pp. 271-281.
Wilson, R. et al., "Counterion-Induced Condensation of Deoxyribonucleic Acid. A Light-Scattering Study," Biochemistry, 1979, vol. 18, No. 11, pp. 2192-2196.
Woodle, M.C. et al., "Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes," Biochimica et Biophysica Acta, 1992, vol. 1105, pp. 193-200.
Zelphati et al., "Stable and Monodisperse Lipoplex Formulations for Gene Delivery," Gene Therapy, 1998, vol. 5, pp. 1272-1282.
Zhu, N. et al., "Systemic Gene Expression After Intravenous DNA Delivery into Adult Mice," Science, 1993, vol. 261, pp. 209-211.
Global Newswire, retrieved from http://globalnewswire.com on Feb. 27, 2013, Tekmira sues Alnylam Pharmaceuticals for repeated misuse of trade secrets and confidential information, Mar. 16, 2011, pp. 1-3.
Jeffs et al. "A Scalable, Extrusion-Free Method for Efficient Liposomal Encapsulation of Plasmid DNA" Pharmaceutical Research, 2005, vol. 22, No. 3 pp. 362-372.
Kee, J., "e-Study guide for: Clinical calculations," Just the Facts 101, Textbook Key Facts, 6th Ed., 2015, 1 page.
Omega Engineering, Inc., "Omegaflex® Peristaltic Pump Model No. FPU500," retrieved online at <https://web.archive.org/web/20010715124628/http://www.omega.com/pdf/tubing/pumps/fpu500/fpu500.asp>, cached Jul. 15, 2001, 2 pages.

* cited by examiner
† cited by third party

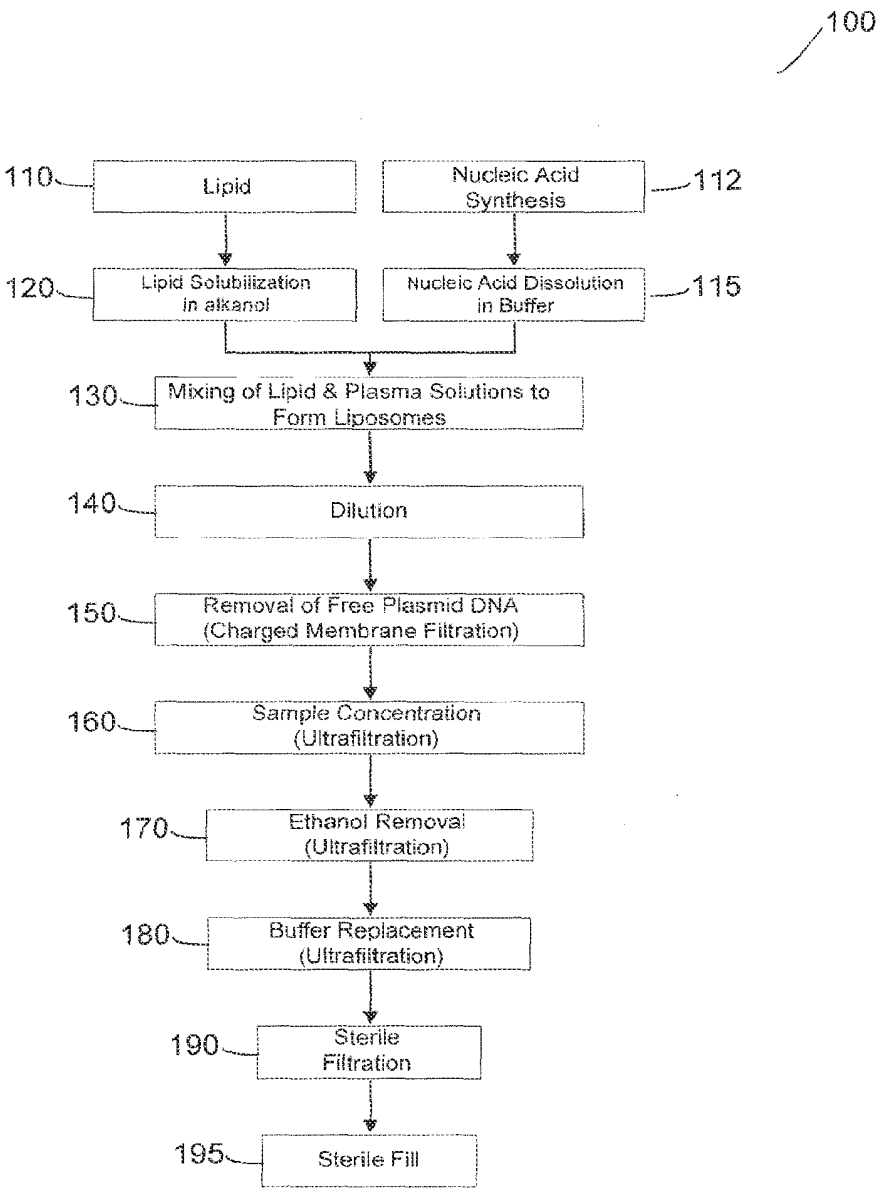

100
110
Lipid
Nucleic Acid Synthesis
112
120
Lipid Solubilization in alkanol
Nucleic Acid Dissolution in Buffer
115
130
Mixing of Lipid & Plasma Solutions to Form Liposomes
140
Dilution
150
Removal of Free Plasmid DNA (Charged Membrane Filtration)
160
Sample Concentration (Ultrafiltration)
170
Ethanol Removal (Ultrafiltration)
180
Buffer Replacement (Ultrafiltration)
190
Sterile Filtration
195
Sterile Fill

FIG. 1

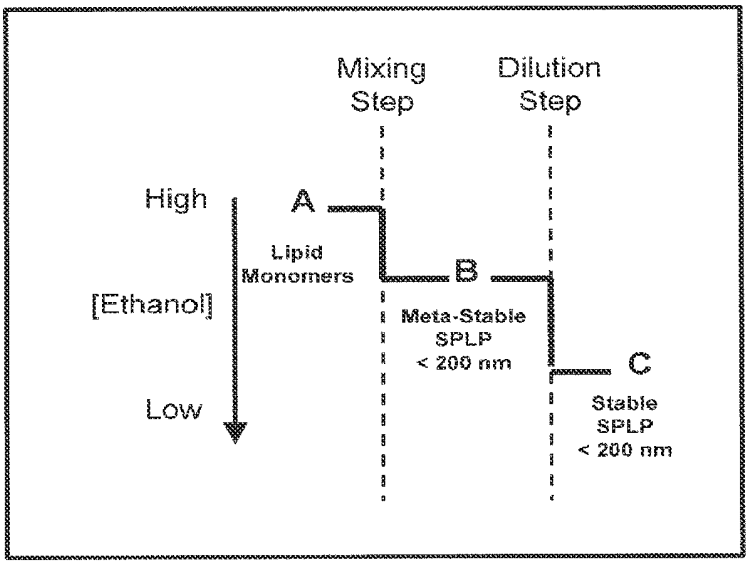

Mixing Step
Dilution Step
High
[Ethanol]
Low
A
Lipid Monomers
B
Meta-Stable SPLP < 200 nm
C
Stable SPLP < 200 nm

FIG. 2

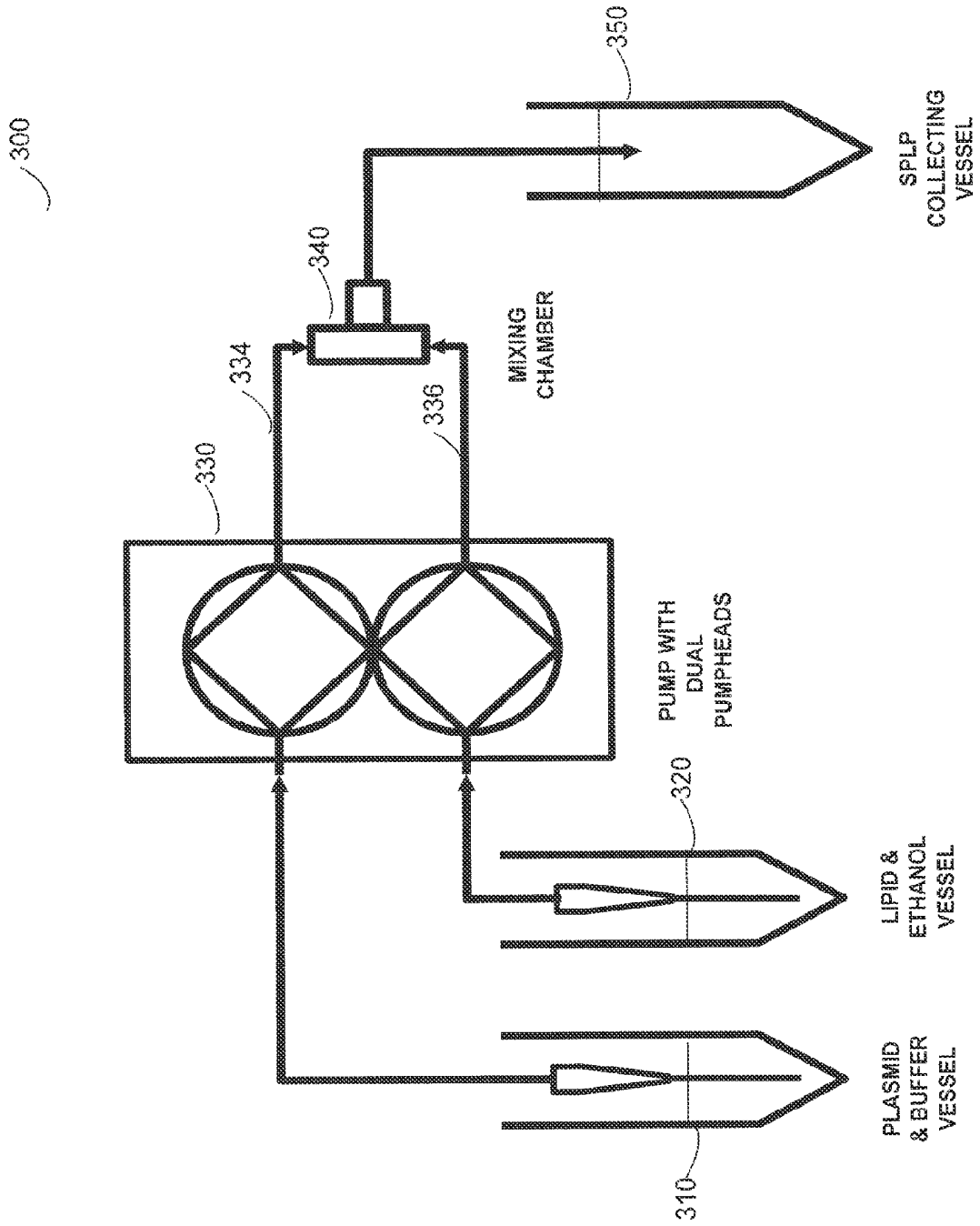

300
350
SPLP COLLECTING VESSEL
340
MIXING CHAMBER
334
336
330
PUMP WITH DUAL PUMPHEADS
320
LIPID & ETHANOL VESSEL
PLASMID & BUFFER VESSEL
310

FIG. 3

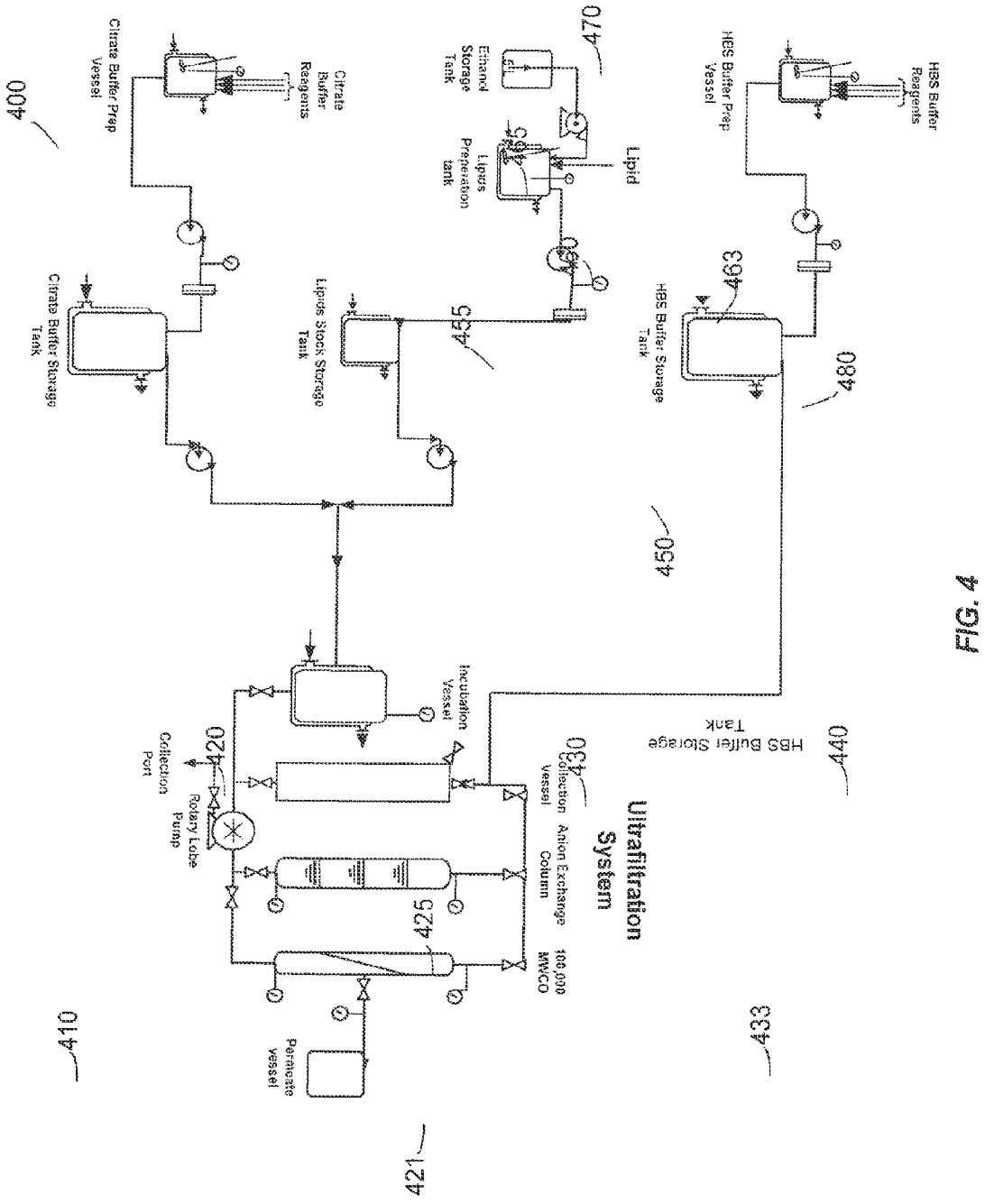

400
Citrate Buffer Prep Vessel
Citrate Buffer Reagents
Citrate Buffer Storage Tank
Ethanol Storage Tank
Lipids Preparation tank
Lipid
470
Lipids Stock Storage Tank
495
HBS Buffer Prep Vessel
HBS Buffer Reagents
HBS Buffer Storage Tank
463
480
450
Incubation Vessel
420
Collection Port
Rotary Lobe Pump
425
Collection Vessel
Anion Exchange Column
100,000 MWCO
Ultrafiltration System
HBS Buffer Storage Tank
440
410
433
Permeate vessel
421

FIG. 4

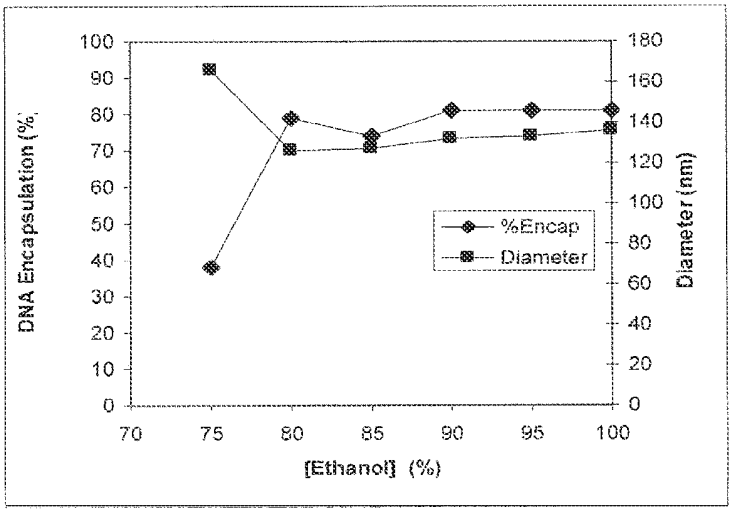

DNA Encapsulation (%)
Diameter (nm)
[Ethanol] (%)
%Encap
Diameter
100
90
80
70
60
50
40
30
20
10
0
180
160
140
120
100
80
60
40
20
0
70
75
80
85
90
95
100

FIG. 5

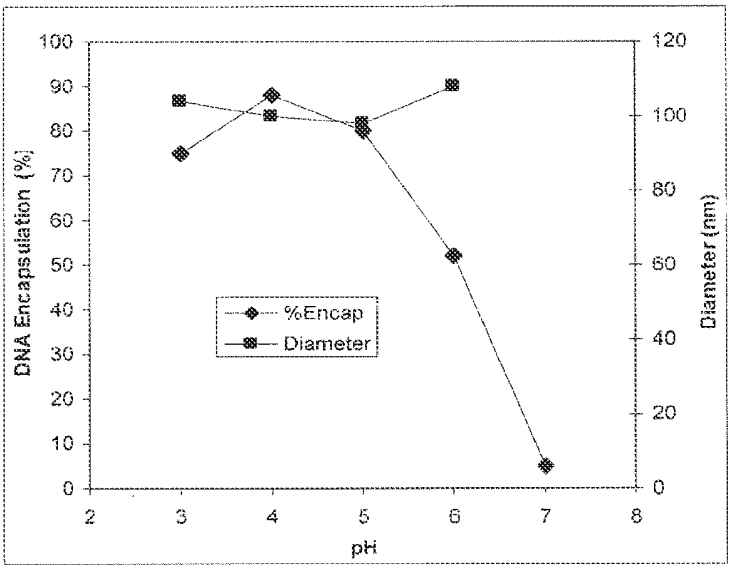

DNA Encapsulation (%)
Diameter (nm)
pH
%Encap
Diameter
100
90
80
70
60
50
40
30
20
10
0
120
100
80
60
40
20
0
2
3
4
5
6
7
8

FIG. 6

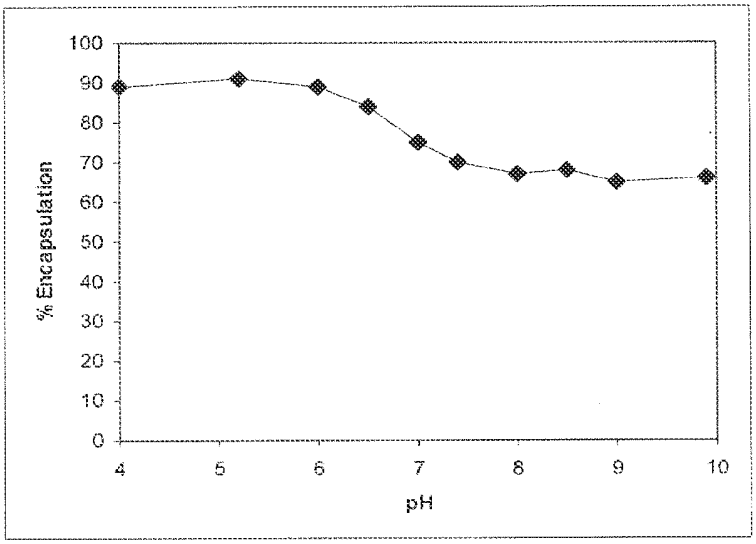


FIG. 7

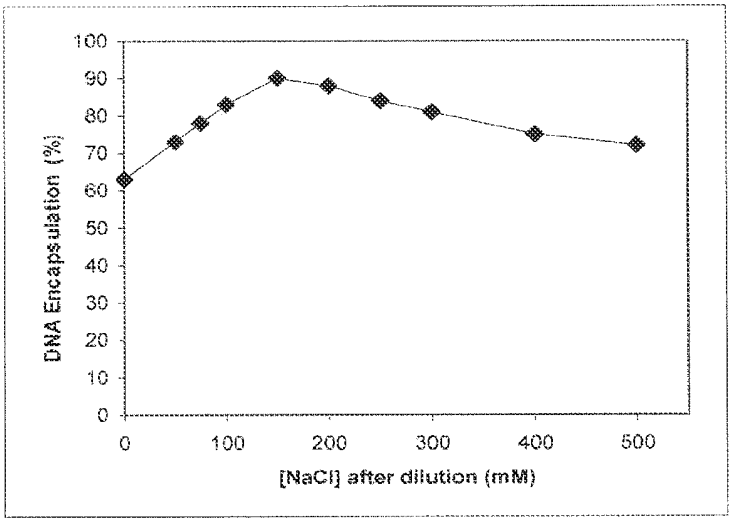

DNA Encapsulation (%)
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
[NaCl] after dilution (mM)

FIG. 8

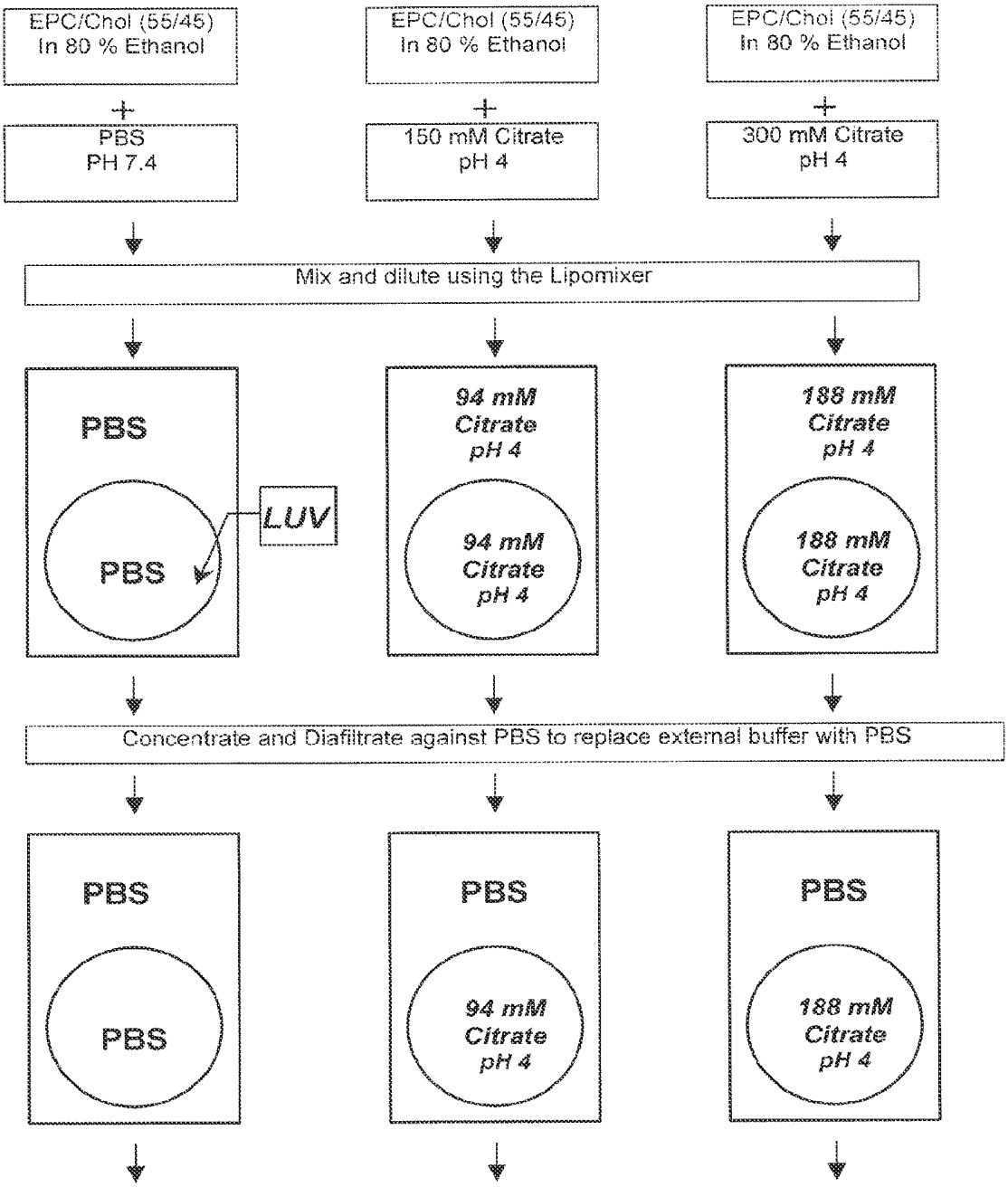

EPC/Chol (55/45) In 80 % Ethanol
EPC/Chol (55/45) In 80 % Ethanol
EPC/Chol (55/45) In 80 % Ethanol
PBS PH 7.4
150 mM Citrate pH 4
300 mM Citrate pH 4
Mix and dilute using the Lipomixer
PBS
PBS
LUV
94 mM Citrate pH 4
94 mM Citrate pH 4
188 mM Citrate pH 4
188 mM Citrate pH 4
Concentrate and Diafiltrate against PBS to replace external buffer with PBS
PBS
PBS
PBS
94 mM Citrate pH 4
PBS
188 mM Citrate pH 4

FIG. 9A

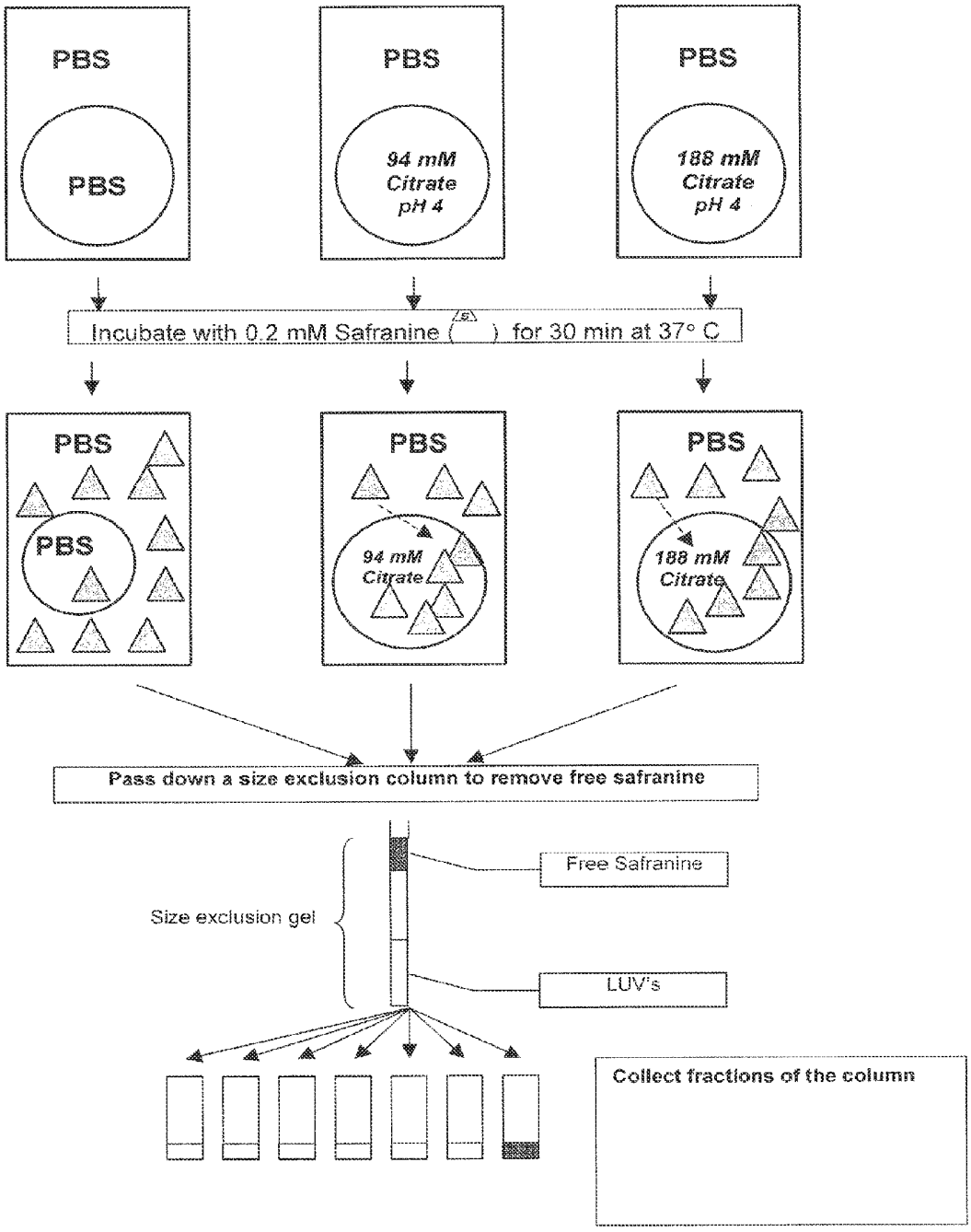

PBS
PBS
PBS
94 mM Citrate pH 4
PBS
188 mM Citrate pH 4
Incubate with 0.2 mM Safranine ( ) for 30 min at 37° C
PBS
PBS
PBS
94 mM Citrate
PBS
188 mM Citrate
Pass down a size exclusion column to remove free safranine
Size exclusion gel
Free Safranine
LUV's
Collect fractions of the column

FIG. 9B

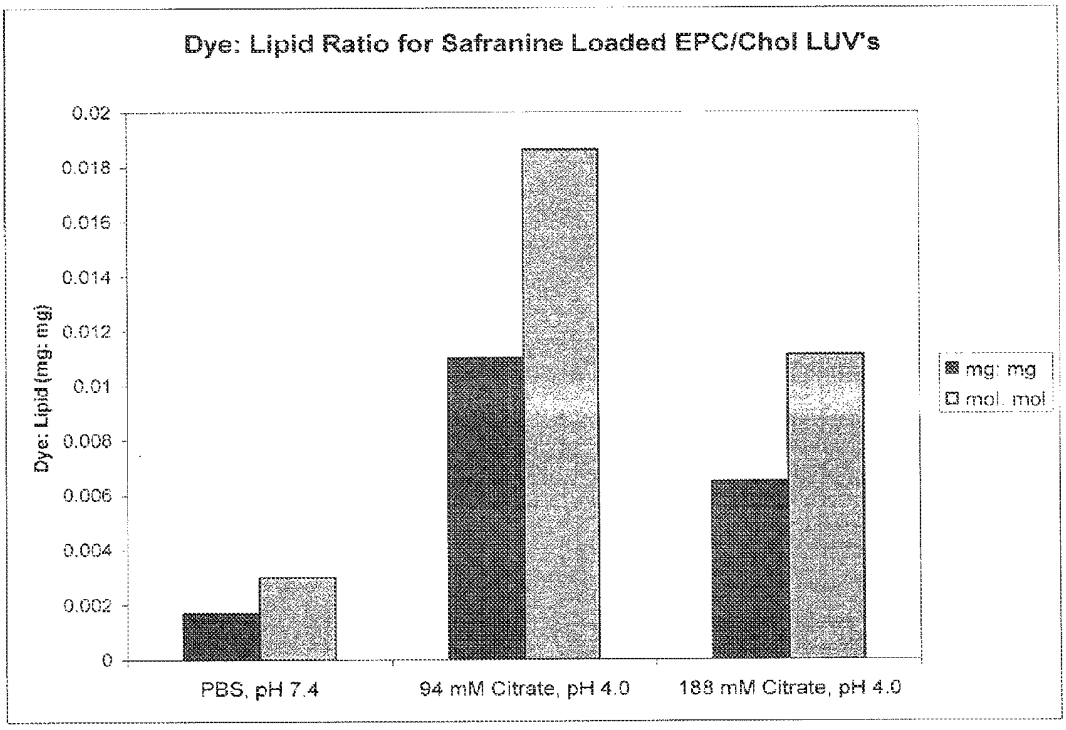
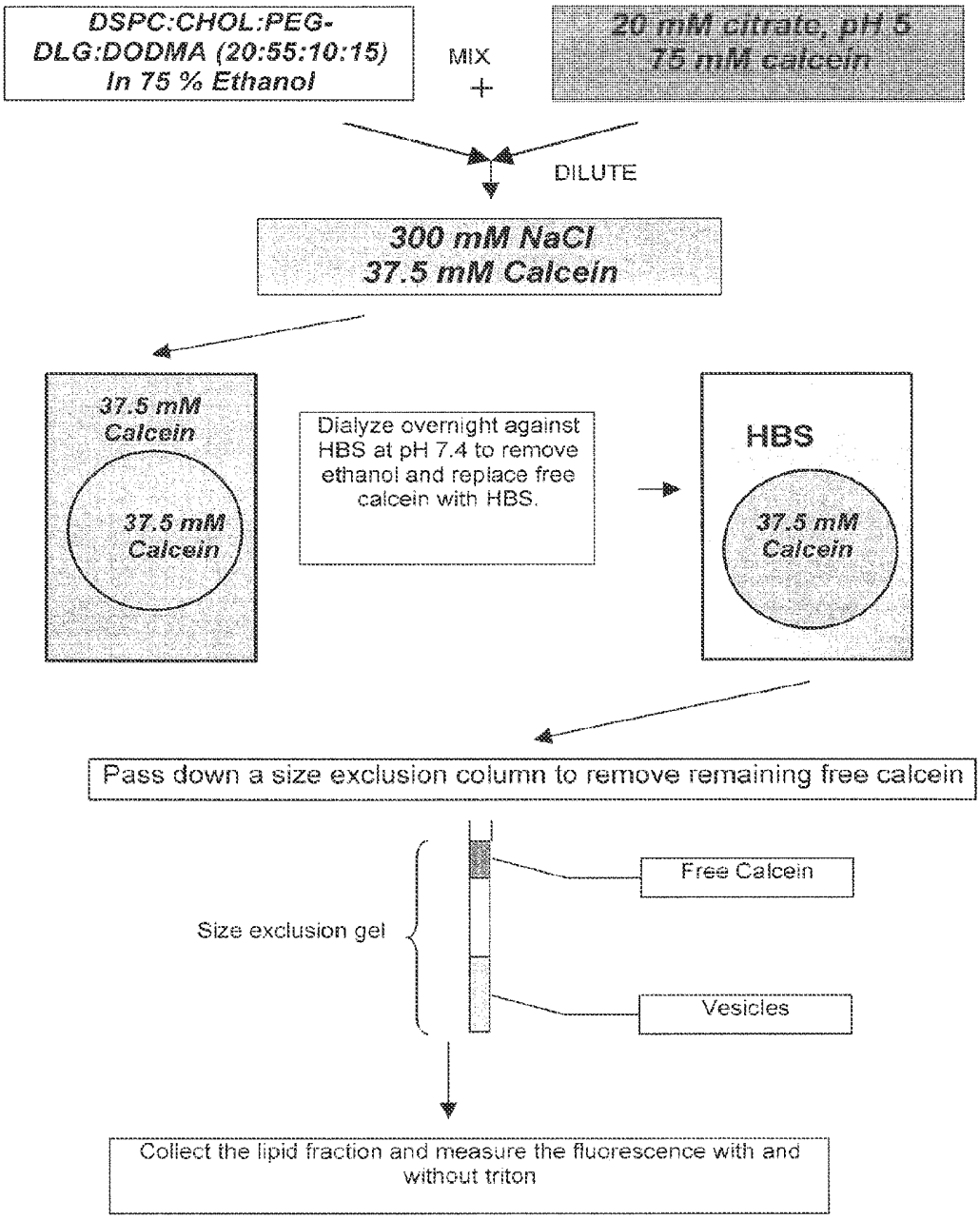

Dye: Lipid Ratio for Safranine Loaded EPC/Chol LUV's
Dye: Lipid (mg: mg)
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
PBS, pH 7.4
94 mM Citrate, pH 4.0
188 mM Citrate, pH 4.0
mg: mg
mol: mol

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

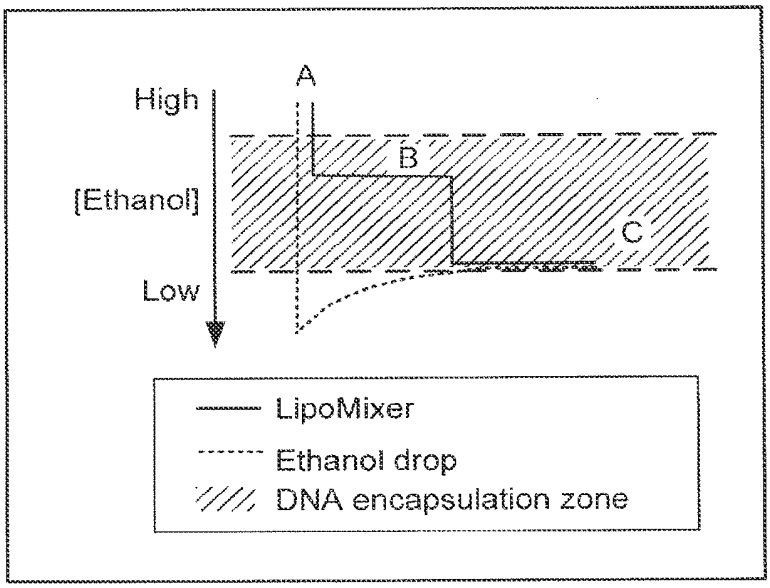

A
High
[Ethanol]
Low
B
C
LipoMixer
Ethanol drop
DNA encapsulation zone

FIG. 12

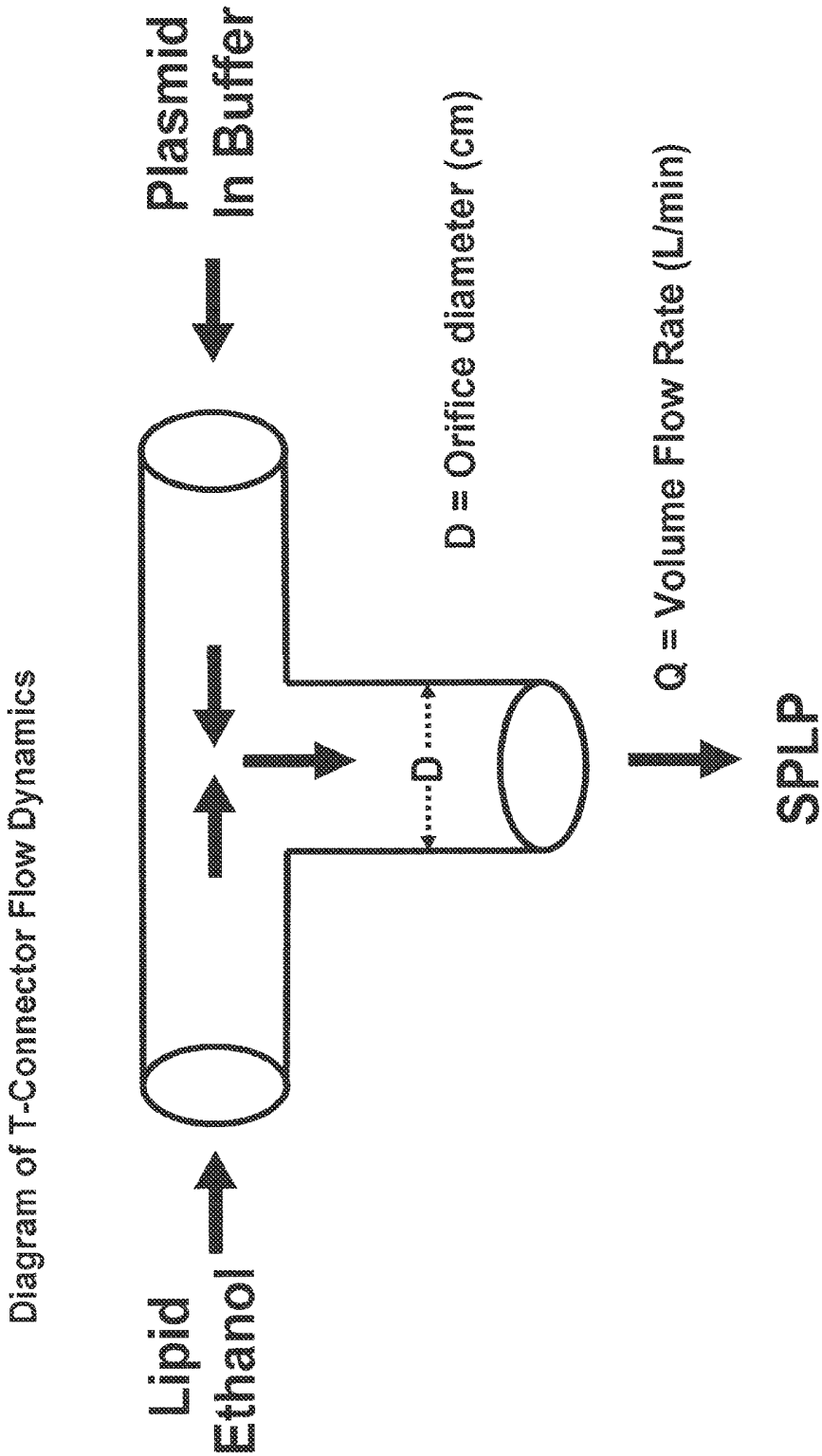


Diagram of T-Connector Flow Dynamics
Lipid
Ethanol
Plasmid
In Buffer
D = Orifice diameter (cm)
D
Q = Volume Flow Rate (L/min)
SPLP

FIG. 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)	χ2	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/d Not Determined. N/dN/d

FIG. 14

LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 13/684,066, filed Nov. 21, 2012, which is a continuation of U.S. application Ser. No. 12/965,555, filed Dec. 10, 2010, which is a divisional application of U.S. patent application Ser. No. 10/611,274, filed Jun. 30, 2003, which application claims priority to U.S. Provisional Application Ser. No. 60/392,887, filed Jun. 28, 2002, the disclosures of which are hereby incorporated by reference in their 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, phosphato, 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 phosphatidyletha-

nolamines, 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-dimethyl-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, methanol, 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-Nal]-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-guaninephosphoribosyl transferase, a p53, a purine nucleoside phosphorylase, a carboxylesterase,

a deoxycytidine kinase, a nitroreductase, a thymidine phosphorylase, or cytochrome P450 2B1.

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-Nal]-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; anti-depressants, e.g., imipramine, amitriptyline and doxepim; anti-conversants, e.g., phenytoin; antihistamines, e.g., diphenhydramine, chlorphenirimine 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 505 L 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	45	<0.1%	0.64	55	14.4	76
Removal**			(0.55)		(12.4)	
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)

TABLE III-continued

	Test	Specification
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	<20%
	Nicked	<2%
	Linear	
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 mM 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 lipid vesicle formulation comprising:

(a) a plurality of lipid vesicles, wherein each lipid vesicle comprises:

a cationic lipid;

an amphipathic lipid; and

a polyethyleneglycol (PEG)-lipid; and

(b) messenger RNA (mRNA), wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

2. The lipid vesicle formulation of claim **1**, wherein the amphipathic lipid is a phospholipid.

3. The lipid vesicle formulation of claim **2**, wherein the phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, di stearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine.

4. The lipid vesicle formulation of claim **1**, wherein each lipid vesicle further comprises a sterol.

5. The lipid vesicle formulation of claim **4**, wherein the sterol is cholesterol.

6. The lipid vesicle formulation of claim **4**, wherein the sterol is cholesterol and the amphipathic lipid is a phospholipid.

7. The lipid vesicle formulation of claim **6**, wherein the phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, di stearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine.

8. The lipid vesicle formulation of claim **1**, wherein each lipid vesicle is a liposome.

9. The lipid vesicle formulation of claim **1**, wherein each lipid vesicle is a lipid-nucleic acid particle.

10. The lipid vesicle formulation of claim **1**, wherein each lipid vesicle is about 150 nm or less in diameter.

11. The lipid vesicle formulation of claim **1**, wherein the cationic lipid only carries a positive charge at below physiological pH.

12. The lipid vesicle formulation of claim **1**, wherein each lipid vesicle is about 100 nm or less in diameter.

13. The lipid vesicle formulation of claim **1**, wherein at least 80% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

14. The lipid vesicle formulation of claim **1**, wherein about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

* * * * *

JOINT APPENDIX 06

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PATENT NUMBER: *11,141,378*
ISSUE DATE: *October 12, 2021*

By Authority of the
Under Secretary of Commerce for Intellectual Property
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W. Montgomery

Wanda Montgomery
Certifying Officer

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

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(54) **LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY**

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

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(63) Continuation of application No. 17/094,724, filed on Nov. 10, 2020, which is a continuation of application No. 16/422,441, filed on May 24, 2019, now abandoned, which is a continuation of application No. 15/840,933, filed on Dec. 13, 2017, now abandoned, which is a continuation of application No. 15/670,742, filed on Aug. 7, 2017, now abandoned, which is a continuation of application No. 15/164,803, filed on May 25, 2016, now abandoned, which is a continuation of application No. 14/462,441, filed on Aug. 18, 2014, now Pat. No. 9,364,435, which is a continuation of application No. 13/928,309, filed on Jun. 26, 2013, now Pat. No. 8,822,668, which is a continuation of application No. 13/253,917, filed on Oct. 5, 2011, now Pat. No. 8,492,359, which is a continuation of application No. 12/424,367, filed on Apr. 15, 2009, now Pat. No. 8,058,069.

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(51) **Int. Cl.**

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CPC C12N 15/113

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,394,448	A	7/1983	Szoka, Jr. et al.
4,438,052	A	3/1984	Weder et al.
4,515,736	A	5/1985	Deamer
4,598,051	A	7/1986	Papahadjopoulos et al.
4,897,355	A	1/1990	Eppstein et al.
5,013,556	A	5/1991	Woodle et al.
5,171,678	A	12/1992	Behr et al.
5,208,036	A	5/1993	Eppstein et al.
5,225,212	A	7/1993	Martin et al.
5,264,618	A	11/1993	Felgner et al.
5,279,833	A	1/1994	Rose
5,283,185	A	2/1994	Epand et al.
5,320,906	A	6/1994	Eley et al.
5,334,761	A	8/1994	Gebeyehu et al.
5,545,412	A	8/1996	Eppstein et al.
5,578,475	A	11/1996	Jessee
5,627,159	A	5/1997	Shih et al.
5,641,662	A	6/1997	Debs et al.

(Continued)

FOREIGN PATENT DOCUMENTS

CA	2309727	A1	4/1999
CA	2271582	A1	11/1999

(Continued)

OTHER PUBLICATIONS

Basarkar et al. (Int J Nanomedicine, 2007, 2(3), 353-360).*

(Continued)

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(57) **ABSTRACT**

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.

30 Claims, 24 Drawing Sheets

Specification includes a Sequence Listing.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,656,743	A	8/1997	Busch et al.
5,674,908	A	10/1997	Haces et al.
5,703,055	A	12/1997	Felgner et al.
5,705,385	A	1/1998	Bally et al.
5,736,392	A	4/1998	Hawley-Nelson et al.
5,753,613	A	5/1998	Ansell et al.
5,820,873	A	10/1998	Choi et al.
5,877,220	A	3/1999	Schwartz et al.
5,885,613	A	3/1999	Holland et al.
5,958,901	A	9/1999	Dwyer et al.
5,976,567	A	11/1999	Wheeler et al.
5,981,501	A	11/1999	Wheeler et al.
6,020,202	A	2/2000	Jessee
6,020,526	A	2/2000	Schwartz et al.
6,034,135	A	3/2000	Schwartz et al.
6,051,429	A	4/2000	Hawley-Nelson et al.
6,075,012	A	6/2000	Gebeyehu et al.
6,165,501	A	12/2000	Tirosh et al.
6,172,049	B1	1/2001	Dwyer et al.
6,251,939	B1	6/2001	Schwartz et al.
6,284,267	B1	9/2001	Aneja
6,287,591	B1	9/2001	Semple et al.
6,339,173	B1	1/2002	Schwartz et al.
6,376,248	B1	4/2002	Hawley-Nelson et al.
6,534,484	B1	3/2003	Wheeler et al.
6,586,410	B1	7/2003	Wheeler et al.
6,638,529	B2	10/2003	Schwartz et al.
6,649,780	B1	11/2003	Eibl et al.
6,671,393	B2	12/2003	Hays et al.
6,696,424	B1	2/2004	Wheeler et al.
6,815,432	B2	11/2004	Wheeler et al.
6,858,224	B2	2/2005	Wheeler et al.
7,166,745	B1	1/2007	Chu et al.
7,422,902	B1	9/2008	Wheeler et al.
7,479,573	B2	1/2009	Chu et al.
7,491,409	B1	2/2009	Meers et al.
7,601,872	B2	10/2009	Chu et al.
7,687,070	B2	3/2010	Gebeyehu et al.
7,745,651	B2	6/2010	Heyes et al.
7,799,565	B2	9/2010	MacLachlan et al.
7,803,397	B2	9/2010	Heyes et al.
7,807,815	B2	10/2010	MacLachlan et al.
7,838,658	B2	11/2010	MacLachlan et al.
7,901,708	B2	3/2011	MacLachlan et al.
7,915,450	B2	3/2011	Chu et al.
7,939,505	B2	5/2011	Quay et al.
7,982,027	B2	7/2011	MacLachlan et al.
8,058,068	B2	11/2011	Hawley-Nelson et al.
8,058,069	B2	11/2011	Yaworski et al.
8,101,741	B2	1/2012	MacLachlan et al.
8,158,601	B2	4/2012	Chen et al.
8,158,827	B2	4/2012	Chu et al.
8,188,263	B2	5/2012	MacLachlan et al.
8,227,443	B2	7/2012	MacLachlan et al.
8,236,943	B2	8/2012	Lee et al.
8,283,333	B2	10/2012	Yaworski et al.
8,455,455	B1	6/2013	Robbins et al.
8,466,122	B2	6/2013	Heyes et al.
8,492,359	B2	7/2013	Yaworski et al.
8,513,403	B2	8/2013	MacLachlan et al.
8,569,256	B2	10/2013	Heyes et al.
8,598,333	B2	12/2013	MacLachlan et al.
8,716,464	B2	5/2014	Geisbert et al.
8,822,668	B2	9/2014	Yaworski et al.
8,865,675	B2	10/2014	Heyes et al.
8,999,950	B2	4/2015	MacLachlan et al.
9,006,191	B2	4/2015	MacLachlan et al.
9,006,417	B2	4/2015	Yaworski et al.
9,018,187	B2	4/2015	Heyes et al.
9,035,039	B2	5/2015	Dhillon et al.
9,126,966	B2	9/2015	Martin et al.
9,139,554	B2	9/2015	Hope et al.
9,187,748	B2	11/2015	Geisbert et al.
9,364,435	B2	6/2016	Yaworski et al.
9,404,127	B2	8/2016	Yaworski et al.
9,504,651	B2	11/2016	MacLachlan et al.
9,518,272	B2	12/2016	Yaworski et al.
9,878,042	B2	1/2018	Yaworski et al.
10,053,689	B2	8/2018	Fitzgerald et al.
10,077,232	B2	9/2018	Heyes et al.
10,415,037	B2	9/2019	Cross et al.
2001/0048940	A1	12/2001	Tousignant et al.
2003/0069173	A1	4/2003	Hawley-Nelson et al.
2003/0072794	A1	4/2003	Boulikas
2003/0077829	A1	4/2003	MacLachlan
2003/0143732	A1	7/2003	Fosnaugh et al.
2004/0063654	A1	4/2004	Davis et al.
2004/0142892	A1	7/2004	Finn et al.
2004/0253723	A1	12/2004	Tachas et al.
2004/0259247	A1	12/2004	Tuschi et al.
2005/0064595	A1	3/2005	MacLachlan et al.
2005/0118253	A1	6/2005	MacLachlan et al.
2005/0260757	A1	11/2005	Gebeyehu et al.
2006/0008910	A1	1/2006	MacLachlan et al.
2006/0134189	A1	6/2006	MacLachlan et al.
2006/0147514	A1	7/2006	Gebeyehu et al.
2006/0228406	A1	10/2006	Chiou et al.
2006/0240093	A1	10/2006	MacLachlan et al.
2006/0240554	A1	10/2006	Chen et al.
2007/0042031	A1	2/2007	MacLachlan et al.
2007/0135372	A1	6/2007	MacLachlan et al.
2007/0202598	A1	8/2007	Chu et al.
2007/0202600	A1	8/2007	Chu et al.
2008/0020058	A1	1/2008	Chen et al.
2008/0317839	A1	12/2008	Quay et al.
2009/0143583	A1	6/2009	Chu et al.
2009/0291131	A1	11/2009	MacLachlan et al.
2010/0130588	A1	5/2010	Yaworski et al.
2010/0159593	A1	6/2010	Chu et al.
2011/0076335	A1	3/2011	Yaworski et al.
2012/0058188	A1	3/2012	MacLachlan et al.
2012/0136073	A1	5/2012	Zhiwei et al.
2012/0183581	A1	7/2012	Yaworski et al.
2012/0238747	A1	9/2012	Chu et al.
2013/0116307	A1	5/2013	Heyes et al.
2013/0122104	A1	5/2013	Yaworski et al.
2013/0303587	A1	11/2013	Yaworski et al.
2014/0065228	A1	3/2014	Yaworski et al.
2014/0294937	A1	10/2014	MacLachlan et al.
2015/0164799	A1	6/2015	Yaworski et al.
2015/0374834	A1	12/2015	Yaworski et al.
2016/0032320	A1	2/2016	Yaworski et al.
2016/0251681	A1	9/2016	Yaworski et al.
2017/0042814	A1	2/2017	Yaworski et al.
2017/0260523	A1	9/2017	Yaworski et al.
2017/0307608	A1	10/2017	Bettencourt
2018/0071397	A1	3/2018	Yaworski et al.
2018/0085312	A1	3/2018	Yaworski et al.
2018/0092848	A1	4/2018	Yaworski et al.
2019/0032051	A1	1/2019	Yaworski et al.
2019/0240339	A1	8/2019	Yaworski et al.
2020/0113832	A1	4/2020	Yaworski et al.
2021/0207140	A1	7/2021	Yaworski et al.
2021/0220274	A1	7/2021	Yaworski et al.

FOREIGN PATENT DOCUMENTS

CA	2330741	A1	11/1999	
CA	2397016	A1	7/2001	
CA	2513623		8/2004	
CA	2637931	*	7/2007	..... C12N 15/113
EP	1 648 519	B1	10/2014	
EP	2 567 693	B1	10/2015	
JP	03-126211		5/1991	
JP	05-202085		8/1993	
JP	06-080560		3/1994	
JP	2002-525063		8/2002	
JP	2003-505401		2/2003	
JP	2007-524349		8/2007	
WO	91/16024	A1	10/1991	
WO	93/05162	A1	3/1993	
WO	93/12240	A1	6/1993	
WO	93/12756	A2	7/1993	
WO	93/24640	A2	12/1993	

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

WO	93/25673	A1	12/1993
WO	95/02698	A1	1/1995
WO	95/18863	A1	7/1995
WO	95/35301	A1	12/1995
WO	96/02655	A1	2/1996
WO	96/10390	A1	4/1996
WO	96/40964	A2	12/1996
WO	96/41873	A1	12/1996
WO	98/51285	A2	11/1998
WO	00/03683	A2	1/2000
WO	00/15820	A1	3/2000
WO	00/62813	A2	10/2000
WO	01/05374	A1	1/2001
WO	01/05873	A1	1/2001
WO	01/75164	A2	10/2001
WO	01/93836		12/2001
WO	02/034236	A2	5/2002
WO	02/087541	A1	11/2002
WO	03/097805	A2	11/2003
WO	2004/065546	A2	8/2004
WO	2004/110499	A1	12/2004
WO	2005/007196	A2	1/2005
WO	2005/026372	A1	3/2005
WO	2005/035764	A1	4/2005
WO	2005/120152	A2	12/2005
WO	2006/002538	A1	1/2006
WO	2006/053430	A1	5/2006
WO	2007/056861	A1	5/2007
WO	2009/086558	A1	7/2009
WO	2009/111658	A2	9/2009
WO	2010/042877	A1	4/2010
WO	2010/048228	A2	4/2010
WO	2010/088537	A2	8/2010
WO	2010/105209	A1	9/2010
WO	2013/090648	A1	6/2013
WO	2017/223135	A1	12/2017
WO	2018/232357	A1	12/2018

OTHER PUBLICATIONS

Shimada et al. (International Journal of Pharmaceutics, 203, 2000, 255-263).*

Gallie (Plant Cell Reports, 1993, 13, 119-122).*

Semple et al. (Biochemistry, 1996, 35, 2521-2525).*

Li et al. (Trends in Pharmacological Sciences, 23, 5, 2002, 206-209).*

Arima et al. (Biomaterials, 29, 2008, 551-560).*

Ahmad, A. et al., "New multivalent cationic lipids reveal bell curve for transfection efficiency versus membrane charge density: lipid-DNA complexes for gene delivery," J. Gene Med., 7(6):739-48, 2005.

Akinc, A. et al., "The Onpattro story and the clinical translation of nanomedicines containing nucleic acid-based drugs," Nature Nanotechnology, 14:1084-1087, 2019.

Alnylam Pharmaceuticals, Inc., ONPATTRO Label, Revised Aug. 2018.

Ambegia, E. et al., "Stabilized plasmid-lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression," Biochimica et Biophysica Acta, 1669:155-163, 2005.

Arbutus Biopharma Corporation, "Arbutus' LNP Licensee Alnylam Announces FDA Approval of ONPATTRO™ (patisiran), for the Treatment of ATTR Amyloidosis," Press Release, https://investor.arbutusbio.com/news-releases/news-release-details/arbutus-lnp-licensee-alnylam-announces-fda-approval-onpattrotm, Aug. 13, 2018, 2 pages.

Arpicco, S. et al., "Preparation and characterization of novel cationic lipids developed for gene transfection," Proceed. Int'l Symp. Control. Rel. Bioact. Mater. (Controlled Release Society, Inc.), 26:759-760, 1999.

Arpicco, S. et al., "Synthesis, characterization and transfection activity of new saturated and unsaturated cationic lipids," IL Farmaco, 59:869-878, 2004.

Bahl, K. et al., "Preclinical and Clinical Demonstration of Immunogenicity by mRNA Vaccines against H10N8 and H7N9 Influenza Viruses," Molecular Therapy, 25(6):1316-1327, 2017.

Ballas, N. et al., "Liposomes bearing a quaternary ammonium detergent as an efficient vehicle for functional transfer of TMV-RNA into plant protoplasts," Biochimica et Biophysica Acta, 939:8-18, 1988.

Barinaga, M., "Step taken toward improved vectors for gene transfer," Science, 266:1326, 1994.

Bass, B. "The short answer," Nature, 411:428-429, 2001.

Beale, G. et al., "Gene silencing nucleic acids designed by scanning arrays: Anti-EGFR activity of siRNA, ribozyme and DNA enzymes targeting a single hybridization-accessible region using the same delivery system," Journal of Drug Targeting, 11(7):449-456, 2003.

Behr, J., "Synthetic gene-transfer vectors," Acc. Chem. Res., 26:274-278, 1993.

Bennett et al., "Cholesterol enhances cationic liposome-mediated DNA transfection of human respiratory epithelial cells," Bioscience Reports, 15:47-53, 1995.

Brigham, K. et al., "Rapid communication: In vivo transfection of murine lungs with a functioning prokaryotic gene using a liposome vehicle," The American Journal of the Medical Sciences, 298(4):278-281, 1989.

Brummelkamp, T. et al., "A system for stable expression of short interfering RNAs in mammalian cells," Science, 296:550-553, 2002.

Cevc, G., "How membrane chain-melting phase-transition temperature is affected by the lipid chain asymmetry and degree of unsaturation: an effective chain-length model," Biochemistry, 30:7186-7193, 1991.

Check, E., "RNA to the Rescue? Disease therapies based on a technique for gene silencing called RNA interference are racing towards the clinic. Erika Check investigates molecular medicine's next big thing," Nature, 425:10-12, 2003.

Chonn, A. et al., "Recent advances in liposomal drug-delivery systems," Current Opinion in Biotechnology, 6:698-708, 1995.

Cortesi, R. et al., "Effect of cationic liposome composition on in vitro cytotoxicity and protective effect on carried DNA," International Journal of Pharmaceutics, 139:69-78, 1996.

Crystal, R., "Transfer of genes to humans: early lessons and obstacles to success," Science, 270:404-410, 1995.

Culver, K., "The first human gene therapy experiment," Gene Therapy: A Handbook for Physicians, 1994, pp. 33-40.

Duzgunes, N., "Membrane fusion," Subcellular Biochemistry, 11:195-286, 1985.

Dwarki, V. et al., "Cationic liposome-mediated RNA transfection," Methods in Enzymology, 217:644-654, 1993.

Elbashir, S. et al., "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells," Nature, 411:494-498, 2001.

Enoch, H. et al., "Formation and properties of 1000-Å-diameter, single-bilayer phospholipid vesicles," Proc. Natl. Acad. Sci. USA, 76(1):145-149, 1979.

European Medicines Agency, "New Medicine for Hereditary Rare Disease," Press Release, https://www.ema.europa.eu/en/news/new-medicine-hereditary-rare-disease, Jul. 27, 2018, 2 pages.

Felgner, J. et al., "Cationic lipid-mediated transfection in mammalian cells: 'Lipofection,'" J. Tiss. Cult. Meth., 15:63-68, 1993.

Felgner, J. et al., "Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations," The Journal of Biological Chemistry, 269(4):2550-2561, 1994.

Felgner, P. et al., "Cationic liposome mediated transfection," Proc. West. Pharmacol. Soc., 32:115-121, 1989.

Felgner, P. et al., "Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure," Proc. Natl. Acad. Sci. USA, 84:7413-7417, 1987.

Filion, M.C. and Phillips, N.C., "Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids towards immune effector cells," Biochim. Biophys. Acta., 1329(2):345-356, 1997.

(56) **References Cited**

OTHER PUBLICATIONS

Gao, X. et al., "A novel cationic liposome reagent for efficient transfection of mammalian cells," Biochem. Biophys. Res. Comm., 179(1):280-285, 1991.
Gao, X. et al., "Nonviral gene Delivery: What we know and what is next," AAPS Journal, 9(1):E92-104, 2007.
Gershon, H. et al., "Mode of formation and structural feature of DNA-cationic liposome complexes used for transfection," Biochemistry, 32:7143-7151, 1993.
Global Newswire, retrieved from http://globalnewswire.com on Feb. 27, 2013, Tekmira sues Alnylam Pharmaceuticals for repeated misuse of trade secrets and confidential information, Mar. 16, 2011, pp. 1-3.
Guy-Caffey, J. et al., "Novel polyaminolipids enhance the cellular uptake of oligonucleotides," The Journal of Biological Chemistry, 270(52):31391-31396, 1995.
Hausseker, D., "The Business of RNAi Therapeutics in 2012,"Molecular Therapy-Nucleic Acids, 2:e8, 2012, 12 pages.
Hawley-Nelson, P. et al., "LipofectAmine™ reagent: a new, higher efficiency polycationic liposome transfection reagent," Focus, 15(3):73-80, 1993.
Heyes, J. et al., "Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids," Journal of Controlled Release, 107:276-287, 2005.
Heyes, J. et al., "Synthesis of novel cationic lipids: effect of structural modification on the efficiency of gene transfer," J. Med. Chem., 45:99-114, 2002.
Huang, L. et al., "Liposomal Gene Delivery: A Complex Package," Nature Biotechnology, 15:620-621, 1997.
Hyde, S. et al., "Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy," Nature, 362:250-255, 1993.
Janoff, A., "Declaration of Andrew S. Janoff, Ph.D. in support of Moderna Therapeutics, Inc.'s Petition for Inter Partes Review of U.S. Pat. No. 9,364,435," Inter Partes Review of U.S. Pat. No. 9,364,435 B2, Case No. IPR2018-00739, filed Mar. 5, 2018, 69 pages.
Jiang, L. et al., "Comparison of protein precipitation methods for sample preparation prior to proteomic analysis," Journal of Chromatography A, 1023:317-320, 2004.
Juliano, R. et al., "The effect of particle size and charge on the clearance rates of liposomes and liposome encapsulated drugs," Biochem. Biophys. Res. Commun., 63(3):651-658, 1975.
Kauffman, K. et al., "Optimization of Lipid Nanoparticle Formulations for mRNA Delivery in Vivo with Fractional Factorial and Definitive Screening Designs," Nano Letters, 15(11):7300-7306, 2015.
Keough, K., "Influence of chain unsaturation and chain position on thermotropism and intermolecular interactions in membranes," Biochem. Soc. Transactions, 18(5):835-837, 1990.
Krichevsky, A. et al., "RNAi functions in cultured mammalian neurons," PNAS, 99(18):11926-29, 2002.
Lawrence, M. et al. "The formation, characterization and stability of non-ionic surfactant vesicles," S.T.P. Pharma Sciences, 6(1):49-60, 1996.
Lawrence, M. et al., "Synthesis and aggregation properties of dialkyl polyoxyethylene glycerol ethers," Chemistry and Physics of Lipids, 82(2):89-100, 1996.
Ledford, H., "Gene-Silencing Drug Approved: US Government Okays First RNA-Interference Drug—After a 20-Year Wait," Nature, 560:291-292, 2018.
Legendre, J.-Y. et al., "Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes," Pharm. Res., 9(10):1235-42, 1992.
Leventis, R. et al., "Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles," Biochem. Biophys. Acta, 1023:124-132, 1990.
Li and Szoka, "Lipid-based nanoparticles for nucleic acid delivery," Pharmaceutical Research, 2483:438-449, 2007.
Lin, A.J. et al., "Three-dimensional imaging of lipid gene-carriers: Membrane charge density controls universal transfection behavior in lamellar cationic liposome-DNA complexes," Biophys J., 84(5):3307-16, 2003.
Liu, Y. et al., "Cationic liposome-mediated intravenous gene delivery", J. Biol. Chem., 270:24864-70, 1995.
MacLachlan, "Liposomal Formulations for Nucleic Acid Delivery," Antisense Drug Technologies, Second Edition; 2007; pp. 237-270.
MacLachlan, I. and Cullis, P., "Diffusible-PEG-Lipid Stabilized Plasmid Lipid Particles," Advances in Genetics, 53:157-188, 2005.
Marshall, E., "Gene therapy's growing pains," Science, 269:1050-1055, 1995.
Middaugh, C. and Ramsey, C., "Analysis of Cationic-Lipid-Plasmid-DNA Complexes," Analytical Chemistry, American Chemical Society, 2007, pp. 7240-7248.
Murahashi, N. et al., "Synthesis and application of neoglycolipid for liposome modification," Biol. Pharm. Bull., 20(6):704-707, 1997.
Orkin, S. et al., NIH Report, Report and Recommendations of the Panel to Assess the NIH Investment in Research on Gene Therapy, 1995, 41 pages.
Ortho Biotech Products, LP, "Doxil® Label—Product Insert, Revised May 2007," https://www.accessdata.fda.gov/drugsatfda_docs/label/2007/ 050718s029lbl.pdf, 33 pages.
Pak, C. et al., "Elastase-activated liposomal delivery to nucleated cells," Biochimica et Biophysica Acta, 1419:111-126, 1999.
Parr, M. et al., "Factors influencing the retention and chemical stability of polly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles," Biochimica et Biophysica Acta, 1195:21-30, 1994.
Paul, C. et al., "Effective expression of small interfering RNA in human cells," Nature Biotech., 20:505-508, 2002.
Puyal, C. et al., "A new cationic liposome encapsulating genetic material: A potential delivery system for polynucleotides," Eur. J. Biochem., 228:697-703, 1995.
Sawada, K. et al., "Microemulsions in supercritical CO2 utilizing the polyethyleneglycol dialkylglycerol and their use for the solubilization of hydrophiles," Dyes and Pigments, 2005, 65:64-74.
Schmidt, C., "Therapeutic Interference: Small RNA Molecules Act as Blockers of Disease Metabolism," Modern Drug Discovery, American Chemical Society, 2003, pp. 37-42.
Sedic. M. et al., "Safety Evaluation of Lipid Nanoparticle-Formulated Modified mRNA in the Sprague-Dawley Rat and Cynomolgus Monkey," Veterinary Pathology, 2017:1-14, 2017.
Semple, S. et al., "Immunogenicity and Rapid Blood Clearance of Liposomes Containing Polyethylene Glycol-Lipid Conjugates and Nucleic Acid," The Journal Of Pharmacology and Experimental Therapeutics, 312(3):1020-1026, 2006.
Semple, S. et al., "Rational Design of Cationic Lipids for siRNA Delivery," Nature Biotechnology, 28(2):172-176, 2010.
Semple, S. et al., "Supplementary Text and Figures, Supplementary Fig. 1, Supplementary Tables 1-4 and Supplementary Syntheses 1 and 2 to Rational Design of Cationic Lipids for siRNA Delivery," Nature Biotechnology, 28(2):172-176, 2010, published online at https://www.nature.com/articles/nbt.1602?draft=collection#supplementary-information, last visited on Nov. 14, 2019, 19 pages.
Shin, J. et al. "Acid-triggered release via dePEGylation of DOPE liposomes containing acid-labile vinyl ether PEG-lipids," Journal of Controlled Release, 91:187-200, 2003.
Song, L. et al., "Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes," Biochimica et Biophysica Acta, 1558:1-13, 2002.
Sorensen, D. et al., "Gene silencing by systemic delivery of synthetic siRNAs in adult mice", J. Biol. Chem., 327:761-766, 2003.
Spagnou, S. et al., "Lipidic carriers of siRNA: differences in the formulation, cellular uptake, and delivery with plasmid DNA," Biochemistry, 43:13348-56, 2004.
Stamatatos, L. et al., "Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes," Biochemistry, 27:3917-3925, 1988.

(56) **References Cited**

OTHER PUBLICATIONS

Szoka, F. et al., "Comparative properties and methods of preparation of lipid vesicles (liposomes)," Ann. Rev. Biophys. Bioeng., 9:467-508, 1980.
Szoka, F. et al., "Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation," Proc. Natl. Acad. Sci. USA, 75(9):4194-98, 1978.
Tam P. et al., "Stabilized plasmid-lipid particles for systemic gene therapy," Gene Therapy, 7:1867-74, 2000.
Templeton, N., "Cationic liposome-mediated gene delivery in vivo", Bioscience Reports, 22(2):283-295, 2002.
Timmerman, L. "Merck's Alan Sachs, on RNAi's Big Challenge: Delivery, Delivery, Delivery," Xconomy, Inc., https://xconomy.com/national/2010/01/21/mercks-alan-sachs-on-rnais-big-challenge-delivery-delivery-delivery/ -2016-, last visited on Jan. 21, 2010, 9 pages.
U.S. Department of Health and Human Services, "Liposome Drug Products: Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation, Guidance for Industry," Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Apr. 2018, 18 pages.
U.S. Food and Drug Administration, "FDA Approves First-of-its Kind Targeted RNA-based Therapy to Treat a Rare Disease," Press Announcement, https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm616518.htm, Aug. 10, 2018, 3 pages.
U.S. Food and Drug Administration, "Orange Book: Approved Drug Products with Therapeutic Equivalence Evaluations, Patent and Exclusivity for: N210922," 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, 3 pages.
U.S. Patent and Trademark Office, Patent Trial and Appeal Board, Inter Partes Review of U.S. Pat. No. 9,364,435 B2, Case No. IPR2018-00739, Final Written Decision, Sep. 11, 2019, pp. 1-52.
Uyechi-O'Brien, L. and Szoka, F., "Mechanisms for Cationic Lipids in Gene Transfer," Pharmaceutical Gene Delivery Systems, Marcel Dekker, Inc., vol. 131, pp. 79-108, 2003.
Van Der Woude, I. et al., "Parameters influencing the introduction of plasmid DNA into cells by the use of synthetic amphiphiles as a carrier system," Biochimica et Biophysica Acta, 1240:34-40, 1995.
Wheeler, J. et al., "Stabilized plasmid-lipid particles: constructions and characterization," Gene Therapy, 6:271-281, 1999.
Wilson, R. et al., "Counterion-induced condensation of deoxyribonucleic acid," A Light-Scattering Study, Biochemistry, 18(11):2192-96, 1979.
Woodle, M. et al., "Versatility in lipid compositions showing prolonged circulation with sterically stabilized liposomes," Biochimica et Biophysica Acta, 1105:193-200, 1992.
Yew, N.S. and Scheule, R.K., "Toxicity of cationic lipid-DNA complexes," Advances in Genetics, 53:189-214, 2005.
Zhang, P. et al., "Use of small RNA as antiaging cosmeceuticals," J. Cosmetic Sci., 64(6):455-68, 2013 (Abstract only).
Zhu, N. et al., "Systemic gene expression after intravenous DNA delivery into adult mice," Science, 261:209-211, 1993.
Zimmermann, T. et al, "RNAi-mediated gene silencing in non-human primates," Nature, Nature Publishing Group, London, GB, 441 (7089):111-114, 2006.

* cited by examiner

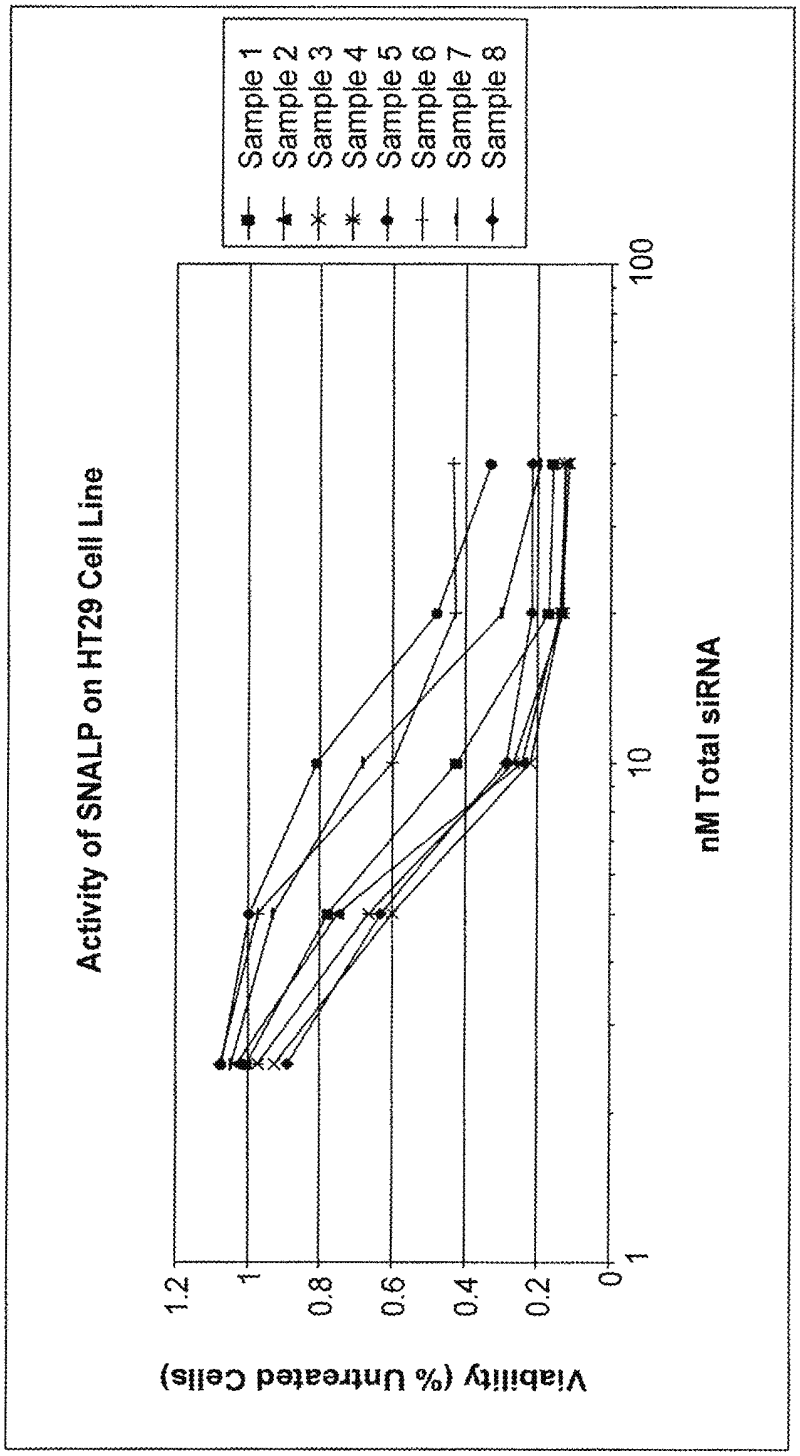

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8

FIG. 1A

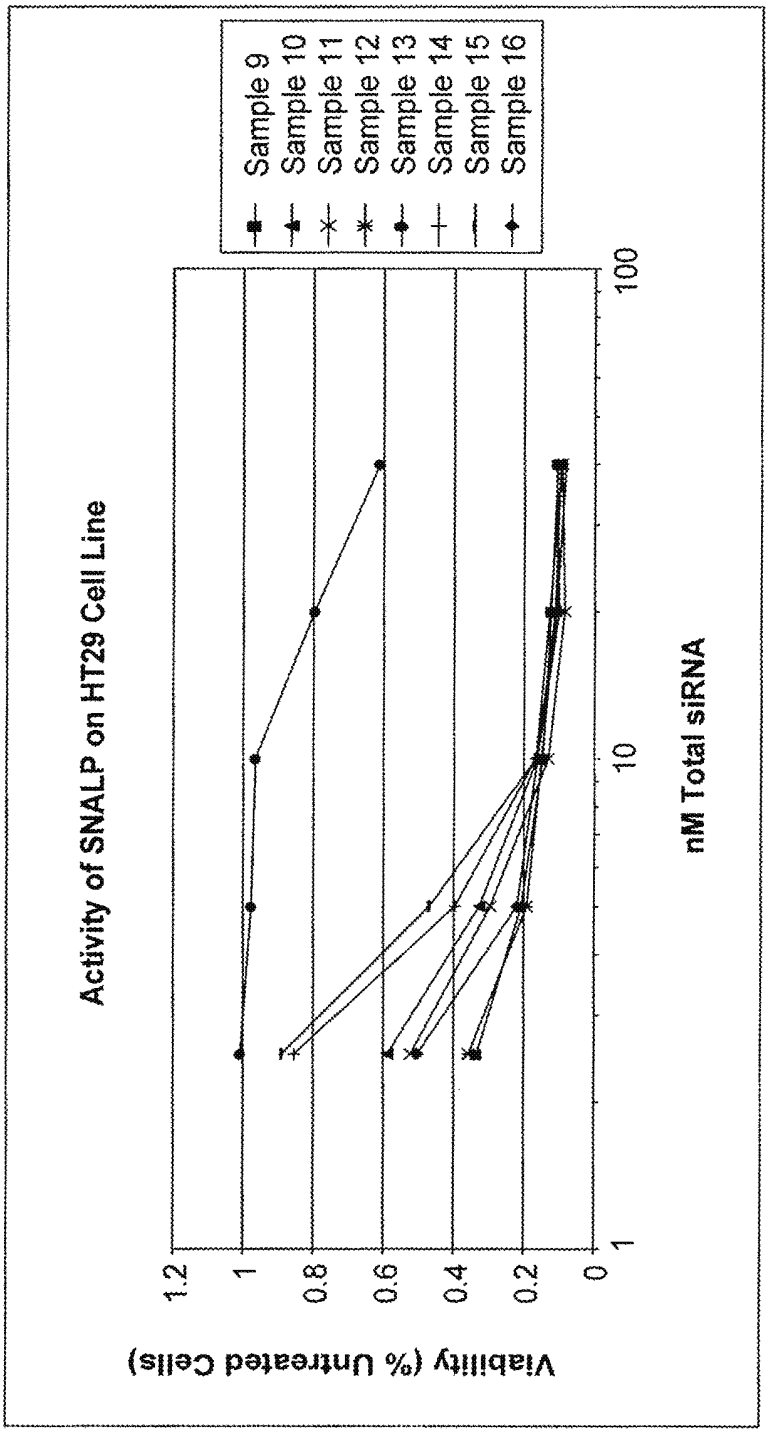

Activity of SNALP on HT29 Cell Line
Viability (% Untreated Cells)
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
nM Total siRNA
Sample 9
Sample 10
Sample 11
Sample 12
Sample 13
Sample 14
Sample 15
Sample 16

FIG. 1B

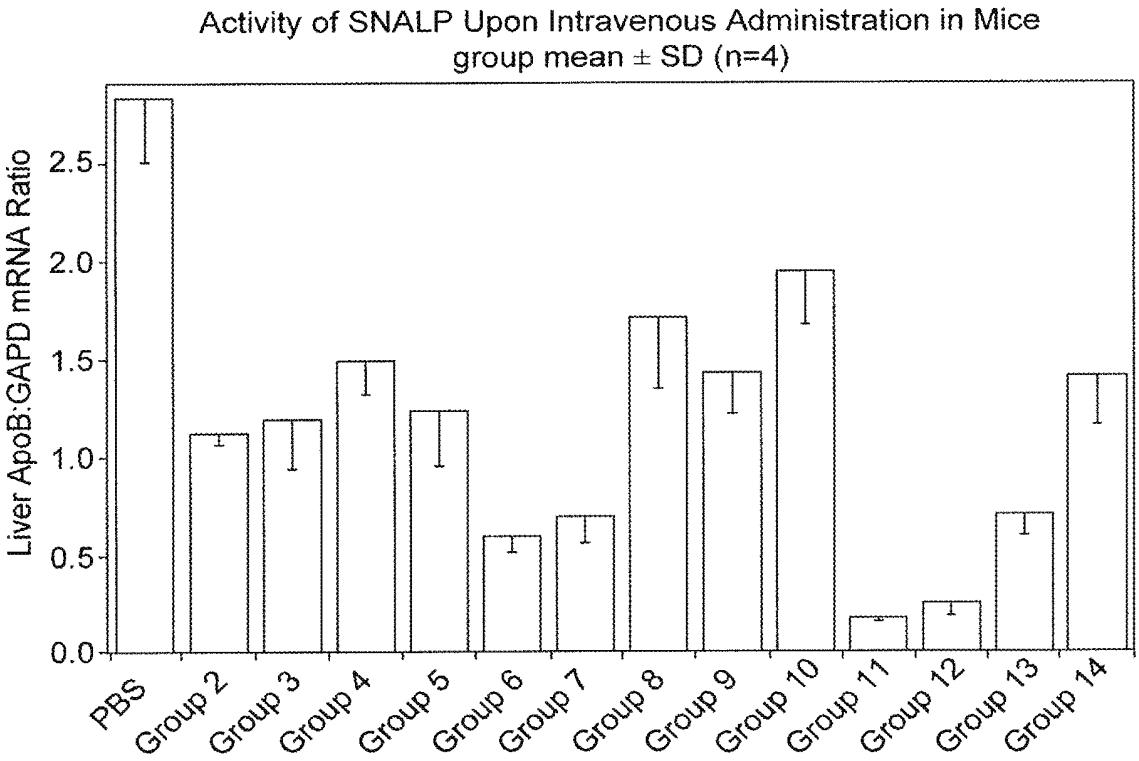

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
0.0
0.5
1.0
1.5
2.0
2.5
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14

FIG. 2

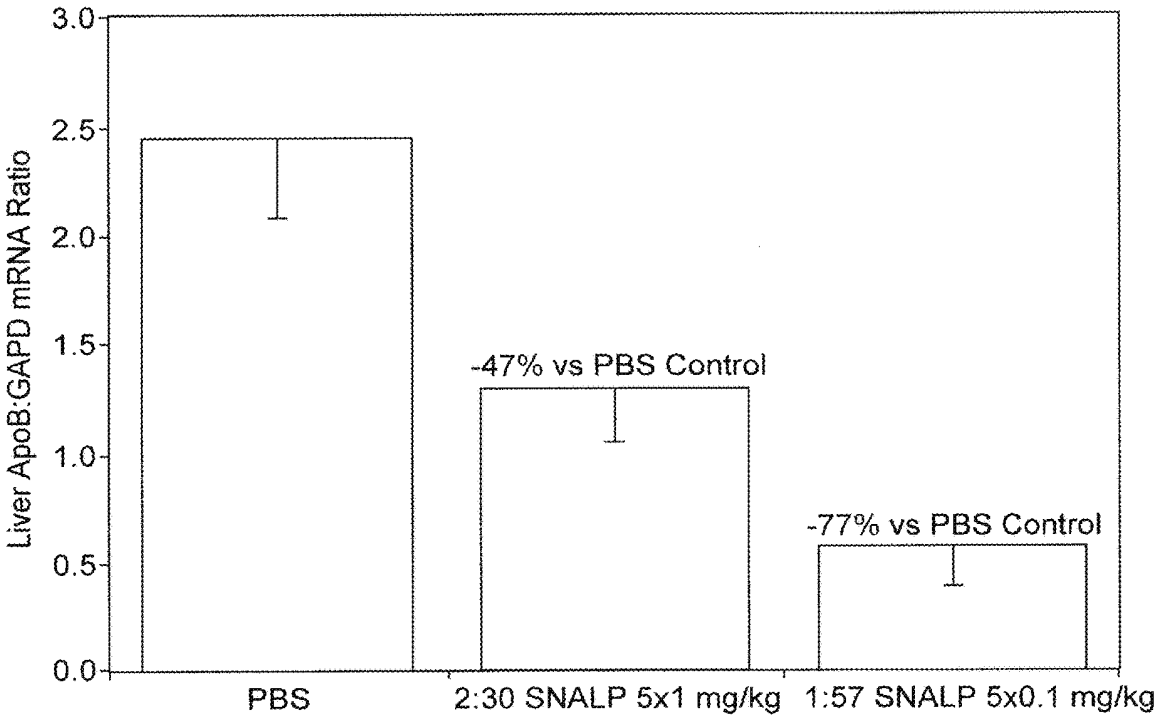

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Liver ApoB:GAPD mRNA Ratio
-47% vs PBS Control
-77% vs PBS Control
PBS
2:30 SNALP 5x1 mg/kg
1:57 SNALP 5x0.1 mg/kg

FIG. 3

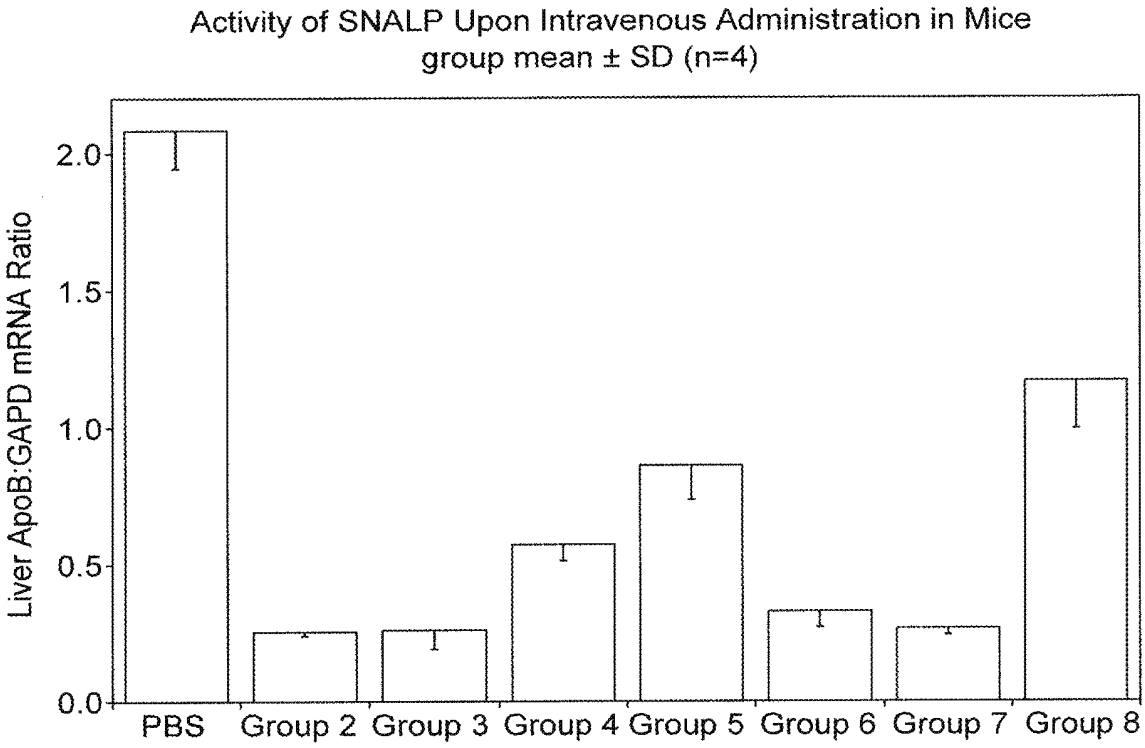

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8

FIG. 4

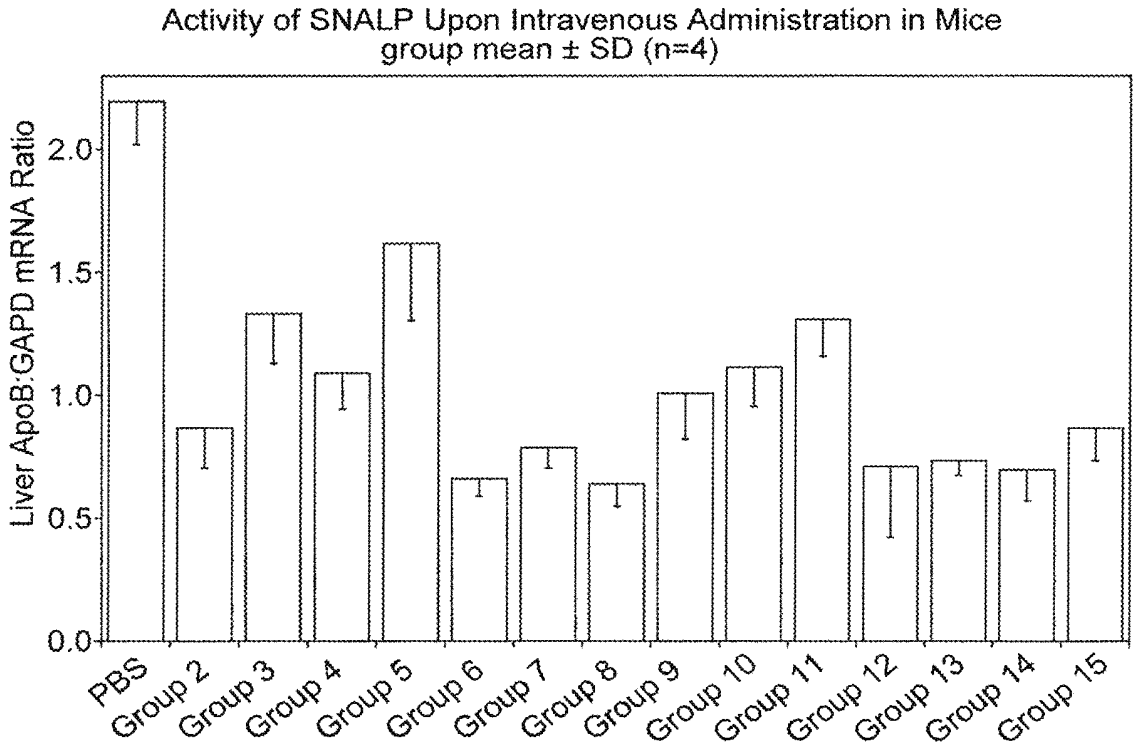

Activity of SNALP Upon Intravenous Administration in Mice
group mean ± SD (n=4)
Liver ApoB:GAPD mRNA Ratio
2.0
1.5
1.0
0.5
0.0
PBS
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
Group 13
Group 14
Group 15

FIG. 5

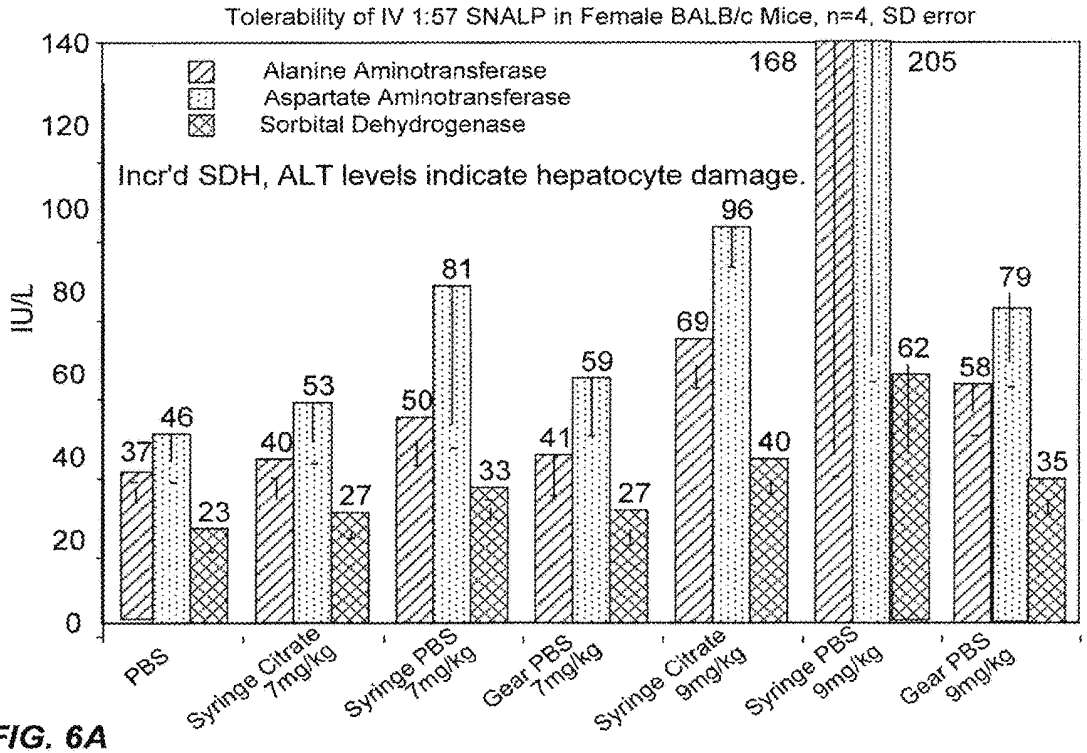

Tolerability of IV 1:57 SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Incr'd SDH, ALT levels indicate hepatocyte damage.
IU/L
140
120
100
80
60
40
20
0
168
205
37
46
23
40
53
27
50
81
33
41
59
27
69
96
40
62
58
79
35
PBS
Syringe Citrate 7mg/kg
Syringe PBS 7mg/kg
Gear PBS 7mg/kg
Syringe Citrate 9mg/kg
Syringe PBS 9mg/kg
Gear PBS 9mg/kg

FIG. 6A

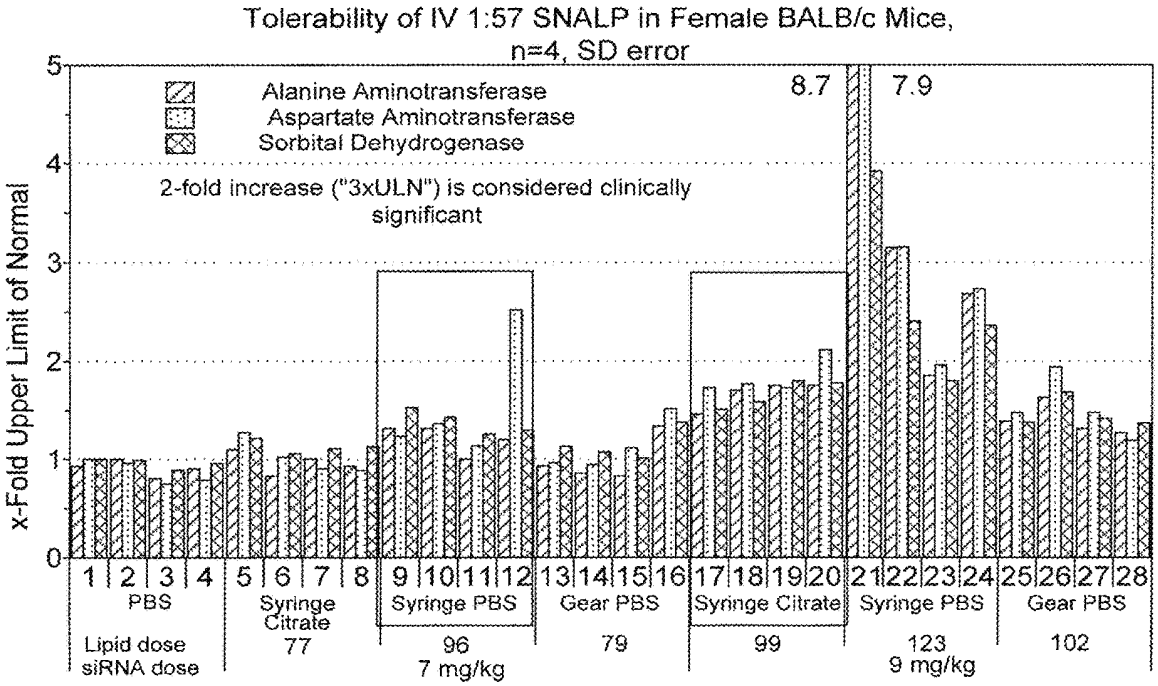

Tolerability of IV 1:57 SNALP in Female BALB/c Mice,
n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
5
4
3
2
1
0
8.7
7.9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 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

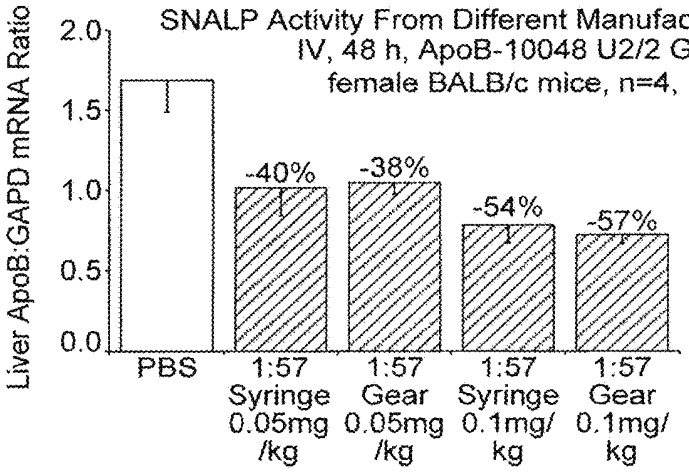


FIG. 7B

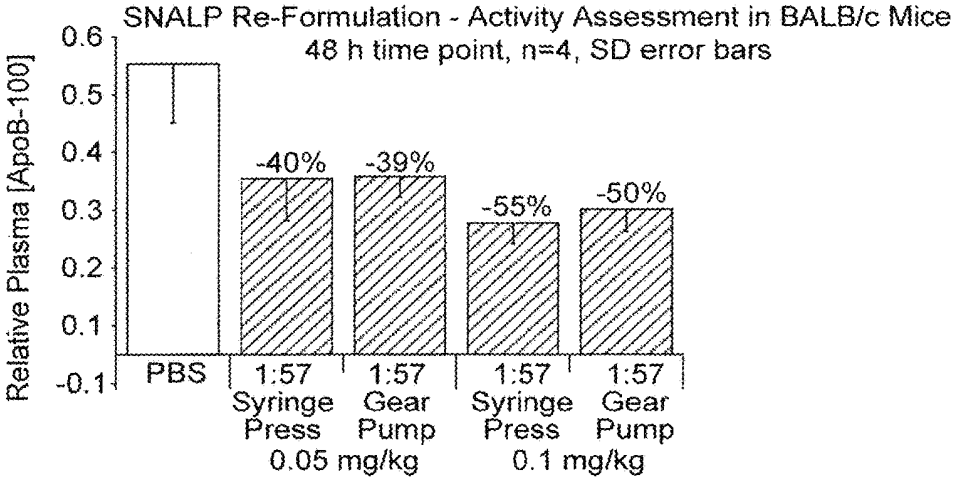
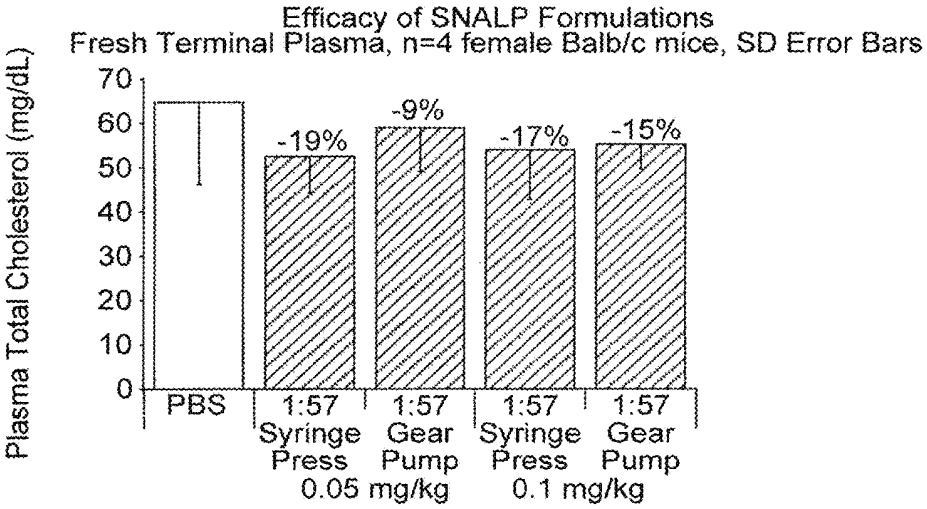


FIG. 7C



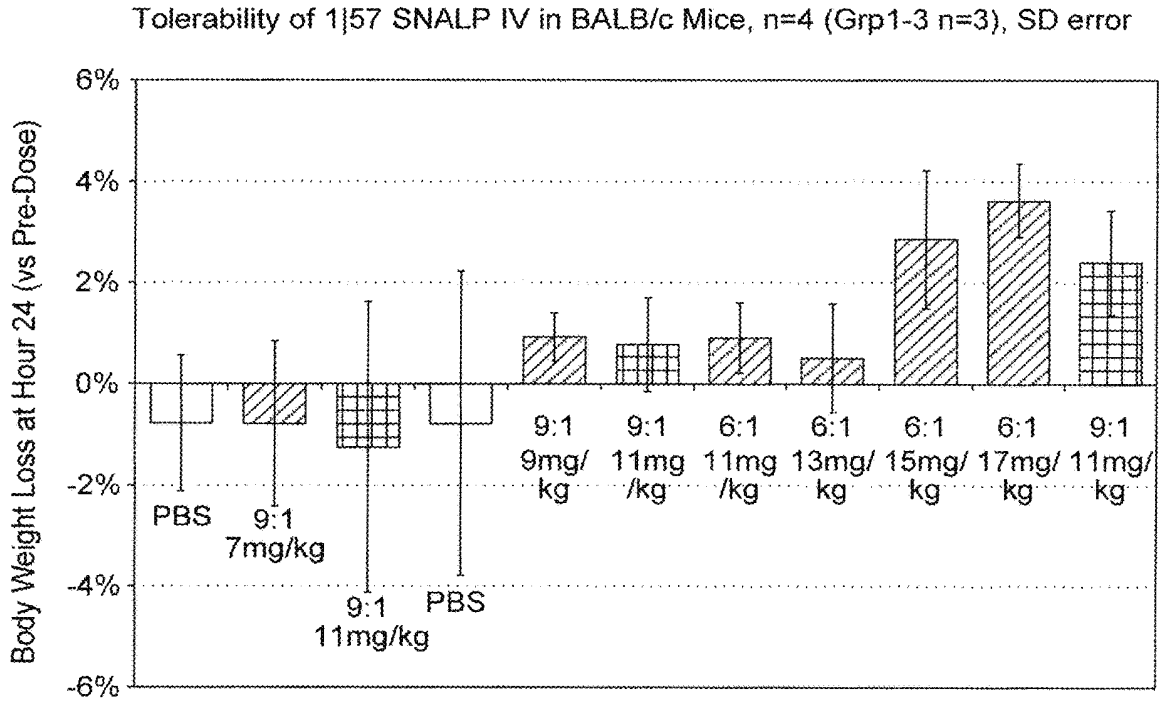

Tolerability of 1|57 SNALP IV in BALB/c Mice, n=4 (Grp1-3 n=3), SD error
Body Weight Loss at Hour 24 (vs Pre-Dose)
6%
4%
2%
0%
-2%
-4%
-6%
PBS
9:1 7mg/kg
9:1 11mg/kg
PBS
9:1 9mg/kg
9:1 11mg/kg
6:1 11mg/kg
6:1 13mg/kg
6:1 15mg/kg
6:1 17mg/kg
9:1 11mg/kg

FIG. 8

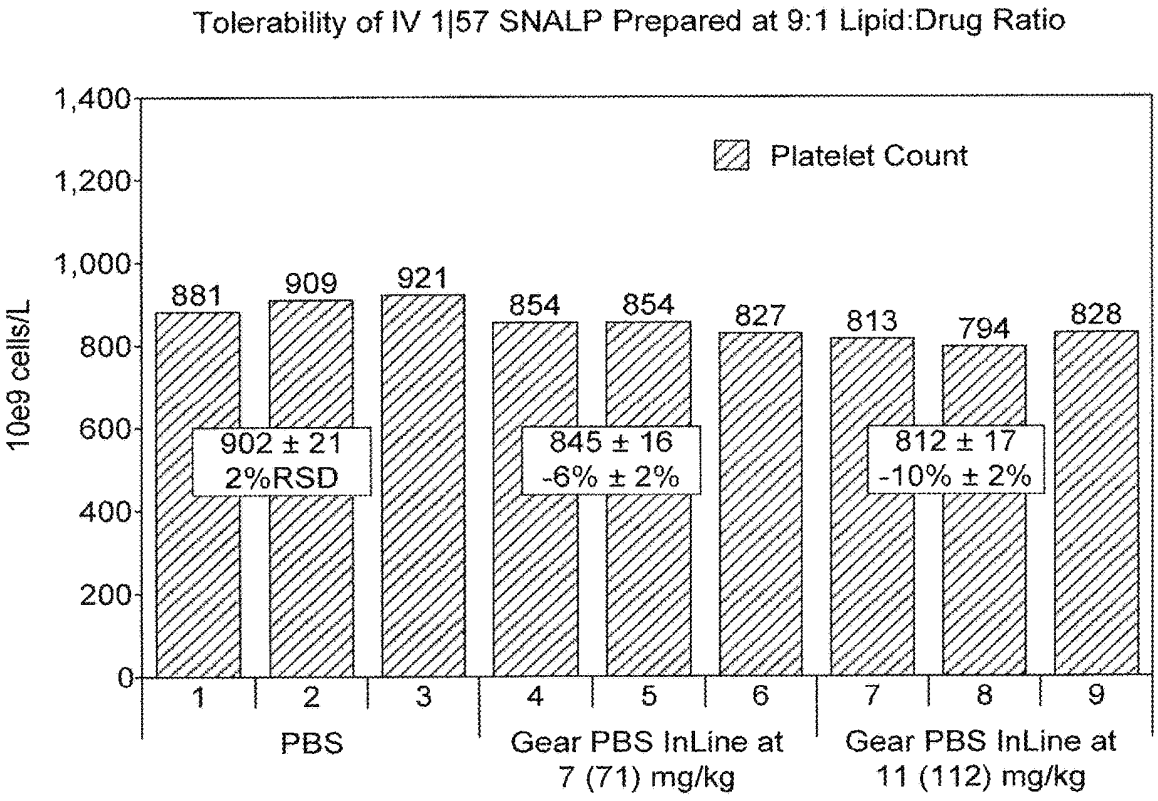

Tolerability of IV 1|57 SNALP Prepared at 9:1 Lipid:Drug Ratio
Platelet Count
10e9 cells/L
1,400
1,200
1,000
800
600
400
200
0
881
909
921
854
854
827
813
794
828
902 ± 21
2%RSD
845 ± 16
-6% ± 2%
812 ± 17
-10% ± 2%
1
2
3
4
5
6
7
8
9
PBS
Gear PBS InLine at
7 (71) mg/kg
Gear PBS InLine at
11 (112) mg/kg

FIG. 9

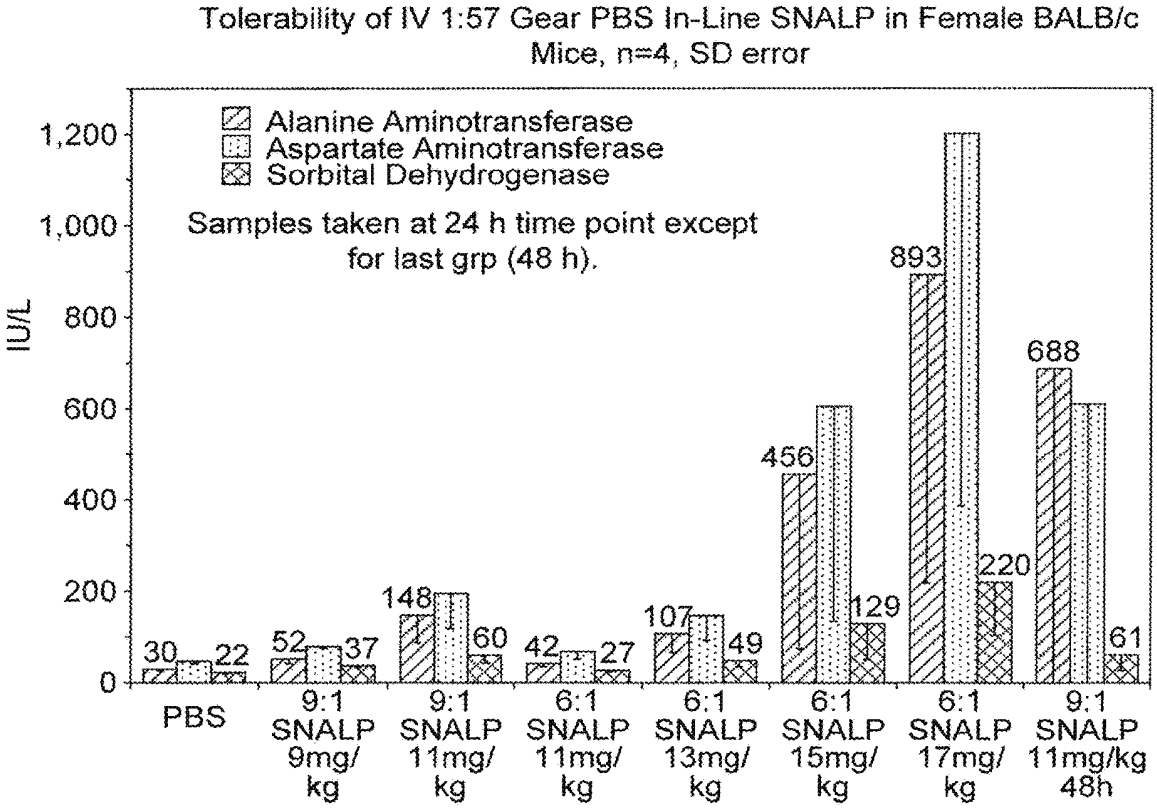

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
Samples taken at 24 h time point except for last grp (48 h).
IU/L
1,200
1,000
800
600
400
200
0
30
22
52
37
148
60
42
27
107
49
456
129
893
220
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

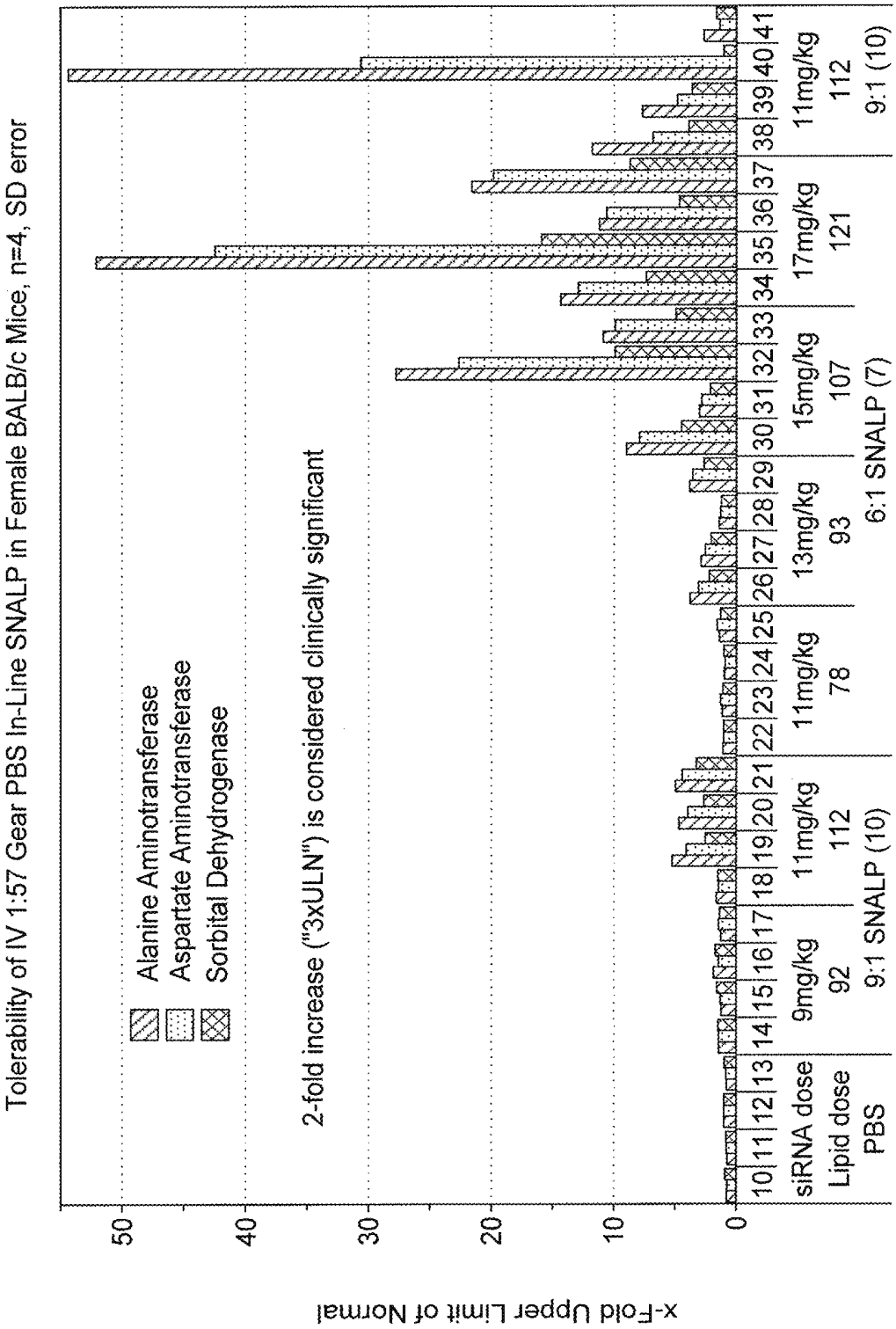

Tolerability of IV 1:57 Gear PBS In-Line SNALP in Female BALB/c Mice, n=4, SD error
Alanine Aminotransferase
Aspartate Aminotransferase
Sorbital Dehydrogenase
2-fold increase ("3xULN") is considered clinically significant
x-Fold Upper Limit of Normal
0
10
20
30
40
50
10 11 12 13 14 15 16 17 18 19 20 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

FIG. 11A

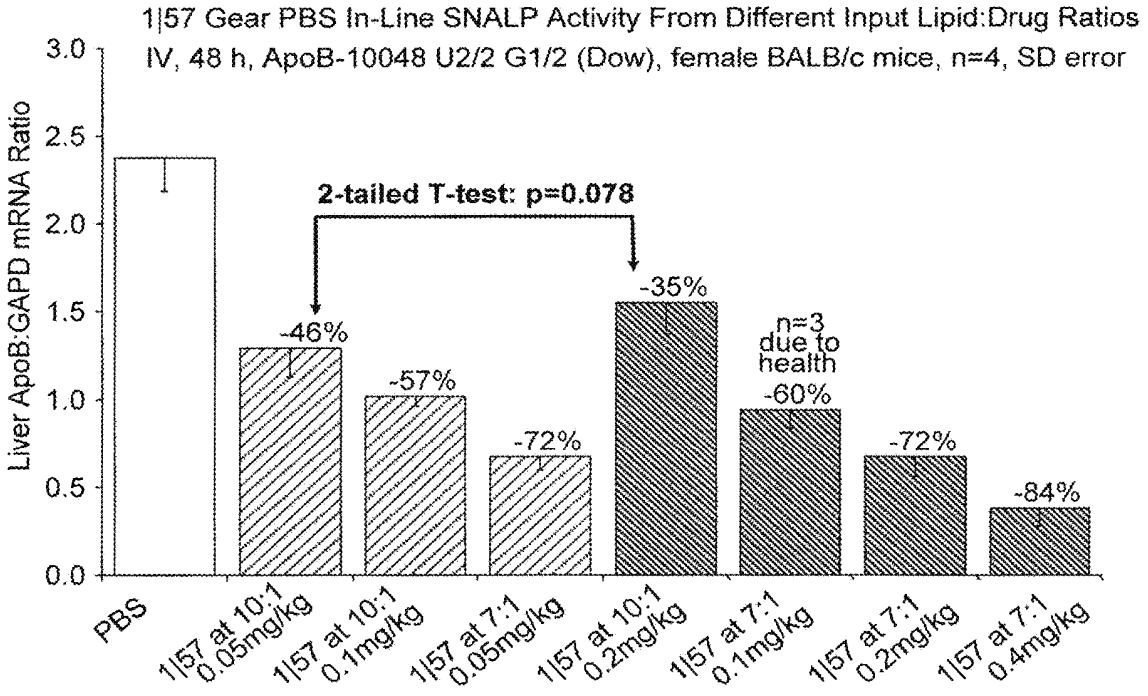
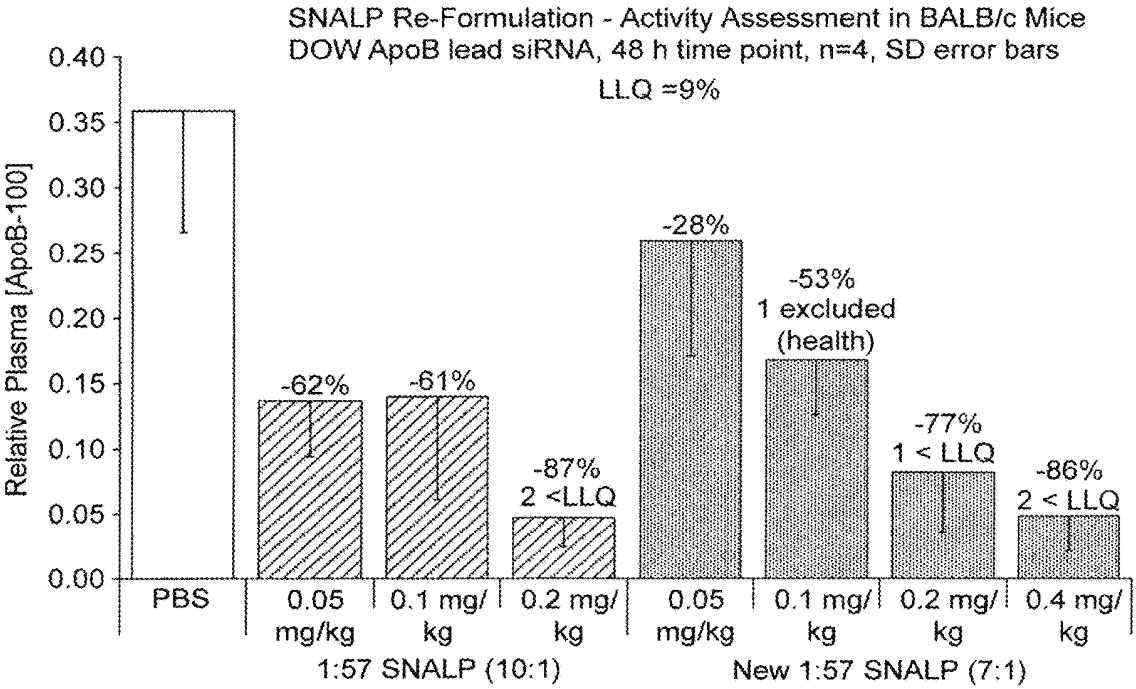


FIG. 11B



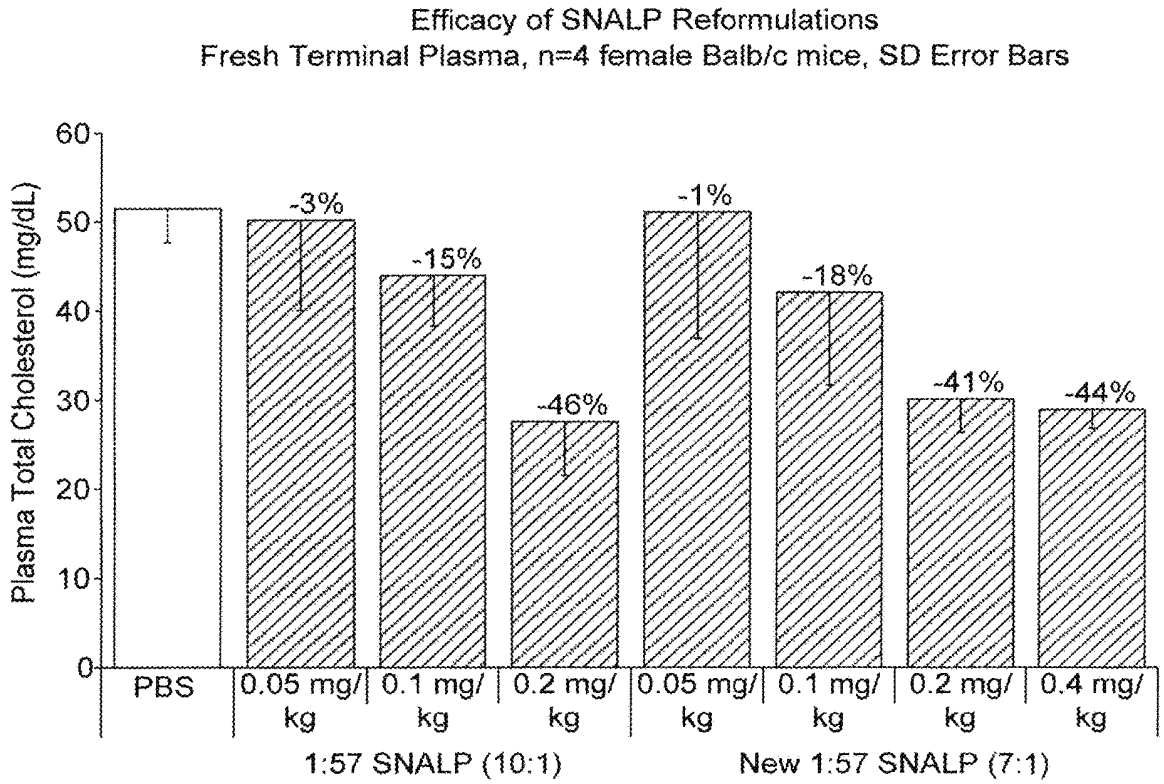

Efficacy of SNALP Reformulations
Fresh Terminal Plasma, n=4 female Balb/c mice, SD Error Bars
Plasma Total Cholesterol (mg/dL)
60
50
40
30
20
10
0
-3%
-15%
-46%
-1%
-18%
-41%
-44%
PBS
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.05 mg/kg
0.1 mg/kg
0.2 mg/kg
0.4 mg/kg
1:57 SNALP (10:1)
New 1:57 SNALP (7:1)

FIG. 12

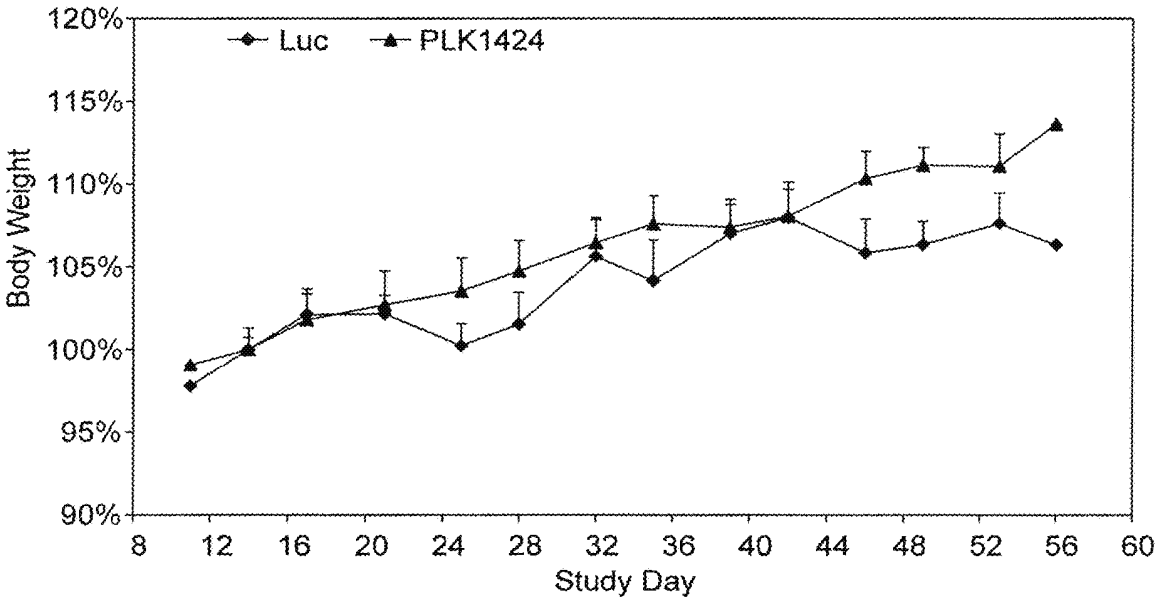

120%
115%
110%
105%
100%
95%
90%
Body Weight
Luc
PLK1424
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Study Day

FIG. 13

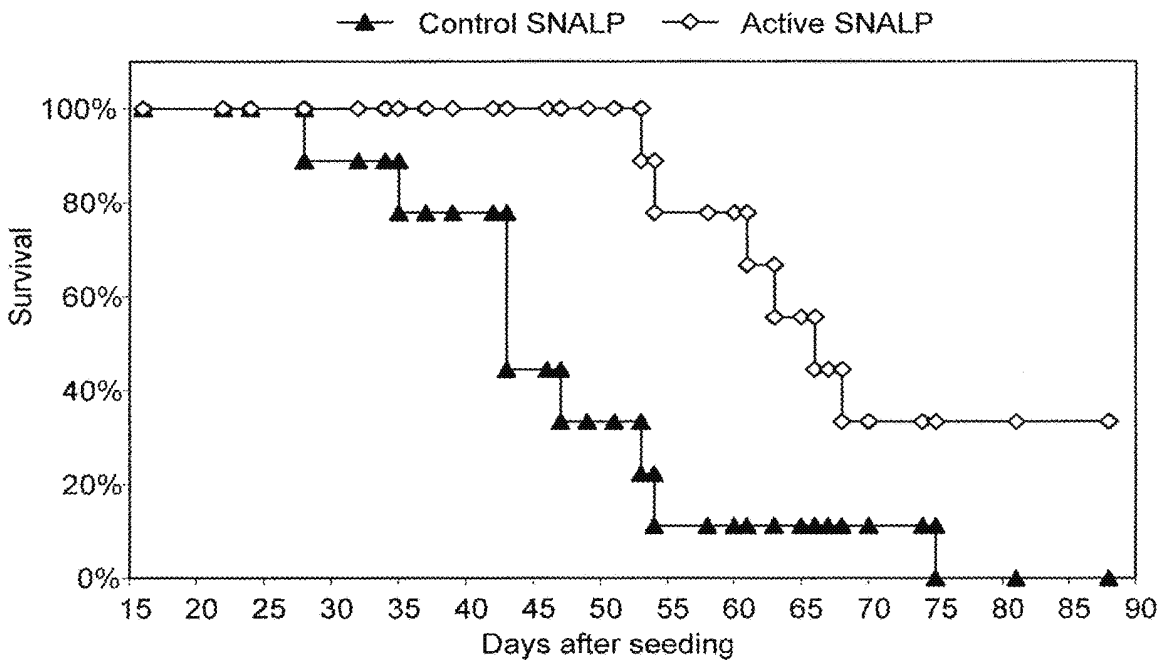

Control SNALP
Active SNALP
100%
80%
60%
40%
20%
0%
Survival
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
Days after seeding

FIG. 14

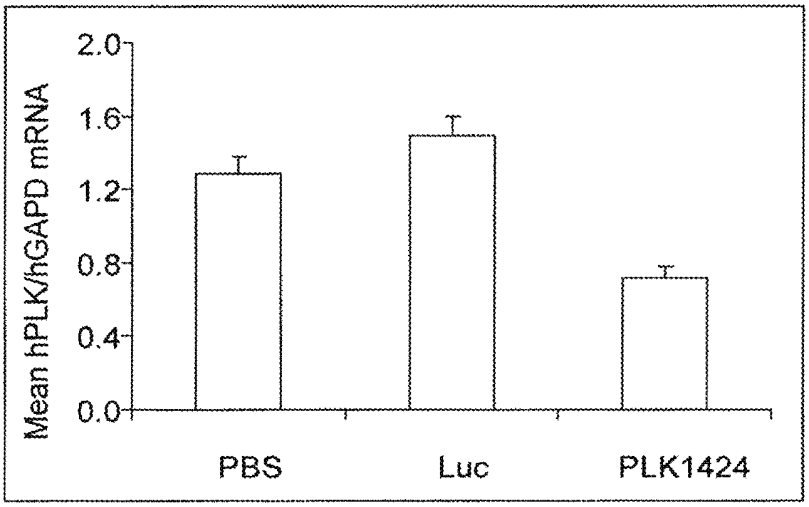


FIG. 15

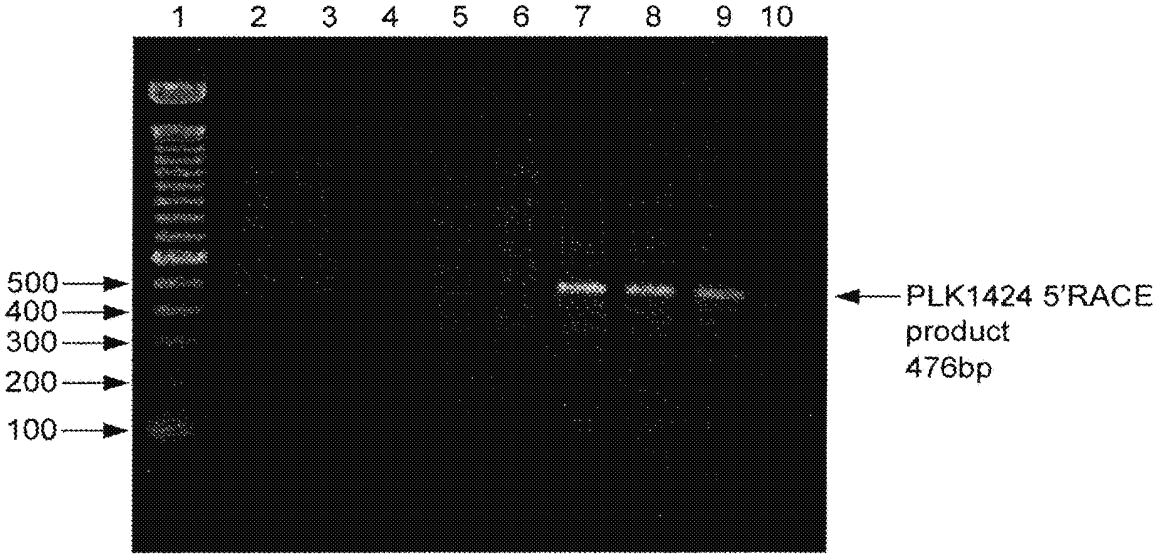
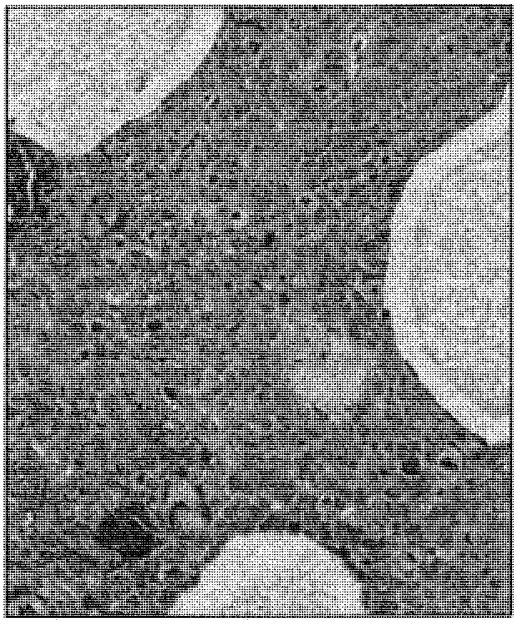
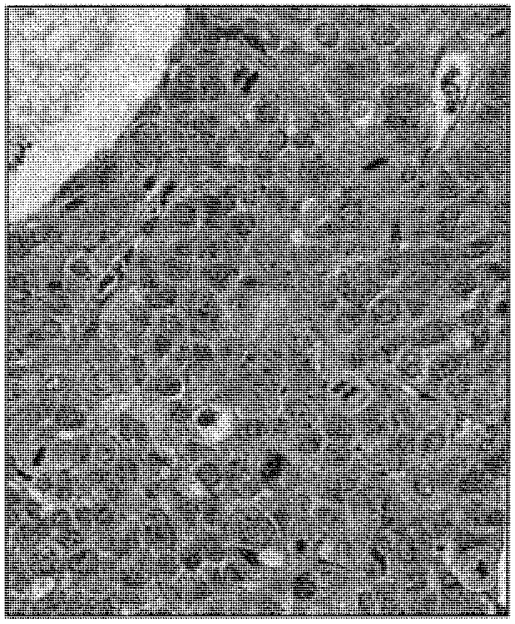

1
2
3
4
5
6
7
8
9
10
500
400
300
200
100
PLK1424 5'RACE
product
476bp

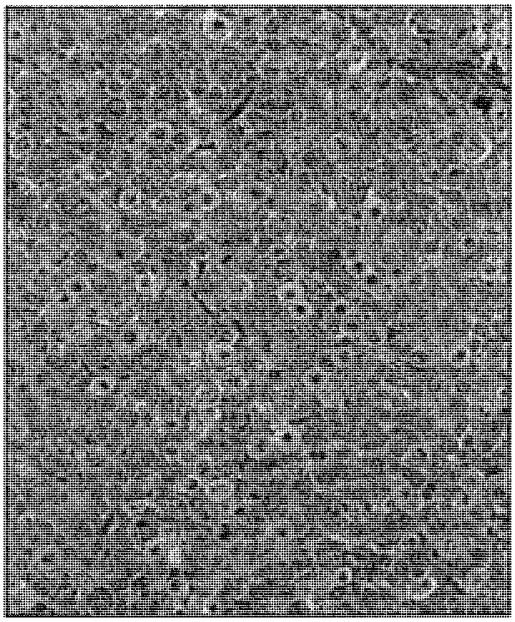
FIG. 16



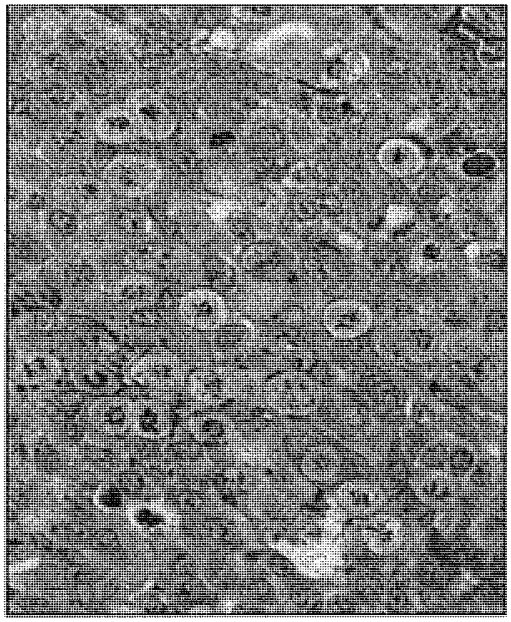
x200 mag



x400 mag



x200 mag



x400 mag

FIG. 17

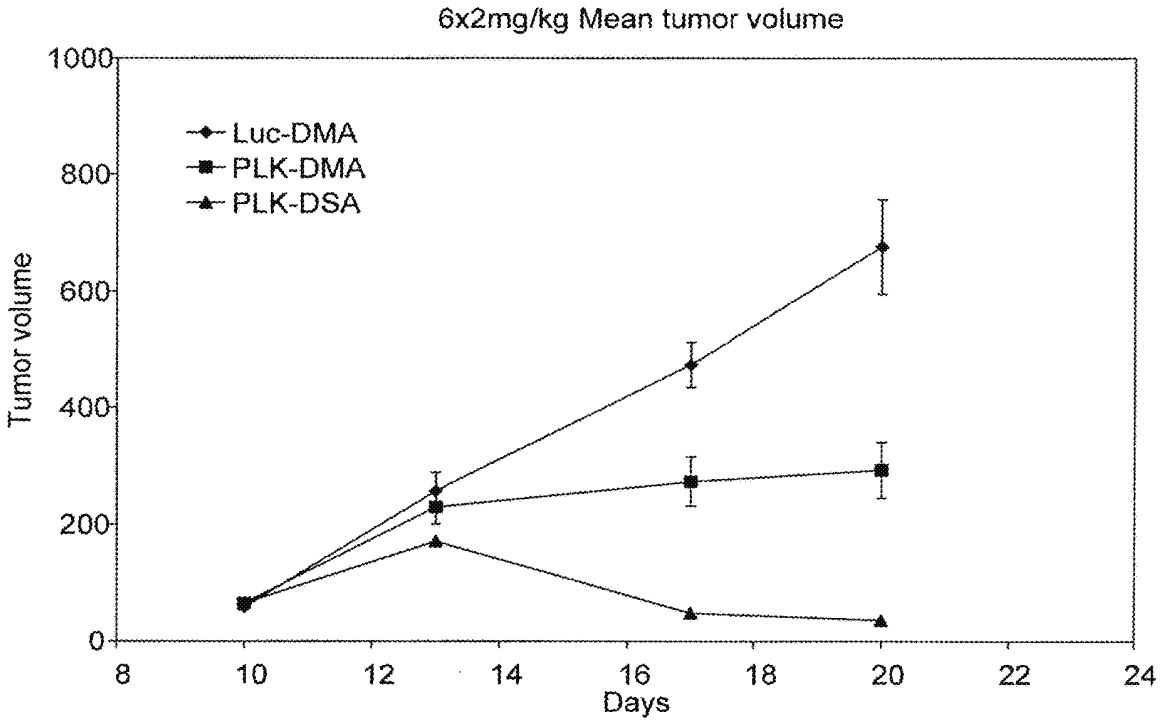

6x2mg/kg Mean tumor volume
Luc-DMA
PLK-DMA
PLK-DSA
Tumor volume
1000
800
600
400
200
0
8
10
12
14
16
18
20
22
24
Days

FIG. 18

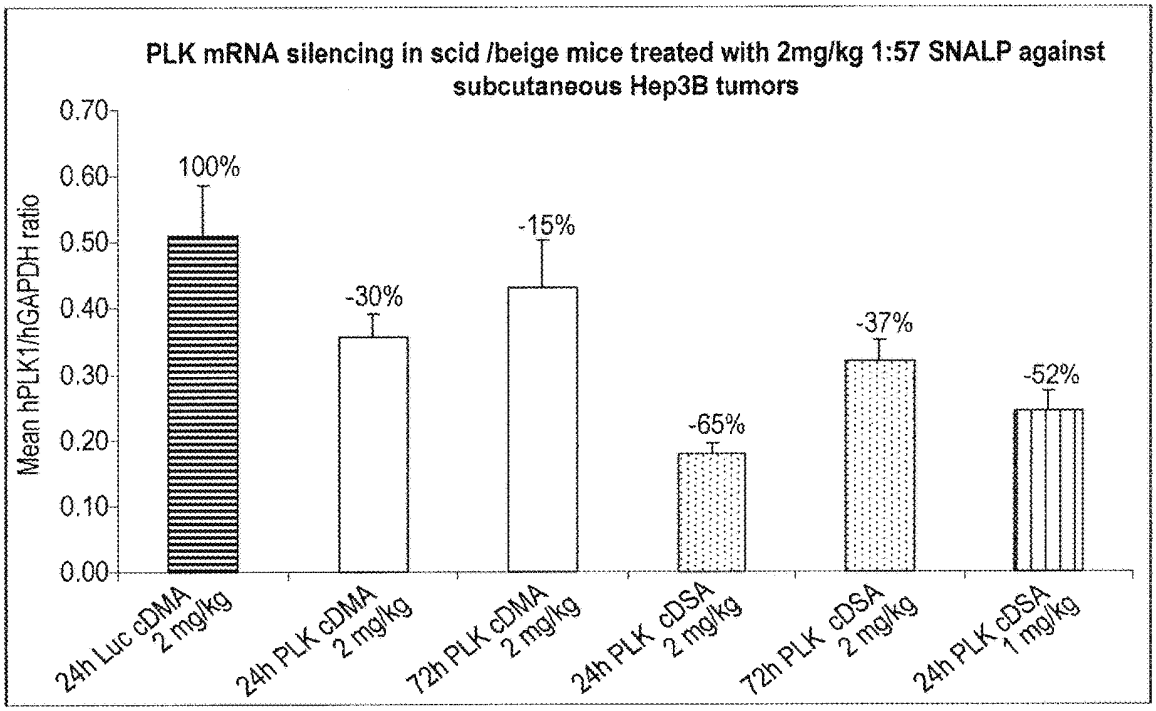

PLK mRNA silencing in scid /beige mice treated with 2mg/kg 1:57 SNALP against subcutaneous Hep3B tumors
Mean hPLK1/hGAPDH ratio
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100%
-30%
-15%
-65%
-37%
-52%
24h Luc cDMA 2 mg/kg
24h PLK cDMA 2 mg/kg
72h PLK cDMA 2 mg/kg
24h PLK cDSA 2 mg/kg
72h PLK cDSA 2 mg/kg
24h PLK cDSA 1 mg/kg

FIG. 19

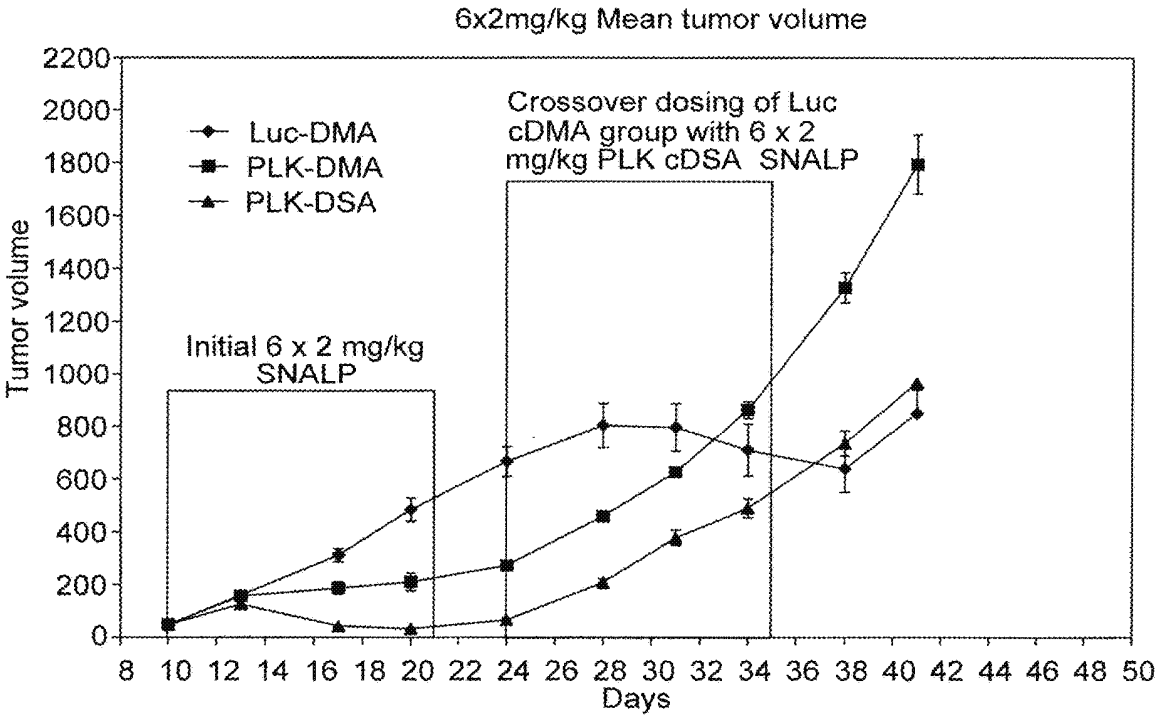

6x2mg/kg Mean tumor volume
Tumor volume
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Luc-DMA
PLK-DMA
PLK-DSA
Initial 6 x 2 mg/kg SNALP
Crossover dosing of Luc cDMA group with 6 x 2 mg/kg PLK cDSA SNALP
8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Days

FIG. 20

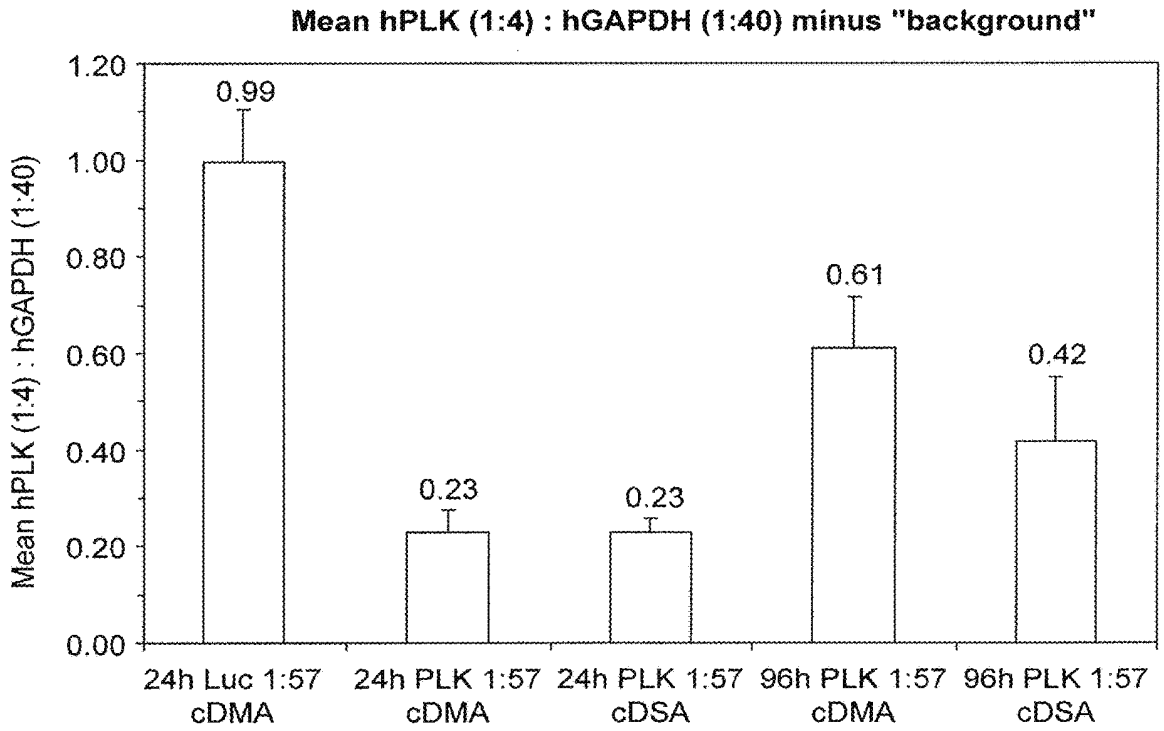

Mean hPLK (1:4) : hGAPDH (1:40) minus "background"
Mean hPLK (1:4) : hGAPDH (1:40)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.99
0.23
0.23
0.61
0.42
24h Luc 1:57 cDMA
24h PLK 1:57 cDMA
24h PLK 1:57 cDSA
96h PLK 1:57 cDMA
96h PLK 1:57 cDSA

FIG. 21

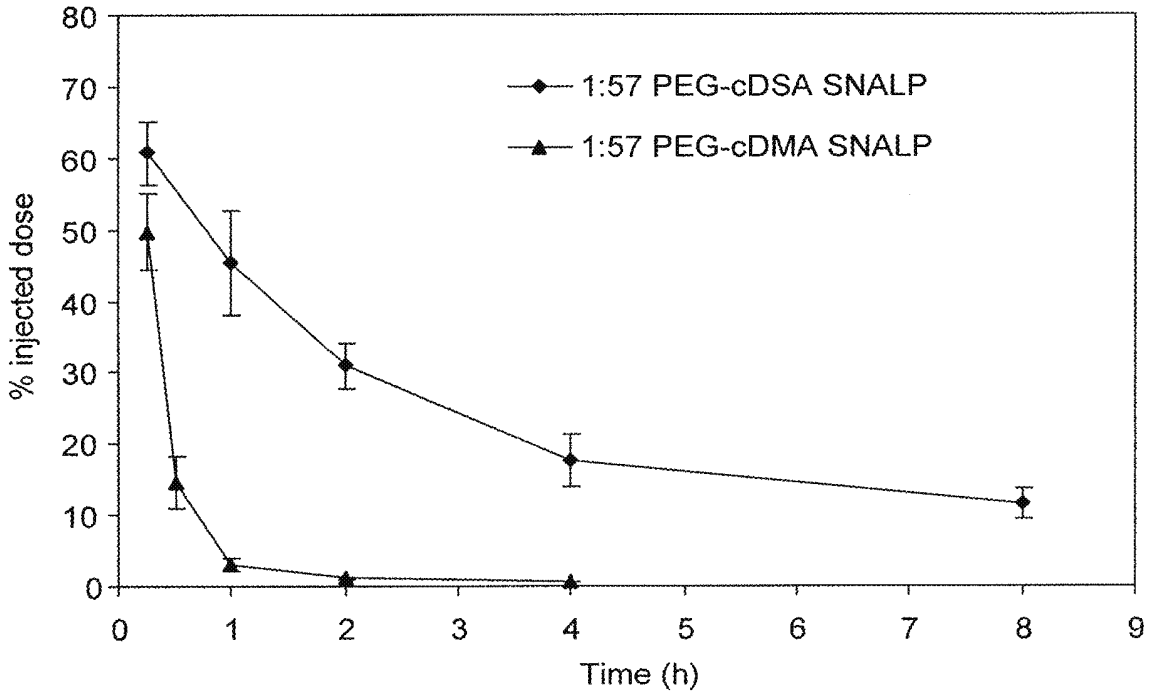

80
70
60
50
40
30
20
10
0
% injected dose
0
1
2
3
4
5
6
7
8
9
Time (h)
1:57 PEG-cDSA SNALP
1:57 PEG-cDMA SNALP

FIG. 22

LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a continuation of U.S. application Ser. No. 17/094,724, filed Nov. 10, 2020; which is a continuation of U.S. application Ser. No. 16/422,441, filed May 24, 2019; which is a continuation of U.S. application Ser. No. 15/840,933, filed Dec. 13, 2017; which is a continuation of U.S. application Ser. No. 15/670,742, filed Aug. 7, 2017; which is a continuation of U.S. application Ser. No. 15/164,803, filed May 25, 2016; which is a continuation of U.S. application Ser. No. 14/462,441, filed Aug. 18, 2014, which issued on Jun. 14, 2016 as U.S. Pat. No. 9,364,435; which is a continuation of U.S. application Ser. No. 13/928,309, filed Jun. 26, 2013, which issued on Sep. 2, 2014 as U.S. Pat. No. 8,822,668; which is a continuation of U.S. application Ser. No. 13/253,917, filed Oct. 5, 2011, which issued on Jul. 23, 2013 as U.S. Pat. No. 8,492,359; which is a continuation of U.S. application Ser. No. 12/424,367, filed Apr. 15, 2009, which issued on Nov. 15, 2011 as U.S. Pat. No. 8,058,069; which claims priority to U.S. Provisional Application No. 61/045,228, filed Apr. 15, 2008, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

STATEMENT REGARDING_FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

NAMES OF PARTIES TO A JOINT RESEARCH AGREEMENT

Not applicable.

REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING_APPENDIX SUBMITTED AS AN ASCII TEXT FILE

The Sequence Listing written in file 104290-007791US-1243808_SequenceListing.txt, created on Apr. 7, 2021, 4,543 bytes, machine format IBM-PC, MS-Windows operating system, is hereby incorporated by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

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.

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.

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.

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. Pat. No. 6,458,382. Cationic liposome complexes are also disclosed in U.S. Patent Publication No. 20030073640.

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)).

Other liposomal delivery systems include, for example, the use of reverse micelles, anionic liposomes, and polymer liposomes. Reverse micelles are disclosed in U.S. Pat. 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.

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.

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.

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

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).

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.

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.

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).

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.

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.

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.

The present invention also provides pharmaceutical compositions comprising a lipid particle described herein (e.g., SNALP) and a pharmaceutically acceptable carrier.

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).

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).

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).

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

FIG. **1**A (Samples 1-8) and FIG. **1**B (Samples 9-16) illustrate data demonstrating the activity of 1:57 SNALP containing Eg5 siRNA in a human colon cancer cell line.

FIG. **2** illustrates data demonstrating the activity of 1:57 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **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.

FIG. **4** illustrates data demonstrating the activity of 1:57 and 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **5** illustrates data demonstrating the activity of 1:62 SNALP containing ApoB siRNA following intravenous administration in mice.

FIG. **6**A (expressed as IU/L) and FIG. **6**B (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.

FIG. **7**A (expressed as liver ApoB:GAPD mRNA ratio), FIG. **7**B (expressed as relative plasma ApoB-100 concentration), and FIG. **7**C (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.

FIG. **8** illustrates data demonstrating that there was very little effect on body weight 24 hours after administration of 1:57 SNALP containing ApoB siRNA.

FIG. **9** illustrates data demonstrating that there were no obvious changes in platelet count after administration of 1:57 SNALP containing ApoB siRNA.

FIG. **10**A (expressed as IU/L) and FIG. **10**B (expressed as x-Fold Upper Limit of Normal) illustrate data demonstrating that clinically significant liver enzyme elevations (3×ULN) occurred at particular drug dosages of 1:57 SNALP containing ApoB siRNA.

FIG. **11**A (expressed as liver ApoB:GAPD mRNA ratio) and FIG. **11**B (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.

FIG. **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).

FIG. **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.

FIG. **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.

FIG. **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.

FIG. **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.

FIG. **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).

FIG. **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.

FIG. **19** illustrates data demonstrating PLK-1 mRNA silencing using 1:57 PLK SNALP in S.C. Hep3B tumors following a single intravenous SNALP administration.

FIG. **20** illustrates data demonstrating that PLK-1 PEG-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

FIG. **21** illustrates data demonstrating tumor-derived PLK-1 mRNA silencing in Hep3B intrahepatic tumors.

FIG. **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

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, FIG. **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, FIG. **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").

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.

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.

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

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

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.

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.

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).

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.

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.

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.

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.

"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.

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 Tijessen, *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.

Exemplary stringent hybridization conditions can be as follows: 50% formamide, 5× SSC, and 1% SDS, incubating at 42° C., or, 5× SSC, 1% SDS, incubating at 65° C., with wash in 0.2× 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).

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 1× 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.

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.

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.

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, Wis.), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology*, Ausubel et al., eds. (1995 supplement)).

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 (www.ncbi.nlm.nih.gov/).

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.

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.

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.

"Gene product," as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

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.

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.

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.

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.

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).

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. Pat. 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.

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.

Representative examples of phospholipids include, but are not limited to, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, di stearoylphosphatidylcholine, 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 amphip-

athic lipids described above can be mixed with other lipids including triglycerides and sterols.

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.

The term "non-cationic lipid" refers to any amphipathic lipid as well as any other neutral lipid or anionic lipid.

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.

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. Pat. 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, C_{18} 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.

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.

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.

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.

"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.

"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.

"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.

"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.

The term "mammal" refers to any mammalian species such as a human, mouse, rat, dog, cat, hamster, guinea pig, rabbit, livestock, and the like.

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

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).

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.

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.

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.

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.

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.

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.

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.

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.

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.

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).

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.

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.

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.

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.

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.

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.

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.

In some embodiments, the anti 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% 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.

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.

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-C_2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)[1,3]-dioxolane (DLin-K-C_3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)[1,3]-dioxolane (DLin-K-C_4-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-C_2-DMA ("XTC2"), or mixtures thereof.

The synthesis of cationic lipids such as DLin-K-C_2-DMA ("XTC2"), DLin-K-C_3-DMA, DLin-K-C_4-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 Oct. 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 Dec. 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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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 (C_{12}), a PEG-dimyristyloxypropyl (C_{14}), a PEG-dipalmityloxypropyl (C16), a PEG-distearyloxypropyl (C_{18}), or mixtures thereof.

Additional PEG-lipid conjugates suitable for use in the invention include, but are not limited to, mPEG2000-1,2-di-O-alkyl-sn3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Application No. PCT/US08/88676, filed Dec. 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. Pat. No. 7,404,969, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

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.

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.

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.

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, Calif.). "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.

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.

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).

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).

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-C_2-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.

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-C_2-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-C_2-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.

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.

The present invention also provides a pharmaceutical composition comprising a lipid particle (e.g., SNALP) described herein and a pharmaceutically acceptable carrier.

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.

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, down-regulation 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, down-regulation 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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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

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.

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

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.

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.

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.

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.

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.

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

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.*, 15188 (2001) or Nykäanen et al., *Cell,* 107:309 (2001)), or may lack overhangs (i.e., have blunt ends).

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.

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.

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.

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

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* 1, 20:6877-6888 (2001) are combined with rational design rules set forth in Reynolds et al., *Nature Biotech.,* 22(3):326-330 (2004).

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.

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., 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.

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.

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 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.

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.

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. Pat. 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. Pat. 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. Pat. 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.

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, N.J.); human IL-6 and TNF-α (eBioscience; San Diego, Calif.); and mouse IL-6, TNFα, and IFN-γ (BD Biosciences; San Diego, Calif.)).

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

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).

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.

To form a long dsRNA, for synthetic RNAs, the complement is also transcribed in vitro and hybridized to form a dsRNA. If a naturally occurring 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.

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. Pat. 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.

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, Calif.). 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.

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

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.

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.

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-((3-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. Pat. 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.

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.

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.

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. Pat. 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

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.

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)).

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. Pat. 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.

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.

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 NSSA protein, and/or the NSSB 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. Pat. No. 7,348,314; and U.S. Provisional Application No. 61/162,127, filed Mar. 20, 2009, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

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 LXRa and LXRI3 (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, 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 Jan. 26, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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); COPS signalosome subunits such as CSN1, CSN2, CSN3, CSN4, CSNS (JAB 1; Genbank Accession No. NM_006837); CSN6, CSN7A, CSN7B, and CSNS; 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, HDACS, 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 Ser. 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 Ser. No. 12/343,342, filed Dec. 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 CSNS gene include those described in U.S. Provisional Application No. 61/045,251, filed Apr. 15, 2008, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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., EGFRI-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 Ser. No. 11/807,872, filed May 29, 2007, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

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.

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 pathologi-

cal development of the disease. Examples of anti-angiogenic genes include, but are not limited to, endostatin (see, e.g., U.S. Pat. No. 6,174,861), angiostatin (see, e.g., U.S. Pat. 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.

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-□⊕, 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)).

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)).

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

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.

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.

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.

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

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.

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.

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.

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.

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.

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.

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

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.

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. Pat. 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); Penis et al., *Brain Res Mot Brain Res.,* 15;57:310-20 (1998); and U.S. Pat. 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. Pat. 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.

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

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.

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.

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. Pat. No. 5,631,359. An example of the hepatitis 6 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. Pat. 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.

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.

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. Pat. 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

Nucleic acids associated with lipid particles 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).

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.

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 Dec. 31, 2008, PCT Publication Nos. WO 02/069369 and WO 01/15726, U.S. Pat. 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

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.

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.

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).

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.).

Examples of conventional radiotherapeutic agents include, but are not limited to, radionuclides such as ^{47}Sc, ^{64}Cu, ^{67}Cu, 89Sr, 86Y, 87Y, 90Y, 105Rh, ^{111}Ag, ^{111}In, ^{117}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.

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.

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.

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

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.

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. Pat. 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.

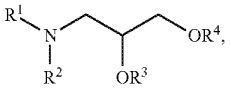
A. Cationic Lipids

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.

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 (DMIRIE), 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. Pat. 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, N.Y., 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, Wis., USA).

Additionally, cationic lipids of Formula I having the following structures are useful in the present invention.

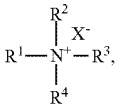
(I)



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).

Furthermore, cationic lipids of Formula II having the following structures are useful in the present invention.

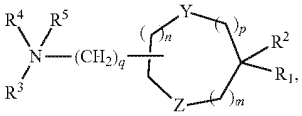
(II)



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.

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 O; q is 0, 1, 2, 3, or 4; and Y and Z are either the same or different and independently O, S, or NH.

In some embodiments, the cationic lipid of Formula III is 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C_2-DMA; "XTC2"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C_3-DMA), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-

C_4-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-C_2-DMA (XTC2).

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.

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

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.

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, di stearoylphosphatidylcholine (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.

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.

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.

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.

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.

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.

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.

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.

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

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.

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. Pat. 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.

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-NH2), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), and monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM). Other PEGs such as those described in U.S. Pat. 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.

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.

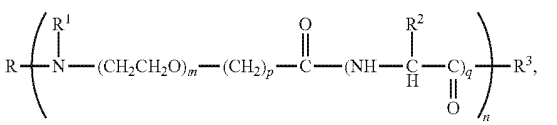
In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, e.g., non-ester containing 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)CCH2CH2C(O)—), succinamidyl (—NHC(O)CH2CH2C(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.

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.

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).

The term "ATTA" or "polyamide" refers to, without limitation, compounds described in U.S. Pat. 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)

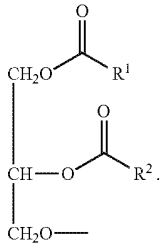


wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R^1 is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R^1 and the nitrogen to which they are bound form an azido moiety; R^2 is a member of the group selected from hydrogen, 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.

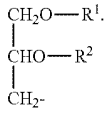
The term "diacylglycerol" refers to a compound having 2 fatty acyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons bonded to the 1- and 2-position of glycerol by ester linkages. The acyl groups can be saturated or have varying degrees of unsaturation. Suitable acyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C16), 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)



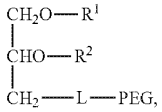
The term "dialkyloxypropyl" refers to a compound having 2 alkyl chains, R^1 and R^2, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkyloxypropyls have the following general formula:

(VI)



In a preferred embodiment, the PEG-lipid is a PEG-DAA conjugate having the following formula:

(VII)

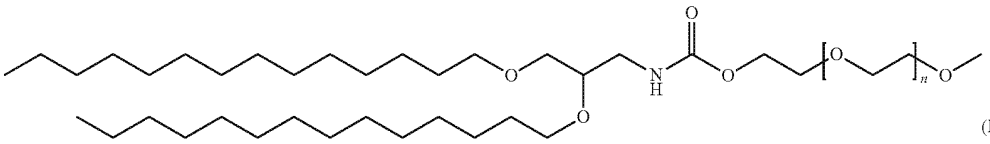


wherein R^1 and R^2 are independently selected and are long-chain alkyl groups having from about 10 to about 22 carbon atoms; PEG is a polyethyleneglycol; and L is a non-ester containing linker moiety or an ester containing linker moiety as described above. The long-chain alkyl groups can be saturated or unsaturated. Suitable alkyl groups include, but are not limited to, lauryl (C_{12}), myristyl (C_{14}), palmityl (C16), stearyl (C_{18}), and icosyl (C20). 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.

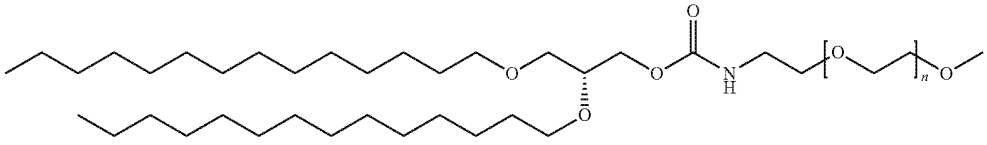
In Formula VII above, the PEG has an average molecular weight ranging from about 550 daltons to about 10,000 daltons. In certain instances, the PEG has an average molecular weight of from about 750 daltons to about 5,000 daltons (e.g., from about 1,000 daltons to about 5,000 daltons, from about 1,500 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.

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).

In particular embodiments, the PEG-lipid conjugate is selected from:



(PEG-C-DMA); and



(PEG-C-DOMG).

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).

Preferably, the PEG-DAA conjugate is a dilauryloxypropyl (C_{12})-PEG conjugate, dimyristyloxypropyl (C_{14})-PEG conjugate, a dipalmityloxypropyl (C16)-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.

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.

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. Pat. 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.

Suitable CPLs include compounds of Formula VIII:

$$A\text{-}W\text{-}Y \qquad \text{(VIII)},$$

wherein A, W, and Y are as described below.

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.

"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.

"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.

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.

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. Pat. 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.

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.

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.

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.

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

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.

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.

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.

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.

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.

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.

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.

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.

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.

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.

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. Pat. 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.

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.

In some embodiments, the nucleic acids in the SNALP are precondensed as described in, e.g., U.S. patent application Ser. No. 09/744,103, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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, Wis., 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.

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.

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.

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. Pat. 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

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.

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).

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

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.

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.

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.

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.

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.

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

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.

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. Pat. 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. Pat. 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.

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.

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. Pat. Nos. 5,756,353 and 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 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. Pat. No. 5,780,045. The disclosures of the above-described patents are herein incorporated by reference in their entirety for all purposes.

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.

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.

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. Pat. 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.

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.

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.

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.

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.

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.

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

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.

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.

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.

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

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.

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).

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, N.Y. (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

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

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 3H, ^{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

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.

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).

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. Pat. No. 4,683,202; PCR Protocols, A Guide to Methods and Applications (Innis et al. eds.) Academic Press Inc. San Diego, Calif. (1990); Arnheim & Levinson (Oct. 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.

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.

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

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

siRNA: All siRNA molecules used in these studies were chemically synthesized by the University of Calgary (Calgary, AB) or Dharmacon Inc. (Lafayette, Colo.). The siRNAs were desalted and annealed using standard procedures.

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

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'-C**U**GAAGACC**U**GAAGACAA**U**dTdT-3'	1	6/42 = 14.3%	6/38 = 15.8%
	3'-dTdTGAC**U**UC**U**GGAC**U**UCUGUUA-5'	2		

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.

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
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

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, Wis.), 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.

FIG. **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, FIG. **1**B, Sample 9).

Example 3. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity In Vivo

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	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 doubld-stranded (DS) region of the siRNA duplex are provided.

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).

nase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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).

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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydroge-

Example 4. ApoB siRNA Formulated as 1:57 SNALP Have Potent Silencing Activity In Vivo

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., *rnal 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

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.

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.

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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.

	Formulation Composition	Lipid/Drug	Finished Product Characterization		
Group	Lipid Name & Mole %	Ratio	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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy,* 13:494 (2006).

FIG. **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

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 | DLinDMA | 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
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

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.

Liver tissues were analyzed for ApoB mRNA levels normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the QuantiGene assay (Panomics; Fremont, Calif.) essentially as described in Judge et al., *Molecular Therapy*, 13:494 (2006).

FIG. **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

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

Animal Model: Female BALB/c mice, 5 wks old, n=4 per group/cage.

siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

Tolerability:

Group	Formulation	IV Injection siRNA mg/kg	IV Injection 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	IV Injection 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:

Formulations are provided at 0.005 to 0.9 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

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.

Formulation Summary:

		Particle Size			
	1:57 (9:1) + DOW siRNA	Zavg (nm)	Poly	% Encap	Final L:D (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

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.

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.

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 16,000×g (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 gin 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.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

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

There was no body weight loss or change in animal appearance/behavior upon administration of the 1:57 SNALP formulations. FIG. **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.

FIG. **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

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

Animal Model: Female BALB/c mice, 7 wks old.

siRNA payload: ApoB10048 U2/2 G1/2 siRNA.

CBC/Diff:

	#		IV Dosage	
Group	Mice	Test Article	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:

	#		IV Dosage	
Group	Mice	Test Article	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:

	#		IV Dosage	
Group	Mice	Test Article	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:

Formulations are provided at 0.005 to 1.7 mg siRNA/mL, 0.22 μm filter sterilized in crimp top vials.

Formulation Details:

"1|157 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.

Formulation Summary:

		Particle Size			
	1|57 SNALP Gear PBS In-Line	Zavg (nm)	Poly	% Encap	Final L:D (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

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.

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).

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.

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 16,000×g & 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.

Groups 12-19: Blood is collected by cardiac puncture and processed for plasma: immediately centrifuge for 5 min at 16,000×g (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.

Termination: Mice are anaesthetized with a lethal dose of ketamine/xylazine; then cardiac puncture is performed followed by cervical dislocation.

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:

FIG. **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.

FIG. **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.

FIG. **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:

FIG. **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.

FIG. **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:

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 FIG. **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

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 is DS Region
PLK1424 U4/GU	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3'	5	6/38 = 15.8%
	3'-NNUC**U**AGUGGGAG**G**AAUUUAU-5'	6	
PLK1424 U4/G	5'-AGA**U**CACCC**U**CCU**U**AAA**U**ANN-3'	5	7/38 = 18.4%
	3'-NNUCUA**G**UG**G**GA**G**GAAUUUAU-5'	7	

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

20 CD1 nu/nu mice were seeded as follows:

Group	# Mice	Tumor seeding	SNALP	# Mice	SNALP dosing IV	SNALP dose	Sacrifice	Assay
A B	20 to seed	I.H. 1.5×10^6 Hep3B	Luc 1:57 PLK 1424 1:57	9 9	Days 11, 14, 17, 21, 25, 28, 32, 35, 39, 42	10 × 2 mg/kg	When moribund	Survival Body Weights

Test Articles

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 (28 mM lipid)
B	PLK1424 U4/GU SNALP 1:57 (28 mM lipid)
	PLK1424 U4/G SNALP 1:57 (28 mM 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 (⅜") 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

FIG. **13** shows the mean body weights of mice during therapeutic dosing of PLK1424 SNALP in the Hep3B intra-

hepatic (I. H.) tumor model. The treatment regimen was well tolerated with no apparent signs of treatment-related toxicity.

FIG. **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

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.

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

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. 1×10^6 Hep3B	PBS	6	1×2 mg/kg Day 20	24 h after treatment	Tumor QG
B			Luc 1:57	7			Tumor RACE-PCR
C			PLK 1424 1:57	7			Histopathology

Test Articles

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. 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 (⅜") 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

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.

FIG. **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.

FIG. **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.

FIG. **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

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

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.

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 6×2 mg/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.

FIG. **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.

FIG. **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 FIG. **18**.

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 FIG. **20**, which shows that PLK1-cDSA SNALP inhibited the growth of large S.C. Hep3B tumors.

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.

FIG. **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.

FIG. **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.

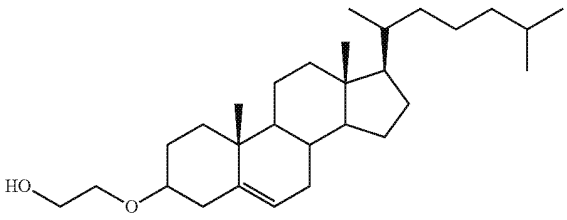
Thus, this study shows that the 1:57 PEG-cDSA SNALP formulation can be used to preferentially target tumors outside of the liver, whereas the 1:57 PEG-cDMA SNALP can be used to preferentially target the liver.

Example 12. Synthesis of Cholesteryl-2'-Hydroxyethyl Ether

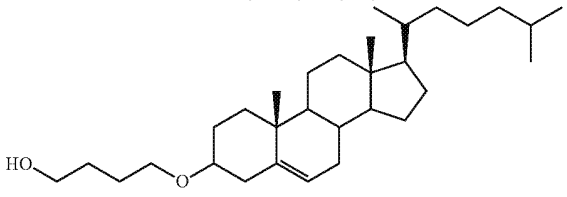
Step 1: A 250 ml round bottom flask containing cholesterol (5.0 g, 12.9 mmol) and a stir bar was sealed and flushed with nitrogen. Toluenesulphonyl chloride (5.0 g, 26.2 mmol) was weighed into a separate 100-mL round bottom flask, also sealed and flushed with nitrogen. Anhydrous pyridine (2×50 ml) was 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%).

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 (3×100 ml). The organic phases were combined, washed with water (2×150 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%).

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

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.

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What is claimed is:

1. A nucleic acid-lipid particle consisting essentially of:

(a) an RNA;

(b) a cationic lipid having a protonatable tertiary amine;

(c) a mixture of a phospholipid and cholesterol of from 30 mol % to 55 mol % of the total lipid present in the particle, wherein the phospholipid consists of from 3 mol % to 15 mol % of the total lipid present in the particle; and

(d) a polyethyleneglycol (PEG)-lipid conjugate consisting of from 0.1 mol % to 2 mol % of the total lipid present in the particle.

2. The nucleic acid-lipid particle of claim **1**, wherein the cholesterol consists of from 25 mol % to 45 mol % of the total lipid present in the particle.

3. The nucleic acid-lipid particle of claim **2**, wherein the phospholipid is distearoylphosphatidylcholine (DSPC).

4. The nucleic acid-lipid particle of claim **3**, wherein the PEG has an average molecular weight of about 2,000 daltons.

5. The nucleic acid-lipid particle of claim **4**, wherein the PEG has a terminal methoxy group.

6. The nucleic acid-lipid particle of claim **5**, wherein the PEG-lipid conjugate is a PEG-diacylglycerol (PEG-DAG) conjugate having the same saturated acyl groups.

7. The nucleic acid-lipid particle of claim **6**, wherein the cholesterol consists of from 35 mol % to 45 mol % of the total lipid present in the particle.

8. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim **6** and a pharmaceutically acceptable carrier.

9. The pharmaceutical composition of claim **8**, wherein the RNA is fully encapsulated in the nucleic acid-lipid particle.

10. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim **7** and a pharmaceutically acceptable carrier.

11. The pharmaceutical composition of claim **10**, wherein the RNA is fully encapsulated in the nucleic acid-lipid particle.

12. The nucleic acid-lipid particle of claim **1**, wherein the RNA is an mRNA.

13. The nucleic acid-lipid particle of claim **12**, wherein the cholesterol consists of from 25 mol % to 45 mol % of the total lipid present in the particle.

14. The nucleic acid-lipid particle of claim **13**, wherein the phospholipid is DSPC.

15. The nucleic acid-lipid particle of claim **14**, wherein the PEG has an average molecular weight of about 2,000 daltons.

16. The nucleic acid-lipid particle of claim **15**, wherein the PEG has a terminal methoxy group.

17. The nucleic acid-lipid particle of claim **16**, wherein the PEG-lipid conjugate is a PEG-DAG conjugate having the same saturated acyl groups.

18. The nucleic acid-lipid particle of claim **17**, wherein the cholesterol consists of from 35 mol % to 45 mol % of the total lipid present in the particle.

19. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim **17** and a pharmaceutically acceptable carrier.

20. The pharmaceutical composition of claim **19**, wherein the mRNA is fully encapsulated in the nucleic acid-lipid particle.

21. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim **18** and a pharmaceutically acceptable carrier.

22. The pharmaceutical composition of claim **21**, wherein the mRNA is fully encapsulated in the nucleic acid-lipid particle.

23. The nucleic acid-lipid particle of claim **5**, wherein the PEG-lipid conjugate comprises an amido linker moiety.

24. The nucleic acid-lipid particle of claim **3**, wherein the cholesterol consists of from 35 mol % to 45 mol % of the total lipid present in the particle.

25. The nucleic acid-lipid particle of claim **24**, wherein the PEG-lipid conjugate consists of from 0.5 mol % to 2 mol % of the total lipid present in the particle.

26. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim **25** and a pharmaceutically acceptable carrier.

27. The pharmaceutical composition of claim **26**, wherein the RNA is fully encapsulated in the nucleic acid-lipid particle.

28. The nucleic acid-lipid particle of claim **16**, wherein the PEG-lipid conjugate comprises an amido linker moiety.

29. The nucleic acid-lipid particle of claim **28**, wherein the DSPC consists of from 4 mol % to 10 mol % of the total lipid present in the particle.

30. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim **29** and a pharmaceutically acceptable carrier.

* * * * *

JOINT APPENDIX 07

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ARBUTUS BIOPHARMA CORPORATION and GENEVANT SCIENCES GMBH *Plaintiffs*, v. MODERNA, INC. and MODERNATX, INC., *Defendants*.	C.A. No. 22-252-MSG
MODERNA, INC. and MODERNATX, INC., *Counterclaim-Plaintiffs*, v. ARBUTUS BIOPHARMA CORPORATION and GENEVANT SCIENCES GMBH *Counterclaim-Defendants.*	**JURY TRIAL DEMANDED**

DECLARATION OF DAVID H. THOMPSON, PH.D.
REGARDING CLAIM CONSTRUCTION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further 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: September 25, 2022

David H. Thompson, Ph.D.

TABLE OF CONTENTS

I, David H. Thompson, Ph.D., declare as follows:

I. QUALIFICATIONS AND EXPERIENCE

1. I am a Professor of Chemistry at Purdue University and the Director of the Medicinal Chemistry Group in the Purdue Center for Cancer Research. My primary research interests include the development of transiently-stable carrier systems for drug and nucleic acid delivery, including fusogenic lipid systems and PEG-lipid formulations, and flow chemistry methods for preparing nucleic acid complexes and small molecules. I have studied, taught, practiced, and conducted research involving the formulation, use, characterization, and delivery of lipid particles. I am an expert in the delivery of therapeutic agents using lipid particles.

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. I conducted post-doctoral research at Oregon Health & Sciences University.

3. I served as an assistant professor at Oregon Health & Sciences University for roughly seven years, after which time I moved over to Purdue University, where I have been a faculty member for over 29 years. I served as associate professor in the Department of Chemistry and was promoted to full professor in 2001. Since that time, I have served as Professor of Organic Chemistry, as well as a professor in Purdue University's Department of Biomedical Engineering. In addition, I have served as a visiting professor at numerous institutions, including: University of Florida, Department of Pharmaceutics; University of British Columbia, Department of Biochemistry; Chulalongkorn University, Department of Pharmaceutics; Technical University of Denmark, Department of Micro & Nanotechnology; Japan Advanced Institute of Science & Technology, Department of Biomaterials; and Osaka University, Department of Applied Chemistry.

4. I serve in leadership roles on numerous committees and programs at Purdue University, including serving as: director of the Medical Chemistry Group, co-director of Chemical & Structural Biology Interdisciplinary Research, head of the Organic Chemistry Division for the Department of Chemistry, course developer for NSF Interdisciplinary Science Education for Engineers, member of the committee for the Department of Chemistry Graduate Studies, and director of the NIH-National Cancer Institute's Experimental Therapeutics – Chemical Biology Consortium. In addition, I have served in many government-service and advisory-board positions, including participating as a panelist on numerous NIH Study sections, such as the study section for Bioengineering and Physiology as well as on Gene and Drug Delivery.

5. I have over 37 years of experience working on lipid-based transport materials and mechanisms. Throughout this time, I have taught, researched, and practiced the formulation, use, characterization, and delivery of lipid particles. In my laboratory, I perform a wide range of studies on nucleic acid biotherapeutic delivery, including the formulation of lipid-based bio-responsive carrier systems. My laboratory also designs and synthesizes materials to improve the efficacy of such carrier systems in human tissue culture and animal models.

6. Since the 1990s, my work has focused on lipid-based drug carrier systems, including evaluating which systems were most efficient for intracellular drug delivery and mechanisms for improving the release of the drugs' contents into cells.[1] Starting around 2000, my research expanded to include not just drug, but nucleic-acid delivery via lipid-based carriers, and

[1] *See, e.g.*, V. C. Anderson and D. H. Thompson, *Triggered Release of Hydrophilic Agents from Plasmalogen Liposomes Using Visible Light or Acid*, Biochimica et Biophysica Acta, vol. 1109, pp. 33-42 (1992); D. H. Thompson et al., *Triggerable Plasmalogen Liposomes: Improvement of System Efficiency*, Biochimica et Biophysica Acta, vol. 1279, pp. 25-34 (1996); Y. Rui et al., *Diplasmenylcholine-Folate Liposomes: An Efficient Vehicle for Intracellular Drug Delivery*, Journal of the American Chemical Society, vol. 120, pp. 11213-11218 (1998).

lipid-mediated transfection for in vitro and in vivo nucleic acid transfer.[2] Since then, I have continued researching and formulating various types of vehicles for delivering therapeutic payloads, including the delivery of nucleic acids and lipid-based carrier systems.[3]

7. I have supervised over twenty-five Ph.D.-level graduate students with a primary emphasis on the development of lipid-based carriers and materials for building lipid-based carriers, including carriers to serve as gene-delivery vectors.

8. I have organized numerous conferences, including: "Materials Science of Phospholipid Assemblies" for the Material Research Society Symposium in 1999, "Drug Delivery" for the American Chemical Society Symposium in 2007, and "Advanced Materials Designs for Drug and Gene Delivery" for the American Chemical Symposium in 2013. I have been invited to speak at over 150 guest lectures for various events, universities, and research organizations regarding my work in various fields of study including the structure and design of gene carriers and lipid-based therapeutic delivery systems. I have also been invited to guest edit a

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[2] J. A. Boomer and D. H. Thompson, *Synthesis of Acid-Labile Diplasmenyl Lipids for Drug and Gene Delivery Applications*, Chemistry and Physics of Lipids, vol. 99, pp. 145-153 (1999); J. A. Boomer et al., *Formation of Plasmid-Based Transfection Complexes with an Acid-Labile Cationic Diplasmenyl Lipid:* In Vitro *and* In Vivo *Gene Transfer*, Pharmaceutical Research, vol. 19, pp. 1289-1298 (2002).

[3] J. A. Boomer et al., *Cytoplasmic Delivery of Liposomal Contents Mediated by an Acid-Labile Cholesterol-Vinyl Ether-PEG Conjugate*, Bioconjugate Chemistry, vol. 20, pp. 47-59 (2009) ("Boomer 2009"); H.-k. Kim et al., *Effective Targeted Gene Delivery to Dendritic Cells via Synergistic Interaction of Mannosylated Lipid with DOPE and BCAT*, Biomacromolecules, vol. 13, pp. 636-644 (2012); A. Kulkarni et al., *Pendant Polymer:Amino-β-Cyclodextrin:siRNA Guest:Host Nanoparticles as Efficient Vectors for Gene Silencing*, Journal of the American Chemical Society, vol. 134, pp. 7596-7599 (2012); V. D. Badwaik, et al., *Structure-Property Relationship for siRNA Delivery Performance of Cationic 2-Hydroxypropyl-β-cyclodextrin: Poly(ethylene glycol)*-Poly*(propylene glycol)-Poly(ethylene glycol) Polyrotaxane Vectors* Biomaterials vol. 84, pp. 86-98 (2016); K. J. Wright et al. *Organocatalytic Synthesis and Evaluation of Polycarbonate Pendant Polymer:β-Cyclodextrin-based Nucleic Acid Delivery Vectors* Macromolecules vol. 51, pp. 670-678 (2018); S. Samaddar et al. *Immunostimulatory Response of RWFV Peptide-Targeted Lipid Nanoparticles on Bladder Tumor Associated Cells* ACS Applied Bio Materials, vol. 4, pp. 3178-3188 (2021).

special issue in Advanced Drug Delivery Reviews on stimulated release from lipid-based carrier systems and published four highly cited reviews in this area.

9. I have served as a reviewer for over 40 journals, including Science, Nature, Journal of the American Chemical Society, Bioconjugate Chemistry, Biophysical Chemistry, Chemistry of Materials, Chemistry & Physics of Lipids, Advanced Drug Delivery Reviews, Pharmaceutical Research, Journal of Controlled Release, Lipids, Journal of Membrane Science, Nanomedicine, and Organic & Biomolecular Chemistry.

10. I am listed as a co-inventor on 10 United States patents covering inventions related to many different types of technologies and materials including lipids, lipid-based carriers, and gene delivery, including for instance, U.S. Patent No. 5,277,913, which is titled "Liposomal delivery system with photoactivatable triggered release," and U.S. Patent No. 6,979,460, which is titled "Vinyl ether lipids with cleavable hydrophilic headgroups," and describes novel PEG-lipids.

11. I have also published more than 170 peer-reviewed scientific papers in the fields of physical chemistry, organic chemistry, bioconjugate chemistry, lipids, drug delivery, viral immunology including DNA-based vaccine development, biochemistry, and biomaterials science.

12. A copy of my curriculum vitae is provided in **Appendix A** and contains further details on my education, experience, publications, and other qualifications to render an expert opinion in this matter.

13. I am being compensated my customary rate of $600 per hour for my consultation in connection with this litigation. My compensation is in no way dependent on the outcome of my analysis or opinions rendered in this litigation.

II. ASSIGNMENT

14. I understand that Plaintiffs Arbutus Biopharma Corporation ("Arbutus") and Genevant Sciences GmbH ("Genevant") have asserted U.S. Patent Nos. 8,058,069 ("the '069

patent"), 8,492,359 ("the '359 patent"), 8,822,668 ("the '668 patent"), 9,364,435 ("the '435 patent"), and 11,141,378 ("the '378 patent") (collectively, the "Lipid Composition Patents"), as well as U.S. Patent No. 9,504,651 ("the '651 patent"), against Defendants Moderna, Inc. and ModernaTX, Inc. (collectively, "Moderna") in this litigation.

15. I previously submitted declarations and was deposed concerning the '435 and '069 patents in *inter partes* review ("IPR") proceedings in Case Nos. IPR2018-00739 and IPR2019-00554 before the Patent Trial & Appeal Board ("PTAB" or "Board") brought by Moderna Therapeutics, Inc.[4] In those IPRs, I responded to Moderna's assertion that the claims of the '435 and '069 patents were unpatentable as either anticipated or obvious. I understand that the Board subsequently determined that claims 7-8, 10-11, 13, and 16-20 of the '435 patent were not unpatentable, that none of the claims of the '069 patent were unpatentable, and that these determinations were not disturbed on appeal by the Court of Appeals for the Federal Circuit.

16. I have now been asked by counsel for Plaintiffs to opine on how the person of ordinary skill in the art ("POSA"), in the context of the Lipid Composition Patents and the '651 patent, would interpret the following claim terms at the priority dates asserted by the parties:[5]

- **"mol % of the total lipid present in the particle"** as recited in claims 1, 8, 15, 20, and 21 of the '069 patent; claims 1, 7, 9, 10, 11, 12, 13, 18, 19 of the '359 patent, claims 1, 8, 10, and 15 of the '668 patent, claims 1, 4, 7, and 8 of the '435 patent; and claims 1, 2, 7, 13, 18, 24, and 25 of the '378 patent; and

[4] I also submitted a declaration and was deposed concerning another patent, U.S. Patent No. 9,404,127 ("the '127 patent')—which I understand has *not* been asserted and is *not* at issue in this case—in another IPR brought by Moderna Therapeutics, Inc., Case No. IPR2018-00680. I was deposed simultaneously concerning my declarations regarding the '127 patent and the '435 patent in the IPRs concerning those patents on February 4 and 5, 2019. I was separately deposed in the IPR concerning the '069 patent on January 15, 2020.

[5] I understand that beyond the filing date of the patents and the provisional applications to which the Lipid Composition Patents and the '651 patent claim priority—which I discuss below, *see infra* § IV.A—Moderna contends August 1, 2013 to be a relevant priority date for the '651 patent, and further contends that the Lipid Composition Patents are not entitled to a priority date earlier than April 15, 2009. My opinions do not change depending on the priority date applied.

- **"wherein at least 70% / at least 80% / about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles"** as recited in claims 1, 13, and 14 of the '651 patent.

17. In reaching the opinions I express herein, I have considered the Lipid Composition Patents, all of which I understand share a common specification, *see infra* § IV.A; the '651 patent; the prosecution histories of the Lipid Composition Patents and the '651 patent; the materials cited in this declaration; as well as my training, general knowledge, basic principles, and experience in the relevant scientific disciplines.

III. TECHNICAL BACKGROUND

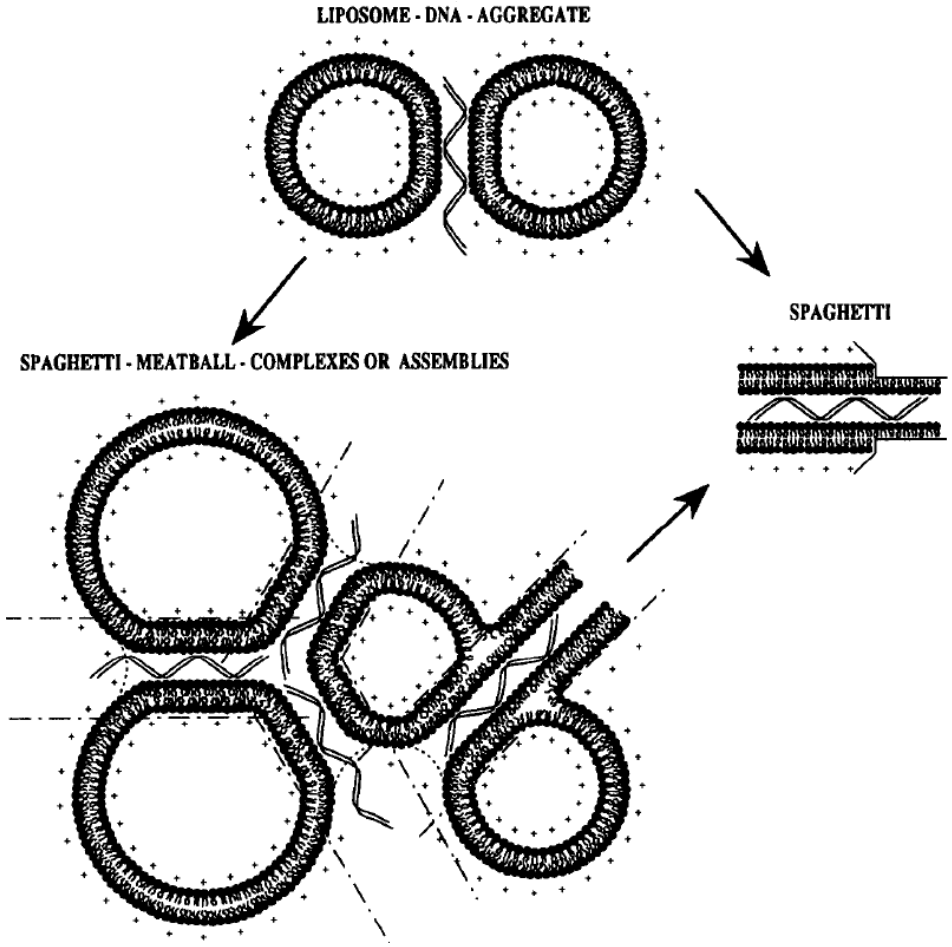
A. Lipid Nanoparticles

18. Gene therapies using nucleic acids such as DNA or RNA to manufacture therapeutic proteins (or to "silence" disease-causing genes) inside human cells have been a focus of sustained research since the discovery of DNA and the genetic code. These therapies hold the promise of targeting disease pathways in highly specific ways, but it has long been known that the promise of gene therapies would only be realized through the development of appropriate delivery vehicles. That is because therapeutic nucleic acids cannot simply be administered to a patient, *e.g.*, by injection. Therapeutic nucleic acids require an effective vehicle to protect them from being destroyed by the human body before they are even able to reach a target cell, and once there, to transit across the cell membrane and deposit them inside the cell, where they can then exert their therapeutic effect. For this reason, the central challenge in gene therapy has long been "delivery, delivery, delivery." Check 2003[6] at 11.

19. Lipid-based formulations for delivering nucleic acids have been explored since the late 1970s, but these early formulations differ markedly from the lipid nanoparticles ("LNP") at

[6] E. Check, *RNA to the rescue?*, Nature, vol. 425, pp. 10-12 (2003) ("Check 2003").

issue in this case. The first generation of nucleic acid delivery systems that were developed included cationic liposome nucleic acid complexes. These formulations have been described structurally as resembling "spaghetti" and "meatballs," where "tubules" of lipid-coated strands of nucleic acids (the "spaghetti") are interspersed with the cationic liposome-nucleic acid "lipoplexes" (the "meatballs"), shown schematically below:



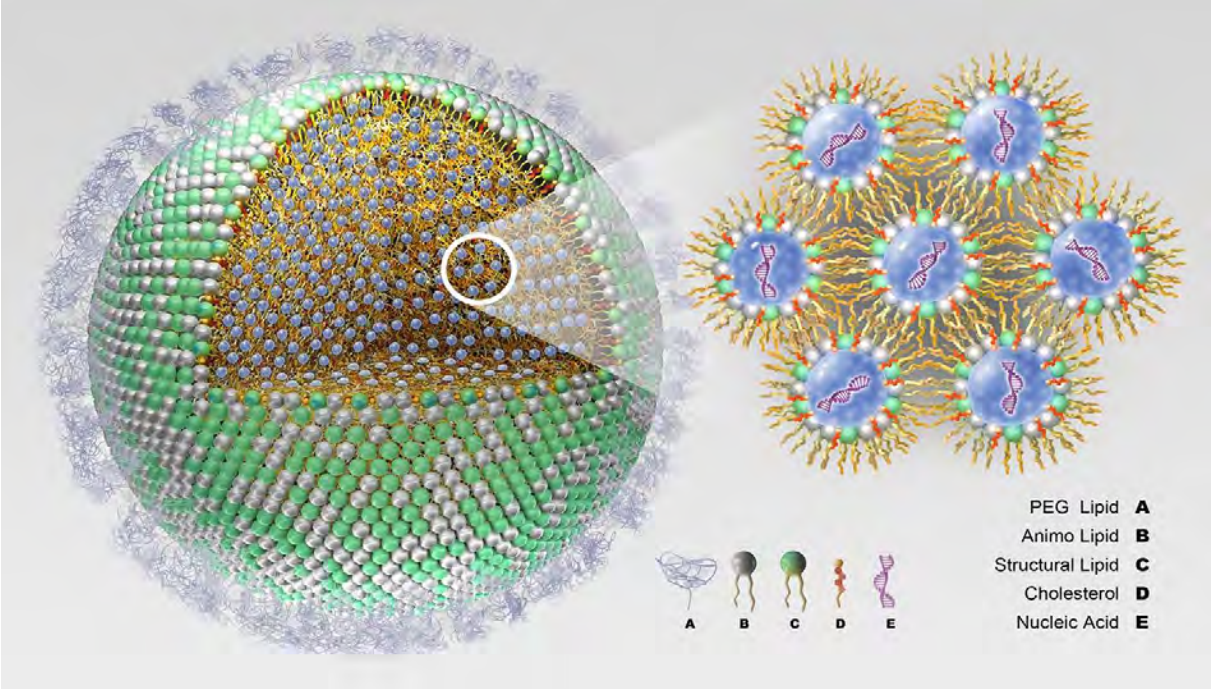
Sternberg 1994[7] at 364 (Figure 2). These cationic lipoplexes "enhance uptake by recipient cells," but are generally understood not to achieve "true encapsulation" of their nucleic payload. Sternberg 1994 at 361, 365.

[7] B. Sternberg et al., *New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy*, FEBS Letters, vol. 356, pp. 361-66 (1994) ("Sternberg 1994").

20. The LNPs at issue in this case differ from cationic lipoplexes and are generally comprised of three or four lipid components: (1) "cationic" (or "ionizable" lipids or "aminolipids") that are neutral or uncharged at physiological pH (approximately pH 7) but which become positively charged at low pH due to the presence of nitrogen- or amine-bearing chemical groups; (2) cholesterol and (3) phospholipids, which are "structural" lipids that maintain the LNP structure; and (4) conjugated lipids that contain a lipid "anchor" attached (or "conjugated") to a hydrophilic component, generally polyethylene glycol or "PEG." The hydrophilic nature of PEG reduces the propensity of LNPs to aggregate or fuse during manufacture and also "shields" them from being rapidly cleared in the bloodstream. However, PEG-lipids can also can interfere with the ability of LNPs to fuse with cell membranes.

21. The LNPs at issue in this case generally are formed by the self-assembly of the lipids and the nucleic acids into particles. In particular, when the lipid components and nucleic acid are combined under certain conditions, they form LNPs due to the electrostatic interaction of the positively charged ionizable lipid (the cationic state of the lipid) with the negatively charged nucleic acid, as well as clustering of the lipids together to minimize their contact with water (the same phenomenon behind the formation of oil droplets in water). The resulting particles have a lipid outer layer surrounded by a "corona" of hydrophilic PEG domains from the conjugated lipid at the particle surface, which are so oriented due to the affinity of the PEG for water. The nucleic acid resides in the interior of the LNP, surrounded by the cationic lipid, phospholipid, and cholesterol. Various schematic or "cartoon" depictions of LNPs from the literature reflect this consensus understanding of their structure, including the following:

Samaridou 2020,[8] Graphical Abstract



The "Amino Lipid" above corresponds to the cationic lipid, while the "Structural Lipid" encompasses the phospholipid. *See* Samaridou 2020 at 38-41; *see also, e.g.*, Kulkarni 2018,[9] Figure 6; Buschmann 2021,[10] Figure 2.

B. Significant Figures & Rounding

22. The concept of significant figures and rounding is a standard scientific convention that is used to understand the meaning and scope of numeric values. As a general matter in my field, the significant figures in a numeric value refer to those digits that carry "practical meaning."

[8] E. Samaridou et al., *Lipid nanoparticles for nucleic acid delivery: Current perspectives*, Advanced Drug Delivery Reviews, vols. 154-55, pp. 37-63 (2020) ("Samaridou 2020"), *available at* https://www.sciencedirect.com/science/article/abs/pii/S0169409X2030048X.
[9] J. A. Kulkarni et al., *On the Formation and Morphology of Lipid Nanoparticles Containing Ionizable Cationic Lipids and siRNA*, ACS Nano, vol. 12, pp. 4787-95 (2018) ("Kulkarni 2018").
[10] Michael D. Buschmann et al., *Nanomaterial Delivery Systems for mRNA Vaccines*, Vaccines (Basel), vol. 9, no. 65 pp. 1-30 (Jan. 19, 2021) ("Buschmann 2021").

Remington 2000[11] at 103. As *Remington*—a widely used and authoritative treatise in the pharmaceutical field—summarizes, "[w]eighing and measuring can be carried out only with a certain maximum degree of accuracy; the result always is approximate due to the many sources of error such as temperature, limitations of the instruments employed, personal factors, and so on." *Id*. In a measured value such as 473 mL (milliliters), there are three significant figures, "the last" or rightmost "significant figure is only approximate," and "it is understood that the measurement had been made somewhere between 472.5 and 473.5 mL." *Id*. The significant figures in a numeric value are thus understood to encompass a range of values having greater precision, or more significant figures, all of which round to the numeric value in question.

23. Rounding also comes into play when comparing values having different significant figures. As explained in the *United States Pharmacopeia* ("USP")—an official compendia of reference standards in the pharmaceutical industry and another authoritative text that reflects the conventional understanding of scientists working in the field—when considering a "stated limit" (such as a range in a patent claim), the limit values "are considered significant to the last digit shown." USP 23[12] at 3. Put another way, the number 473 has three significant digits, as does the number 470, since the "4" is significant, and all ensuing digits are significant to the "last digit shown," whether a 3 or a 0. And because analytical results (whether "observed" or "calculated") generally contain more significant figures than the "stated limits," "the observed or calculated result" must be "rounded off to the number of places that is in agreement with the limit expression" using the following procedure:

[11] *Remington: The Science and Practice of Pharmacy Twentieth Edition* (Alfonso R. Gennaro et al. eds., 2000) ("Remington 2000").
[12] *United States Pharmacopeia, Twenty-third Revision* (1995) ("USP 23").

When rounding off is required, consider only one digit in the decimal place to the right of the last place in the limit expression. If this digit is smaller than 5, it is eliminated and the preceding digit is unchanged. If this digit is greater than 5, it is eliminated and the preceding digit is increased by one. If this digit equals 5, the 5 is eliminated and the preceding digit is increased by one.

Illustration of Rounding Numerical Values for Comparison with Requirements

Compendial Requirement	Unrounded Value	Rounded Result	Conforms
Assay limit ≥98.0%	97.96%	98.0%	Yes
	97.92%	97.9%	No
	97.95%	98.0%	Yes
Assay limit ≤101.5%	101.55%	101.6%	No
	101.46%	101.5%	Yes
	101.45%	101.5%	Yes
Limit test ≤0.02%	0.025%	0.03%	No
	0.015%	0.02%	Yes
	0.027%	0.03%	No
Limit test ≤3 ppm	0.00035%	0.0004%	No
	0.00025%	0.0003%	Yes
	0.00028%	0.0003%	Yes

USP 23 at 4. To explain the above procedure, which would be well understood by those working in the field, for the first limit shown—the range "≥98.0%" (greater than or equal to "98.0%")—the POSA would understand there to be 3 significant figures, where the last significant digit is the "0" in the tenths decimal place, the last digit shown. The zero after the decimal point is called a trailing zero. This last significant digit (in this case, a trailing zero) is important to ascertaining whether values fall within this limit, because any value stated with one or more additional digits that rounds up to "98.0%" would be within the limit. "97.95%" for instance contains one additional digit compared to "98.0%," in the hundredths decimal place. To compare "97.95%" to "≥98.0%," the POSA would determine whether the additional digit is greater than or equal to 5, and if so add 1 to the preceding digit. Here, 9 plus 1 is 10 and so "97.95%" rounds up to "98.0%" and would be within the limit. However, in "97.92%," the additional digit is 2, which is less than 5, and does not round up, and thus "97.92%" is simply "97.9%" when compared to "98.0%" and is thus not within the limit "≥98.0%."

24. As a comparison, in the limit range "≤0.02%" (less than or equal to "0.02%"), the POSA would understand that the leading zeros before the 2 are not significant. Thus, only the last digit shown ("2") is significant, and "0.02%" contains only one significant figure. "0.025%" contains an additional digit ("5") that is greater than or equal to 5, and thus in comparing this value to "0.02%," the preceding digit is rounded up to "0.03%" which is not less than or equal to "0.02%" and accordingly "0.025%" is not within the limit.

IV. PATENTS-IN-SUIT

A. Lipid Composition Patents

25. I understand from counsel that the five Lipid Composition Patents belong to the same "family" in that all of them claim priority to U.S. Provisional Application No. 61/045,228 filed April 15, 2008, and/or are direct or indirect continuations of U.S. Patent Application No. 12/424,367, filed April 15, 2009, which was granted and issued as the '069 patent.

26. I further understand from counsel that the Lipid Composition Patents share a common specification based on their relationship as continuing applications but that the claims found at the end of each patent differ. The following table summarizes the dates and application numbers associated with the Lipid Composition Patents:

U.S. Patent No.	Issue Date:	U.S. Application No.	Filing Date:
		61/045,228	April 15, 2008
8,058,069 ("the '069 patent)	November 15, 2011	12/424,367	April 15, 2009
8,492,359 ("the '359 patent)	July 23, 2013	13/253,917	October 5, 2011
8,822,668 ("the '668 patent)	September 2, 2014	13/928,309	June 26, 2013
9,364,435 ("the '435 patent)	June 14, 2016	14/462,441	August 18, 2014
11,141,378 ("the '378 patent)	October 12, 2021	17/227,802	April 12, 2021

27. The Lipid Composition Patents provide for "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." '069 patent,[13] Abstract. The Lipid Composition Patents in particular describe "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." '069 patent, 5:51-56.

28. Claim 1 of the '069 patent, which is one of the claims reciting the disputed term, "mol % of the total lipid present in the particle," is reproduced below:

> **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 a mixture of a phospholipid and cholesterol or a derivative thereof, wherein the phospholipid comprises from 4 mol % to 10 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.

B. The '651 Patent

29. The '651 patent is titled "Lipid Compositions for Nucleic Acid Delivery" and teaches "processes and apparatus for making lipid vesicles that optionally contain a therapeutic agent." '651 patent, 2:12-14. The '651 patent was filed on June 13, 2014, and claims priority to U.S. Provisional Application No. 60/392,887 filed on June 28, 2002.

[13] For convenience, I have generally only cited to one of the Lipid Composition Patents in referring to the shared specification, but my opinions regarding citations to that patent's specification apply equally to all of the Lipid Composition Patents.

30. The '651 patent discloses that the "invention can be used to form lipid vesicles that contain encapsulated plasmid DNA or small molecule drugs." *Id.* at 2:16-18. The inventors of the patent discovered new ways to synthesize these vesicles, including with a device called a "T-connector." *See* '651 patent, Figure 13. The patent discloses that the inventors obtained vesicles with various beneficial properties, including the ability to encapsulate a surprisingly high percentage of the nucleic acid inside vesicles, thereby protecting the nucleic acid from degradation when the vesicles are administered to patients. *Id.* at 15:19-56, 18:30-43. The '651 patent claims particular lipid compositions having those improved properties.

31. Claims 1, 13, and 14 of the '651 patent are reproduced below:

> **1**. A lipid vesicle formulation comprising:
> (a) a plurality of lipid vesicles, wherein each lipid vesicle comprises:
> a cationic lipid;
> an amphipathic lipid; and
> a polyethyleneglycol (PEG)-lipid; and
> (b) messenger RNA (mRNA), wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.
>
> **13**. The lipid vesicle formulation of claim **1**, wherein at least 80% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.
>
> **14**. The lipid vesicle formulation of claim **1**, wherein about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

V. LEGAL STANDARDS

32. I understand from counsel that patent claim terms are generally given the plain and ordinary meaning that they would have had to the POSA as of the relevant date. I have been asked to assume the relevant date of the Lipid Composition Patents to be April 15, 2008, and the relevant date of the '651 patent to be June 28, 2002. As I discuss above, my opinions in this declaration would not change if I were to assume a different date. *See supra* § II & note 5.

33. I further understand from counsel that the plain and ordinary meaning of claim terms must be considered in the context of the entire patent claims in which they appear, as well as in the context of the other claims, the specification, and the prosecution or file history of the patent—that is, the complete record of the proceedings, filings, and communications exchanged between the patent applicant and the U.S. Patent & Trademark Office ("Patent Office" or "PTO") during examination—taken as a whole. I understand that the patent specification is the single best guide to the meaning of a claim term. I further understand that the claims, specification, and the file history constitute the "intrinsic evidence" of the patent relevant to claim construction.

34. I understand that an applicant may depart from the plain and ordinary meaning of a claim term in two instances. *First*, an applicant may provide a definition in the specification that differs from the plain and ordinary meaning, in which case the inventor's definition governs. However, I understand that any such definition must be clearly stated. *Second*, an applicant may disclaim the full scope of a term, such that the invention must include or not include a particular feature. I understand, however, that any such disclaimer must be clear and unmistakable in the specification and/or the prosecution history.

35. In addition to the "intrinsic evidence" above, I understand that certain "extrinsic evidence," such as expert and inventor testimony, dictionaries, and learned treatises, also may shed useful light on the relevant art and the way in which the POSA might use the claim term. However, I further understand that such extrinsic evidence must always be considered in the context of the patent's intrinsic evidence when determining the meaning of a claim term. In particular, I understand that extrinsic evidence may not be used to contradict a claim meaning that is unambiguous in light of the intrinsic evidence.

VI. THE PERSON OF ORDINARY SKILL IN THE ART

36. I understand that in IPR2018-00739, the Board adopted the following definition of the POSA with respect to the '435 patent:

> Accordingly, we find in the record as a whole that a person of ordinary skill in the art would have specific experience with, and/or be generally familiar with, lipid particle formation and use in the context of delivering therapeutic payloads, and would have a Ph.D., an M.D., or a similar advanced degree in an allied field (e.g., biophysics, microbiology, biochemistry) or an equivalent combination of education and experience.

37. I understand that the Board adopted the same definition in IPR2018-00554 with respect to the '069 patent. I further understand that the Board found me to possess at least the level of ordinary skill in the art under this definition, and I also note that I possessed the qualifications of the POSA as of any of the possible priority dates. *See supra* § II.16 & note 5. In this declaration, I have been asked to apply this definition and to explain the meaning of disputed terms to the POSA in the context of the specification and, where relevant, the file history. The opinions expressed in this report are provided from the vantage point of the POSA.

38. As I have discussed, I understand that the '435 patent shares a common specification with the other Lipid Composition Patents, including the '069 patent, and that Moderna previously applied this definition in the previous IPR proceedings. For the purposes of this declaration, I have applied this definition of the POSA adopted by the Board with respect to both the Lipid Composition Patents and the '651 patent. My opinions in this declaration would not change if I were to adopt a slightly different definition of the POSA. I reserve the right to opine further on the POSA's qualifications in light of any evidence offered by Moderna regarding the level of the ordinary skill in the art.

VII. CLAIM CONSTRUCTION

A. Lipid Composition Patents: "mol % of the total lipid present in the particle"

Plaintiffs' Construction	Moderna's Construction
Plain and ordinary meaning, *i.e.*, "mol % of the total lipid present in the particle" The recited "mol %" ranges are understood to encompass their standard variation based on the number of significant figures recited in the claim.	"__ mol % of the total lipid present in the finished lipid particle" Where the asserted claims do not recite "**about** __ mol %," Moderna contends that the recited "__mol%" ranges are understood as the exact ranges recited in the claim.
'069 Patent, Claims 1, 8, 15, 20, 21; '359 Patent, Claims 1, 7, 9, 10, 11, 12, 13, 18, 19; '668 Patent, Claims 1, 8, 10, 15; '435 Patent, Claims 1, 4, 7, 8; '378 Patent, Claims 1, 2, 7, 13, 18, 24, 25	

39. I understand that while Plaintiffs contend that the term "mol % of the total lipid present in the particle" recited in the claims of the Lipid Composition Patents should be construed in accordance with its plain and ordinary meaning, Moderna has proposed that the term instead be construed to mean "__ mol % of the total lipid present in the ***finished lipid*** particle," which adds the words "finished" and "lipid" to the claim text. Moderna also contends that the POSA would understand the recited mol % ranges "as the exact ranges recited in the claim." I understand that Moderna has clarified that "the exact ranges" referenced in its construction means that a value such as 49.999 mol % would not fall within the range of "from 50 mol % to 65 mol %" recited in claim 1 of the '069 patent, for example. For the reasons below, I disagree with both aspects of Moderna's proposed construction.

1. The meaning of "particle" in the context of the specification and file history is not limited to a "finished" particle, as Moderna uses the word "finished."

40. I understand that Moderna's construction is similar to the one Plaintiffs proposed but adds the term "finished" to the construction. I understand that Moderna has reasoned that the term "finished" must be read into the claims because, in their view, the asserted claims of the Lipid Composition Patents only encompass particles that are the "finished product," or a "final nucleic

acid-lipid particle" that does not undergo any further manufacturing steps. Moderna Invalidity Contentions at 137-39. I disagree. In my opinion, the POSA would understand that the claims encompass a nucleic acid-lipid particle without limitation as to when in the manufacturing process the particle is formed.

41. The term "particle" as used in the claims clearly refers to the "nucleic acid-lipid particle" set forth previously in the claim and described in the Lipid Composition Patents. I understand that the PTAB has already 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." '435 Patent Final Written Decision, IPR2018-00739, Paper No. 51 at 13 (P.T.A.B. Sept. 11, 2019). I understand that neither party is presently contesting that construction. I note that entirely absent from that definition is any limitation that these particles be "finished," as Moderna uses that term.

42. The specification describes that "the lipid particles of the present invention" can be formed by any method including, but not limited to, a continuous mixing method or a direct dilution process." *E.g.*, '378 patent, 59:27-33. For example, the specification teaches that "[b]y mixing [an] 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." *Id*. at 59:66-60:12. The specification makes clear that nucleic acid lipid particles formed as a result of this process are particles that are embodiments within the scope of the claims. The specification also discloses, however, additional embodiments for which "the methods will further comprise" additional processing to refine the particle. *Id*. at 61:37-62:27. These resulting particles, which have undergone subsequent processing after formation, are also disclosed to be within the scope of the claims. Contrary to

Moderna's proposed construction, the POSA would recognize that the specification does not include a limitation related to whether a particle will or will not undergo these post-formation modifications.

43. The POSA reading the asserted claims of the Lipid Composition Patents would understand they cover nucleic acid-lipid particles without any restrictions on whether the particles will be subjected to any further manufacturing steps. Neither "finished" nor any other language imposing such a restriction is present in any of the claims. The POSA would understand the term "particle" as used in the claims to refer to any formed particle, irrespective of whether it was the particle in the final drug product or whether it would be subjected to additional manufacturing and processing steps, including those that can change the particle and its composition.

44. The specification of the Lipid Composition Patents uses the term particle consistently with its ordinary meaning in the field. In the context of the specification, the term "mol % of the total lipid present in the particle" is not limited by whether the nucleic acid-lipid particle is formed mid-way through manufacturing and subject to further processing or manufacturing steps, or at the end of the manufacturing process. Particles that are formed at each of those stages are "particles" within the ordinary meaning of that term to the POSA in the context of the specification. *E.g.*, '378 patent, 59:25-62:27.

45. The POSA would recognize that the specification provides definitions of the nucleic acid-lipid particles recited in the claims. *E.g.*, '378 patent, 11:42-12:8, 57:60-67, 59:25-32. The specification describes a "lipid particle" as a "lipid formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid." *Id.* The specification explains that "stable nucleic acid-lipid particles" or "SNALPs" are within the scope of the invention, *e.g.*, '378 patent, 12:8-10 ("The lipid particles of the invention (e.g., SNALP)"), and further defines a

SNALP as "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 . . . is fully encapsulated within the lipid." *Id.* at 11:51-55.

46. As discussed above, I understand that during the IPR of the '435 patent, the PTAB adopted a definition of a "nucleic acid-lipid particle" consistent with that disclosed in the specification. '435 Patent Final Written Decision, IPR2018-00739, Paper No. 51 at 13 (P.T.A.B. Sept. 11, 2019). Consistent with the term's ordinary meaning, these definitions lack any limitations as to how the particle is formed, when in the manufacturing process it is formed, or whether it is subjected to further processing. Rather, the specification recites that the particles can be "formed by any method known in the art." '378 patent, 59:25-32. The POSA would understand the claims at issue in the Lipid Composition Patents are product claims that do not require any particular manufacturing process or that any manufacturing process be completed in order to constitute a "particle" within the meaning of the claims. Therefore, the claimed particle need not be "finished" in the sense that Moderna uses that word, as not being subject to further processing. The claimed "particle" need only be "formed," and until it is, there is no particle.

47. Moderna's use of the term "finished" is not synonymous with the term "formed" used in the specification's definition. Rather, Moderna's use of "finished" adds a temporal limitation to the claims because, under its construction, only the last-in-time particle of a manufacturing process would be within the scope of the claims. That interpretation is contrary to the ordinary meaning of particle in the field, in the context of the specification. The POSA would recognize that the specification expressly discloses embodiments that contravene Moderna's interpretation and teaches that particles within the claims can be modified and subject to further processing, "if needed." *Id*. at 61:4-62:27. Whether those further processing steps are performed

does not affect whether the particle, before further processing, is a "particle" within the meaning of the claim; it plainly is, per the specification's disclosures. The specification's embodiments would inform the POSA and confirm that the patent claims, in referring to a particle, do not require a "*finished*" particle (as I understand Moderna uses that term to refer to a particle not subject to further manufacturing steps), but rather just a *formed* particle.

48. As I discussed above, the specification describes several manufacturing methods for producing the "lipid particles of the claimed invention," including by a "continuous mixing method" or by a "direct dilution process." *Id*. at 59:27-65. The patent discloses that the "SNALP formed" by these processes can have particular properties and are within the claims. *Id*. at 59:66-61:4. The use of the term SNALP to refer to these structures confirms clearly that they are particles; in the SNALP acronym, the "P" stands for "particle," the precise word used in the claim. *E.g.*, *id*. at 11:51-55 ("As used herein, the term 'SNALP' refers to a stable nucleic acid-lipid particle."). The specification then teaches that "if needed," these particles can undergo further processing like homogenization, sonication, or extrusion. *Id*. at 61:4-62:27. Homogenization is a technique for sizing particles wherein an initial heterodisperse liposome preparation is pumped under high pressure through a small orifice or reaction chamber until a desired average size of liposome particles is achieved. U.S. Patent No. 4,737,323 (issued Apr. 12, 1988) at 1:44-2:44. Sonication is another technique for sizing particles and can be done using probe tips to deliver high energy input to the lipid suspension in order to break particles apart. *Id*. Extrusion is a technique in which a lipid suspension is forced through a polycarbonate filter with a defined pore size to yield particles of a desired size. These processes can result in new particles that may have lipid molar ratios that are different from the particles prior to these processes. *E.g.*, '378 patent, 61:16-18 ("Homogenization is another method which relies on shearing energy to fragment larger

particles into smaller ones."). Consistent with these teachings, the POSA would understand that particles formed before any such further processing, as well as particles resulting from these additional manufacturing steps, are both identified as SNALPs—"particles"—and are thus within the scope of the term "particle" in the claims (of course, the particles must meet the claims' additional limitations to fall within the scope of the claims as a whole).

49. As an additional example, the specification discloses methods for forming "SNALP-CPLs." *E.g.*, '378 patent, 62:8-62:27. SNALP-CPLs are described as "lipid particles of the invention" that contain cationic polymer lipid conjugates, or "CPLs." *Id.* at 22:28-46. The specification teaches that the POSA can create these particles by either using the "standard" technique—wherein "the CPL is included in the lipid mixture during, for example, the SNALP formation steps," or the "post-insertion" technique—which is "insertion of a CPL into, for example, a pre-formed SNALP." *Id*. at 62:8-27. In the "post-insertion" technique, a SNALP (a "particle") is formed, and then it is subjected to a further manufacturing process—called the "post-insertion" technique, in which a CPL is inserted "into . . . a pre-formed SNALP," resulting in a different SNALP. *Id*. In other words, the specification expressly teaches that "the lipid particles of the invention" "*may* comprise" this additional lipid, and refers to the particles before the insertion of this additional lipid as "SNALPs" within the scope of the claims. *Id*. at 22:28-46, 62:8-27. Likewise, the specification teaches that the resulting "SNALP-CPLs," which may have altered lipid ratios following the insertion of the additional lipid, is equally within the scope of the claims. Under Moderna's construction, however, only one of these categories of particles—those that did not undergo subsequent processing—would be encompassed by the claims' use of the word "particle."

50. The specification also explains that certain methods of the invention may include

"adding non-lipid polycations which are useful to effect the lipofection of cells using the present compositions," and that "[a]ddition of these salts is preferably after the particles have been formed." '378 patent, 61:38-47. Under Moderna's construction, however, these pre-addition particles would be outside the scope of the claims, even though they are explicitly referred to as "particles" by the specification.

51. There is nothing in the specification that would suggest to the POSA that the term "finished" (as Moderna uses it to require that a particle is not subject to further manufacturing steps, as opposed to synonymously with "formed") should be read into the claims. The word "finished" never modifies "lipid particles" anywhere in the specification. That term is used only in the context of the "Finished Product Characterization" in Tables 2, 4, 6, and 7. There, the POSA would understand that the term "finished" is used to distinguish the starting materials and the formed particles, consistent with other disclosures in the specification drawing a similar distinction. '378 patent, 25:55-59, 61:48-62:8 (distinguishing "nucleic acid to lipid ratios . . . in a formed SNALP" from "ratio of the starting materials"), 70:32-43. That is also consistent with the understanding in the field, since the manufacturing process can result in a deviation in the lipid ratios of the formed particles as compared to the ratio of the starting material inputs. The POSA would understand that the claims do not encompass the lipid starting materials—they are not a "particle" that has been formed.

52. Regardless of what "finished" means in the tables, the term "particle" in the claim is not limited to a particle that is not subject to further manufacturing steps. Neither the inclusion of the term "finished" nor anything else in the specification would lead the POSA to conclude that the claims are limited to lipid particles that undergo no subsequent manufacturing steps and somehow exclude formed "particles" that are further modified or processed. Such an interpretation

is contrary to the POSA's understanding of the plain meaning of the term "particle" and the clear disclosure of the specification, which deems structures to be "particles" once they are formed, even if they undergo further manufacturing or processing. *E.g.*, '378 patent, 22:28-46, 61:4-62:27.

53. I understand that Moderna has cited statements made by Plaintiffs and by me as an expert during the IPR of the '435 patent, as well as the appeal that followed, as support for reading into the claim language a requirement that the particle not be subject to further manufacturing steps. As I stated above, I submitted a declaration in those proceedings in which I provided expert opinions on the patentability of the claims of the '435 patent in view of the asserted grounds and the cited prior art. In particular, I responded to Moderna's claim that a patent publication (US 2006/0240554 or the "'554 publication") anticipated the challenged claims in view of its disclosure of a formulation termed L054. I opined that the '554 publication failed to anticipate the challenged claims in part because "[t]he 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." IPR2018-00739, Exhibit No. 2009 ("Thompson '435 IPR Decl.") ¶ 110. I have excerpted the relevant portion of Table 4 from the '554 publication below:

TABLE IV

Lipid Nanoparticle (LNP) Formulations

Formulation #	Composition	Molar Ratio
L051	CLinDMA/DSPC/Chol/PEG-n-DMG	48/40/10/2
L053	DMOBA/DSPC/Chol/PEG-n-DMG	30/20/48/2
L054	DMOBA/DSPC/Chol/PEG-n-DMG	50/20/28/2
L069	CLinDMA/DSPC/Cholesterol/PEG-Cholesterol	48/40/10/2
L073	pCLinDMA or CLin DMA/DMOBA/DSPC/Chol/PEG-n-DMG	25/25/20/28/2

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—the input starting materials—not a nucleic acid-lipid particle as claimed in the Lipid Composition Patents. There is no disclosure in the '554 publication regarding the composition of particles generated or formed using the L054

formulation. In distinguishing the '435 patent claims from the prior art, I distinguished the "claimed" "particle" from "components or mixture of lipids used to form particles"—in other words, the difference between the formed particle and the starting materials used to form it. *Id*.

54. Further explaining this distinction, I noted that Moderna's argument "confuse[d] the composition of the input formulation (i.e., lipids of Table 4 [of the asserted prior art '554 publication]) with something different—i.e., the output formulation (i.e. lipid particles)" and that Moderna's anticipation argument necessarily failed because "there is no disclosure in the '554 publication regarding the composition of particles generated using the L054 formulation." Thompson '435 IPR Decl. ¶ 110. As I explained, "[t]his 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." *Id.* I also noted that both the '435 patent and the '554 publication presented data that "input and output formulations are not identical" and 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." *Id.* ¶ 111. As such, I discussed that "[t]esting 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." *Id.* ¶ 110.

55. As is evident from this discussion, my IPR declaration, and Plaintiffs' materials that cite my declaration, I used the term "finished product" to refer to a nucleic acid-lipid particle that was actually formed, as distinguished from the starting materials used to form it. As I discussed above, that is consistent with the use of the term "finished" in the context of the patent specification and in the field more generally. The final product administered to patients, at the end

of the manufacturing process, is indeed a particle within the scope of the claim. But so too are particles formed during the manufacturing process that are subject to further manufacturing or processing steps; those particles are likewise formed or finished particles, as distinguished from starting materials that can have a different lipid composition and ratio. My statements during the IPR distinguished starting materials from formed particles; they did not suggest that the nucleic acid-lipid particle must be free from any additional processing in order to fall within the scope of the claims, and I disagree with Moderna's contention that the claims contain such a limitation.

2. The POSA would interpret the claimed mol % ranges using significant figures and rounding.

56. As I discuss above, significant figures and rounding are standard scientific conventions that the POSA would have been aware of and would have applied in interpreting the claims of the Lipid Composition Patents. With respect to the recited mol % ranges, the POSA would have known that lipid concentrations could be experimentally determined, for example, using high-performance liquid chromatography ("HPLC"). *See, e.g.*, Tam 2000[14] at 1872 ("The final lipid concentration was determined by HPLC analysis."); Heyes 2006[15] at 282, 284 ("To ascertain lipid stability, we studied lipid concentrations over time in SPLP samples stored at different temperatures. Formulations . . . were examined by HPLC for degradation at 5, 25 and 40 °C."). Accordingly, the POSA would have known that mol % values, as with measured values more generally in the field, are subject to numerical uncertainty, and would have interpreted the claimed mol % ranges, in the context of the patent specification, using the standard convention of significant figures and rounding. *See supra* § III.B.

[14] P. Tam et al., *Stabilized plasmid-lipid particles for systemic gene therapy*, Gene Therapy, vol. 7, pp. 1867-74 (2000) ("Tam 2000").

[15] J. Heyes et al., *Synthesis and characterization of novel poly(ethylene glycol)-lipid conjugates suitable for use in drug delivery*, Journal of Controlled Release, vol. 112, pp. 280-90 (2006) ("Heyes 2006").

57. The intrinsic evidence establishes that the POSA would have understood the inventors to have expressed the claimed mol % ranges using significant figures. In particular, the specification shows that the inventors used significant figures to express mol % with varying precision, consistent with how significant figures are used and applied in the field. Table 2 of the specification, for instance, lists mol % values for different compositions in terms of a ratio of the PEG-lipid PEG(2000)-C-DMA to cationic lipid DLinDMA to phospholipid DPPC to cholesterol. Several examples in Table 2 state mol % for each of the lipid components with no digits in the tenths position, but others include values where the inventors used digits, including trailing zeros, to indicate precision to tenths. That distinction is a clear indication that the inventors, consistent with standard convention, used significant digits to convey the degree of precision and rounding to apply to the percentages of lipids in the claimed particles. The use of trailing zeros (shown below as zeros after the last non-zero digit following a decimal point) in particular further confirms to the POSA that the inventors were aware of and used significant figures to express mol % values with different degrees of precision. I have highlighted these trailing zeros in Table 2 below:

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
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

'069 patent, 69:24-45.

58. Table 4 similarly reflects the inventors' use of trailing zeros and significant digits to state mol % values with different degrees of precision:

TABLE 4

Characteristics of the SNALP formulations used in this study.

	Formulation Composition	Lipid/Drug	Finished Product Characterization		
Group	Lipid Name & Mole %	Ratio	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

'069 patent, 71:24-53.

59. Simply put, there is no reason to use numbers like "40" (in the first example in the table) and then numbers like 60.0 (in group number 10), other than to convey that the latter has more significant digits for rounding purposes than the former. Were the inventors not applying significant digits, they would have reported the values in the same way, such as 40 and 60; the POSA would have understood the specification to use significant figures and rounding in accordance with their usual convention. The specification clearly conveys the number of significant digits used with each reported value, and the POSA would have credited that disclosure in interpreting the values in the claims.

60. The plain and ordinary meaning of the mol % ranges recited in the claims of the Lipid Composition Patents thus would be interpreted by the POSA using the ordinary rules of significant figures and rounding. Illustratively, claim 1 of the '069 patent recites "a cationic lipid comprising from 50 mol % to 65 mol %." '069 patent, 91:26-27. The lower endpoint of the range "50 mol %" is "significant to the last digit shown," USP 23 at 3, and thus contains two significant figures and has precision (or is significant to) to the units place. A value of "49.**4** mol %" has an additional significant figure (".4") compared to "50 mol %." "[T]his digit is smaller than 5," and thus "it is eliminated and the preceding digit is unchanged," leaving the rounded value for comparison "49 mol %." A value of "49.**5** mol %" also contains an additional significant figure (".5") but it "equals 5," and so "the 5 is eliminated and preceding digits is increased by 1, leading to a rounded value of "50 mol %." Thus, under the ordinary rules of rounding, "50 mol %" would be understood by the POSA to encompass values of 49.5 mol % or higher. The following table summarizes this analysis with respect to the mol % ranges in claim 1 of the '069 patent.[16]

'069 Patent, Claim 1	Recited Range	Lower Limit	Upper Limit
Cationic Lipid	"50 mol % to 65 mol %"	49.5 mol %	65.4 mol %
Phospholipid	"4 mol % to 10 mol %"	3.5 mol %	10.4 mol %
Cholesterol	"30 mol % to 40 mol %"	29.5 mol %	40.4 mol %
Conjugated Lipid	"0.5 mol % to 2 mol %"	0.45 mol %	2.4 mol %

61. Moderna's approach, in contrast, requires "50 mol %" to be read quite differently than this plain and ordinary meaning. I understand from counsel that under Moderna's interpretation of the "exact ranges" set forth in its construction, a value of "49.999 mol %" would *not* fall within the claimed range of "from 50 mol % to 65 mol %." Moderna contends that any

[16] Counterfactually, if "50.0 mol %" were recited instead of "50 mol %," then "49.5 mol % has the same precision as "50.0 mol %" and does not round. Instead, following the same protocol above, *see supra* § III.B, the value "49.95 mol % would round up to "50.0 mol %" and the value "49.94 %" would round to "49.9 mol %."

value numerically less than "50" falls would fall outside this range, thereby rewriting "50 mol %" instead to have an *infinite* number of trailing zeros after the decimal point. This may be denoted, by standard convention, with a horizontal bar over the trailing zero in 50.0. No such denotation or other indication of infinite precision is present in the claims.

62. I disagree with Moderna's construction. Based on my review of the specification and prosecution history, there is no support for reading in additional precision into the claimed mol % values. The specification in fact reflects, to the contrary, that the inventors described mol % values with the precision reflected in the claims; when more precision was intended, additional significant figures were used. I have discussed Tables 2 and 4 above, and note additionally the formulations with "2" mol % conjugated lipid, "40" mol % cationic lipid, "10" mol % phospholipid, and "48" mol % cholesterol. *See* '069 patent, 69:24-45 (Table 2, Sample Nos. 1 and 16), 71:24-53 (Table 4, Groups 2, 4, 5, 7, 13). The specification also provides a general description of the mol % ranges for the lipid components that reflects consistent precision. For instance, the specification states that

> 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.

'069 patent, 18:40-46. This illustrative excerpt shows that the inventors found it perfectly acceptable to use two significant figures to describe the mol % of cationic lipid; I am not aware of any disclosure in the specification that supports reading "50 mol %" as "50.0 mol %," or "50.000 mol %" or with infinite trailing zeros after the decimal as I understand Moderna to contend.

63. Similarly, in describing the mol % of the conjugated lipid, the specification states:

> 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.

'069 patent, 22:30-42. Here too, the specification reinforces that when the inventors recited the value "2 mol %" or "1.5 mol %" for the conjugated lipid, that was the precision they intended. Notably, "2 mol %" would encompass "1.5 mol %" to "2.4 mol %," but "1.5 mol %" would encompass "1.45 mol %" to "1.54 mol %" which is a distinct (if overlapping) range.

64. Moreover, I am not aware of any established convention in the field requiring mol percentages to be expressed with more precision than what the inventors provided in the claims and specification. Nor am I aware of any data or statements in the specification, prosecution history, or otherwise, establishing, for example, that a particle with "50 mol %" cationic lipid versus one with "49.9 mol %" cationic to be a critical difference between them.

65. Finally, I understand that Moderna's construction relies on the absence of the term "about" in the claims where the disputed "mol % of the total lipid present in the particle" terms appears. The absence of "about" or other terms of approximation in the claims does not change my opinion that the POSA would read the claimed mol % ranges in accordance with the standard scientific convention of significant figures and rounding. As I explain above, *see supra* § III.B, rounding based on significant figures is not a matter of approximation or variance; it is the way that numbers are expressed and interpreted when measurement or some other experimental imprecision applies, which is the case here.

66. Moreover, in this case, I understand that the patentee amended the claims to remove the term "comprising about" from then-pending claims based on the Examiner's construction of "comprising about" to mean "an amount +/- 10, 20, 30 mol % of a lipid component." *See* '069 File History, Jan. 31, 2011 Reply to Office Action; '069 File History, May 12, 2011 Non-Final Rejection; '069 File History, Aug. 11, 2011 Reply to Office Action. The POSA would recognize the file history to use the term "comprising about" to refer *not* to significant figures and rounding—the Examiner plainly provided a different understanding. Thus, the POSA viewing the file history would understand that the removal of "comprising about" does not reflect that the inventors intended to exclude rounding from the claims. There is no indication, or any "clear and unmistakable" statement, that in removing the term "comprising about" from the claims, the applicant sought to depart from the standard convention of significant figures and rounding or to impose the infinite precision that Moderna proposes to read into the claim.

67. In particular, I understand that during prosecution of the '069 patent, claim 1 submitted by the applicant originally recited mol % ranges using "about":

> 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.

'069 File History, Jan. 31, 2011 Reply to Office Action at 2.

68. The Examiner rejected then-pending claim 1 of the '069 patent as allegedly anticipated by U.S. Patent Application Publication No. 2006/0008910 (published Jan. 12, 2006) ("MacLachlan '910"). In making this rejection, however, the Examiner stated:

> Claims 1-4, 9, 14, 17-26, 47, and 48 are rejected under 35 U.S.C. 102(b) as being anticipated by MacLachlan, *et al.* (US 2006/0008910, of record) ("MacLachlan").
>
> The claims are to a nucleic acid lipid particle comprising a nucleic acid, a cationic lipid, a non-cationic lipid mixture of phospholipid and cholesterol, and a conjugated lipid. The claims are further directed to the particle wherein the nucleic acid is a siRNA, the relative amounts of components read on a broad range of amounts because of the term "comprising about". The applicants do not provide a definition of the term in the specification. Thus, "comprising about" could embrace an amount +/- 10, 20, 30 mol % of a lipid component.

'069 File History, May 12, 2011 Non-Final Rejection at 2.

69. The applicant responded by amending claim 1 to remove the word "about":

> 1. (Currently amended) 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.

'069 File History, Aug. 11, 2011 Reply to Office Action at 2.

70. The applicant further explained that these amended claims not including "about"—and therefore not susceptible to the Examiner's expansive interpretation of "about" to mean "+/- 10, 20, 30 mol % of a lipid component"—were not anticipated by the broad ranges disclosed by MacLachlan '910 because the reference did not teach the claimed ranges with "sufficient specificity." '069 File History, Aug. 11, 2011 Reply to Office Action at 6. The Examiner subsequently allowed the claims to issue as the '069 patent. '069 File History, Sept. 12, 2011 Notice of Allowance.

71. The POSA reading the file history of the Lipid Composition Patents would therefore not understand the applicant's amendment to have disclaimed the application of significant figures and rounding to the claims. Rather, the prosecution history shows that the Examiner construed "about" to mean "+/- 10, 20, 30 mol %," and the applicant's amendment addressed that construction. The prosecution history of the Lipid Composition Patents thus makes clear that the meaning of "about" as "+/- 10, 20, 30 mol %" is unconnected to whether the POSA would have interpreted the claimed mol % ranges using the ordinary rules of significant figures and rounding. The prosecution history does not at all support that the applicant intended the claimed ranges to be infinitely precise under Moderna's position that ranges would be understood to be "exact." I therefore disagree with Moderna's construction.

B. The '651 Patent: "wherein at least 70% / at least 80% / about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles"

Plaintiffs' Construction	Moderna's Construction
"wherein at least 70% / at least 80% / about 90% of the mRNA in the formulation is contained inside the lipid vesicles"	"fully, as distinct from partially, encapsulated"
'651 Patent, Claims 1, 13, 14	

72. I understand that Moderna has argued that the phrase "fully encapsulated" as used in claims 1, 13, and 14 of the '651 patent should be construed as "fully, as distinct from partially,

encapsulated." I disagree. In my opinion, the POSA would have understood the term "fully encapsulated" within the context of the full claim limitations in which it appears: "wherein at least 70% / at least 80% / about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles." Taking into account this context and the claims and specification as a whole, the POSA would have understood the whole claim limitation to mean "wherein at least 70% / at least 80% / about 90% of the mRNA in the formulation is contained inside the lipid vesicles."

73. In my opinion, the POSA reading the claim limitation "wherein at least 70% / at least 80% / about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles" would have understood that the full claim term at issue delineates (1) the proportion of mRNA encapsulated in lipid vesicles (at least 70% / 80% or about 90%), and (2) the location of the mRNA in the vesicles (the mRNA must be *contained inside* the vesicles).

74. Regarding the first point above, the POSA reviewing the plain language of claims 1, 13, and 14 of the '651 patent would have understood that numerical references to at least 70% / at least 80% / about 90% to refer to the proportion of mRNA encapsulated by lipid vesicles—this value is typically referred to in the field as "encapsulation efficiency."

75. At the time of the invention, encapsulation efficiency was a standard measurement for assessing the properties of a lipid vesicle. The encapsulation efficiency of a lipid vesicle formulation refers to the measurement of the total percent of nucleic acid that is encapsulated by lipid vesicles, as a proportion of the total amount of nucleic acid in the composition.

76. As explained above and further below, this encapsulation or protection of nucleic acid can occur, for example, "within a relatively disordered lipid mixture," '651 patent, 5:35-37, where the nucleic acid is present outside the lipids and encapsulated in a complex web of lipids. *See supra* § III.A. Alternatively, this encapsulation or protection can occur where the nucleic acid

is contained inside a lipid vesicle. This distinction is addressed in further detail below.

77. A standard method of measuring this encapsulation efficiency was conducting an assay utilizing a dye that fluoresces when it binds to nucleic acid. The intrinsic evidence confirms that the POSA would have recognized the applicability of this assay.

78. Initially, the POSA would have recognized the various disclosures of encapsulation percentages in the specification as reporting encapsulation efficiency. The written description of the invention frequently refers to the improved encapsulation efficiency of the invention. For example, the specification discloses that "[a]dvantageously, 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%." '651 patent, 8:5-10, 12:64-13:1.

79. Separately, the written description of the invention discloses numerous measurements of encapsulation efficiency that correspond to the percentages included in claims 1, 13, and 14 of the '651 patent ("at least 70%," "at least 80%," and "about 90%"). For example, the patent discloses that "[i]n certain aspects, the initial therapeutic product encapsulation efficiency is as high as about 90%." '651 patent, 2:51-54. This corresponds to the percentage included in claim 14 ("wherein about 90% of the mRNA in the formulation is fully encapsulated"). The patent also discloses a preference for "70-95% plasmid DNA ("pDNA) encapsulation after mixing, and ethanol removal steps." *Id*. at 12:59-61. This corresponds to the claimed percentages in claims 1, 13, and 14. The specification further discloses 70-80% rates of encapsulation, *id.* at 9:24-26, 36-38, which correspond with claims 1 and 13; "77-90% DNA encapsulation" rates, *id.* at 15:32-33, which corresponds with the percentages in claims 1, 13, and 14; and that "the SPLP formed according to the processes of the present invention had 85% DNA encapsulation," *id.* at 18:40-42,

which corresponds at least to the claimed percentage in claim 13.

80. Further, the figures in the '651 patent, which show measurements of encapsulation percentages, would further inform the POSA's understanding of the claims. For example, Figures 5, 6, 7, and 8 show measures of "DNA Encapsulation (%)" or "% Encapsulation" as various aspects of the processes disclosed in the specification are modulated, including the ethanol concentration of the initial lipid solution (Fig. 5), the pH of initial plasmid solution and the buffer solution (Figs. 6 & 7), and the concentration of salt (NaCl) after dilution (Fig. 8). *See* '651 patent, 3:17-29. The POSA reviewing these figures and their descriptions would understand that this encapsulation percent is referring to encapsulation efficiency, as measured by a standard assay, such as a fluorescence assay.

81. When reading the claims in light of the written description and the figures in the specification, including the emphasis on the invention's advance in leading to improved encapsulation efficiencies, the inclusion of disclosures of specific percentages of encapsulation efficiency that are tied to the specific claimed percentages of fully encapsulated mRNA, and the figures in the specification, the POSA would understand the numerical values in the percentage terms to refer to encapsulation efficiency percentages.

82. Reviewing the file history would provide further confirmation to the POSA that the percent fully encapsulated referred to encapsulation efficiency. During prosecution, a third-party submitted a declaration to the Examiner contending that WO 98/51278 (published Nov. 19, 1998) ("Semple") disclosed a process for preparing "[l]ipid vesicles, such as liposomes or lipid-nucleic acid particles, fully encapsulating nucleic acids." '651 File History, Jan. 5, 2015 Third Party Submission at 10. Semple reports the use of a "fluorescent dye binding assay" to assess encapsulation efficiency. Semple at 36:1-4. Using that assay, Semple reports encapsulation

efficiencies of greater than 50%. *Id*. at 13:24-29. The Examiner then rejected all the claims in the application over Semple, which the Examiner argued taught lipid vesicles, specifically lipid-nucleic acid particles and liposomes, "which comprise mRNA, [and the specific lipid components claimed in the '651 patent], wherein the nucleic acids are enclosed by the lipid vesicle (i.e., protected from degradation)." '651 File History, Feb. 13, 2015 Non-Final Rejection at 10.

83. The applicant then responded by arguing that "the method described in Semple *et al.* for preparing liposomes is not able to produce the population of smaller lipid vesicles of the present invention with high mRNA encapsulation efficiency that is desirable for *in vivo* and clinical applications." '651 File History, May 12, 2015 Response at 5.

84. The Examiner then issued a Final Rejection, arguing that the rejection was not overcome by the applicant's statements or the accompanying declaration from Dr. Heyes, in part because even though the applicant argued "that the encapsulation of the mRNA using the method of Semple is not as effective and/or does not show the high mRNA encapsulation which is desired in the instant invention, . . . the features upon which applicant relies (i.e., encapsulation efficiency and lower PDI) are not recited in the rejected claim(s)." '651 File History, June 19, 2015 Final Rejection at 14.

85. The applicant then filed a Request for Continued Examination ("RCE"), along with amendments to the claims to include the limitations "wherein at least 50% / at least 70% / at least 80% / about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles." '651 File History, June 19, 2015 RCE at 2-3. The applicant made these amendments to overcome the Examiner's rejections over Semple. *Id.* at 8 ("[T]he Examiner notes that this feature of encapsulation efficiency is not recited in the rejected claims. In an earnest effort to advance prosecution, but without acquiescing on the merits of the present rejections, Applicants have

amended claim 1 to recite a lipid vesicle formulation wherein at least 50% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.").

86. The applicant described the limitations at issue, numerous times, as limitations on the encapsulation efficiency of the lipid vesicles of the claims and noted that the claimed encapsulation efficiency was greater than the encapsulation efficiency produced by the methods of Semple. *E.g.*, *id.* at 7 ("Heyes Declaration clearly shows that the method described in Semple *et al.* for preparing liposomes containing antisense oligonucleotide is not suitable for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed."); *id.* at 8 ("the method of Semple *et al.* for preparing liposomes containing antisense oligonucleotide is not suitable for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed"); *id.* ("the very low encapsulation efficiency associated with the method described in Semple *et al.* is well below the minimum mRNA encapsulation efficiency required in the presently claimed formulations."); *id.* at 9 ("Applicants have provided sufficient objective evidence in the Heyes Declaration to demonstrate that the method described in Semple *et al.* for preparing liposomes is simply not suitable for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed.").

87. The Examiner then found that "Applicant's amendments to the claims, filed 8/18/15, have rendered the previous 103 rejections moot." '651 File History, Oct. 9, 2015 Non-Final Rejection at 8. However, the Examiner found that a different reference, U.S. Patent No. 6,734,171 ("Saravolac") taught processes for forming liposomes and lipid-nucleic acid particles, "wherein the encapsulation efficiency of the mRNA in the vesicles is about 80% and can even approach 90% which reads upon the instantly claimed at least 70%, more specifically at least 80%, and about 90%." *Id.* at 7. In other words, just as the applicant had equated the amendments to the

claims to include the percent fully encapsulated limitations with a measurement of encapsulation efficiency, the Examiner also understood the limitations of the amended claims to refer to encapsulation efficiency.

88. To overcome the Examiner's rejections, the applicant submitted further responses and declarations from Dr. Heyes, detailing that the process disclosed in Saravolac would not produce a lipid vesicle fully encapsulating mRNA with the claimed encapsulation efficiencies. '651 File History, Dec. 14, 2015 Response at 4-7; '651 File History May 19, 2016 Response at 4-7. Saravolac disclosed encapsulation of DNA plasmid, but not a process for the encapsulation of mRNA. *Id.* Again, the applicant and Dr. Heyes equated numerous times the claimed limitations for the percent fully encapsulated with measures of encapsulation efficiencies. '651 File History, Dec. 14, 2015 Response at 4-7; '651 File History, Dec. 14, 2015 Heyes Decl. at 2-5; '651 File History, May 19, 2016 Response at 4-7; '651 File History, May 19, 2016, Heyes Decl. at 2-5.

89. The Examiner ultimately found that the applicant's remarks and Dr. Heyes's affidavits overcame the rejections based on Saravolac, specifically, because the applicant demonstrated that "Saravolac does not encapsulate mRNA with the same efficiency as other nucleic acids and as such does not function as effectively the claimed lipid vesicles which applicants have demonstrated do effectively encapsulate mRNA with efficiencies of greater than 70%." '651 File History, Oct. 3, 2016 Non-Final Rejection at 6.

90. Subsequently, the Examiner issued a Notice of Allowance ("NOA"), providing as a reason for allowance that "applicants have been able to demonstrate that their method of making liposomes allows for liposomes which more effectively encapsulate mRNA over the methods/liposomes of the prior art and have shown this effective encapsulation through the submission of several declarations under 1.132. No further art was found which had these high

encapsulation efficiencies of mRNA." '651 File History, Oct. 19, 2016 NOA at 2. Reviewing this prosecution history—including the consistent understanding of the claim limitation shared by the Examiner, the applicant, and Dr. Heyes—would confirm for the POSA that the numerical values in the percent fully encapsulated terms refer to encapsulation efficiency percentages.

91. Technical literature further confirms that the POSA would have known how to measure encapsulation efficiency with a fluorescent dye and would have understood the percent fully encapsulated term as referring to encapsulation efficiency. First, a specified amount of the dye would be added to a formulation of the lipid vesicles. The dye that is added to the formulation is lipid impermeable—that is, it cannot pass through a lipid barrier—and thus it cannot access or bind to nucleic acid that is encapsulated by the lipid and only binds to nucleic acid that is *not* encapsulated by the lipid. After adding the dye, the POSA would then measure the formulation's fluorescence intensity. Subsequently, a detergent is added to the formulation to break down the lipid barrier, which releases the encapsulated nucleic acid (which the dye, before the detergent was added, could not reach), and the fluorescence intensity is again measured. The second measurement identifies all of the nucleic acid that was in the formulation (including the nucleic acid that was previously inaccessible to the dye). With these measurements, the POSA could calculate the encapsulation efficiency as follows:

$$\text{Encapsulation efficiency (\%)} = (I_o - I)/I_o \times 100$$

where I and I_o refer to the fluorescence intensities before and after the lipid vesicles are broken down. *See, e.g.*, Zhang 1999[17] at 1439, 1445; MacLachlan 2007[18] at 253-254; Heyes 2005[19] at

[17] Y.P. Zhang et al., *Stabilized plasmid-lipid particles for regional gene therapy: formulation and transfection properties*, Gene Therapy, vol. 6, pp. 1438-1447 (1999) ("Zhang 1999").
[18] I. MacLachlan, *Liposomal Formulations for Nucleic Acid Delivery*, Antisense Drug Techs. (2d ed. 2007) ("MacLachlan 2007").
[19] J. Heyes et al., *Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids*, J. Controlled Release, vol. 107, pp. 276-87 (2005) ("Heyes 2005").

279; WO 00/03683 (filed Jan. 27, 2000) at 24:1-8. For example, if there were no difference between the fluorescence intensities measured in the first step and the fluorescence measured in the second step, that would indicate that 0% of the nucleic acid was encapsulated by the lipid system, whereas if there were no fluorescence at all at the first step and then fluorescence after the detergent was added, that would indicate that 100% of the nucleic acid was encapsulated by the lipid system. The POSA would have recognized that these differential fluorescence-based assays were used extensively by the priority date to measure encapsulation efficiency and would have understood the process for measuring encapsulation efficiency.

92. The POSA would further understand that the full claim limitations "wherein at least 70% / at least 80% / about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles" also limit the invention of the '651 patent based on *where* the mRNA is located, *i.e.*, the mRNA must be ***contained inside*** the vesicle.

93. At the time of the invention, the POSA would have understood that different lipid vesicle systems that could be used for the delivery of therapeutic agents such as nucleic acids would have the nucleic acid in different locations of the vesicle system. The '651 patent describes some of these locations in lipid vesicle systems including (1) SPLPs, in which a "vesicle of lipids coating an interior comprising a nucleic acid such as a plasmid with a reduced aqueous interior," (2) 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"; and (3) "lipid aggregates or micelles, wherein the encapsulated component is contained within a relatively disordered lipid mixture." *See* '651 patent, 5:30-37. These different locations of nucleic acid in various lipid systems were well-understood to the POSA at the time of the invention, for example the "meatballs" and "spaghetti" arrangement I

discussed above, *see supra* § III.A; Sternberg 1994 at 364, Figure 2, as well as others. *See, e.g.*, Wheeler 1999;[20] Chonn 1995;[21] Mok 1999.[22]

94. Immediately following the '651 patent's description of the different locations in which the vesicles can retain nucleic acids, the specification refers to lipid formulations that provide "a compound with full encapsulation, partial encapsulation, or both." '651 patent, 5:38-40. The POSA would understand that this reference to "full" and "partial" encapsulation was in reference to the different potential locations of the encapsulated nucleic acid (such as mRNA)—"within a relatively disordered lipid mixture" or in the "interior." *See* '651 patent, 5:30-44; *supra* § III.A. In view of this context, the POSA would understand that a partially encapsulated nucleic acid is one that is contained within the relatively disordered lipid mixture (but not actually inside any lipid vesicles), whereas a fully encapsulated nucleic acid is inside the lipid vesicle. *See, e.g.*, Sternberg 1994 at 364, Figure 2; *supra* § III.A.

95. The mRNA's location can have substantial practical effect, as when nucleic acid is not contained inside a lipid vesicle, it can result in toxic side effects, degradation, and rapid clearance. *See* Wheeler 1999 at 271 ("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 lung. Plasmid DNA-cationic lipid complexes can also result in toxic side-effects both in vitro and in vivo."). Given the POSA's understanding of the difference

[20] J. J. Wheeler et al., *Stabilized plasmid-lipid particles: construction and characterization*, Gene Therapy, vol. 6, pp. 271-281 (1999) ("Wheeler 1999").
[21] A. Chonn et al., *Recent advances in liposome drug delivery systems*, Curr. Opin. Biotech, vol. 6, pp. 698-708 (1995) ("Chonn 1995").
[22] K. Mok et al., *Stabilized plasmid–lipid particles: factors influencing plasmid entrapment and transfection properties*, *Biochim Biophys Acta*, vol. 1419, pp. 137-150 (1999) ("Mok 1999").

between nucleic acid being contained inside the lipid vesicle (in the "interior"), compared to being on the outside of the lipid vesicle ("within a relatively disordered lipid mixture") the POSA would understand that the reference to "fully encapsulated" in the claims of the '651 patent refers to the location of nucleic acid—inside the vesicle. *See* '651 patent, 5:30-44.

96. The file history further confirms that the POSA would understand the limitation "wherein at least 70% / at least 80% / about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles" related to nucleic acid being contained inside the lipid vesicle. The Examiner rejected the initial claims, which included the "fully encapsulated" language, over U.S. Patent No. 5,830,430 ("Unger"), which the Examiner argued taught a lipid vesicle that encapsulates nucleic acid. '651 File History, Aug. 14, 2014 Non-Final Rejection at 5-6. The applicant distinguished Unger, noting that "*none* of the examples in Unger et al. . . . discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA," including because it concerned a lipid system "in which little, if any, of the DNA payload is encapsulated within the preformed cationic liposomes," and instead "is merely associated with the surface of the preformed liposome." '651 File History, Oct. 22, 2014 Response at 5-7. That differed from "the encapsulated mRNA present within the lipid vesicles of the present invention." *Id.*

97. Dr. Heyes drew the same distinction during prosecution. '651 File History, Oct. 22, 2014 Decl. ¶ 10 ("[T]he cationic lipid compounds and cationic liposome formulations disclosed in Unger *et al.* are exemplified in the context of their use for preparing preformed liposomes to form complexes with DNA called lipoplexes. However, lipoplexes are electrostatic complexes in which little, if any, of the DNA payload is encapsulated within the preformed cationic liposomes. . . . Importantly, the encapsulated mRNA present *within the lipid vesicles of the present invention* will be protected from nuclease degradation upon systemic administration, while *nucleic acid that*

is merely associated with the surface of a preformed liposome (such as the DNA of the lipoplexes of Unger *et al.)* will be more readily degraded by serum nucleases.") (emphasis added).

98. It would be clear to the POSA that the experiments disclosed in Unger prepared vesicles where the nucleic acid was on the surface of the vesicle (*i.e.*, lipoplexes) rather than vesicles where the nucleic acid is contained inside the lipid vesicles and was thus fully encapsulated. Examples 6A and 6B of Unger disclose that the lipid vesicles of that invention were formed by mixing in solution DNA and preformed liposomes. Unger at 29:11-40:25. In these examples, Unger does not disclose a process whereby the preformed liposomes would be disrupted to allow for the encapsulation of the nucleic acid inside the liposomes. Accordingly, the POSA would understand that the DNA in the Examples disclosed in Unger would have formed lipoplexes, where the DNA was associated with the surface of the lipid vesicles.

99. These statements by the applicant would confirm the POSA's understanding that the "full encapsulation" language in the specification requires the nucleic acid to be in a specific location. Consistent with the specification, the statements differentiate between the systems in which nucleic acid is encapsulated "within a relatively disordered lipid mixture"—such as those of Unger that the applicant distinguished—and lipid vesicles with full encapsulation in which a nucleic acid is in the "interior" and *contained inside* the lipid vesicle. '651 patent, 5:30-40.

Appendix A

DAVID H. THOMPSON

Professor of Chemistry

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EDUCATION

B.A., Biology	**University of Missouri, Columbia**	1978
B.S., Chemistry	**University of Missouri, Columbia**	1978
	Research Advisor: *John P. McCormick*	
Ph.D., Organic Chemistry	**Colorado State University**	1984
	Thesis Advisor: *Louis S. Hegedus*	
	Dissertation: *Mechanistic Study of π-Methallyl Nickel Bromide Cross Coupling Reactions with Organic Halides*	

POSITIONS HELD

Postdoctoral Research Associate, Oregon Health & Sciences University (OHSU/OGI)	1984-1987
Research Advisor: *James K. Hurst*	
Assistant Professor, OHSU/OGI – Dept. of Chemical & Biological Sciences	1987-1994
Visiting Professor, University of British Columbia – Dept. of Biochemistry	1992
Associate Professor, Purdue University – Dept. of Chemistry	1994-2001
Professor, Purdue University – Dept. of Chemistry	2001-present
Visiting Professor, University of Florida – Dept. of Pharmaceutics	2003
Visiting Professor, Osaka University – Dept. of Applied Chemistry	2003
Visiting Professor, Japan Advanced Institute of Science & Technology – Dept. of Biomaterials	2005
Professor, Purdue University – Dept. of Biomedical Engineering	2008
Visiting Professor, Technical University of Denmark – Dept. of Micro & Nanotechnology	2012
Visiting Professor, Chulalongkorn University – Dept. of Pharmaceutics	2013 & 2016

DISTINCTIONS

Colorado Fellowship	1983-1984
Fall MRS Gold Paper Award	1999
Special Issue Editor, *Advanced Drug Delivery Reviews*	2001
Head, Organic Chemistry Division, Department of Chemistry, Purdue University	2003-2010
Top Ten Outstanding Teachers in the College of Science, Purdue U.	2004-2005
Chair, *Chemistry of Supramolecules & Assemblies* Gordon Conference	2005
JSPS Fellow, Japan Society for the Promotion of Science	2006
University Faculty Scholar, Purdue University	2006-2011
Award for Undergraduate Advising, College of Science, Purdue U.	2008
Co-Director, *Chemical & Structural Biology Group*, Purdue Center for Cancer Research	2008-2018
Director, *Targets, Structures & Drugs Group*, Purdue Center for Cancer Research	2018-2021
Alternate Counselor, American Chemical Society – Division of Colloid & Surface Science	2012-2015
Chair, HC Brown Symposium in Organic Chemistry	2014
Director, NIH-National Cancer Institute, Experimental Therapeutics – Chemical Biology Consortium, Purdue University Specialized Center	2016-2022

GOVERNMENT SERVICE & ADVISORY BOARDS

NIH Panelist, P50 *Cancer Imaging* Program (ad hoc)	1999
NSF Reviewer, *IGERT* Program (ad hoc)	1996-2000
Editorial Advisory Board, ***Langmuir***	2000-2005
NIH Study Section, *Bioorganic & Natural Products Chemistry* (ad hoc)	2001
Editorial Advisory Board, ***Bioconjugate Chemistry***	2004-2010
Associate Editor, ***WIREs: Nanomedicine & Nanobiotechnology***	2005-2018
NIH Study Section Panelist, *Bioengineering & Physiology SBIR*	2001-2005
NIH Study Section Panelist, *Nanoscience & Nanotechnology in Biology & Medicine*	2004-2006
NIH Advisory Board, *Nanomedicine Development Center* Initiative	2004-2006
NIH Study Section Charter Member, *Gene & Drug Delivery*	2006-2010
NIH Study Section, *COBRE Type I* (ad hoc)	2008
NIH Study Section, *Major Research Instrumentation - Flow Cytometry Grants*	2009-2010
NIH Study Section, P01 Review	2009-2010
NIH-NCI *Nanobiology* Site-Visit Program Review	2010
NIH Study Section, *Nano* (ad hoc)	2010
NIH Study Section, *MBRS SCORE* (ad hoc)	2012
NSF Division of Materials Research – Biomaterials Panelist	2018
NSF MRSEC Site Visit Reviewer	2018
NSF CAREER Award Panelist	2018
NIH Study Section, NCI *K99/R00* Program	2022

RESEARCH INTERESTS

- Transiently-stable carrier system development for intracellular drug & nucleic acid delivery
- Applications of energy-efficient, analytics-guided continuous synthesis
- Materials development for accelerated protein structure elucidation

PROFESSIONAL & SCHOLARLY ASSOCIATIONS

- American Association for the Advancement of Science
- American Chemical Society (Organic and Colloid & Surface Science Divisions)
- American Society for Gene Therapy
- Materials Research Society

PUBLICATIONS

1. L. S. Hegedus & D. H. Thompson, "Reactions of Organic Halides with (π-Allyl)nickel Halide Complexes: A Mechanistic Study" ***Journal of the American Chemical Society*** **1985** *107*, 5663-5669.

2. J. K. Hurst & D. H. Thompson, "Mechanisms of Oxidation-Reduction Across Vesicle Bilayer Membranes: An Overview" ***Journal of Membrane Science*** **1986** *28*, 3-29.

3. J. K. Hurst, D. H. Thompson & J. S. Connolly, "Oxidative Quenching of Photoexcited $ZnTPPS^{4-}$ Ion by Dihexadecylphosphate Vesicle-Bound Viologens" ***Journal of the American Chemical Society*** **1987** *109*, 507-515.

4. J. K. Hurst & D. H. Thompson, "One-Electron Reduction of Dihexadecylphosphate-Bound Viologens by Pentacyanocobaltate Ions" ***Inorganic Chemistry*** **1987** *26*, 39-43.

5. D. H. Thompson, W. C. Barrette & J. K. Hurst, "One-Electron Reduction of Dihexadecylphosphate Vesicle-Bound Viologens by Dithionite Ions" ***Journal of the American Chemical Society*** **1987** *109*, 2003-2009.

6. M. J. Colaneri, L. Kevan, D. H. Thompson & J. K. Hurst, "Variation of Alkylmethylviologen Radical Cation-Water Interactions in Micelles and Vesicles from ESEM Spectroscopy: Effect of Alkyl Chain Length" ***Journal of Physical Chemistry*** **1987** *91*, 4072-4077.

7. T. Lu, T. M. Cotton, J. K. Hurst & D. H. Thompson, "A Raman and Surface-Enhanced Raman Study of Asymmetrically-Substituted Viologens" ***Journal of Physical Chemistry*** **1988** *92*, 6978-6985.

8. T. Lu, T. M. Cotton, J. K. Hurst & D. H. Thompson, "A Voltammetric Study of Asymmetric Viologen in an Organic Solvent, Aqueous Solution and Vesicle Systems" ***Journal of Electroanalytical Chemistry*** **1988** *246*, 337-347.

9. D. H. Thompson & J. K. Hurst, "Intermolecular Transmembrane Redox--Electron Tunneling or Molecular Diffusion?" in ***Molecular Electronic Devices III***, F. L. Carter, R. Siatkowski, and H. Wohltjen, Eds., Elsevier, Amsterdam, **1988**, pp. 413-425.

10. B. C. Patterson, D. H. Thompson & J. K. Hurst, "Methyl Viologen-Mediated Oxidation-Reduction Across Dihexadecylphosphate Vesicles Involves Transmembrane Diffusion" ***Journal of the American Chemical Society*** **1988** *110*, 3656-3657.

11. B. C. Patterson, D. H. Thompson & J. K. Hurst, "Pathways of Transmembrane Redox Reactions for Dihexadecylphosphate Vesicle-Bound Viologens" in ***Molecular Electronics—Science and Technology***, Aviram, A., Ed., Engineering Foundation Publications, New York, **1989**, pp. 385-392.

12. P. L. Camacho, E. Geiger, G. Vigh, R. Webster & D. H. Thompson, "Separation of the Enantiomeric Intermediates of Some Platelet-Activating Factor Analogues on a Naphthylalanine-Type Pirkle Column" ***Journal of Chromatography*** **1990** *506*, 611-616.

13. R. Humphry-Baker, D. H. Thompson, Y. Lei, M. Hope & J. K. Hurst, "Structural Investigations of Dihexadecylphosphate Small Unilamellar Vesicles" ***Langmuir*** **1991** *7*, 2592-2601.

14. J. M. Kim & D. H. Thompson, "Tetraether Bolaform Amphiphiles as Models of Archaebacterial Membrane Lipids: Synthesis, Differential Scanning Calorimetry, and Monolayer Studies" ***Langmuir*** **1992** *8*, 637-644.

15. A. Heuer, D. Fink, V. Laraia, J. Arias, P. Calvert, K. Kendall, G. Messing, J. Blackwell, P. Rieke, D. H. Thompson, A. Wheeler, A. Veis & A. Caplan, "Innovative Materials Processing Strategies: A Biomimetic Approach" ***Science*** **1992** *255*, 1098-1105.

16. V. C. Anderson & D. H. Thompson, "Photoinduced Morphology Changes in Plasmalogen Liposomes Using Visible Light" in Macromolecular Assemblies, P. Stroeve & A. C. Balazs, Eds., ***ACS Symposium Series*** **1992** *493*, 154-170.

17. V. C. Anderson & D. H. Thompson, "Triggered Release of Hydrophilic Agents from Plasmalogen Liposomes Using Visible Light or Acid" ***Biochimica et Biophysica Acta*** **1992** *1109*, 33-42.

18. D. H. Thompson & J.-M. Kim, "Photoinduced Charge Transfer Studies in Bolaamphiphile-Gramicidin-Porphyrin Membranes" ***MRS Symposium Proceedings***, Macromolecular Host-Guest Complexes: Optical & Optoelectronic Properties and Applications **1992** *277*, 93-98.

19. D. H. Thompson, K. Wong, R. Humphry-Baker, J. Wheeler, J. M. Kim & S. B. Rananavare, "Tetraether Bolaform Amphiphiles as Models of Archaebacterial Membrane Lipids: Raman Spectroscopy, ^{31}P-NMR, X-Ray Scattering and Electron Microscopy" ***Journal of the American Chemical Society*** **1992** *114*, 9035-9042.

20. D. H. Thompson, J.-M. Kim & C. DiMeglio, "Photoinduced Charge Transfer Properties of Bolaamphiphile Membrane-Gramicidin Diad and Triad Composites" ***SPIE Proceedings***, Organic and Biological Optoelectronics **1993** *1853*, 142-147.

21. P. L. Camacho-Torralba, G. Vigh & D. H. Thompson, "High Performance Chiral Displacement Chromatographic Separations in the Normal Phase Mode. Part 1. Retention and Adsorption Studies of Potential Displacers Developed for the Pirkle-Type Naphthylalanine Silica Stationary Phase" ***Journal of Chromatography*** **1993** *641*, 31-38.

22. P. L. Camacho-Torralba, M. D. Beeson, G. Vigh & D. H. Thompson, "High Performance Chiral Displacement Chromatographic Separations in the Normal Phase Mode. Part 2. Separation of the Enantiomers of 1,2-*O*-Dihexadecyl-*rac*-glycerol-3-*O*-(3,5-dinitrophenyl)carbamate Using the Pirkle-Type Naphthylalanine Silica Stationary Phase" ***Journal of Chromatography*** **1993** *646*, 259-266.

23. D. H. Thompson, C. B. Svendsen, C. DiMeglio & V. C. Anderson, "Synthesis of Chiral Diether and Tetraether Phospholipids. Regiospecific Ring Opening of Epoxides Derived from Asymmetric Epoxidation" ***Journal of Organic Chemistry*** **1994** *59*, 2945-2955.

24. Y. Rui & D. H. Thompson, "Stereocontrolled Synthesis of Plasmalogen-Type Lipids from Glyceryl Ester Precursors" ***Journal of Organic Chemistry*** **1994** *59*, 5758-5762.

25. D. H. Thompson, O. V. Gerasimov, J. J. Wheeler & V. C. Anderson, "Triggerable Plasmalogen Liposomes: Improvement of System Efficiency" ***Biochimica et Biophysica Acta*** **1996** *1279*, 25-34.

26. Y. Rui & D. H. Thompson, "Efficient Stereoselective Synthesis of Plasmenylcholines" ***Chemistry—A European Journal*** **1996** *2*, 1505-1508.

27. D. H. Thompson, Y. Rui & O. V. Gerasimov, "Triggered Release from Liposomes Mediated by Physically- and Chemically-Induced Phase Transitions" *Surfactant Science Series:* **Vesicles**, M. Rosoff, Ed.; Marcel Dekker: New York, NY, **1996**, pp. 679-746.

28. O. Gerasimov, A. Schwan & D. H. Thompson, "Acid-Catalyzed Plasmenylcholine Hydrolysis and its Effect on Bilayer Permeability: A Quantitative Study" ***Biochimica et Biophysica Acta*** **1997** *1324*, 200-214.

29. E. Barklis, J. McDermott, S. Wilkens, E. Schabtach, M. Schmid, S. Fuller, S. Karanjia, Z. Love, R. Jones, X. Zhao, Y. Rui & D. H. Thompson, "Structural Analysis of Membrane-Bound Retrovirus Capsid Proteins" ***EMBO Journal*** **1997** *16*, 1199-1213.

30. I. Szleifer, O. V. Gerasimov & D. H. Thompson, "Spontaneous Liposome Formation Induced by Grafted Poly(ethylene oxide) Layers: Theoretical Prediction and Experimental Verification" ***Proceedings of the National Academy of Sciences USA*** **1998** *95*, 1032-1037.

31. N. Wymer, O. V. Gerasimov & D. H. Thompson, "Cascade Liposomal Triggering: Light-Induced Ca^{2+} Release from Plasmenylcholine Liposomes Triggers PLA_2-Catalyzed Hydrolysis and Contents Leakage from DPPC Liposomes" ***Bioconjugate Chemistry*** **1998** *9*, 305-308.

32. E. Barklis, J. McDermott, S. Wilkens, S. Fuller, & D. H. Thompson, "Organization of HIV-1 Capsid Proteins on a Lipid Monolayer" ***Journal of Biological Chemistry*** **1998** *273*, 7177-7180.

33. S. Svenson & D. H. Thompson, "Facile and Efficient Synthesis of Bolaamphiphilic Tetraether Phosphocholines" ***Journal of Organic Chemistry*** **1998** *63*, 7180-7182.

34. Y. Rui, S. Wang, P. S. Low & D. H. Thompson, "Diplasmenylcholine-Folate Liposomes: An Efficient Vehicle for Intracellular Drug Delivery" ***Journal of the American Chemical Society*** **1998** *120*, 11213-11218.

35. O. V. Gerasimov, N. Wymer, D. Miller, Y. Rui, & D. H. Thompson, "Intracellular Delivery of Liposomal Contents Using pH- and Light-Activated Plasmenyl-Type Liposomes" S.M. Dinh, J.D. DeNuzzio, A.R. Comfort, Eds., ***ACS Symposium Series*** **1999** *728*, 164-178.

36. J. A. Boomer & D. H. Thompson, "Synthesis of Acid-Labile Diplasmenyl Lipids for Drug and Gene Delivery Applications" ***Chemistry and Physics of Lipids*** **1999** *99*, 145-153.

37. A. Patwardhan & D. H. Thompson, "Efficient Synthesis of 40- and 48-Membered Tetraether Macrocyclic Bisphosphocholines" ***Organic Letters*** **1999** *1*, 241-244.

38. O. Gerasimov, J. Boomer, M. Qualls & D. H. Thompson, "Cytosolic Drug Delivery Using pH- and Light-Sensitive Liposomes" ***Advanced Drug Delivery Reviews*** **1999** *38*, 317-338.

39. C. DiMeglio, S. B. Rananavare, S. Svenson & D. H. Thompson, "Phosphocholine Analogs of Bolaamphiphiles: Phase Structure and Mesomorphism" ***Langmuir*** **2000** *16*, 128-133.

40. A. Patwardhan & D. H. Thompson, "Novel Flexible and Rigid Tetraether Acyclic and Macrocyclic Bisphosphocholines: Synthesis and Monolayer Properties" ***Langmuir*** **2000**, *16*, 10340-10350.

41. J. Shin, M. M. Qualls, J. A. Boomer, J. Robarge & D. H. Thompson, "An Efficient New Route to Plasmenyl-Type Lipids: Synthesis and Cytotoxicity of a Plasmenylcholine Analog of the Antitumor Ether Lipid ET-18-OCH_3" ***Journal of the American Chemical Society*** **2001** *123*, 508-509.

42. M. M. Qualls & D. H. Thompson, "Synergistic Phototoxicity of Chloroaluminum Phthalocyanine Tetrasulfonate Delivered via Acid-Labile Diplasmenylcholine-Folate Liposomes" ***International Journal of Cancer*** **2001** *93*, 384-392.

43. J.-M. Kim & D. H. Thompson, "Acid- & Oxidatively-Labile Vinyl Ether Lipids: Synthesis & Drug Delivery Applications" *Surfactant Science Series:* **Reactions & Synthesis in Surfactant Systems**, J. Texter, Ed.; Marcel Dekker: New York, NY, 2001, pp. 145-154.

44. J. H. Collier, B.-H. Hu, J. W. Ruberti, Z.-Y. Zhang, P. Shum, D. H. Thompson & P. B. Messersmith, "Thermally and Photochemically Triggered Self-Assembly of Peptide Hydrogels" ***Journal of the American Chemical Society*** **2001** *123*, 9463-9464.

45. J.-M. Kim, J. Shin, P. Shum & D. H. Thompson, "Acid- and Oxidatively-Labile Vinyl Ether Surfactants: Synthesis and Drug Delivery Applications" ***Journal of Dispersion Science and Technology*** **2001** *22*, 399-407.

46. P. Shum, J.-M. Kim & D. H. Thompson, "Phototriggering of Liposomal Drug Delivery Systems" ***Advanced Drug Delivery Reviews*** **2001** *53*, 273-284. *(JOURNAL COVER, ISSUE #3, DEC 31, 2001)*

47. Z.-Y. Zhang, P. Shum, M. Yates, P. B. Messersmith & D. H. Thompson, "Formation of Fibrinogen-Based Hydrogels Using Phototriggerable Diplasmalogen Liposomes" ***Bioconjugate Chemistry*** **2002** *13*, 640-646. *(JOURNAL COVER, ISSUE #3, APR 4, 2002)*

48. J. A. Boomer, D. H. Thompson & S. Sullivan, "Formation of Plasmid-Based Transfection Complexes with an Acid-Labile Cationic Diplasmenyl Lipid: *In Vitro* and *In Vivo* Gene Transfer" ***Pharmaceutical Research*** **2002** *19*, 1289-1298.

49. J. Shin, O. Gerasimov & D. H. Thompson, "Facile Synthesis of Plasmalogens via Barbier-type Reactions of Vinyl Dioxolanes with Alkyl Halides in LiDBB Solution" ***Journal of Organic Chemistry*** **2002** *67*, 6503-6508.

50. M. Rovira-Bru, D. H. Thompson & I. Szleifer, "Size and Structure of Spontaneously Forming Liposomes in Lipid:PEG-Lipid Mixtures" ***Biophysical Journal*** **2002** *82*, 2419-2439.

51. J.-C. Kim, K. Park & D. H. Thompson, "Synthesis of Tris(amino acid)-Substituted α-Cyclodextrins Derivatives", ***Macromolecular Chemistry Symposium*** **2002** *15*, 303-312.

52. N. Bergstrand, M. C. Arfvidsson, J.-M. Kim, D. H. Thompson & K. Edwards, "Interactions Between pH-Sensitive Liposomes and Model Membranes", ***Biophysical Chemistry*** **2003** *104*, 361-379.

53. J. A. Boomer, H. D. Inerowicz, Z.-Y. Zhang, N. Bergstrand, K. Edwards, J.-M. Kim & D. H. Thompson, "Acid-Triggered Release from Sterically-Stabilized Fusogenic Vesicles: A Novel DePEGylation Strategy", ***Langmuir*** **2003** *19*, 6408-6415.

54. J. Shin, P. Shum & D. H. Thompson, "Acid-Triggered Release via DePEGylation of DOPE Liposomes Containing Acid-Labile Vinyl Ether PEG-Lipids", ***Journal of Controlled Release*** **2003** *91*, 187-200.

55. J. Shin & D. H. Thompson, "Direct Synthesis of Plasmalogens from Allyl Substituted Glycerols", ***Journal of Organic Chemistry*** **2003** *68*, 6760-6766.

56. J. Shin & D. H. Thompson, "Intracellular Delivery of DNA and Proteins Using Vinyl Ether-Based Drug Delivery Vehicles" ***ACS Symposium Series*** **2003** *879*, 50-60.

57. D. H. Thompson, H. D. Inerowicz, J. Groves & T. Sarna "Characterization of Plasmalogen Photooxidation Products", ***Photochemistry and Photobiology*** **2003** *78*, 323-330.

58. H. Seong, W.-M. Choi, J.-C. Kim, D. H. Thompson & K. Park, "Preparation of Liposomes With Glucose Binding Sites: Liposomes Containing Di-branched Amino Acid Derivatives", ***Biomaterials*** **2003** *24*, 4487-4493.

59. M. D. Kennedy, K. N. Jallad, D. H. Thompson, P. S. Low & D. Ben-Amotz, "Optical Imaging of Folate-Receptor Positive Tissues and Tumors Targeted with a Folate-Fluorescein Conjugate", ***Journal of Biomedical Optics*** **2003** *8*, 636-641.

60. J.-M. Kim, A. Patwardhan, A. Bott & D. H. Thompson, "Preparation and Electrochemical Behavior of Gramicidin Bipolar Lipid Monolayer Membranes Supported on Gold Electrodes", ***Biochimica et Biophysica Acta*** **2003** *1617*, 10-21.

61. D. H. Thompson, J. Shin, J. A. Boomer & J.-M. Kim, "Preparation of Plasmenylcholine Lipids and Plasmenyl-Type Liposome Dispersions", ***Methods in Enzymology*** **2004** *387*, 153-168.

62. M. Zhou, S. Haldar, J. Franses, J.-M. Kim & D. H. Thompson, "Synthesis and Self-Assembly Properties of Acylated Cyclodextrins and Nitrilotriacetic Acid (NTA)-Modified Inclusion Ligands for Interfacial Protein Crystallization", ***Supramolecular Chemistry*** **2005** *17*, 101-111.

63. C. Wang, S. Leffler, D. H. Thompson & C. A. Hrycyna, "A General Assay for S-adenosyl Methionine–dependent Enzymes Using a Thiol-activated Fluorescent Molecular Beacon", ***Biochemical and Biophysical Research Communications*** **2005** *331*, 351-356.

64. H. B. Hodges, M. Zhou, J. L. Anderson, D. H. Thompson & C. A. Hrycyna, "Inhibition of Membrane-Spanning Metalloproteins by Hydrophobic Metal Chelators", ***Bioconjugate Chemistry*** **2005** *16*, 490-493.

65. G. Acharya, K. Park & D. H. Thompson, "Synthesis and Evaluation of α-Cyclodextrin-Aldonamide Conjugates for D-Glucose Recognition", ***Journal of Drug Delivery Science and Technology*** **2006** *16*, 45-48.

66. V. Dixit, J. Van den Bossche, D. M. Sherman, D. H. Thompson & R. P. Andres, "Synthesis and Grafting of Thioctic Acid-PEG-Folate Conjugates onto Au Nanoparticles for Selective Targeting of Cancer Cells", ***Bioconjugate Chemistry*** **2006** *17*, 603-609.

67. T. Ooya, D. Inoue, H. S. Choi, Y. Kobayashi, S. Loethen, D. H. Thompson, Y. Ho Ko, K. Kim & N. Yui, "pH-Responsive Movement of Cucurbit[7]uril in a Dual Polypseudorotaxane: Contribution of Dimethyl β-Cyclodextrin", ***Organic Letters*** **2006** *8*, 3159-3162.

68. S. Loethen, T. Ooya, H. S. Choi, N. Yui & D. H. Thompson, "Synthesis, Characterization and pH-Triggered Dethreading of α-Cyclodextrin Polyethylene Glycol Polyrotaxanes Bearing Cleavable Stoppers", ***Biomacromolecules*** **2006** *7*, 2501-2506.

69. C. Huguet, E. C. Hopmans, W. Febo-Ayala, D. H. Thompson, J. S. Sinninghe Damsté & S. Schouten, "An Improved Method to Determine the Absolute Abundance of Glycerol Dibiphytanyl Glycerol Tetraether Lipids", ***Organic Geochemistry*** **2006** *37*, 1036-1041.

70. R. Wampler, M. Zhou, D. H. Thompson & G. J. Simpson, "Mechanism of the Chiral SHG Activity of Bacteriorhodopsin Films", ***Journal of the American Chemical Society*** **2006** *128*, 10994-10995.

71. W. Febo-Ayala, S. Morera-Felix, C. A. Hrycyna & D. H. Thompson, "Functional Reconstitution of the Integral Membrane Enzyme, Isoprenylcysteine Carboxyl Methyltransferase, in Synthetic Bolalipid Membrane Vesicles", ***Biochemistry*** **2006** *45*, 14683-14694.

72. W. Febo-Ayala, D. P. Holland, S. A. Bradley, D. H. Thompson, "Lateral Diffusion Coefficients of an Eicosanyl-Based Bisglycerophosphocholine Determined by PFG-NMR and FRAP", ***Langmuir*** **2007** *23*, 6276-6280.

73. E. Kang, J.-w. Park, S. McClellan, J.-M. Kim, D. P. Holland, G. U. Lee, E. Franses, K. Park, D. H. Thompson, "Specific Adsorption of Histidine-Tagged Proteins on Silica Surfaces Modified with Ni^{2+}:NTA-Derivatized Poly(ethylene glycol)", ***Langmuir*** **2007** *23*, 6281-6288.

74. J. Van den Bossche, J. Shin, D. H. Thompson, "Improved Plasmalogen Synthesis Using Organobarium Intermediates", ***Journal of Organic Chemistry*** **2007** *72*, 5005-5007.

75. S. Loethen, J.-M. Kim, D. H. Thompson, "Biomedical Applications of Cyclodextrin-Based Polyrotaxanes", ***Journal of Macromolecular Science C—Polymer Reviews*** **2007** *47*, 383-418.

76. D. H. Thompson, M. Zhou, J. Grey, H.-k. Kim, "Design, Synthesis and Performance of NTA-Modified Lipids as Templates for Histidine-Tagged Protein Crystallization", ***Chemistry Letters*** **2007** *36*, 956-975.

77. G. S. Longo, D. H. Thompson, I. Szleifer, "Stability and Phase Separation in Mixed Bola-Linear Lipid Layers" ***Biophysical Journal*** **2007** *93*, 2609-2621.

78. G. Acharya, C.-L. Chang, D. Holland, D. H. Thompson, C. A. Savran, "Rapid Detection of *S*-Adenosyl Homocysteine Using Self-Assembled Optical Diffraction Gratings", ***Angewandte Chemie, International Edition*** **2008** *47*, 1051-1053.

79. D. P. Holland, A. V. Struts, M. F. Brown, D. H. Thompson, "Bolalipid Membrane Structure Revealed by Solid-State ^{2}H NMR Spectroscopy", ***Journal of the American Chemical Society*** **2008** *130*, 4584-4585.

80. D. H. Thompson, "Adenovirus in a Synthetic Membrane Wrapper: An Example of Hybrid Vigor?", ***ACS Nano*** **2008** *2*, 821-826.

81. G. Longo, D. H. Thompson, I. Szleifer, "Ligand-Receptor Interactions Between Surfaces: The Role of Binary Polymer Spacers", ***Langmuir*** **2008** *24*, 10324-10333.

82. J. J. Bozell, N. C. Tice, N. Sanyal, D. H. Thompson, J.-M. Kim & S. Vidal, "Synthesis and Self-Assembly of Glycal-based Bolaforms", ***Journal of Organic Chemistry*** **2008** *73*, 8763-8771.

83. R. D. Wampler, D. J. Kissick, C. J. Dehen, E. J. Gualtieri, J. L. Grey, H.-F. Wang, D. H. Thompson, J.-X. Cheng & G. J. Simpson, "Selective Detection of Protein Crystals by Second Harmonic Microscopy", ***Journal of the American Chemical Society*** **2008** *130*, 14076-14077.

84. J. A. Boomer, M. M. Qualls, H. D. Inerowicz, R. Haynes, G. V. Patri, J.-M. Kim & D. H. Thompson, "Cytoplasmic Delivery of Liposomal Contents Mediated by an Acid-Labile Cholesterol-Vinyl Ether-PEG Conjugate", ***Bioconjugate Chemistry*** **2009** *20*, 47-59.

85. H. Chen, R. Ahn, J. Van den Bossche, D. H. Thompson & T. V. O'Halloran, "Folate-mediated Intracellular Drug Delivery Increases the Anti-cancer Efficacy of Nanoparticulate Formulation of Arsenic Trioxide", ***Molecular Cancer Therapeutics*** **2009** *8*, 1955-1963.

86. D. P. Brownholland, G. S. Longo, A. V. Struts, M. J. Justice, I. Szleifer, H. I. Petrache, M. F. Brown, D. H. Thompson, "Phase Separation in Binary Mixtures of Bipolar and Monopolar Lipid Dispersions Revealed by ^{2}H NMR Spectroscopy, Small Angle X-ray Scattering, and Molecular Theory", ***Biophysical Journal*** **2009** *97*, 2700-2709.

87. K. Mulligan, D. Brownholland, A. Carnini, D. H. Thompson, L. Johnston, "AFM Investigations of Phase Separation in Supported Membranes of Binary Mixtures of POPC and an Eicosanyl-based Bisphosphocholine Bolalipid", ***Langmuir*** **2010** *26*, 8525-8533.

88. W. Deng & D. H. Thompson, "pH and Cation-responsive Supramolecular Gels Formed by Cyclodextrin Amines in DMSO", ***Soft Matter*** **2010** *6*, 1884-1887.

89. T. L. Andresen, D. H. Thompson, T. Kaasgaard, "Enzyme-triggered Nanomedicine: Drug Release Strategies in Cancer Therapy", ***Molecular Membrane Biology*** **2010** *27*, 353-363.

90. J. Grey, D. H. Thompson, "Challenges and Opportunities for New Protein Crystallization Strategies in Structure-Based Drug Design", ***Current Opinion in Drug Discovery*** **2010** *5*, 1039-1045.

91. S.-H. Hyun, H.-k. Kim, J.-M. Kim, D. H. Thompson, "Oriented Insertion of phi29 *N*-Hexahistidine-tagged gp10 Connector Protein Assemblies into C_{20}BAS Bolalipid Membrane Vesicles", ***Journal of the American Chemical Society*** **2010** *132*, 17053-17055. *(JOURNAL COVER, ISSUE #48, DEC 8, 2010)*

92. A. Broniec, R. Klosinski, A. Pawlak, M. Wrona-Krol, D. H. Thompson, T. Sarna, "Interactions of Plasmalogens and Their Diacyl Analogs with Singlet Oxygen in Selected Model Systems", ***Free Radical Biology & Medicine*** **2011** *50*, 892-898.

93. B. G. Coon, S. Crist, A. González-Bonet, H.-k. Kim, J. Sowa, D. H. Thompson, T. L. Ratliff, R. C. Aguilar, "Fibronectin Attachment Protein (FAP) from Bacillus Calmette-Guerin as a Targeting Agent for Bladder Tumor Cells", ***International Journal of Cancer*** **2012** *131*, 591-600.

94. H.-k. Kim, H. Wei, A. Kulkarni, R. M. Pogranichniy, D. H. Thompson, "Effective Targeted Gene Delivery to Dendritic Cells via Synergistic Interaction of Mannosylated Lipid with DOPE and BCAT", ***Biomacromolecules*** **2012** *13*, 636-644.

95. D. Wei, J. Chen, A. Kulkarni, D. H. Thompson, "Poly(ethylene glycol)-Poly(vinyl alcohol)-Adamantanate: Synthesis and Stimuli-Responsive Micelle Properties", ***Soft Matter*** **2012** *8*, 5843-5846.

96. A. Kulkarni, D. Wei, S.-H. Hyun, D. H. Thompson, "Development of a Safe and Effective pDNA Vector Based on Non-covalent Assembly of Bioresponsive Amino-β-Cyclodextrin:Adamantane-Poly(vinyl alcohol)-Poly(ethylene glycol) Transfection Complexes", ***Bioconjugate Chemistry*** **2012** *23*, 933-940.

97. A. Kulkarni, K. DeFrees, S.-H. Hyun, D. H. Thompson, "Pendant Polymer:Amino-β-Cyclodextrin:siRNA Guest:Host Nanoparticles as Efficient Vectors for Gene Silencing", ***Journal of the American Chemical Society*** **2012** *134*, 7596-7599.

98. H. Wei, S.D. Lenz, D. H. Thompson, R. M. Pogranichniy, "DNA Vaccine Development Against H1N1 Subtype Swine Influenza Viruses", ***Viral Immunology*** **2012** *25*, 297-305.

99. J. Shin, P. Shum, J. Grey, G. S. Malhotra, S. Fujiwara, A. M. González-Bonet, E. Moase, T. M. Allen, D. H. Thompson, "Acid-labile PEG-Vinyl Ether-Lipids with Tunable pH Sensitivity: Synthesis and Structural Effects on Hydrolysis Rates, Calcein Release Performance and Biodistribution of Their DOPE Dispersions", ***Molecular Pharmaceutics*** **2012** *9*, 3266-3276.

100. H.-k. Kim, J. Van den Bossche, S.-H. Hyun, D. H. Thompson, "Acid-triggered Release via DePEGylation of Fusogenic Liposomes Mediated by Tunable pH Sensitive Phenyl Substituted Vinyl Ether PEG-Lipids", ***Bioconjugate Chemistry*** **2012** *23*, 2071-2077.

101. A. Kulkarni, K. DeFrees, R. A. Schuldt, A. Vlahu, R. VerHeul, S.-H. Hyun, D. Wei, D. H. Thompson, "Multi-Armed Cationic Cyclodextrin:Poly(ethylene glycol) Polyrotaxanes as Efficient Gene Silencing Vectors", ***Integrative Biology*** **2013** *5*, 115-121.

102. N. Morimoto, S. Hirano, H. Takahashi, S. Loethen, D. H. Thompson, K. Akiyoshi, "Self-assembled pH-sensitive Cholesteryl Pullulan Nanogel as a Protein Delivery Vehicle", ***Biomacromolecules*** **2013** *14*, 56-63.

103. A. Kulkarni, K. DeFrees, R. A. Schuldt, S.-H. Hyun, K. J. Wright, C. K. Yerneni, R. VerHeul, D. H. Thompson, "Cationic α-Cyclodextrin:Poly(ethylene glycol) Polyrotaxanes for siRNA Delivery", ***Molecular Pharmaceutics*** **2013** *10*, 1299-1305.

104. C. J. Collins, L. McCauliff, S.-H. Hyun, Z. Zhang, L. N. Paul, A. Kulkarni, K. Zick, M. Wirth, J. Storch, D. H. Thompson, "Synthesis, Characterization, and Evaluation of Pluronic-based β-Cyclodextrin Polyrotaxanes for Mobilization of Accumulated Cholesterol from Niemann-Pick Type C Fibroblasts", ***Biochemistry*** **2013** *52*, 3242-3253.

105. A. Kulkarni, R. VerHeul, K. DeFrees, C. J. Collins, R. A. Schuldt, A. Vlahu, D. H. Thompson, "Microfluidic Assembly of Cationic β-Cyclodextrin:Hyaluronic Acid-Adamantane Host:Guest pDNA Nanoparticles", ***Biomaterials Science*** **2013** *1*, 1029-1033.

106. H.-k. Kim, D. H. Thompson, H. S. Jang, Y. J. Chung, J. Van den Bossche, "pH-Responsive Biodegradable Assemblies Containing Tunable Phenyl Substituted Vinyl Ethers for Use as Efficient Gene Delivery Vehicles", ***ACS Applied Materials & Interfaces*** **2013** *5*, 5648-5658.

107. Y. A. Mondjinou, L. McCauliff, A. Kulkarni, L. Paul, S.-H. Hyun, Z. Zhang, Z. Wu, M. Wirth, J. Storch, D. H. Thompson, "Synthesis of 2-Hydroxypropyl-β-Cyclodextrin and Pluronic Based Polyrotaxanes in Heterogeneous Reactions for Niemann-Pick Type C Therapy", ***Biomacromolecules*** **2013** *14*, 4189-4197.

108. T. C. Flynn, D. H. Thompson, S.-H. Hyun, "Molecular Weight Analyses and Enzymatic Degradation Profiles of the Soft-Tissue Fillers Belotero Balance, Restylane, and Juvéderm Ultra", ***Plastic and Reconstructive Surgery*** **2013** *132*, 22S-32S.

109. A. Kulkarni, V. Badwaik, K. DeFrees, R. A. Schuldt, D. S. Gunasekera, C. Powers, A. Vlahu, R. VerHeul, D. H. Thompson, "Effect of Pendant Group on pDNA Delivery by Cationic-β-Cyclodextrin:Alkyl-PVA-PEG Pendant Polymer Complexes", ***Biomacromolecules*** **2014** *15*, 12-19.

110. H. Wei, S.D. Lenz, D. H. Thompson, R. M. Pogranichniy, "DNA-Epitope Vaccine Provided Efficient Protection to Mice Against Lethal Dose of Influenza A Virus H1N1", ***Viral Immunology*** **2014** *27*, 14-19.

111. G. Yu, F. Vago, D. Zhang, J. E. Snyder, R. Yan, C. Zhang, C. Benjamin, X. Jiang, R. J. Kuhn, P. Serwer, D. H. Thompson, W. Jiang, "Single-step Antibody-based Affinity Cryo-Electron Microscopy for Imaging and Structural Analysis of Macromolecular Assemblies", ***Journal of Structural Biology*** **2014** *187*, 1-9.

112. J. H. Byeon, A. Kulkarni, H.-k. Kim, D. H. Thompson, J. Roberts, "Photoassisted One-Step Aerosol Fabrication of Zwitterionic Chitosan Nanoparticles", ***Biomacromolecules*** **2014** *15*, 2320-2325.

113. J. H. Byeon, H.-k. Kim, D. H. Thompson, J. Roberts, "Aerosol-based Fabrication of Modified Chitosans and Their Application for Gene Transfection", ***ACS Applied Materials & Interfaces*** **2014** *6*, 4597-4602.

114. Y. Cui, A. Naz, D. H. Thompson, J. Irudayaraj, "Decitabine Nanoconjugate Sensitizes Human Glioblastoma Cells to Temozolomide", ***Molecular Pharmaceutics*** **2015** *12*, 1279-1288.

115. Y. A. Mondjinou, S.-H. Hyun, M. Xiong, C. J. Collins, P. L. Thong, D. H. Thompson, "Impact of Mixed β-Cyclodextrin Feeds on Pluronic Rotaxanation Efficiency and Product Solubility", ***ACS Applied Materials & Interfaces*** **2015** *7*, 23831-23836.

116. Z. Zhou, Y. Mondjinou, S.-H. Hyun, A. Kulkarni, Z.-R. Lu, D. H. Thompson, "Gd^{3+}-1,3,7,10-Tetraazacyclododecane-1,4,7-triacetic-2-Hydroxypropyl-β-Cyclodextrin/Pluronic Polyrotaxane as a Long Circulating High Relaxivity MRI Contrast Agent", ***ACS Applied Materials & Interfaces*** **2015** *7*, 22272-22276.

117. T. C. Flynn, D. H. Thompson, S.-H. Hyun, D. J. Howell, "Ultrastructural Analysis of 3 Hyaluronic Acid Soft-Tissue Fillers Using Scanning Electron Microscopy" ***Dermatologic Surgery*** **2015** Suppl 1:S143-52. PMID: 25828039.

118. V. Badwaik, Y. Mondjinou, A. Kulkarni, L. Liu, A. Demoret, D. H. Thompson, "Efficient pDNA Delivery Using Cationic 2-Hydroxypropyl-β-Cyclodextrin Pluronic based Polyrotaxanes", ***Macromolecular Bioscience*** **2016** *16*, 63-73. *(JOURNAL COVER, ISSUE #1, JAN 12, 2016)*

119. F. W. S. Stetter, S.-H. Hyun, S. Brander, J. M. Urban, D. H. Thompson, T. Hugel "Nanomechanical Characterization of Lipid Bilayers With AFM-based Methods", ***Polymer*** **2016** *102*, 326-332.

120. C. J. Benjamin, K. J. Wright, S.-H. Hyun, K. Krynski, G. Yu, R. Bajaj, F. Guo, C. V. Stauffacher, W. Jiang, D. H. Thompson, "Non-fouling NTA-PEG-based TEM Grid Coatings for Selective Capture of Histidine-tagged Protein Targets from Cell Lysates", ***Langmuir*** **2016** *32*, 551-559.

121. V. D. Badwaik, E. Aicart, Y. A. Mondjinou, M. A. Johnson, V. D. Bowman, D. H. Thompson, "Structure-Property Relationship for siRNA Delivery Performance of Cationic 2-Hydroxypropyl-β-cyclodextrin: Poly(ethylene glycol)-Poly(propylene glycol)-Poly(ethylene glycol) Polyrotaxane Vectors", ***Biomaterials*** **2016** *84*, 86-98.

122. V. Badwaik, L. Liu, D. Gunasekera, A. Kulkarni, D. H. Thompson, "Mechanistic Insight into Receptor-mediated Delivery of Cationic-β-Cyclodextrin:Hyaluronic Acid-Adamantane Host:Guest pDNA Nanoparticles to CD44+ Cells", ***Molecular Pharmaceutics*** **2016** *13*, 1176-1184.

123. A. Lee, H.-k. Kim, D. H. Thompson, "A Facile Method for the Preparation of Unsymmetrical Ureas Utilizing Zirconium(IV) Chloride", ***Bulletin of the Korean Chemical Society*** **2016** *37*, 154-160.

124. J. W. Boley, S.-H. Hyun, E. L. White, D. H. Thompson, R. K. Kramer, "Hybrid Self-Assembly During Evaporation Enables Drop-on-Demand Thin Film Devices", ***ACS Applied Materials & Interfaces*** **2016** *8*, 34171-34178.

125. C. J. Collins, Y. Mondjinou, B. Loren, S. Torregrosa-Allen, C. J. Simmons, B. D. Elzey, N. Ayat, Z.-R. Lu, D. H. Thompson, "Influence of Molecular Structure on the *In Vivo* Performance of Flexible Rod Polyrotaxanes", ***Biomacromolecules*** **2016** *17*, 2777-2786.

126. W. Gao, Y. Chen, D. H. Thompson, K. Park, T. Li, "Impact of Surfactant Treatment of Paclitaxel Nanocrystals on Biodistribution and Tumor Accumulation in Tumor-bearing Mice", ***Journal of Controlled Release*** **2016** *237*, 168-176.

127. C. J. Benjamin, K. J. Wright, S. Bolton, S.-H. Hyun, K. Krynski, M. Grover, G. Yu, F. Guo, T. Kinzer-Ursem, W. Jiang, D. H. Thompson, "Selective Capture of Histidine-tagged Proteins from Cell Lysates Using TEM Grids Modified with NTA-Graphene Oxide", ***Scientific Reports*** **2016** *6*, 32500.

128. M. Wleklinski, C. Falcone, B. P. Loren, Z. Jaman, K. Iyer, H. S. Ewan, S.-H. Hyun, D. H. Thompson, R. G. Cooks, "Can Accelerated Reactions in Droplets Guide Chemistry at Scale?", ***European Journal of Organic Chemistry*** **2016**, 5480-5484.

129. T. R. Lear, S.-H. Hyun, J. W. Boley, E. L. White, D. H. Thompson, R. K. Kramer, "Liquid Metal Particle Popping: Nanoscale to Macroscale", ***Extreme Mechanics Letters*** **2017** *13*, 126-134.

130. C. J. Collins, B. P. Loren, M. S. Alam, Y. A. Mondjinou, J. L. Skulsky, C. Chaplain, K. Haldar, D. H. Thompson, "Pluronic based β–Cyclodextrin Polyrotaxanes for Treatment of Niemann-Pick Type C Disease", ***Scientific Reports*** **2017** *7*, 46737.

131. B. P. Loren, M. Wleklinski, A. Koswara, K. Yammine, Y. Hu, Z. K. Nagy, D. H. Thompson, R. G. Cooks, "Mass Spectrometry Directed System for the Continuous-Flow Synthesis and Purification of Diphenhydramine", ***Chemical Science*** **2017** *8*, 4363-4370.

132. Y. Lee, E. Kischuk, S. Crist, T. Ratliff, D. H. Thompson, "Targeting and Internalization of Liposomes by Bladder Tumor Cells Using a Fibronectin Attachment Protein-Derived Peptide-Lipopolymer Conjugate", ***Bioconjugate Chemistry*** **2017** *28*, 1481-1490.

133. A. Broniec, A. Zadlo, A. Pawlak, B. Fuchs, R. Klosinski, D. H Thompson, T. Sarna, "Interaction of Plasmenylcholine with Free Radicals in Selected Model Systems", ***Free Radical Biology & Medicine*** **2017** *106*, 368-378.

134. A. Naz, Y. Cui, C. J. Collins, D. H. Thompson, J. Irudayaraj, "PLGA-PEG Nano-delivery System for Epigenetic Therapy", ***Biomedicine & Pharmacotherapy*** **2017** *90*, 586-597.

135. C. E. Falcone, Z. Jaman, M. Wleklinski, A. Koswara, D. H. Thompson, R. G. Cooks, "Reaction Screening and Optimization of Continuous-Flow Atropine Synthesis by Preparative Electrospray Mass Spectrometry", ***Analyst*** **2017** *142*, 2836-2845.

136. Y. Lee, D. H. Thompson, "Stimuli-responsive Liposomes for Drug Delivery" ***WIREs: Nanomedicine & Nanobiotechnology*** **2017** *9*, e1450.

137. H. S. Ewan, K. Iyer, S.-H. Hyun, M. Wleklinski, R. G. Cooks, D. H. Thompson, "Multistep Flow Synthesis of Diazepam Guided by Droplet Reaction Screening With Mechanistic Insights from Rapid MS Analysis", ***Organic Process Research & Development*** **2017** *21*, 1566-1570.

138. B. Hudson, S.-H. Hyun, D. H. Thompson, A. M. Lyon, "Phospholipase Cβ3 Membrane Adsorption and Activation is Regulated by its C-terminal Domains and PIP_2", ***Biochemistry*** **2017** *56*, 5604-5614.

139. K. J. Wright, S. Samaddar, V. Badwaik, S.-H. Hyun, K. Glauninger, T. Eom, D. H. Thompson, "Organocatalytic Synthesis and Evaluation of Polycarbonate Pendant Polymer:β-Cyclodextrin-based Nucleic Acid Delivery Vectors", ***Macromolecules*** **2018** *51*, 670-678.

140. M. Wleklinski, B. P. Loren, C. Ferreira, Z. Jaman, L. Avramova, T. J. P. Sobreira, D. H. Thompson, R. G. Cooks, "High Throughput Reaction Screening Using Desorption Electrospray Ionization Mass Spectrometry", ***Chemical Science*** **2018** *9*, 1647-1653.

141. R. VerHeul, C. Sweet, D. H. Thompson, "Rapid and Simple Purification of Elastin-like Polypeptides Directly from Whole Cells and Cell Lysates by Organic Solvent Extraction", ***Biomaterials Science*** **2018** *6*, 863-876.

142. J. Payne, V. Badwaik, H. Waghwan, H. Moolani, S. Tockstein, D. H. Thompson, D. Rajalingam, "Development of Dihydrochalcone-functionalized Gold Nanoparticles for Augmented Antineoplastic Activity", ***International Journal of Nanomedicine*** **2018** *13*, 1917-1926.

143. Y. Lee, S. B. Pai, R. V. Bellamkonda, D. H. Thompson, J. Singh, "Cerivastatin Nano-Liposome as a Potential Disease Modifying Approach for the Treatment of Pulmonary Arterial Hypertension", ***Journal of Pharmacology and Experimental Therapeutics*** **2018** *366*, 66-74.

144. Z. Jaman, A. Mufti, S. Sah, L. Avramova, D. H. Thompson, "High-throughput Experimentation and Continuous Flow Validation of Suzuki-Miyaura Cross-Coupling Reactions", ***Chemistry – A European Journal*** **2018** *24*, 9546-9554.

145. Y. A. Mondjinou, B. P. Loren, V. Badwaik, S.-H. Hyun, A. Demoret, J. Skulsky, C. Chaplain, D. H. Thompson, "Gd^{3+}:DOTA-modified 2-Hydroxypropyl-β-Cyclodextrin/4-Sulfobutyl Ether-β-Cyclodextrin-Based Polyrotaxanes as Long Circulating High Relaxivity MRI Contrast Agents", ***Bioconjugate Chemistry*** **2018** *29*, 3550-3560.

146. B. Szilagyi, A. Koswara, B. Loren, C. Ferreira, D. H. Thompson, Z. K. Nagy, "Piezoelectric-Based High Performance Spray Solvent Delivery System for Desorption Electrospray Ionization Mass Spectrometry: Systematic Design and Case Studies for High Throughput Screening of N-Alkylation Reactions", ***Chemical Engineering Science*** **2019** *195*, 1010-1020.

147. K. Egele, N. Baudendistel, S. Samaddar, D. H. Thompson, G. Wenz, "Synthesis of Hydroxypropyl-β-Cyclodextrin:Poly(decamethylenephosphate) and Evaluation of its Cholesterol Efflux Potential in Niemann-Pick C1 Cells", ***Journal of Materials Chemistry B*** **2019** *7*, 528-537.

148. Z. Jaman, T. J. P. Sobreira, A. Mufti, C. R. Ferreira, R. G. Cooks, D. H. Thompson, "On-demand Rapid Synthesis of Lomustine Under Continuous Flow Conditions", ***Organic Process Research & Development***, **2019** *23*, 334-341.

149. A. Kim, J. Zhou, S. Samaddar, S. H. Song, D. H. Thompson, B. Ziaie, "An Implantable Ultrasonically-Powered Micro-Light-Source (µLight) for Photodynamic Therapy", ***Scientific Reports*** **2019** *9*, 1395.

150. B. P. Loren, H. S. Ewan, L. Avramova, C. R. Ferreira, T. J. P. Sobreira, K. Yammine, H. Liu, R. G. Cooks, D. H. Thompson, "High Throughput Experimentation Using DESI-MS to Guide Continuous-Flow Synthesis", ***Scientific Reports*** **2019** *9*, 14745.

151. S. Samaddar, J. Mazur, D. Boehm, D. H. Thompson, "Development and In Vitro Characterization of Bladder Tumor Cell Targeted Lipid-Coated Polyplex for Dual Delivery of Plasmids and Small Molecules", ***International Journal of Nanomedicine*** **2019** *14*, 9547-9561.

152. Z. Jaman, D. L. Logsdon, B. Szilagyi, T. J. P. Sobreira, D. Aremu, L. Avramova, R. G. Cooks, D. H. Thompson, "High-Throughput Experimentation and Continuous Flow Evaluation of Nucleophilic Aromatic Substitution Reactions", ***ACS Combinatorial Science***, **2020** *4*, 184-196.

153. Z. Struzik, A. Weerts, J. Storch, D. H. Thompson, "Stereospecific Synthesis of Phosphatidylglycerol Using a Cyanoethyl Phosphoramidite Precursor", ***Chemistry & Physics of Lipids*** **2020** *231*, 104933.

154. D. L. Logsdon, Y. Li, T. J. P. Sobreira, C. R. Ferreira, D. H. Thompson, R. G. Cooks, "High Throughput Screening of Reductive Amination Reactions Using Desorption Electrospray Ionization (DESI) Mass Spectrometry", ***Organic Process Research & Development*** **2020** *24*, 1647-1657.

155. T. Sobreira, L. Avramova, B. Szilagyi, D. Logsdon, B. Loren, Z. Jaman, R. Hilger, R. S. Hosler, C. Ferreira, A. Koswara, D. H. Thompson, R. G. Cooks, Z. Nagy, "High-throughput Screening of Organic Reactions in Microdroplets Using Desorption Electrospray Ionization Mass Spectrometry (DESI-MS): Hardware and Software Implementation", ***Analytical Methods*** **2020** *12*, 3654-3669.

156. S. Biyani, Q. Qi, J. Wu, Y. Moriuchi, E. A. Larocque, H. O. Sintim, D. H. Thompson, "Use of High-throughput Tools for Telescoped Continuous Flow Synthesis of an Alkynylnapthyridine Anticancer Agent, HSN608", ***Organic Process Research & Development*** **2020** *24*, 2240-2251.

157. H. S. Ewan, S. Biyani, J. DiDomenico, D. Logsdon, T. J. P. Sobreira, L. Avramova, R. G. Cooks, D. H. Thompson, "Aldol Reactions of Bio-renewable Triacetic Acid Lactone Precursor Evaluated Using Desorption Electrospray Ionization Mass Spectrometry High Throughput Experimentation", ***ACS Combinatorial Science*** **2020** *22*, 796-803.

158. S. Samaddar, J. Mazur, J. Sargent, D. H. Thompson, "Immunostimulatory Response of RWFV Peptide-Targeted Lipid Nanoparticles on Bladder Tumor Associated Cells", ***ACS Applied Bio Materials*** **2021** *4*, 3178-3188.

159. C. Sweet, A. Aayush, L. Readnour, K. V. Solomon, D. H. Thompson, "Development of a Fast Organic Extraction-Precipitation Method for Enhanced Purification of Elastin-like Polypeptides Independent of Charge and Size", ***Biomacromolecules*** **2021** *22*, 1990-1998.

160. Md. R. Hoq, F. S. Vago, K. Li, M. Kovaliov, R. J. Nicholas, D. M. Huryn, P. Wipf, W. Jiang, D. H. Thompson, "Affinity Capture with Small Molecule Bait for CryoEM: New Sample Preparation Method Reveals a 3.6Å Double-ring p97 Structure", ***ACS Nano*** **2021**, *15*, 8376-8385.

161. S. A. Biyani, Y. Moriuchi, D. H. Thompson, "Advancement in Organic Synthesis Through High-Throughput Experimentation", ***Chemistry – Methods*** **2021** *1*, 323-339.

162. C. Pan, K. Banerjee, G. Lehmann, D. Almeida, K. A. Hajjar, I. Benedicto, Z. Jiang, R. A. Radu, D. H. Thompson, E. Rodriguez-Boulan, M. M. Nociari "Lipofuscin Causes Atypical Necroptosis Through Lysosomal Membrane Permeabilization", ***Proceedings of the National Academy of Sciences USA*** **2021** *118*, e2100122118.

163. E. Gonzalez Solveyra, D. H. Thompson, I. Szleifer, "Proteins Adsorbing Onto Surface Modified Nanoparticles: Effect of Surface Curvature, pH and the Interplay of Polymer and Proteins Acid-Base Equilibria", ***Polymers*** **2022** *14*, 739.

164. G. Murbach-Oliveira, K. Banerjee, M. M. Nociari, D. H. Thompson, "Continuous Flow Synthesis of A2E Guided by Design of Experiments and High Throughput Studies", ***ACS Bio & Med Chem*** **2022** *2*, 297-306.

165. S. Samaddar, D. Bose, B. P. Loren, J. L. Skulsky, O. Ilnytska, Z. J. Struzik, J. Storch, D. H. Thompson, "Structure-Function Relationships of Cholesterol Mobilization From the Endo-Lysosome Compartment of NPC1-deficient Human Cells by β-CD Polyrotaxanes", ***PLoS ONE*** **2022** *17*, e0268613.

166. Z. J. Struzik, S. Biyani, T. Grotzer, J. Storch, D. H. Thompson, "Synthesis of Phosphatidyl Glycerol Containing Asymmetric Acyl Chains Using H-Phosphonate Methodology", ***Molecules*** **2022** *27*, 2199.

167. R. J. Nicholas, M. A. McGuire, S.-H. Hyun, M. N. Cullison, D. H. Thompson, "Development of an Efficient, High Purity Continuous Flow Synthesis of Diazepam", ***Frontiers in Chemical Engineering*** **2022** *4*, 877498.

168. S. A. Biyani, C. Lytle, S.-H. Hyun, M. McGuire, R. Pendyala, D. H. Thompson, "Development of a Continuous Flow Synthesis of Lorazepam", ***Organic Process Research & Development*** **2022** *26*, 2715-2727.

169. Aayush, S. Darji, D. Dhawan, A. Enstrom, M. M. Broman, M. T. Idrees, H. Kaimakliotis, T. Ratliff, D. Knapp, D. H. Thompson, "Targeted Elastin-like Polypeptide Fusion Protein for Near-Infrared Imaging of Human and Canine Transitional Cell Carcinoma in Bladder Tumors", ***Oncotarget*** **2022** *13*, 1004-1016.

170. S. C. Bolton, D. H. Thompson, T. L. Kinzer-Ursem, "Method Optimization for the Expression and Purification of Human Calcium Calmodulin-Dependent Protein Kinase II Alpha", ***PLoS ONE*** **2023**, in press.

171. S. Darji, A. Aayush, K. M. Estes, J. D. Strock, D. H. Thompson, "Unravelling the Mechanism of Elastin-like Polypeptide-Enzyme Fusion Stabilization in Organic Solvent", ***Biomacromolecules*** **2023**, submitted.

172. K. M. Estes, D. H. Thompson, "Synthesis and Characterization of One-Dimensional Water-Soluble Copolymer Brushes: A Review", ***Polymer Reviews*** **2023**, submitted.

IN PREPARATION

173. Aayush, S. Darji, D. H. Thompson, "Development of an Elastin-like Polypeptide-based Nucleic Acid Delivery System Targeted to EGFR+ Bladder Cancer Cells Using a Layer-by-Layer Approach".

174. J. Mackey, I. Akturk, A. Mufti, A. Harris, J. Prahlow, D. Weinstein, S. Godoshian, D. H. Thompson, Z. K. Nagy, "End to End Synthesis and Purification of Lomustine".

175. Z. Struzik, B. Cooper, O. Ilnytska, J. Storch, D. H. Thompson, "Elucidation of the Phosphatidylglycerol Lipid Biosynthetic Pathway Using Multiple Reaction Monitoring".

BOOK CHAPTERS, REVIEWS & OPINION ARTICLES

1. "Comprehensive Organic Transformations: A Guide to Functional Group Preparations, 2nd Edition" (R. C. Larock, Wiley, 1999) *Pharmaceutical Research* **2001** *18*, 1386.

2. "Solid-Phase Organic Synthesis" (K. Burgess, Ed., Wiley, 2000) ***Pharmaceutical Research*** **2001** *18*, 1386-1387.

3. "Solid-Phase Synthesis and Combinatorial Technologies" (P. Seneci, Ed., Wiley, 2000) ***Pharmaceutical Research*** **2001** *18*, 1387.

4. "Active Transport of Ca^{2+} by an Artificial Photosynthetic Membrane" News & Views, ***Nature Materials*** **2002**, *1*, 214-215; ibid, pp. 398-401.

5. D. H. Thompson, P. Shum, O. V. Gerasimov, M. M. Qualls, "Photosensitive Liposomes as Potential Targeted Therapeutic Agents", in Nanoparticulate Drug Delivery Systems: Strategies, Technologies, and Applications, Y. Yeo, Ed., John Wiley & Sons, Hoboken, NJ, 2013, pp. 81-99.

6. S. Samaddar, D. H. Thompson, "Nucleic Acid Anticancer Agents", in Biomaterials for Cancer Therapeutics: Evolution and Innovation, 2nd Edition, K. Park, Ed., Elsevier, 2020, pp. 165-185.

PATENTS

1. U. S. Patent #**5,277,913** (Issued 1/11/94): D. Thompson, V.C. Anderson, "Liposomal Delivery System With Photoactivatable Triggered Release." (Japanese Patent No. 502261/95)

2. U.S. Patent #**6,979,460** (Issued 12/27/05): D. Thompson, J.A. Boomer, R. Haynes, "Vinyl Ether Lipids with Cleavable Hydrophilic Headgroups"

3. US Patent #**7,374,944** (Issued 5/20/2008): O. Basaran, C. Hrycyna, K. Park, D. Thompson, "Device and Bioanalytical Method Utilizing Asymmetric Biofunctionalized Membrane"

4. US Patent **#8,916,697** (Issued 12/23/2014): D. Thompson, "Nucleic Acid Complexes, Methods of Preparation Thereof, and Uses Thereof for Delivery Nucleic Acid Into A Cell"

5. US Patent **#9,341,621 B2** (Issued 5/17/16): C. Savran, P. Low, D. Thompson, W. Henne, D. Doornweerd, C.-L. Chang, G. Acharya, D. Brownholland: "Ultrasensitive Detection of Biomolecules Using Immunoseparation and Diffractometry"

6. US Patent #**9,518,114 B2** (Issued 12/13/16): R.C. Aguilar, T. Ratliff, D. Thompson, S. Crist, "Treating Bladder Tumor Cells Using Fibronectin Protein As A Target"

7. U.S. Patent #**9,526,802 B2** (Issued 12/27/16): D. Thompson, A. Kulkarni, W. Deng, "Nucleic Acid Complexes".

8. U.S. Patent #**10,000,581 B2** (Issued 6/19/18): D. Thompson, A. Kulkarni, C. Collins, Y. Mondjinou: "Therapeutic and Imaging Compositions and Uses Thereof".

9. U.S. Patent #**10,118,996 B2** (Issued 11/6/18): D. Thompson, S.-H. Hyun, K. J. Wright: "Polyrotaxanes and Uses Thereof".

10. U.S. Patent #**11,434,330 B2** (Issued 9/6/22): D. Thompson, S.-H. Hyun, Y. Mondijnou, C. Collins: "Polyrotaxanes Bearing Mixed Cyclodextrin Species and Uses Thereof".

11. WO 2019/165390: D. H. Thompson, R. VerHeul, C. Sweet, "Rapid and Simple Purification of Elastin-like Polypeptides Directly from Whole Cells and Cell Lysates by Organic Solvent Extraction".

12. 2019-THOM-68395: D. H. Thompson, Z. Jaman, R. G. Cooks, C. A. Ferreira, "Low Cost Synthesis of Lomustine Under Continuous Flow Conditions".

13. 2020-THOM-69095: D. H. Thompson, S. Biyani, Q. Qi, H. Sintim, "New Method for Synthesizing HSN608 Anticancer Agent, HSN608s".

14. 2020-THOM-69096: D. H. Thompson, Z. Nagy, A. Mufti, J. Mackey, "Scaled-Up Synthesis of Lomustine Under Continuous Flow Conditions"

15. 2021-THOM-69651: D. H. Thompson, "A Fast and Efficient Process for the Preparation of N-Retinylidene-N-Retinylethanolamine (A2E)".

16. 2022-THOM-69904: D. H. Thompson, S. Biyani, M. A. McGuire, "Continuous Flow Synthesis of Lorazepam".

17. 2023-THOM-xxxxx: D. H. Thompson, A. Aayush, S. Darji, "Delivery of Nucleic Acids Using a Targeted Peptide Carrier System".

CONFERENCES ORGANIZED

American Chemical Society Symposium: "Intelligent Materials & Novel Concepts for Controlled Release Technology: Liposome Technology", 4/13-18/97, San Francisco, CA.

Materials Research Society Symposium: "Materials Science of Phospholipid Assemblies", 11/29-12/3/99, Boston, MA.

Gordon Research Conference, *Chemistry of Supramolecules and Assemblies*, 6/12-6/17/05, Colby College, Waterville, ME; www.grc.uri.edu/programs/2005/supramol.htm.

American Chemical Society Symposium: "Drug Delivery", MEDI, 8/21/07, Boston, MA.
American Chemical Society Symposium: "Advanced Materials Designs for Drug and Gene Delivery", MEDI, 9/10/13, Indianapolis, IN.
American Chemical Society Symposium: "Smart Polymers and Materials from Cyclodextrins", POLY, Spring 2017, San Francisco, CA.

INVITED PRESENTATIONS

FASEB Archaebacteria Research Meeting, Williamsburg, VA, 10/24/89, Lecture.
Oregon Materials Symposium, Eugene, OR, 5/9/92, Lecture.
GRC: *Chemistry of Supramolecules & Assemblies,* Henniker, NH, 7/5/93, Discussion Leader.
GRC: *Chemistry of Supramolecules & Assemblies,* Henniker, NH, 7/28/95, Lecture.
American Oil Chemist's Society, *Gene Therapy Symposium*, Indianapolis, IN, 4/30/96, Lecture.
NSF Workshop on Physical Organic Chemistry, Whidbey Island, WA, 6/96, Lecture.
9th Int'l Symp. on Molecular Recognition and Inclusion, Lyon, France, 9/7-12/96, Lecture.
Artificial Self-Assembling Systems for Gene Delivery, Coronado, CA, 11/16/96, Lecture.
ACS Symposium: *Intelligent Materials for Drug Delivery*, San Francisco, CA, 4/13-18/97, Lecture.
1st Int'l Symp. on Ordered Proteins at Interfaces, U. Washington, Seattle, WA, 8/17-19/97, Lecture.
ACS Symposium: *Syn. Design and Char. Surf. & Interfac.*, Las Vegas, NV 9/7-11/97, Lecture.
Ohio State University, Department of Chemistry, Columbus, OH, 1/29/98
Renaissance in Dermatology, Montecatini, Italy, 3/25-28/98, Lecture.
6th Int'l Liposome Research Days Conference, Isle d'Hyeres, France, 5/28-31/98, Lecture.
1st Int'l Supramolecular Chemistry Conference, Zakopane, Poland, 9/27-10/2/98, Lecture.
University of Utrecht-Department of Pharmaceutics, Utrecht, Holland, 10/6/98, Lecture.
Eindhoven University-Department of Chemistry, Eindhoven, Holland, 10/7/98, Lecture.
1st Int'l Academy of Cosmetic Dermatology, St. Julian, Malta, 1/27-31/99, Lecture.
Structure and Design of Synthetic Gene Carriers, San Francisco, CA, 2/24-26/99, Lecture.
Bürgenstock Conference on Stereochemistry, Burgenstock, Switzerland, 4/25-30/99, Poster.
GRC: *Chemistry of Supramolecules and Assemblies*, Henniker, NH, 8/1-6/99, Discussion Leader.
ACS Symposium: *Novel Surfactants*, New Orleans, LA, 8/22-26/99, Lecture.
IUPUI Physics Colloquium, Indianapolis, IN, 9/16/99
National Institute of Standards & Technology, Rockville, MD, 10/19/99, Lecture.
ARO Workshop, *Templated Nanoscale Synthesis & Reactivity*, Edgewood, MD, 10/20-21/99, Lecture.
University of Toronto-Dept. Medical Biophys. & Biochem., Toronto, ON, 12/9/99, PENCE Lecture.
GRC: *Drug Carriers in Medicine & Biology*, Ventura, CA, 2/20-25/00, Lecture.
Liposome Research Days, Napa, CA, 4/12-15/00, Lecture.
Central Regional ACS Meeting Symposium, Cincinnati, OH, 5/16-19/00, Lecture.
NIH Workshop, *Nanoscience & Nanotechnology*, Bethesda, MD, 6/25-26/00, Poster.
XVIII IUPAC Symposium on Photochemistry, Dresden, Germany, 7/22-25/00, Poster.
10th Int'l Conf. on Colloid and Interfacial Science, Bristol, England, 7/25-28/00, Lecture.
NSF Workshop on Materials Chemistry, Mt. Hood, OR, 10/12-15/00, Lecture.
Washington State University-Department of Chemistry, Pullman, WA, 11/27/00, Lecture.
North Carolina State University-Department of Chemistry, Raleigh, NC, 1/29-30/01, Lecture.
Particles 2001, Orlando, FL, 2/24-28/01, Lecture.
Antioch College-Department of Chemistry, Antioch, OH, 3/16/01, Lecture.
Interface of Biology & Mat. Sci., Purdue MATCON Symposium, West Lafayette, IN, 3/28/02, Lecture.
ACS Symposium: *Drug Delivery Systems*, Orlando, FL, 4/7-9/02, Lecture.
Particles 2002, Orlando, FL, 4/21-23/02, Keynote Lecture.
Bowling Green State University-Department of Chemistry, Bowling Green, OH, 5/1/02, Lecture.

ACS Symposium: *Polymeric Bioconjugates*, Boston, MA, 8/22/02, Lecture.
Elmhurst College-Department of Chemistry, Elmhurst, IL, 9/18/02, Lecture.
2nd Conf. on Tumor Targeted Drug Delivery, National Cancer Inst., Bethesda, MD, 9/24/02, Lecture.
Brown University-Department of Chemistry, Providence, RI, 3/7/03, Lecture.
University of Florida-Department of Pharmaceutics, Gainesville, FL, 4/24/03, Lecture.
University of Wisconsin-Department of Pharmacy, Madison, WI, 5/6/03, Lecture.
Colorado State University-Department of Chemistry, Ft. Collins, CO, 5/10/03, Lecture.
GRC: *Bioorganic Chemistry*, Andover, NH, 6/19/03, Lecture.
GRC: *Chemistry of Supramolecules & Assemblies*, Ventura, CA, 7/7/03, Discussion Leader.
ACS Symposium: *Polymeric Drug Delivery* Symposium, New York, NY, 9/7/03, Lecture.
11th Int'l Conf. on Surface & Colloid Science, Iguassu Falls, Brazil, 9/15-19/03, Lecture.
9th Int'l Kyoto Conference on New Aspects of Organic Chemistry, Kyoto, JP, 11/12/03, Lecture.
Osaka Prefecture University-Department of Applied Materials Science, Osaka, JP, 11/12/03, Lecture.
Japan Advanced Institute of Science & Tech., School of Mat. Sci., Komatsu, JP, 11/14/03, Lecture.
Osaka Dental University, Osaka, JP, 11/17/03, Lecture.
Osaka University-Department of Molecular Chemistry, Osaka, JP, 11/20-21/03, 3 Lectures.
University of Victoria-Department of Chemistry, Victoria, BC, Canada, 1/5/04, Lecture.
Rutgers University-Department of Chemistry, New Brunswick, NJ, Departmental Colloquium.
9th Liposome Research Days, Taipei, Taiwan, 5/12/04, Lecture.
Purdue Nanomedicine Symposium, West Lafayette, IN, 7/26/04, Lecture.
13th Int'l Symposium on Supramolecular Chemistry, South Bend, IN, 7/27/04, Lecture.
ARO Workshop, Jackson Hole, WY, 10/7/04, Lecture.
National Renewable Energy Laboratory, Golden, CA, 1/24/05, Lecture.
University of Colorado-Department of Chemistry, Boulder, CA, 1/25/05, Lecture.
Max Planck Institute-Biophysics Institute, Frankfurt, Germany, 1/30/05, Lecture.
University of Twente-Department of Chemistry, Enschede, Netherlands, 2/2/05, Lecture.
ACS Symposium: *Surfactant Self-Assembly*, San Diego, CA, 3/13-3/17/05, 3 Lectures.
MRS Symp.: *Nano-Bio Interfac.; Smart Surf.; Dyn. Self-Assem.*, San Francisco, CA, 3/29-30/05, 3 Lect.
Scanning 2005, Monterrey, CA, 4/7/05, Lecture.
University of North Carolina, Chapel Hill-Department of Chemistry, Chapel Hill, NC, 4/28/05, Lecture.
GRC: *Chemistry of Supramolecules & Assemblies,* Ventura, CA, 6/17/05, Discussion Leader.
Particles 2005, San Francisco, CA, 8/15/05, Keynote Lecture.
NanoTechnology 2005, JAIST-Komatsu, JP, 9/15-9/19/05, Lecture.
Illinois State University, Department of Chemistry, Normal, IL, 1/27/06, Lecture.
Frontiers in Nanotechnology, Northwestern University, Evanston, IL, 2/23/06, Lecture.
University of Arizona, Department of Chemistry, Tuczon, AZ, 2/27/06, Lecture.
Particles 2006, Orlando, FL, 5/14/06, Keynote Lecture.
Purdue University Cancer Center Retreat, West Lafayette, IN, 9/7/06, Lecture.
Osaka University, Department of Applied Chemistry, Osaka, JP, 11/20/06, Lecture.
Hokkaido University, Department of Pharmaceutical Sciences, Hokkaido, JP, 11/22/06, Lecture.
Nara Advanced Institute of Science & Technology, School of Chem. Sci, Nara, JP, 11/27/06, Lecture.
Osaka University, Department of Polymer Science, Osaka, JP, 11/28/06, Lecture.
Japan Advanced Institute of Science & Tech., School of Mat. Sci., Komatsu, JP, 11/29/06, Lecture.
RIKEN, Tokyo, JP, 12/4/06, Lecture.
University of Tokyo-Hongo, Tokyo, JP, 12/6/06, Lecture.
Tokyo Institute of Technology, Tokyo, JP, 12/7/06, Lecture.
33rd Symposium on Main Group Chemistry, Fukuoka University, Kyushu, JP, 12/9/06, Lecture.
Kyushu University, Dept. of Chemistry & Biochemistry, Kyushu, JP, 12/11/06, Lecture.
Tokyo Medical & Dental University, Inst. of Biomaterials & Bioengr., Tokyo, JP, 12/12/06, Lecture.

Kyoto University, Graduate School of Engineering, Kyoto, JP, 12/13/06, Lecture.
Kyoto University, Graduate School of Human & Environmental Studies, Kyoto, JP, 12/15/06, Lecture.
National Meeting, American Oil Chemist's Society, Quebec City, Quebec, 5/14/07, Lecture.
Steacie Institute for Molecular Science, NRC, Ottawa, Canada, 5/17/07, Lecture.
ACS Symposium: *Drug Delivery*, Boston, MA, 8/21/07, Lecture.
Polytechnic University, Dept. of Chemical & Biological Engineering, Brooklyn, NY, 11/16/07, Lecture.
International Liposome Society 2007 Annual Meeting, London, UK, 12/8-11/07, Lecture.
City College of New York, Dept. of Chemical Engineering, New York, NY, 3/3/08, Lecture.
Particles 2008, Orlando, FL, 5/10-13/08, Keynote Lecture.
Technical University of Denmark, Dept. of Micro & Nanotechnology, Roskilde, DK, 7/4/08, Lecture.
Tokyo Medical & Dental University, Inst. of Biomatl's & Bioengineering, Tokyo, JP, 7/17/08, Lecture.
11th Liposome Research Days, Yokohama, JP, 7/20-22/08, Lecture.
GRC: *Drug Carriers in Medicine & Biology*, Big Sky, MT, 8/24-29/08, Lecture.
University of Pennsylvania, Dept. of Pharmacology, Philadelphia, PA, 11/12/08, Lecture.
National Cancer Institute, Nanobiology Seminar Series, Frederick, MD, 1/16/09, Lecture.
ARO Workshop, Napa, CA, 3/11/09, Lecture.
Nanotechnology, Liposomes and Health, Ilha Itaparica, Brazil, 4/18/09, Lecture.
Rutgers University-Department of Chemistry, New Brunswick, NJ, 10/6/09, Lecture.
U. Southern California, Dept. of Pharmacology & Pharmaceutical Sci, Los Angeles, CA, 12/09, Lecture.
ACS Symposium in Honor of Clifford Bunton, San Francisco, CA, 3/21/10, Lecture.
Brazilian Biochemistry & Cell Biology Annual Meeting, Iguassu Falls, Brazil, 5/16-20/10, Lecture.
Particles 2010, Orlando, FL, 5/23/10, Keynote Lecture.
Bio/Abio Interface Symposium, U. Canterbury, Christchurch, NZ, 6/22-24/10, Lecture.
KIST-Purdue Symposium, Seoul, ROK, 6/28/10, Lecture.
Pohang University of Science & Technology (POSTECH), Pohang, ROK, 7/1/10, Lecture.
ACS Symposium on Drug & Gene Delivery, Boston, MA, 8/22-24/10, Lecture.
GRC: *Biointerfacial Science*, 9/5-10/10, Les Diablerets, Switzerland, Lecture.
University of Basel-Department of Chemistry, Basel, Switzerland, 9/10/10, Lecture.
University of Geneva-Department of Chemistry, Geneva, Switzerland, 9/14/10, Lecture.
Case Western Reserve University, Dept. of Biomedical Engineering, Cleveland, OH, 2/24/11, Lecture.
Parseghian Scientific Conf. for Niemann-Pick Type C Research, South Bend, IN, 6/10/11, Lecture.
Technical University of Denmark, Dept. of Micro- and Nanotechnology, Lyngby, DK, 8/11/11, Lecture.
President's Council Back-to-Class, West Lafayette, IN, 9/30/11, Lecture.
U. Michigan-Department of Chemistry, Ann Arbor, MI, 11/10/11, Lecture.
Purdue U.-Department of Biological Sciences, West Lafayette, IN, 11/16/11, Lecture.
Georgetown U., Dept. of Chemistry, Georgetown, DC, 2/2/12, Lecture.
Parseghian Scientific Conf. for Niemann-Pick Type C Research, South Bend, IN, 6/9/12, Lecture.
Shanghai University, Department of Chemistry, Shanghai, China, 10/7/12, Lecture.
Southwest University for the Nationalities, Chengdu, China, 10/9/12, Lecture.
University of Illinois-Chicago, Dept. of Pharmacy, Chicago, IL, 3/27/13, Lecture.
GRC: *Self-Assembly and Supramolecular Chemistry*, 5/5-10/13, Les Diablerets, Switzerland, Lecture.
16th Annual Meeting, American Society of Gene & Cell Therapy, Salt Lake City, UT, 5/15/13, Lecture.
National University of Singapore, Dept. of Chemical & Biomolecular Engineering, Singapore, 7/26/13
SABIC, Bangalore, India, 7/30/13, Lecture.
Indian Institute of Science, Bangalore, India, 8/1/13, Lecture.
Chulalongkorn University, Bangkok, Thailand, 8/5 – 8/12/13, Short Course.
Contrast Media Research 2013, Beijing, China, 11/3/13, Lecture.
Institute of Chemical Technology-Mumbai, Mumbai, India, 11/28/13, Lecture.
Southwest University for the Nationalities, Chengdu, China, 4/8/14, Lecture.

17th Cyclodextrin Symposium, Saarbrücken, Germany, 5/29 – 5/31/14, Plenary Lecture.
MM&C Parseghian Scientific Conference for NPC Research, South Bend, IN, 6/14/14, Lecture.
U. Pennsylvania-CT3N Symposium, Philadelphia, PA, 11/12/14, Lecture.
Rare and Neglected Disease Symposium, South Bend, IN, 2-13-15, Lecture.
U. Missouri-Columbia, Columbia, MO, 3-2-15, Lecture.
4th European Conference on Cyclodextrins, Lille, France, 10/8/15, Lecture.
Goethe University, Department of Pathobiology, Frankfurt, Germany, 10/13/15, Lecture.
Contrast Media Research 2015, Berlin, Germany, 11/3/15, Lecture.
University of California – Los Angeles, Department of Chemistry, Los Angeles, CA, 2/29/16, Lecture.
Carthage College, Department of Chemistry, Kenosha, WI, 9/19/16, Lecture.
University of Notre Dame – Purdue University Polymer Symposium, South Bend, IN, 10/8/16, Lecture.
Northeastern University, Department of Pharmaceutics, Boston, MA, 4/20/17, Lecture.
Tulane University, Department of Chemistry, New Orleans, LA, 4/24/17, Lecture.
Purdue University, Dept. of Industrial & Physical Pharmacy, West Lafayette, IN, 11/20/17, Lecture.
Oregon Health & Science University, Portland, OR, 6/19/18, Lecture.
GRC: *Drug Carriers in Medicine & Biology*, 8/12 – 8/17/18, Lecture.
University of Washington, Molecular Engineering & Sciences Institute, Seattle, WA, 10/23/18, Lecture.
Pharmaceutica 2019, Edinburgh, Scotland, 3/19/19, Lecture.
SOAR-NPC Cyclodextrin Meeting, New York, NY, 4/23/19, Lecture.
Commercializing Flow Chemistry Summit 2020, Virtual Meeting, 8/26/20, Lecture.
Drug Discovery, Development & Lead Optimization 2020, Virtual Mtg, 10/5/20, Lecture & Session Chair.
2020 Nat'l Inst. for Pharmaceutical Tech. & Education Research Conf., Virtual Meeting, 12/9/20, Lecture.
eMolecules Webinar, Virtual Meeting, 4/21/21, Lecture.
2nd Applied Biocatalysis Summit 2021, Virtual Meeting, 11/4/21, Lecture.
Flow Chemistry Summit 2022, Boston, MA, 3/17-18/22, Lecture.
ACS National Meeting, Innovative Technologies to Support 21st Century Agriculture Symposium, Indianapolis, IN, 3/27/23, Lecture.
Commercializing Flow Chemistry Summit 2023, Boston, MA, 10/16-18/23, Lecture.
API Innovation Center Summit: Pathway to Build Supply Chain Resilience for Critical Drugs, 11/8/23, Workshop Panelist.
8th International Conference on Catalysis and Chemical Engineering, Boston, MA, 2/26-28/24, Lecture.

SERVICE

Reviewer for the following journals:

Science
Nature
Nature-Biotechnology
Nature-Materials
Science Translational Medicine
Journal of the American Chemical Society
Angewandte Chemie, International Edition
Langmuir
Bioconjugate Chemistry
Journal of Organic Chemistry
Organic Letters
Journal of Medicinal Chemistry
Journal of Physical Chemistry B
Macromolecules
Biophysical Journal
Biophysical Chemistry
Chemistry-A European Journal
Chemistry & Biology
Advanced Drug Delivery Reviews
Biochimica et Biophysica Acta
Pharmaceutical Research
Bioorganic & Medicinal Chemistry Letters
Journal of Controlled Release
Biochemistry
Biomacromolecules
Photochemistry & Photobiology
Journal of Colloid & Interfacial Science
Tetrahedron

European Journal of Pharmaceutics & Biopharmaceutics
Nanomedicine
Chemistry of Materials
ACS Nano
Chemistry & Physics of Lipids
Biotechnology & Bioengineering
Journal of Chemical Education
Soft Matter
Chemical Communications
Protein Science
Lipids
Journal of Membrane Science
Supramolecular Chemistry
Organic & Biomolecular Chemistry

Ad-hoc reviewer for new faculty & international grant programs:

Cottrell Scholar, Research Corporation
Dreyfus Teacher-Scholar Program
NATO Former Soviet Union-US Research Program
Saskatchewan Medical Research Foundation
UK Biotechnology & Biological Sciences Research Council
Swiss National Science Foundation
Petroleum Research Foundation Program
Israeli-US Bilateral Research Program
Biomedical Research Council-Singapore

Purdue Service:

Center for Cancer Research *Targets, Structures & Drugs* Interdisciplinary Research Director: 2018-2021
Center for Cancer Research *Chemical & Structural Biology* Interdisciplinary Research Co-Director: 2008-2018
Center for Cancer Research Faculty Screening Committee: 2002
Center for Cancer Research Grant Review Committee: 2001-present
College of Science Faculty Council: 2000-2004
College of Science Educational Policy Committee: 2000-2004
College of Science Membrane Science COALESCE Faculty Recruiting Committee: 2003-2008
Life Science Education (PULSe) Admissions Committee: 2004-2007, 2011-2014
Life Science Microscopy Facility Advisory Committee: 2007-2018
Library Committee: 2000-2003
Department of Chemistry Primary Promotions Committee: 2005-2009
Department of Chemistry Executive Committee: 2001-2011
Department of Chemistry Faculty Recruiting & Screening Committees: 2001 (Chemical Biology), 2010-2017 (Drug Discovery), 2011 (Organic Chemistry), 2013 (Organic Materials), 2022 (Organic Chemistry)
Department of Chemistry Graduate Studies Committee: 1998-2003, 2011-2015
Department of Chemistry Industrial Associates Committee: 1994-2000, 2009-2011
Horizons Program: 1997-2001, 2011
ACS Student Affiliates Advisor: 1994-1997
Course Developer, NSF Interdisciplinary Science Education for Engineers: 2005-2015

INVOLVEMENT IN EDUCATIONAL PROGRAMS

Graduate Students Receiving Degree

Aayush, April 20, 2023
Ph.D. Thesis: *Developing Elastin-like Polypeptide-based Imaging and Drug Delivery Systems*

Vivek Badwaik, April 19, 2016
Ph.D. Thesis: *Development and Evaluation of Cyclodextrin Based Materials for Applications in Gene Therapeutics*

Christopher Benjamin, August 17, 2015
Ph.D. Thesis: *Non-Fouling Affinity Platforms For Protein Immobilization In Electron Microscopy*

Shruti Biyani, June 14, 2022
Ph.D. Thesis: *High-Throughput Experimentation and Continuous Flow Synthesis of Active Pharmaceutical Ingredients*

Scott C. Bolton, November 26, 2019
Ph.D. Thesis: *Quaternary Structure Analysis of Calcium/Calmodulin-Dependent Protein Kinase II Alpha by Cryo-electron Microscopy*

Jeremy A. Boomer, January 25, 2000

Ph.D. Thesis: *Synthesis of Acid-Sensitive Lipids and Their Application in Drug & Gene Delivery*

Christopher J. Collins, May 19, 2015

Ph.D. Thesis: *Synthesis, Characterization, In Vitro Evalution, And Preclinical Profiling Of β-Cyclodextrin Polyrotaxane Families For Use As Potential Niemann-Pick Type C Therapeutics*

Damien Dobson, May 2022

M.S. Thesis: *High-throughput Experimentation of the Buchwald-Hartwig Amination for Reaction Scouting and Guided Synthesis*

H. Sam Ewan, November 8, 2019

Ph.D. Thesis: *High-Throughput Experimentation with Desorption Electrospray Ionization Mass Spectrometry to Guide Continuous-Flow Synthesis*

Wilma Febo-Ayala, August 14, 2006

Ph.D. Thesis: *Synthesis and Structure-Function Relationships of Tetraether Bisglyceryl Bolalipids for Development of a High-Throughput Membrane Protein Biosensor*

Jessica Grey, October 1, 2010

Ph.D. Thesis: *Development of NTA-modified Ligands and Non-covalent Cyclodextrin-based Templates for Crystallization of Histidine-tagged Proteins*

Andres Gonzalez-Bonet, June 28, 2013

Ph.D. Thesis: *Development of Fusogenic Liposomes Based on Flavivirus-derived Lipopeptides*

Maggie Gunnerson, December 15, 2006

M.S. Thesis: *Design & Synthesis of NTA-Polyrotaxanes for 1D Crystallization of His-Tag Proteins*

Minji Ha, May 25, 2015

M.S. Thesis: *Non-covalent Affinity Materials For Protein Structure Determination Via Single Particle Reconstruction Analysis Cryo-Electron Microscopy*

David P. Holland, July 14, 2008

Ph.D. Thesis: *The Development of a Membrane-Based Biosensor of ICMT: Membrane Synthesis, Properties and Methods of Detection*

Robin Hyder, December 1, 1998

M.S. Thesis: *Synthesis of Lipids for Two-Dimensional Crystallization*

Zinia Jaman, April 8, 2019

Ph.D. Thesis: *High Throughput Experimentation as a Guide to the Continuous Flow Synthesis of Active Pharmaceutical Ingredients*

Hee-kwon Kim, July 14, 2011

Ph.D. Thesis: *Synthesis of Biofunctional Compounds for Protein Crystallization and Gene Delivery*

Aditya Kulkarni, November 16, 2012

Ph.D. Thesis: *Development of Cyclodextrin Based Materials for Gene Delivery*

Young Lee, March 2, 2017

Ph.D. Thesis: *Development of Targeted Liposomes for Bladder Tumor Imaging and Treatment*

Scott Loethen, March 24, 2008

Ph.D. Thesis: *Cyclodextrin-Based Pseudopolyrotaxanes and Polyrotaxanes for Biological Applications*

Brad Loren, April 16, 2018

Ph.D. Thesis: *Part I - Polyrotaxanes as MRI Contrast Agents and NPC Therapeutics. Part II – Development of an Analytic-Directed Synthesis System*

Jonathan Merrell, M.D., Indiana School of Medicine, August 2016

M.S. Thesis: *Development of Degradable Polymer Particles for Administration of Drugs to the GI Tract*

Yawo Mondjinou, July 21, 2015

Ph.D. Thesis: *β-Cyclodextrin Derivatives Based Molecular Machines for Biomedical Applications and Magnetic Resonance Imaging*

Robert J. Nicholas, May 2022

M.S. Thesis: *Process Development for the Synthesis of Essential Medicines in Continuous Flow*

Patrick Palafox, May 10, 2010

M.S. Thesis: *Modification of Conductive Surfaces for Imaging and Detecting of Biological Activity*

Aniruddha Patwardhan, August 25, 2000

Ph.D. Thesis: *Development of Novel Membrane Materials and Their Application to Coupled Transport Phenomena*

Lizhen Peng, April 27, 2012

M.S. Thesis: *Controlled Nucleation of His-tag Protein Assemblies Using Rigid Symmetric Multivalent Nitrilotriacetic Acid Chelating Ligands*

Marquita M. Qualls, February 2, 2001

Ph.D. Thesis: *Targeted and Triggerable Lipid Carriers for the In vitro Delivery of Water Soluble Molecules*

Yuanjin Rui, November 6, 1996

Ph.D. Thesis: *Synthesis and Drug Delivery Applications of Plasmenylcholine Liposomes*

Shayak Samaddar, July 17, 2019

Ph.D. Thesis: *Delivery Strategies for Gene Therapy*

Junhwa Shin, November 24, 2002

Ph.D. Thesis: *Synthesis and Acid-Triggered Release Activity of PEG Lipid Conjugates in DOPE Liposomes*

Pochi Shum, April 24, 2002

M.S. Thesis: *Formation of Fibrinogen-Based Hydrogels Using Phototriggerable Liposomes*

Nicholas Snead, July 9, 2008

M.S. Thesis: *Developing Polyrotaxanes as siRNA Delivery Vectors*

Zachary Struzik, April 12, 2022

Ph.D. Thesis: *Synthesis and Evaluation of Labeled Phosphatidylglycerol Probes to Elucidate Mechanisms Behind Cholesterol Trafficking in Niemann-Pick Type C Disease*

Craig Sweet, November 24, 2020

Ph.D. Thesis: *Applications of Elastin-like Polypeptides in Imaging and Drug Delivery for Bladder Cancer Using Facile Organic Extraction Purification*

Brandi Thomas, March 27, 2001

M.S. Thesis: *Preparation of NTA Chelating Lipids and Green Fluorescent Protein for the Characterization of Two-Dimensional Crystallization*

Jeroen Van den Bossche, March 21, 2008

Ph.D. Thesis: *Polyethylene Glycol Conjugates and Vinyl Ether Constructs for Programmed Gene and Drug Delivery*

Kyle J. Wright, November 30, 2016

Ph.D. Thesis: *Synthesis and Performance af Novel Supramolecular Tools for Single-Particle Cryogenic Electron Microscopy and Drug and Gene Delivery*

Yi Xiao, November 29, 2007

M.S. Thesis: *Oxidation of Vinyl Ether Compounds: Behavior and Kinetics*

Mingkang Zhou, July 14, 2006

Ph.D. Thesis: *Design, Synthesis and Performance of Cyclodextrin-Based Noncovalent Templates for Crystallization of Histidine-Tagged Proteins*

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Mr. Rasmus Jølck, Technical University of Denmark, 9/10 – 12/10
Mr. Anthony Marshal, University of Arizona, 1/07
Dr. Nobuyuki Morimoto, Tokyo Medical & Dental University, 10/07 – 1/08
Ms. Erika Murase, Osaka University, 6/07 – 8/07

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JOINT APPENDIX 08

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on January 31, 2011

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By: /Linda Lim/

PATENT
Attorney Docket No.: 020801-007710US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 1406
Edward Yaworski	Examiner: Jennifer S. Pitrak
Application No.: 12/424,367	Technology Center/Art Unit: 1635
Filed: April 15, 2009	AMENDMENT
For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY	
Customer No.: 20350	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Office Action mailed July 30, 2010, 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/Arguments 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.

49-55. (Canceled)

REMARKS/ARGUMENTS

With entry of this Amendment, claims 1-5, 9, 14, 17-26, 38, 47 and 48 are pending in the present application and are currently under examination. In order to expedite prosecution, claim 55 has been canceled without prejudice to future prosecution. No claim amendments have been made and, thus, no new matter has been introduced. Reconsideration is respectfully requested.

I. PRIORITY CLAIM

In the Office Action, the Examiner indicated that all of the claims, except for claim 55, are entitled to the priority date of previously filed U.S. Provisional Application No. 61/046,228. As noted above, without acquiescing to the merits of the Examiner's position, claim 55 has been canceled without prejudice to future prosecution. As such, all of the currently pending claims are, in fact, entitled to the priority date of U.S. Provisional Application No. 61/046228, which is April 15, 2008.

II. REJECTIONS UNDER 35 U.S.C. § 103(a)

Claims 1-4, 9, 14, 17-26, 38, 47, and 48 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over MacLachlan *et al.* (US 2006/0008910). In addition, claims 1-5, 9, 14, 17-26, 38, 47, and 48 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over MacLachlan *et al.* as applied to claims 1-4, 9, 14, 17-26, 38, 47, and 48, and further in view of Fosnaugh *et al.* (US 2003/0143732). Applicants respectfully traverse.

A. The Legal Standard for Obviousness.

The U.S. Supreme Court has affirmed the analysis set forth in *Graham* for the determination of obviousness. *See KSR International Co. v. Teleflex Inc.*, 127 S. Ct. 1727 (2007). Specifically, the Court, quoting from *Graham*, stated:

> Under §103, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the

> pertinent art resolved. Against this background the obviousness or nonobviousness of the subject is determined. Such secondary considerations as commercial success, long felt but unresolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented. *Id.* at 1734.

Among the secondary considerations specifically discussed in *KSR*, the Court emphasized surprising or unexpected results as being indicative of non-obviousness. *Id.* at 1740, citing to *U.S. v. Adams*, 383 U.S. 39, 40 (1996) ("The fact that the elements worked together in an unexpected and fruitful manner supported the conclusion that Adams' design was not obvious to those skilled in the art.") The Federal Circuit has emphatically and repeatedly held that objective evidence of nonobviousness must always be taken into account and not just when the decision-maker is in doubt: "objective evidence such as commercial success, failure of others, long-felt need, and unexpected results must be considered before a conclusion on obviousness is reached." *Hybridtech Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81 (Fed. Cir. 1986). *See also Bausch & Lomb, Inc. v. Barnes Hindes, Inc.*, 230 USPQ 416 (Fed. Cir. 1986); *Jones v. Hardy*, 220 USPQ 1021 (Fed. Cir. 1984). M.P.E.P. § 716.01(a) further states that "[a]ffidavits or declarations, when timely presented, containing evidence of criticality or unexpected results, commercial success, long-felt but unsolved needs, failure of others, skepticism of experts, *etc.*, must be considered by the Patent Office in determining the issue of obviousness of claims for patentability under 35 U.S.C. 103. Thus, the *Graham* factors, including the use of objective evidence of secondary considerations to rebut a *prima facie* case of obviousness, remains the framework to be followed for a determination of obviousness.

With regard to objective evidence of unexpected results, M.P.E.P. § 716.02(a) states that "[a] greater than expected result is an evidentiary factor pertinent to the legal conclusion of obviousness . . . of the claims at issue." M.P.E.P. § 716.02(a) also states that "[e]vidence of unobvious or unexpected advantageous properties, such as superiority in a property the claimed compound shares with the prior art, can rebut *prima facie* obviousness." Furthermore, M.P.E.P. § 716.02(b) states that "[e]vidence of unexpected properties may be in the

form of a direct or indirect comparison of the claimed invention with the closest prior art which is commensurate in scope with the claims."

B. **MacLachlan *et al.* (U.S. Patent Application Publication No. 2006/0008910, co-pending U.S. Patent Application No. 11/148,152)**

Claims 1-4, 9, 14, 17-26, 38, 47 and 48 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over MacLachlan, *et al.* (U.S. Patent Application Publication No. 2006/0008910, co-pending U.S. Patent Application No. 11/148,152). In support of this rejection, the Examiner points out that:

> 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. . . .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).

(*See*, pages 3 and 4 of the Office Action). Based on these teachings, the Examiner alleges that it "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" (*see*, page 5 of the Office Action).

Assuming, *arguendo*, that the Examiner has established a *prima facie* case of obviousness, Applicants submit that the presently claimed SNALP formulations, which are referred to in the specification as "1:57 SNALP," have new and unexpected results. As set forth in M.P.E.P § 2144.05, such new and unexpected results should be more than sufficient to overcome a presumption of obviousness (*see*, *e.g.*, M.P.E.P. § 2144.05, wherein it is stated: "Applicant can rebut a presumption of obviousness based on a claimed invention that falls within

a prior art range by showing '(1) [t]hat the prior art taught aware from the claimed invention. . .**or** (2) that there are new and unexpected results relative to the prior art' (emphasis added)).

It is clear from the specification that the present invention is based, in part, on the surprising discovery that 1:57 SNALP formulations provide ***new and unexpected results*** 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). More particularly, Applicants have found that SNALP formulations having **increased** amounts of cationic lipid, *e.g.*, one or more cationic lipids comprising from about 50 mol % to about 65 mol % of the total lipid present in the particle, provide ***unexpectedly superior 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).

Claim 1 is illustrative of the pending claims and recites:

1. 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.

As demonstrated by the Examples provided in the specification, Applicants have found that the presently claimed 1:57 SNALP formulations 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 1:57 SNALP formulations of the present invention are stable in circulation, *e.g.*, resistant to degradation by nucleases in serum, and are substantially non-toxic to mammals such as humans.

For instance, Figure 3 of Example 4 demonstrates that 1:57 SNALP formulations were significantly ***more 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***. It is noted that the 2:30 SNALP formulation is disclosed in the cited MacLachlan *et al.* reference, and that of all the SNALP formulations prepared and tested in the cited MacLachlan *et al.* reference, the 2:30 SNALP formulation contains the greatest amount of cationic lipid (*i.e.*, 30 mol % cationic lipid). Similarly, Figure 2 of Example 3 demonstrates that the 1:57 SNALP formulations were substantially ***more effective*** at silencing the expression of a target gene as compared to nucleic acid-lipid particles previously described ("2:40 SNALP," wherein the cationic lipid is present in the formulation at about 40 mol %).

In view of the foregoing, Applicants submit that the Examples provided in the specification demonstrate that the presently claimed 1:57 SNALP formulations, which have increased amounts of cationic lipid, are highly effective in down-regulating the mRNA and/or protein levels of target genes. Applicants further submit that the Examples provided in the specification demonstrate that the presence of increased amounts of cationic lipids results in improved and/or enhanced activity of the presently claimed 1:57 SNALP formulations. Such new and unexpected results unequivocally rebut the Examiner's *prima facie* case of obviousness. Therefore, Applicants respectfully request withdrawal of the present rejection under 35 U.S.C. § 103(a).

C. **MacLachlan *et al.* (U.S. Patent Application Publication No. 2006/0008910) and Fosnaugh, *et al.* (U.S. Patent Application Publication No. 2003/0143732)**

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over MacLachlan *et al.* as applied to claims 1-4, 9, 14, 17-26, 38, 47, and 48, above, and further in view of Fosnaugh *et al.* (US Patent Application Publication No. 2003/0143732).

As set forth above, the presently claimed 1:57 SNALP formulations, which have increased amounts of cationic lipid, 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. Applicants respectfully submit that such new and unexpected results are more than sufficient to rebut a presumption of obviousness based on MacLachlan *et al.* Fosnaugh *et al.* does ***not*** teach or suggest the presently claimed 1:57 SNALP formulations nor their new and unexpected results. Therefore, Applicants respectfully request withdrawal of the present rejection under 35 U.S.C. § 103(a).

III. REJECTION UNDER 35 U.S.C. § 102

Claim 55 was rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by MacLachlan *et al.* (US 2009/0291131) ("MacLachlan"). As mentioned above, in order to expedite prosecution and without acquiescing to the merits of the present rejection, Applicants have canceled, without prejudice, claim 55. In view of the cancellation of claim 55, the present §102(e) rejection is rendered moot. Accordingly, Applicants request that the Examiner withdraw this rejection.

IV. DOUBLE PATENTING REJECTIONS

The Examiner has provisionally rejected claims 1-5, 9, 14, 17-26, 38, 47, 48 and 55, in various combinations, under the judicially created doctrine of obviousness-type double patenting over claims of co-pending Application Nos. 12/343,342, 11/148,152, 11/174,453, 11/283,550, 11/426,907, 11/511,855, 11/584,341, 11/092,756, 11/807,872 and 12/359,119. Because such obviousness-type double patenting rejections are provisional rejections, Applicants respectfully request that the Examiner hold these rejections in abeyance until there is an indication of allowable subject matter. *See,* M.P.E.P. § 714.02.

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.

Further, the Commissioner is hereby authorized to charge any additional fees or credit any overpayment in connection with this paper to Deposit Account No. 20-1430.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 925-472-5000.

Respectfully submitted,

/Eugenia Garrett-Wackowski, Reg. No. 37,330/

Eugenia Garrett-Wackowski
Reg. No. 37,330

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63128186 v1

JOINT APPENDIX 09

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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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 05/12/2011
KILPATRICK TOWNSEND & STOCKTON LLP
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EXAMINER	
WHITEMAN, BRIAN A	

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
05/12/2011	ELECTRONIC

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.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

Docket@kilpatricktownsend.com
ipefiling@kilpatricktownsend.com
jlhice@kilpatrick.foundationip.com

Office Action Summary	Application No.	Applicant(s)	
	12/424,367	YAWORSKI ET AL.	
	Examiner	Art Unit	
	BRIAN WHITEMAN	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 *31 January 2011*.

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 and 48* 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) *1-5,14,17-26,38,47,48* 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 _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

The examiner of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Brian Whiteman, Art Unit 1635.

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 –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1-4, 9, 14, 17-26, 47, and 48 are rejected under 35 U.S.C. 102(b) as being anticipated by MacLachlan, *et al.* (US 2006/0008910, of record) ("MacLachlan").

The claims are to a nucleic acid lipid particle comprising a nucleic acid, a cationic lipid, a non-cationic lipid mixture of phospholipid and cholesterol, and a conjugated lipid. The claims are further directed to the particle wherein the nucleic acid is a siRNA, the relative amounts of components read on a broad range of amounts because of the term "comprising about". The applicants do not provide a definition of the term in the specification. Thus, "comprising about" could embrace an amount +/- 10, 20, 30 mol % of a lipid component.

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).

Below is a comparison of the lipid components:

instant claims of '367
1) cationic lipid comprising from about 50-65 mol %

2) phospholipid comprises from about 4-10 mol %

3) cholesterol comprising from about 30-40 mol%

4) conjugated lipid comprising from about 0.5-2 mol%

pre-grant US publication (paragraph 0085)
1) cationic lipid 2-60, 5-50, 10-45, 20-40, 30 mol%

2) phospholipid 5-90 mol%

3) cholesterol 20-55 mol %

4) conjugated lipid 1-20 mol %

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.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein

were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claim 5 is rejected under 35 U.S.C. 103(a) as being unpatentable over MacLachlan, *et al.* (US 2006/0008910, of record) 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).

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.

Applicant's arguments filed 1/31/11 have been fully considered but they are not persuasive.

In response to applicant's argument that Fosnaugh and MacLachlan do not teach or suggest 1:57 SNALP formulation and their new and unexpected results, the argument is not found persuasive because while it is acknowledged that 1:57 shows a new an unexpected result, the product recited in the instant claims read on broad range of SNALP formulations, including 2:30 and 2:40 because of the term "comprising from about". The term is broad because the specification does not provide a definition of the term and the term could read on SNALP formulations other than 1:57, e.g., 2:30 and 2:40. "For example, a showing of unexpected results for a single member of a claimed subgenus, or a narrow portion of a claimed range would be sufficient to rebut a prima facie case of obviousness if a skilled artisan "could ascertain a trend in the exemplified data that would allow him to reasonably extend the probative value thereof." See In re Clemens, 622 F.2d 1029, 1036, 206 USPQ 289, 296 (CCPA 1980). In view of Figure 2 and Example 3, one of ordinary skill in the art could not ascertain a trend in the exemplified data that would allow him/her to reasonably extend the probative value thereof.

"In the case where the claimed ranges "overlap or lie inside ranges disclosed by the prior art" a prima facie case of obviousness exists." See In re Wertheim, 541 F.2d 257, 191 USPQ 90 (CCPA 1976); In re Woodruff, 919 F.2d 1575, 16 USPQ2d 1934

(Fed. Cir. 1990). This is the case here since the term "comprising from about" recited in the instant claims embraces a broad range of SNALP formulations.

Furthermore, the claimed invention is directed to a product not a method of using a product. One of ordinary skill in the art would have a reasonable expectation of success for making the claimed product and would have been motivated to study the antisense oligonucleotides in a cell culture. "However, where the claims are not limited to a particular use, and where the prior art provides other motivation to select a particular species or subgenus, a showing of a new use may not be sufficient to confer patentability." See Dillon, 919 F.2d at 692, 16 USPQ2d at 1900-01. "Mere recognition of latent properties in the prior art does not render non-obvious an otherwise known invention." See In re Wiseman, 596 F.2d 1019, 201 USPQ 658 (CCPA 1979).

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).

Claims 1-5, 14, 17-19, 23-26, and 38 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.

Claims 1-4, 9, 14, 17-26, 38, 47, and 48 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-15 of U.S. Patent No. 7,799,565. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '565 application are directed to siRNA-lipid particles comprising the instantly claimed components.

Claims 1-5, 9, 14, 17-19, 21, 22, 23, 25, 26, 38, 47, and 48 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-17 of U.S. Patent No. 7,807,815 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 '815 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 '815 claims with a 2'-O-methyl modification to improve the stability of the siRNA.

This is a provisional obviousness-type double patenting rejection.

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.

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.

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.

Claims 1-5, 9, 14, 17-26, 38, 47, and 48 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-45 of U.S. Patent No. 7,838,658. Although the conflicting claims are not identical, they are not patentably distinct from each other because the claims of the '658 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.

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.

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.

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.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Brian Whiteman whose telephone number 571-272-

0764. The examiner can normally be reached on Monday-Thursday from 6:30 to 4:00 (Eastern Standard Time). The examiner can also be reached on alternate Fridays. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor Heather Calamita can be reached on 571 272-2876. 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).

/Brian Whiteman/

Primary Examiner, Art Unit 1635

JOINT APPENDIX 10

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on August 11, 2011

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Judith Cotham/

PATENT

Attorney Docket No.: 86399-766878(007710US)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Edward Yaworski

Application No.: 12/424,367

Filed: April 15, 2009

For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

Customer No.: 20350

Confirmation No. 1406

Examiner: Brian A. Whiteman

Technology Center/Art Unit: 1635

AMENDMENT

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Office Action mailed May 12, 2011, 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 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. (Currently amended) 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. (Currently amended) 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. (Currently amended) 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. (Currently amended) 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. (Currently amended) 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.

49-55. (Canceled)

56. (New) The nucleic acid-lipid particle of claim 2, wherein said siRNA is about 19 to about 25 base pairs in length.

57. (New) The nucleic acid-lipid particle of claim 2, wherein said siRNA comprises 3' overhangs.

REMARKS

I. STATUS OF THE CLAIMS

After entry of this amendment, claims 1-5, 9, 14, 17-26, 38, 47, 48, 56, and 57 are pending in this application and are presented for examination. Claims 6-8, 10-13, 15, 16, 27-37, and 49-55 have been canceled without prejudice to future prosecution. Claims 1, 9, 21, 47, and 48 have been amended to remove each instance of "about" from the claims. Claims 56 and 57 are newly added and find support, for example, in paragraph [0054] on pages 8 and 9 of the specification as filed.

As such, no new matter has been introduced. Reconsideration is respectfully requested.

II. EXAMINER INTERVIEWS

Applicants' representatives thank Examiner Whiteman for the series of telephonic interviews conducted after issuance of the Office Action mailed May 12, 2011. In particular, on June 14, 2011, Applicants' representatives discussed the art rejections of record with Examiner Whiteman. However, technical difficulties with the U.S. Patent Office phone system resulted in Examiner Whiteman rescheduling the interview. During the next telephonic interview conducted on July 6, 2011, Applicants' representatives discussed the rejection of record under 35 U.S.C. § 102(b), M.P.E.P. 2131.03 (II), and *Atofina v. Great Lakes Chem. Corp*, 441 F.3d 991, 999, 78 USPQ2d 1417, 1423 (Fed. Cir. 2006) with Examiner Whiteman and his supervisor, Examiner Calamita. During the interview, Applicants' representatives proposed amending the claims to delete the word "about" from the ranges of lipid components and argued that the claimed ranges were not anticipated by MacLachlan *et al.* (US 2006/0008910) because that reference failed to disclose the claimed ranges with sufficient specificity as required by M.P.E.P. 2131.03 (II) and *Atofina*. Examiners Whiteman and Calamita indicated that they would present the arguments to Examiner Bennett and notify Applicants' representatives of his decision.

During a subsequent telephonic interview conducted on July 13, 2011, Examiner Whiteman indicated that Examiner Bennett found the arguments of Applicants' representatives

to be persuasive. As a result, Examiner Whiteman agreed to withdraw both rejections under 35 U.S.C. § 102(b) and 35 U.S.C. § 103(a) when Applicants' representatives make the arguments of record. The obviousness-type double patenting rejections of record were also discussed during the interview. In particular, Applicants' representatives argued that the comparative data set forth in the instant specification, which Examiner Whiteman acknowledged as showing a new and unexpected result between the exemplary 1:57 SNALP formulation of the present invention and the 2:30 and 2:40 SNALP formulations previously described (*see,* Office Action mailed May 12, 2011 at page 6), was sufficient to overcome these rejections. Examiner Whiteman found the arguments to be persuasive and agreed to withdraw all of the obviousness-type double patenting rejections when Applicants' representatives make the arguments of record.

III. REJECTIONS UNDER 35 U.S.C. § 102(b) & 35 U.S.C. § 103(a)

Claims 1-4, 9, 14, 17-26, 47, and 48 were rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by MacLachlan *et al.* In addition, claim 5 was rejected under 35 U.S.C. § 103(a) as allegedly being obvious over MacLachlan *et al.* as applied to claims 1-4, 9, 14, 17-26, 38,[1] 47, and 48, and further in view of Fosnaugh *et al.* (US 2003/0143732). To the extent these rejections apply to the amended claims, Applicants respectfully traverse.

In making both rejections, the Examiner alleges that the term "comprising from about" recited in the instant claims embraces a broad range of lipid components. In an earnest effort to expedite prosecution, but without acquiescing on the merits of the rejection, Applicants have amended the claims to delete "about" from the ranges of lipid components recited therein.

As set forth in M.P.E.P. 2131.03 (II):

> When the prior art discloses a range which touches or overlaps the claimed range, but no specific examples falling within the claimed range are disclosed, a case by case determination must be made as to anticipation. In order to anticipate the claims, the claimed subject matter must be disclosed in the reference with "sufficient specificity to constitute an anticipation under the statute." What constitutes a "sufficient specificity" is fact dependent. If the claims are directed to a narrow range, and the reference teaches a broad range, depending on the other facts of the case, it may be reasonable to conclude that the narrow range is not

[1] Applicants note that claim 38 was not rejected under 35 U.S.C. § 102(b) by the Examiner.

disclosed with "sufficient specificity" to constitute an anticipation of the claims. See, e.g., *Atofina v. Great Lakes Chem. Corp*, 441 F.3d 991, 999, 78 USPQ2d 1417, 1423 (Fed. Cir. 2006) wherein the court held that a reference temperature range of 100-500 degrees C did not describe the claimed range of 330-450 degrees C with sufficient specificity to be anticipatory. Further, while there was a slight overlap between the reference's preferred range (150-350 degrees C) and the claimed range, that overlap was not sufficient for anticipation. "[T]he disclosure of a range is no more a disclosure of the end points of the range than it is each of the intermediate points." *Id.* at 1000, 78 USPQ2d at 1424. Any evidence of unexpected results within the narrow range may also render the claims unobvious. The question of "sufficient specificity" is similar to that of "clearly envisaging" a species from a generic teaching. See MPEP § 2131.02. A 35 U.S.C. 102/103 combination rejection is permitted if it is unclear if the reference teaches the range with "sufficient specificity." The examiner must, in this case, provide reasons for anticipation as well as a *>reasoned< statement regarding obviousness. *Ex parte Lee*, 31 USPQ2d 1105 (Bd. Pat. App. & Inter. 1993) (expanded Board). For a discussion of the obviousness of ranges see MPEP § 2144.05.

Applicants provide a comparison of the ranges of lipid components between claim 1 as amended and the lipid ranges of MacLachlan *et al.* as quoted by the Examiner:

Lipid Component	Claim 1 as Amended	US 2006/0008910*
Cationic Lipid	50-65 mol %	"2-60, 5-50, 10-45, 20-40, 30 mol%"
Phospholipid	4-10 mol %	"5-90 mol%"
Cholesterol	30-40 mol %	"20-55 mol %"
Conjugated Lipid	0.5-2 mol %	"1-20 mol %"

*The ranges set forth in this column are reproduced from page 4 of the Office Action mailed May 12, 2011.

Applicants respectfully point out that claim 1 as presently amended recites narrow ranges for each of the lipid components compared to the substantially broader ranges taught by MacLachlan *et al.* Thus, per M.P.E.P. 2131.03 (II), it is reasonable to conclude that the claimed narrow ranges are not disclosed with "sufficient specificity" to constitute an anticipation of claim 1 and its dependent claims. *See, e.g., Atofina*, wherein the Federal Circuit held: (1) a reference temperature range of 100-500°C did not describe the claimed range of 330-450°C with sufficient specificity to be anticipatory; and (2) while there was a slight overlap between the reference's

preferred temperature range of 150-350°C and the claimed range of 330-450°C, that overlap was not sufficient for anticipation.

For the foregoing reasons, Applicants respectfully request that the Examiner withdraw the present rejections under 35 U.S.C. § 102(b) and 35 U.S.C. § 103(a). Indeed, the Examiner indicated that both rejections would be withdrawn when the arguments discussed above were made of record.

IV. DOUBLE PATENTING REJECTIONS

The Examiner has provisionally rejected claims 1-5, 9, 14, 17-26, 38, 47, 48 and 55, in various combinations, under the judicially created doctrine of obviousness-type double patenting over claims of co-pending Application Nos. 12/343,342; 11/283,550; 11/426,907; 11/511,855; 11/092,756; 11/807,872; and 12/359,119. The Examiner has also rejected claims 1-5, 9, 14, 17-26, 38, 47, 48 and 55, in various combinations, under the judicially created doctrine of obviousness-type double patenting over claims of U.S. Patent Nos. 7,799,565; 7,807,815; and 7,838,658.

As discussed above, the Examiner found the comparative data set forth in the instant specification to be sufficient to overcome all of the obviousness-type double patenting rejections of record. In particular, Applicants reiterate that it is clear from the specification that the present invention is based, in part, on the surprising discovery that 1:57 SNALP formulations provide ***new and unexpected results*** 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 fact, Applicants have found that SNALP formulations having increased amounts of cationic lipid, *e.g.*, one or more cationic lipids comprising from 50 mol % to 65 mol % of the total lipid present in the particle, provide ***unexpectedly superior 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).

Indeed, Figure 3 of Example 4 demonstrates that 1:57 SNALP formulations were significantly ***more 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 demonstrates that the 1:57 SNALP formulations were substantially ***more effective***

at silencing the expression of a target gene as compared to nucleic acid-lipid particles previously described ("2:40 SNALP," wherein the cationic lipid is present in the formulation at 40 mol %).

It is noted that the cited references disclose the preparation and testing of SNALP formulations such as the 2:30, 2:40, and 10:15 SNALP formulations as exemplified formulations containing the greatest amount of cationic lipid (*i.e.*, 30 mol %, 40 mol %, and 15 mol % cationic lipid, respectively). As set forth in the instant specification and acknowledged by the Examiner, SNALP formulations having increased amounts of cationic lipid such as, *e.g.*, the 1:57 SNALP formulation, provide ***unexpectedly superior advantages*** over previously exemplified SNALP formulations containing lower amounts of cationic lipid.

Per the Examiner's request during the telephonic interview conducted on July 13, 2011, Applicants submit that the presently claimed invention and the invention claimed in co-pending Application No. 12/343,342 ("the '342 application") were, at the time the invention claimed in the '342 application was made, owned by or under an obligation of assignment to Protiva Biotherapeutics, Inc. In particular, the Examiner indicated during the interview that such a statement of common ownership would be sufficient to overcome the present double patenting rejection over the '342 application in view of the SNALP formulations exemplified in its priority document, U.S. Provisional Application No. 61/017,075, filed December 27, 2007, which is the only priority document filed before the effective filing date of the instant application.

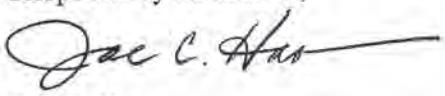
Accordingly, Applicants respectfully request that the Examiner withdraw all of the present obviousness-type double patenting rejections. Indeed, the Examiner indicated that such rejections would be withdrawn when the arguments discussed above were made of record.

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,



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63610531 v1

JOINT APPENDIX 11

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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NOTICE OF ALLOWANCE AND FEE(S) DUE

20350 7590 09/12/2011
KILPATRICK TOWNSEND & STOCKTON LLP
TWO EMBARCADERO CENTER
EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

EXAMINER	
WHITEMAN, BRIAN A	
ART UNIT	PAPER NUMBER
1635	

DATE MAILED: 09/12/2011

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/424,367	04/15/2009	Edward Yaworski	020801-007710US	1406

TITLE OF INVENTION: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1510	$300	$0	$1810	12/12/2011

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

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I. Review the SMALL ENTITY status shown above.

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(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/424,367	04/15/2009	Edward Yaworski	020801-007710US	1406

TITLE OF INVENTION: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1510	$300	$0	$1810	12/12/2011

EXAMINER	ART UNIT	CLASS-SUBCLASS
WHITEMAN, BRIAN A	1635	435-458000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
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- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

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2 _____
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5. **Change in Entity Status** (from status indicated above)
- ☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.
- ☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

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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 09/12/2011

KILPATRICK TOWNSEND & STOCKTON LLP
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EIGHTH FLOOR
SAN FRANCISCO, CA 94111-3834

EXAMINER	
WHITEMAN, BRIAN A	

ART UNIT	PAPER NUMBER
1635	

DATE MAILED: 09/12/2011

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

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The information provided by you in this form will be subject to the following routine uses:

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No.	Applicant(s)	
	12/424,367	YAWORSKI ET AL.	
	Examiner	Art Unit	
	BRIAN WHITEMAN	1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *the amendment filed on 8/11/11*.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are *1-5,9,14,17-26,38,47,48,56 and 57*.

4. ☐ 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 the:
      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)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 8/11/11
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
7. ☒ Examiner's Amendment/Comment
8. ☐ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

Examiner's Comments

The prior art of record is considered pertinent to applicant's disclosure. US 6,815,432, cited on an IDS discloses lipid formulations but does not appear to disclose the ranges for each of the lipids recited in the instant claims.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Brian Whiteman whose telephone number 571-272-0764. The examiner can normally be reached on Monday-Thursday from 6:30 to 4:00 (Eastern Standard Time). The examiner can also be reached on alternate Fridays. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor Heather Calamita can be reached on 571 272-2876. 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).

/Brian Whiteman/

Primary Examiner, Art Unit 1635

JOINT APPENDIX 12

WHAT IS CLAIMED IS:

1. A nucleic acid-lipid particle consisting essentially of:

(a) an RNA;

(b) a cationic lipid having a protonatable tertiary amine;

(c) a mixture of a phospholipid and cholesterol of from 30 mol % to 55 mol % of the total lipid present in the particle, wherein the phospholipid consists of from 3 mol % to 15 mol % of the total lipid present in the particle; and

(d) a polyethyleneglycol (PEG)-lipid conjugate consisting of from 0.1 mol % to 2 mol % of the total lipid present in the particle.

2. The nucleic acid-lipid particle of claim 1, wherein the cholesterol consists of from 25 mol % to 45 mol % of the total lipid present in the particle.

3. The nucleic acid-lipid particle of claim 2, wherein the phospholipid is distearoylphosphatidylcholine (DSPC).

4. The nucleic acid-lipid particle of claim 3, wherein the PEG has an average molecular weight of about 2,000 daltons.

5. The nucleic acid-lipid particle of claim 4, wherein the PEG has a terminal methoxy group.

6. The nucleic acid-lipid particle of claim 5, wherein the PEG-lipid conjugate is a PEG-diacylglycerol (PEG-DAG) conjugate having the same saturated acyl groups.

7. The nucleic acid-lipid particle of claim 6, wherein the cholesterol consists of from 35 mol % to 45 mol % of the total lipid present in the particle.

8. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 6 and a pharmaceutically acceptable carrier.

9. The pharmaceutical composition of claim 8, wherein the RNA is fully encapsulated in the nucleic acid-lipid particle.

10. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 7 and a pharmaceutically acceptable carrier.

11. The pharmaceutical composition of claim 10, wherein the RNA is fully encapsulated in the nucleic acid-lipid particle.

12. The nucleic acid-lipid particle of claim 1, wherein the RNA is an mRNA.

13. The nucleic acid-lipid particle of claim 12, wherein the cholesterol consists of from 25 mol % to 45 mol % of the total lipid present in the particle.

14. The nucleic acid-lipid particle of claim 13, wherein the phospholipid is DSPC.

15. The nucleic acid-lipid particle of claim 14, wherein the PEG has an average molecular weight of about 2,000 daltons.

16. The nucleic acid-lipid particle of claim 15, wherein the PEG has a terminal methoxy group.

17. The nucleic acid-lipid particle of claim 16, wherein the PEG-lipid conjugate is a PEG-DAG conjugate having the same saturated acyl groups.

18. The nucleic acid-lipid particle of claim 17, wherein the cholesterol consists of from 35 mol % to 45 mol % of the total lipid present in the particle.

19. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 17 and a pharmaceutically acceptable carrier.

20. The pharmaceutical composition of claim 19, wherein the mRNA is fully encapsulated in the nucleic acid-lipid particle.

21. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 18 and a pharmaceutically acceptable carrier.

22. The pharmaceutical composition of claim 21, wherein the mRNA is fully encapsulated in the nucleic acid-lipid particle.

23. The nucleic acid-lipid particle of claim 5, wherein the PEG-lipid conjugate comprises an amido linker moiety.

24. The nucleic acid-lipid particle of claim 3, wherein the cholesterol consists of from 35 mol % to 45 mol % of the total lipid present in the particle.

25. The nucleic acid-lipid particle of claim 24, wherein the PEG-lipid conjugate consists of from 0.5 mol % to 2 mol % of the total lipid present in the particle.

26. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 25 and a pharmaceutically acceptable carrier.

27. The pharmaceutical composition of claim 26, wherein the RNA is fully encapsulated in the nucleic acid-lipid particle.

28. The nucleic acid-lipid particle of claim 16, wherein the PEG-lipid conjugate comprises an amido linker moiety.

29. The nucleic acid-lipid particle of claim 28, wherein the DSPC consists of from 4 mol % to 10 mol % of the total lipid present in the particle.

30. A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 29 and a pharmaceutically acceptable carrier.

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.

JOINT APPENDIX 13

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
17/227,802	04/12/2021	Edward Yaworski	104290-007791US-1243808	1046

20350 7590 06/14/2021

Kilpatrick Townsend & Stockton LLP - West Coast
Mailstop: IP Docketing - 22
1100 Peachtree Street
Suite 2800
Atlanta, GA 30309

EXAMINER	
BOWMAN, AMY HUDSON	

ART UNIT	PAPER NUMBER
1635	

NOTIFICATION DATE	DELIVERY MODE
06/14/2021	ELECTRONIC

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.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

KTSDocketing2@kilpatrick.foundationip.com
ipefiling@kilpatricktownsend.com

Office Action Summary	Application No. 17/227,802	Applicant(s) Yaworski et al.	
	Examiner Amy H Bowman	Art Unit 1635	AIA (FITF) Status No

-- 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 MONTHS 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 4/12/21.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ 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*

5) ☑ Claim(s) 1-30 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-30 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 4/12/21 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).

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).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

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) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Notice of Pre-AIA or AIA Status

The present application is being examined under the pre-AIA first to invent provisions.

Claim Rejections - 35 USC § 103

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of pre-AIA 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, 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.

The factual inquiries for establishing a background for determining obviousness under pre-AIA 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

Claim 1-30 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Basarkar et al. (Int J Nanomedicine, 2007, 2(3), 353-360), Shimada et al. (International Journal of Pharmaceutics, 203, 2000, 255-263), Gallie (Plant Cell Reports, 1993, 13, 119-122), Semple et al. (Biochemistry, 1996, 35, 2521-2525), Li et al. (Trends in Pharmacological Sciences, 23, 5, 2002, 206-209), Arima et al. (Biomaterials, 29, 2008, 551-560), and Tarcha et al. (CA 2637931 7/26/2007).

Basarkar et al. teach that PEI has a high density of protonable amino groups, every third atom being amino nitrogen, which imparts it a high buffering ability at practically any pH (page 356).

Basarkar et al. teach that polymethacrylates are cationic vinyl-based polymers that possess the ability to condense polynucleotides into nanometer size particles. Several polymethacrylates such as poly [2-(dimethylamino) ethyl methacrylate] and its co-polymers have been used for polynucleotide delivery. Presence of protonable tertiary amine groups in their structure provides buffering ability similar to that of PEI. A range of Polymethacrylates, differing in molecular weights and chemical structures, have been

evaluated for their potential as gene delivery vector. Polymethacrylates containing only tertiary amine groups were found to be similar to PEI in terms of transfection efficiency while displaying much better biocompatibility profile. Nanoparticles with a methacrylate core and PEI shell prepared via graft copolymerization have also been employed lately for gene delivery. Such conjugation resulted in nanoparticles with a higher transfection efficiency and lower toxicity as compared to PEI alone. We have recently formulated cationic nanoparticles with commercially available polymethacrylate Eudragit® E100 in combination with PLGA/PLA using cationic surfactant, cetyltrimethylammonium bromide (CTAB), and achieved much improved transfection efficiency as compared to PLA/CTAB and PLGA/CTAB nanoparticles (pages 356-357).

Basarkar et al. teach that cationic polymers by virtue of their positive charge can efficiently condense the anionic polynucleotides into nanometer range complexes (polyplexes) thereby masking their negative charge. Polynucleotides are polyanionic molecules with a large hydrodynamic diameter which presents a significant barrier towards efficient cellular uptake. Cationic polymers, apart from condensing it to a several fold smaller size, also provide a net positive charge to the complex which helps in attachment on cellular membrane. Also, most cationic polymers bear amine groups that are protonable at acidic pH. Thus, once inside the endosome, these polymers accept proton thereby resisting a drop in pH. This causes influx of counterions (chloride ions) resulting in osmotic swelling and subsequent rupture of endosome. This phenomenon, first explained by Behr, is known as proton sponge effect. The efficiency of polyplexes has been found to be dependent on the ratio of nitrogen atoms of the

polymer to the phosphate groups present in the polynucleotide (N/P ratio). A variety of natural and synthetic cationic polymers have been used for gene delivery (page 356).

Therefore, it was known that the presence of protonable tertiary amine groups in their structure provides buffering ability similar to that of PEI. One would have been motivated to incorporate such groups into a cationic lipid with the expectation of the benefits taught by Basarkar et al.

Basarkar et al. teach that liposomes are spherical vesicles made of phospholipids used to deliver drugs or genes inside the cells. They can range in size from 20 nm to a few microns (page 358).

Basarkar et al. teach that investigators have studied conjugation of polyethylene glycol (PEG) to PEI to form diblock or triblock copolymers to reduce PEI-associated toxicity. PEG also shields the positive charge of the polyplexes, thereby providing steric stability to the complex. Such stabilization prevents non-specific interaction with blood components during systemic delivery (page 356).

Basarkar et al. teach that conjugation of PLL with poly (ethylene glycol (PEG) has been performed to shield charge on the surface thereby providing *in vivo* stability, delaying body clearance, and protecting DNA from nuclease degradation. Therefore, the delivery benefits of PEG-lipid conjugates were known in the art. Additionally, it was known that PEG of 2000 daltons molecular weight can be attached to a liposome surface for delivery, as taught by Li (page 208). It was also known in the art that the presence of terminal methoxy groups in PEG strongly activate the complement system via the alternative pathway, as taught by Arima et al. (page 552). Therefore, if desiring

to activate the complement system, it would be obvious to incorporate a terminal methoxy in the PEG.

It would have been obvious to incorporate an amido linker into the PEG-lipid conjugate because Tarcha et al. recite a polymer for non-viral delivery of nucleic acids (claim 1), wherein the nucleic acid is an siRNA (claim 3), wherein the polymer comprises PEG (claim 5), and wherein the polymer comprises an amido linker (claim 26). One would reasonably expect that each of the components known to be a part of nucleic acid delivery polymers would result in successful delivery of a RNA.

It would have been obvious to utilize the cationic lipid particle of Basarkar et al. to deliver RNA because Basarkar et al. teach that gene therapy involves delivering DNA, RNA, antisense oligonucleotides, and siRNA, either systemically or locally and teaches the benefits of cationic lipid particles for delivery (page 353). Basarkar et al. teach that siRNAs can be delivered from nanoparticles, PLL, and chitosan.

Shimada et al. teach PEG-DAG incorporation into liposomes including DSPC (100 µmol), cholesterol (100 µmol), DSPG (60 µmol), and PEG-DAG (15 µmol) (page 257). The PEG-DAGs are incorporated into liposomes, which are pharmaceutically acceptable carriers.

Shimada et al. teach that acyl chain length in PEG-DAGs is an important factor in their incorporation into liposomes. Shimada et al. teach that shorter acyl chains results in a higher amount of PEG-DAG incorporated (page 262). Shimada et al. teach that the PEG-DAGs have diacylglyceride anchors with PEG heads and tend to form micelles in aqueous media (page 262). Shimada et al. teach that percent incorporation of PEG-DAGs into liposomes is suggested to influence the density of PEG on the liposome

surfaces and hence affect the biodistribution of such liposomes (page 262). Therefore, it would have been obvious and a matter of design choice for the PEG-DAGs to have the same saturated acyl groups.

Shimada et al. teach that the PEG-DAG is 5.45 mol % to total lipids (page 257) and teach PEG at 2 mol % (page 262), which is considered to be a matter of design choice.

Gallie teach that delivery of mRNA with PEG results in levels of expression that are comparable to those obtained with electroporation and that the use of PEG to deliver mRNA is an inexpensive alternative (page 119).

Therefore, it would have been obvious to deliver mRNA via the nucleic acid-lipid particle with a reasonable expectation of successful delivery. Selection of mRNA as the nucleic acid is considered to be a matter of design choice.

Semple et al. teach that liposomes composed of long-chain saturated phospholipids including DSPC bound large quantities of blood proteins, whereas the incorporation of cholesterol into DSPC liposomes resulted in significantly decreased P_B values and enhanced circulation lifetimes for this lipid system. The cholesterol effect plateaued at 30 mol % cholesterol (abstract). Semple et al. teach incorporation at various percentages (Figure 4). Therefore, incorporation of cholesterol at up to 30% is considered to be a matter of routine optimization and it was known to be beneficial up to 30%.

Semple et al. teach incorporation of DSPC:Chol at 9:1, 8:2, 7:3, 6:4, and 5:5 with successful delivery (Table 2).

There was motivation in the art to utilize a cationic lipid having a protonatable tertiary amine; a mixture of DSPC and cholesterol; and PEG for delivery of nucleic acids. Combining these compounds that were each known to add benefits to nucleic acid delivery is considered obvious and one would reasonably expect for the composition to have the benefits taught in the prior art for each element.

Applicant has not demonstrated any unexpected result for the instantly recited genus of ranges in combination with the genus of agents (i.e. any cationic lipid, any RNA, any phospholipid). The specification does not disclose even a single species of cationic lipids having a protonatable tertiary amine.

Therefore the invention as a whole would have been prima facie obvious to one ordinary skill in the art at the time the invention was made.

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 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); *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 nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP § 2146 *et seq.* for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

MPEP 804 II B.2.(a) states:

The specification can be used as a dictionary to learn the meaning of a term in the patent claim. Toro Co. v. White Consol. Indus., Inc., 199 F.3d 1295, 1299, 53 USPQ2d 1065, 1067 (Fed. Cir. 1999)... Further, those portions of the specification which provide support for the patent claims may also be examined and considered when addressing

the issue of whether a claim in the application defines an obvious variation of an invention claimed in the patent. In re Vogel,

422 F.2d 438, 441-42, 164 USPQ 619, 622 (CCPA 1970). The portion of the reference disclosure that describes the subject matter that falls within the scope of a reference claim may be relied upon to properly construe the scope of that claim.

Claims 1-30 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-14 of U.S. Patent No. 9,364,435 B2. Although the claims at issue are not identical, they are not patentably distinct from each other because both set of claims are directed to a lipid formulation having a nucleic acid and a cationic lipid, non-cationic lipid and a conjugated lipid. The proportions in the formulation for each lipid in both set of claims overlap. The conjugated lipid can be a PEG-lipid conjugate. A PEG comprising a terminal methoxy group is embraced by the term "PEG" and PEG can be linked to the lipid via an amido linker. The instant claims require RNA which is a species of nucleic acids of '435 B2. Both claim sets recite incorporation of cholesterol, DSPC, mRNA, PEG, PEG-DAG, and carriers. The claims are obvious variations of each other.

Claims 1-30 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-17 of U.S. Patent No. 8,822,668 B2. Although the claims at issue are not identical, they are not patentably distinct from each other because both set of claims are directed to a lipid formulation having a nucleic acid and a cationic lipid, non-cationic lipid and a conjugated lipid. The proportions in the formulation for each lipid in both set of claims overlap. The conjugated lipid can be a PEG-lipid conjugate. A PEG comprising a terminal methoxy group is embraced by the term "PEG" and PEG can be linked to the lipid via an amido linker. The instant claims require RNA which is a species

of nucleic acids of '435 B2. Both claim sets recite incorporation of cholesterol, DSPC, mRNA, PEG, PEG-DAG, and carriers. The claims are obvious variations of each other.

Claims 1-30 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-21 of U.S. Patent No. 8,492,359 B2. Although the claims at issue are not identical, they are not patentably distinct from each other because both set of claims are directed to a lipid formulation having a nucleic acid and a cationic lipid, non-cationic lipid and a conjugated lipid. The proportions in the formulation for each lipid in both set of claims overlap. The conjugated lipid can be a PEG-lipid conjugate. A PEG comprising a terminal methoxy group is embraced by the term "PEG" and PEG can be linked to the lipid via an amido linker. The instant claims require RNA which is a species of nucleic acids of '435 B2. Both claim sets recite incorporation of cholesterol, DSPC, mRNA, PEG, PEG-DAG, and carriers. The claims are obvious variations of each other.

Claims 1-30 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-22 of U.S. Patent No. 8,058,069 B2. Although the claims at issue are not identical, they are not patentably distinct from each other because both set of claims are directed to a lipid formulation having a nucleic acid and a cationic lipid, non-cationic lipid and a conjugated lipid. The proportions in the formulation for each lipid in both set of claims overlap. The conjugated lipid can be a PEG-lipid conjugate. A PEG comprising a terminal methoxy group is embraced by the term "PEG" and PEG can be linked to the lipid via an amido linker. The instant claims require RNA which is a species

of nucleic acids of '435 B2. Both claim sets recite incorporation of cholesterol, DSPC, mRNA, PEG, PEG-DAG, and carriers. The claims are obvious variations of each other.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Amy Hudson Bowman whose telephone number is (571)272-0755. The examiner can normally be reached on M-F 8:00am-6:00pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ram Shukla can be reached on 571-272-0735. 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 https://ppair-my.uspto.gov/pair/PrivatePair. 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.

/AMY H BOWMAN/
Primary Examiner, Art Unit 1635

JOINT APPENDIX 14

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on August 20, 2021

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Sharleen Lane/
Sharleen Lane

PATENT
Attorney Docket No.: 104290-007791US-1243808

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Edward Yaworski
Application No.: 17/227,802
Filed: April 12, 2021
For: NOVEL LIPID FORMULATIONS FOR NUCLEIC ACID DELIVERY
Customer No.: 20350

Confirmation No.: 1046
Examiner: Amy Hudson Bowman
Art Unit: 1635

AMENDMENT / RESPONSE TO OFFICE ACTION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Office Action mailed June 14, 2021, please enter the following amendments and consider the following remarks:

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. (Original) A nucleic acid-lipid particle consisting essentially of:

(a) an RNA;

(b) a cationic lipid having a protonatable tertiary amine;

(c) a mixture of a phospholipid and cholesterol of from 30 mol % to 55 mol % of the total lipid present in the particle, wherein the phospholipid consists of from 3 mol % to 15 mol % of the total lipid present in the particle; and

(d) a polyethyleneglycol (PEG)-lipid conjugate consisting of from 0.1 mol % to 2 mol % of the total lipid present in the particle.

2. (Original) The nucleic acid-lipid particle of claim 1, wherein the cholesterol consists of from 25 mol % to 45 mol % of the total lipid present in the particle.

3. (Original) The nucleic acid-lipid particle of claim 2, wherein the phospholipid is distearoylphosphatidylcholine (DSPC).

4. (Original) The nucleic acid-lipid particle of claim 3, wherein the PEG has an average molecular weight of about 2,000 daltons.

5. (Original) The nucleic acid-lipid particle of claim 4, wherein the PEG has a terminal methoxy group.

6. (Original) The nucleic acid-lipid particle of claim 5, wherein the PEG-lipid conjugate is a PEG-diacylglycerol (PEG-DAG) conjugate having the same saturated acyl groups.

7. (Original) The nucleic acid-lipid particle of claim 6, wherein the cholesterol consists of from 35 mol % to 45 mol % of the total lipid present in the particle.

8. (Original) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 6 and a pharmaceutically acceptable carrier.

9. (Original) The pharmaceutical composition of claim 8, wherein the RNA is fully encapsulated in the nucleic acid-lipid particle.

10. (Original) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 7 and a pharmaceutically acceptable carrier.

11. (Original) The pharmaceutical composition of claim 10, wherein the RNA is fully encapsulated in the nucleic acid-lipid particle.

12. (Original) The nucleic acid-lipid particle of claim 1, wherein the RNA is an mRNA.

13. (Original) The nucleic acid-lipid particle of claim 12, wherein the cholesterol consists of from 25 mol % to 45 mol % of the total lipid present in the particle.

14. (Original) The nucleic acid-lipid particle of claim 13, wherein the phospholipid is DSPC.

15. (Original) The nucleic acid-lipid particle of claim 14, wherein the PEG has an average molecular weight of about 2,000 daltons.

16. (Original) The nucleic acid-lipid particle of claim 15, wherein the PEG has a terminal methoxy group.

17. (Original) The nucleic acid-lipid particle of claim 16, wherein the PEG-lipid conjugate is a PEG-DAG conjugate having the same saturated acyl groups.

18. (Original) The nucleic acid-lipid particle of claim 17, wherein the cholesterol consists of from 35 mol % to 45 mol % of the total lipid present in the particle.

19. (Original) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 17 and a pharmaceutically acceptable carrier.

20. (Original) The pharmaceutical composition of claim 19, wherein the mRNA is fully encapsulated in the nucleic acid-lipid particle.

21. (Original) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 18 and a pharmaceutically acceptable carrier.

22. (Original) The pharmaceutical composition of claim 21, wherein the mRNA is fully encapsulated in the nucleic acid-lipid particle.

23. (Original) The nucleic acid-lipid particle of claim 5, wherein the PEG-lipid conjugate comprises an amido linker moiety.

24. (Original) The nucleic acid-lipid particle of claim 3, wherein the cholesterol consists of from 35 mol % to 45 mol % of the total lipid present in the particle.

25. (Original) The nucleic acid-lipid particle of claim 24, wherein the PEG-lipid conjugate consists of from 0.5 mol % to 2 mol % of the total lipid present in the particle.

26. (Original) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 25 and a pharmaceutically acceptable carrier.

27. (Original) The pharmaceutical composition of claim 26, wherein the RNA is fully encapsulated in the nucleic acid-lipid particle.

28. (Original) The nucleic acid-lipid particle of claim 16, wherein the PEG-lipid conjugate comprises an amido linker moiety.

29. (Original) The nucleic acid-lipid particle of claim 28, wherein the DSPC consists of from 4 mol % to 10 mol % of the total lipid present in the particle.

30. (Original) A pharmaceutical composition comprising a nucleic acid-lipid particle of claim 29 and a pharmaceutically acceptable carrier.

REMARKS

I. STATUS OF THE CLAIMS

Upon entry of the present amendment, claims 1-30 are pending in this application and are presented for examination.

Based on the following remarks, Applicant respectfully requests reconsideration and allowance of the pending claims.

II. EXAMINER INTERVIEW

Applicant's representative thanks Examiner Bowman for the interview conducted on August 3, 2021, in which the rejections raised in the Office Action of June 14, 2021 were discussed. Agreement was reached that a reply would be filed with the Office.

III. REJECTION UNDER 35 U.S.C. § 103(a)

Claims 1-30 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Basarkar *et al.* (*Int. J. Nanomedicine*, 2(3):353-360 (2007); "Basarkar"), Shimada *et al.* (*Int. J. Pharmaceutics*, 203:255-263 (2000); "Shimada"), Gallie (*Plant Cell Reports*, 13:119-122 (1993); "Gallie"), Semple *et al.* (*Biochemistry*, 35:2521-2525 (1996); "Semple"), Li *et al.* (*TRENDS in Pharmacological Sciences*, 23(5):206-209 (2002); "Li"), Arima *et al.* (*Biomaterials*, 29:551-560 (2008); "Arima"), and Tarcha *et al.* (CA 2637931; "Tarcha").

Applicant respectfully traverses the rejection and submits that the cited references do not teach or suggest the claimed invention as a whole, nor do they do teach or suggest the claimed combination of components present in the lipid particles at the recited concentration ranges for at least the reasons provided below. As such, a person of ordinary skill in the art would have had no motivation to combine the cited references to arrive at the claimed lipid particles.

A. *Cationic lipid*

According to the Examiner, Basarkar teaches that polyethyleneimine (PEI) has a high density of protonatable amino groups, polymethacrylates are cationic vinyl-based polymers that possess the ability to condense polynucleotides into nanometer size particles, and nanoparticles with a methacrylate core and PEI shell prepared via graft copolymerization have been employed lately for gene delivery. Office Action at pages 3-4.

However, Basarkar focuses on cationic polymers, not cationic lipids as recited in the claims. For example, Basarkar teaches that "[p]olyethyleneimine is the most commonly used cationic polymer and is widely regarded as a gold standard, amongst non-viral vectors." Basarkar at page 356. Since PEI and polymethacrylate are not lipids, but are instead homopolymers of repeating units, particles made with them are not lipid particles and are not the claimed invention. Basarkar provides no motivation to replace cationic polymers such as PEI and polymethacrylate with cationic lipids in the nanoparticulate systems taught by Basarkar.

As noted, Basarkar contains a separate discussion of cationic liposomes. That discussion teaches that the toxicity of cationic liposomes "is still a concern" and "[s]everal lipoplex formulations have caused moderate to severe toxicities in animal models." Basarkar at page 358. According to Basarkar, "[a]t [the] cellular level, lipoplexes have been reported to cause cell shrinking, reduced mitoses, and vacuolization of [the] cytoplasm." *Id.* Combined with the teaching that PEI was regarded as the "gold standard," a person of ordinary skill in the art would not have been motivated to start with Basarkar's polymeric nanoparticles and then replace that cationic polymer with a cationic lipid.

B. *PEG-lipid conjugate*

According to the Examiner, the delivery benefits of PEG-lipid conjugates were known in the art because Basarkar teaches conjugation of PEG to PEI to reduce PEI-associated toxicity and conjugation of poly-L-lysine (PLL) with PEG to provide in vivo stability, delay body clearance, and protect DNA from nuclease degradation. Office Action at page 5. However, Basarkar provides no disclosure of PEG-*lipid* conjugates, which typically comprise long-chain acyl or alkyl groups attached to the PEG moiety. In contrast, PEI and PLL are both homopolymers of repeating units, not lipid conjugates. As such, there is no motivation to replace PEG-conjugated PEI and PLL with PEG-lipid conjugates in the nanoparticulate systems taught by Basarkar. Put another way, if a person of ordinary skill were motivated to replace Basarkar's cationic polymer with a cationic lipid (as posited in III.A above), there would no longer be a cationic polymer to modify with PEG in order to ameliorate its toxicity. And if a person of ordinary skill were then to extrapolate the toxicity of a cationic polymer to the cationic lipid (and no reason has been provided for why that would be so), and based on that toxicity, decide to conjugate PEG to the cationic lipid, the resulting particle would not contain *both* a cationic lipid *and* a PEG-lipid.

The Examiner further alleges that it would have been obvious to incorporate an amido linker into the PEG-lipid conjugate because Tarcha recites a polymer comprising PEG and an amido linker. Claim 26 of Tarcha is cited by the Examiner for teaching that the polymer comprises an amido linker. However, claim 26 of Tarcha depends from claim 24, which recites a polycation polymer of PEI units linked by non-ester linkers. As explained above, PEI is a homopolymer of repeating units, not a lipid conjugate. Thus, there is no motivation to replace polycation PEI polymers with PEG-lipid conjugates in the non-viral carriers taught by Tarcha.

The Examiner also cites Gallie for teaching that delivery of mRNA with PEG results in levels of expression that are comparable to those obtained with electroporation and that the use of PEG to deliver mRNA is an inexpensive alternative. Office Action at page 7. However, Gallie does not disclose particles with any lipid components as presently claimed. Gallie teaches only mRNA delivery using PEG *solutions*. Notably, Gallie discloses only PEG, without any disclosure of lipid particles or incorporation of a PEG-lipid conjugate into a lipid particle at the recited concentration. In fact, Gallie teaches that "[t]he concentration of PEG was also a critical parameter influencing mRNA delivery" and "a PEG concentration between 22 and 24% is optimal." Gallie at page 120. In contrast, the claims recite a PEG-lipid conjugate consisting of from 0.1 mol % to 2 mol % of the total lipid present in the particle. As such, Gallie has no relevance to whether a person of ordinary skill would include a PEG-lipid conjugate in a particle or the concentration of PEG-lipid conjugate recited in the claimed lipid particles.

In addition, the Examiner alleges that it would have been obvious to incorporate a terminal methoxy in the PEG if desiring to activate the complement system because Arima teaches that the presence of terminal methoxy groups in PEG strongly activate the complement system via the alternative pathway. Office Action at pages 5-6. However, Arima teaches away from the claimed lipid particles by disclosing that the complement system is associated with "unanticipated body reactions such as hypersensitivity reactions caused by PEG-modified liposomes ..., and rapid clearance of PEG-modified liposomes from blood have been reported." Arima at page 551. As such, a person of ordinary skill in the art would not have been motivated to use a PEG-lipid conjugate, let alone one with a terminal methoxy group, in view of the discouraging disclosure in Arima that PEG-modified liposomes cause unanticipated body reactions and rapid blood clearance.

C. *Lipid Components and Concentrations*

According to the Examiner, Li teaches that PEG of 2000 daltons can be attached to a liposome surface for delivery. Office Action at page 5. However, Li only discloses that PEG 2000 can be attached to the surface of neutral immunoliposomes carrying small molecule drugs or plasmid DNA. Li at page 208. Notably, Li is silent on RNA delivery by a lipid particle containing a cationic lipid with phospholipid, cholesterol, and PEG-lipid components at the recited concentration ranges. As such, Li provides no disclosure of a four-component lipid system as claimed or the recited concentrations of lipid components.

Furthermore, Li discloses that antibodies are conjugated to the PEG on the liposome surface (see Figure 2). However, the claimed lipid particles recite particles "consisting essentially of" the recited components, which excludes particles containing conjugated antibodies. As such, the antibody-conjugated liposomes taught by Li are excluded from the claims.

The Examiner cites Shimada for teaching the incorporation of PEG-DAG into liposomes. Office Action at page 6. However, Shimada is an analytical paper that describes a method for determining incorporated amounts of PEG-lipids in liposomes where the percent incorporation of PEG-lipids into liposomes would be a good parameter of quality control of PEG-liposomes in manufacturing. Shimada at page 255. As such, Shimada is silent on lipid particles containing RNA and cationic lipid with phospholipid, cholesterol, and PEG-lipid components at the recited concentration ranges and provides no motivation to modify the particle taught or suggested by the other references cited by the Examiner.

The Examiner cites Semple for teaching that the incorporation of cholesterol ("CHOL") into DSPC liposomes resulted in significantly decreased P_B values and enhanced circulation lifetimes for this lipid system. Office Action at page 7. In particular, the Examiner points to Table 2 of Semple as teaching incorporation of DSPC:CHOL at 9:1, 8:2, 7:3, 6:4, and 5:5 with successful delivery. *Id.* However, Semple discloses a two-component lipid system, and does not teach or suggest particles with a four-component lipid system as claimed. Moreover, Semple teaches away from the claimed phospholipid:CHOL concentrations and molar ratios (i.e., 30 mol % to 55 mol % mixture of phospholipid and cholesterol with 3 mol % to 15 mol % phospholipid). Instead, Table 2 of Semple discloses neutral liposomes containing only DSPC:CHOL where optimal P_B values were obtained at a DSPC:CHOL ratio of 7:3, i.e., 70 mol % DSPC:30 mol % CHOL. Thus,

whereas the claims recite less phospholipid than cholesterol (or at most an equal amount of the two) in the molar ratio, Semple discloses the opposite, teaching that more phospholipid than cholesterol is optimal. As such, a person of ordinary skill in the art would not have been motivated to modify the two-component neutral liposomes taught by Semple to arrive at the claimed four-component lipid particles with the recited lipid components and molar ratios.

D. *No Motivation to Combine Cited References*

For at least the foregoing reasons, Applicant submits that there is no motivation to combine the cited references as alleged by the Examiner to arrive at the claimed invention because none of the references teaches or suggests: (i) a four-component lipid particle with RNA as the payload; (ii) a cationic lipid having a protonatable tertiary amine; or (iii) RNA delivery using a lipid particle containing a cationic lipid with phospholipid, cholesterol, and PEG-lipid components at the recited concentration ranges.

As explained above, Basarkar discourages the use of cationic liposomes due to their toxicity and both Basarkar and Tarcha emphasize the use of cationic homopolymers of repeating units for nucleic acid delivery. Gallie discloses mRNA delivery using PEG solutions (not lipid particles) with high PEG concentrations, but fails to teach or suggest a nucleic acid-lipid particle with PEG-lipid conjugates at the recited concentration. Semple teaches away from the concentrations and molar ratios of phospholipid and cholesterol recited in the claimed lipid particles. Arima teaches away from PEG-modified liposomes due to unanticipated body reactions and rapid blood clearance. Therefore, a person of ordinary skill in the art would not have been motivated to combine the disclosures of the cited references to arrive at the claimed lipid particles, and any resulting particles would fail to meet each and every feature of the claimed lipid particles.

Accordingly, Applicant submits that the cited references do not render the instant claims obvious to one of ordinary skill in the art and respectfully requests that this rejection be withdrawn.

IV. SPECIFICATION'S DISCLOSURE

The Examiner alleges that the instant specification does not disclose even a single species of cationic lipids having a protonatable tertiary amine. Office Action at page 8. Applicant respectfully disagrees and points out that the application as filed discloses numerous cationic lipids with tertiary amines. For example, paragraph [0242] of the specification discloses that "[c]ationic

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" and provides a number of exemplary species. In addition, paragraph [0243] discloses additional cationic lipids having a protonatable tertiary amine and provides exemplary species. Likewise, paragraph [0245] discloses additional cationic lipids having a protonatable tertiary amine (when R^5 is absent) and paragraph [0246] provides a number of exemplary species. As such, the instant specification discloses numerous examples of cationic lipids having a protonatable tertiary amine.

V. NONSTATUTORY DOUBLE PATENTING

Claims 1-30 were rejected for nonstatutory double patenting over claims 1-14 of U.S. Patent No. 9,364,435, claims 1-17 of U.S. Patent No. 8,822,668, claims 1-21 of U.S. Patent No. 8,492,359, and claims 1-22 of U.S. Patent No. 8,058,069.

Without conceding in the basis of these rejections, and solely to expedite prosecution of the instant application, Applicant submits herewith terminal disclaimers to U.S. Patent Nos. 9,364,435, 8,822,668, 8,492,359, and 8,058,069. Accordingly, Applicant respectfully requests that the present nonstatutory double patenting rejections be withdrawn.

CONCLUSION

In view of the foregoing, Applicant believes 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.

Except for the issue fees payable under 37 C.F.R. § 1.18, the Director is authorized to charge any additional fees during pendency of this application, including any required extension of time fees, or credit any overpayment to Deposit Account Number 20-1430. This paragraph is intended to be a constructive petition for extension of time in accordance with 37 C.F.R. § 1.136(a)(3).

If the Examiner believes a telephone conference would expedite prosecution of this application, please contact the undersigned at (925) 472-5000.

Respectfully submitted,

/Joe C. Hao/
Joe C. Hao
Registration No. 55,246

KILPATRICK TOWNSEND & STOCKTON LLP

75097505v.1

JOINT APPENDIX 15

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/304,578	06/13/2014	Ian MacLachlan	86399-001220US-911148	1042

20350 7590 08/14/2014

KILPATRICK TOWNSEND & STOCKTON LLP
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EXAMINER	
HIRT, ERIN E	

ART UNIT	PAPER NUMBER
1616	

NOTIFICATION DATE	DELIVERY MODE
08/14/2014	ELECTRONIC

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.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipefiling@kilpatricktownsend.com
jlhice@kilpatrick.foundationip.com

Office Action Summary	Application No. 14/304,578	Applicant(s) MACLACHLAN ET AL.	
	Examiner ERIN HIRT	Art Unit 1616	AIA (First Inventor to File) Status No

-- 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 MONTHS 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 _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ 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*

5)☒ Claim(s) 1-10 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) 1-10 is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on 06/13/2014 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).

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).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

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) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Claims 1-10 are pending in this application.

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 double patenting rejection is appropriate where the claims at issue 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 reference 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. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1, 9, 10 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 9-10, 17, and 19 of U.S. Patent No. 8058069 (‘069). Although the conflicting claims are not identical, they are not patentably distinct from each other because both '069 and the instant invention claim nucleic acid particles comprising phospholipids, PEG-lipids, cationic lipids and nucleic acids. ‘069 merely teaches that their nucleic acids are siRNA instead of the instantly claimed mRNA. However, it would have been obvious to one of ordinary skill in the art at the time of the instant invention that the particles of '069 are merely obvious variants of the particles instantly claimed.

Claims 1-7, and 9-10 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4-6, 12, 21 of US Patent 8283333 for the same reasons discussed above.

Claims 1 and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4, 10, and 18 of U.S. Patent No. 7799565 for the same reasons discussed above.

Claims 1-7, and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-6, 8, 10-11 of U.S. Patent No. 8466122 for essentially the same reasons as discussed above.

Claims 1-3, and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 8, 14, and 20 of U.S. Patent No. 8492359 for essentially the same reasons as discussed above.

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.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 1-10 are rejected under 35 U.S.C. 103(a) as being unpatentable over Unger et al. (US5830430), Bally et al. (US5705385), Lasic (*Tibtech*, **1998**, *16,* 307-321), and Meyer et al. (*J. Biol. Chem.*, **1998**, *273*, 15621-15627).

Applicant claims:

> A lipid vesicle comprising:
> a messenger RNA (mRNA);
> a cationic lipid;
> an amphipathic lipid; and
> a polyethyleneglycol (PEG)-lipid,
> wherein the mRNA is fully encapsulated in the lipid vesicle.

Determination of the scope and content of the prior art

(MPEP 2141.01)

Unger teaches cationic liposomes/lipid vesicles which comprise: a cationic lipid, a phospholipid, specifically the instantly claimed dioleoylphosphatidyl choline, a PEG-lipid, cholesterol, and genetic material, specifically RNA, which obviously includes mRNA which is a type of RNA (see entire document; esp. Col. 20, ln. 15-40; Col. 25, ln. 47-58; Col. 20, ln. 55-Col. 21, ln. 40; Claims 103-106, 110-111, 118, 120-123, 135-141)

(Claims 1 (in part), 2-8). Unger further teaches wherein the liposome encapsulates the genetic material (Col. 9, ln. 66-Col. 10, ln. 9; Col. 10, ln. 15-38; Col. 20, ln. 15-40; Col. 20, ln. 55-Col. 21, ln. 40; Col. 25, ln. 47-58;) (Claim 1). Unger then teaches that the size of the liposomes can be adjusted by a variety of techniques, but that preferably the size of the liposomes are less than 100 nm in diameter, which reads upon the less than 150 nm that is instantly claimed (Col. 22, ln. 28-42) (Claim 10). Unger does not specifically state that the lipid vesicle is a lipid-nucleic acid particle but as it is a liposome which encapsulates genetic material, i.e. RNA, it is obviously a lipid-nucleic acid particle (Claim 9). Unger then expressly teaches that the amount of stabilizing material, i.e. amphipathic compounds/lipids which are combined with the cationic lipid may vary depending on a variety of factors including, the specific cationic lipids, the specific stabilizing material(s) selected (i.e. more than one type or combination), the particular use for which it is being employed, the mode of delivery, and the like (Col. 21, ln. 45-51). Further, Unger states, "The amount of stabilizing material to be combined with the present cationic lipid compounds in a particular situation and the ratio of stabilizing material to cationic lipid, will vary and is **readily determinable by one skilled in the art based on the present disclosure"** (emphasis added) (i.e. one of ordinary skill in the art) (Col. 21, ln. 51-56).

Ascertainment of the difference between prior art and the claims

(MPEP 2141.02)

Unger merely does not teach an example wherein the lipid vesicle comprises all of these things and wherein the RNA is mRNA. However, mRNA is a type of RNA i.e.

RNA is the genus and mRNA is the species. Therefore, by teaching that RNA can be carried by the lipid vesicles, Unger does render the instantly claimed mRNA obvious because it was already known in the art to use lipid vesicles to encapsulate the genus.

However, this deficiency in Unger is addressed by Bally.

Bally teaches lipid vesicles which comprise mRNA, cationic lipid(s), phospholipid(s), cholesterol, and PEG-lipids wherein the nucleic acids are enclosed by the lipid vesicle (i.e. protected from degradation) (see entire document; Abstract; Col. 2, ln. 34-Col. 3, ln. 11; Col. 5, ln. 32-45; Col. 6, ln. 19-35; Col. 5, ln. 46-65; Col. 7, ln. 9-17; Col. 7, ln. 18-37; Col. 9, ln. 14-38; Col. 11, ln. 19-27; Col. 12, ln. 66-Col. 13, ln. 7). Bally also teaches wherein the cholesterol and PEG-lipids improve the pharmacokinetics of this lipid vesicles (Col. 6, ln. 19-35; Col. 12, ln. 66-Col. 13, ln. 7).

Also, further motivation to include the specific lipids instantly claimed in the liposomes of Unger is provided by Meyer and Lasic.

Meyer teaches cationic liposomes coated with PEG and PEG-PE (a PEG-lipid) for increased stabilization of the liposomes which are used to carry oligonucleotides (i.e. nucleic acids, similar to RNA) (Abstract; pg. 15621, right col. Last paragraph).

Lasic teaches that liposomes/phospholipid vesicles are useful for drug delivery, specifically the delivery of DNA, RNA, oligonucleotides, etc. because the liposome allow for the protection of these sensitive drug molecules (Pg. 314, right col., 2nd paragraph). Lasic also teaches wherein it is known to have these liposomes comprise PEG-lipids for steric stabilization (pg. 314, right col. 1st para.). Lasic also teaches that liposomes are preferably between 80-200 nm because this size range is a compromise between

loading efficiency of the liposomes, the stability of the liposomes, and the ability of the liposomes to extravasate (pg. 309, right col. 1st full paragraph). Lasic further states that, "If the liposomes are larger than ~150 nm, sterile filtering is probably not possible and so the whole process must be performed aseptically, which is not very practical from the engineering or economical viewpoints." (pg. 313, left col., 2nd full paragraph). Lasic further teaches wherein the nucleic acid, is/can be encapsulated (i.e. fully encapsulated) in the lipid vesicle and wherein cholesterol is used to stabilize the lipid vesicle comprising nucleic acids (i.e. DNA or RNA) (pg. 318, left column, last paragraph-right column end of paragraph).

Finding of prima facie obviousness

Rationale and Motivation (MPEP 2142-2143)

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to make liposomes having a cationic lipid, a PEG-lipid, cholesterol, and a phospholipid, specifically dioleylphosphatidyl choline, which encapsulate mRNA because Unger teaches liposomes comprising cationic lipids, PEG-lipids, cholesterol, and phospholipids, specifically dioleylphosphatidyl choline are useful for delivering genetic material, specifically RNA. Furthermore, it was known that PEG-lipids stabilize the liposomes and cholesterol

In light of the forgoing discussion, the Examiner concludes that the subject matter defined by the above claims would have been obvious to one of ordinary skill in the art within the meaning of 35 USC 103(a).

From the teachings of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

Claims 1-7, and 9-10 are rejected under 35 U.S.C. 103(a) as being unpatentable over Bally et al (US5705385).

Applicant claims:

A lipid vesicle comprising:
a messenger RNA (mRNA);
a cationic lipid;
an amphipathic lipid; and
a polyethyleneglycol (PEG)-lipid,
wherein the mRNA is fully encapsulated in the lipid vesicle.

Determination of the scope and content of the prior art

(MPEP 2141.01)

Bally teaches lipid vesicles, specifically lipid-nucleic acid particles, which comprise mRNA, cationic lipid(s), phospholipid(s), cholesterol, and PEG-lipids wherein the nucleic acids are enclosed by the lipid vesicle (i.e. protected from degradation) (see entire document; Abstract; Col. 2, ln. 34-Col. 3, ln. 11; Col. 5, ln. 32-45; Col. 6, ln. 19-35; Col. 5, ln. 46-65; Col. 7, ln. 9-17; Col. 7, ln. 18-37; Col. 9, ln. 14-38; Col. 11, ln. 19-27; Col. 12, ln. 66-Col. 13, ln. 7) (Claims 1-2, 4-6, 9). Bally also teaches wherein the cholesterol and PEG-lipids improve the pharmacokinetics of lipid vesicles (Col. 6, ln. 19-35; Col. 12, ln. 66-Col. 13, ln. 7). Bally finally teaches wherein the phospholipids are

preferably, dioleylphosphatidyl choline etc., and wherein particles of less than 150 nm in size can be made (Abstract; Col. 9, ln. 14-38; Col. 6, ln. 19-34; Col. 10, ln. 48-53;) (Claims 3, 7, 10)

Ascertainment of the difference between prior art and the claims

(MPEP 2141.02)

Bally merely does not teach a specific example comprising all of the claimed features of the instantly claimed lipid vesicle.

Finding of prima facie obviousness

Rationale and Motivation (MPEP 2142-2143)

It would have been obvious to one of ordinary skill in the art to make a lipid vesicle of the instant claims by looking to Bally because Bally teaches lipid vesicles of the same size and comprising the same components as instantly claimed for delivering and protecting mRNA from degradation. One of ordinary skill in the art would want to develop the lipid vesicles of Bally with the same features as instantly claimed because adding cholesterol and PEG-lipids to lipid nucleic acid particles comprising mRNA, cationic lipids and phospholipids, were known to improve the pharmacokinetics of these particles. Thereby making a more effective lipid vesicle for delivering the mRNA to cells.

Conclusion

Claims 1-10 are rejected.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Erin Hirt whose telephone number is (571)270-1077.

The examiner can normally be reached on Monday through Friday 9:00am to 6:30pm. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sue X. Liu can be reached on 571-272-5539. 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.

Erin Hirt
Examiner, Art Unit 1616

/Mina Haghighatian/
Primary Examiner, Art Unit 1616

JOINT APPENDIX 16

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on October 22, 2014.

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Judith Cotham/
Judith Cotham

PATENT
Attorney Docket No.: 86399-001220US-911148

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Ian MacLachlan et al.

Application No.: 14/304,578

Filed: June 13, 2014

For: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY

Customer No.: 20350

Confirmation No. 1042

Examiner: Hirt, Erin E.

Art Unit: 1616

AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Office Action mailed August 14, 2014, please enter the following amendments and remarks. A Declaration of Dr. James Heyes under 37 C.F.R. § 1.132 accompanies the present response.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks begin on page 4 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. (Original) A lipid vesicle comprising:
a messenger RNA (mRNA);
a cationic lipid;
an amphipathic lipid; and
a polyethyleneglycol (PEG)-lipid,
wherein the mRNA is fully encapsulated in the lipid vesicle.

2. (Original) The lipid vesicle of claim 1, wherein the amphipathic lipid is a phospholipid.

3. (Original) The lipid vesicle of claim 2, wherein the phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine.

4. (Original) The lipid vesicle of claim 1, wherein the lipid vesicle further comprises a sterol.

5. (Original) The lipid vesicle of claim 4, wherein the sterol is cholesterol.

6. (Original) The lipid vesicle of claim 4, wherein the sterol is cholesterol and the amphipathic lipid is a phospholipid.

7. (Original) The lipid vesicle of claim 6, wherein the phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine.

8. (Original) The lipid vesicle of claim 1, wherein the lipid vesicle is a liposome.

9. (Original) The lipid vesicle of claim 1, wherein the lipid vesicle is a lipid-nucleic acid particle.

10. (Original) The lipid vesicle of claim 1, wherein the lipid vesicle is about 150 nm or less in diameter.

11. (New) The lipid vesicle of claim 1, wherein the cationic lipid only carries a positive charge at below physiological pH.

12. (New) The lipid vesicle of claim 1, wherein the lipid vesicle is about 100 nm or less in diameter.

REMARKS

I. STATUS OF THE CLAIMS

Upon entry of this amendment, claims 1-12 are pending in this application and are presented for examination. Claims 11 and 12 are newly added. Support for new claim 11 is found, for example, on page 7, lines 23-24 of the specification as filed. Support for new claim 12 is found, for example, on page 10, lines 2-5 and page 17, lines 25-27 of the specification as filed. Accordingly, no new matter has been introduced. Reconsideration is respectfully requested.

II. DOUBLE PATENTING REJECTIONS

Claims 1, 9, and 10 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 9, 10, 17, and 19 of U.S. Patent No. 8,058,069. Claims 1-7, 9, and 10 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 4-6, 12, and 21 of U.S. Patent No. 8,283,333. Claims 1 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 4, 10, and 18 of U.S. Patent No. 7,799,565. Claims 1-7 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1-6, 8, 10, and 11 of U.S. Patent No. 8,466,122. Claims 1-3 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 8, 14, and 20 of U.S. Patent No. 8,492,359.

Applicants respectfully request that the Examiner hold these rejections in abeyance until there is an indication of allowable subject matter. *See*, M.P.E.P. § 714.02.

III. FIRST REJECTION UNDER 35 U.S.C. § 103(a)

Claims 1-10 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Unger *et al.* (US Patent No. 5,830,430), Bally *et al.* (US Patent No. 5,705,385), Lasic (*Trends in Biotech.*, 16:307-21 (1998)), and Meyer *et al.* (*J. Biol. Chem.*, 273:15621-7 (1998)). Applicants respectfully traverse this rejection.

In the Office Action, the Examiner alleges that it would have been obvious to one of ordinary skill in the art to look to the teachings of these references and make liposomes

comprising the same components as presently claimed to encapsulate and deliver genetic material such as mRNA. *See,* Office Action at page 8.

In response, Applicants respectfully submit herewith a Declaration of Dr. James Heyes under 37 C.F.R. § 1.132 (hereinafter, "Heyes Declaration") to clarify the teachings of Unger *et al.* and Bally *et al.* by explaining that these references only provide general guidance about the types of components that can be included in lipid vesicles, without providing any clear and specific teaching with regard to the selection of the particular components recited in the present claims or the successful delivery of mRNA. *See*, Heyes Declaration ¶ 7. In particular, Dr. Heyes explains that none of the examples in Unger *et al.* and Bally *et al.* discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming smaller, physically stable lipid vesicles that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells. *See*, *id.*

As explained by Dr. Heyes, Unger *et al.* is primarily directed to "cationic lipid compounds which comprise at least two cationic groups" and Unger *et al.* asserts that "the cationic lipid compounds are also particularly suitable for use in the formulation of cationic vesicles, including micelles and liposomes," which are allegedly "particularly suitable for use as carriers for the intracellular delivery of bioactive agents." *See*, Heyes Declaration ¶ 8. Nonetheless, Dr. Heyes explains that Unger *et al.* only provides a general teaching about the components that are useful in lipid vesicles in addition to cationic lipid compounds with at least two cationic groups, and methods of making and using such lipid vesicles, for example, to treat disease. *See*, *id.* Importantly, Dr. Heyes points out that there is no specific teaching in Unger *et al.* regarding the desirability of the combination of elements set forth in the lipid vesicles of the present invention. *See*, *id.* Indeed, Dr. Heyes notes that Unger *et al.* fails to exemplify a lipid vesicle comprising all of the components recited in the present claims or the successful encapsulation and delivery of mRNA or any other payload. *See*, *id.*

Instead, Dr. Heyes explains that Examples 1-5 of Unger *et al.* are directed to the synthesis of cationic lipid compounds comprising at least two cationic groups; Examples 6A and 6B are directed to the *in vitro* transfection of HeLa cells in culture with DNA using the cationic lipid compounds prepared in Examples 5C and 5D or the commercially available cationic

liposome formulations LIPOFECTAMINE, LIPOFECTIN, and TRANSFECTAM; Example 7 describes the use of the cationic lipid compound prepared in Example 5D for delivery of plasmid DNA to rats; and Examples 8-12 are directed to catheters or stents coated with a cationic lipid formulation containing the cationic lipid compound prepared in Example 5D. *See*, Heyes Declaration ¶ 9. However, Dr. Heyes states that none of these examples describes or suggests the use of a lipid vesicle of the present invention to deliver mRNA to a living cell. *See*, *id.* Indeed, Dr. Heyes points out that the examples of Unger *et al.* describe the cationic lipid compounds of Unger *et al.* as being sufficient, in themselves, to deliver bioactive agents to cells. *See*, *id.* For instance, Example 6A of Unger *et al.* states that "the experiments performed in this example demonstrate that the cationic lipid compounds of the present invention provide useful and improved transfection of cells with bioactive agents as compared to compounds of the prior art." *See*, *id.* Similarly, Example 7 of Unger *et al.* states that the "localization of gene expression can be achieved with the compounds and methods of the present invention." *See*, *id.* As such, Applicants submit that Unger *et al.* actually teaches away from the present invention because the experimental data and statements in this reference show that its cationic lipid compounds are, by themselves, capable of delivering DNA to cells. Thus, one of ordinary skill in the art would not have been motivated to combine the cationic lipid compounds of Unger *et al.* with other components to facilitate nucleic acid delivery because Unger *et al.* teaches that other components are not necessary to "provide useful and improved transfection of cells with bioactive agents." *See,* col. 39, lines 46-50.

Furthermore, Dr. Heyes points out that the cationic lipid compounds and cationic liposome formulations disclosed in Unger *et al.* are exemplified in the context of their use for preparing preformed liposomes to form complexes with DNA called lipoplexes. *See*, Heyes Declaration ¶ 10. However, Dr. Heyes explains that lipoplexes are electrostatic complexes in which little, if any, of the DNA payload is encapsulated within the preformed cationic liposomes. *See*, *id.* According to Dr. Heyes, Examples 6A and 6B of Unger *et al.* teach that the DNA as well as the cationic lipid compounds and cationic liposome formulations were each diluted in aqueous buffer (*i.e.,* HEPES buffered saline or HBS) prior to mixing. *See*, *id.* The diluted DNA and "lipid solutions" were then mixed by inverting and incubating at room temperature for 15

minutes. *See*, *id.* However, Dr. Heyes explains that the term "lipid solutions" is not an accurate description of the suspension of preformed liposomes that is produced when the cationic lipid compounds or cationic liposome formulations were diluted in aqueous buffer. *See*, *id.* In other words, Dr. Heyes clarifies that a liposome suspension instead of a "lipid solution" is formed by diluting the cationic lipids in aqueous buffer because the cationic lipids are not solubilized in an organic medium such as ethanol. *See*, *id.* According to Dr. Heyes, the resulting products formed by mixing the suspension of preformed liposomes with DNA (also in aqueous buffer) are lipoplexes. *See*, *id.* However, Dr. Heyes points out that lipoplexes are heterogeneous, metastable aggregates that are effective when used to transfect cells in culture, but have relatively poor performance *in vivo*. *See*, *id.* Importantly, Dr. Heyes explains that the encapsulated mRNA present within the lipid vesicles of the present invention will be protected from nuclease degradation upon systemic administration, while nucleic acid that is merely associated with the surface of a preformed liposome (such as the DNA of the lipoplexes of Unger *et al.*) will be more readily degraded by serum nucleases. *See*, *id.*

With regard to Bally *et al.*, Dr. Heyes explains that this reference purports to provide *in vitro* and *in vivo* methods for treatment of diseases, in which a nucleic acid encoding a protein is formulated through a hydrophobic intermediate into a lipid-nucleic acid particle, and the particles are administered to patients in need thereof. *See*, Heyes Declaration ¶ 11. According to Dr. Heyes, Bally *et al.* describes detergent dialysis methods for making the lipid-nucleic acid particles. *See*, *id.* Nonetheless, Dr. Heyes explains that Bally *et al.* only provides a general teaching about the components that are useful in lipid vesicles, and there is no specific teaching regarding the desirability of the combination of elements set forth in the lipid vesicles of the present invention. *See*, *id.* Indeed, as with Unger *et al.*, Dr. Heyes notes that Bally *et al.* fails to exemplify a lipid vesicle comprising all of the components recited in the present claims or the successful encapsulation and delivery of mRNA or any other payload. *See*, *id.*

Instead, Dr. Heyes explains that all of the examples of Bally *et al.* are directed to the use of commercially available cationic lipid formulations containing DOTMA, DOSPA, DOGS, or DODAC to make lipid-nucleic acid complexes (Examples 1-7) or lipid-nucleic acid particles (Examples 8-12). *See*, Heyes Declaration ¶ 12. Notably, Dr. Heyes points out that all

of the cationic lipids used in these formulations are permanently charged, and therefore carry a net positive charge at physiological pH. *See*, *id.* According to Dr. Heyes, there are numerous disadvantages of using permanently charged cationic lipids to make lipid particles and deliver nucleic acid, including: (1) limited utility *in vivo* because they are rapidly opsonized (*i.e.*, coated with protein) and cleared from the bloodstream, diminishing their potency; (2) instability in the blood, where they aggregate, increase in size, and accumulate in the fine capillary beds of the lung, which renders them toxic; (3) significant toxicity issues because they are not only more immune stimulatory, but are also membrane lytic, which can produce hemolysis in the blood; (4) difficulty to work with because they aggregate easily and bind rather indiscriminately, preventing effective concentration, buffer exchange, and filtration; and (5) promoting the generation of heterogeneous particle populations with large particle size, and not small, stable, discrete populations of particles that are desirable for *in vivo* applications. *See*, *id.*

Indeed, Dr. Heyes clarifies that Example 8 of Bally *et al.* demonstrates that formulating lipid-nucleic acid particles with a permanently charged cationic lipid such as DODAC using a detergent dialysis method was associated with two populations of particles, with one group sized between 50 to 150 nm and the other group sized between 500 to 1000 nm. *See*, Heyes Declaration ¶ 13. As explained above by Dr. Heyes, particles in the second group are too large for *in vivo* applications, while the heterogeneous nature of the particles as a whole limits their utility *in vivo* due to significant issues with toxicity and reduced potency. *See*, *id.* According to Dr. Heyes, although Bally *et al.* states that the particles in the first group accounted for over 90% of the total particle number, these smaller sized particles were not characterized or shown to have encapsulated the plasmid DNA payload, and so it is indeed possible that the larger particles in the second group contained the majority of the plasmid DNA. *See*, *id.*

As explained by Dr. Heyes in his Declaration, he and his colleagues attempted to reproduce the experiments described in Example 8 of Bally *et al.* in order to characterize and compare the different particles found in the two particle populations. *See*, Heyes Declaration ¶ 14. However, Dr. Heyes states that they were unable to replicate the bimodal distribution of lipid-nucleic acid particles described in this example, irrespective of whether plasmid DNA or mRNA was used as the nucleic acid payload. *See*, *id.* For these experiments, five formulations

were prepared in accordance with the detergent dialysis method described in Example 8 and characterized for particle size and polydispersity. *See*, Heyes Declaration ¶¶ 14 & 15. The results of the experiments are shown in Table 1 of the Heyes Declaration, which is reproduced below for the Examiner's convenience:

Table 1

Formulation	Size	Polydispersity Index
1	745 nm	0.48
2	550 nm	0.51
3	Aggregation in Dialysis Bag	
4	Aggregation in Dialysis Bag	
5	Aggregation in Dialysis Bag	

As explained by Dr. Heyes in his Declaration, given the sizes of the particles and their polydispersity indexes, they were unable to produce lipid particles of reasonable size using the detergent dialysis method described in Example 8 of Bally *et al.* *See*, Heyes Declaration ¶ 16. Indeed, Dr. Heyes points out that they had no success in preparing any lipid particles below 500 nm using this technique, irrespective of whether plasmid DNA or mRNA was used as the payload (Formulations #1 and #2), and thus were unable to reproduce the bimodal distribution of lipid-nucleic acid particles described in Example 8, with one group of particles sized between 50 to 150 nm and the other group of particles sized between 500 to 1000 nm. *See*, *id.* In fact, Dr. Heyes notes that when they tried to scale up the method in an attempt to detect the first group of smaller particles by increasing the total number of particles formed, either by increasing the concentrations (Formulations #3 and #4) or the total volume (Formulation #5) of the components of the system, the particles aggregated in the dialysis bag and precipitated during the dialysis procedure. *See*, *id.* As such, Dr. Heyes explains that this method of Bally *et al.* is not amenable to the concentration, buffer exchange, and filtration steps that are necessary for scaling up these formulations. *See*, *id.* Based on these experiments, Dr. Heyes concludes that the detergent dialysis method described in Example 8 of Bally *et al.* is not able to produce the smaller, more

stable, and more uniform populations of particles that are desirable for *in vivo* and clinical applications. *See*, *id.*

For the foregoing reasons, Dr. Heyes submits that the teachings of Unger *et al.* and Bally *et al.* only provide general guidance about the types of components that can be included in lipid vesicles, but fail to clearly and specifically direct one skilled in the art to select the particular lipid components recited in the present claims to fully encapsulate and successfully deliver mRNA. *See*, Heyes Declaration ¶ 17. In fact, Dr. Heyes points out that none of the examples in Unger *et al.* and Bally *et al.* discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming smaller, physically stable, and uniform lipid vesicles that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells and thus more desirable for *in vivo* and clinical applications. *See*, *id.* Consequently, Dr. Heyes concludes that there is no motivation for one of ordinary skill in the art to take the teachings of Unger *et al.* and Bally *et al.* and make lipid vesicles using a specific combination of lipid components as claimed that fully encapsulates mRNA with any reasonable expectation of success. *See*, *id.*

Applicants assert that the teachings of Lasic and Meyer *et al.* do not remedy the deficiencies in the disclosures of Unger *et al.* and Bally *et al.* Indeed, Lasic teaches lipoplexes as DNA-carrier systems for gene therapy. *See,* page 318, paragraph bridging left and right columns. In particular, Lasic teaches that cationic liposomes have been shown to complex DNA, and such complexes were able to transfect cells *in vitro*, resulting in the expression of the protein encoded in the DNA plasmid in target cells. *See*, *id.* In addition, Lasic teaches that cationic lipid-based DNA complexes can transfect cells *in vivo* upon localized or systemic administration. *See*, *id.* Moreover, Lasic teaches that improvements including developments in DNA-plasmid design, the synthesis of novel cationic lipids, and the cholesterol stabilization of complexes have resulted in more than a thousand-fold increase in gene expression over initial experiments, and that PEG-lipids may be used to coat DNA-lipid complexes to improve such lipid-based carriers. *See*, *id.* As such, one of ordinary skill in the art would have been motivated to use lipoplexes as lipid-based carrier systems for gene therapy in view of the teachings of Lasic.

In fact, Applicants submit that Meyer *et al.* supports the teachings of Lasic and provides further motivation for the skilled artisan to use lipoplexes for the *in vivo* delivery of nucleic acid. Indeed, as with Unger *et al.*, Meyer *et al.* teaches the formation of lipoplexes between preformed cationic liposomes and DNA (*i.e.,* oligodeoxyribonucleotides (ODN)). In particular, Meyer *et al.* teaches that these "[l]iposome-ODN complexes were produced by overnight incubation of the liposomes with ODN in HBS [HEPES buffered saline]." *See,* page 15622, left column; emphasis added. In addition, the Abstract of Meyer *et al.* repeatedly refers to complexes between the liposome and ODN components. *See also,* Figure 1 of Meyer *et al.*, which describes the binding of ODN to PEG-modified cationic liposomes and the resulting complexes. Importantly, Meyer *et al.* teaches that its PEG-modified cationic liposomes offer numerous advantages as carriers for cellular delivery of ODN (*e.g.,* the resulting complexes are stable against aggregation and retain both their ODN load in blood plasma and the ability to enhance cellular delivery of ODN in the presence of serum) and concludes that "PEG-modified cationic liposomes may provide a useful step in the development of an efficient pharmaceutical carrier for systemic *in vivo* delivery of ODN." *See,* page 15627, left column. Thus, Meyer *et al.* corroborates the teachings of Lasic and provides further motivation for one of ordinary skill in the art to use lipoplexes as lipid-based carrier systems for the *in vivo* delivery of nucleic acid.

In view of the foregoing, Applicants assert that there is no rational underpinning to combine the cited references to support a legal conclusion of obviousness because these references, whether alone or in combination, do not teach or suggest each of the features recited in the claims. Indeed, as explained by Dr. Heyes in his Declaration, there is no motivation for one of ordinary skill in the art to take the teachings of Unger *et al.* and Bally *et al.* and make lipid vesicles using a specific combination of lipid components as claimed that fully encapsulates mRNA with any reasonable expectation of success because none of these references discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming smaller, physically stable, and uniform lipid vesicles that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells and thus more desirable for *in vivo* and clinical applications. Furthermore, the teachings of Lasic and Meyer *et al.* do not remedy the clear deficiencies in the disclosures of both Unger *et al.* and Bally

et al. since the skilled artisan would be motivated to use complexes formed between preformed liposomes and nucleic acid for gene therapy based on their numerous advantages as carriers for *in vivo* cellular delivery. Accordingly, Applicants respectfully request that the Examiner withdraw the present rejection under 35 U.S.C. § 103(a).

IV. SECOND REJECTION UNDER 35 U.S.C. § 103(a)

Claims 1-7, 9, and 10 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Bally *et al.* (US Patent No. 5,705,385). Applicants respectfully traverse this rejection.

In the Office Action, the Examiner alleges that it would have been obvious to one of ordinary skill in the art to look to the teachings of Bally *et al.* and make a lipid vesicle comprising the same components as presently claimed for delivering and protecting mRNA from degradation. *See,* Office Action at page 10.

As explained by Dr. Heyes in his Declaration, Bally *et al.* only provides general guidance about the types of components that can be included in lipid vesicles, without providing any clear and specific teaching with regard to the selection of the particular components recited in the present claims or the successful delivery of mRNA. *See*, Heyes Declaration ¶ 7. In particular, Dr. Heyes explains that none of the examples in Bally *et al.* discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming smaller, physically stable lipid vesicles that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells. *See*, *id.* Indeed, Dr. Heyes notes that Bally *et al.* fails to exemplify a lipid vesicle comprising all of the components recited in the present claims or the successful encapsulation and delivery of mRNA or any other payload. *See*, Heyes Declaration ¶ 11.

Instead, Dr. Heyes explains that all of the examples of Bally *et al.* are directed to the use of commercially available cationic lipid formulations containing DOTMA, DOSPA, DOGS, or DODAC to make lipid-nucleic acid complexes (Examples 1-7) or lipid-nucleic acid particles (Examples 8-12). *See*, Heyes Declaration ¶ 12. According to Dr. Heyes, all of the cationic lipids used in these formulations are permanently charged, and therefore carry a net

positive charge at physiological pH. *See*, *id.* In his Declaration, Dr. Heyes points out that there are numerous disadvantages of using permanently charged cationic lipids to make lipid particles and deliver nucleic acid. *See*, *id.* Indeed, Dr. Heyes clarifies that Example 8 of Bally *et al.* demonstrates that formulating lipid-nucleic acid particles with a permanently charged cationic lipid such as DODAC using a detergent dialysis method was associated with two populations of particles, with one group sized between 50 to 150 nm and the other group sized between 500 to 1000 nm. *See*, Heyes Declaration ¶ 13. Dr. Heyes states that particles in the second group are too large for *in vivo* applications, while the heterogeneous nature of the particles as a whole limits their utility *in vivo* due to significant issues with toxicity and reduced potency. *See*, *id.* As explained by Dr. Heyes, although Bally *et al.* states that the particles in the first group accounted for over 90% of the total particle number, these smaller sized particles were not characterized or shown to have encapsulated the plasmid DNA payload, and so it is indeed possible that the larger particles in the second group contained the majority of the plasmid DNA. *See*, *id.*

Although Dr. Heyes and his colleagues attempted to reproduce the experiments described in Example 8 of Bally *et al.* in order to characterize and compare the different particles found in the two particle populations, they were unable to replicate the bimodal distribution of lipid-nucleic acid particles described in this example, irrespective of whether plasmid DNA or mRNA was used as the nucleic acid payload. *See*, Heyes Declaration ¶ 14. For these experiments, five formulations were prepared in accordance with the detergent dialysis method described in Example 8 of Bally *et al.* and characterized for particle size and polydispersity. *See*, Heyes Declaration ¶¶ 14 & 15. The results of the experiments are shown in Table 1 of the Heyes Declaration, which is also reproduced above on page 9.

As explained by Dr. Heyes in his Declaration, given the sizes of the particles and their polydispersity indexes, they were unable to produce lipid particles of reasonable size using the detergent dialysis method described in Example 8 of Bally *et al.* *See*, Heyes Declaration ¶ 16. Indeed, Dr. Heyes points out that they had no success in preparing any lipid particles below 500 nm using this technique, irrespective of whether plasmid DNA or mRNA was used as the payload (Formulations #1 and #2), and thus were unable to reproduce the bimodal distribution of lipid-nucleic acid particles described in Example 8, with one group of particles sized between 50

to 150 nm and the other group of particles sized between 500 to 1000 nm. *See*, *id.* In fact, Dr. Heyes notes that when they tried to scale up the method in an attempt to detect the first group of smaller particles by increasing the total number of particles formed, either by increasing the concentrations (Formulations #3 and #4) or the total volume (Formulation #5) of the components of the system, the particles aggregated in the dialysis bag and precipitated during the dialysis procedure. *See*, *id.* As such, Dr. Heyes explains that this method is not amenable to the concentration, buffer exchange, and filtration steps that are necessary for scaling up these formulations. *See*, *id.* Based on these experiments, Dr. Heyes concludes that the detergent dialysis method described in Example 8 of Bally *et al.* is not able to produce the smaller, more stable, and more uniform populations of particles that are desirable for *in vivo* and clinical applications. *See*, *id.*

For the foregoing reasons, Dr. Heyes submits that the teachings of Bally *et al.* only provide general guidance about the types of components that can be included in lipid vesicles, but fail to clearly and specifically direct one skilled in the art to select the particular lipid components recited in the present claims to fully encapsulate and successfully deliver mRNA. *See*, Heyes Declaration ¶ 17. In fact, Dr. Heyes points out that none of the examples in Bally *et al.* discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming smaller, physically stable, and uniform lipid vesicles that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells and thus more desirable for *in vivo* and clinical applications. *See*, *id.* Consequently, Dr. Heyes concludes that there is no motivation for one of ordinary skill in the art to take the teachings of Bally *et al.* and make lipid vesicles using a specific combination of lipid components as claimed that fully encapsulates mRNA with any reasonable expectation of success. *See*, *id.*

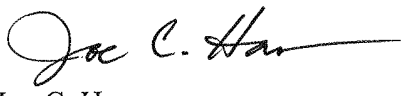
Therefore, Applicants assert that there is simply no rational underpinning to use the teachings of Bally *et al.* to support a legal conclusion of obviousness. Accordingly, Applicants respectfully request that the Examiner withdraw the present rejection under 35 U.S.C. § 103(a).

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,



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JCH
Attachments

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Ian MacLachlan et al. Application No.: 14/304,578 Filed: June 13, 2014 For: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY Customer No.: 20350	Confirmation No. 1042 Examiner: Hirt, Erin E. Art Unit: 1616 DECLARATION UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, James Heyes, Ph.D., being duly warned 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 patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I hold a Ph.D. (2001) in Medicinal Chemistry from the Institute of Cancer Research (Surrey, UK). I am presently the Director of Formulation Chemistry at Tekmira Pharmaceuticals Corp. (Burnaby, Canada). The assignee of the above-referenced application, Protiva Biotherapeutics Inc., is a wholly-owned subsidiary of Tekmira.

3. My expertise lies in the development of lipid particle formulations and the design of novel compounds as components of lipid particles. Attached hereto as Exhibit A is a copy of my *Curriculum Vitae*.

4. I have reviewed the above-referenced patent application, and I am familiar with the contents therein. I have also reviewed the contents of the Office Action dated August 14, 2014.

5. The present invention is directed to a lipid vesicle comprising: a messenger RNA (mRNA); a cationic lipid; an amphipathic lipid; and a polyethyleneglycol (PEG)-lipid, wherein the mRNA is fully encapsulated in the lipid vesicle. In certain embodiments, the cationic lipid only carries a positive charge at below physiological pH. In other embodiments, the lipid vesicle is about 150 nm or less in diameter.

6. In the Office Action, the Examiner relies on Unger *et al.* (US Patent No. 5,830,430) and Bally *et al.* (US Patent No. 5,705,385) in alleging that it would have been obvious to one of ordinary skill in the art to look to the teachings of these references and make a lipid vesicle comprising the same components as presently claimed to encapsulate mRNA and protect it from degradation.

7. I submit this Declaration to clarify the teachings of Unger *et al.* and Bally *et al.* by explaining that these references only provide general guidance about the types of components that can be included in lipid vesicles, without providing any clear and specific teaching with regard to the selection of the particular components recited in the present claims or the successful delivery of mRNA. In fact, none of the examples in Unger *et al.* and Bally *et al.* discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming smaller, physically stable lipid vesicles that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells.

8. Unger *et al.* is primarily directed to "cationic lipid compounds which comprise at least two cationic groups." *See, e.g.,* Abstract. Unger *et al.* states that "the cationic lipid compounds are also particularly suitable for use in the formulation of cationic vesicles, including micelles and liposomes," which are allegedly "particularly suitable for use as carriers for the intracellular delivery of bioactive agents." *See, e.g.,* col 17, lines 41-46. Nonetheless,

Unger *et al.* only provides a general teaching about the components that are useful in lipid vesicles in addition to cationic lipid compounds with at least two cationic groups, and methods of making and using such lipid vesicles, for example, to treat disease. There is no specific teaching regarding the desirability of the combination of elements set forth in the lipid vesicles of the present invention. Indeed, Unger *et al.* fails to exemplify a lipid vesicle comprising all of the components recited in the present claims or the successful encapsulation and delivery of mRNA or any other payload.

9. Instead, Examples 1-5 of Unger *et al.* are directed to the synthesis of cationic lipid compounds comprising at least two cationic groups. Examples 6A and 6B of Unger *et al.* are directed to the *in vitro* transfection of HeLa cells in culture with DNA using the cationic lipid compounds prepared in Examples 5C and 5D or the commercially available cationic liposome formulations LIPOFECTAMINE, LIPOFECTIN, and TRANSFECTAM. Example 7 describes the use of the cationic lipid compound prepared in Example 5D for delivery of plasmid DNA to rats, while Examples 8-12 are directed to catheters or stents coated with a cationic lipid formulation containing the cationic lipid compound prepared in Example 5D. None of these examples describes or suggests the use of a lipid vesicle of the present invention to deliver mRNA to a living cell. Indeed, the examples describe the cationic lipid compounds of Unger *et al.* as being sufficient, in themselves, to deliver bioactive agents to cells. For instance, Example 6A states that "the experiments performed in this example demonstrate that the cationic lipid compounds of the present invention provide useful and improved transfection of cells with bioactive agents as compared to compounds of the prior art." *See,* col. 39, lines 46-50. Similarly, Example 7 states that the "localization of gene expression can be achieved with the compounds and methods of the present invention." *See,* col. 40, lines 49-51.

10. Further, the cationic lipid compounds and cationic liposome formulations disclosed in Unger *et al.* are exemplified in the context of their use for preparing preformed liposomes to form complexes with DNA called lipoplexes. However, lipoplexes are electrostatic complexes in which little, if any, of the DNA payload is encapsulated within the preformed

cationic liposomes. In particular, Examples 6A and 6B teach that the DNA as well as the cationic lipid compounds and cationic liposome formulations were each diluted in aqueous buffer (*i.e.,* HEPES buffered saline or HBS) prior to mixing. The diluted DNA and "lipid solutions" were then mixed by inverting and incubating at room temperature for 15 minutes. However, the term "lipid solutions" is not an accurate description of the suspension of preformed liposomes that is produced when the cationic lipid compounds or cationic liposome formulations were diluted in aqueous buffer. In other words, a liposome suspension instead of a "lipid solution" is formed by diluting the cationic lipids in aqueous buffer because the cationic lipids are not solubilized in an organic medium such as ethanol. The resulting products formed by mixing the suspension of preformed liposomes with DNA (also in aqueous buffer) are lipoplexes. However, lipoplexes are heterogeneous, metastable aggregates that are effective when used to transfect cells in culture, but have relatively poor performance *in vivo*. Importantly, the encapsulated mRNA present within the lipid vesicles of the present invention will be protected from nuclease degradation upon systemic administration, while nucleic acid that is merely associated with the surface of a preformed liposome (such as the DNA of the lipoplexes of Unger *et al.*) will be more readily degraded by serum nucleases.

11. Bally *et al.* purports to provide *in vitro* and *in vivo* methods for treatment of diseases, in which a nucleic acid encoding a protein is formulated through a hydrophobic intermediate into a lipid-nucleic acid particle, and the particles are administered to patients in need thereof. *See, e.g.,* col 3, lines 12-22. Bally *et al.* describes detergent dialysis methods for making the lipid-nucleic acid particles. *See, e.g.,* columns 8-10. Nonetheless, Bally *et al.* only provides a general teaching about the components that are useful in lipid vesicles, and there is no specific teaching regarding the desirability of the combination of elements set forth in the lipid vesicles of the present invention. Indeed, Bally *et al.* fails to exemplify a lipid vesicle comprising all of the components recited in the present claims or the successful encapsulation and delivery of mRNA or any other payload.

12. Instead, all of the examples of Bally *et al.* are directed to the use of commercially available cationic lipid formulations containing DOTMA, DOSPA, DOGS, or DODAC to make lipid-nucleic acid complexes (Examples 1-7) or lipid-nucleic acid particles (Examples 8-12). *See,* col. 17-22. Notably, all of the cationic lipids used in these formulations are permanently charged, and therefore carry a net positive charge at physiological pH. There are numerous disadvantages of using permanently charged cationic lipids to make lipid particles and deliver nucleic acid. For example, permanently charged lipid particles have limited utility *in vivo* because they are rapidly opsonized (*i.e.,* coated with protein) and cleared from the bloodstream, diminishing their potency. Permanently charged lipid particles are also unstable in the blood, where they aggregate, increase in size, and accumulate in the fine capillary beds of the lung, which renders them toxic. In addition, permanently charged lipid particles have significant toxicity issues because they are not only more immune stimulatory, but are also membrane lytic, which can produce hemolysis in the blood. Further, permanently charged lipid particles are difficult to work with because they aggregate easily and bind rather indiscriminately, preventing effective concentration, buffer exchange, and filtration. Moreover, the use of permanently charged lipid particles promotes generation of heterogeneous particle populations with large particle size, and not small, stable, discrete populations of particles that are desirable for *in vivo* applications.

13. Indeed, Example 8 of Bally *et al.* demonstrates that formulating lipid-nucleic acid particles with a permanently charged cationic lipid such as DODAC using a detergent dialysis method was associated with two populations of particles, with one group sized between 50 to 150 nm and the other group sized between 500 to 1000 nm. *See also,* Figures 10A and 10B. As explained above, particles in the second group are too large for *in vivo* applications, while the heterogeneous nature of the particles as a whole limits their utility *in vivo* due to significant issues with toxicity and reduced potency. Although Bally *et al.* states that the particles in the first group accounted for over 90% of the total particle number, these smaller sized particles were not characterized or shown to have encapsulated the plasmid DNA payload.

Indeed, it is possible that the larger particles in the second group contained the majority of the plasmid DNA.

14. My colleagues and I attempted to reproduce the experiments described in Example 8 of Bally *et al.* in order to characterize and compare the different particles found in the two particle populations. However, we were unable to replicate the bimodal distribution of lipid-nucleic acid particles described in this example, irrespective of whether plasmid DNA or mRNA was used as the nucleic acid payload. For these experiments, five formulations were prepared in accordance with the detergent dialysis method described in Example 8 as follows:

- Formulation #1 was prepared using the same concentrations of the lipids DODAC and ESM (160 nmol) diluted in the same volume and concentration of octyl glucopyranoside (OGP) detergent solution (400 µl of 200 mM OGP) as described in Example 8, except that 10 µg input luciferase mRNA was diluted in 200 µl of 200 mM OGP instead of 10 µg input β-gal plasmid DNA.
- Formulation #2 was prepared using the same concentrations of the lipids DODAC and ESM (160 nmol) diluted in the same volume and concentration of OGP detergent solution (400 µl of 200 mM OGP) as described in Example 8, and using the same amount of input β-gal plasmid DNA (10 µg) diluted in the same volume and concentration of OGP detergent solution (200 µl of 200 mM OGP) as described in Example 8.
- Formulation #3 provides a scale-up preparation to increase the concentrations of the components of the system which was identical to Formulation #1 except that the input mRNA and lipid components were increased 10-fold, *i.e.,* 100 µg input mRNA and 1600 nmol each of DODAC and ESM.
- Formulation #4 provides a scale-up preparation to increase the concentration and volume of OGP detergent solution which was identical to Formulation #3 except that the concentration of OGP used to dilute the lipid components was

increased to 500 mM and the volume of OGP used to dilute the input mRNA was increased to 500 µl.

- Formulation #5 provides a scale-up preparation to increase the total volume of the components of the system which was identical to Formulation #3 except that the volume of OGP used to dilute the lipid components was increased from 400 µl to 4.0 ml and the volume of OGP used to dilute the input mRNA was increased from 200 µl to 2.0 ml. The resulting solution was to be concentrated following the dialysis procedure.

15. The formulations were characterized for particle size and polydispersity using a Malvern ZetaSizer. Polydispersity is a measure of the heterogeneity of sizes of particles in a mixture. A larger Polydispersity Index Value indicates a larger amount of heterogeneity of particle size in the mixture. Table 1 shows the results of these experiments.

Table 1

Formulation	**Size**	**Polydispersity Index**
1	745 nm	0.48
2	550 nm	0.51
3	Aggregation in Dialysis Bag	
4	Aggregation in Dialysis Bag	
5	Aggregation in Dialysis Bag	

16. Given the sizes of the particles and their polydispersity indexes, we were unable to produce lipid particles of reasonable size using the detergent dialysis method described in Example 8 of Bally *et al.* Indeed, we had no success in preparing any lipid particles below 500 nm using this technique, irrespective of whether plasmid DNA or mRNA was used as the payload (Formulations #1 and #2). As a result, using this technique, we were unable to reproduce the bimodal distribution of lipid-nucleic acid particles described in Example 8, with one group of particles sized between 50 to 150 nm and the other group of particles sized between

500 to 1000 nm. In fact, when we tried to scale up the method in an attempt to detect the first group of smaller particles by increasing the total number of particles formed, either by increasing the concentrations (Formulations #3 and #4) or the total volume (Formulation #5) of the components of the system, the particles aggregated in the dialysis bag and precipitated during the dialysis procedure. As such, this method is not amenable to the concentration, buffer exchange, and filtration steps that are necessary for scaling up these formulations. Based on these experiments, I conclude that the detergent dialysis method described in Example 8 of Bally *et al.* is not able to produce the smaller, more stable, and more uniform populations of particles that are desirable for *in vivo* and clinical applications.

17. For the foregoing reasons, I submit that the teachings of Unger *et al.* and Bally *et al.* only provide general guidance about the types of components that can be included in lipid vesicles, but fail to clearly and specifically direct one skilled in the art to select the particular lipid components recited in the present claims to fully encapsulate and successfully deliver mRNA. In fact, none of the examples in Unger *et al.* and Bally *et al.* discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming smaller, physically stable, and uniform lipid vesicles that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells and thus more desirable for *in vivo* and clinical applications. Consequently, there is no motivation for one of ordinary skill in the art to take the teachings of Unger *et al.* and Bally *et al.* and make lipid vesicles using a specific combination of lipid components as claimed that fully encapsulates mRNA with any reasonable expectation of success.

18. The declarant has nothing further to say.

Oct 20th, 2014
Date

James Heyes, Ph.D.

EXHIBIT A

James Anthony Heyes

Experience

Dec 2010 - Present — Tekmira Pharmaceuticals — Burnaby, BC

Director, Formulation Chemistry

- Direct research of multidisciplinary team of scientists
- Planning and execution of experiments according to company objectives
- Supervise scientific collaborations with external parties
- Drive technology development of proprietary lipid nanoparticle (LNP) platform

Dec 2009 – Dec 2010 — Tekmira Pharmaceuticals — Burnaby, BC

Senior Scientist

- Reporting to CSO, supervise research activities of chemistry team
- Conceptualize / design novel compounds for proprietary LNP platform
- Provide guidance in formulation selection for corporate scientific collaborations

May 2008 – Dec 2009 — Tekmira Pharmaceuticals — Burnaby, BC

Scientist II

- Conceptualize / design novel compounds for proprietary LNP platform
- Troubleshoot problematic syntheses

April 2001 – May 2008 — Protiva Biotherapeutics — Burnaby, BC

Research Scientist

- Conceptualize / design novel compounds for proprietary LNP platform
- Troubleshoot problematic syntheses

Education

Sept 1996 – Jan 2001 — Institute of Cancer Research — Surrey, UK

Doctor of Philosophy (Medicinal Chemistry)

- Hunter Scholarship

Sept 1992 – Jun 1995 — The University of Manchester — Lancs, UK

Bachelor of Science (Chemistry)

- Honours, First Class

James Anthony Heyes

Publications

Lipid encapsulation enables the effective systemic delivery of polyplex plasmid DNA; Heyes et al, *Mol. Ther.*, **2007**, 15, 713-720

RNAi-mediated gene silencing in non-human primates; Zimmermann et al, *Nature,* **2006**, 441, 111-114

Synthesis and characterization of novel poly (ethylene glycol)-lipid conjugates suitable for use in drug delivery; Heyes et al, *J. Controlled Rel.,* **2006**, 112, 280-290

Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids; Heyes et al, *J. Controlled Rel.,* **2005**, 107, 276-287

Stabilized plasmid–lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression; Ambegia et al, *BBA-Biomembranes*, **2005**, 1669, 155-163

Structure-activity relationship in cationic lipid mediated gene transfection; Niculescu-Duvaz et al, *Curr. Med. Chem.*, **2003**, 10, 1233-1261

Synthesis of novel cationic lipids: effect of structural modification on the efficiency of gene transfer; Heyes et al, *J. Med. Chem.*, **2002**, 45, 99-114

Recent developments in gene-directed enzyme prodrug therapy (GDEPT) for cancer; Niculescu-Duvaz et al, *Curr. Opin. Mol. Ther.*, 1999, 1, 4810-486

Issued Patents

US Patent 8,569,256 – Cationic lipids and methods for the delivery of nucleic acids (Issued 10/29/2013).

US Patent 8,466,122 – Trialkyl cationic lipids and methods of use thereof (Issued 6/18/2013)

US Patent 8,865,675 – Compositions and methods for silencing Apolipoprotein B (Issued 10/21/2014)

US Patent 7,799,565 – Lipid encapsulated interfering RNA (Issued 21/9/2010)

US Patent 7,982,027 – Lipid encapsulated interfering RNA (Issued 19/7/2011)

US Patent 7,803,397 – Polyethyleneglycol-modified lipid compounds and uses thereof (Issued 28/9/2010)

US Patent 7,745,651 – Cationic lipids and methods of use (Issued 29/6/2010)

US Patent 7,807,815 – Compositions comprising immunostimulatory siRNA molecules and DLinDMA or DLenDMA (Issued 10/5/2010)

James Anthony Heyes

US Patent 8,283,333 – Lipid formulations for nucleic acid delivery (Issued 10/9/2012)

Patent Applications

US Application US20140134260 – Improved cationic lipids and methods for the delivery of therapeutic agents (Filed 10/1/2013)

US Application US20130022649 – SNALP Formulations containing antioxidants (Filed 12/1/2010)

US Application US20130123338 – Novel cationic lipids and methods of use thereof (Filed 5/12/2011)

US Application US20140288146 – Novel trialkyl cationic lipids and methods of use thereof (Filed 4/25/2013)

US Application US20130116307 – Novel cyclic cationic lipids and methods of use thereof (Filed 11/8/2012)

US Application US20130064894 – Novel cationic lipids and methods of use thereof (Filed 8/30/2012)

US Application US20110060032 – Lipid encapsulated interfering RNA (Filed 8/6/2010)

US Application US20120058188 – Lipid encapsulated interfering RNA (Filed 6/24/2011)

US Application US20110262527 – Cationic lipids and methods of use (Filed 10/5/2010)

US Application US20130122104 – Novel lipid formulations for delivery of therapeutic agents to solid tumors (Filed 8/3/2012)

JOINT APPENDIX 17

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Protiva Biotherapeutics, Inc.
Inventors:	Ian MacLachlan, *et al.*
Serial No.:	14/304,578
Filed:	June 13, 2014
Status:	Published as US 2014-0294937 on October 2, 2014 Response to Non-Final Office action filed on October 22, 2014

THIRD PARTY SUBMISSION UNDER 37 CFR § 1.290

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
ATTENTION: Director, Technology Center 1600 (1616)

Dear Commissioner:

The undersigned hereby submits one public document ("Document 1") of potential relevance to the examination of the above-identified patent application ("the '578 Application"), in accordance with 37 C.F.R. § 1.290 and 35 U.S.C. § 122(e).

This submission is made before the date a notice of allowance is given or mailed in the '578 Application, and before six months after the date on which the '578 Application was first published by the Office, and therefore, is timely under 37 C.F.R. §§ 1.290(b)(1) and 1.290(b)(2)(i). The undersigned submits that she is not an individual who has a duty to disclose information with respect to the above-identified application under 37 C.F.R. § 1.56.

I. Document being submitted

The following Document 1 is being submitted in accordance with 37 C.F.R. § 1.290(d)(1) and (d)(3):

Document 1 – **WO 98/51278**, filed on May 14, 1998 by Semple S. C., *et al.* (hereinafter "Semple"), claiming priority to May 14, 1997, and published on November 19, 1998. Thus, Semple is prior art to the '578 Application under pre-AIA 35 U.S.C. 102(b).

II. Concise description of the relevance of the Document

A concise explanation of the relevance of Document 1 is provided below, in accordance with 37 C.F.R. § 1.290(d)(2).

Document 1

Document 1, Semple, entitled "High Efficiency Encapsulation of Charged Therapeutic Agents in Lipid Vesicles," discloses lipid-nucleic acid compositions in which nucleic acids, such as mRNA, are encapsulated within a lipid vesicle comprising a cationic lipid, an amphipathic lipid (*e.g.*, phospholipid), and a polyethyleneglycol (PEG) lipid, and methods of preparing the same. Because the claims of the '578 Application are specifically directed to a lipid vesicle composition including a messenger RNA (mRNA), a cationic lipid, an amphipathic lipid, and a polyethyleneglycol (PEG) lipid, where the mRNA is fully encapsulated in the lipid vesicle, the teachings of Document 1 (which was published in 1998) is highly relevant to the patentability of the '578 Application.

For example, Semple teaches: "Methods for the preparation of a lipid-nucleic acid composition are provided. According to the methods, a mixture of lipids containing a protonatable or deprotonatable lipids, for example an amino lipid and a lipid such as a PEG- or polyamide oligomer-modified lipid is combined with a buffered aqueous solution of a charged therapeutic agent, for example, polyanionic nucleic acids, to produce particles in which the therapeutic agent is encapsulated in a lipid vesicle." Semple, Abstract; *see also id.*, Summary of the Invention.

Cationic lipids, also referred to as amino lipids, are described at least on pages 15-17 of Semple. Specifically, Semple teaches: "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 Figure 2A)." Semple, page 15, lines 9-14 (emphasis added). Exemplary cationic lipids, such as DODAP, DODMA, and others, are specifically disclosed on pages 16 and 17 of Semple. These same cationic lipids are also disclosed in paragraph [0037] of the '578 Application.

PEG-lipids are described on pages 17-18 of Semple. Specifically, Semple teaches: "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, . . . 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 Figure 2B, structures A and B), PEG-modified diacylglycerols and dialkylglycerols (see Figure 2B, structures C and D), PEG-modified dialkylamines (Figure 2B, structure E) and PEG-modified 1 ,2-diacyloxypropan-3-amines (Figure 2B, structure F). Particularly preferred are PEG-ceramide conjugates (e.g., PEG-CerC14 or PEG-CerC20) which are described in copending USSN 08/486,214, incorporated herein by reference." Semple, page 17, lines 43-47, and page 18, lines 8-16; *see also id.*, lines 17-24.

On pages 18 and 19, Semple teaches that a disclosed lipid vesicle "may contain additional lipids. These additional lipids may be, for example, neutral or sterols." Semple, page 18, lines 28-30.

Semple describes neutral lipids to include various amphipathic lipids disclosed in the '578 Application. Specifically, Semple teaches: "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. . . . 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. . . . 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." Semple 1, page 19, lines 1-17 (emphasis

added). The term "zwitterionic lipids" used in Semple appears to be interchangeable with the term "amphipathic lipids" used in the '578 Application. Indeed, those highlighted zwitterionic lipids above (*e.g.*, phospholipids, phosphatidylcholine, diacylphosphatidylcholine, and diacylphosphatidylethanolamine) are specifically disclosed as amphipathic lipids in paragraph [0035] and recited in claims 2, 3, 6 and 7 of the '578 Application.

Sterols are described on page 19 of Semple. Specifically, Semple teaches: "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." Semple, page 19, lines 18-20.

Semple teaches lipid-nucleic acid combinations at least on pages 20-28. For example, on page 20, lines 8 and 9, Semple teaches: "In accordance with the invention, the lipid mixture is combined with a buffered aqueous solution of charged therapeutic agent, preferably nucleic acids." On page 24, lines 24-26, Semple specifically defines nucleic acids to include mRNA: "Accordingly, the nucleic acids, or nucleotide polymers, can be polymers of nucleic acids including genomic DNA, cDNA, ***mRNA*** or oligonucleotides containing nucleic acid analogs, . . ." (Emphasis added).

On page 31, lines 6-8, Semple further teaches that typical applications of disclosed lipid-nucleic acid compositions "include using well known transfection procedures to provide intracellular delivery of DNA or ***mRNA*** sequences which code for therapeutically useful polypeptides." (Emphasis added).

Lipid vesicles, such as liposomes or lipid-nucleic acid particles, fully encapsulating nucleic acids are specifically disclosed in section III, entitled "Methods of Preparing Liposome/Nucleic Acid Complexes" and other parts of Semple. For example, on page 13, lines 1-14, Semple teaches: "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 pKa 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." Semple, page 13, lines 1-10 (emphasis added).

Lipid vesicles of a size less than about 150 nm are disclosed on page 13, lines 11-14, of Semple. Specifically, Semple discloses: "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***." (Emphasis added).

Lipid vesicles of a size less than about 100 nm are disclosed on page 27, lines 4-6. Specifically, Semple teaches the following: "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***." (Emphasis added).

Dated: January 5, 2015

Respectfully submitted,

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JOINT APPENDIX 18

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/304,578	06/13/2014	Ian MacLachlan	86399-001220US-911148	1042

20350 7590 02/13/2015

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EXAMINER	
HIRT, ERIN E	

ART UNIT	PAPER NUMBER
1616	

NOTIFICATION DATE	DELIVERY MODE
02/13/2015	ELECTRONIC

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.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipefiling@kilpatricktownsend.com
jlhice@kilpatrick.foundationip.com

Office Action Summary	Application No. 14/304,578	Applicant(s) MACLACHLAN ET AL.	
	Examiner ERIN HIRT	Art Unit 1616	AIA (First Inventor to File) Status No

-- 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* MONTHS 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 *10/22/14*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ 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*

5)☒ Claim(s) *1-12* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-12* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *06/13/14* 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).

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).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

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) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Status of Action

Currently claims 1-12 are pending in this application. Claims 11-12 are new claims

Status of Claims

Accordingly, claims 1-12 are presented for examination on the merits for patentability. Rejection(s) not reiterated from the previous Office Action are hereby withdrawn. The following rejections are either reiterated or newly applied. They constitute the complete set of rejections presently being applied to the instant application. **This is a second non-final office action.**

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 double patenting rejection is appropriate where the claims at issue 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 reference 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. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1, 9, 10 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 9-10, 17, and 19 of U.S. Patent No. 8058069 (‘069). Although the conflicting claims are not identical, they are not patentably distinct from each other because both '069 and the instant invention claim nucleic acid particles comprising phospholipids, PEG-lipids, cationic lipids and nucleic

acids. '069 merely teaches that their nucleic acids are siRNA instead of the instantly claimed mRNA. However, it would have been obvious to one of ordinary skill in the art at the time of the instant invention that the particles of '069 are merely obvious variants of the particles instantly claimed.

Claims 1-7, and 9-10 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4-6, 12, 21 of US Patent 8283333 for the same reasons discussed above.

Claims 1 and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4, 10 and 18 of U.S. Patent No. 7799565 for the same reasons discussed above.

Claims 1-7 and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-6, 8, 10-11 of U.S. Patent No. 8466122 for essentially the same reasons as discussed above.

Claims 1-3 and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 8, 14, and 20 of U.S. Patent No. 8492359 for essentially the same reasons as discussed above.

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.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 1-12 are rejected under 35 U.S.C. 103(a) as being unpatentable over Unger et al. (US5830430), Semple et al. (WO98/51278), Lasic (*Tibtech*, **1998**, *16,* 307-321), and Meyer et al. (*J. Biol. Chem.*, **1998**, *273*, 15621-15627).

Applicant claims:

A lipid vesicle comprising:
a messenger RNA (mRNA);
a cationic lipid;
an amphipathic lipid; and
a polyethyleneglycol (PEG)-lipid,
wherein the mRNA is fully encapsulated in the lipid vesicle.

Determination of the scope and content of the prior art

(MPEP 2141.01)

Unger teaches cationic liposomes/lipid vesicles which comprise combination of a cationic lipid, a phospholipid, specifically the instantly claimed dioleoylphosphatidyl choline, a PEG-lipid, cholesterol, and genetic material, specifically RNA, which obviously includes mRNA which is a type of RNA (see entire document; esp. Col. 20, ln. 15-40; Col. 25, ln. 47-58; Col. 20, ln. 55-Col. 21, ln. 40; Claims 103-106, 110-111, 118, 120-123, 135-141) (Claims 1 (in part), 2-8). Unger further teaches wherein the liposome encapsulates the genetic material (Col. 9, ln. 66-Col. 10, ln. 9; Col. 10, ln. 15-38; Col. 20, ln. 15-40; Col. 20, ln. 55-Col. 21, ln. 40; Col. 25, ln. 47-58;) (Claim 1). Unger then teaches that the size of the liposomes can be adjusted by a variety of techniques, but that preferably the size of the liposomes are less than 100 nm in diameter, which reads upon the less than 150 nm that is instantly claimed (Col. 22, ln. 28-42) (Claim 10). Unger does not specifically state that the lipid vesicle is a lipid-nucleic acid particle but as it is a liposome which encapsulates genetic material, i.e. RNA, it is obviously a lipid-nucleic acid particle (Claim 9). Unger then expressly teaches that the amount of stabilizing material, i.e. amphipathic compounds/lipids which are combined with the cationic lipid may vary depending on a variety of factors including, the specific cationic lipids, the specific stabilizing material(s) selected (i.e. more than one type or combination), the particular use for which it is being employed, the mode of delivery, and the like (Col. 21, ln. 45-51). Further, Unger states, “The amount of stabilizing material to be combined with the present cationic lipid compounds in a particular

situation and the ratio of stabilizing material to cationic lipid, will vary and is **readily determinable by one skilled in the art based on the present disclosure"** (emphasis added) (i.e. one of ordinary skill in the art) (Col. 21, ln. 51-56).

Ascertainment of the difference between prior art and the claims

(MPEP 2141.02)

Unger merely does not teach an example wherein the lipid vesicle comprises all of these things and wherein the RNA is mRNA. However, mRNA is a type of RNA i.e. RNA is the genus and mRNA is the species. Therefore, by teaching that RNA can be carried by the lipid vesicles, Unger does render the instantly claimed mRNA obvious because it was already known in the art to use lipid vesicles to encapsulate the genus. However, this deficiency in Unger is also addressed by Semple.

Semple teaches lipid vesicles which comprise mRNA, cationic lipid(s) (DODAP, DODMA), phospholipid(s), cholesterol, and PEG-lipids wherein the nucleic acids are enclosed by the lipid vesicle (i.e. protected from degradation) (see entire document; Abstract; pg. 1, ln. 10-22; pg. 4, ln. 15-pg. 5, ln. 2; pg. 9, ln. 20-pg. 11, ln. 10; pg.11, ln. 27-pg. 13, ln. 14; pg. 13, ln. 15-pg. 16, ln. 24; pg. 17, ln. 43-46; pg. 19 ln. 1-20; pg. 19, ln. 28-pg. 20, ln. 7; pg. 20, ln. 8-pg. 21, ln. 6; pg. 24, ln. 22-30; pg. 31, ln. 6-12; Examples; pg. 19, ln. 28-pg. 21, ln. 6; pg. 13, ln. 11-14; Claims 11, 37). Semple further teaches wherein the particles are less than 100 nm and are only positively charged at below physiological pH (Claim 11).

Also, further motivation to include the specific lipids instantly claimed in the liposomes of Unger is provided by Meyer and Lasic.

Meyer teaches cationic liposomes coated with PEG and PEG-PE (a PEG-lipid) for increased stabilization of the liposomes which are used to carry oligonucleotides (i.e. nucleic acids, similar to RNA) (Abstract; pg. 15621, right col. Last paragraph).

Lasic teaches that liposomes/phospholipid vesicles are useful for drug delivery, specifically the delivery of DNA, RNA, oligonucleotides, etc. because the liposome allow for the protection of these sensitive drug molecules (Pg. 314, right col., 2nd paragraph). Lasic also teaches wherein it is known to have these liposomes comprise PEG-lipids for steric stabilization (pg. 314, right col. 1st para.). Lasic also teaches that liposomes are preferably between 80-200 nm because this size range is a compromise between loading efficiency of the liposomes, the stability of the liposomes, and the ability of the liposomes to extravasate (pg. 309, right col. 1st full paragraph). Lasic further states that, "If the liposomes are larger than ~150 nm, sterile filtering is probably not possible and so the whole process must be performed aseptically, which is not very practical from the engineering or economical viewpoints." (pg. 313, left col., 2nd full paragraph). Lasic further teaches wherein the nucleic acid, is/can be encapsulated (i.e. fully encapsulated) in the lipid vesicle and wherein cholesterol is used to stabilize the lipid vesicle comprising nucleic acids (i.e. DNA or RNA) (pg. 318, left column, last paragraph-right column end of paragraph).

Finding of prima facie obviousness

Rationale and Motivation (MPEP 2142-2143)

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to make liposomes having a cationic lipid, a PEG-lipid, cholesterol, and a phospholipid, specifically dioleylphosphatidyl choline, which encapsulate mRNA because Unger teaches liposomes comprising cationic lipids, PEG-lipids, cholesterol, and phospholipids, specifically dioleylphosphatidyl choline are useful for delivering genetic material, specifically RNA. Furthermore, it was known that PEG-lipids stabilize the liposomes and cholesterol and it would have been obvious to an ordinary skilled artisan to have the particles be about 100 nm in diameter and only carry a positive charge when at below physiological pH because Semple teaches lipid vesicles comprising mRNA, cationic lipids specifically DODAP which is charged at below physiological pH, PEG-lipid, cholesterol, and phospholipids to deliver the mRNA to cells in patients in need thereof.

In light of the forgoing discussion, the Examiner concludes that the subject matter defined by the above claims would have been obvious to one of ordinary skill in the art within the meaning of 35 USC 103(a).

From the teachings of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

Claims 1-12 are rejected under 35 U.S.C. 103(a) as being unpatentable over Semple et al (WO98/51278, from 3rd party IDS submission).

Applicant claims:

A lipid vesicle comprising:
a messenger RNA (mRNA);
a cationic lipid;
an amphipathic lipid; and
a polyethyleneglycol (PEG)-lipid,
wherein the mRNA is fully encapsulated in the lipid vesicle.

Determination of the scope and content of the prior art

(MPEP 2141.01)

Semple teaches lipid vesicles, specifically lipid-nucleic acid particles and liposomes, which comprise mRNA, cationic lipid(s), specifically DODAP or DODMA which are only charged at below physiological pH, phospholipid(s) such as distearoylphophatidyl choline, cholesterol (i.e. a sterol), and PEG-lipids wherein the nucleic acids are enclosed by the lipid vesicle (i.e. protected from degradation) (see entire document; pg. 1, ln. 10-22; pg. 4, ln. 15-pg. 5, ln. 2; pg. 9, ln. 20-pg. 11, ln. 10; pg.11, ln. 27-pg. 13, ln. 14; pg. 13, ln. 15-pg. 16, ln. 24; pg. 17, ln. 43-46; pg. 19 ln. 1-20; pg. 19, ln. 28-pg. 20, ln. 7; pg. 20, ln. 8-pg. 21, ln. 6; pg. 24, ln. 22-30; pg. 31, ln. 6-12; Examples; Claims 37) (Claims 1-9, 11). Semple also teaches an exemplary embodiment wherein the mixture of lipids forming the lipid vesicle include amino lipids, i.e. phopsholipids, distearoylphosphatidyl choline, palmitoyloleoylphsphatidyl choline, etc., cholesterol and PEG-lipids which is combined with a charged therapeutic agent, preferably nucleic acids (pg. 19, ln. 28-pg. 21, ln. 6). Semple finally teaches wherein the phospholipids are preferably, distearoylphosphatidyl choline, palmitoyloleoylphsphatidyl

choline, etc., and wherein particles of less than 150 nm, specifically about 100 nm or less in size can be made (pg. 13, ln. 11-14; Claim 11) (Claims 3, 7, 10, 12).

Ascertainment of the difference between prior art and the claims

(MPEP 2141.02)

Semple merely does not teach a specific lipid vesicle which comprises all of the instantly claimed features.

Finding of prima facie obviousness

Rationale and Motivation (MPEP 2142-2143)

It would have been obvious to one of ordinary skill in the art to make a lipid vesicle of the instant claims by looking to Semple because Semple teaches lipid vesicles of the same size and comprising the same components as instantly claimed for delivering and protecting mRNA from degradation when delivered to patients in need thereof. One of ordinary skill in the art would want to develop the lipid vesicles of Semple with the same features as instantly claimed because adding cholesterol and PEG-lipids to lipid nucleic acid particles comprising mRNA, cationic lipids and phospholipids, were known to improve the pharmacokinetics of these particles and/or the delivery of the nucleic acid/mRNA. Thereby, allowing one of ordinary skill in the art to make a more effective lipid vesicle for delivering the mRNA to cells to patients.

Response to Arguments/Remarks

Applicant's arguments with respect to the 103 rejections have been fully considered and were persuasive with respect to Bally as applicants have demonstrated

that Bally does not work in their affidavit. Therefore, the rejections including Bally have been dropped. However, these arguments and new claims have prompted the new grounds of rejection presented in this second non-final office action. Applicant's arguments insofar as they pertain to the new grounds of rejection are addressed herein.

Applicant's arguments appear to merely restate the rejections supplied in the affidavit submitted by Dr. James Heynes which was filed 10/22/14 and dated 10/20/14. Rather than address these arguments more than once they will be addressed in regards to the affidavit.

Declaration under 1.132

Dr. James Heynes provided a declaration under 1.132, filed October 22, 2014 and dated 20 October 2014. The Declaration meets the formal requirements. In the most relevant part, the Declaration demonstrates that the prior art Bally does not work as reported. A Declaration is due full consideration and weight for all that it discloses. Declarations are reviewed for the following considerations: 1) whether the Declaration presents a nexus such as a side-by-side or single-variable comparison (In re Huang, 40 USPQ2d 1685, 1689 (Fed. Cir. 1996)), 2) whether the Declaration presents a comparison to the closest art, 3) whether the Declaration is commensurate in scope with the scope of the claims (In re Kulling, 14 USPQ2d 1056, 1058 (Fed. Cir. 1990)), 4) whether the Declaration shows a difference in kind rather than merely a difference in degree (In re Waymouth, 182 USPQ 290, 293 (C.C.P.A. 1974)), and 5)whether the prima facie case is sufficiently strong that allegedly superior results are insufficient to overcome the case for obviousness (Pfizer Inc. v. Apotex, Inc., 82 USPQ2d 1321, 1339

(Fed. Cir. 2007)). The relevant criterion here is No. 5, whether the prima facie case is sufficiently strong that allegedly superior results are insufficient to overcome the case for obviousness. The examiner has carefully reviewed the Declaration, including the data presented in the Declaration. The data show that the prior art Bally does not function as reported. Thus, because Bally does not allow for applicants to product lipid particles of reasonable size using their method, the rejections over Bally have been withdrawn.

With respect to the prior art Unger, applicants argue that it is merely a general teaching reference and does not provide guidance to each of the instantly claimed components for making the lipid vesicles. In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.,* 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986). In the instant case, Unger teaches forming lipid vesicles comprising the same lipids instantly claimed but does not teach an example which comprises each of these. Semple teaches lipid vesicles which comprise all of the same lipids as instantly claimed and wherein the nucleic acids which are encapsulated can be mRNA.

Applicants then argue that Unger does not teach encapsulating the nucleic acids/mRNA into the lipid vesicle, and actually teaches away from combining the lipids to form a vesicle to deliver the nucleic acids/mRNA. The examiner respectfully disagrees because at Col. 10, ln. 15, Unger states that, “In combination with” refers to

the incorporation of the bioactive agent with a cationic lipid compound....The cationic lipid compound can be combined with the bioactive agent in any of a variety of different ways...the bioactive agent can be entrapped within the internal void of the vesicle, i.e. it is encapsulated within the vesicle. Further, the examiner believes that Unger does not teach away from using the combination of lipids instantly claimed because at Col. 20, ln. 55-Col. 21, ln. 44, Unger teaches lipids that can be used in combination with the cationic lipid compounds and expressly teaches cholesterol, dioleylphosphatidyl choline, and PEG-lipids. Using these lipids to form the lipid vesicles is further supported by the addition of the prior art Semple.

Thus, the examiner believes that the combination of the prior art does render the composition/lipid vesicles of the instant claims prima facie obvious.

Conclusion

Claims 1-12 are rejected.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ERIN HIRT whose telephone number is (571)270-1077. The examiner can normally be reached on Monday through Friday 9:00am to 6:30pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sue X. Liu can be reached on 571-272-5539. 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.

Erin Hirt
Examiner, Art Unit 1616

/Mina Haghighatian/
Primary Examiner, Art Unit 1616

JOINT APPENDIX 19

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on May 12, 2015.

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Judith Cotham/
Judith Cotham

PATENT
Attorney Docket No.: 86399-001220US-911148

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 1042
Ian MacLachlan et al.	Examiner: Hirt, Erin E.
Application No.: 14/304,578	Art Unit: 1616
Filed: June 13, 2014	AMENDMENT
For: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY	
Customer No.: 20350	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Office Action mailed February 13, 2015, please enter the following amendments and remarks. A Declaration of Dr. James Heyes under 37 C.F.R. § 1.132 accompanies the present response.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks begin on page 4 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. (Original) A lipid vesicle comprising:

a messenger RNA (mRNA);

a cationic lipid;

an amphipathic lipid; and

a polyethyleneglycol (PEG)-lipid,

wherein the mRNA is fully encapsulated in the lipid vesicle.

2. (Original) The lipid vesicle of claim 1, wherein the amphipathic lipid is a phospholipid.

3. (Original) The lipid vesicle of claim 2, wherein the phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine.

4. (Original) The lipid vesicle of claim 1, wherein the lipid vesicle further comprises a sterol.

5. (Original) The lipid vesicle of claim 4, wherein the sterol is cholesterol.

6. (Original) The lipid vesicle of claim 4, wherein the sterol is cholesterol and the amphipathic lipid is a phospholipid.

7. (Original) The lipid vesicle of claim 6, wherein the phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine.

8. (Original) The lipid vesicle of claim 1, wherein the lipid vesicle is a liposome.

9. (Original) The lipid vesicle of claim 1, wherein the lipid vesicle is a lipid-nucleic acid particle.

10. (Original) The lipid vesicle of claim 1, wherein the lipid vesicle is about 150 nm or less in diameter.

11. (Previously presented) The lipid vesicle of claim 1, wherein the cationic lipid only carries a positive charge at below physiological pH.

12. (Previously presented) The lipid vesicle of claim 1, wherein the lipid vesicle is about 100 nm or less in diameter.

REMARKS

I. STATUS OF THE CLAIMS

Upon entry of this amendment, claims 1-12 are pending in this application and are presented for examination. No new matter has been introduced. Reconsideration is respectfully requested.

II. DOUBLE PATENTING REJECTIONS

Claims 1, 9, and 10 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 9, 10, 17, and 19 of U.S. Patent No. 8,058,069. Claims 1-7, 9, and 10 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 4-6, 12, and 21 of U.S. Patent No. 8,283,333. Claims 1 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 4, 10, and 18 of U.S. Patent No. 7,799,565. Claims 1-7 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1-6, 8, 10, and 11 of U.S. Patent No. 8,466,122. Claims 1-3 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 8, 14, and 20 of U.S. Patent No. 8,492,359.

Applicants respectfully request that the Examiner hold these rejections in abeyance until there is an indication of allowable subject matter. *See*, M.P.E.P. § 714.02.

III. FIRST REJECTION UNDER 35 U.S.C. § 103(a)

Claims 1-12 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Unger *et al.* (US Patent No. 5,830,430), Semple *et al.* (WO 98/51278), Lasic (*Trends in Biotech.*, 16:307-21 (1998)), and Meyer *et al.* (*J. Biol. Chem.*, 273:15621-7 (1998)). Applicants respectfully traverse this rejection.

In the Office Action, the Examiner alleges that it would have been obvious to one of ordinary skill in the art to make liposomes comprising the same components as presently claimed to encapsulate mRNA from the teachings of the references. *See,* Office Action at page 9. In particular, the Examiner relies on Semple *et al.* for allegedly teaching lipid vesicles comprising mRNA with the same lipid components as presently claimed for delivering the

mRNA to cells in patients in need thereof. *See, id.* As such, the Examiner concludes that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention as evidenced by the references in the absence of evidence to the contrary. *See, id.*

In response, Applicants respectfully submit herewith a Declaration of Dr. James Heyes under 37 C.F.R. § 1.132 (hereinafter, "Heyes Declaration") to present evidence that the method described in Semple *et al.* for preparing liposomes is not able to produce the population of smaller lipid vesicles of the present invention with high mRNA encapsulation efficiency that is desirable for *in vivo* and clinical applications.

As explained by Dr. Heyes in his Declaration, he and his colleagues used the method for preparing liposomes containing antisense oligonucleotide described in the "Materials and Methods" on pages 33-34 of Semple *et al.* to determine the suitability of this method for formulating mRNA in lipid vesicles. *See*, Heyes Declaration ¶¶ 8 & 9.

Based on the results of the experiment (*see,* Heyes Declaration ¶¶ 10-12), Dr. Heyes states that the method for preparing liposomes described in Semple *et al.* is not suitable for formulating mRNA in the lipid vesicles as presently claimed. *See*, Heyes Declaration ¶13. Indeed, Dr. Heyes points out that they had little success in producing lipid vesicles with encapsulated mRNA due to the high degree of degradation of the starting mRNA payload and the very low encapsulation efficiency that were associated with the method described in Semple *et al.* *See, id.* Given the sizes of the particles and their polydispersity indexes, they were unable to produce lipid particles of reasonable size and homogeneity using the method described in Semple *et al.* *See, id.* As a result, Dr. Heyes explains that this method produced lipid particles that failed all of the formulation parameters measured, as the final product consisted of a population of particles with only a minute amount of the starting mRNA being successfully encapsulated and with larger and more heterogeneous particles sizes that would likely invoke an unwanted innate immune response upon systemic administration. *See, id.* Thus, Dr. Heyes concludes that the method described in Semple *et al.* is not amenable to the production of the

population of smaller lipid vesicles of the present invention with high mRNA encapsulation efficiency that is desirable for *in vivo* and clinical applications. *See, id.*

For the foregoing reasons, Dr. Heyes submits that there is no motivation for one of ordinary skill in the art to take the teaching of Semple *et al.* and make lipid vesicles using a specific combination of lipid components as claimed that fully encapsulates mRNA with any reasonable expectation of success. *See*, Heyes Declaration ¶14. In fact, Dr. Heyes points out that none of the examples in Semple *et al.* discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming smaller lipid vesicles that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells and thus more desirable for *in vivo* and clinical applications. *See, id.* Indeed, the experiment described in the Heyes Declaration clearly demonstrates that the method for preparing liposomes described in Semple *et al.* is not suitable for successfully encapsulating mRNA and formulating the lipid vesicles as presently claimed. *See, id.*

Applicants assert that the teaching of Unger *et al.* does not remedy the deficiencies in the disclosure of Semple *et al.* In fact, Unger *et al.* fails to provide any clear and specific teaching with regard to the successful encapsulation and delivery of mRNA. Indeed, none of the examples in Unger *et al.* discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming smaller lipid vesicles that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells. The cationic lipid compounds and cationic liposome formulations disclosed in Unger *et al.* are only exemplified in the context of their use for preparing preformed liposomes to form complexes with DNA called lipoplexes. As explained by Dr. Heyes in paragraph 10 of his Declaration submitted on October 22, 2014, lipoplexes are electrostatic complexes in which little, if any, of the DNA payload is encapsulated within the preformed cationic liposomes.

The Examiner alleges that Unger *et al.* teaches that bioactive agents can be entrapped within the internal void of a lipid vesicle, but Applicants point out that Unger *et al.* still fails to exemplify encapsulation of nucleic acid of any kind, let alone mRNA. Although Semple *et al.* discloses a method for preparing liposomes containing encapsulated antisense

oligonucleotide, Dr. Heyes presents clear evidence in his Declaration that this method is not suitable for successfully encapsulating mRNA in lipid vesicles. As such, contrary to the Examiner's allegation, one of ordinary skill in the art would not have had a reasonable expectation of success in producing the claimed invention based on the combined disclosures of Unger *et al.* and Semple *et al.*

Furthermore, Applicants assert that the teachings of Lasic and Meyer *et al.* do not remedy the deficiencies in the disclosures of Unger *et al.* and Semple *et al.* Indeed, Lasic teaches lipoplexes as DNA-carrier systems for gene therapy. *See,* page 318, paragraph bridging left and right columns. In particular, Lasic teaches that cationic liposomes have been shown to complex DNA, and such complexes were able to transfect cells *in vitro*, resulting in the expression of the protein encoded in the DNA plasmid in target cells. *See*, *id.* In addition, Lasic teaches that cationic lipid-based DNA complexes can transfect cells *in vivo* upon localized or systemic administration. *See*, *id.* Moreover, Lasic teaches that improvements including developments in DNA-plasmid design, the synthesis of novel cationic lipids, and the cholesterol stabilization of complexes have resulted in more than a thousand-fold increase in gene expression over initial experiments, and that PEG-lipids may be used to coat DNA-lipid complexes to improve such lipid-based carriers. *See*, *id.* At most, one of ordinary skill in the art might have been motivated to use lipoplexes as lipid-based carrier systems for gene therapy in view of the teachings of Lasic.

In fact, Applicants submit that Meyer *et al.* supports the teachings of Lasic and provides further motivation for the skilled artisan to use lipoplexes for the *in vivo* delivery of nucleic acid. Indeed, as with Unger *et al.*, Meyer *et al.* teaches the formation of lipoplexes between preformed cationic liposomes and DNA (*i.e.,* oligodeoxyribonucleotides (ODN)). In particular, Meyer *et al.* teaches that these "[l]iposome-ODN complexes were produced by overnight incubation of the liposomes with ODN in HBS [HEPES buffered saline]." *See,* page 15622, left column; emphasis added. In addition, the Abstract of Meyer *et al.* repeatedly refers to complexes between the liposome and ODN components. *See also,* Figure 1 of Meyer *et al.*, which describes the binding of ODN to PEG-modified cationic liposomes and the resulting

complexes. Importantly, Meyer *et al.* teaches that its PEG-modified cationic liposomes offer numerous advantages as carriers for cellular delivery of ODN (*e.g.,* the resulting complexes are stable against aggregation and retain both their ODN load in blood plasma and the ability to enhance cellular delivery of ODN in the presence of serum) and concludes that "PEG-modified cationic liposomes may provide a useful step in the development of an efficient pharmaceutical carrier for systemic *in vivo* delivery of ODN." *See,* page 15627, left column. Thus, Meyer *et al.* corroborates the teachings of Lasic and provides further motivation for one of ordinary skill in the art to use lipoplexes as lipid-based carrier systems for the *in vivo* delivery of nucleic acid.

In view of the foregoing, Applicants assert that there is no rational underpinning to combine the cited references to support a legal conclusion of obviousness because these references, whether alone or in combination, do not teach or suggest each of the features recited in the claims. Indeed, as explained by Dr. Heyes in his Declaration, there is no motivation for one of ordinary skill in the art to take the teaching of Semple *et al.* and make lipid vesicles using a specific combination of lipid components as claimed that fully encapsulates mRNA with any reasonable expectation of success. Furthermore, Unger *et al.* fails to provide any clear and specific teaching whatsoever with regard to the successful encapsulation and delivery of mRNA. Indeed, none of these references discloses or suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming a population of smaller lipid vesicles with high mRNA encapsulation efficiency that are more effective at delivering the mRNA payload to living cells and thus more desirable for *in vivo* and clinical applications. Moreover, the teachings of Lasic and Meyer *et al.* do not remedy the clear deficiencies in the disclosures of both Unger *et al.* and Semple *et al.* since the skilled artisan would be motivated to use complexes formed between preformed liposomes and nucleic acid for gene therapy based on their numerous advantages, according to the cited references, as carriers for *in vivo* cellular delivery.

Accordingly, Applicants respectfully request that the Examiner withdraw the present rejection under 35 U.S.C. § 103(a).

IV. SECOND REJECTION UNDER 35 U.S.C. § 103(a)

Claims 1-12 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Semple *et al.* (WO 98/51278). Applicants respectfully traverse this rejection.

In the Office Action, the Examiner alleges that it would have been obvious to one of ordinary skill in the art to make a lipid vesicle of the same size and comprising the same components as presently claimed for delivering and protecting mRNA from degradation when delivered to patients in need thereof. *See,* Office Action at page 11.

As explained by Dr. Heyes in his Declaration, the method for preparing liposomes described in Semple *et al.* is not suitable for formulating mRNA in the lipid vesicles as presently claimed. *See*, Heyes Declaration ¶13. Notably, Dr. Heyes states that this method produced lipid particles that failed all of the formulation parameters measured, as the final product consisted of a population of particles with only a minute amount of the starting mRNA being successfully encapsulated and with larger and more heterogeneous particles sizes that would likely invoke an unwanted innate immune response upon systemic administration. *See, id.* Dr. Heyes concludes that the method described in Semple *et al.* is not amenable to the production of the population of smaller lipid vesicles of the present invention with high mRNA encapsulation efficiency that is desirable for *in vivo* and clinical applications. *See, id.* As such, there is no motivation for one of ordinary skill in the art to take the teaching of Semple *et al.* and make lipid vesicles using a specific combination of lipid components as claimed that fully encapsulates mRNA with any reasonable expectation of success. *See*, Heyes Declaration ¶14.

Therefore, Applicants assert that there is simply no rational underpinning to use the teaching of Semple *et al.* to support a legal conclusion of obviousness. Accordingly, Applicants respectfully request that the Examiner withdraw the present rejection under 35 U.S.C. § 103(a).

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/

Joe C. Hao
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Attachments

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 1042
Ian MacLachlan et al.	Examiner: Hirt, Erin E.
Application No.: 14/304,578	Art Unit: 1616
Filed: June 13, 2014	DECLARATION UNDER 37 C.F.R. § 1.132
For: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY	
Customer No.: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, James Heyes, Ph.D., being duly warned 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 patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I hold a Ph.D. (2001) in Medicinal Chemistry from the Institute of Cancer Research (Surrey, UK). I am presently the Director of Formulation Chemistry at Tekmira Pharmaceuticals Corp. (Burnaby, Canada). The assignee of the above-referenced application, Protiva Biotherapeutics Inc., is a wholly-owned subsidiary of Tekmira.

3. My expertise lies in the development of lipid particle formulations and the design of novel compounds as components of lipid particles. A copy of my *Curriculum Vitae* is of record.

4. I have reviewed the above-referenced patent application, and I am familiar with the contents therein. I have also reviewed the contents of the Office Action dated February 13, 2015.

5. The present invention is directed to a lipid vesicle comprising: a messenger RNA (mRNA); a cationic lipid; an amphipathic lipid; and a polyethyleneglycol (PEG)-lipid, wherein the mRNA is fully encapsulated in the lipid vesicle. In certain embodiments, the lipid vesicle is about 100 nm or less in diameter.

6. In the Office Action, the Examiner relies on Semple *et al.* (WO 98/51278) in alleging that it would have been obvious to one of ordinary skill in the art to make a lipid vesicle of the same size and comprising the same components as presently claimed for delivering and protecting mRNA from degradation when delivered to patients in need thereof.

7. I submit this Declaration to present evidence that the method described in Semple *et al.* for preparing liposomes is not able to produce the population of smaller lipid vesicles of the present invention with high mRNA encapsulation efficiency that is desirable for *in vivo* and clinical applications.

8. My colleagues and I used the method for preparing liposomes containing antisense oligonucleotide described in the "Materials and Methods" on pages 33-34 of Semple *et al.* to determine the suitability of this method for formulating mRNA in lipid vesicles. The method described in Semple *et al.* was followed, except for a couple of minor, enforced modifications as indicated below. The lipid composition selected was that which appeared to give optimal formulation characteristics in Semple *et al.*

9. The experiment was performed as follows:

mRNA Stock Prep: To stock mLuc (510µL) at 1 mg/mL in 10 mM Tris-HCl, pH 7.5 was added 90 µL of 2 M Citrate, pH 3.62 to achieve 600 µL of 0.85 mg/mL mLuc in 300 mM Citrate at pH 3.8. It was not possible to purchase mRNA at a high enough concentration to

follow the method of Semple *et al.* exactly. We therefore purchased the highest concentration available (1 mg/mL from TriLink BioTechnologies) and conducted the formulating with an accordingly (4-fold) more dilute lipid solution. This ensures that the important mass ratio of lipids to nucleic acid is not affected. Other important parameters such as pH were similarly maintained.

Lipid Stock Prep: DSPC, cholesterol (Chol), and DODAP individual stock solutions were prepared at 20 mg/mL in 95% EtOH, while PEG_{2000}-CerC16 and PEG_{2000}-C-DMA were prepared at 50 mg/mL. Two combined lipid stocks at 6.3 mg/mL (8.2 mM) were prepared in 95% EtOH with the following compositions:

- (A) DSPC:Chol:DODAP:PEG_{2000}-CerC16 (25:45:20:10 molar ratio); and
- (B) DSPC:Chol:DODAP:PEG_{2000}-C-DMA (25:45:20:10 molar ratio).

The PEG_{2000}-CerC14 used in the method of Semple *et al.* was not available for purchase from regular lipid suppliers (*e.g.*, Avanti Polar Lipids). We therefore opted to use PEG_{2000}-CerC16, an analogous PEG lipid with a minor structural difference that is not expected to affect the formulating process, as only the C14 alkyl chain has been replaced with a C16 alkyl chain. As a further control, we prepared a second composition using PEG-C-DMA, which is a similar PEG lipid having a C14 alkyl chain, but different linker chemistry. PEG-C-DMA is also expected to behave similarly in the formulating process.

Prep of Lipid Particles: The mRNA stock was heated to 65°C for 10 min. 0.4 mL of the combined lipid stock was then added dropwise to 0.6 mL of the mLuc stock which was being vortexed. The resulting MLV mRNA particles were then subjected to freeze/thaw cycles in liquid N_2/65°C five times, followed by extrusion ten times through an extruder containing 3 stacked 100 nm filters at 400 psi and heated to 65°C. The resulting mixture was then diluted with 1 mL of 300 mM Citrate, pH 3.8.

Removal of EtOH from Lipid Particles: To remove the EtOH from the particles, the mixture was transferred to a 3 mL Slide-A-Lyzer (MWCO 10,000) and was dialyzed for 4 hours against an appropriate amount of 300 mM citrate, pH 3.8.

Buffer Change of Lipid Particles: To change the buffer and thus the pH of the mixture, the particles were dialyzed for 12-18 hours against an appropriate amount of HEPES Buffered Saline (HBS), pH 7.5 (20 mM HEPES, 145 mM NaCl, pH 7.5). The resulting mixture should contain the final lipid particles.

10. The results of the experiment are summarized in Table 1. Four formulation parameters were measured and followed throughout the process:

- % Encapsulation of mRNA (*i.e.*, how much mRNA has been successfully encapsulated in the particle);
- % Recovery (*i.e.*, how much of the starting mRNA remains);
- Particle size; and
- Polydispersity (*i.e.*, a measure of the heterogeneity of sizes of particles in a mixture).

Table 1. Size, encapsulation, and recovery at various steps during formulation

Step	Measurement	Sample	
		PEG-C-DMA	PEG-CerC16
Before Extrusion	% Encapsulation	13%	5%
	~% Recovery (Total mRNA)	100%	100%
Before Dialysis	Diameter (PDI) in Citrate	188nm (0.36)	150nm (0.25)
	% Encapsulation	10%	3%
	~% Recovery (Total mRNA)	22%	27%
Final Product	Diameter (PDI) in HBS	138nm (0.22)	131nm (0.17)
	% Encapsulation	17%	8%
	~% Recovery (Total mRNA)	20%	26%
	~Final [Total mRNA]	64µg/mL	89µg/mL

11. A number of deficiencies in the lipid particles prepared by the method described in Semple *et al.* were observed. First, the vast majority of the mRNA payload (75-80%) was either lost or degraded during the process. Second, what little mRNA that remained had a very low degree of encapsulation (17% and 8% for PEG-C-DMA and PEG-CerC16, respectively). When combining these two readouts, it became apparent that only **3.4%** (PEG-C-DMA) or **2.1%** (PEG-CerC16) of the starting mRNA had been successfully encapsulated into lipid particles.

12. Further shortcomings were revealed in the particle sizing data. The average size of the lipid particles prepared by the method described in Semple *et al.* was well over 100 nm, and therefore likely to invoke an innate immune response upon systemic administration. Additionally, the polydispersity index (PDI) was quite high (~0.2). This measurement reflects how homogeneous a formulation is from a size perspective, with a lower number (*i.e.*, PDI ~0.1 or less) being desirable and reflecting a more homogeneous particle population. A larger PDI value indicates a larger amount of heterogeneity of particle size in the mixture.

13. This experiment clearly demonstrates that the method for preparing liposomes described in Semple *et al.* is not suitable for formulating mRNA in the lipid vesicles as presently claimed. Indeed, we had little success in producing lipid vesicles with encapsulated mRNA due to the high degree of degradation of the starting mRNA payload and the very low encapsulation efficiency that were associated with the method described in Semple *et al.* Given the sizes of the particles and their polydispersity indexes, we were unable to produce lipid particles of reasonable size and homogeneity using the method described in Semple *et al.* As a result, this method produced lipid particles that failed all of the formulation parameters described above, as the final product consisted of a population of particles with only a minute amount of the starting mRNA being successfully encapsulated and with larger and more heterogeneous particles sizes that would likely invoke an unwanted innate immune response upon systemic administration. Based on this experiment, I conclude that the method described in Semple *et al.* is not amenable to the production of the population of smaller lipid vesicles of the present invention with high mRNA encapsulation efficiency that is desirable for *in vivo* and clinical applications.

14. For the foregoing reasons, I submit that there is no motivation for one of ordinary skill in the art to take the teaching of Semple *et al.* and make lipid vesicles using a specific combination of lipid components as claimed that fully encapsulates mRNA with any reasonable expectation of success. In fact, none of the examples in Semple *et al.* discloses or

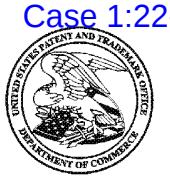
suggests a lipid vesicle of the present invention comprising fully encapsulated mRNA or the desirability of forming smaller lipid vesicles that are more effective at delivering encapsulated nucleic acid molecules such as mRNA to living cells and thus more desirable for *in vivo* and clinical applications. Indeed, our experiment clearly shows that the method for preparing liposomes described in Semple *et al.* is not suitable for successfully encapsulating mRNA and formulating the lipid vesicles as presently claimed.

15. The declarant has nothing further to say.

May 4th 2015
Date

James Heyes, Ph.D.

JOINT APPENDIX 20



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14/304,578	06/13/2014	Ian MacLachlan	86399-001220US-911148	1042

20350 7590 06/19/2015

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HIRT, ERIN E

ART UNIT	PAPER NUMBER
1616	

NOTIFICATION DATE	DELIVERY MODE
06/19/2015	ELECTRONIC

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.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipefiling@kilpatricktownsend.com
jlhice@kilpatrick.foundationip.com

Office Action Summary	Application No. 14/304,578	Applicant(s) MACLACHLAN ET AL.	
	Examiner ERIN HIRT	Art Unit 1616	AIA (First Inventor to File) Status No

-- 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 MONTHS 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 *05/12/2015*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ 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*

5)☒ Claim(s) *1-12* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-12* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ 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).

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).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

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) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

DETAILED ACTION

Status of Action

Currently claims 1-12 are pending in this application.

Status of Claims

Accordingly, claims 1-12 are presented for examination on the merits for patentability. Rejection(s) not reiterated from the previous Office Action are hereby withdrawn. The following rejections are either reiterated or newly applied. They constitute the complete set of rejections presently being applied to the instant application.

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 double patenting rejection is appropriate where the claims at issue 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 reference 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. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1, 9, 10 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 9-10, 17, and 19 of U.S. Patent No. 8058069 (‘069). Although the conflicting claims are not identical, they are not patentably distinct from each other because both '069 and the instant invention claim nucleic acid particles comprising phospholipids, PEG-lipids, cationic lipids and nucleic acids. ‘069 merely teaches that their nucleic acids are siRNA instead of the instantly

claimed mRNA. However, it would have been obvious to one of ordinary skill in the art at the time of the instant invention that the particles of '069 are merely obvious variants of the particles instantly claimed.

Claims 1-7, and 9-10 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4-6, 12, 21 of US Patent 8283333 for the same reasons discussed above.

Claims 1 and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4, 10 and 18 of U.S. Patent No. 7799565 for the same reasons discussed above.

Claims 1-7 and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-6, 8, 10-11 of U.S. Patent No. 8466122 for essentially the same reasons as discussed above.

Claims 1-3 and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 8, 14, and 20 of U.S. Patent No. 8492359 for essentially the same reasons as discussed above.

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.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 1-12 are rejected under 35 U.S.C. 103(a) as being unpatentable over Unger et al. (US5830430), Semple et al. (WO98/51278), Lasic (*Tibtech*, **1998**, *16,* 307-321), and Meyer et al. (*J. Biol. Chem.*, **1998**, *273*, 15621-15627).

Applicant claims:

A lipid vesicle comprising:
a messenger RNA (mRNA);
a cationic lipid;
an amphipathic lipid; and
a polyethyleneglycol (PEG)-lipid,
wherein the mRNA is fully encapsulated in the lipid vesicle.

Determination of the scope and content of the prior art

(MPEP 2141.01)

Unger teaches cationic liposomes/lipid vesicles which comprise combination of a cationic lipid, a phospholipid, specifically the instantly claimed dioleoylphosphatidyl choline, a PEG-lipid, cholesterol, and genetic material, specifically RNA, which obviously includes mRNA which is a type of RNA (see entire document; esp. Col. 20, ln. 15-40; Col. 25, ln. 47-58; Col. 20, ln. 55-Col. 21, ln. 40; Claims 103-106, 110-111, 118, 120-123, 135-141) (Claims 1 (in part), 2-8). Unger further teaches wherein the liposome encapsulates the genetic material (Col. 9, ln. 66-Col. 10, ln. 9; Col. 10, ln. 15-38; Col. 20, ln. 15-40; Col. 20, ln. 55-Col. 21, ln. 40; Col. 25, ln. 47-58;) (Claim 1). Unger then teaches that the size of the liposomes can be adjusted by a variety of techniques, but that preferably the size of the liposomes are less than 100 nm in diameter, which reads upon the less than 150 nm that is instantly claimed (Col. 22, ln. 28-42) (Claim 10). Unger does not specifically state that the lipid vesicle is a lipid-nucleic acid particle but as it is a liposome which encapsulates genetic material, i.e. RNA, it is obviously a lipid-nucleic acid particle (Claim 9). Unger then expressly teaches that the amount of stabilizing material, i.e. amphipathic compounds/lipids which are combined with the cationic lipid may vary depending on a variety of factors including, the specific cationic lipids, the specific stabilizing material(s) selected (i.e. more than one type or combination), the particular use for which it is being employed, the mode of delivery, and the like (Col. 21, ln. 45-51). Further, Unger states, “The amount of stabilizing material to be combined with the present cationic lipid compounds in a particular

situation and the ratio of stabilizing material to cationic lipid, will vary and is **readily determinable by one skilled in the art based on the present disclosure"** (emphasis added) (i.e. one of ordinary skill in the art) (Col. 21, ln. 51-56).

Ascertainment of the difference between prior art and the claims

(MPEP 2141.02)

Unger merely does not teach an example wherein the lipid vesicle comprises all of these things and wherein the RNA is mRNA. However, mRNA is a type of RNA i.e. RNA is the genus and mRNA is the species. Therefore, by teaching that RNA can be carried by the lipid vesicles, Unger does render the instantly claimed mRNA obvious because it was already known in the art to use lipid vesicles to encapsulate the genus. However, this deficiency in Unger is also addressed by Semple.

Semple teaches lipid vesicles which comprise mRNA, cationic lipid(s) (DODAP, DODMA), phospholipid(s), cholesterol, and PEG-lipids wherein the nucleic acids are enclosed by the lipid vesicle (i.e. protected from degradation) (see entire document; Abstract; pg. 1, ln. 10-22; pg. 4, ln. 15-pg. 5, ln. 2; pg. 9, ln. 20-pg. 11, ln. 10; pg.11, ln. 27-pg. 13, ln. 14; pg. 13, ln. 15-pg. 16, ln. 24; pg. 17, ln. 43-46; pg. 19 ln. 1-20; pg. 19, ln. 28-pg. 20, ln. 7; pg. 20, ln. 8-pg. 21, ln. 6; pg. 24, ln. 22-30; pg. 31, ln. 6-12; Examples; pg. 19, ln. 28-pg. 21, ln. 6; pg. 13, ln. 11-14; Claims 11, 37). Semple further teaches wherein the particles are less than 100 nm and are only positively charged at below physiological pH (Claim 11).

Also, further motivation to include the specific lipids instantly claimed in the liposomes of Unger is provided by Meyer and Lasic.

Meyer teaches cationic liposomes coated with PEG and PEG-PE (a PEG-lipid) for increased stabilization of the liposomes which are used to carry oligonucleotides (i.e. nucleic acids, similar to RNA) (Abstract; pg. 15621, right col. Last paragraph).

Lasic teaches that liposomes/phospholipid vesicles are useful for drug delivery, specifically the delivery of DNA, RNA, oligonucleotides, etc. because the liposome allow for the protection of these sensitive drug molecules (Pg. 314, right col., 2nd paragraph). Lasic also teaches wherein it is known to have these liposomes comprise PEG-lipids for steric stabilization (pg. 314, right col. 1st para.). Lasic also teaches that liposomes are preferably between 80-200 nm because this size range is a compromise between loading efficiency of the liposomes, the stability of the liposomes, and the ability of the liposomes to extravasate (pg. 309, right col. 1st full paragraph). Lasic further states that, "If the liposomes are larger than ~150 nm, sterile filtering is probably not possible and so the whole process must be performed aseptically, which is not very practical from the engineering or economical viewpoints." (pg. 313, left col., 2nd full paragraph). Lasic further teaches wherein the nucleic acid, is/can be encapsulated (i.e. fully encapsulated) in the lipid vesicle and wherein cholesterol is used to stabilize the lipid vesicle comprising nucleic acids (i.e. DNA or RNA) (pg. 318, left column, last paragraph-right column end of paragraph).

Finding of prima facie obviousness

Rationale and Motivation (MPEP 2142-2143)

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to make liposomes having a cationic lipid, a PEG-lipid, cholesterol, and a phospholipid, specifically dioleylphosphatidyl choline, which encapsulate mRNA because Unger teaches liposomes comprising cationic lipids, PEG-lipids, cholesterol, and phospholipids, specifically dioleylphosphatidyl choline are useful for delivering genetic material, specifically RNA. Furthermore, it was known that PEG-lipids stabilize the liposomes and cholesterol and it would have been obvious to an ordinary skilled artisan to have the particles be about 100 nm in diameter and only carry a positive charge when at below physiological pH because Semple teaches lipid vesicles comprising mRNA, cationic lipids specifically DODAP which is charged at below physiological pH, PEG-lipid, cholesterol, and phospholipids to deliver the mRNA to cells in patients in need thereof.

In light of the forgoing discussion, the Examiner concludes that the subject matter defined by the above claims would have been obvious to one of ordinary skill in the art within the meaning of 35 USC 103(a).

From the teachings of the references, it is apparent that one of ordinary skill in the art would have had a reasonable expectation of success in producing the claimed invention. Therefore, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

Claims 1-12 are rejected under 35 U.S.C. 103(a) as being unpatentable over Semple et al (WO98/51278, from 3rd party IDS submission).

Applicant claims:

A lipid vesicle comprising:
a messenger RNA (mRNA);
a cationic lipid;
an amphipathic lipid; and
a polyethyleneglycol (PEG)-lipid,
wherein the mRNA is fully encapsulated in the lipid vesicle.

Determination of the scope and content of the prior art

(MPEP 2141.01)

Semple teaches lipid vesicles, specifically lipid-nucleic acid particles and liposomes, which comprise mRNA, cationic lipid(s), specifically DODAP or DODMA which are only charged at below physiological pH, phospholipid(s) such as distearoylphophatidyl choline, cholesterol (i.e. a sterol), and PEG-lipids wherein the nucleic acids are enclosed by the lipid vesicle (i.e. protected from degradation) (see entire document; pg. 1, ln. 10-22; pg. 4, ln. 15-pg. 5, ln. 2; pg. 9, ln. 20-pg. 11, ln. 10; pg.11, ln. 27-pg. 13, ln. 14; pg. 13, ln. 15-pg. 16, ln. 24; pg. 17, ln. 43-46; pg. 19 ln. 1-20; pg. 19, ln. 28-pg. 20, ln. 7; pg. 20, ln. 8-pg. 21, ln. 6; pg. 24, ln. 22-30; pg. 31, ln. 6-12; Examples; Claims 37) (Claims 1-9, 11). Semple also teaches an exemplary embodiment wherein the mixture of lipids forming the lipid vesicle include amino lipids, i.e. phopsholipids, distearoylphosphatidyl choline, palmitoyloleoylphsphatidyl choline, etc., cholesterol and PEG-lipids which is combined with a charged therapeutic agent, preferably nucleic acids (pg. 19, ln. 28-pg. 21, ln. 6). Semple finally teaches wherein the phospholipids are preferably, distearoylphosphatidyl choline, palmitoyloleoylphsphatidyl

choline, etc., and wherein particles of less than 150 nm, specifically about 100 nm or less in size can be made (pg. 13, ln. 11-14; Claim 11) (Claims 3, 7, 10, 12).

Ascertainment of the difference between prior art and the claims

(MPEP 2141.02)

Semple merely does not teach a specific lipid vesicle which comprises all of the instantly claimed features.

Finding of prima facie obviousness

Rationale and Motivation (MPEP 2142-2143)

It would have been obvious to one of ordinary skill in the art to make a lipid vesicle of the instant claims by looking to Semple because Semple teaches lipid vesicles of the same size and comprising the same components as instantly claimed for delivering and protecting mRNA from degradation when delivered to patients in need thereof. One of ordinary skill in the art would want to develop the lipid vesicles of Semple with the same features as instantly claimed because adding cholesterol and PEG-lipids to lipid nucleic acid particles comprising mRNA, cationic lipids and phospholipids, were known to improve the pharmacokinetics of these particles and/or the delivery of the nucleic acid/mRNA. Thereby, allowing one of ordinary skill in the art to make a more effective lipid vesicle for delivering the mRNA to cells to patients.

Response to Arguments/Remarks

Applicant's IDS filed, 5/12/15 prompted the addition of another double patenting rejection. Applicant's arguments with respect to the 103 rejections have been fully considered but were not persuasive.

Applicant's arguments appear to merely restate the rejections supplied in the affidavit submitted by Dr. James Heynes which was filed 10/22/14 and dated 10/20/14. Rather than address these arguments more than once they will be addressed in regards to the affidavit.

Declaration under 1.132

Dr. James Heynes provided a declaration under 1.132, filed May 12, 2015 and dated 4 May 2015. The Declaration meets the formal requirements. In the most relevant part, the Declaration demonstrates that Semple does work to encapsulate mRNA giving liposomes which are ~130 nm. A Declaration is due full consideration and weight for all that it discloses. Declarations are reviewed for the following considerations: 1) whether the Declaration presents a nexus such as a side-by-side or single-variable comparison (In re Huang, 40 USPQ2d 1685, 1689 (Fed. Cir. 1996)), 2) whether the Declaration presents a comparison to the closest art, 3) whether the Declaration is commensurate in scope with the scope of the claims (In re Kulling, 14 USPQ2d 1056, 1058 (Fed. Cir. 1990)), 4) whether the Declaration shows a difference in kind rather than merely a difference in degree (In re Waymouth, 182 USPQ 290, 293 (C.C.P.A. 1974)), and 5)whether the prima facie case is sufficiently strong that allegedly superior results are insufficient to overcome the case for obviousness (Pfizer Inc. v. Apotex, Inc., 82 USPQ2d 1321, 1339 (Fed. Cir. 2007)). The relevant criterion here is No. 2, whether the

Declaration presents a comparison to the closest art and No. 3, whether the Declaration is commensurate in scope with the scope of the claims. The examiner has carefully reviewed the Declaration, including the data presented in the Declaration. The data show that the prior art Semple encapsulates mRNA but not at a high efficiency and the particle sizes from the experiments are ~130 nm, which is less than the 150 nm instantly claimed but not less than the 100 nm instantly claimed. However, the rejection of claim 1 is not solely over Semple but is instead over the combination of Unger and Semple as applicants appear to be asserting. Therefore, the examples and data presented by applicant's are not the closest prior art to instant claims because that would be Unger in view of Semple as the instant rejection above explains.

Regarding, the second 103 rejection which is solely over Semple, the data has been explained above. However, the data was not commensurate with the claims. The claims claim that the liposomes are 100 nm. Applicant's experiments showed that the liposomes of Semple were ~130 nm. However, Semple further teaches at pg. 26, ln. 28-pg. 27, ln. 11, "Optionally, the lipid-encapsulated therapeutic agent complexes which are produced by combining the lipid mixture and the buffered aqueous solution of therapeutic agents can be sized to achieve a desired size range and relatively narrow distribution of lipid particles sizes…..Several techniques are available for sizing liposomes to a desired size." Thus, the liposomes that are formed in Semple can be sized to the desired size using further techniques which are disclosed in Semple. Thus, it would have been well within the skill of an ordinary artisan to further treat the liposomes of Semple to a technique which allows one of ordinary skill in the art to size

the liposomes to the desired size of 70-200nm, preferably 90-130 nm as are disclosed in Semple and still read upon applicants claimed diameter of 100 nm.

Applicants further argue in the declaration that the encapsulation of the mRNA using the method of Semple is not as effective and/or does not show high mRNA encapsulation which is desired in the instant invention and that the polydispersity index (PDI) is quite high and that the instant invention prefers a more homogeneous formula (i.e. a lower PDI). The examiner respectfully notes that the features upon which applicant relies (i.e., encapsulation efficiency and lower PDI) are not recited in the rejected claim(s). Although the claims are interpreted in light of the specification, limitations from the specification are not read into the claims. See *In re Van Geuns*, 988 F.2d 1181, 26 USPQ2d 1057 (Fed. Cir. 1993).

Applicants then argue that the liposomes of Semple are not as small as those instantly claimed because when they performed Semple's process of making the particles they got a diameter of ~130 nm. The examiner respectfully points out that ~130 nm is less than 150 nm and as such still reads upon claim 10 of the instant invention. Furthermore, Semple expressly states at pg. 26, ln. 28-pg. 27, ln. 11, "Optionally, the lipid-encapsulated therapeutic agent complexes which are produced by combining the lipid mixture and the buffered aqueous solution of therapeutic agents can be sized to achieve a desired size range and relatively narrow distribution of lipid particles sizes…..Several techniques are available for sizing liposomes to a desired size." Thus, the liposomes that are formed in Semple can be sized to the desired size using further techniques which are disclosed in Semple. Thus, it would have been well within the skill

of an ordinary artisan to further treat the liposomes of Semple to a technique which allows one of ordinary skill in the art to size the liposomes to the desired size of 70-200nm, preferably 90-130 nm as are disclosed in Semple and still read upon applicants claimed diameter of 100 nm.

With respect to the prior art Unger, applicants argue that it is merely a general teaching reference and does not provide guidance to each of the instantly claimed components for making the lipid vesicles. In response to applicant's arguments against the references individually, one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. See *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.,* 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986). In the instant case, Unger teaches forming lipid vesicles comprising the same lipids instantly claimed but does not teach an example which comprises each of these. Semple teaches lipid vesicles which comprise all of the same lipids as instantly claimed and wherein the nucleic acids which are encapsulated can be mRNA.

Applicants then argue that Unger does not teach encapsulating the nucleic acids/mRNA into the lipid vesicle, and actually teaches away from combining the lipids to form a vesicle to deliver the nucleic acids/mRNA. The examiner respectfully disagrees because at Col. 10, ln. 15, Unger states that, “In combination with” refers to the incorporation of the bioactive agent with a cationic lipid compound....The cationic lipid compound can be combined with the bioactive agent in any of a variety of different ways...the bioactive agent can be entrapped within the internal void of the vesicle, i.e. it

is encapsulated within the vesicle. Further, the examiner believes that Unger does not teach away from using the combination of lipids instantly claimed because at Col. 20, ln. 55-Col. 21, ln. 44, Unger teaches lipids that can be used in combination with the cationic lipid compounds and expressly teaches cholesterol, dioleylphosphatidyl choline, and PEG-lipids. Using these lipids to form the lipid vesicles is further supported by the addition of the prior art Semple which teaches forming liposomes which contain mRNA. Whether or not it is as efficient at encapsulating this mRNA as the instantly claimed liposomes is not as issue because applicants have never claimed the efficiency of the encapsulation in their claims. Thus, Semple does teach encapsulating the mRNA in liposomes. Thus, when Semple is combined with Unger the combination does teach the liposomes of the instant claims.

Finally, applicants argue that the teachings of Lasic and Meyer do not remedy the deficiencies they believe exist in Unger and Semple. The examiner respectfully disagrees because they state that Lasic teaches lipoplexes as DNA carrier systems for gene therapy. Lasic does teach phospholipid vesicles/liposomes which are useful for drug delivery, specifically the delivery of DNA, RNA, oligonucleotides, etc. because the liposome allow for the protection of these sensitive drug molecules which are stabilized by the incorporation of PEG and PEG-PE (a PEG-lipid). These references are merely used to provide further motivation for selecting these specific lipids which are already disclosed in Semple for use in encapsulating mRNA in liposomes.

Thus, the examiner believes that the combination of the prior art does render the composition/lipid vesicles of the instant claims prima facie obvious.

Conclusion

Claims 1-12 are rejected.

Applicant's submission of an information disclosure statement under 37 CFR 1.97(c) with the fee set forth in 37 CFR 1.17(p) on 05/12/15 prompted the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 609.04(b). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ERIN HIRT whose telephone number is (571)270-1077. The examiner can normally be reached on Monday through Friday 9:00am to 6:30pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sue X. Liu can be reached on 571-272-5539. 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.

Erin Hirt
Examiner, Art Unit 1616

/Mina Haghighatian/
Primary Examiner, Art Unit 1616

JOINT APPENDIX 21

Attorney Docket No.: 86399-001220US-911148

Doc Code: TRACK1.REQ
Document Description: TrackOne Request

PTO/AIA/424 (04-14)

CERTIFICATION AND REQUEST FOR PRIORITIZED EXAMINATION UNDER 37 CFR 1.102(e) (Page 1 of 1)

First Named Inventor:	Ian MacLachlan	Nonprovisional Application Number (if known):	14/304,578
Title of Invention:	LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY		

APPLICANT HEREBY CERTIFIES THE FOLLOWING AND REQUESTS PRIORITIZED EXAMINATION FOR THE ABOVE-IDENTIFIED APPLICATION.

1. The processing fee set forth in 37 CFR 1.17(i)(1) and the prioritized examination fee set forth in 37 CFR 1.17(c) have been filed with the request. The publication fee requirement is met because that fee, set forth in 37 CFR 1.18(d), is currently $0. The basic filing fee, search fee, and examination fee are filed with the request or have been already been paid. I understand that any required excess claims fees or application size fee must be paid for the application.

2. I understand that the application may not contain, or be amended to contain, more than four independent claims, more than thirty total claims, or any multiple dependent claims, and that any request for an extension of time will cause an outstanding Track I request to be dismissed.

3. The applicable box is checked below:

I. ☐ **Original Application (Track One) - Prioritized Examination under § 1.102(e)(1)**

i. (a) The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a). This certification and request is being filed with the utility application via EFS-Web.

---OR---

(b) The application is an original nonprovisional plant application filed under 35 U.S.C. 111(a). This certification and request is being filed with the plant application in paper.

ii. An executed inventor's oath or declaration under 37 CFR 1.63 or 37 CFR 1.64 for each inventor, **or** the application data sheet meeting the conditions specified in 37 CFR 1.53(f)(3)(i) is filed with the application.

II. ☑ **Request for Continued Examination - Prioritized Examination under § 1.102(e)(2)**

i. A request for continued examination has been filed with, or prior to, this form.
ii. If the application is a utility application, this certification and request is being filed via EFS-Web.
iii. The application is an original nonprovisional utility application filed under 35 U.S.C. 111(a), or is a national stage entry under 35 U.S.C. 371.
iv. This certification and request is being filed prior to the mailing of a first Office action responsive to the request for continued examination.
v. No prior request for continued examination has been granted prioritized examination status under 37 CFR 1.102(e)(2).

Signature /Joe C. Hao/	Date August 18, 2015
Name (Print/Typed) Joe C. Hao	Practitioner Registration Number 55,246

*Note: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.**

☐ *Total of _____ forms are submitted.

I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on August 18, 2015.

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Jose Luna/
Jose Luna

PATENT
Attorney Docket No.: 86399-001220US-911148

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 1042
Ian MacLachlan et al.	Examiner: Hirt, Erin E.
Application No.: 14/304,578	Art Unit: 1616
Filed: June 13, 2014	AMENDMENT
For: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY	
Customer No.: 20350	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

In response to the Final Office Action mailed June 19, 2015, please enter the following amendments and remarks. A Request for Continued Examination (RCE) accompanies the present response.

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. (Currently amended) A lipid vesicle formulation comprising:

(a) a plurality of lipid vesicles, wherein each lipid vesicle comprises:

~~**a messenger RNA (mRNA);**~~

a cationic lipid;

an amphipathic lipid; and

a polyethyleneglycol (PEG)-lipid; and [[,]]

(b) messenger RNA (mRNA), wherein at least 50% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

~~**wherein the mRNA is fully encapsulated in the lipid vesicle.**~~

2. (Currently amended) The lipid vesicle formulation of claim 1, wherein the amphipathic lipid is a phospholipid.

3. (Currently amended) The lipid vesicle formulation of claim 2, wherein the phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine.

4. (Currently amended) The lipid vesicle formulation of claim 1, wherein each **[[the]]** lipid vesicle further comprises a sterol.

5. (Currently amended) The lipid vesicle formulation of claim 4, wherein the sterol is cholesterol.

6. (Currently amended) The lipid vesicle formulation of claim 4, wherein the sterol is cholesterol and the amphipathic lipid is a phospholipid.

7. (Currently amended l) The lipid vesicle formulation of claim 6, wherein the phospholipid is selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, and dilinoleoylphosphatidylcholine.

8. (Currently amended) The lipid vesicle formulation of claim 1, wherein each **[[the]]** lipid vesicle is a liposome.

9. (Currently amended) The lipid vesicle formulation of claim 1, wherein each **[[the]]** lipid vesicle is a lipid-nucleic acid particle.

10. (Currently amended) The lipid vesicle formulation of claim 1, wherein each **[[the]]** lipid vesicle is about 150 nm or less in diameter.

11. (Currently amended) The lipid vesicle formulation of claim 1, wherein the cationic lipid only carries a positive charge at below physiological pH.

12. (Currently amended) The lipid vesicle formulation of claim 1, wherein each **[[the]]** lipid vesicle is about 100 nm or less in diameter.

13. (New) The lipid vesicle formulation of claim 1, wherein at least 70% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

14. (New) The lipid vesicle formulation of claim 1, wherein at least 80% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

15. (New) The lipid vesicle formulation of claim 1, wherein about 90% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

REMARKS

I. STATUS OF THE CLAIMS

Upon entry of this amendment, claims 1-15 are pending in this application and are presented for examination. Claims 1-12 have been amended. Support for the amendments to claim 1 is found, for example, in original claim 33 and paragraphs [45], [47] of the specification as filed. Claims 2-12 have been amended in view of the amendments made to claim 1.

Claims 13-15 are newly added. Support for new claims 13-15 is found, for example, in original claim 34 and paragraph [59] of the specification as filed.

As such, no new matter has been introduced. Based on the following remarks, Applicants respectfully request reconsideration and allowance of the pending claims.

II. EXAMINER INTERVIEW

Applicants' representative thanks Examiner Hirt and Examiner Haghighatian for the telephonic interview conducted on July 29, 2015, in which the Declaration of Dr. James Heyes under 37 C.F.R. § 1.132 submitted on May 12, 2015 (hereinafter, "Heyes Declaration") and proposed amendments to claim 1 were discussed. Applicants' representative notes that Examiner Hirt and Examiner Haghighatian responded favorably to the proposed claim amendments in view of the evidence presented in the Heyes Declaration demonstrating that the method described in Semple *et al.* for preparing liposomes is not able to produce the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed.

III. DOUBLE PATENTING REJECTIONS

Claims 1, 9, and 10 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 9, 10, 17, and 19 of U.S. Patent No. 8,058,069. Claims 1-7, 9, and 10 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 4-6, 12, and 21 of U.S. Patent No. 8,283,333. Claims 1 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 4, 10, and 18 of U.S. Patent No. 7,799,565. Claims 1-7 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1-6, 8, 10, and 11 of U.S. Patent No. 8,466,122.

Claims 1-3 and 9 were rejected on the ground of nonstatutory obviousness-type double patenting over claims 1, 8, 14, and 20 of U.S. Patent No. 8,492,359.

Applicants respectfully request that the Examiner hold these rejections in abeyance until there is an indication of allowable subject matter. *See*, M.P.E.P. § 714.02.

IV. REJECTIONS UNDER 35 U.S.C. § 103(a)

Claims 1-12 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Unger *et al.* (US Patent No. 5,830,430), Semple *et al.* (WO 98/51278), Lasic (*Trends in Biotech.*, 16:307-21 (1998)), and Meyer *et al.* (*J. Biol. Chem.*, 273:15621-7 (1998)). Claims 1-12 were also rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Semple *et al.* Applicants respectfully traverse these rejections.

In the Office Action, the Examiner states that Declarations are reviewed for considerations including whether the Declaration presents a comparison to the closest art. *See,* Office Action at page 12. The Examiner acknowledges that the data in the Heyes Declaration show that the method of Semple *et al.* does not encapsulate mRNA at a high efficiency. *See,* Office Action at page 13. However, the Examiner points out that the rejection of claim 1 is not solely over Semple *et al.* but is instead over the combination of Unger *et al.* and Semple *et al. See, id.* As a result, the Examiner alleges that the examples and data presented in the Heyes Declaration are not directed to the closest prior art to the instant claims because that would be Unger *et al.* in view of Semple *et al. See, id.*

As explained during the telephonic interview, M.P.E.P. § 716.02(e), Section III, states:

> Although evidence of unexpected results must compare the claimed invention with the closest prior art, ***applicant is not required to compare the claimed invention with subject matter that does not exist in the prior art***. *In re Geiger*, 815 F.2d 686,689, 2 USPQ2d 1276,1279 (Fed. Cir. 1987) (Newman, J., concurring) (Evidence rebutted *prima facie* case by comparing claimed invention with the most relevant prior art. Note that the majority held the Office failed to establish a *prima facie* case of obviousness.); *In re Chapman*, 357 F.2d 418, 148 USPQ 711 (CCPA 1966) (Requiring applicant to compare claimed invention with polymer suggested by the combination of references relied

> upon in the rejection of the claimed invention under 35 U.S.C. § 103 "***would be requiring comparison of the results of the invention with the results of the invention***." 357 F.2d at 422, 148 USPQ at 714). (Emphasis added.)

As M.P.E.P. § 716.02(e), Section III, makes clear, Applicants are **not** required to compare the claimed invention with subject matter that does **not** exist in the prior art. Applicants respectfully point out that the composition that the Examiner would like Applicants now to compare the present invention to **does not exist** in the prior art. Instead, it is a composition that the Examiner has **suggested** based on a combination of teachings that the Examiner has pieced together in an effort to render the present invention obvious. In fact, the Examiner makes this clear by stating in the Office Action:

> [T]he rejection of claim 1 is not solely over Semple but is instead over the combination of Unger and Semple .... Therefore, the examples and data presented by applicants are not the closest prior art to [the] instant claims because that would be Unger in view of Semple .... *See,* Office Action at page 13.

Thus, in order to arrive at the composition that the Examiner would like Applicants now to compare the present invention against, one must combine the teachings of Unger *et al.* and Semple *et al.* However, M.P.E.P. § 716.02(e), Section III, makes it clear that Applicants are **not** required to compare the claimed invention with subject matter that **does not exist** in the prior art. In support of this position, M.P.E.P. § 716.02(e), Section III, cites *In re Chapman, supra*, which held that requiring applicant to compare the claimed invention to a composition suggested by the combination of references relied upon in the rejection under 35 U.S.C. § 103 "***would be requiring comparison of the results of the invention with the results of the invention***" — this would clearly be ridiculous.

As such, in accordance with M.P.E.P. § 716.02(e), Section III, the Heyes Declaration clearly shows that the method described in Semple *et al.* for preparing liposomes containing antisense oligonucleotide is **not suitable** for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed. Indeed, Dr. Heyes provides in his Declaration objective evidence that demonstrates that the method described in

Semple *et al.* produced formulations with a **very low degree of encapsulation**. *See,* Heyes Declaration at Table 1 and ¶11. As the Examiner will readily appreciate, the **very low encapsulation efficiency** associated with the method described in Semple *et al.* is **well below** the minimum mRNA encapsulation efficiency required in the presently claimed formulations. None of the other cited references remedies this notable deficiency in the method of Semple *et al*.

In the Office Action, the Examiner further states that Declarations are reviewed for considerations including whether the Declaration is commensurate in scope with the scope of the claims. *See,* Office Action at page 12. The Examiner acknowledges that the Heyes Declaration shows that the encapsulation of mRNA using the method of Semple *et al.* is not as effective and does not show high mRNA encapsulation efficiency. *See,* Office Action at page 14. However, the Examiner notes that this feature of encapsulation efficiency is not recited in the rejected claims. *See, id.*

In an earnest effort to advance prosecution, but without acquiescing on the merits of the present rejections, Applicants have amended claim 1 to recite a lipid vesicle formulation wherein at least 50% of the mRNA in the formulation is fully encapsulated in the lipid vesicles. As explained during the telephonic interview and clearly demonstrated in the Heyes Declaration, the method of Semple *et al.* for preparing liposomes containing antisense oligonucleotide is **not suitable** for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed. As explained above, Dr. Heyes provides in his Declaration objective evidence that shows that the method described in Semple *et al.* produced formulations with a **very low degree of encapsulation**. *See,* Heyes Declaration at Table 1 and ¶11. Importantly, the **very low encapsulation efficiency** associated with the method of Semple *et al.* is **well below** the minimum mRNA encapsulation efficiency required in the presently claimed formulations. None of the other cited references remedies this notable deficiency in the method of Semple *et al*.

In view of the foregoing, Applicants assert that the cited references, whether alone or in combination, do **not** teach or suggest each of the features recited in the instant claims and thus fail to support a legal conclusion of obviousness. Indeed, **none** of these references discloses or suggests a lipid vesicle formulation of the present invention comprising a plurality of lipid

vesicles and mRNA, wherein at least 50% of the mRNA in the formulation is fully encapsulated in the lipid vesicles. To the contrary, Applicants have provided sufficient objective evidence in the Heyes Declaration to demonstrate that the method described in Semple *et al.* for preparing liposomes is simply **not suitable** for producing the population of lipid vesicles with an mRNA encapsulation efficiency as presently claimed. The teachings of the other cited references do **not** remedy this deficiency in the method of Semple *et al*. Accordingly, Applicants respectfully request that the Examiner withdraw the present rejections under 35 U.S.C. § 103(a).

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/

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I hereby certify that this correspondence is being filed via EFS-Web with the United States Patent and Trademark Office on August 18, 2015.

KILPATRICK TOWNSEND & STOCKTON LLP

By: /Jose Luna/
Jose Luna

PATENT
Attorney Docket No.: 86399-001220US-911148

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Confirmation No.: 1042
Ian MacLachlan et al.	Examiner: Hirt, Erin E.
Application No.: 14/304,578	Art Unit: 1616
Filed: June 13, 2014	
For: LIPID COMPOSITIONS FOR NUCLEIC ACID DELIVERY	SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT
Customer No.: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

The references cited on the attached form PTO/SB/08A&B are being called to the attention of the Examiner. Copies of the references [in compliance with the requirements of 37 CFR §1.98(a)(2)] are enclosed. It is respectfully requested that the cited references be expressly considered during the prosecution of this application, and the references be made of record therein and appear among the "references cited" on any patent to issue therefrom.

Some of the references cited in this Information Disclosure Statement were cited in an office action mailed in connection with related U.S. Application No. 13/684,066. Copies of the Office Actions in that application are available on PAIR and are believed to be readily accessible to the Examiner.

Commonly-owned patent applications are cited in this Information Disclosure Statement. Copies of any office actions in such patent applications are available on PAIR and are believed to be readily accessible to the Examiner. Thus, although Applicant may submit copies of some such office actions, Applicant does not represent that copies of all, or the most significant, office actions are being supplied. If the Examiner desires copies of all such office actions, the Examiner should contact the undersigned.

As provided for by 37 CFR § 1.97(g) and (h), no inference should be made that the information and references cited are prior art merely because they are in this statement and no representation is being made that a search has been conducted or that this statement encompasses all the possible relevant information.

Applicant believes that no fee is required for submission of this statement. However, if a fee is required, the Commissioner is authorized to deduct such fee from the undersigned's Deposit Account No. 20-1430. Please deduct any additional fees from, or credit any overpayment to, the above-noted Deposit Account.

Respectfully submitted,

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JOINT APPENDIX 22

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/304,578	06/13/2014	Ian MacLachlan	86399-001220US-911148	1042

20350 7590 10/09/2015

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EXAMINER	
HIRT, ERIN E	

ART UNIT	PAPER NUMBER
1616	

NOTIFICATION DATE	DELIVERY MODE
10/09/2015	ELECTRONIC

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.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

ipefiling@kilpatricktownsend.com
jlhice@kilpatrick.foundationip.com

Office Action Summary	Application No. 14/304,578	Applicant(s) MACLACHLAN ET AL.	
	Examiner ERIN HIRT	Art Unit 1616	AIA (First Inventor to File) Status No

-- 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* MONTHS 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 *09/02/15*.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ 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*

5)☒ Claim(s) *1-15* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1-15* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ 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).

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).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

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) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .

4) ☐ Other: _____.

The present application is being examined under the pre-AIA first to invent provisions.

Continued Examination Under 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 18 August 2015 has been entered.

Status of Action

Currently claims 1-15 are pending in this application; claims 13-15 are new claims.

Status of Claims

Accordingly, claims 1-15 are presented for examination on the merits for patentability. Rejection(s) not reiterated from the previous Office Action are hereby withdrawn. The following rejections are either reiterated or newly applied. They constitute the complete set of rejections presently being applied to the instant application.

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 double patenting rejection is appropriate where the claims at issue 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 reference 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. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO internet Web site contains terminal disclaimer forms which may be used. Please visit http://www.uspto.gov/forms/. The filing date of the application will determine what form should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1, 9, and 10 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 9-10, 17, and 19 of U.S. Patent No. 8058069 (‘069). Although the conflicting claims are not identical, they are not patentably distinct from each other because both '069 and the instant invention claim nucleic acid particles comprising phospholipids, PEG-lipids, cationic lipids and fully encapsulated nucleic acids. ‘069 merely teaches that their nucleic acids are siRNA instead of the instantly claimed mRNA. However, it would have been obvious to one of ordinary skill in the art at the time of the instant invention that the particles of '069 are merely obvious variants of the particles instantly claimed.

Claims 1-7 and 9-10 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4-6, 12, 21 of US Patent 8283333 for the same reasons discussed above.

Claims 1 and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 4, 10 and 18 of U.S. Patent No. 7799565 for the same reasons discussed above.

Claims 1-7 and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-6, 8, 10-11 of U.S. Patent No. 8466122 for essentially the same reasons as discussed above.

Claims 1-3 and 9 are also rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1, 8, 14, and 20 of U.S. Patent No. 8492359 for essentially the same reasons as discussed above.

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.

The factual inquiries set forth in *Graham* v. *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

Claims 1-15 are rejected under 35 U.S.C. 103(a) as being unpatentable over Saravolac et al. (US6734171) and Yoshioka et al. (US5593622).

Applicant's claim:

> A lipid vesicle comprising:
> a messenger RNA (mRNA);
> a cationic lipid;
> an amphipathic lipid; and
> a polyethyleneglycol (PEG)-lipid; and mRNA, wherein at least 50% of the mRNA in the formulation is fully encapsulated in the lipid vesicles.

Determination of the scope and content of the prior art

(MPEP 2141.01)

Regarding claims 1-3 and 6-7, Saravolac teaches methods of encapsulating mRNA/nucleic acids in lipid bilayers to form liposomes and lipid-nucleic acid particles (see entire document; Col. 2, ln. 23-44; Col. 12, ln. 8-28). **Further regarding claims 1-3 and 6-7,** Saravolac teaches lipid vesicles comprising a cationic lipid (i.e. DODAC), an amphipathic/fusogenic lipid, specifically a phospholipid such as DOPE (dioleylphosphatidylethanolamine) or lysophosphatidylcholine or lysophosphatidylethanolamine, preferably a PEG-lipid, and mRNA/plasmid, specifically wherein at least 50% of the mRNA is fully encapsulated in the lipid vesicles (see entire document; Col. 8, ln. 33-Col. 9, ln. 25; Col. 10, ln. 12-Col. 11, ln. 8; Col. 11, ln. 26-35; Col. 12, ln. 29-Col. 14, ln. 33; Col. 14, ln. 60-Col. 15, ln. 12).

Regarding claim 4, Saravolac teaches wherein the lipid vesicles can further comprise a sterol (see entire document; Col. 8, ln. 55-57).

Regarding claims 10 and 12, Saravolac teaches wherein the diameter of the lipid vesicles are about 100 nm or less in diameter which reads upon the instantly

claimed 150 nm or less and 100 nm or less (see entire document; e.g. Claim 15; Examples).

Regarding claims 8-9, Saravolac teaches wherein the lipid vesicle is either a liposome or a lipid-nucleic acid particle (see entire document; Col. 12, ln. 8-28).

Regarding claims 13-15, Saravolac teaches wherein the encapsulation efficiency of the mRNA in the vesicles is about 80% and can even approach 90% which reads upon the instantly claimed at least 70%, more specifically at least 80%, and about 90% (see entire document; Col 2, ln. 45-54; see Fig. 8).

Ascertainment of the difference between prior art and the claims

(MPEP 2141.02)

Regarding claim 5, Saravolac does not specifically teach wherein the sterol is cholesterol. However, this deficiency in Saravolac is addressed by Yoshioka.

Yoshioka teaches incorporating cholesterol into liposomes as membrane stabilizing agents (see entire document; Col. 5, ln. 20-23).

Regarding claims 3 and 7, Saravolac does not specifically teach an example wherein the fusogenic lipid is other than DOPE. However, Saravolac teaches that lysolipids, specifically the instantly claimed lysophosphatidylcholine or lysophosphatidylethanolamine are useful as lysolipids/fusogenic lipids for forming the instantly claimed lipid vesicles (see entire document; Col. 13, ln. 22-Col. 14, ln. 48).

Finding of prima facie obviousness

Rationale and Motivation (MPEP 2142-2143)

It would have been obvious to one of ordinary skill in the art at the time of the instant invention to make liposomes having a cationic lipid, a PEG-lipid, a sterol, and a phospholipid, specifically lysophosphatidylcholine or lysophosphatidylethanolamine as instantly claimed because Saravolac teaches that it was known in the art to make liposomes and lipid-nucleic acid particles from cationic lipids, PEG-lipids, a sterol, and fusogenic lipids such as the instantly claimed lysophosphatidylcholine or lysophosphatidylethanolamine which allow for increased encapsulation efficiencies of over 80%, specifically up to about 86%.

Regarding the selection of cholesterol as the sterol in the liposomes and lipid-nucleic acid particles, it would have been obvious to select cholesterol as the sterol for inclusion in the liposome/lipid-nucleic acid particles of Saralovac because Yoshioka teaches that the addition of cholesterol helps to stabilize the membrane of the liposomes.

Response to Arguments/Remarks

Applicant's amendments to the claims, filed 08/18/15, have rendered the previous 103 rejections moot. Therefore, these rejections have been withdrawn by the examiner. However, applicant's amendments have prompted the new grounds of rejection under 103 presented herein.

Conclusion

Claims 1-15 are rejected.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to ERIN HIRT whose telephone number is (571)270-1077. The examiner can normally be reached on Monday through Friday 9:00am to 6:30pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sue X. Liu can be reached on 571-272-5539. 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.

/ERIN HIRT/
Examiner, Art Unit 1616