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**Lee et al.**

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(54) **COMPOSITIONS AND METHODS FOR SILENCING APOLIPOPROTEIN B**

2011/0076335 A1 3/2011 Yaworski et al.  
2011/0262527 A1 10/2011 Heyes et al.  
2012/0058188 A1 3/2012 MacLachlan et al.

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**FOREIGN PATENT DOCUMENTS**

WO WO 01/05374 A1 1/2001  
WO WO 02/304236 A 5/2002  
WO WO 2004/091515 A2 10/2004  
WO WO 2005/007196 A2 1/2005  
WO WO 2009/086558 A1 \* 7/2009  
WO WO 2009/127060 A1 10/2009  
WO WO 2009/132131 A1 10/2009  
WO WO 2010/042877 A1 4/2010  
WO WO 2010/054401 A1 5/2010

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**OTHER PUBLICATIONS**

Arpicco, S., et al., "Synthesis, characterization and transfection activity of new saturated and unsaturated cationic lipids," *IL Farmaco*, 2004, vol. 59, pp. 869-878.  
Heyes, J., et al., "Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids," *Journal of Controlled Release*, 2005, vol. 107, pp. 276-287.  
Jaeger et al. "Preparation and characterization of glycerol-based cleavable surfactants and derived vesicles," *Journal of the American Chemical Society*, 1989, vol. 111, pp. 3001-3006.  
Kiefer et al., "Transfection efficiency and cytotoxicity of nonviral gene transfer reagents in human smooth muscle and endothelial cells," *Journal of Pharmaceutical Research*, vol. 21, pp. 1009-1017.  
Leifer, C., et al., "Heterogeneity in the Human Response to Immunostimulatory CpG Oligodeoxynucleotides," *Journal of Immunotherapy*, Jul./Aug. 2003, vol. 26, pp. 313-319.  
Lu et al., *In Vivo application of RNA interference: From functional genomics to therapeutics*, 2005, *Advances in Genetics*, vol. 54, pp. 117-142.  
Madry et al., "Efficient lipid-mediated gene transfer to articular chondrocytes," 2000, *Gene Therapy*, vol. 7, pp. 286-291.  
Mashek et al., "Short Communication: Net uptake of nonesterified long chain fatty acids by the perfused caudate lobe of the caprine liver," 2003, *Journal of Dairy Science*, vol. 86, pp. 1218-1220.  
Morrissey, D. V., et al., "Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs," *Nature Biotechnology*, 2005, vol. 23, No. 8, pp. 1002-1007.  
Prakash, T. P., et al., "Position effects of chemical modification on siRNA activity—MEDI 175," *General Oral Session, Division of Medicinal Chemistry, The 227th ACS National Meeting*, 2004, 1 page.  
Reynolds et al., "Rational siRNA design for RNA interference," *Nature Biotechnology*, 2004, vol. 22, pp. 326-330.

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(51) **Int. Cl.**

**C07H 21/02** (2006.01)  
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**C12N 15/00** (2006.01)

(52) **U.S. Cl.** ..... **536/24.5; 435/450; 435/455; 514/44; 977/800; 977/816**

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

(56) **References Cited**

**U.S. PATENT DOCUMENTS**

5,968,909 A 10/1999 Agrawal et al.  
6,417,326 B1 7/2002 Cullis et al.  
6,680,068 B2 1/2004 Jain et al.  
7,745,651 B2 6/2010 Heyes et al.  
7,799,565 B2 9/2010 MacLachlan et al.  
7,807,815 B2 10/2010 MacLachlan et al.  
7,838,658 B2 11/2010 MacLachlan et al.  
7,982,027 B2 7/2011 MacLachlan et al.  
8,058,069 B2 11/2011 Yaworski et al.  
8,101,741 B2 1/2012 MacLachlan et al.  
2003/0077829 A1 4/2003 MacLachlan  
2003/0125263 A1 7/2003 Gold et al.  
2004/0192626 A1 9/2004 McSwiggen et al.  
2005/0064595 A1 3/2005 MacLachlan et al.  
2005/0107325 A1 5/2005 Manoharan et al.  
2005/0282188 A1 12/2005 Haeberli et al.  
2006/0105976 A1 5/2006 Soutschek et al.  
2006/0134189 A1 6/2006 MacLachlan et al.  
2006/0142230 A1 6/2006 Quay et al.  
2006/0211642 A1 9/2006 McSwiggen et al.  
2006/0217330 A1 9/2006 Hartmann et al.  
2007/0042983 A1 2/2007 Haeberli et al.  
2007/0135372 A1 \* 6/2007 MacLachlan et al. .... 514/44  
2007/0218122 A1 9/2007 MacLachlan et al.  
2009/0012021 A1 1/2009 Sood et al.

(Continued)

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(57) **ABSTRACT**

The present invention provides compositions and methods for the delivery of interfering RNAs that silence APOB expression to liver cells. In particular, the nucleic acid-lipid particles provide efficient encapsulation of nucleic acids and efficient delivery of the encapsulated nucleic acid to cells in vivo. The compositions of the present invention are highly potent, thereby allowing effective knock-down of APOB at relatively low doses. In addition, the compositions and methods of the present invention are less toxic and provide a greater therapeutic index compared to compositions and methods previously known in the art.

**26 Claims, 17 Drawing Sheets**

**ARBUTUS - EXHIBIT 2010**

**Moderna Therapeutics, Inc. v. Arbutus Biopharma Corporation - IPR2019-00554**

OTHER PUBLICATIONS

Semple et al. "Rational design of cationic lipids for siRNA delivery," Nature Biotechnology, 2010, vol. 28, pp. 172-176.

Sioud, siRNA delivery in vivo, 2005, Methods in Molecular Biology, vol. 309, pp. 237-249.

Soutschek, J., et al., "Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs," Nature, 2004, vol. 432, pp. 173-178.

Vigh et al., Does the membrane's physical state control the expression of heat shock and other genes? 1998, Trends in Biochemical Sciences, vol. 23, pp. 369-374.

Wang et al. "Preparation, properties and applications of vesicle-forming cleavable surfactants with a 1,3-dioxane ring," Journal of Colloidal and Interface Science, 1995, vol. 173, pp. 49-59.

\* cited by examiner

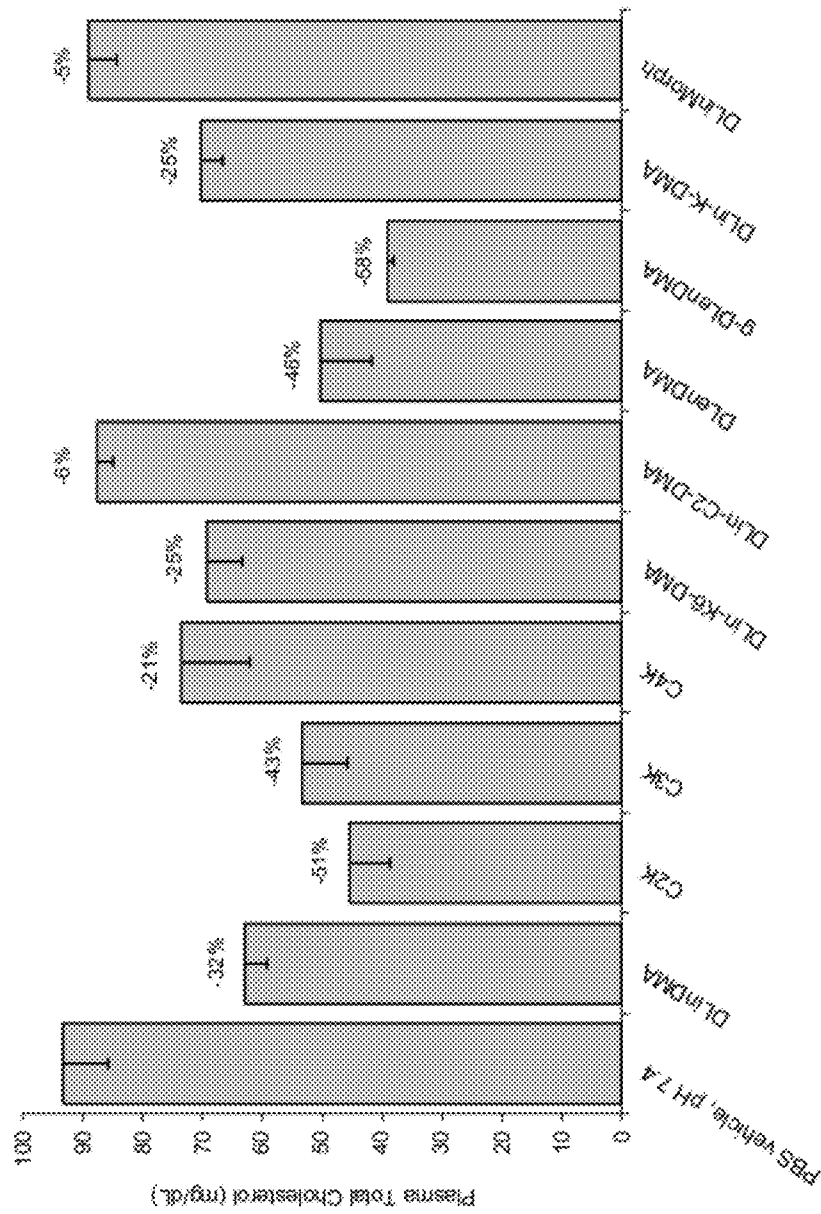
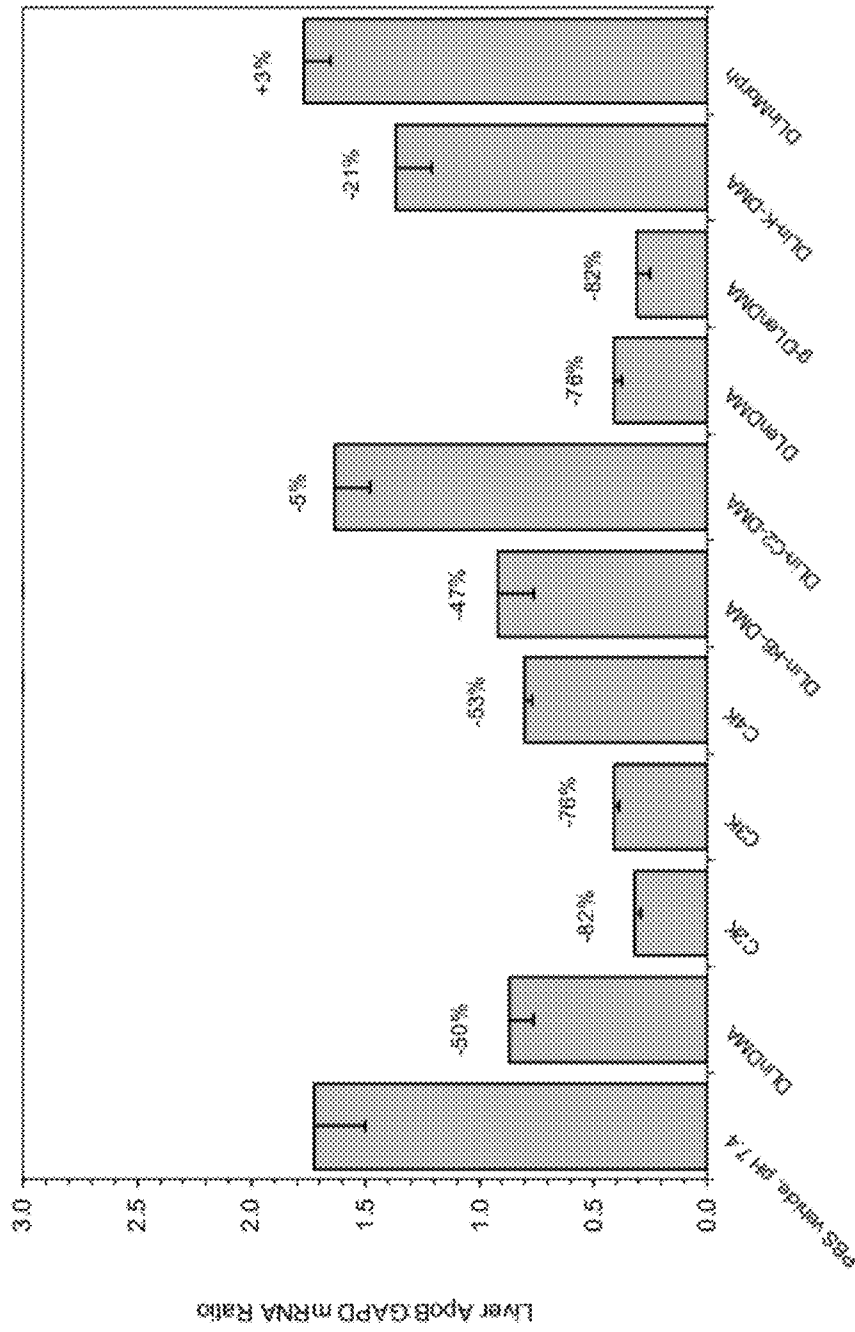


FIG. 1





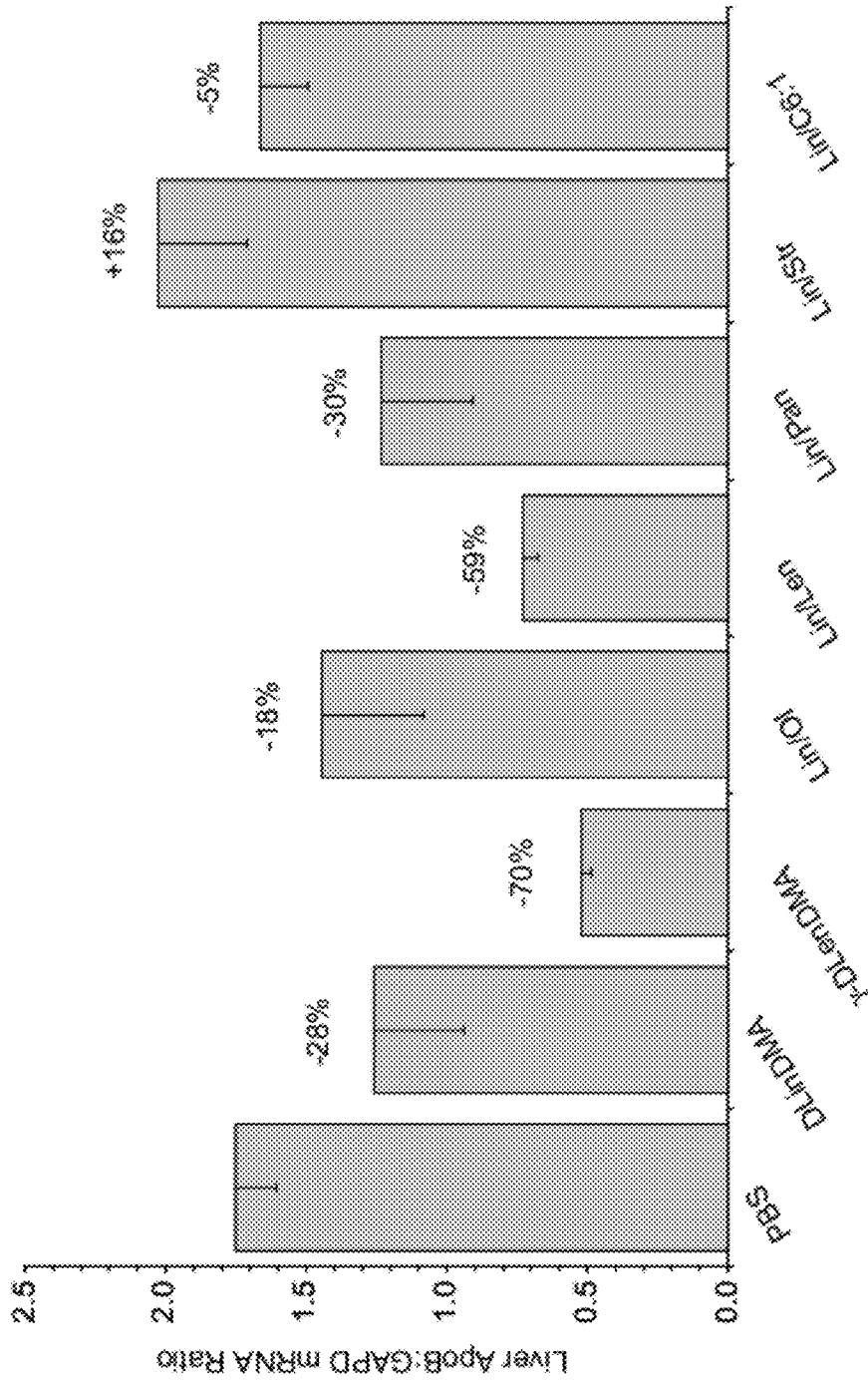


FIG. 3

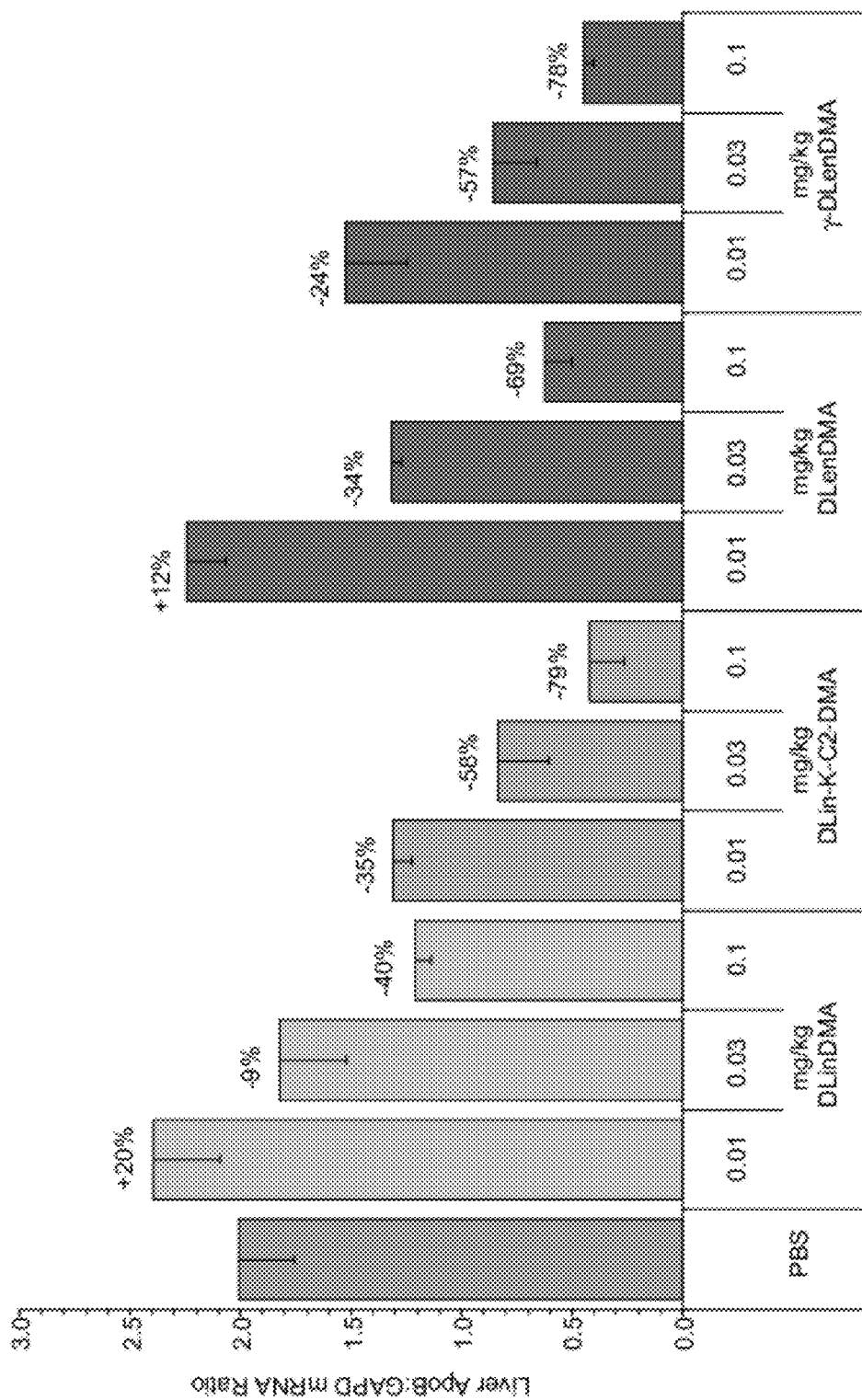


FIG. 4

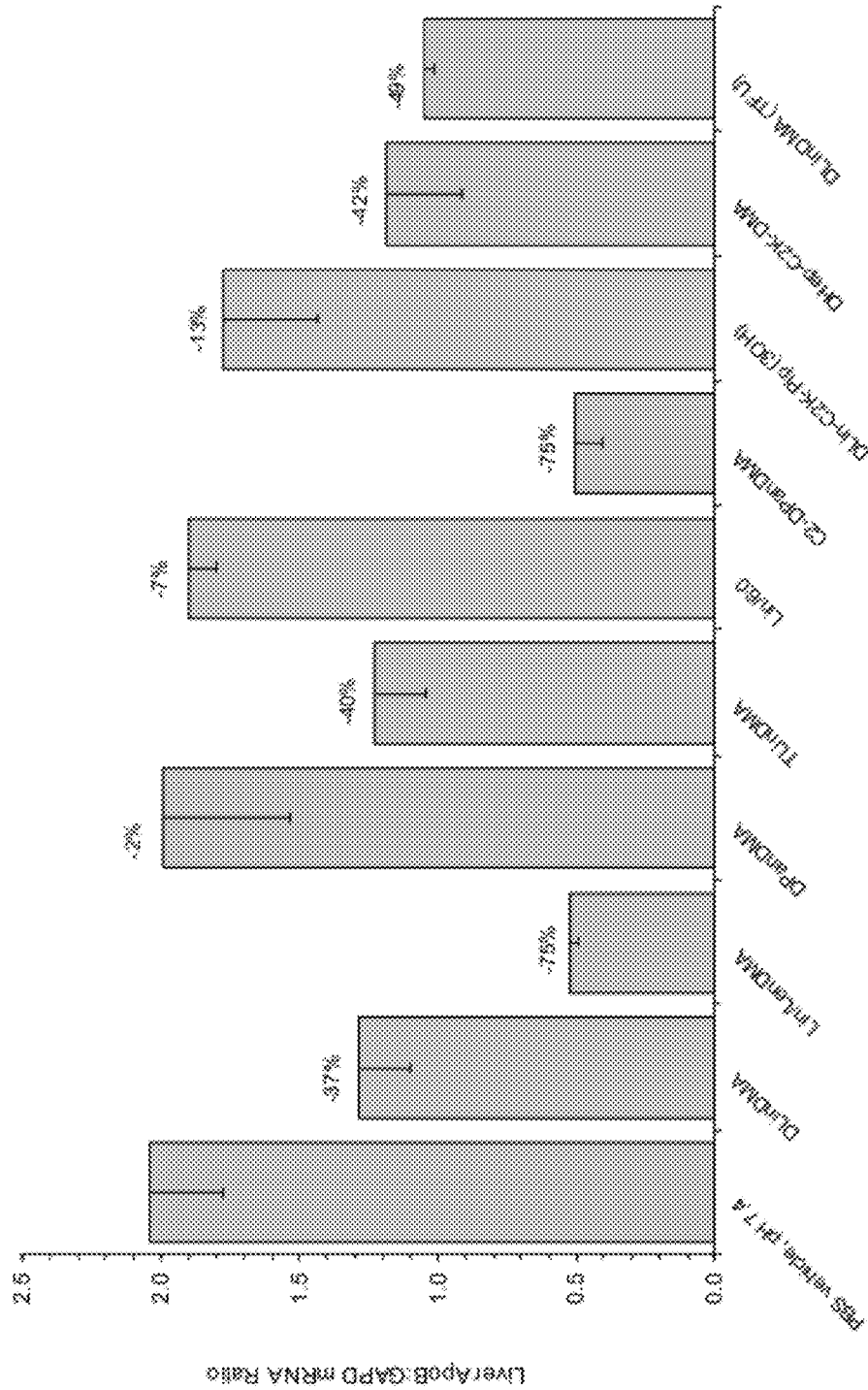


FIG. 5

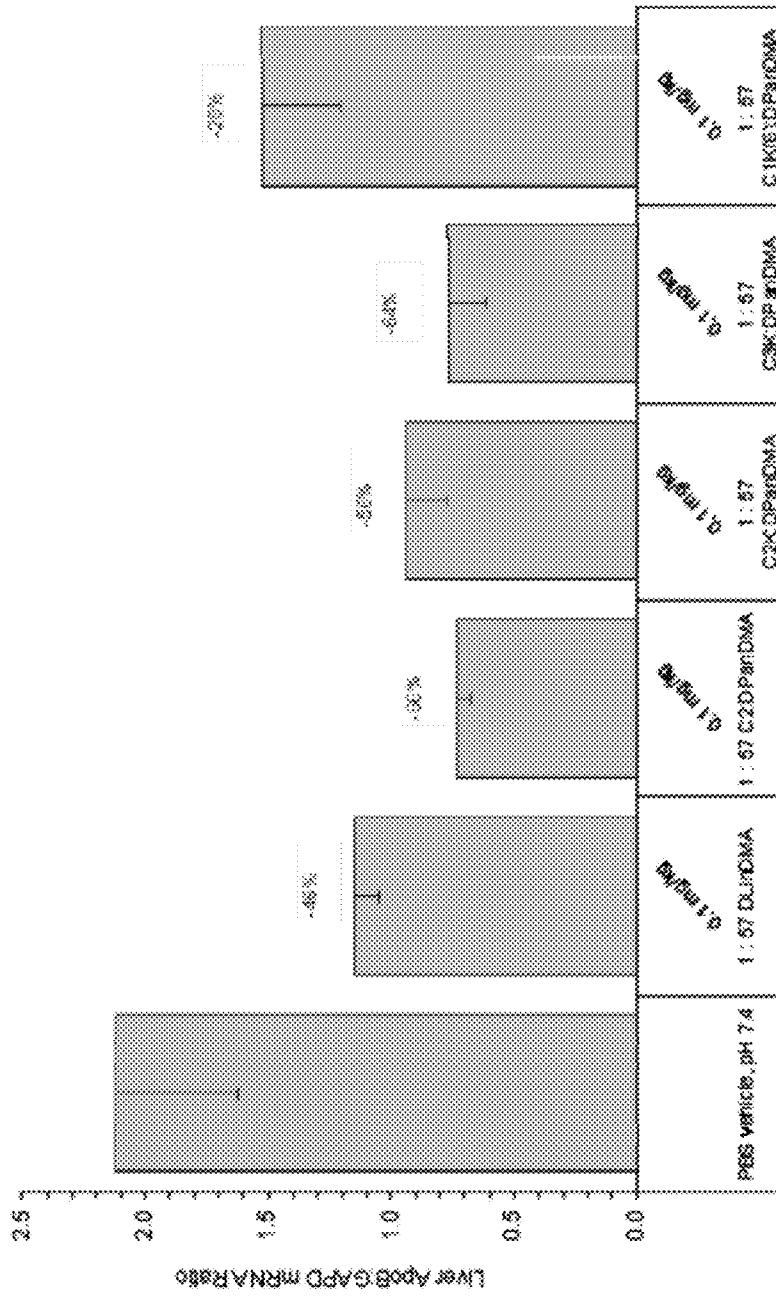


FIG. 6

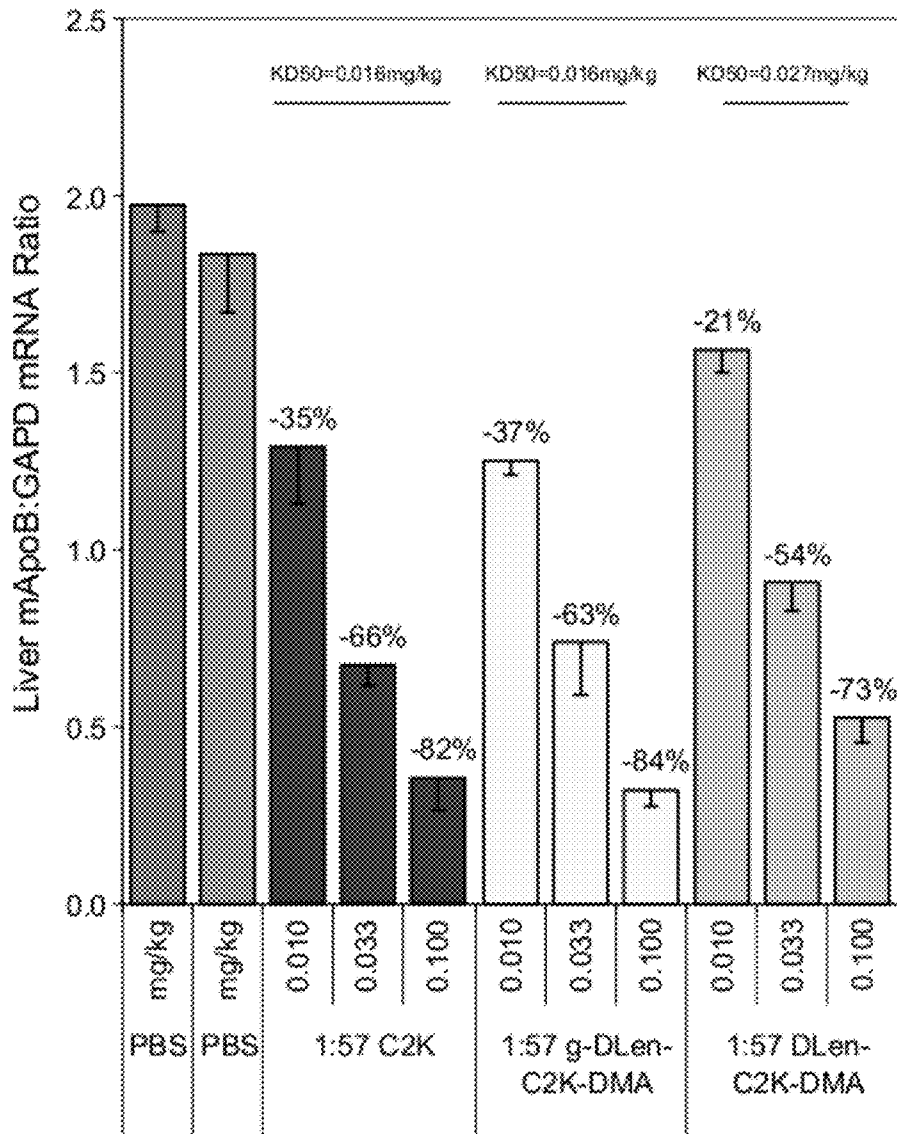


FIG. 7

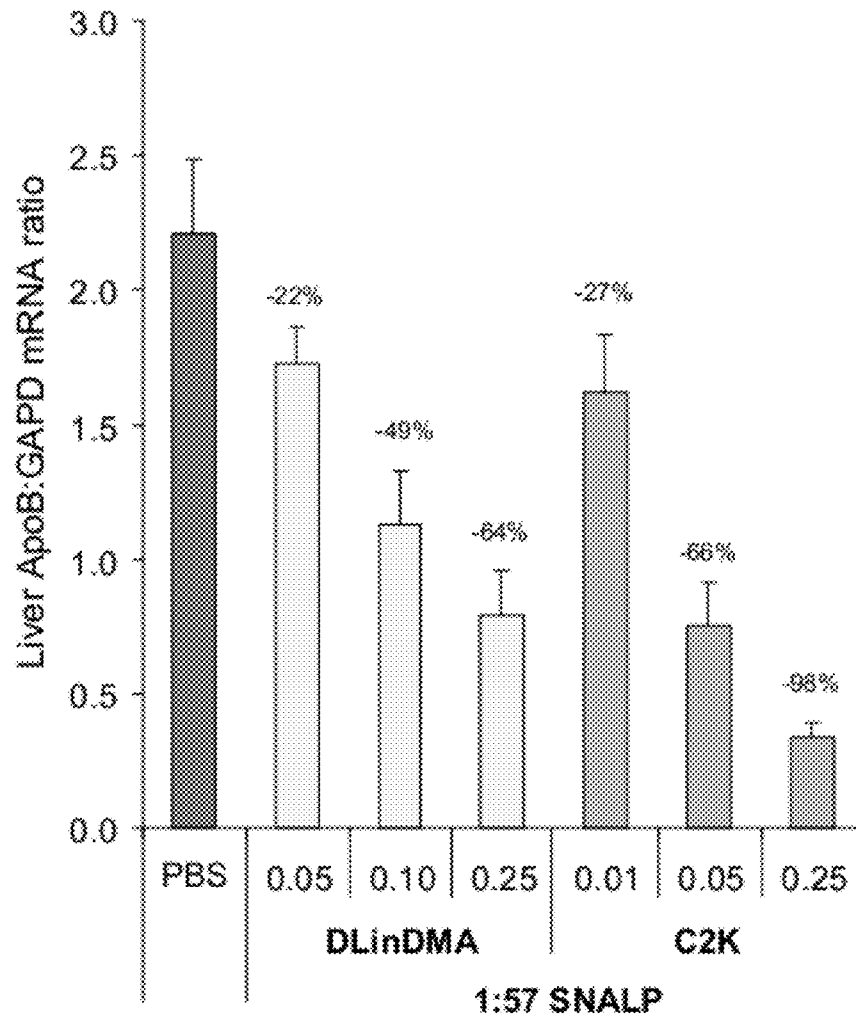


FIG. 8

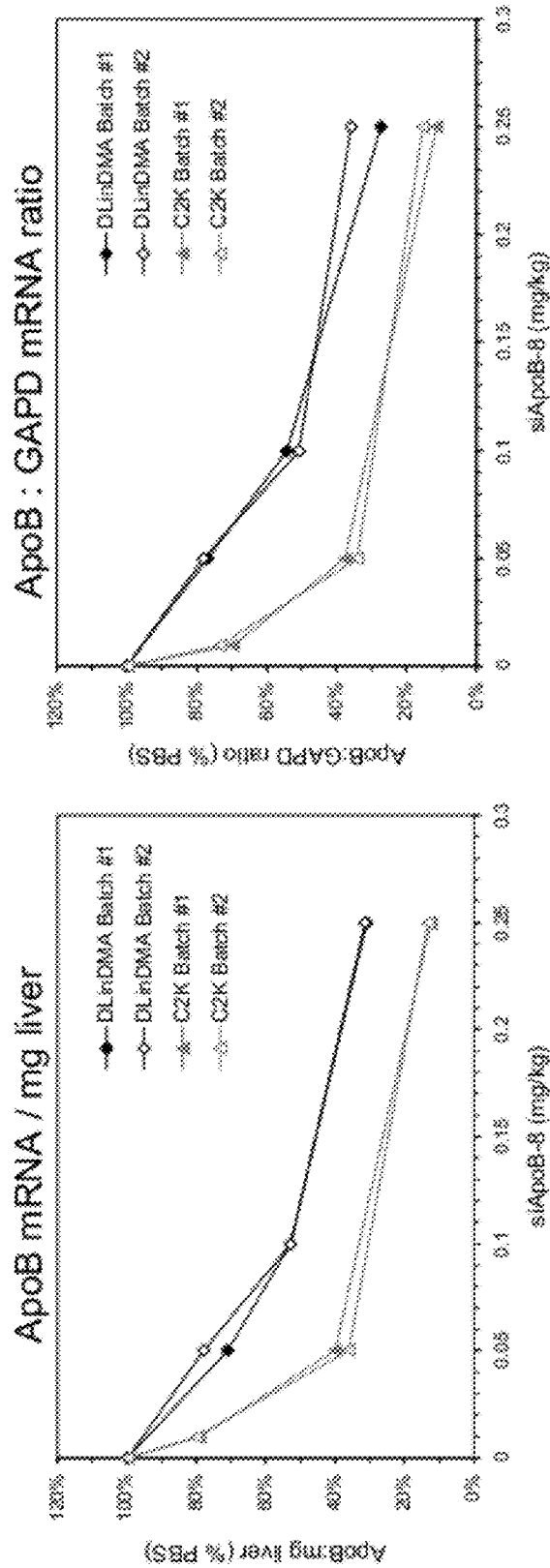


FIG. 9

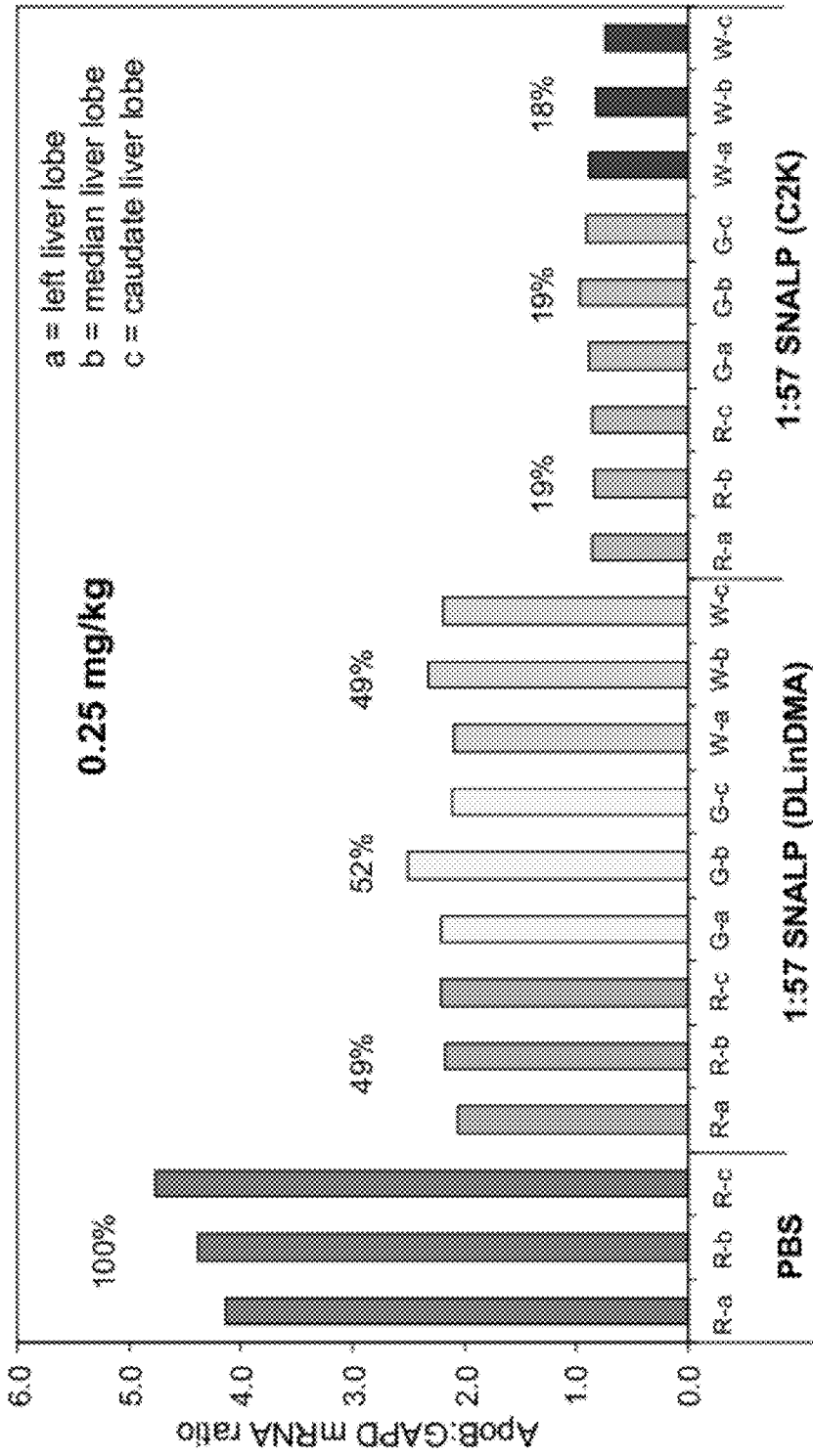


FIG. 10



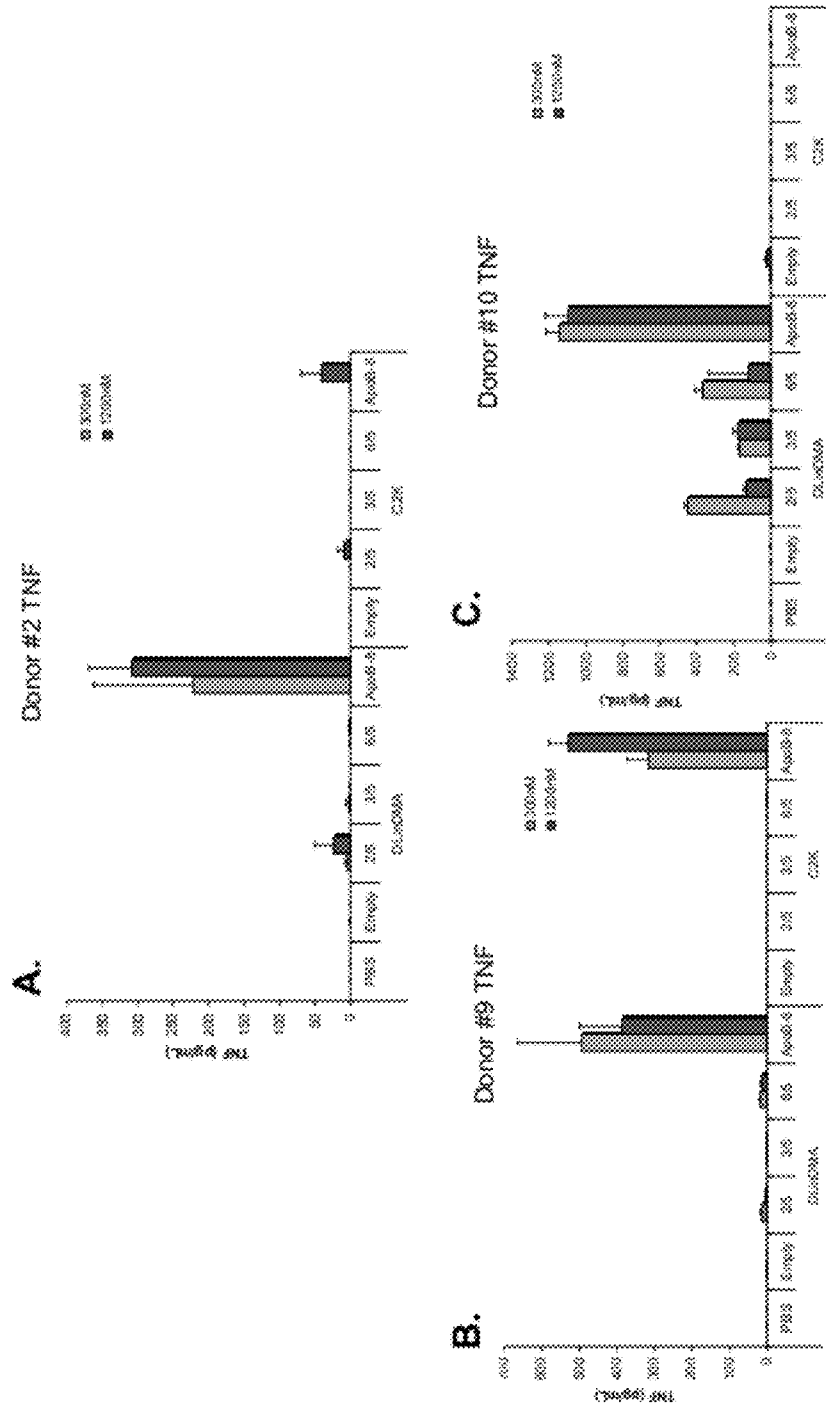


FIG. 11

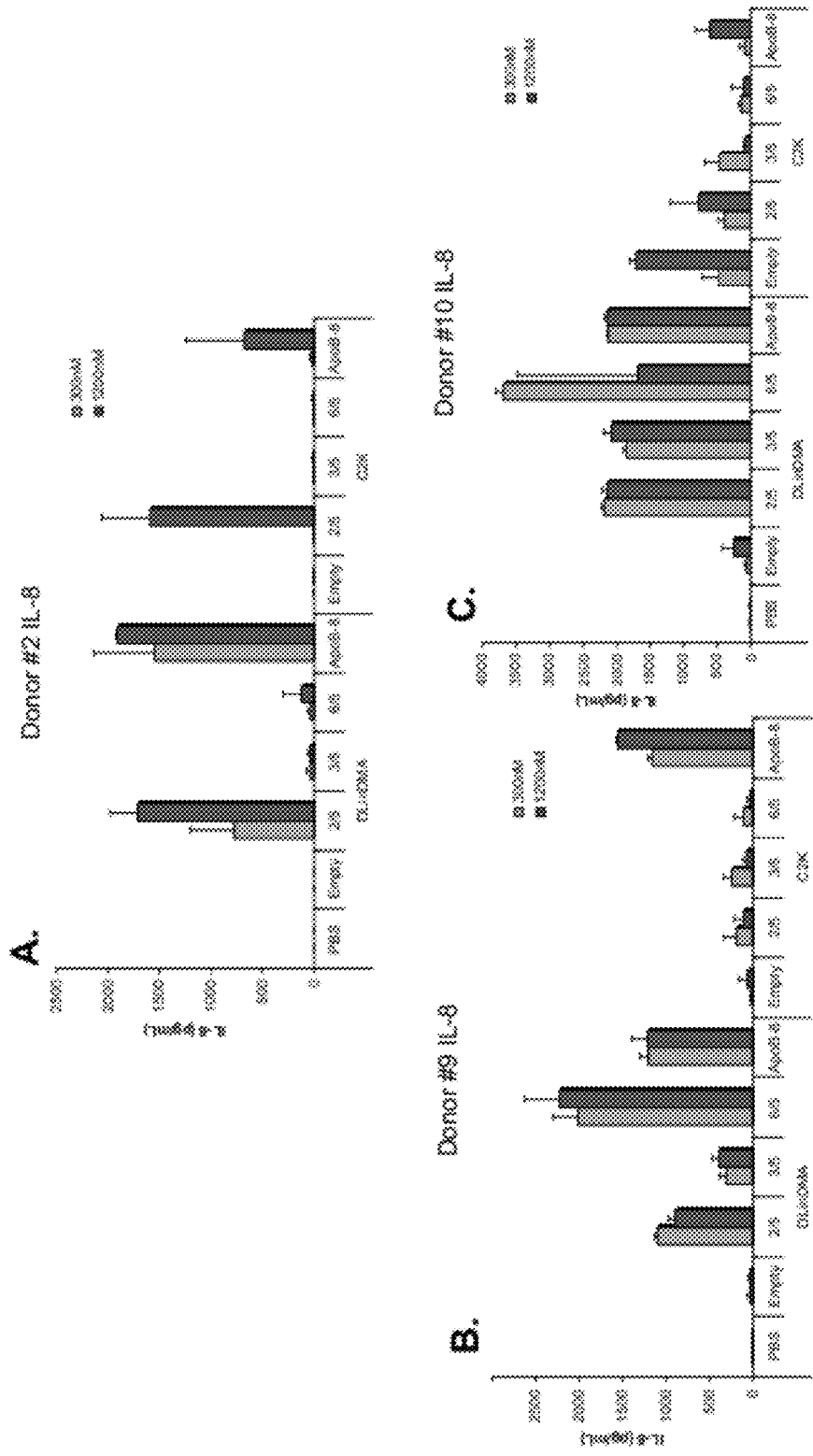


FIG. 12

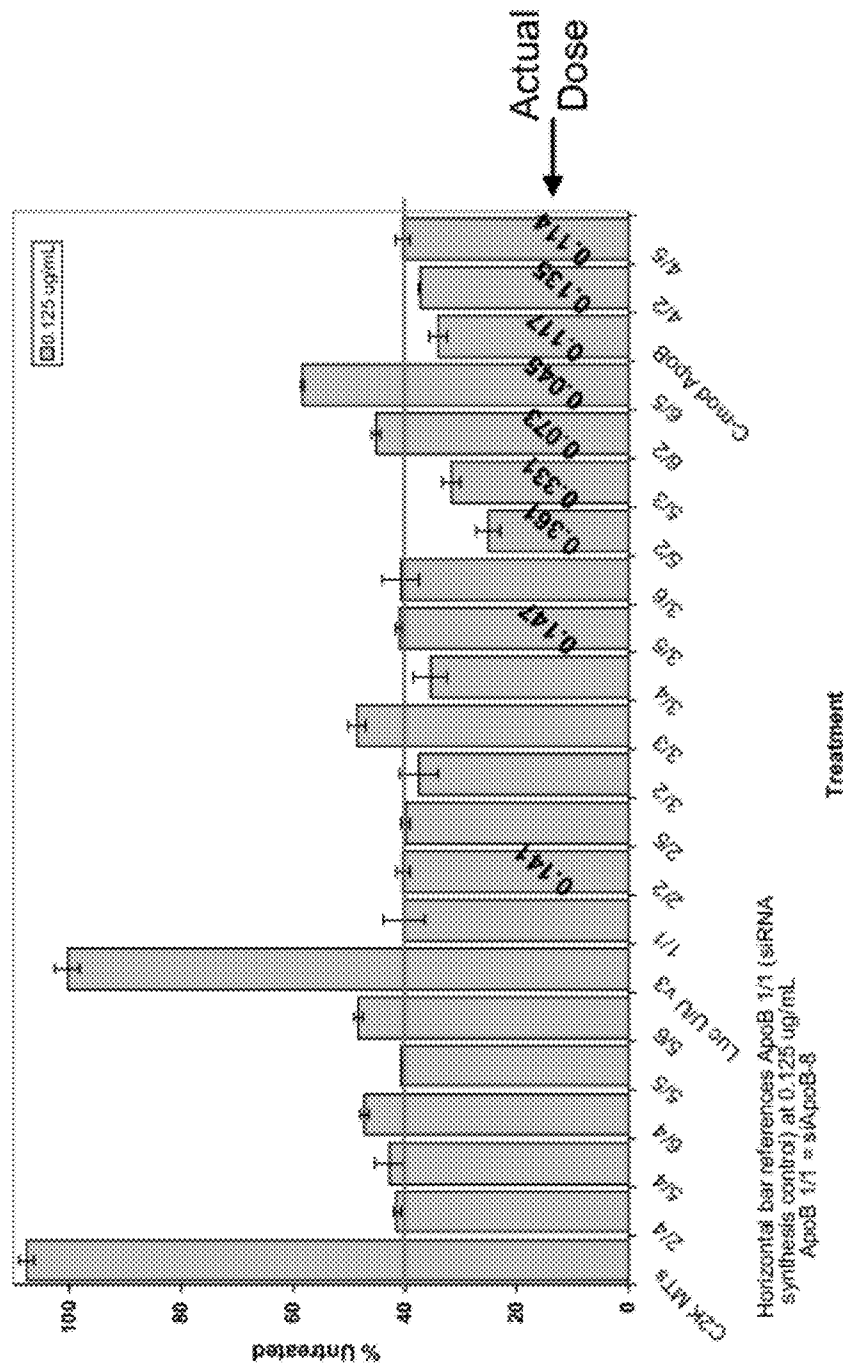


FIG. 13

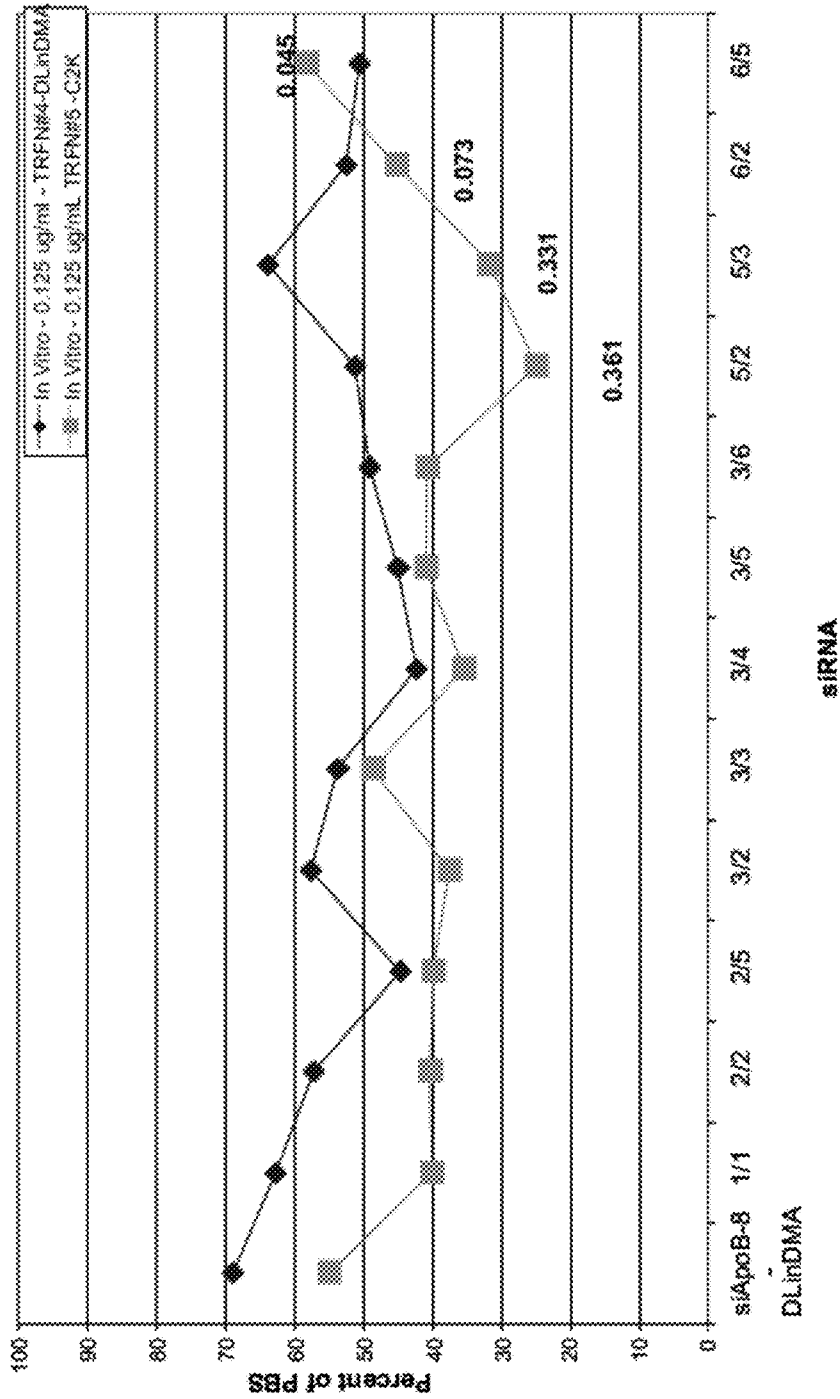


FIG. 14

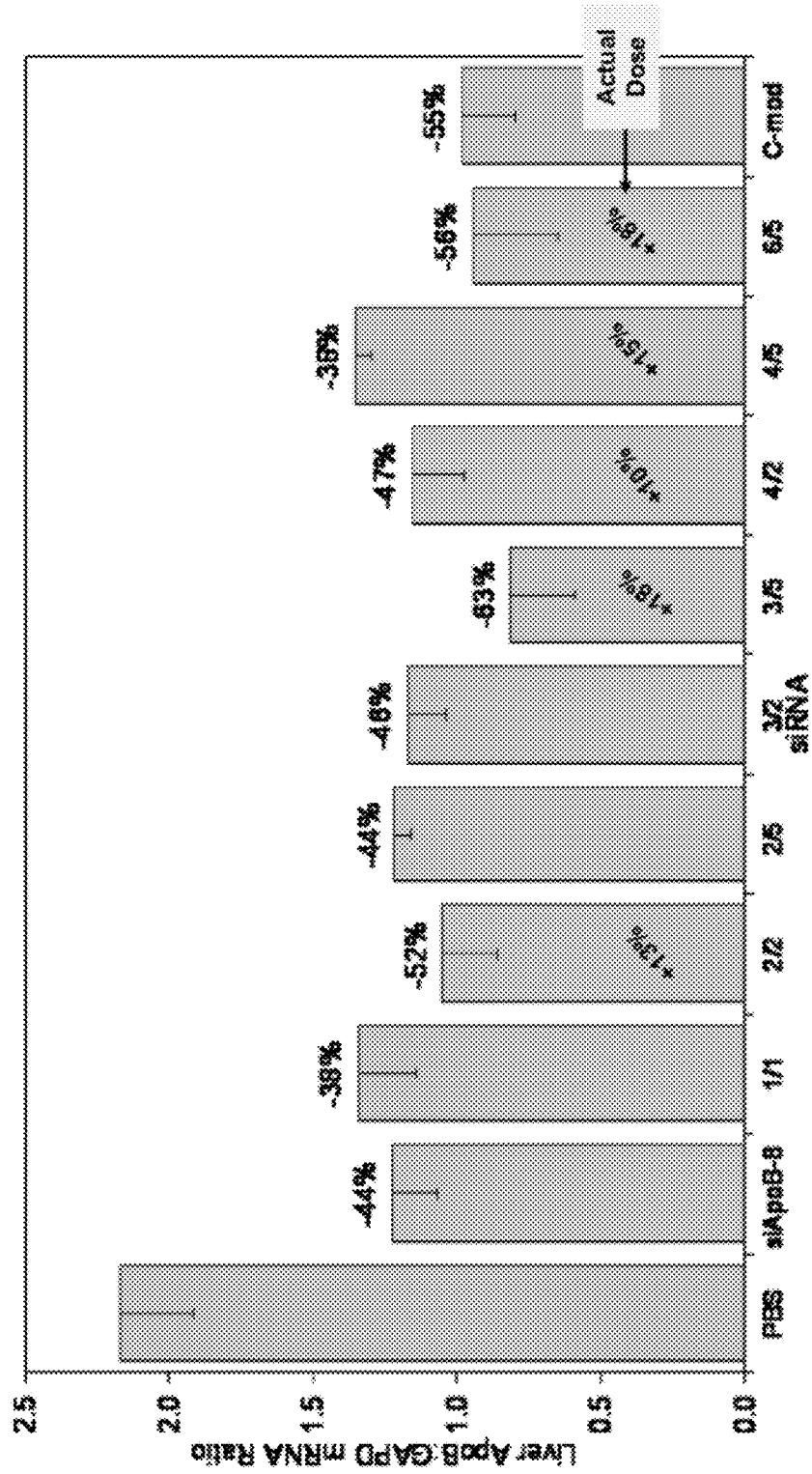


FIG. 15

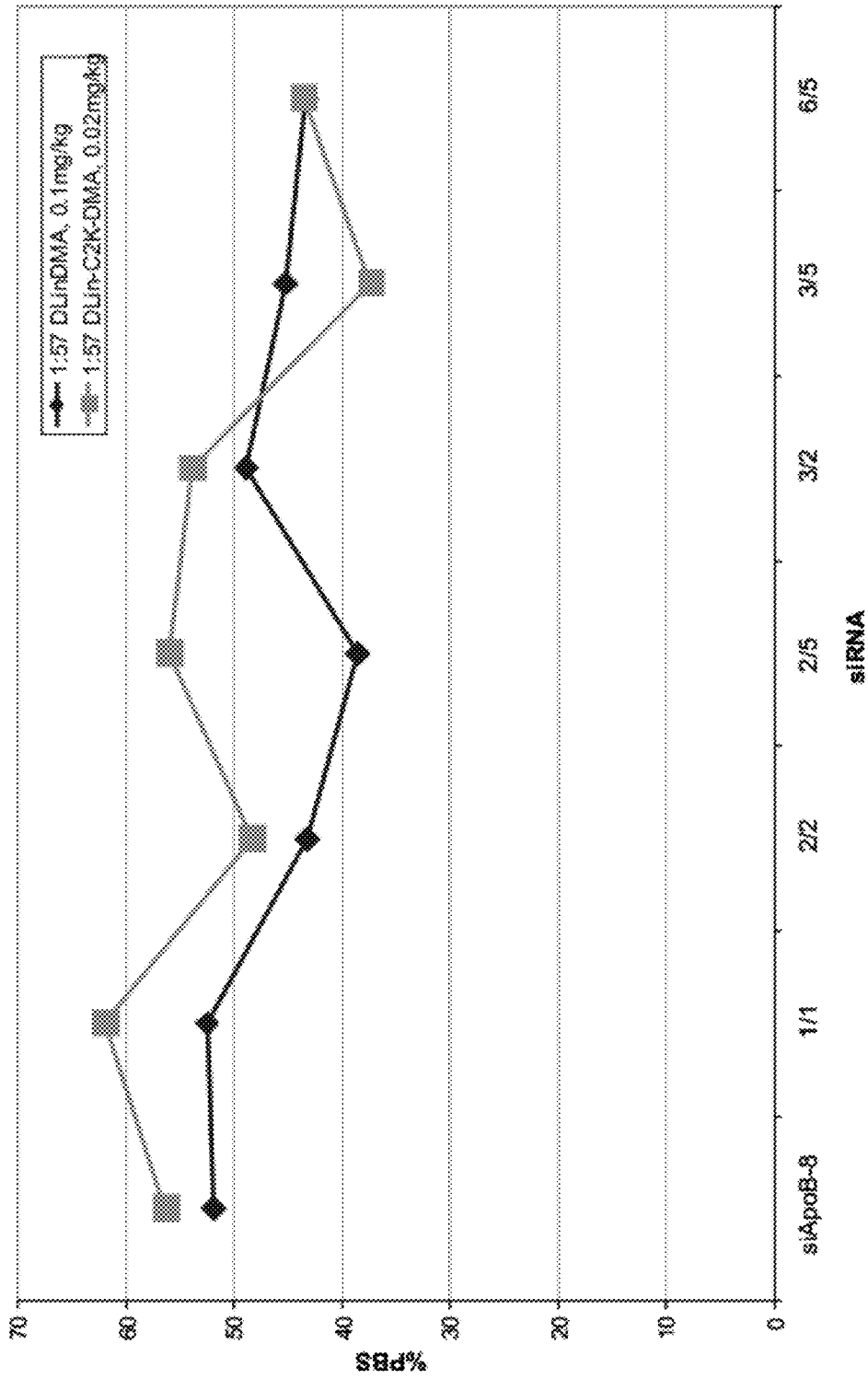


FIG. 16

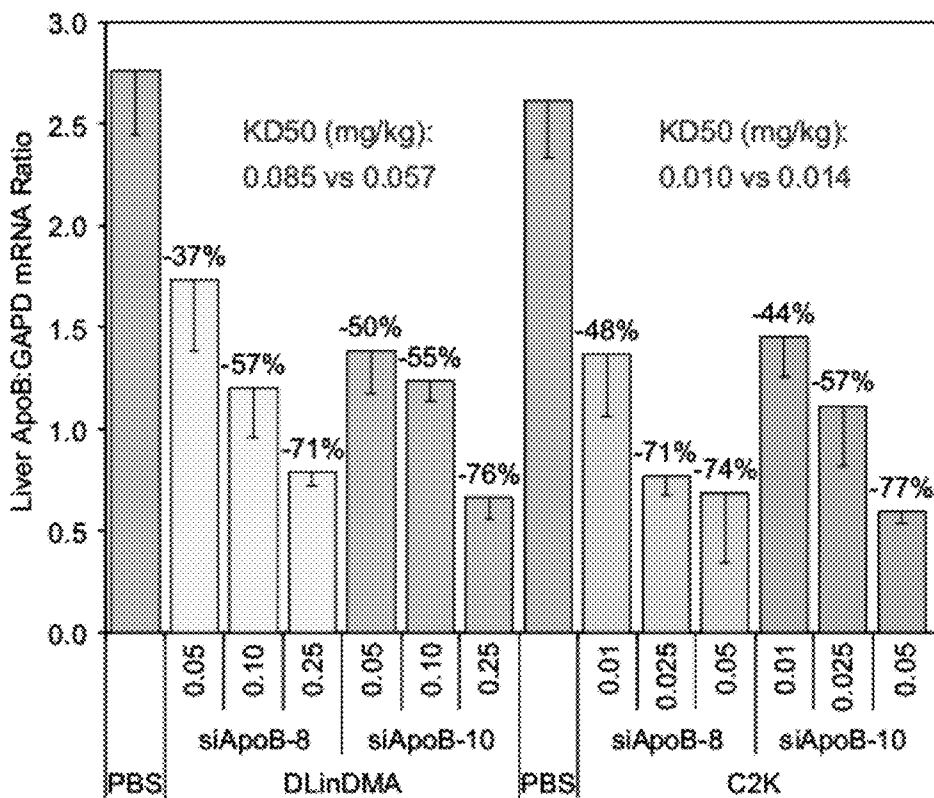


FIG. 17

## COMPOSITIONS AND METHODS FOR SILENCING APOLIPOPROTEIN B

### CROSS-REFERENCES TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Application No. 61/222,464, filed Jul. 1, 2009, and U.S. Provisional Application No. 61/351,275, filed Jun. 3, 2010, the disclosures of which are hereby incorporated by reference in their entirety for all purposes.

### BACKGROUND OF THE INVENTION

Apolipoprotein B (also known as ApoB, apolipoprotein B-100; ApoB-100, apolipoprotein B-48; ApoB-48 and Ag(x) antigen), is a large glycoprotein that serves an indispensable role in the assembly and secretion of lipids and in the transport and receptor-mediated uptake and delivery of distinct classes of lipoproteins. Apolipoprotein B was cloned (Law et al., *PNAS USA* 82:8340-8344 (1985)) and mapped to chromosome 2p23-2p24 in 1986 (Deeb et al., *PNAS USA* 83, 419-422 (1986)). ApoB has a variety of functions, from the absorption and processing of dietary lipids to the regulation of circulating lipoprotein levels (Davidson and Shelness, *Annu. Rev. Nutr.*, 20:169-193 (2000)). Two forms of ApoB have been characterized: ApoB-100 and ApoB-48. ApoB-100 is the major protein component of LDL, contains the domain required for interaction of this lipoprotein species with the LDL receptor, and participates in the transport and delivery of endogenous plasma cholesterol (Davidson and Shelness, 2000, supra). ApoB-48 circulates in association with chylomicrons and chylomicron remnants which are cleared by the LDL-receptor-related protein (Davidson and Shelness, 2000, supra). ApoB-48 plays a role in the delivery of dietary lipid from the small intestine to the liver.

Susceptibility to atherosclerosis is highly correlated with the ambient concentration of apolipoprotein B-containing lipoproteins (Davidson and Shelness, 2000, supra). Elevated plasma levels of the ApoB-100-containing lipoprotein Lp(a) are associated with increased risk for atherosclerosis and its manifestations, which may include hypercholesterolemia (Seed et al., *N. Engl. J. Med.* 322:1494-1499 (1990)), myocardial infarction (Sandkamp et al., *Clin. Chem.* 36:20-23 (1990)), and thrombosis (Nowak-Gottl et al., *Pediatrics*, 99:E11 (1997)).

Apolipoprotein B knockout mice (bearing disruptions of both ApoB-100 and ApoB-48) have been generated which are protected from developing hypercholesterolemia when fed a high-fat diet (Farese et al., *PNAS USA*. 92:1774-1778 (1995) and Kim and Young, *J. Lipid Res.*, 39:703-723 (1998)). The incidence of atherosclerosis has been investigated in mice expressing exclusively ApoB-100 or ApoB-48 and susceptibility to atherosclerosis was found to be dependent on total cholesterol levels.

In view of such findings, significant efforts have been made to modulate serum cholesterol levels by modulating ApoB expression using therapeutic nucleic acids, e.g., antisense oligonucleotides, ribozymes, etc. (see, e.g., U.S. Pat. No. 7,407,943, which is directed to modulation of ApoB using antisense oligonucleotides). More recent efforts have focused on the use of interfering RNA molecules, such as siRNA and miRNA, to modulate ApoB (see, Zimmermann et al., *Nature*, 441: 111-114 (2006), U.S. Patent Publication Nos. 20060134189 and 20060105976, and PCT Publication No. WO 04/091515). Interfering RNA molecules can down-regulate intracellular levels of specific proteins, such as ApoB,

through a process termed RNA interference (RNAi). Following introduction of interfering RNA into the cell cytoplasm, these double-stranded RNA constructs can bind to a protein termed RISC. The sense strand of the interfering RNA is displaced from the RISC complex, providing a template within RISC that can recognize and bind mRNA with a complementary sequence to that of the bound interfering RNA. Having bound the complementary mRNA, the RISC complex cleaves the mRNA and releases the cleaved strands. RNAi can provide down-regulation of specific proteins, such as ApoB, by targeting specific destruction of the corresponding mRNA that encodes for protein synthesis.

Despite the high therapeutic potential of RNAi, two problems currently faced by interfering RNA constructs are, first, their susceptibility to nuclease digestion in plasma and, second, their limited ability to gain access to the intracellular compartment where they can bind RISC when administered systemically as free interfering RNA molecules. These double-stranded constructs can be stabilized by the incorporation of chemically modified nucleotide linkers within the molecule, e.g., phosphothioate groups. However, such chemically modified linkers provide only limited protection from nuclease digestion and may decrease the activity of the construct.

In an attempt to improve efficacy, investigators have employed various lipid-based carrier systems to deliver chemically modified or unmodified therapeutic nucleic acids, including anionic (conventional) liposomes, pH sensitive liposomes, immunoliposomes, fusogenic liposomes, and cationic lipid/nucleic acid aggregates. In particular, one lipid-based carrier system, i.e., the stable nucleic-acid lipid particle (SNALP) system, has been found to be particularly effective for delivering interfering RNA (see, U.S. Patent Publication No. 20050064595 and U.S. Patent Publication No. 20060008910 (collectively referred to as "MacLachlan et al.")). MacLachlan et al. have demonstrated that interfering RNA, such as siRNA, can be effectively systemically administered using nucleic acid-lipid particles containing a cationic lipid, and that these nucleic acid-lipid particles provide improved down-regulation of target proteins in mammals including non-human primates (see, Zimmermann et al., *Nature*, 441: 111-114 (2006)).

Even in spite of this progress, there remains a need in the art for improved SNALPs that are useful for delivering therapeutic nucleic acids, such as siRNA and miRNA, to the liver of a mammal (e.g., a human), and that result in increased silencing of target genes of interest in the liver, such as ApoB. Preferably, these compositions would encapsulate nucleic acids with high-efficiency, have high drug:lipid ratios, protect the encapsulated nucleic acid from degradation and clearance in serum, be suitable for systemic delivery, and provide intracellular delivery of the encapsulated nucleic acid. In addition, these nucleic acid-lipid particles should be well-tolerated and provide an adequate therapeutic index, such that patient treatment at an effective dose of the nucleic acid is not associated with significant toxicity and/or risk to the patient. The present invention provides such compositions, methods of making the compositions, and methods of using the compositions to introduce nucleic acids, such as siRNA and miRNA, into the liver, including for the treatment of diseases, such as hypercholesterolemia (e.g., atherosclerosis, angina pectoris or high blood pressure).

### BRIEF SUMMARY OF THE INVENTION

The present invention is based, in part, on the discovery that the use of certain cationic (amino) lipids in nucleic acid-



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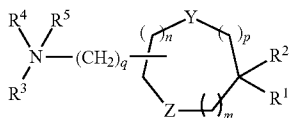
lipid particles provides advantages when the particles are used for the in vivo delivery of therapeutic nucleic acids, such as siRNA, into the liver of a mammal. In particular, it has been unexpectedly found that the nucleic acid-lipid particles of the present invention comprising at least one cationic lipid of Formula I-XIV and at least one interfering RNA as described herein demonstrate increased potency (i.e., increased silencing activity) and/or increased tolerability (e.g., a more favorable toxicity profile) when targeting a gene of interest in the liver such as APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 when compared to other nucleic acid-lipid particle compositions previously described. In preferred embodiments, the present invention provides nucleic acid-lipid particles (e.g., SNALP) comprising APOB siRNA 3/5 and the cationic lipid DLin-K-C2-DMA and methods of use thereof, which nucleic acid-lipid particles unexpectedly possess increased potency and increased tolerability when silencing APOB expression in vivo compared to other nucleic acid-lipid particle compositions previously described.

In particular embodiments, the present invention provides cationic lipids that enable the formulation of compositions for the in vitro and in vivo delivery of interfering RNA, such as siRNA, to the liver that result in increased silencing of the target gene of interest, such as APOB. It is shown herein that these improved lipid particle compositions are particularly effective in down-regulating (e.g., silencing) the protein levels and/or mRNA levels of target genes in the liver, such as APOB. Furthermore, it is shown herein that the activity of these improved lipid particle compositions is dependent on the presence of the cationic lipids of Formula I-XIV of the invention.

In one aspect, the present invention provides a nucleic acid-lipid particle (e.g., SNALP) comprising:

(a) an interfering RNA that silences Apolipoprotein B (APOB) expression and/or the expression of another liver target gene such as APOC3, PCSK9, DGAT1, and/or DGAT2;

(b) a cationic lipid of Formula I having the following structure:



or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkenyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkynyl, or optionally substituted C<sub>12</sub>-C<sub>24</sub> acyl, with the proviso that at least one of R<sup>1</sup> and R<sup>2</sup> has at least two sites of unsaturation; R<sup>3</sup> and R<sup>4</sup> are either the same or different and are independently optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, optionally substituted C<sub>2</sub>-C<sub>6</sub> alkenyl, or optionally substituted C<sub>2</sub>-C<sub>6</sub> alkynyl or R<sup>3</sup> and R<sup>4</sup> 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<sup>5</sup> is either absent or hydrogen or C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; m, n and p are either the same or different and are independently either 0, 1 or 2, with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; Y and Z are either the same or different and are independently O, S, or NH; and

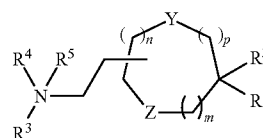
(c) a non-cationic lipid.

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In another aspect, the present invention provides a nucleic acid-lipid particle (e.g., SNALP) comprising:

(a) an interfering RNA that silences Apolipoprotein B (APOB) expression and/or the expression of another liver target gene such as APOC3, PCSK9, DGAT1, and/or DGAT2;

(b) a cationic lipid of Formula II having the following structure:



or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkenyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkynyl, or optionally substituted C<sub>12</sub>-C<sub>24</sub> acyl; R<sup>3</sup> and R<sup>4</sup> are either the same or different and are independently optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, optionally substituted C<sub>2</sub>-C<sub>6</sub> alkenyl, or optionally substituted C<sub>2</sub>-C<sub>6</sub> alkynyl or R<sup>3</sup> and R<sup>4</sup> 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<sup>5</sup> is either absent or is hydrogen or C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; m, n, and p are either the same or different and are independently either 0, 1 or 2, with the proviso that m, n, and p are not simultaneously 0; Y and Z are either the same or different and are independently O, S, or NH; and

(c) a non-cationic lipid.

In some embodiments, cationic lipids falling within the scope of Formulas I and/or II that are useful in the nucleic acid-lipid particles of the present invention include, but are not limited to, the following: 2,2-dilinoleyl-4-(2-dimethylaminoethyl)[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2" or "C2K"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA; "C3K"), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA; "C4K"), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpiperazine-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 2,2-dioleoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DO-K-DMA), 2,2-distearoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DS-K-DMA), 2,2-dilinoleyl-4-N-morpholino-[1,3]-dioxolane (DLin-K-MA), 2,2-Dilinoleyl-4-trimethylamino-[1,3]-dioxolane chloride (DLin-K-TMA.Cl), 2,2-dilinoleyl-4,5-bis(dimethylaminomethyl)[1,3]-dioxolane (DLin-K<sup>2</sup>-DMA), 2,2-dilinoleyl-4-methylpiperazine-[1,3]-dioxolane (D-Lin-K-N-methylpiperazine), analogs thereof, salts thereof, and mixtures thereof.

In yet another aspect, the present invention provides a nucleic acid-lipid particle (e.g., SNALP) comprising: (a) an interfering RNA that silences Apolipoprotein B (APOB) expression and/or the expression of another liver target gene such as APOC3, PCSK9, DGAT1, DGAT2, etc.); (b) a cationic lipid having the structure of Formula III-XIV; and (c) a non-cationic lipid. Examples of cationic lipids falling within the scope of Formula III-XIV that are useful in the nucleic acid-lipid particles of the present invention include, but are not limited to, the following: 1,2-di-γ-linolenyloxy-N,N-dimethylaminopropane (γ-DLenDMA), γ-DLen-C2K-DMA,

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DLen-C2K-DMA, DPan-C2K-DMA, DPan-C3K-DMA, 1,2-dilinoleoyloxy-3-piperidinopropylamine (DLinPip), 1,2-dilinoleoyloxy-3-(3'-hydroxypiperidino)-propylamine (DLinPip-3OH), 1,2-dilinoleoyloxy-3-(4'-hydroxypiperidino)-propylamine (DLinPip-4OH), 1,2-dilinoleoyloxy-3-(N,N-dimethyl)-propylamine (DLinDEA), N1-((2,3-linoleoyloxy)propyl)-N1,N3,N3-trimethylpropane-1,3-diamine (2N-DLinDMA), 1,2-Dilinoleoyloxy-3-(1-imidazole)propylamine (DLinIm), 1,2-dilinoleoyloxy-(N,N-dimethyl)-butyl-4-amine (C2-DLinDMA), 1,2-diphytanyloxy-(N,N-dimethyl)-butyl-4-amine (C2-DPanDMA), 1,2-dilinoleoyloxy-(N,N-dimethyl)-butyl-4-amine (C2-DLinDAP), Linoleyl/Oleyl DMA, Linoleyl/Phytanyl DMA, Linoleyl/Linolenyl DMA, Linoleyl/Stearyl DMA, Linoleyl/C<sub>6</sub>:0 DMA, Linoleyl/C<sub>6</sub>:1 DMA, 1-(2,3-linoleoyloxypropoxy)-2-(linoleoyloxy)-(N,N-dimethyl)-propyl-3-amine (TLinDMA), C2-TLinDMA, DHep-C2K-DMA, DLin-C2K-Pip-3OH, 1,2-diarachidonyloxy-(N,N-dimethyl)-propyl-3-amine (DaraDMA), 1,2-didocosahexaenyloxy-(N,N-dimethyl)-propyl-3-amine (DDocDMA), 1,2-diphytanyloxy-3-(N,N-dimethyl)-propylamine (DPanDMA), 6-membered ketal lipids such as DPan-C1K6-DMA, analogs thereof, salts thereof, and mixtures thereof.

In some embodiments, the lipid particles of the invention preferably comprise an interfering RNA that silences APOB and/or other liver target genes such as APOC3, PCSK9, DGAT1, DGAT2, or combinations thereof, a cationic lipid of Formula I-XIV as disclosed herein, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles.

In certain embodiments, the non-cationic lipid component of the lipid particle may comprise a phospholipid, cholesterol (or cholesterol derivative), or a mixture thereof. In one particular embodiment, the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof. In some embodiments, the conjugated lipid component of the lipid particle comprises a polyethyleneglycol (PEG)-lipid conjugate. In certain instances, the PEG-lipid conjugate comprises a PEG-dialkylglycerol (PEG-DAG) conjugate, a PEG-dialkylxypropyl (PEG-DAA) conjugate, or a mixture thereof.

In some embodiments, the interfering RNA is fully encapsulated within the lipid portion of the lipid particle such that the interfering RNA in the lipid particle is resistant in aqueous solution to enzymatic degradation, e.g., by a nuclease or protease. Non-limiting examples of interfering RNA include siRNA, aiRNA, miRNA, Dicer-substrate dsRNA, shRNA, and mixtures thereof. In other embodiments, the lipid particles described herein are substantially non-toxic to mammals such as humans.

In other embodiments, the nucleic acid-lipid particle comprises an interfering RNA (e.g., siRNA) that targets APOB, wherein the interfering RNA comprises an antisense strand comprising the sequence 5'-UAUUCAGUGUGAUGACACU-3' (SEQ ID NO:13). In still other embodiments, the nucleic acid-lipid particle further comprises a sense strand comprising the sequence 5'-AGUGUCAUACACUGAAUA-3' (SEQ ID NO:14). In certain embodiments, the interfering RNA comprises a 3' overhang in one or both strands of the interfering RNA molecule. In certain embodiments, the interfering RNA comprises an antisense strand comprising a 5'-UG-3' overhang and/or a sense strand comprising a 5'-CC-3' overhang.

In yet other embodiments, the nucleic acid-lipid particle comprises an interfering RNA (e.g., siRNA) that targets APOB, wherein the interfering RNA comprises at least one modified nucleotide. In certain embodiments, one or more of the nucleotides in the double-stranded region of the interfer-

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ing RNA comprise modified nucleotides. In certain other embodiments, one or more of the nucleotides in the 3' overhang in one or both strands of the interfering RNA comprise modified nucleotides. In particular embodiments; the modified nucleotides comprise 2'-O-methyl (2'OMe) nucleotides.

In further embodiments, the nucleic acid-lipid particle comprises an interfering RNA (e.g., siRNA) that targets APOB, wherein the interfering RNA comprises an antisense strand comprising the sequence 5'-UAUUCAGUGUGAUGACACU-3' (SEQ ID NO:15), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides. In other embodiments, the particle further comprises a sense strand comprising the sequence 5'-AGUGUCAUACACUGAAUA-3' (SEQ ID NO:16), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides. In certain embodiments, the interfering RNA comprises a 3' overhang in one or both strands of the interfering RNA molecule. In some embodiments, the interfering RNA comprises an antisense strand comprising a 5'-UG-3' overhang and/or a sense strand comprising a 5'-CC-3' overhang, wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides. In other embodiments, the nucleic acid-lipid particle comprises an interfering RNA consisting of the following sequences:

(SEQ ID NO: 4)  
5' -**AGUGUCAU**CACACUGAAUACC-3'  
and

(SEQ ID NO: 11)  
3' -GUUCACAGUAGUGACUUAU-5'

wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides.

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

In another aspect, the present invention provides methods for introducing one or more interfering RNA molecules (e.g., siRNAs) that silence APOB expression and/or the expression of other liver target genes such as APOC3, PCSK9, DGAT1, and/or DGAT2) into a cell (e.g., a liver cell), the method comprising contacting the cell with a nucleic acid-lipid particle described herein (e.g., SNALP). In one embodiment, the cell is in a mammal and the mammal is a human.

In yet another aspect, the present invention provides methods for the in vivo delivery of one or more interfering RNA molecules (e.g., siRNAs) to liver cells, the method comprising administering to a mammal a nucleic acid-lipid particle described herein (e.g., SNALP). Advantageously, the nucleic acid-lipid particles of the invention are particularly effective at silencing target gene expression in the liver and, thus, are well suited for targeting genes such as APOB, APOC3, PCSK9, DGAT1, DGAT2, and combinations thereof. In certain embodiments, the nucleic acid-lipid particles (e.g., SNALP) are administered by one of the following routes of administration: oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. In particular embodiments, the nucleic acid-lipid particles (e.g., SNALP) are administered systemically, e.g., via enteral or parenteral routes of administration. In preferred embodiments, the mammal is a human.

In certain embodiments, the present invention provides methods for treating a liver disease or disorder by administering an interfering RNA (e.g., one or more siRNAs targeting APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 expres-

sion) in nucleic acid-lipid particles (e.g., SNALP) as described herein, alone or in combination with a lipid-lowering agent. Examples of lipid diseases and disorders include, but are not limited to, dyslipidemia (e.g., hyperlipidemia such as elevated triglyceride levels (hypertriglyceridemia) and/or elevated cholesterol levels (hypercholesterolemia)), atherosclerosis, coronary heart disease, coronary artery disease, atherosclerotic cardiovascular disease (CVD), fatty liver disease (hepatic steatosis), abnormal lipid metabolism, abnormal cholesterol metabolism, diabetes (including Type 2 diabetes), obesity, cardiovascular disease, and other disorders relating to abnormal metabolism. Non-limiting examples of lipid-lowering agents include statins, fibrates, ezetimibe, thiazolidinediones, niacin, beta-blockers, nitroglycerin, calcium antagonists, and fish oil.

In one particular embodiment, the present invention provides a method for lowering or reducing cholesterol levels in a mammal (e.g., human) in need thereof (e.g., a mammal with elevated blood cholesterol levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (e.g., a SNALP formulation) described herein comprising one or more interfering RNAs (e.g., siRNAs) that target one or more genes associated with metabolic diseases and disorders (e.g., APOB, APOC3, PCSK9, DGAT1, and/or DGAT2). In another particular embodiment, the present invention provides a method for lowering or reducing triglyceride levels in a mammal (e.g., human) in need thereof (e.g., a mammal with elevated blood triglyceride levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (e.g., a SNALP formulation) described herein comprising one or more interfering RNAs (e.g., siRNAs) that target one or more genes associated with metabolic diseases and disorders (e.g., APOB, APOC3, PCSK9, DGAT1, and/or DGAT2). These methods can be carried out in vitro using standard tissue culture techniques or in vivo by administering the interfering RNA (e.g., siRNA) using any means known in the art. In preferred embodiments, the interfering RNA (e.g., siRNA) is delivered to a liver cell (e.g., hepatocyte) in a mammal such as a human.

Additional embodiments related to treating a liver disease or disorder using a lipid particle are described in, e.g., PCT Application No. PCT/CA2010/000120, filed Jan. 26, 2010, and U.S. Patent Publication No. 20060134189, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

In a further aspect, the present invention provides methods for treating a disease or disorder associated with overexpression of APOB in a mammal (e.g., human) in need thereof, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (e.g., SNALP) comprising one (or more) interfering RNA that silences APOB expression. Diseases and disorders associated with overexpression of APOB include, but are not limited to, atherosclerosis, angina pectoris, high blood pressure, diabetes, and hypothyroidism. In preferred embodiments, the mammal (e.g., human) has a disease or disorder involving hypercholesterolemia and serum cholesterol levels are lowered when expression of APOB is silenced by the interfering RNA delivered using the nucleic acid-lipid particles of the present invention.

The nucleic acid-lipid particles of the invention (e.g., SNALP) comprising one or more cationic lipids of Formula I-XIV or salts thereof are particularly advantageous and suitable for use in the administration of interfering RNA 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.

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 shows a comparison of the plasma total cholesterol knockdown efficacy of exemplary APOB SNALP formulations containing various cationic lipids described herein.

FIG. 2 shows a comparison of the liver ApoB mRNA knockdown activity of exemplary APOB SNALP formulations containing various cationic lipids described herein.

FIG. 3 shows a comparison of the liver ApoB mRNA knockdown activity of additional exemplary APOB SNALP formulations containing various cationic lipids described herein.

FIG. 4 shows a dose response evaluation of three different doses of exemplary APOB SNALP formulations containing various cationic lipids described herein on liver ApoB mRNA knockdown activity.

FIG. 5 shows a comparison of the liver ApoB mRNA knockdown activity of additional exemplary APOB SNALP formulations containing various cationic lipids described herein.

FIG. 6 shows a comparison of the liver ApoB mRNA knockdown activity of additional exemplary APOB SNALP formulations containing various cationic lipids described herein.

FIG. 7 shows a comparison of the liver ApoB mRNA knockdown activity of additional exemplary APOB SNALP formulations containing various cationic lipids described herein.

FIG. 8 shows a dose response evaluation of three different doses of exemplary APOB SNALP formulations containing either DLinDMA or C2K on liver ApoB mRNA knockdown activity.

FIG. 9 shows the reproducibility of liver ApoB mRNA knockdown using two independent SNALP batches.

FIG. 10 shows a comparison of the liver ApoB mRNA knockdown activity of exemplary APOB SNALP formulations containing either DLinDMA or C2K in rats.

FIG. 11 shows a comparison of the TNF inflammatory response in donors to exemplary APOB SNALP formulations containing either DLinDMA or C2K.

FIG. 12 shows a comparison of the IL-8 inflammatory response in donors to exemplary APOB SNALP formulations containing either DLinDMA or C2K.

FIG. 13 shows a comparison of APOB mRNA knockdown activity of exemplary 2'OMe-modified APOB SNALP formulations containing C2K in human primary hepatocytes.

FIG. 14 shows a comparison of APOB mRNA knockdown activity of exemplary 2'OMe-modified APOB SNALP formulations containing either DLinDMA or C2K in human primary hepatocytes.

FIG. 15 shows a comparison of the liver ApoB mRNA knockdown activity of exemplary 2'OMe-modified APOB SNALP formulations containing C2K in mice.

FIG. 16 shows a comparison of the liver ApoB mRNA knockdown activity of exemplary 2'OMe-modified APOB SNALP formulations containing either DLinDMA or C2K in mice.

FIG. 17 shows a dose response evaluation of three different doses of exemplary APOB SNALP formulations containing either DLinDMA or C2K and either APOB siRNA 1/1 ("siApoB-8") or APOB siRNA 3/5 ("siApoB-10") on liver ApoB mRNA knockdown activity.

## DETAILED DESCRIPTION OF THE INVENTION

## I. Introduction

The present invention is based, in part, on the discovery that the use of certain cationic (amino) lipids in nucleic acid-lipid particles provide advantages when the particles are used for the in vivo delivery of therapeutic nucleic acids, such as siRNA, into the liver of a mammal. In particular, it has been unexpectedly found that the nucleic acid-lipid particles of the present invention (i.e., SNALP formulations) containing at least one cationic lipid of Formula I-XIV and at least one interfering RNA (e.g., siRNA) as described herein demonstrate increased potency (i.e., increased silencing activity) and/or increased tolerability (e.g., a more favorable toxicity profile) when targeting a gene of interest in the liver, such as APOB, when compared to other nucleic acid-lipid particle compositions previously described.

In particular embodiments, the present invention provides cationic lipids that enable the formulation of compositions for the in vitro and in vivo delivery of interfering RNA, such as siRNA, to the liver that result in increased silencing of the target gene of interest in the liver. It is shown herein that these improved lipid particle compositions are particularly effective in down-regulating (e.g., silencing) the protein levels and/or mRNA levels of target genes in the liver, such as APOB. Furthermore, it is shown herein that the activity of these improved lipid particle compositions is dependent on the presence of the cationic lipids of the invention.

The lipid particles and compositions of the present invention may be used for a variety of purposes, including the delivery of encapsulated interfering RNA, such as siRNA, to liver cells (e.g., hepatocytes), both in vitro and in vivo. Accordingly, the present invention further provides methods of treating metabolic diseases or disorders in a subject in need thereof by contacting the subject with a lipid particle that encapsulates or is associated with a suitable therapeutic agent, wherein the lipid particle comprises one or more of the novel cationic lipids described herein.

In particular, the lipid particles and compositions of the present invention are useful for silencing APOB expression to treat diseases or disorders associated with expression or over-expression of APOB. Such diseases include, e.g., atherosclerosis, angina pectoris, high blood pressure, diabetes, hypothyroidism, and hypercholesterolemia. In view of their enhanced potency, the nucleic acid-lipid particles of the present invention comprising an siRNA sequence that targets APOB can effectively be used to lower serum cholesterol levels.

As described herein, the lipid particles of the present invention have been found to provide more potent silencing when used to deliver interfering RNA molecules, such as siRNA, to the liver, when compared to lipid particle compositions previously described. As such, in addition to being useful for silencing APOB, the lipid particles of the present invention are also used for targeting other genes of interest in the liver. Such genes of interest include, but are not limited to, APOC3, PCSK9, DGAT1, DGAT2, and combinations thereof.

As explained herein, it has surprisingly been found that the lipid particles of the present invention (e.g., SNALP) containing at least one cationic lipid of Formulas I-XIV, either alone or in combination with other cationic lipids, show increased potency and/or increased tolerability when targeting a gene of interest in the liver, such as, e.g., APOB, APOC3, PCSK9, DGAT1, and/or DGAT2, when compared to other SNALP formulations. For instance, as set forth in the Examples below, it has been found that a lipid particle (e.g., SNALP)

containing, e.g., DLin-K-C2-DMA (“C2K”),  $\gamma$ -DLenDMA, Linoleyl/Linolenyl DMA (“Lin/Len”), C2-DPanDMA, DPan-C2K-DMA, DPan-C3K-DMA,  $\gamma$ -DLen-C2K-DMA, DLen-C2K-DMA, or C2-TLinDMA was unexpectedly more potent in silencing APOB expression in vivo compared to SNALP containing DLinDMA or DLenDMA. In addition, as set forth in the Examples below, it has been found that a lipid particle (e.g., SNALP) comprising an APOB siRNA described herein and containing, e.g., DLin-K-C2-DMA, displayed an unexpectedly more favorable toxicity profile in vivo compared to SNALP formulations containing DLinDMA. As such, in certain preferred embodiments, the lipid particles of the present invention (e.g., SNALP) comprise a 1:57, 1:62, 7:54, or 7:58 lipid particle (e.g., SNALP) containing one or more cationic lipids of Formulas I-XIV, such as C2K,  $\gamma$ -DLenDMA, Linoleyl/Linolenyl DMA (“Lin/Len”), C2-DPanDMA, DPan-C2K-DMA, DPan-C3K-DMA,  $\gamma$ -DLen-C2K-DMA, DLen-C2K-DMA, and/or C2-TLinDMA.

Various exemplary embodiments of the cationic lipids of the present invention, lipid particles and compositions comprising the same, and their use to deliver therapeutic nucleic acids, such as siRNA, to modulate gene and protein expression and to treat metabolic diseases and disorders, 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 “Apolipoprotein B” or “ApoB” refers to 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. ApoB48 contains 2,152 residues compared to 4,535 residues in apoB100. Cloning and characterization of APOB is described by, e.g., Glickman et al., *PNAS USA* 83:5296-5300 (1986); Chen et al., *J. Biol. Chem.* 261: 2918-12921 (1986); and Hospattankar et al., *J. Biol. Chem.* 261:9102-9104 (1986). APOB sequences are set forth in, e.g., Genbank Accession Nos. NM\_000384 and BC051278. siRNA sequences that target APOB are set forth herein as well as in U.S. Patent Publication Nos. 20060134189 and 20060105976, PCT Publication No. WO 04/091515, Soutschek et al., *Nature* 432:173-178 (2004), and Zimmermann et al., *Nature*, 441: 111-114 (2006).

The term “interfering RNA” or “RNAi” or “interfering RNA sequence” as used herein includes single-stranded RNA (e.g., mature miRNA, ssRNAi oligonucleotides, ssDNAi oligonucleotides), double-stranded RNA (i.e., duplex RNA such as siRNA, Dicer-substrate dsRNA, shRNA, aiRNA, or premiRNA), a DNA-RNA hybrid (see, e.g., PCT Publication No. WO 2004/078941), or a DNA-DNA hybrid (see, e.g., PCT Publication No. WO 2004/104199) 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. Preferably, the interfering RNA molecules are chemically synthesized. The disclosures of each of the above patent documents are herein incorporated by reference in their entirety for all purposes.

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. As used herein, the term “siRNA” includes RNA-RNA duplexes as well as DNA-RNA hybrids (see, e.g., PCT Publication No. WO 2004/078941).

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) 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.

The phrase “inhibiting expression of a target gene” refers to the ability of an interfering RNA (e.g., siRNA) to silence, reduce, or inhibit the expression of a target gene (e.g., APOB, APOC3, PCSK9, DGAT1, and/or DGAT2). To examine the

extent of gene silencing, a test sample (e.g., a sample of cells in culture expressing the target gene) or a test mammal (e.g., a rodent (e.g., mouse) or a non-human primate (e.g., monkey) model) is contacted with an interfering RNA (e.g., siRNA) that silences, reduces, or inhibits expression of the target gene. Expression of the target gene in the test sample or test animal is compared to expression of the target gene in a control sample (e.g., a sample of cells in culture expressing the target gene) or a control mammal (e.g., a mammal such as a human or an animal model such as a rodent (e.g., mouse) or non-human primate (e.g., monkey) model) that is not contacted with or administered the interfering RNA (e.g., siRNA). The expression of the target gene in a control sample or a control mammal may be assigned a value of 100%. In particular embodiments, silencing, inhibition, or reduction of expression of a target gene is achieved when the level of target gene expression in the test sample or the test mammal relative to the level of target gene expression in the control sample or the control mammal is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. In other words, the interfering RNAs (e.g., siRNAs) of the present invention are capable of silencing, reducing, or inhibiting the expression of a target gene (e.g., APOB, APOC3, PCSK9, DGAT1, and/or DGAT2) by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in a test sample or a test mammal relative to the level of target gene expression in a control sample or a control mammal not contacted with or administered the interfering RNA. Suitable assays for determining the level of target gene expression include, without limitation, examination of protein or mRNA levels using techniques known to those of skill in the art, such as, e.g., dot blots, Northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

An “effective amount” or “therapeutically effective amount” of 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 95%, 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., IFN $\gamma$ , IFN $\alpha$ , TNF $\alpha$ , IL-6, IL-8, 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- $\alpha$ , IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, TGF, and combinations thereof. Detectable immune responses also include, e.g., induction of interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) mRNA.

“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, 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 $\times$ SSC, and 1% SDS, incubating at 42° C., or, 5 $\times$ SSC, 1% SDS, incubating at 65° C., with wash in 0.2 $\times$ 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 condi-

tions. 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 $\times$ 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)).

Non-limiting examples 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 (1997) 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. Another example is a global alignment algorithm for determining

percent sequence identity such as the Needleman-Wunsch algorithm for aligning protein or nucleotide (e.g., RNA) sequences.

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, RNA, and hybrids thereof. DNA may be in the form of, e.g., antisense molecules, plasmid DNA, DNA-DNA duplexes, pre-condensed DNA, PCR products, 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 small interfering RNA (siRNA), Dicer-substrate dsRNA, small hairpin RNA (shRNA), 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 (e.g., ApoB).

"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.

The term "lipid particle" includes 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 (e.g., cell, tissue, organ, and the like). In preferred embodiments, the lipid particle of the invention is a nucleic acid-lipid particle, which is typically formed from a cationic lipid, a non-cationic lipid, and optionally a conjugated lipid that prevents aggregation of the particle. In other preferred embodiments, the active agent or therapeutic agent, such as a nucleic acid, may be encapsulated in the lipid portion of the particle, thereby protecting it 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 optionally a conjugated lipid that prevents aggregation of the particle), wherein the nucleic acid (e.g., an interfering RNA) is fully encapsulated within the lipid. In certain instances, SNALP 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 silencing of target gene expression at these distal sites. The nucleic acid may be complexed with a condensing agent and encapsulated within a SNALP 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 30 nm to about 150 nm, 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, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm, and are substantially non-toxic. In addition, nucleic acids, when present in the lipid particles of the present 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 that targets APOB), 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 a 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, PEG-lipid conjugates such as, e.g., PEG coupled to dialkylxypropyls (e.g., PEG-DAA conjugates), PEG coupled to diacylglycerols (e.g., PEG-DAG conjugates), PEG coupled to cholesterol, PEG coupled to phosphatidylethanolamines, and PEG conjugated to ceramides (see, e.g., U.S. Pat. No. 5,885,613), cationic PEG lipids,



polyoxazoline (POZ)-lipid conjugates (e.g., POZ-DAA conjugates; see, e.g., U.S. Provisional Application No. 61/294, 828, filed Jan. 13, 2010, and U.S. Provisional Application No. 61/295, 140, filed Jan. 14, 2010), polyamide oligomers (e.g., ATTA-lipid conjugates), and mixtures thereof. Additional examples of POZ-lipid conjugates are described in PCT Publication No. WO 2010/006282. PEG or POZ 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 or the POZ to a lipid can be used including, e.g., non-ester containing linker moieties and ester-containing linker moieties. In certain preferred embodiments, non-ester containing linker moieties, such as amides or carbamates, are used. The disclosures of each of the above patent documents are herein incorporated by reference in their entirety for all purposes.

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  $\beta$ -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, cerebroside, 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 “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 such as an interfering RNA (e.g., siRNA) 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 such as an interfering RNA (e.g., siRNA) directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site 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.

### III. Description of the Embodiments

The present invention provides novel, serum-stable lipid particles comprising one or more therapeutic nucleic acids, 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 certain embodiments, the therapeutic nucleic acid comprises an interfering RNA molecule such as, e.g., an siRNA, Dicer-substrate dsRNA, shRNA, aiRNA, miRNA, or mixtures thereof. In preferred embodiment, the interfering RNA targets a gene of interest in the liver. Examples of target genes of interest that are in the liver include, but are not limited to, APOB, APOC3, PCSK9, DGAT1, DGAT2, and combinations thereof.

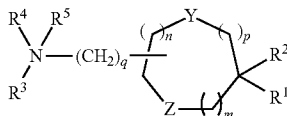


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In one aspect, the present invention provides a nucleic acid-lipid particle (e.g., SNALP) comprising:

(a) an interfering RNA that silences Apolipoprotein B (APOB) expression and/or the expression of another liver target gene such as APOC3, PCSK9, DGAT1, and/or DGAT2;

(b) a cationic lipid of Formula I having the following structure:



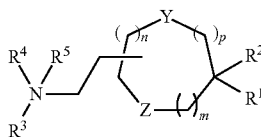
or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkenyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkynyl, or optionally substituted C<sub>12</sub>-C<sub>24</sub> acyl, with the proviso that at least one of R<sup>1</sup> and R<sup>2</sup> has at least two sites of unsaturation; R<sup>3</sup> and R<sup>4</sup> are either the same or different and are independently optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, optionally substituted C<sub>2</sub>-C<sub>6</sub> alkenyl, or optionally substituted C<sub>2</sub>-C<sub>6</sub> alkynyl or R<sup>3</sup> and R<sup>4</sup> 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<sup>5</sup> is either absent or hydrogen or C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; m, n and p are either the same or different and are independently either 0, 1 or 2, with the proviso that m, n, and p are not simultaneously 0; q is 0, 1, 2, 3, or 4; Y and Z are either the same or different and are independently O, S, or NH; and

(c) a non-cationic lipid.

In another aspect, the present invention provides a nucleic acid-lipid particle (e.g., SNALP) comprising:

(a) an interfering RNA that silences Apolipoprotein B (APOB) expression and/or the expression of another liver target gene such as APOC3, PCSK9, DGAT1, and/or DGAT2;

(b) a cationic lipid of Formula II having the following structure:



or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkenyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkynyl, or optionally substituted C<sub>12</sub>-C<sub>24</sub> acyl; R<sup>3</sup> and R<sup>4</sup> are either the same or different and are independently optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, optionally substituted C<sub>2</sub>-C<sub>6</sub> alkenyl, or optionally substituted C<sub>2</sub>-C<sub>6</sub> alkynyl or R<sup>3</sup> and R<sup>4</sup> 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<sup>5</sup> is either absent or is hydrogen or C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; m, n, and p are either the same or different and are

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independently either 0, 1 or 2, with the proviso that m, n, and p are not simultaneously 0; Y and Z are either the same or different and are independently O, S, or NH; and

(c) a non-cationic lipid.

In some embodiments, cationic lipids falling within the scope of Formulas I and/or II that are useful in the nucleic acid-lipid particles of the present invention (e.g., SNALP) include, but are not limited to, the following: 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2" or "C2K"), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-K-C3-DMA; "C3K"), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-K-C4-DMA; "C4K"), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K6-DMA), 2,2-dilinoleyl-4-N-methylpiperazine-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 2,2-di-oleoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DO-K-DMA), 2,2-distearoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DS-K-DMA), 2,2-dilinoleyl-4-N-morpholino-[1,3]-dioxolane (DLin-K-MMA), 2,2-Dilinoleyl-4-trimethylamino-[1,3]-dioxolane chloride (DLin-K-TMA.Cl), 2,2-dilinoleyl-4,5-bis(dimethyl aminomethyl)-[1,3]-dioxolane (DLin-K<sup>2</sup>-DMA), 2,2-dilinoleyl-4-methylpiperazine-[1,3]-dioxolane (D-Lin-K-N-methylpiperazine), analogs thereof, salts thereof, and mixtures thereof. In preferred embodiments, the cationic lipid comprises DLin-K-C2-DMA ("C2K").

In particular embodiments, the interfering RNA (e.g., siRNA) that targets APOB and/or other target genes such as APOC3, PCSK9, DGAT1, and/or DGAT2 comprises a sense strand and a complementary antisense strand, and the interfering RNA comprises a double-stranded region of about 15 to about 60 nucleotides in length (e.g., about 15-60, 15-30, 15-25, 19-30, 19-25, 20-60, 20-55, 20-50, 20-45, 20-40, 20-35, 20-30, 20-25, 21-30, 21-29, 22-30, 22-29, 22-28, 23-30, 23-28, 24-30, 24-28, 25-60, 25-55, 25-50, 25-45, 25-40, 25-35, or 25-30 nucleotides in length, or about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 nucleotides in length). In one embodiment, the interfering RNA is chemically synthesized. The interfering RNA molecules of the invention are capable of silencing the expression of a target sequence such as APOB in vitro and/or in vivo.

In certain embodiments, the interfering RNA (e.g., siRNA) of the present invention may comprise at least one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides such as 2'OMe nucleotides, e.g., in the sense and/or antisense strand of the double-stranded region of the interfering RNA. Preferably, uridine and/or guanosine nucleotides in the interfering RNA are modified with 2'OMe nucleotides. In certain instances, the interfering RNA contains 2'OMe nucleotides in both the sense and antisense strands and comprises at least one 2'OMe-uridine nucleotide and at least one 2'OMe-guanosine nucleotide in the double-stranded region. In some embodiments, the sense and/or antisense strand of the interfering RNA may further comprise modified (e.g., 2'OMe-modified) adenosine and/or modified (e.g., 2'OMe-modified) cytosine nucleotides, e.g., in the double-stranded region of the interfering RNA.

In some embodiments, the sense and/or antisense strand sequences may comprise at least one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides such as 2'OMe nucleotides. In certain embodiments, the sense and/or antisense strand sequences may each independently comprise or consist of a modified (e.g., 2'OMe) and/or

unmodified 3' overhang of 1, 2, 3, or 4 nucleotides, or one or both ends of the double-stranded molecule may be blunt-ended.

One of skill in the art will understand that unmodified sense and/or antisense strand sequences can be modified in accordance with the selective modification patterns described herein (e.g., at selective uridine and/or guanosine nucleotides, and optionally at adenosine and/or cytosine nucleotides, within the RNA duplex), and screened for RNAi activity as well as immune stimulation, such that the degree of chemical modifications introduced into the interfering RNA molecule strikes a balance between reduction or abrogation of the immunostimulatory properties of the interfering RNA and retention of RNAi activity.

In particular embodiments, the interfering RNA (e.g., siRNA) molecules of the present invention comprise a 3' overhang of 1, 2, 3, or 4 nucleotides in one or both strands. In certain instances, the interfering RNA may contain at least one blunt end. In particular embodiments, the 3' overhangs in one or both strands of the interfering RNA may each independently comprise 1, 2, 3, or 4 modified and/or unmodified deoxythymidine ("T" or "dT") nucleotides, 1, 2, 3, or 4 modified (e.g., 2'OMe) and/or unmodified uridine ("U") ribonucleotides, or 1, 2, 3, or 4 modified (e.g., 2'OMe) and/or unmodified ribonucleotides or deoxyribonucleotides having complementarity to the target sequence or the complementary strand thereof.

In another embodiment, the present invention provides a composition comprising a cocktail (e.g., at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more) of unmodified and/or modified interfering RNA (e.g., siRNA) sequences that target APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 expression. The cocktail of interfering RNA (e.g., 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 particular embodiments, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more (e.g., all) of these sequences are chemically modified (e.g., 2'OMe-modified) as described herein.

In certain embodiments, the sense strand comprises or consists of a sequence that is at least about 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to the target sequence or a portion thereof. In certain other embodiments, the sense strand comprises or consists of at least about 15 contiguous nucleotides (e.g., at least about 15, 16, 17, 18, or 19 contiguous nucleotides) of a sequence that is identical to the target sequence or a portion thereof. In preferred embodiments, the interfering RNA (e.g., siRNA) comprising such a sense strand sequence is capable of mediating target-specific RNAi.

In some embodiments, the antisense strand comprises or consists of a sequence that is at least about 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% complementary to the target sequence or a portion thereof. In other embodiments, the antisense strand comprises or consists of at least about 15 contiguous nucleotides (e.g., at least about 15, 16, 17, 18, or 19 contiguous nucleotides) of a sequence that is complementary to the target sequence or a portion thereof. In further embodiments, the antisense strand comprises or consists of a sequence that specifically hybridizes to the target sequence or a portion thereof. In preferred embodiments, the interfering RNA (e.g., siRNA) comprising such an antisense strand sequence is capable of mediating target-specific RNAi.

In one preferred embodiment, the APOB siRNA comprises an antisense strand comprising the following sequence:

5'-UAUUCAGUGUGAUGACACU-3' (SEQ ID NO:13). In another preferred embodiment, the APOB siRNA further comprises a sense strand comprising the following sequence: 5'-AGUGUCAUCACACUGAAUA-3' (SEQ ID NO:14). In some embodiments, the APOB siRNA comprises at least one 2'OMe nucleotide, e.g., at least one 2'OMe-guanosine and/or 2'OMe-uridine nucleotide. In certain instances, the APOB siRNA comprises an antisense strand comprising at least one, at least two, at least three, at least four, at least five, at least six, at least seven, or more 2'OMe nucleotides, e.g., 2'OMe-guanosine and/or 2'OMe-uridine nucleotides. In certain other instances, the APOB siRNA comprises a sense strand comprising at least one, at least two, at least three, at least four, at least five, at least six, at least seven, or more 2'OMe nucleotides, e.g., 2'OMe-guanosine and/or 2'OMe-uridine nucleotides.

In particular embodiments, from about 20%-40%, 25%-40%, 30%-40%, 20%-35%, 25%-35%, 20%-30%, 25%-30%, 26%-34%, 27%-33%, 28%-32%, or about 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, or 40% of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides such as, e.g., 2'OMe nucleotides (e.g., 2'OMe-guanosine and/or 2'OMe-uridine nucleotides).

In some embodiments, the APOB siRNA of the invention comprises a 3' overhang in one or both strands of the siRNA. In one particular embodiment, the antisense strand comprises a 5'-UC-3' overhang and the sense strand comprises a 5'-CC-3' overhang. In certain instances, the 3' overhangs on one or both strands of the siRNA comprise at least one 2'OMe nucleotide, e.g., at least one 2'OMe-guanosine and/or 2'OMe-uridine nucleotide. In other embodiments, the 3' overhangs on one or both strands of the siRNA molecule comprise 1-4 deoxythymidine (dT) nucleotides, 1-4 modified and/or unmodified uridine (U) ribonucleotides, or 1-2 additional ribonucleotides having complementarity to the target sequence or the complementary strand thereof.

In a first embodiment, the APOB siRNA comprises the following sense strand sequence: 5'-AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO:1) ("**5-1**"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides. In a second embodiment, the APOB siRNA comprises the following sense strand sequence: 5'-AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO:3) ("**S-2**"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides. In a third embodiment, the APOB siRNA comprises the following sense strand sequence: 5'-AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO:4) ("**S-3**"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides. In a fourth embodiment, the APOB siRNA comprises the following sense strand sequence: 5'-AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO:5) ("**S-4**"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides. In a fifth embodiment, the APOB siRNA comprises the following sense strand sequence: 5'-AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO:6) ("**S-5**"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides. In a sixth embodiment, the APOB siRNA comprises the following sense strand sequence: 5'-AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO:7) ("**S-6**"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In a first embodiment, the APOB siRNA comprises the following antisense strand sequence: 5'-UAUUCAGUGAUGACACUUG-3' (SEQ ID NO:2) ("**AS-1**"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides. In a second embodiment, the APOB siRNA comprises the following antisense strand sequence: 5'-UAUUCAGUGAUGAUGACACUUG-3' (SEQ ID NO:8) ("**AS-2**"), wherein the bolded and underlined nucleotides are 2'OMe nucle-

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otides. In a third embodiment, the APOB siRNA comprises the following antisense strand sequence: 5'-UAUUCAGUGU GAUGACACUUG-3' (SEQ ID NO:9) (“AS-3”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides. In a fourth embodiment, the APOB siRNA comprises the following antisense strand sequence: 5'-UAUUCAGUGU GAUGACACUUG-3' (SEQ ID NO:10) (“AS-4”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides. In a fifth embodiment, the APOB siRNA comprises the following antisense strand sequence: 5'-UAUUCAG UGUGAUGACACUUG-3' (SEQ ID NO:11) (“AS-5”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides. In a sixth embodiment, the APOB siRNA comprises the following antisense strand sequence: 5'-UAUUCAG UGUGAUGACACUUG-3' (SEQ ID NO:12) (“AS-6”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides.

In one preferred embodiment, the APOB siRNA comprises: an antisense strand comprising the sequence 5'-UAUUCAGUGAUGACACU-3' (SEQ ID NO:13) and at least one, two, three, four, five, six, or more 2'OMe nucleotides, e.g., at least one, two, three, four, five, six, or more 2'OMe-guanosine and/or 2'OMe-uridine nucleotides; and a sense strand comprising the sequence 5'-AGUGUCAUCA CACUGAAUA-3' (SEQ ID NO:14) and at least one, two, three, four, five, six, or more 2'OMe nucleotides, e.g., at least one, two, three, four, five, six, or more 2'OMe-guanosine and/or 2'OMe-uridine nucleotides. In another preferred embodiment, the APOB siRNA of the invention comprises: a sense strand comprising nucleotides 1-19 of S-1, S-2, S-3, S-4, S-5, or S-6; and an antisense strand comprising nucleotides 1-19 of AS-1, AS-2, AS-3, AS-4, AS-5, or AS-6. In a particularly preferred embodiment, the APOB siRNA consists of: a sense strand selected from S-1, S-2, S-3, S-4, S-5, and S-6; and an antisense strand selected from AS-1, AS-2, AS-3, AS-4, AS-5, and AS-6.

In one particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 1)  
5' -AGUGUCAUCACACUGAAUACC-3'

(SEQ ID NO: 2)  
3' -GUUCACAGUAGUGGACUUUAU-5'

(“S-1+AS-1”, “1/1”, or “ApoB-8”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides.

In another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 3)  
5' -AGUGUCAUCACACUGAAUACC-3'

(SEQ ID NO: 8)  
3' -GUUCACAGUAGUGGACUUUAU-5'

(“S-2+AS-2” or “2/2”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides.

In yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 3)  
5' -AGUGUCAUCACACUGAAUACC-3'

(SEQ ID NO: 9)  
3' -GUUCACAGUAGUGGACUUUAU-5'

(“S-2+AS-3” or “2/3”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides.

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In still yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 4)  
5' -AGUGUCAUCACACUGAAUACC-3'

(SEQ ID NO: 8)  
3' -GUUCACAGUAGUGGACUUUAU-5'

(“S-3+AS-2” or “3/2”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides.

In another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 4)  
5' -AGUGUCAUCACACUGAAUACC-3'

(SEQ ID NO: 9)  
3' -GUUCACAGUAGUGGACUUUAU-5'

(“S-3+AS-3” or “3/3”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides.

In yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 5)  
5' -AGUGUCAUCACACUGAAUACC-3'

(SEQ ID NO: 8)  
3' -GUUCACAGUAGUGGACUUUAU-5'

(“S-4+AS-2” or “4/2”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides.

In still yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 5)  
5' -AGUGUCAUCACACUGAAUACC-3'

(SEQ ID NO: 9)  
3' -GUUCACAGUAGUGGACUUUAU-5'

(“S-4+AS-3” or “4/3”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides.

In another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 6)  
5' -AGUGUCAUCACACUGAAUACC-3'

(SEQ ID NO: 8)  
3' -GUUCACAGUAGUGGACUUUAU-5'

(“S-5+AS-2” or “5/2”), wherein the **bolded** and underlined nucleotides are 2'OMe nucleotides.

In yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

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(SEQ ID NO: 6)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 9)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-5+AS-3" or "5/3"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In still yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 7)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 8)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-6+AS-2" or "6/2"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 7)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 9)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-6+AS-3" or "6/3"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 3)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 10)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-2+AS-4" or "2/4"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In still yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 3)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 11)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-2+AS-5" or "2/5"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 3)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 12)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-2+AS-6" or "2/6"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

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(SEQ ID NO: 4)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 10)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-3+AS-4", or "3/4"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In still yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 4)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 11)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-3+AS-5", "3/5", or "ApoB-10"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 4)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 12)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-3+AS-6" or "3/6"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 5)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 10)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-4+AS-4" or "4/4"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In still yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 5)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 11)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-4+AS-5" or "4/5"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 5)  
5' - **AGUGUCAU**CACACUGAAUACC - 3'

(SEQ ID NO: 12)  
3' - GUUCACAGUAGUG**GACUU**AU - 5'

("S-4+AS-6" or "4/6"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

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(SEQ ID NO: 6)  
5' - **AGUGUCAU**CACACU**GAAU**ACC-3'

(SEQ ID NO: 10)  
3' - **GUUCACAGUAGUG****GACUU**AU-5',

("S-5+AS-4" or "5/4"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In still yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 6)  
5' - **AGUGUCAU**CACACU**GAAU**ACC-3'

(SEQ ID NO: 11)  
3' - **GUUCACAGUAGUG****GACUU**AU-5',

("S-5+AS-5" or "5/5"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 6)  
5' - **AGUGUCAU**CACACU**GAAU**ACC-3'

(SEQ ID NO: 12)  
3' - **GUUCACAGUAGUG****GACUU**AU-5',

("S-5+AS-6" or "5/6"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 7)  
5' - **AGUGUCAU**CACACU**GAAU**ACC-3'

(SEQ ID NO: 10)  
3' - **GUUCACAGUAGUG****GACUU**AU-5',

("S-6+AS-4" or "6/4"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In still yet another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 7)  
5' - **AGUGUCAU**CACACU**GAAU**ACC-3'

(SEQ ID NO: 11)  
3' - **GUUCACAGUAGUG****GACUU**AU-5',

("S-6+AS-5" or "6/5"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

In another particular embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 7)  
5' - **AGUGUCAU**CACACU**GAAU**ACC-3'

(SEQ ID NO: 12)  
3' - **GUUCACAGUAGUG****GACUU**AU-5',

("S-6+AS-6" or "6/6"), wherein the bolded and underlined nucleotides are 2'OMe nucleotides.

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In a further embodiment, the APOB siRNA consists of the following sense and antisense strand sequences:

(SEQ ID NO: 17)  
5' - GUCAUCACACUGAAU**ACCAU**-3'

(SEQ ID NO: 18)  
3' - CACAGUAGUG**GACUU**AUGGUUA-5'.

It will be readily apparent to those of skill in the art that the foregoing APOB siRNA can also be chemically modified, if desired, to reduce its immunostimulatory properties, while maintaining its silencing activities.

The nucleic acid-lipid particles (e.g., SNALP) typically comprise one or more (e.g., a cocktail) of the interfering RNAs described herein, a cationic lipid, and a non-cationic lipid. In certain instances, the nucleic acid-lipid particles (e.g., SNALP) further comprise a conjugated lipid that inhibits aggregation of particles. Preferably, the nucleic acid-lipid particles (e.g., SNALP) comprise one or more (e.g., a cocktail) of the interfering RNAs described herein, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In particular embodiments, the nucleic acid-lipid particles (e.g., SNALP) of the invention comprise 1, 2, 3, 4, 5, 6, 7, 8, or more unmodified and/or modified interfering RNAs that silence 1, 2, 3, 4, 5, 6, 7, 8, or more different genes associated with liver diseases or disorders, a cationic lipid, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles.

In some embodiments, the interfering RNAs (e.g., siRNAs) are fully encapsulated in the nucleic acid-lipid particle (e.g., SNALP). With respect to formulations comprising an interfering RNA cocktail, the different types of interfering RNA species present in the cocktail (e.g., interfering RNA compounds with different sequences) may be co-encapsulated in the same particle, or each type of interfering RNA species present in the cocktail may be encapsulated in a separate particle. The interfering RNA cocktail may be formulated in the particles described herein using a mixture of two or more individual interfering RNAs (each having a unique sequence) at identical, similar, or different concentrations or molar ratios. In one embodiment, a cocktail of interfering RNAs (corresponding to a plurality of interfering RNAs with different sequences) is formulated using identical, similar, or different concentrations or molar ratios of each interfering RNA species, and the different types of interfering RNAs are co-encapsulated in the same particle. In another embodiment, each type of interfering RNA species present in the cocktail is encapsulated in different particles at identical, similar, or different interfering RNA concentrations or molar ratios, and the particles thus formed (each containing a different interfering RNA payload) are administered separately (e.g., at different times in accordance with a therapeutic regimen), or are combined and administered together as a single unit dose (e.g., with a pharmaceutically acceptable carrier). The particles described herein are serum-stable, are resistant to nuclease degradation, and are substantially non-toxic to mammals such as humans.

The cationic lipid in the nucleic acid-lipid particles of the invention (e.g., SNALP) may comprise, e.g., one or more cationic lipids of Formula I and II described herein and/or any other cationic lipid species. In one particular embodiment, the cationic lipid comprises 2,2-dilinoleyl-4-(2-dimethylaminoethyl)[1,3]-dioxolane (DLin-K-C2-DMA or "C2K").

In addition to the cationic lipids of Formula I and II, the cationic lipids in the nucleic acid-lipid particles of the invention (e.g., SNALP) may comprise, e.g., one or more cationic

lipids of Formula III-XIV (or salts thereof) described herein, either alone or in combination with other known cationic lipids. In one particular embodiment, the cationic lipid comprises 2,2-dilinoleyl-4-(2-dimethylaminoethyl)[1,3]-dioxolane (DLin-K-C2-DMA), 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-K-DMA), 1,2-di- $\gamma$ -linolenyloxy-N,N-dimethylaminopropane ( $\gamma$ -DLenDMA), or a mixture thereof.

The non-cationic lipid in the nucleic acid-lipid particles of the present invention (e.g., SNALP) may comprise, e.g., one or more anionic lipids and/or neutral lipids. In some embodiments, the non-cationic lipid comprises one of the following neutral lipid components: (1) a mixture of a phospholipid and cholesterol or a derivative thereof; (2) cholesterol or a derivative thereof; or (3) a phospholipid. In certain preferred embodiments, the phospholipid comprises dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), or a mixture thereof. In a particularly preferred embodiment, the non-cationic lipid is a mixture of DPPC and cholesterol.

The lipid conjugate in the nucleic acid-lipid particles of the invention (e.g., SNALP) inhibits aggregation of particles and may comprise, e.g., one or more of the lipid conjugates described herein. In one particular embodiment, the lipid conjugate comprises a PEG-lipid conjugate. Examples of PEG-lipid conjugates include, but are not limited to, PEG-DAG conjugates, PEG-DAA conjugates, and mixtures thereof. In certain embodiments, the PEG-DAA conjugate in the lipid particle may comprise a PEG-didecyloxypropyl (C<sub>10</sub>) conjugate, a PEG-dilauryloxypropyl (C<sub>12</sub>) conjugate, a PEG-dimyristyloxypropyl (C<sub>14</sub>) conjugate, a PEG-dipalmitoyloxypropyl (C<sub>16</sub>) conjugate, a PEG-distearoyloxypropyl (C<sub>18</sub>) conjugate, or mixtures thereof. In another embodiment, the lipid conjugate comprises a POZ-lipid conjugate such as a POZ-DAA conjugate.

In exemplary aspects of these embodiments, the cationic lipid is DLin-K-C2-DMA ("C2K"), the non-cationic lipid is a mixture of a phospholipid (e.g., DPPC) and cholesterol, and the PEG-lipid conjugate is a PEG-DAA conjugate such as a PEG2000-DMA and/or a PEG750-DMA conjugate. In a particularly preferred embodiment, the APOB siRNA is APOB siRNA 3/5 ("ApoB-10"), the cationic lipid is DLin-K-C2-DMA ("C2K"), the non-cationic lipid is a mixture of a phospholipid (e.g., DPPC) and cholesterol, and the PEG-lipid conjugate is a PEG-DMA conjugate such as PEG2000-C-DMA.

In certain embodiments, the present invention provides nucleic acid-lipid particles (e.g., SNALP) comprising: (a) one or more interfering RNA molecules that target APOB expression and/or the expression of other liver target genes such as APOC3, PCSK9, DGAT1, DGAT2, or combinations thereof; (b) one or more cationic lipids of Formula I-XIV or salts thereof 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 one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) an interfering RNA that targets APOB expression and/or the expression of another liver target gene such as APOC3, PCSK9, DGAT1, or DGAT2; (b) a cationic lipid of Formula I-XIV or a salt thereof 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 embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:57" formulation. In one particular embodiment, the 1:57 formulation is a four-component system comprising about 1.4 mol % PEG-lipid conjugate (e.g., PEG2000-C-DMA), about 57.1 mol % cationic lipid of Formula I-XIV or a salt thereof, about 7.1 mol % DPPC (or DSPC), and about 34.3 mol % cholesterol (or derivative thereof). In certain embodiments of the 1:57 formulation, the cationic lipid is DLin-K-C2-DMA.

In another aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) an interfering RNA that targets APOB expression and/or the expression of another liver target gene such as APOC3, PCSK9, DGAT1, or DGAT2; (b) a cationic lipid of Formula I-XIV or a salt thereof 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 embodiment of nucleic acid-lipid particle is generally referred to herein as the "1:62" formulation. In one particular embodiment, the 1:62 formulation is a three-component system which is phospholipid-free and comprises about 1.5 mol % PEG-lipid conjugate (e.g., PEG2000-C-DMA), about 61.5 mol % cationic lipid of Formula I-XIV or a salt thereof, and about 36.9 mol % cholesterol (or derivative thereof). In certain embodiments of the 1:62 formulation, the cationic lipid is DLin-K-C2-DMA.

Additional embodiments related to the 1:57 and 1:62 formulations are described in PCT Publication No. WO 09/127, 060 and U.S. application Ser. No. 12/794,701, filed Jun. 4, 2010, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

In other embodiments, the present invention provides nucleic acid-lipid particles (e.g., SNALP) comprising: (a) one or more interfering RNA molecules that target APOB expression and/or the expression of other liver target genes such as APOC3, PCSK9, DGAT1, DGAT2, or combinations thereof; (b) one or more cationic lipids of Formula I-XIV or salts thereof comprising from about 2 mol % to about 50 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 5 mol % to about 90 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 20 mol % of the total lipid present in the particle.

In one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) an interfering RNA that targets APOB expression and/or the expression of another liver target gene such as APOC3, PCSK9, DGAT1, or DGAT2; (b) a cationic lipid of Formula I-XIV or a salt thereof comprising from about 30 mol % to about 50 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 47 mol % to about 69 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 1 mol % to about 3 mol % of the total lipid present in the particle. This embodiment of nucleic acid-lipid particle is generally referred to herein as the "2:40" formulation. In one particular embodiment, the 2:40 formulation is a four-component system which comprises about 2 mol % PEG-lipid conjugate (e.g., PEG2000-C-DMA), about 40 mol % cationic lipid of Formula I-XIV or a salt thereof, about 10 mol % DPPC (or DSPC), and about 48 mol % cholesterol (or derivative

thereof). In certain embodiments of the 2:40 formulation, the cationic lipid is DLin-K-C2-DMA.

In further embodiments, the present invention provides nucleic acid-lipid particles (e.g., SNALP) comprising: (a) one or more interfering RNA molecules that target APOB expression and/or the expression of other liver target genes such as APOC3, PCSK9, DGAT1, DGAT2, or combinations thereof; (b) one or more cationic lipids of Formula I-XIV or salts thereof comprising from about 50 mol % to about 65 mol % of the total lipid present in the particle; (c) one or more non-cationic lipids comprising from about 25 mol % to about 45 mol % of the total lipid present in the particle; and (d) one or more conjugated lipids that inhibit aggregation of particles comprising from about 5 mol % to about 10 mol % of the total lipid present in the particle.

In one aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) an interfering RNA that targets APOB expression and/or the expression of another liver target gene such as APOC3, PCSK9, DGAT1, or DGAT2; (b) a cationic lipid of Formula I-XIV or a salt thereof comprising from about 50 mol % to about 60 mol % of the total lipid present in the particle; (c) a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 35 mol % to about 45 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 5 mol % to about 10 mol % of the total lipid present in the particle. This embodiment of nucleic acid-lipid particle is generally referred to herein as the "7:54" formulation. In certain instances, the non-cationic lipid mixture in the 7:54 formulation comprises: (i) a phospholipid of from about 5 mol % to about 10 mol % of the total lipid present in the particle; and (ii) cholesterol or a derivative thereof of from about 25 mol % to about 35 mol % of the total lipid present in the particle. In one particular embodiment, the 7:54 formulation is a four-component system which comprises about 7 mol % PEG-lipid conjugate (e.g., PEG750-C-DMA), about 54 mol % cationic lipid of Formula I-XIV or a salt thereof, about 7 mol % DPPC (or DSPC), and about 32 mol % cholesterol (or derivative thereof). In certain embodiments of the 7:54 formulation, the cationic lipid is DLin-K-C2-DMA.

In another aspect of this embodiment, the nucleic acid-lipid particle comprises: (a) an interfering RNA that targets APOB expression and/or the expression of another liver target gene such as APOC3, PCSK9, DGAT1, or DGAT2; (b) a cationic lipid of Formula I-XIV or a salt thereof comprising from about 55 mol % to about 65 mol % of the total lipid present in the particle; (c) cholesterol or a derivative thereof comprising from about 30 mol % to about 40 mol % of the total lipid present in the particle; and (d) a PEG-lipid conjugate comprising from about 5 mol % to about 10 mol % of the total lipid present in the particle. This embodiment of nucleic acid-lipid particle is generally referred to herein as the "7:58" formulation. In one particular embodiment, the 7:58 formulation is a three-component system which is phospholipid-free and comprises about 7 mol % PEG-lipid conjugate (e.g., PEG750-C-DMA), about 58 mol % cationic lipid of Formula I-XIV or a salt thereof, and about 35 mol % cholesterol (or derivative thereof). In certain embodiments of the 7:58 formulation, the cationic lipid is DLin-K-C2-DMA.

Additional embodiments related to the 7:54 and 7:58 formulations are described in U.S. application Ser. No. 12/828,189, entitled "Novel Lipid Formulations for Delivery of Therapeutic Agents to Solid Tumors," filed Jun. 30, 2010, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

The present invention also provides pharmaceutical compositions comprising a nucleic acid-lipid particle such as a SNALP and a pharmaceutically acceptable carrier.

The nucleic acid-lipid particles of the invention are useful for the therapeutic delivery of interfering RNA (e.g., siRNA) molecules that silence the expression of one or more genes associated with liver diseases or disorders (e.g., APOB, APOC3, PCSK9, DGAT1, and/or DGAT2). In some embodiments, a cocktail of siRNAs that target one or more genes expressed in the liver is formulated into the same or different nucleic acid-lipid particles, and the particles are administered to a mammal (e.g., a human) requiring such treatment. In certain instances, a therapeutically effective amount of the nucleic acid-lipid particles can be administered to the mammal, e.g., for treating, preventing, reducing the risk of developing, or delaying the onset of a lipid disorder such as dyslipidemia (e.g., elevated triglyceride and/or cholesterol levels) or atherosclerosis.

Non-limiting examples of lipid disorders suitable for prevention and/or treatment with the nucleic acid-lipid particles of the invention (e.g., SNALP) include dyslipidemia (e.g., hyperlipidemias such as elevated triglyceride levels (hypertriglyceridemia) and/or elevated cholesterol levels (hypercholesterolemia)), atherosclerosis, low HDL-cholesterol, high LDL-cholesterol, coronary heart disease, coronary artery disease, atherosclerotic cardiovascular disease (CVD), fatty liver disease (hepatic steatosis), abnormal lipid metabolism, abnormal cholesterol metabolism, pancreatitis (e.g., acute pancreatitis associated with severe hypertriglyceridemia), diabetes (including Type 2 diabetes), obesity, cardiovascular disease, and other disorders relating to abnormal metabolism.

As described in the Examples below, it has surprisingly been found that the SNALP formulations of the present invention containing at least one cationic lipid of Formulas I-XIV, either alone or in combination with other cationic lipids, show increased potency when targeting a gene of interest in the liver, such as APOB, when compared to other SNALP formulations. Thus, the present invention provides methods for treating a disease or disorder associated with overexpression of APOB in a mammal (e.g., human) in need thereof, the method comprising administering to the mammal a therapeutically effective amount of a lipid particle (e.g., SNALP) comprising one or more interfering RNAs that silence APOB expression. Diseases and disorders associated with overexpression of APOB are described herein and include, but are not limited to, atherosclerosis, angina pectoris, high blood pressure, diabetes, and hypothyroidism. In certain instances, the mammal (e.g., human) has a disease or disorder involving hypercholesterolemia and serum cholesterol levels are lowered when expression of APOB is silenced by the interfering RNA.

In some embodiments, the interfering RNA (e.g., siRNA) molecules described herein are used in methods for silencing APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene expression, e.g., in a cell such as a liver cell. In particular, it is an object of the invention to provide methods for treating, preventing, reducing the risk of developing, or delaying the onset of a lipid disorder in a mammal by downregulating or silencing the transcription and/or translation of the APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene. In certain embodiments, the present invention provides a method for introducing one or more interfering RNA (e.g., siRNA) molecules described herein into a cell by contacting the cell with a nucleic acid-lipid particle described herein (e.g., a SNALP formulation). In one particular embodiment, the cell is a liver cell such as, e.g., a hepatocyte present in the liver tissue of a mammal (e.g., a human). In another embodiment, the present

invention provides a method for the in vivo delivery of one or more interfering RNA (e.g., siRNA) molecules described herein to a liver cell (e.g., hepatocyte) by administering to a mammal (e.g., human) a nucleic acid-lipid particle described herein (e.g., a SNALP formulation).

In some embodiments, the nucleic acid-lipid particles described herein (e.g., SNALP) are administered by one of the following routes of administration: oral, intranasal, intravenous, intraperitoneal, intramuscular, intra-articular, intralesional, intratracheal, subcutaneous, and intradermal. In particular embodiments, the nucleic acid-lipid particles are administered systemically, e.g., via enteral or parenteral routes of administration.

In particular embodiments, the nucleic acid-lipid particles of the invention (e.g., SNALP) can preferentially deliver a payload such as an interfering RNA (e.g., siRNA) to the liver as compared to other tissues, e.g., for the treatment of a liver disease or disorder such as dyslipidemia or atherosclerosis.

In certain aspects, the present invention provides methods for silencing APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene expression in a mammal (e.g., human) in need thereof, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (e.g., a SNALP formulation) comprising one or more interfering RNAs (e.g., siRNAs) described herein (e.g., one or more siRNAs targeting the APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene). In some embodiments, administration of nucleic acid-lipid particles comprising one or more siRNAs described herein reduces liver mRNA levels of the target gene (e.g., in a human or in an animal model such as a mouse model or monkey model) by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% (or any range therein) relative to liver mRNA levels of the target gene detected in the absence of the siRNA (e.g., buffer control or irrelevant siRNA control). In other embodiments, administration of nucleic acid-lipid particles comprising one or more siRNAs described herein reduces liver mRNA levels of the target gene (e.g., in a human or in an animal model such as a mouse model or monkey model) for at least 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, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 days or more (or any range therein) relative to a negative control such as, e.g., a buffer control or an irrelevant siRNA control.

In certain other aspects, the present invention provides methods for treating, preventing, reducing the risk or likelihood of developing (e.g., reducing the susceptibility to), delaying the onset of, and/or ameliorating one or more symptoms associated with a lipid disorder in a mammal (e.g., human) in need thereof, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (e.g., a SNALP formulation) comprising one or more interfering RNA molecules (e.g., siRNAs) described herein (e.g., one or more siRNAs targeting the APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene). Non-limiting examples of lipid disorders are described above and include dyslipidemia and atherosclerosis.

In a related aspect, the present invention provides a method for treating and/or ameliorating one or more symptoms associated with atherosclerosis or a dyslipidemia such as hyperlipidemia (e.g., elevated levels of triglycerides and/or cholesterol) in a mammal (e.g., human) in need thereof (e.g., a mammal with atheromatous plaques, elevated triglyceride levels, and/or elevated cholesterol levels), the method comprising administering to the mammal a therapeutically effective

amount of a nucleic acid-lipid particle (e.g., a SNALP formulation) comprising one or more interfering RNAs (e.g., siRNAs) described herein (e.g., one or more siRNAs targeting the APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene). In some embodiments, administration of nucleic acid-lipid particles comprising one or more siRNA molecules described herein reduces the level of atherosclerosis (e.g., decreases the size and/or number of atheromatous plaques or lesions) or blood (e.g., serum and/or plasma) triglyceride and/or cholesterol levels (e.g., in a human or in an animal model such as a mouse model or monkey model) by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (or any range therein) relative to the level of atherosclerosis, blood triglyceride levels, or blood cholesterol levels detected in the absence of the siRNA (e.g., buffer control or irrelevant siRNA control).

In another related aspect, the present invention provides a method for reducing the risk or likelihood of developing (e.g., reducing the susceptibility to) atherosclerosis or a dyslipidemia such as hyperlipidemia (e.g., elevated levels of triglycerides and/or cholesterol) in a mammal (e.g., human) at risk of developing atherosclerosis or dyslipidemia, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (e.g., a SNALP formulation) comprising one or more interfering RNAs (e.g., siRNAs) described herein (e.g., one or more siRNAs targeting the APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene). In some embodiments, administration of nucleic acid-lipid particles comprising one or more siRNA molecules described herein reduces the risk or likelihood of developing atherosclerosis or dyslipidemia (e.g., in a human or in an animal model such as a mouse model or monkey model) by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (or any range therein) relative to the risk or likelihood of developing atherosclerosis or dyslipidemia in the absence of the siRNA (e.g., buffer control or irrelevant siRNA control).

In yet another related aspect, the present invention provides a method for preventing or delaying the onset of atherosclerosis or a dyslipidemia such as hyperlipidemia (e.g., elevated levels of triglycerides and/or cholesterol) in a mammal (e.g., human) at risk of developing atherosclerosis or dyslipidemia, the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (e.g., a SNALP formulation) comprising one or more interfering RNAs (e.g., siRNAs) described herein (e.g., one or more siRNAs targeting the APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene).

In a further related aspect, the present invention provides a method for lowering or reducing cholesterol levels in a mammal (e.g., human) in need thereof (e.g., a mammal with elevated blood cholesterol levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (e.g., a SNALP formulation) comprising one or more interfering RNAs (e.g., siRNAs) described herein (e.g., one or more siRNAs targeting the APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene). In particular embodiments, administration of nucleic acid-lipid particles (e.g., SNALP) comprising one or more siRNA molecules described herein lowers or reduces blood (e.g., serum and/or plasma) cholesterol levels. In some embodiments, administration of nucleic acid-lipid particles (e.g., SNALP) comprising one or more siRNAs described herein reduces blood cholesterol levels (e.g., in a human or in an animal model such as a mouse model or monkey model) by at



least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (or any range therein) relative to blood cholesterol levels detected in the absence of the siRNA (e.g., buffer control or irrelevant siRNA control). In certain instances, administration of nucleic acid-lipid particles (e.g., SNALP) comprising one or more siRNA molecules described herein elevates HDL-cholesterol levels and/or reduces LDL-cholesterol levels.

In another related aspect, the present invention provides a method for lowering or reducing triglyceride levels in a mammal (e.g., human) in need thereof (e.g., a mammal with elevated blood triglyceride levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (e.g., a SNALP formulation) comprising one or more interfering RNAs (e.g., siRNAs) described herein (e.g., one or more siRNAs targeting the APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene). In particular embodiments, administration of nucleic acid-lipid particles (e.g., SNALP) comprising one or more siRNA molecules described herein lowers or reduces blood (e.g., serum and/or plasma) triglyceride levels. In certain embodiments, administration of nucleic acid-lipid particles comprising one or more siRNA molecules described herein reduces blood triglyceride levels (e.g., in a human or in an animal model such as a mouse model or monkey model) by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (or any range therein) relative to blood triglyceride levels detected in the absence of the siRNA (e.g., buffer control or irrelevant siRNA control). In other embodiments, administration of nucleic acid-lipid particles of the invention lowers or reduces hepatic (i.e., liver) triglyceride levels.

In an additional related aspect, the present invention provides a method for lowering or reducing glucose levels in a mammal (e.g., human) in need thereof (e.g., a mammal with elevated blood glucose levels), the method comprising administering to the mammal a therapeutically effective amount of a nucleic acid-lipid particle (e.g., a SNALP formulation) comprising one or more interfering RNAs (e.g., siRNAs) described herein (e.g., one or more siRNAs targeting the APOB, APOC3, PCSK9, DGAT1, and/or DGAT2 gene). In particular embodiments, administration of nucleic acid-lipid particles (e.g., SNALP) comprising one or more siRNAs described herein lowers or reduces blood (e.g., serum and/or plasma) glucose levels. In some embodiments, administration of nucleic acid-lipid particles comprising one or more siRNAs described herein reduces blood glucose levels (e.g., in a human or in an animal model such as a mouse model or monkey model) by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% (or any range therein) relative to blood glucose levels detected in the absence of the siRNA (e.g., buffer control or irrelevant siRNA control).

#### IV. Lipid Particles

The present invention provides lipid particles comprising one or more of the cationic (amino) lipids of Formula I-XIV as described herein. In some embodiments, the lipid particles of the invention further comprise one or more non-cationic lipids. In other embodiments, the lipid particles further comprise one or more conjugated lipids capable of reducing or inhibiting particle aggregation. In additional embodiments, the lipid particles further comprise one or more therapeutic nucleic acids (e.g., interfering RNA such as siRNA).

Lipid particles include, but are not limited to, lipid vesicles such as liposomes. As used herein, a lipid vesicle includes a structure having lipid-containing membranes enclosing an aqueous interior. In particular embodiments, lipid vesicles comprising one or more of the cationic lipids described herein are used to encapsulate nucleic acids within the lipid vesicles. In other embodiments, lipid vesicles comprising one or more of the cationic lipids described herein are complexed with nucleic acids to form lipoplexes.

The lipid particles of the present invention preferably comprise an active agent or therapeutic agent such as a therapeutic nucleic acid (e.g., an interfering RNA), a cationic lipid of Formula I-XIV, a non-cationic lipid, and a conjugated lipid that inhibits aggregation of particles. In some embodiments, the therapeutic nucleic acid is fully encapsulated within the lipid portion of the lipid particle such that the therapeutic nucleic acid 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 30 nm to about 150 nm, 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. The lipid particles of the invention also typically have a lipid:therapeutic agent (e.g., lipid:nucleic acid) ratio (mass/mass ratio) of from about 1:1 to about 100:1, from about 1:1 to about 50:1, from about 2:1 to about 25:1, from about 3:1 to about 20:1, from about 5:1 to about 15:1, or from about 5:1 to about 10:1.

In preferred embodiments, the lipid particles of the invention are serum-stable nucleic acid-lipid particles (SNALP) which comprise an interfering RNA (e.g., dsRNA such as siRNA, Dicer-substrate dsRNA, shRNA, aiRNA, and/or miRNA), a cationic lipid (e.g., one or more cationic lipids of Formula I-XIV or salts thereof as set forth herein), a non-cationic lipid (e.g., 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 (e.g., siRNA) that target one or more of the genes described herein. 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.

In the nucleic acid-lipid particles of the invention, the nucleic acid may be fully encapsulated within the lipid portion of the particle, thereby protecting the nucleic acid from nuclease degradation. In preferred embodiments, a SNALP comprising a nucleic acid such as an interfering RNA 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 nucleic acid is complexed with the lipid portion of the particle. One of the benefits of the formulations of the present invention is that the nucleic acid-lipid particle compositions are substantially non-toxic to mammals such as humans.

The term “fully encapsulated” indicates that the nucleic acid in the nucleic acid-lipid particle is not significantly degraded after exposure to serum or a nuclease assay that would significantly degrade free DNA or RNA. In a fully encapsulated system, preferably less than about 25% of the nucleic acid in the particle is degraded in a treatment that would normally degrade 100% of free nucleic acid, more preferably less than about 10%, and most preferably less than about 5% of the nucleic acid in the particle is degraded. “Fully encapsulated” also indicates that the nucleic acid-lipid particles are serum-stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

In the context of nucleic acids, full encapsulation may be determined by performing a membrane-impermeable fluorescent dye exclusion assay, which uses a dye that has enhanced fluorescence when associated with nucleic acid. Specific dyes such as OliGreen® and RiboGreen® (Invitrogen Corp.; Carlsbad, Calif.) are available for the quantitative determination of plasmid DNA, single-stranded deoxyribonucleotides, and/or single- or double-stranded ribonucleotides. Encapsulation is determined by adding the dye to a liposomal formulation, measuring the resulting fluorescence, and comparing it to the fluorescence observed upon addition of a small amount of nonionic detergent. Detergent-mediated disruption of the liposomal bilayer releases the encapsulated nucleic acid, allowing it to interact with the membrane-impermeable dye. Nucleic acid encapsulation may be calculated as  $E = (I_o - I) / I_o$ , where *I* and *I<sub>o</sub>* refer to the fluorescence intensities before and after the addition of detergent (see, Wheeler et al., *Gene Ther.*, 6:271-281 (1999)).

In other embodiments, the present invention provides a nucleic acid-lipid particle (e.g., SNALP) composition comprising a plurality of nucleic acid-lipid particles.

In some instances, the SNALP composition comprises nucleic acid that is fully encapsulated within the lipid portion of the particles, 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 particles have the nucleic acid encapsulated therein.

In other instances, the SNALP composition comprises nucleic acid that is fully encapsulated within the lipid portion of the particles, 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 input nucleic acid is encapsulated in the particles.

Depending on the intended use of the lipid particles of the invention, 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.

In particular embodiments, the present invention provides a lipid particle (e.g., SNALP) composition comprising a plurality of lipid particles described herein and an antioxidant. In certain instances, the antioxidant in the lipid particle composition reduces, prevents, and/or inhibits the degradation of a cationic lipid present in the lipid particle. In instances wherein the active agent is a therapeutic nucleic acid such as an interfering RNA (e.g., siRNA), the antioxidant in the lipid particle composition reduces, prevents, and/or inhibits the degradation of the nucleic acid payload, e.g., by reducing, preventing, and/or inhibiting the formation of adducts between the nucleic acid and the cationic lipid. Non-limiting examples of antioxidants include hydrophilic antioxidants such as chelating agents (e.g., metal chelators such as ethylenediaminetetraacetic acid (EDTA), citrate, and the like), lipophilic antioxidants (e.g., vitamin E isomers, polyphenols, and the like), salts thereof; and mixtures thereof. If needed, the antioxidant is typically present in an amount sufficient to prevent, inhibit, and/or reduce the degradation of the cationic lipid and/or active agent present in the particle, e.g., at least about 20 mM EDTA or a salt thereof, or at least about 100 mM citrate or a salt thereof. An antioxidant such as EDTA and/or citrate may be included at any step or at multiple steps in the lipid particle formation process described in Section V (e.g., prior to, during, and/or after lipid particle formation).

Additional embodiments related to methods of preventing the degradation of cationic lipids and/or active agents (e.g., therapeutic nucleic acids) present in lipid particles, compositions comprising lipid particles stabilized by these methods, methods of making these lipid particles, and methods of delivering and/or administering these lipid particles are described in U.S. Provisional Application No. 61/265,671, entitled “SNALP Formulations Containing Antioxidants,” filed Dec. 1, 2009, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

#### A. Therapeutic Nucleic Acids

The 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. Similarly, when used to treat diseases and disorders involving hypercholesterolemia, the nucleic acid, such as the interfering RNA, can be administered alone or co-administered (i.e., concurrently or consecutively) with conventional agents used to treat, e.g., a disease or disorder involving hypercholesterolemia. Such agents include statins such as, e.g., Lipitor®, Mevacor®, Zocor®, Lescol®, Crestor®, and Advicor®.

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 nucleic acid-lipid 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. In preferred embodiments, the nucleic acids are double-stranded RNA. Examples of double-stranded RNA are described herein and include, e.g., siRNA and other RNAi agents such as Dicer-substrate dsRNA, shRNA, aiRNA, and pre-miRNA. In other preferred embodiments, the nucleic acids are single-stranded nucleic acids. Single-stranded nucleic acids include, e.g., antisense oligonucleotides, ribozymes, mature miRNA, and triplex-forming oligonucleotides. In further embodiments, the nucleic acids are double-stranded DNA. Examples of double-stranded DNA include, e.g., DNA-DNA hybrids comprising a DNA sense strand and a DNA antisense strand as described in PCT Publication No. WO 2004/104199, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

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.

#### a) 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, such as APOB, APOC3, PCSK9, DGAT1, DGAT2, or combinations thereof. 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. The modified nucleotides can be present in one strand (i.e., sense or antisense) or both strands of the siRNA. In some preferred embodiments, one or more of the uridine and/or guanosine nucleotides are modified (e.g., 2'OMe-modified) in one strand (i.e., sense or antisense) or both strands of the siRNA. In these embodiments, the modified siRNA can further comprise one or more modified (e.g., 2'OMe-modified) adenosine and/or modified (e.g., 2'OMe-modified) cytosine nucleotides. In other preferred embodiments, only uridine and/or guanosine nucleotides are modified (e.g., 2'OMe-modified) 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).

In particular embodiments, the selective incorporation of modified nucleotides such as 2'OMe uridine and/or guanosine nucleotides into the double-stranded region of either or both strands of the siRNA reduces or completely abrogates the immune response to that siRNA molecule. In certain instances, the immunostimulatory properties of specific siRNA sequences and their ability to silence gene expression can be balanced or optimized by the introduction of minimal and selective 2'OMe modifications within the double-stranded region of the siRNA duplex. This can be achieved at therapeutically viable siRNA doses without cytokine induction, toxicity, and off-target effects associated with the use of unmodified siRNA.

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 certain other embodiments, some or all of the modified nucleotides in the double-stranded region of the siRNA are 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more nucleotides apart from each other. In one preferred embodiment, none of the modified nucleotides in the double-stranded region of the siRNA are adjacent to each other (e.g., there is a gap of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 unmodified nucleotides between each modified nucleotide). In another preferred embodiment, at least two of the modified nucleotides in the double-stranded region of the siRNA are adjacent to each other (e.g., there are no unmodified nucleotides between two or more modified nucle-

otides). In other preferred embodiments, at least three, at least four, or at least five of the modified nucleotides in the double-stranded region of the siRNA are adjacent to each other.

In some embodiments, less than about 50% (e.g., less than about 49%, 48%, 47%, 46%, 45%, 44%, 43%, 42%, 41%, 40%, 39%, 38%, 37%, or 36%, preferably less than about 35%, 34%, 33%, 32%, 31%, or 30%) of the nucleotides in the double-stranded region of the siRNA comprise modified (e.g., 2'OMe) nucleotides. In one aspect of these embodiments, less than about 50% of the uridine and/or guanosine nucleotides in the double-stranded region of one or both strands of the siRNA are selectively (e.g., only) modified. In another aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein 2'OMe-guanosine nucleotides and 2'OMe-uridine nucleotides are the only 2'OMe nucleotides present in the double-stranded region. In yet another aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In a further aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In another aspect of these embodiments, less than about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the 2'OMe nucleotides in the double-stranded region are not adjacent to each other.

In other embodiments, from about 1% to about 50% (e.g., from about 5%-50%, 10%-50%, 15%-50%, 20%-50%, 25%-50%, 30%-50%, 35%-50%, 40%-50%, 45%-50%, 5%-45%, 10%-45%, 15%-45%; 20%-45%, 25%-45%, 30%-45%, 35%-45%, 40%-45%, 5%-40%, 10%-40%, 15%-40%, 20%-40%, 25%-40%, 25%-39%, 25%-38%, 25%-37%, 25%-36%, 26%-39%, 26%-38%, 26%-37%, 26%-36%, 27%-39%, 27%-38%, 27%-37%, 27%-36%, 28%-39%, 28%-38%, 28%-37%, 28%-36%, 29%-39%, 29%-38%, 29%-37%, 29%-36%, 30%-40%, 30%-39%, 30%-38%, 30%-37%, 30%-36%, 31%-39%, 31%-38%, 31%-37%, 31%-36%, 32%-39%, 32%-38%, 32%-37%, 32%-36%, 33%-39%, 33%-38%, 33%-37%, 33%-36%, 34%-39%, 34%-38%, 34%-37%, 34%-36%, 35%-40%, 5%-35%, 10%-35%, 15%-35%, 20%-35%, 21%-35%, 22%-35%, 23%-35%, 24%-35%, 25%-35%, 26%-35%, 27%-35%, 28%-35%, 29%-35%, 30%-35%, 31%-35%, 32%-35%, 33%-35%, 34%-35%, 30%-34%, 31%-34%, 32%-34%, 33%-34%, 30%-33%, 31%-33%, 32%-33%, 30%-32%, 31%-32%, 25%-34%, 25%-33%, 25%-32%, 25%-31%, 26%-34%, 26%-33%, 26%-32%, 26%-31%, 27%-34%,

27%-33%, 27%-32%, 27%-31%, 28%-34%, 28%-33%, 28%-32%, 28%-31%, 29%-34%, 29%-33%, 29%-32%, 29%-31%, 5%-30%, 10%-30%, 15%-30%, 20%-34%, 20%-33%, 20%-32%, 20%-31%, 20%-30%, 21%-30%, 22%-30%, 23%-30%, 24%-30%, 25%-30%, 25%-29%, 25%-28%, 25%-27%, 25%-26%, 26%-30%, 26%-29%, 26%-28%, 26%-27%, 27%-30%, 27%-29%, 27%-28%, 28%-30%, 28%-29%, 29%-30%, 5%-25%, 10%-25%, 15%-25%, 20%-29%, 20%-28%, 20%-27%, 20%-26%, 20%-25%, 5%-20%, 10%-20%, 15%-20%, 5%-15%, 10%-15%, or 5%-10%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides. In one aspect of these embodiments, from about 1% to about 50% of the uridine and/or guanosine nucleotides in the double-stranded region of one or both strands of the siRNA are selectively (e.g., only) modified. In another aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the siRNA, wherein the siRNA comprises at least one 2'OMe-guanosine nucleotide and at least one 2'OMe-uridine nucleotide, and wherein 2'OMe-guanosine nucleotides and 2'OMe-uridine nucleotides are the only 2'OMe nucleotides present in the double-stranded region. In yet another aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In a further aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the siRNA does not comprise 2'OMe-cytosine nucleotides in the double-stranded region. In another aspect of these embodiments, from about 1% to about 50% of the nucleotides in the double-stranded region of the siRNA comprise 2'OMe nucleotides, wherein the siRNA comprises 2'OMe nucleotides in both strands of the modified siRNA, wherein the siRNA comprises 2'OMe nucleotides selected from the group consisting of 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, and mixtures thereof, and wherein the 2'OMe nucleotides in the double-stranded region are not adjacent to each other.

In certain embodiments, the siRNA component of the nucleic acid-lipid particles of the present invention (e.g., SNALP) comprises an asymmetric siRNA duplex as described in PCT Publication No. WO 2004/078941, which comprises a double-stranded region consisting of a DNA sense strand and an RNA antisense strand (e.g., a DNA-RNA hybrid), wherein a blocking agent is located on the siRNA duplex. In some instances, the asymmetric siRNA duplex can be chemically modified as described herein. Other non-limiting examples of asymmetric siRNA duplexes are described in PCT Publication No. WO 2006/074108, which discloses self-protected oligonucleotides comprising a region having a sequence complementary to one, two, three, or more same or different target mRNA sequences (e.g., multivalent siRNAs) and one or more self-complementary regions. Yet other non-limiting examples of asymmetric siRNA duplexes are

described in PCT Publication No. WO 2009/076321, which discloses self-forming asymmetric precursor polynucleotides comprising a targeting region comprising a polynucleotide sequence complementary to a region of one, two, three, or more same or different target mRNA sequences (e.g., multivalent siRNAs); a first self-complementary region; and a second self-complementary region, wherein the first and second self-complementary regions are located one at each end of the targeting region and both self-complementary regions form stem-loop structures, wherein the first self-complementary region is capable of being cleaved by a RNase III endoribonuclease that is not a class IV DICER endoribonuclease, and wherein both self-complementary regions comprise a nucleotide sequence that is complementary to a region of the target gene sequence, but wherein a portion of the target sequence present in the targeting region does not have a complementary sequence in either of the self-complementary regions. The disclosures of each of the above patent documents are herein incorporated by reference in their entirety for all purposes.

Additional ranges, percentages, and patterns of modifications that may be introduced into siRNA are described in U.S. Patent Publication No. 20070135372, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

#### (1) 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).

As a non-limiting example, the nucleotide sequence 3' of the AUG start codon of a transcript from the target gene of interest may be 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://ihome.ust.hk/~bokcmho/siRNA/siRNA.html>. 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://mfold.burnet.edu.au/rna\\_form](http://mfold.burnet.edu.au/rna_form)) 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- $\alpha$ , IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , IL-6, IL-8, 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- $\alpha$  (PBL Biomedical; Piscataway, N.J.); human IL-6 and TNF- $\alpha$  (eBioscience; San Diego, Calif.); and mouse IL-6, TNF- $\alpha$ , and IFN- $\gamma$  (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.

## (2) 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. In some embodiments, siRNA may be produced enzymatically or by partial/total organic synthesis, and modified ribonucleotides can be introduced by in vitro enzymatic or organic synthesis. In certain instances, each strand is prepared chemically. Methods of synthesizing RNA

molecules are known in the art, e.g., the chemical synthesis methods as described in Verma and Eckstein (1998) or as described herein.

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); Krieglger, *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  $\mu$ mol scale protocol. Alternatively, syntheses at the 0.2  $\mu$ mol 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.

### (3) 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 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-( $\beta$ -D-erythrofuranosyl) nucleotides, 4'-thio nucleotides, carbocyclic nucleotides, 1,5-anhydrohexitol nucleotides, L-nucleotides,  $\alpha$ -nucle-

otides, 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-aminoethyl 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, modified (e.g., 2'OMe) and/or unmodified uridine ribonucleotides, and/or any other combination of modified (e.g., 2'OMe) 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 choles-



terol, 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'- $\beta$ -alkoxyalkyl amine, polyamine, C5-cationic modified pyrimidine, cationic peptide, guanidinium group, amidinium 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.

#### (4) Target Genes

The siRNA component of the nucleic acid-lipid particles of the present invention (e.g., SNALP) can be used to downregulate or silence the translation (i.e., expression) of a gene of interest. As previously mentioned, the present invention is based, in part, on the discovery that the use of certain cationic (amino) lipids in nucleic acid-lipid particles provide advantages when the particles are used for the in vivo delivery of therapeutic nucleic acids, such as siRNA, into the liver of a mammal. In particular, it has been unexpectedly found that the nucleic acid-lipid particles of the present invention (i.e., SNALP formulations) containing at least one cationic lipid of Formula I-XIV and at least one interfering RNA as disclosed herein show increased potency (i.e., increased silencing) and/or increased tolerability (e.g., decreased toxicity) when targeting a gene of interest in the liver, such as APOB, when compared to other nucleic acid-lipid particle compositions previously described. As such, genes of interest include, but are not limited to, genes associated with metabolic diseases and disorders (e.g., liver diseases and disorders).

Genes associated with metabolic diseases and disorders (e.g., disorders in which the liver is the target and liver diseases and disorders) include, but are not limited to, genes expressed in dyslipidemia, such as, e.g., apolipoprotein B (APOB) (Genbank Accession No. NM\_000384), apolipoprotein CIII (APOC3) (Genbank Accession Nos. NM\_000040 and NG\_008949 REGION: 5001.8164), apolipoprotein E (APOE) (Genbank Accession Nos. NM\_000041 and NG\_007084 REGION: 5001.8612), pro-

protein convertase subtilisin/kexin type 9 (PCSK9) (Genbank Accession No. NM\_174936), diacylglycerol O-acyltransferase type 1 (DGAT1) (Genbank Accession No. NM\_012079), diacylglycerol O-acyltransferase type 2 (DGAT2) (Genbank Accession No. NM\_032564), liver X receptors such as LXR $\alpha$  and LXR $\beta$  (Genbank Accession No. NM\_007121), farnesoid X receptors (FXR) (Genbank Accession No. NM\_005123), sterol-regulatory element binding protein (SREBP), site-1 protease (S1P), 3-hydroxy-3-methylglutaryl coenzyme-A reductase (HMG coenzyme-A reductase); and genes expressed in diabetes, such as, 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.

In a presently preferred embodiment, the SNALP formulations of the present invention are used to deliver to the liver an siRNA molecule that silences APOB gene expression. Non-limiting examples of siRNA molecules targeting the APOB gene include, but are not limited to, those described in U.S. Patent Publication Nos. 20060134189, 20060105976, and 20070135372, and PCT Publication No. WO 04/091515, the disclosures of which are herein incorporated by reference in their entirety for all purposes. In another preferred embodiment, the SNALP formulations of the present invention are used to deliver to the liver an siRNA molecule that silences APOC3 gene expression. Non-limiting examples of siRNA molecules targeting the APOC3 gene include, but are not limited to, those described in PCT Application No: PCT/CA2010/000120, filed Jan. 26, 2010, the disclosure of which is herein incorporated by reference in its entirety for all purposes. In yet another preferred embodiment, the SNALP formulations of the present invention are used to deliver to the liver an siRNA molecule that silences PCSK9 gene expression. Non-limiting examples of siRNA molecules targeting the PCSK9 gene include those described in U.S. Patent Publication Nos. 20070173473, 20080113930, and 20080306015, the disclosures of which are herein incorporated by reference in their entirety for all purposes. In still another preferred embodiment, the SNALP formulations of the present invention are used to deliver to the liver siRNA molecules that silence DGAT1 and/or DGAT2 gene expression. Exemplary siRNA molecules targeting the DGAT1 gene may be designed using the antisense compounds described in U.S. Patent Publication No. 20040185559, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Exemplary siRNA molecules targeting the DGAT2 gene may be designed using the antisense compounds described in U.S. Patent Publication No. 20050043524, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In addition to being particularly useful for silencing any of APOB, APOC3, PCSK9, DGAT1 and DGAT2, either alone or in various combinations, the SNALP formulations of the



present invention are also useful for treating hepatitis. 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, but are not limited to, those described in U.S. Patent Publication Nos. 20060281175, 20050058982, and 20070149470; U.S. Pat. No. 7,348,314; and PCT Application No. PCT/CA2010/000444, entitled "Compositions and Methods for Silencing Hepatitis C Virus Expression," filed Mar. 19, 2010, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

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.

#### (5) Exemplary siRNA Embodiments

In some embodiments, each strand of the siRNA molecule comprises from 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). In one particular embodiment, the siRNA is chemically synthesized. The siRNA molecules of the invention are capable of silencing the expression of a target sequence *in vitro* and/or *in vivo*.

In other embodiments, the siRNA comprises at least one modified nucleotide. In certain embodiments, the siRNA

comprises one, two, three, four, five, six, seven, eight, nine, ten, or more modified nucleotides in the double-stranded region. In particular embodiments, less than about 50% (e.g., less than about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, or 5%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides. In preferred embodiments, from about 1% to about 50% (e.g., from about 5%-50%, 10%-50%, 15%-50%, 20%-50%, 25%-50%, 30%-50%, 35%-50%, 40%-50%, 45%-50%, 5%-45%, 10%-45%, 15%-45%, 20%-45%, 25%-45%, 30%-45%, 35%-45%, 40%-45%, 5%-40%, 10%-40%, 15%-40%, 20%-40%, 25%-40%, 30%-40%, 35%-40%, 5%-35%, 10%-35%, 15%-35%, 20%-35%, 25%-35%, 30%-35%, 5%-30%, 10%-30%, 15%-30%, 20%-30%, 25%-30%, 5%-25%, 10%-25%, 15%-25%, 20%-25%, 5%-20%, 10%-20%, 15%-20%, 5%-15%, 10%-15%, or 5%-10%) of the nucleotides in the double-stranded region of the siRNA comprise modified nucleotides.

In further embodiments, the siRNA 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, e.g., 2'OMe-guanosine nucleotides, 2'OMe-uridine nucleotides, 2'OMe-adenosine nucleotides, 2'OMe-cytosine nucleotides, or mixtures thereof. In one particular embodiment, the siRNA comprises at least one 2'OMe-guanosine nucleotide, 2'OMe-uridine nucleotide, or mixtures thereof. In certain instances, the siRNA does not comprise 2'OMe-cytosine nucleotides. In other embodiments, the siRNA comprises a hairpin loop structure.

In certain embodiments, the siRNA comprises 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 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 intro-

duction 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- $\alpha$  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 other 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 another embodiment, an unmodified or 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%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the expression of the target sequence relative to a negative control (e.g., buffer only, an siRNA sequence that targets a different gene, a scrambled siRNA sequence, etc.).

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%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 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. In certain embodiments, the 3' overhang on the sense and/or antisense strand independently comprises one, two, three, four, or more modified nucleotides such as 2'OMe nucleotides and/or any other modified nucleotide described herein or known in the art.

In particular embodiments, siRNAs are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more siRNA molecules targeting APOB, APOC3, PCSK9, DGAT1 and/or DGAT2 gene expression; (b) a cationic lipid of Formula I-XIV or a salt thereof; and (c) a non-cationic lipid (e.g., DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (e.g., PEG-DAA).

#### b) Dicer-Substrate dsRNA

As used herein, the term "Dicer-substrate dsRNA" or "precursor RNAi molecule" is intended to include any precursor molecule that is processed in vivo by Dicer to produce an active siRNA which is incorporated into the RISC complex for RNA interference of a target gene, such as APOB, APOC3, PCSK9, DGAT1, DGAT2, or combinations thereof.

In one embodiment, the Dicer-substrate dsRNA has a length sufficient such that it is processed by Dicer to produce an siRNA. According to this embodiment, the Dicer-substrate dsRNA comprises (i) a first oligonucleotide sequence (also termed the sense strand) that is between about 25 and about 60 nucleotides in length (e.g., about 25-60, 25-55, 25-50, 25-45, 25-40, 25-35, or 25-30 nucleotides in length), preferably between about 25 and about 30 nucleotides in length (e.g., 25, 26, 27, 28, 29, or 30 nucleotides in length), and (ii) a second oligonucleotide sequence (also termed the antisense strand) that anneals to the first sequence under biological conditions, such as the conditions found in the cytoplasm of a cell. The second oligonucleotide sequence may be between about 25 and about 60 nucleotides in length (e.g., about 25-60, 25-55, 25-50, 25-45, 25-40, 25-35, or 25-30 nucleotides in length), and is preferably between about 25 and about 30 nucleotides in length (e.g., 25, 26, 27, 28, 29, or 30 nucleotides in length). In addition, a region of one of the sequences, particularly of the antisense strand, of the Dicer-substrate dsRNA has a sequence length of at least about 19 nucleotides, for example, from about 19 to about 60 nucleotides (e.g., about 19-60, 19-55, 19-50, 19-45, 19-40, 19-35, 19-30, or 19-25 nucleotides), preferably from about 19 to about 23 nucleotides (e.g., 19, 20, 21, 22, or 23 nucleotides) that are sufficiently complementary to a nucleotide sequence of the RNA produced from the target gene to trigger an RNAi response.

In a second embodiment, the Dicer-substrate dsRNA has several properties which enhance its processing by Dicer. According to this embodiment, the dsRNA has a length sufficient such that it is processed by Dicer to produce an siRNA and has at least one of the following properties: (i) the dsRNA is asymmetric, e.g., has a 3'-overhang on the antisense strand; and/or (ii) the dsRNA has a modified 3'-end on the sense strand to direct orientation of Dicer binding and processing of the dsRNA to an active siRNA. According to this latter embodiment, the sense strand comprises from about 22 to about 28 nucleotides and the antisense strand comprises from about 24 to about 30 nucleotides.

In one embodiment, the Dicer-substrate dsRNA has an overhang on the 3'-end of the antisense strand. In another embodiment, the sense strand is modified for Dicer binding and processing by suitable modifiers located at the 3'-end of the sense strand. Suitable modifiers include nucleotides such as deoxyribonucleotides, acyclonucleotides, and the like, and sterically hindered molecules such as fluorescent molecules and the like. When nucleotide modifiers are used, they replace ribonucleotides in the dsRNA such that the length of the dsRNA does not change. In another embodiment, the Dicer-substrate dsRNA has an overhang on the 3'-end of the antisense strand and the sense strand is modified for Dicer processing. In another embodiment, the 5'-end of the sense strand has a phosphate. In another embodiment, the 5'-end of the antisense strand has a phosphate. In another embodiment, the antisense strand or the sense strand or both strands have one or more 2'-O-methyl (2'OMe) modified nucleotides. In another embodiment, the antisense strand contains 2'OMe modified nucleotides. In another embodiment, the antisense strand contains a 3'-overhang that is comprised of 2'OMe modified nucleotides. The antisense strand could also include additional 2'OMe modified nucleotides. The sense and antisense strands anneal under biological conditions, such as the conditions found in the cytoplasm of a cell. In addition, a region of one of the sequences, particularly of the antisense strand, of the Dicer-substrate dsRNA has a sequence length of at least about 19 nucleotides, wherein these nucleotides are in the 21-nucleotide region adjacent to the 3'-end of the antisense strand and are sufficiently complementary to a nucleotide sequence of the RNA produced from the target gene, such as APOB. Further, in accordance with this embodiment, the Dicer-substrate dsRNA may also have one or more of the following additional properties: (a) the antisense strand has a right shift from the typical 21-mer (i.e., the antisense strand includes nucleotides on the right side of the molecule when compared to the typical 21-mer); (b) the strands may not be completely complementary, i.e., the strands may contain simple mismatch pairings; and (c) base modifications such as locked nucleic acid(s) may be included in the 5'-end of the sense strand.

In a third embodiment, the sense strand comprises from about 25 to about 28 nucleotides (e.g., 25, 26, 27, or 28 nucleotides), wherein the 2 nucleotides on the 3'-end of the sense strand are deoxyribonucleotides. The sense strand contains a phosphate at the 5'-end. The antisense strand comprises from about 26 to about 30 nucleotides (e.g., 26, 27, 28, 29, or 30 nucleotides) and contains a 3'-overhang of 1-4 nucleotides. The nucleotides comprising the 3'-overhang are modified with 2'OMe modified ribonucleotides. The antisense strand contains alternating 2'OMe modified nucleotides beginning at the first monomer of the antisense strand adjacent to the 3'-overhang, and extending 15-19 nucleotides from the first monomer adjacent to the 3'-overhang. For example, for a 27-nucleotide antisense strand and counting the first base at the 5'-end of the antisense strand as position number 1, 2'OMe modifications would be placed at bases 9, 11, 13, 15, 17, 19, 21, 23, 25, 26, and 27. In one embodiment, the Dicer-substrate dsRNA has the following structure:

5' -pXXXXXXXXXXXXXXXXXXXXXXXXXDD-3'

3' - YXXXXXXXXXXXXXXXXXXXXXXXXXp-5'

wherein "X"=RNA, "p"=a phosphate group, "X"=2'OMe RNA, "Y" is an overhang domain comprised of 1, 2, 3, or 4 RNA monomers that are optionally 2'OMe RNA monomers,

and "D"=DNA. The top strand is the sense strand, and the bottom strand is the antisense strand.

In a fourth embodiment, the Dicer-substrate dsRNA has several properties which enhance its processing by Dicer. According to this embodiment, the dsRNA has a length sufficient such that it is processed by Dicer to produce an siRNA and at least one of the following properties: (i) the dsRNA is asymmetric, e.g., has a 3'-overhang on the sense strand; and (ii) the dsRNA has a modified 3'-end on the antisense strand to direct orientation of Dicer binding and processing of the dsRNA to an active siRNA. According to this embodiment, the sense strand comprises from about 24 to about 30 nucleotides (e.g., 24, 25, 26, 27, 28, 29, or 30 nucleotides) and the antisense strand comprises from about 22 to about 28 nucleotides (e.g., 22, 23, 24, 25, 26, 27, or 28 nucleotides). In one embodiment, the Dicer-substrate dsRNA has an overhang on the 3'-end of the sense strand. In another embodiment, the antisense strand is modified for Dicer binding and processing by suitable modifiers located at the 3'-end of the antisense strand. Suitable modifiers include nucleotides such as deoxyribonucleotides, acyclonucleotides, and the like, and sterically hindered molecules such as fluorescent molecules and the like. When nucleotide modifiers are used, they replace ribonucleotides in the dsRNA such that the length of the dsRNA does not change. In another embodiment, the dsRNA has an overhang on the 3'-end of the sense strand and the antisense strand is modified for Dicer processing. In one embodiment, the antisense strand has a 5'-phosphate. The sense and antisense strands anneal under biological conditions, such as the conditions found in the cytoplasm of a cell. In addition, a region of one of the sequences, particularly of the antisense strand, of the dsRNA has a sequence length of at least 19 nucleotides, wherein these nucleotides are adjacent to the 3'-end of antisense strand and are sufficiently complementary to a nucleotide sequence of the RNA produced from the target gene, such as APOB. Further, in accordance with this embodiment, the Dicer-substrate dsRNA may also have one or more of the following additional properties: (a) the antisense strand has a left shift from the typical 21-mer (i.e., the antisense strand includes nucleotides on the left side of the molecule when compared to the typical 21-mer); and (b) the strands may not be completely complementary, i.e., the strands may contain simple mismatch pairings.

In a preferred embodiment, the Dicer-substrate dsRNA has an asymmetric structure, with the sense strand having a 25-base pair length, and the antisense strand having a 27-base pair length with a 2 base 3'-overhang. In certain instances, this dsRNA having an asymmetric structure further contains 2 deoxynucleotides at the 3'-end of the sense strand in place of two of the ribonucleotides. In certain other instances, this dsRNA having an asymmetric structure further contains 2'OMe modifications at positions 9, 11, 13, 15, 17, 19, 21, 23, and 25 of the antisense strand (wherein the first base at the 5'-end of the antisense strand is position 1). In certain additional instances, this dsRNA having an asymmetric structure further contains a 3'-overhang on the antisense strand comprising 1, 2, 3, or 4 2'OMe nucleotides (e.g., a 3'-overhang of 2'OMe nucleotides at positions 26 and 27 on the antisense strand).

In another embodiment, Dicer-substrate dsRNAs may be designed by first selecting an antisense strand siRNA sequence having a length of at least 19 nucleotides. In some instances, the antisense siRNA is modified to include about 5 to about 11 ribonucleotides on the 5'-end to provide a length of about 24 to about 30 nucleotides. When the antisense strand has a length of 21 nucleotides, 3-9, preferably 4-7, or more preferably 6 nucleotides may be added on the 5'-end.

Although the added ribonucleotides may be complementary to the target gene sequence, full complementarity between the target sequence and the antisense siRNA is not required. That is, the resultant antisense siRNA is sufficiently complementary with the target sequence. A sense strand is then produced that has about 22 to about 28 nucleotides. The sense strand is substantially complementary with the antisense strand to anneal to the antisense strand under biological conditions. In one embodiment, the sense strand is synthesized to contain a modified 3'-end to direct Dicer processing of the antisense strand. In another embodiment, the antisense strand of the dsRNA has a 3'-overhang. In a further embodiment, the sense strand is synthesized to contain a modified 3'-end for Dicer binding and processing and the antisense strand of the dsRNA has a 3'-overhang.

In a related embodiment, the antisense siRNA may be modified to include about 1 to about 9 ribonucleotides on the 5'-end to provide a length of about 22 to about 28 nucleotides. When the antisense strand has a length of 21 nucleotides, 1-7, preferably 2-5, or more preferably 4 ribonucleotides may be added on the 3'-end. The added ribonucleotides may have any sequence. Although the added ribonucleotides may be complementary to the target gene sequence, full complementarity between the target sequence, and the antisense siRNA is not required. That is, the resultant antisense siRNA is sufficiently complementary with the target sequence. A sense strand is then produced that has about 24 to about 30 nucleotides. The sense strand is substantially complementary with the antisense strand to anneal to the antisense strand under biological conditions. In one embodiment, the antisense strand is synthesized to contain a modified 3'-end to direct Dicer processing. In another embodiment, the sense strand of the dsRNA has a 3'-overhang. In a further embodiment, the antisense strand is synthesized to contain a modified 3'-end for Dicer binding and processing and the sense strand of the dsRNA has a 3'-overhang.

Suitable Dicer-substrate dsRNA sequences can be identified, synthesized, and modified using any means known in the art for designing, synthesizing, and modifying siRNA sequences. In particular embodiments, Dicer-substrate dsRNAs are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more Dicer-substrate dsRNA molecules targeting APOB, APOC3, PCSK9, DGAT1 and/or DGAT2 gene expression; (b) a cationic lipid of Formula I-XIV or a salt thereof; and (c) a non-cationic lipid (e.g., DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (e.g., PEG-DAA).

Additional embodiments related to the Dicer-substrate dsRNAs of the invention, as well as methods of designing and synthesizing such dsRNAs, are described in U.S. Patent Publication Nos. 20050244858, 20050277610, and 20070265220, and U.S. application Ser. No. 12/794,701, filed Jun. 4, 2010, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

#### c) Small Hairpin RNA (shRNA)

A "small hairpin RNA" or "short hairpin RNA" or "shRNA" includes a short RNA sequence that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. The shRNAs of the invention may be chemically synthesized or transcribed from a transcriptional cassette in a DNA plasmid. The shRNA hairpin structure is cleaved by the cellular machinery into siRNA, which is then bound to the RNA-induced silencing complex (RISC).

The shRNAs of the invention are typically 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 are preferably about 20-24, 21-22, or 21-23 (duplex) nucleotides in length (e.g., each complementary sequence of the double-stranded shRNA 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 shRNA 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). shRNA duplexes may comprise 3' overhangs of about 1 to about 4 nucleotides or about 2 to about 3 nucleotides on the antisense strand and/or 5'-phosphate termini on the sense strand. In some embodiments, the shRNA comprises a sense strand and/or antisense strand sequence of from about 15 to about 60 nucleotides in length (e.g., about 15-60, 15-55, 15-50, 15-45, 15-40, 15-35, 15-30, or 15-25 nucleotides in length), preferably from about 19 to about 40 nucleotides in length (e.g., about 19-40, 19-35, 19-30, or 19-25 nucleotides in length), more preferably from about 19 to about 23 nucleotides in length (e.g., 19, 20, 21, 22, or 23 nucleotides in length).

Non-limiting examples of shRNA include 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; and a double-stranded polynucleotide molecule with a hairpin secondary structure having self-complementary sense and antisense regions. In preferred embodiments, the sense and antisense strands of the shRNA are linked by a loop structure comprising from about 1 to about 25 nucleotides, from about 2 to about 20 nucleotides, from about 4 to about 15 nucleotides, from about 5 to about 12 nucleotides, or 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, or more nucleotides.

Additional shRNA sequences include, but are not limited to, asymmetric shRNA precursor polynucleotides such as those described in PCT Publication Nos. WO 2006/074108 and WO 2009/076321, the disclosures of which are herein incorporated by reference in their entirety for all purposes. For example, PCT Publication No. WO 2006/074108 discloses self-protected oligonucleotides comprising a region having a sequence complementary to one, two, three, or more same or different target mRNA sequences (e.g., multivalent shRNAs) and one or more self-complementary regions. Similarly, PCT Publication No. WO 2009/076321 discloses self-forming asymmetric precursor polynucleotides comprising a targeting region comprising a polynucleotide sequence complementary to a region of one, two, three, or more same or different target mRNA sequences (e.g., multivalent shRNAs); a first self-complementary region; and a second self-complementary region, wherein the first and second self-complementary regions are located one at each end of the targeting region and both self-complementary regions form stem-loop structures, wherein the first self-complementary region is capable of being cleaved by a RNase III endoribonuclease that is not a class IV DICER endoribonuclease, and wherein both self-complementary regions comprise a nucleotide sequence that is complementary to a region of the target gene sequence, but wherein a portion of the target sequence present in the targeting region does not have a complementary sequence in either of the self-complementary regions.

Suitable shRNA sequences can be identified, synthesized, and modified using any means known in the art for designing, synthesizing, and modifying siRNA sequences. In particular embodiments, shRNAs are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred

embodiment, the nucleic acid-lipid particle comprises: (a) one or more shRNA molecules targeting APOB, APOC3, PCSK9, DGAT1 and/or DGAT2 gene expression; (b) a cationic lipid of Formula I-XIV or a salt thereof; and (c) a non-cationic lipid (e.g., DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (e.g., PEG-DAA).

Additional embodiments related to the shRNAs of the invention, as well as methods of designing and synthesizing such shRNAs, are described in U.S. application Ser. No. 12/794,701, filed Jun. 4, 2010, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

#### d) 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 particular embodiments, aiRNAs are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more aiRNA molecules targeting APOB, APOC3, PCSK9, DGAT1 and/or DGAT2

gene expression; (b) a cationic lipid of Formula I-XIV or a salt thereof; and (c) a non-cationic lipid (e.g., DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (e.g., PEG-DAA).

Suitable aiRNA sequences can be identified, synthesized, and modified using any means known in the art for designing, synthesizing, and modifying siRNA sequences. Additional embodiments related to the aiRNA molecules of the invention are described in U.S. Patent Publication No. 20090291131 and PCT Publication No. WO 09/127,060, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

#### e) 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 particular embodiments, miRNAs are administered using a carrier system such as a nucleic acid-lipid particle. In a preferred embodiment, the nucleic acid-lipid particle comprises: (a) one or more miRNA molecules targeting APOB, APOC3, PCSK9, DGAT1 and/or DGAT2 gene expression; (b) a cationic lipid of Formula I-XIV or a salt thereof; and (c) a non-cationic lipid (e.g., DPPC, DSPC, DSPE, and/or cholesterol). In certain instances, the nucleic acid-lipid particle may further comprise a conjugated lipid that prevents aggregation of particles (e.g., PEG-DAA).

In other embodiments, one or more agents that block the activity of an miRNA targeting APOB, APOC3, PCSK9, DGAT1 and/or DGAT2 mRNA are administered using a lipid particle of the invention (e.g., a nucleic acid-lipid particle such as SNALP). 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.

Additional embodiments related to the miRNA molecules of the invention are described in U.S. Patent Publication No. 20090291131 and PCT Publication No. WO 09/127,060, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

#### B. Cationic Lipids

Any of the cationic lipids of Formulas I-XIV or salts thereof as set forth herein may be used in the lipid particles of the present invention (e.g., SNALP), either alone or in combination with one or more other cationic lipid species or non-cationic lipid species. The cationic lipids include the (R) and/or (S) enantiomers thereof.

In some embodiments, the cationic lipid comprises a racemic mixture. In other embodiments, the cationic lipid comprises a mixture of one or more diastereomers. In certain embodiments, the cationic lipid is enriched in one enantiomer, such that the cationic lipid comprises at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% enantiomeric excess. In certain other embodiments, the cationic lipid is enriched in one diastereomer, such that the cationic lipid comprises at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% diastereomeric excess. In certain additional embodiments, the cationic lipid is chirally pure (e.g., comprises a single optical isomer). In further embodiments, the cationic lipid is enriched in one optical isomer (e.g., an optically active isomer), such that the cationic lipid comprises at least about 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% isomeric excess. The present invention provides the synthesis of the cationic lipids of Formulas I-XIV as a racemic mixture or in optically pure form.

The terms "cationic lipid" and "amino lipid" are used interchangeably herein to include those lipids and salts thereof having one, two, three, or more fatty acid or fatty alkyl chains and a pH-titratable amino head group (e.g., an alkylamino or dialkylamino head group). The cationic lipid is typically protonated (i.e., positively charged) at a pH below the  $pK_a$  of the

cationic lipid and is substantially neutral at a pH above the  $pK_a$ . The cationic lipids of the invention may also be termed titratable cationic lipids.

The term "salts" includes any anionic and cationic complex, such as the complex formed between a cationic lipid disclosed herein and one or more anions. Non-limiting examples of anions include inorganic and organic anions, e.g., hydride, fluoride, chloride, bromide, iodide, oxalate (e.g., hemioxalate), phosphate, phosphonate, hydrogen phosphate, dihydrogen phosphate, oxide, carbonate, bicarbonate, nitrate, nitrite, nitride, bisulfite, sulfide, sulfite, bisulfate, sulfate, thiosulfate, hydrogen sulfate, borate, formate, acetate, benzoate, citrate, tartrate, lactate, acrylate, polyacrylate, fumarate, maleate, itaconate, glycolate, gluconate, malate, mandelate, tiglate, ascorbate, salicylate, polymethacrylate, perchlorate, chlorate, chlorite, hypochlorite, bromate, hypobromite, iodate, an alkylsulfonate, an arylsulfonate, arsenate, arsenite, chromate, dichromate, cyanide, cyanate, thiocyanate, hydroxide, peroxide, permanganate, and mixtures thereof. In particular embodiments, the salts of the cationic lipids disclosed herein are crystalline salts.

The term "alkyl" includes a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms. Representative saturated straight chain alkyls include, but are not limited to, methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, and the like, while saturated branched alkyls include, without limitation, isopropyl, sec-butyl, isobutyl, tert-butyl, isopentyl, and the like. Representative saturated cyclic alkyls include, but are not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like, while unsaturated cyclic alkyls include, without limitation, cyclopentenyl, cyclohexenyl, and the like.

The term "alkenyl" includes an alkyl, as defined above, containing at least one double bond between adjacent carbon atoms. Alkenyls include both cis and trans isomers. Representative straight chain and branched alkenyls include, but are not limited to, ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 2,3-dimethyl-2-butenyl, and the like.

The term "alkynyl" includes any alkyl or alkenyl, as defined above, which additionally contains at least one triple bond between adjacent carbons. Representative straight chain and branched alkynyls include, without limitation, acetylenyl, propynyl, 1-butyne, 2-butyne, 1-pentyne, 2-pentyne, 3-methyl-1 butyne, and the like.

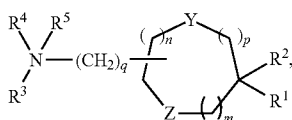
The term "acyl" includes any alkyl, alkenyl, or alkynyl wherein the carbon at the point of attachment is substituted with an oxo group, as defined below. The following are non-limiting examples of acyl groups:  $-C(=O)alkyl$ ,  $-C(=O)alkenyl$ , and  $-C(=O)alkynyl$ .

The term "heterocycle" includes a 5- to 7-membered monocyclic, or 7- to 10-membered bicyclic, heterocyclic ring which is either saturated, unsaturated, or aromatic, and which contains from 1 or 2 heteroatoms independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen heteroatom may be optionally quaternized, including bicyclic rings in which any of the above heterocycles are fused to a benzene ring. The heterocycle may be attached via any heteroatom or carbon atom. Heterocycles include, but are not limited to, heteroaryls as defined below, as well as morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizonyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranlyl, tetrahydropyranlyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranlyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranlyl, and the like.

The terms “optionally substituted alkyl”, “optionally substituted alkenyl”, “optionally substituted alkynyl”, “optionally substituted acyl”, and “optionally substituted heterocycle” mean that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo substituent (=O), two hydrogen atoms are replaced. In this regard, substituents include, but are not limited to, oxo, halogen, heterocycle, —CN, —OR<sup>x</sup>, —NR<sup>x</sup>R<sup>y</sup>, —NR<sup>x</sup>C(=O)R<sup>y</sup>, —NR<sup>x</sup>SO<sub>2</sub>R<sup>y</sup>, —C(=O)R<sup>x</sup>, —C(=O)OR<sup>x</sup>, —C(=O)NR<sup>x</sup>R<sup>y</sup>, —SO<sub>n</sub>R<sup>x</sup>, and —SO<sub>n</sub>NR<sup>x</sup>R<sup>y</sup>, wherein n is 0, 1, or 2, R<sup>x</sup> and R<sup>y</sup> are the same or different and are independently hydrogen, alkyl, or heterocycle, and each of the alkyl and heterocycle substituents may be further substituted with one or more of oxo, halogen, —OH, —CN, alkyl, —OR<sup>x</sup>, heterocycle, —NR<sup>x</sup>R<sup>y</sup>, —NR<sup>x</sup>C(=O)R<sup>y</sup>, —NR<sup>x</sup>SO<sub>2</sub>R<sup>y</sup>, —C(=O)R<sup>x</sup>, —C(=O)OR<sup>x</sup>, —C(=O)NR<sup>x</sup>R<sup>y</sup>, —SO<sub>n</sub>R<sup>x</sup>, and —SO<sub>n</sub>NR<sup>x</sup>R<sup>y</sup>. The term “optionally substituted,” when used before a list of substituents, means that each of the substituents in the list may be optionally substituted as described herein.

The term “halogen” includes fluoro, chloro, bromo, and iodo.

In one aspect, cationic lipids of Formula I having the following structure (or salts thereof) are useful in the present invention:



wherein R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, C<sub>12</sub>-C<sub>24</sub> alkenyl, C<sub>12</sub>-C<sub>24</sub> alkynyl, or C<sub>12</sub>-C<sub>24</sub> acyl; R<sup>3</sup> and R<sup>4</sup> are either the same or different and are independently an optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, or C<sub>2</sub>-C<sub>6</sub> alkynyl, or R<sup>3</sup> and R<sup>4</sup> 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<sup>5</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; m, n, and p are either the same or different and are independently either 0, 1, or 2, 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 are independently O, S, or NH.

In some embodiments, R<sup>3</sup> and R<sup>4</sup> are independently an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>2</sub>-C<sub>4</sub> alkenyl, or C<sub>2</sub>-C<sub>4</sub> alkynyl. In a preferred embodiment, R<sup>3</sup> and R<sup>4</sup> are both methyl groups. In one embodiment, q is 1 or 2. In another embodiment, q is 1-2, 1-3, 1-4, 2-3, or 2-4. In further embodiments, R<sup>5</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>5</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is

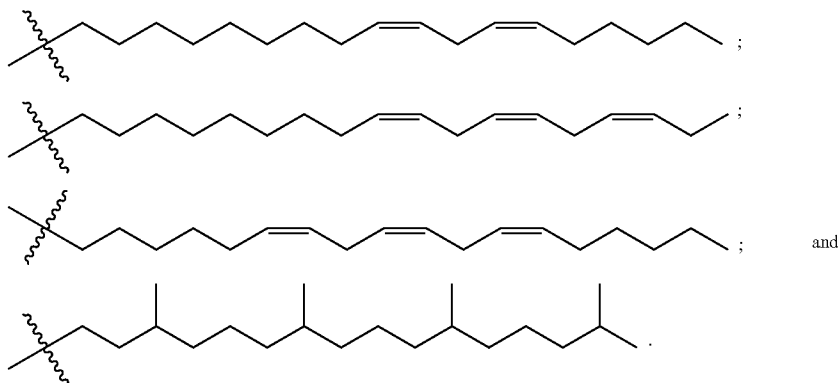
protonated. In an alternative embodiment, R<sup>5</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine. In additional embodiments, Y and Z are both O.

In other embodiments, R<sup>1</sup> and R<sup>2</sup> are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub>, C<sub>12</sub>-C<sub>22</sub>, C<sub>12</sub>-C<sub>20</sub>, C<sub>14</sub>-C<sub>24</sub>, C<sub>14</sub>-C<sub>22</sub>, C<sub>14</sub>-C<sub>20</sub>, C<sub>16</sub>-C<sub>24</sub>, C<sub>16</sub>-C<sub>22</sub>, or C<sub>16</sub>-C<sub>20</sub> alkyl, alkenyl, alkynyl, or acyl group (i.e., C<sub>12</sub>, C<sub>13</sub>, C<sub>14</sub>, C<sub>15</sub>, C<sub>16</sub>, C<sub>17</sub>, C<sub>18</sub>, C<sub>19</sub>, C<sub>20</sub>, C<sub>21</sub>, C<sub>22</sub>, C<sub>23</sub>, or C<sub>24</sub> alkyl, alkenyl, alkynyl, or acyl group). In certain embodiments, at least one or both R<sup>1</sup> and R<sup>2</sup> independently comprises at least 1, 2, 3, 4, 5, or 6 sites of unsaturation (e.g., 1-2, 1-3, 1-4, 1-5, 1-6, 2-3, 2-4, 2-5, or 2-6 sites of unsaturation) or a substituted alkyl or acyl group. In certain instances, the unsaturated side-chain may comprise a myristoleyl moiety, a palmitoleyl moiety, an oleyl moiety, a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradecatrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, or an acyl derivative thereof (e.g., linoleoyl, linolenoyl, γ-linolenoyl, etc.). In some instances, the octadecadienyl moiety is a linoleyl moiety. In particular embodiments, R<sup>1</sup> and R<sup>2</sup> are both linoleyl moieties. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a γ-linolenyl moiety. In particular embodiments, R<sup>1</sup> and R<sup>2</sup> are both linolenyl moieties or γ-linolenyl moieties.

In embodiments where one or both R<sup>1</sup> and R<sup>2</sup> independently comprises at least 1, 2, 3, 4, 5, or 6 sites of unsaturation, the double bonds present in one or both R<sup>1</sup> and R<sup>2</sup> may be in the cis and/or trans configuration. In certain instances, R<sup>1</sup> and R<sup>2</sup> are both the same, e.g., R<sup>1</sup> and R<sup>2</sup> are both linoleyl (C<sub>18</sub>) moieties, etc. In certain other instances, R<sup>1</sup> and R<sup>2</sup> are different, e.g., R<sup>1</sup> is a tetradecatrienyl (C<sub>14</sub>) moiety and R<sup>2</sup> is a linoleyl (C<sub>18</sub>) moiety. In a preferred embodiment, the cationic lipid of Formula I is symmetrical, i.e., R<sup>1</sup> and R<sup>2</sup> are both the same. In another preferred embodiment, at least one or both R<sup>1</sup> and R<sup>2</sup> comprises at least two sites of unsaturation (e.g., 2, 3, 4, 5, 6, 2-3, 2-4, 2-5, or 2-6 sites of unsaturation).

In embodiments where one or both R<sup>1</sup> and R<sup>2</sup> independently comprises a branched alkyl or acyl group (e.g., a substituted alkyl or acyl group), the branched alkyl or acyl group may comprise a C<sub>12</sub>-C<sub>24</sub> alkyl or acyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C<sub>1</sub>-C<sub>6</sub> alkyl substituents. In particular embodiments, the branched alkyl or acyl group comprises a C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl or acyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C<sub>1</sub>-C<sub>4</sub> alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. In some embodiments, the branched alkyl group comprises a phytanyl (3,7,11,15-tetramethyl-hexadecanyl) moiety and the branched acyl group comprises a phytanoyl (3,7,11,15-tetramethyl-hexadecanoyl) moiety. In particular embodiments, R<sup>1</sup> and R<sup>2</sup> are both phytanyl moieties.

In some groups of embodiments to the cationic lipids of Formula I, R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently selected from the group consisting of:





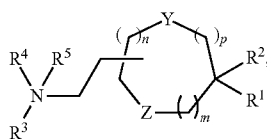
In certain embodiments, cationic lipids falling within the scope of Formula I include, but are not limited to, the following: 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2" or "C2K"), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), 2,2-dilinoleyl-4-(3-dimethylaminopropyl)[1,3]-dioxolane (DLin-K-C3-DMA; "C3K"), 2,2-dilinoleyl-4-(4-dimethylaminobutyl)[1,3]-dioxolane (DLin-K-C4-DMA; "C4K"), 2,2-dilinoleyl-5-dimethylaminomethyl-[1,3]-dioxane (DLin-K-6-DMA), 2,2-dilinoleyl-4-N-methylpiperazine-[1,3]-dioxolane (DLin-K-MPZ), 2,2-dioleoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DO-K-DMA), 2,2-distearoyl-4-dimethylaminomethyl-[1,3]-dioxolane (DS-K-DMA), 2,2-dilinoleyl-4-N-morpholino-[1,3]-dioxolane (DLin-K-MA), 2,2-Dilinoleyl-4-trimethylamino-[1,3]-dioxolane chloride (DLin-K-TMA.Cl), 2,2-dilinoleyl-4,5-bis(dimethylaminomethyl)-[1,3]-dioxolane (DLin-K<sup>2</sup>-DMA), 2,2-dilinoleyl-4-methylpiperazine-[1,3]-dioxolane (D-Lin-K-N-methylpiperazine), DLen-C2K-DMA,  $\gamma$ -DLen-C2K-DMA, DPan-C2K-DMA, DPan-C3K-DMA, or mixtures thereof. In preferred embodiments, the cationic lipid of Formula I comprises DLin-K-C2-DMA and/or DLin-K-DMA.

In some embodiments, the cationic lipids of Formula I form a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula I is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

The synthesis of cationic lipids such as DLin-K-C2-DMA, DLin-K-C3-DMA, DLin-K-C4-DMA, DLin-K-6-DMA, DLin-K-MPZ, DO-K-DMA, DS-K-DMA, DLin-K-MA, DLin-K-TMA.Cl, DLin-K<sup>2</sup>-DMA, D-Lin-K-N-methylpiperazine, as well as additional cationic lipids, is described in PCT Publication No. WO 2010/042877, the disclosure of which is incorporated herein by reference in its entirety for all purposes.

The synthesis of cationic lipids such as DLin-K-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 09/086,558, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In a preferred embodiment, cationic lipids of Formula II having the following structure (or salts thereof) are useful in the present invention:



wherein R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, C<sub>12</sub>-C<sub>24</sub> alkenyl, C<sub>12</sub>-C<sub>24</sub> alkynyl, or C<sub>12</sub>-C<sub>24</sub> acyl; R<sup>3</sup> and R<sup>4</sup> are either the same or different and are independently an optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, or C<sub>2</sub>-C<sub>6</sub> alkynyl, or R<sup>3</sup> and R<sup>4</sup> 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<sup>5</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; m, n, and p are either the same or different and are independently either 0, 1, or 2, with the proviso that m, n, and

p are not simultaneously 0; and Y and Z are either the same or different and are independently O, S, or NH.

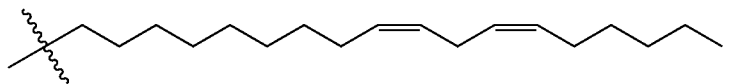
In some embodiments, R<sup>3</sup> and R<sup>4</sup> are independently an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>2</sub>-C<sub>4</sub> alkenyl, or C<sub>2</sub>-C<sub>4</sub> alkynyl. In a preferred embodiment, R<sup>3</sup> and R<sup>4</sup> are both methyl groups. In further embodiments, R<sup>5</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>5</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R<sup>5</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine. In additional embodiments, Y and Z are both O.

In other embodiments, R<sup>1</sup> and R<sup>2</sup> are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub>, C<sub>12</sub>-C<sub>22</sub>, C<sub>12</sub>-C<sub>20</sub>, C<sub>14</sub>-C<sub>24</sub>, C<sub>14</sub>-C<sub>22</sub>, C<sub>14</sub>-C<sub>20</sub>, C<sub>16</sub>-C<sub>24</sub>, C<sub>16</sub>-C<sub>22</sub>, or C<sub>16</sub>-C<sub>20</sub> alkyl, alkenyl, alkynyl, or acyl group (i.e., C<sub>12</sub>, C<sub>13</sub>, C<sub>14</sub>, C<sub>15</sub>, C<sub>16</sub>, C<sub>17</sub>, C<sub>18</sub>, C<sub>19</sub>, C<sub>20</sub>, C<sub>21</sub>, C<sub>22</sub>, C<sub>23</sub>, or C<sub>24</sub> alkyl, alkenyl, alkynyl, or acyl group). In certain embodiments, at least one or both R<sup>1</sup> and R<sup>2</sup> independently comprises at least 1, 2, 3, 4, 5, or 6 sites of unsaturation (e.g., 1-2, 1-3, 1-4, 1-5, 1-6, 2-3, 2-4, 2-5, or 2-6 sites of unsaturation) or a substituted alkyl or acyl group. In certain instances, the unsaturated side-chain may comprise a myristoleyl moiety, a palmitoleyl moiety, an oleyl moiety, a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradecatrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, or an acyl derivative thereof (e.g., linoleoyl, linolenoyl,  $\gamma$ -linolenoyl, etc.). In some instances, the octadecadienyl moiety is a linoleyl moiety. In particular embodiments, R<sup>1</sup> and R<sup>2</sup> are both linoleyl moieties. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a  $\gamma$ -linolenyl moiety. In particular embodiments, R<sup>1</sup> and R<sup>2</sup> are both linolenyl moieties or  $\gamma$ -linolenyl moieties.

In embodiments where one or both R<sup>1</sup> and R<sup>2</sup> independently comprises at least 1, 2, 3, 4, 5, or 6 sites of unsaturation, the double bonds present in one or both R<sup>1</sup> and R<sup>2</sup> may be in the cis and/or trans configuration. In certain instances, R<sup>1</sup> and R<sup>2</sup> are both the same, e.g., R<sup>1</sup> and R<sup>2</sup> are both linoleyl (C<sub>18</sub>) moieties, etc. In certain other instances, R<sup>1</sup> and R<sup>2</sup> are different, e.g., R<sup>1</sup> is a tetradecatrienyl (C<sub>14</sub>) moiety and R<sup>2</sup> is a linoleyl (C<sub>18</sub>) moiety. In a preferred embodiment, the cationic lipid of Formula II is symmetrical, i.e., R<sup>1</sup> and R<sup>2</sup> are both the same. In another preferred embodiment, at least one or both R<sup>1</sup> and R<sup>2</sup> comprises at least two sites of unsaturation (e.g., 2, 3, 4, 5, 6, 2-3, 2-4, 2-5, or 2-6 sites of unsaturation).

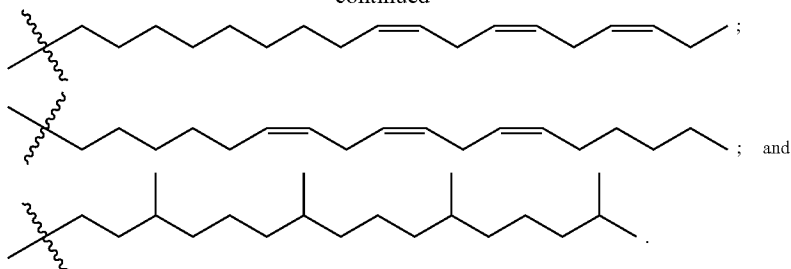
In embodiments where one or both R<sup>1</sup> and R<sup>2</sup> independently comprises a branched alkyl or acyl group (e.g., a substituted alkyl or acyl group), the branched alkyl or acyl group may comprise a C<sub>12</sub>-C<sub>24</sub> alkyl or acyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C<sub>1</sub>-C<sub>6</sub> alkyl substituents. In particular embodiments, the branched alkyl or acyl group comprises a C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl or acyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C<sub>1</sub>-C<sub>4</sub> alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. In some embodiments, the branched alkyl group comprises a phytanyl (3,7,11,15-tetramethyl-hexadecanyl) moiety and the branched acyl group comprises a phytanoyl (3,7,11,15-tetramethyl-hexadecanoyl) moiety. In particular embodiments, R<sup>1</sup> and R<sup>2</sup> are both phytanyl moieties.

In some groups of embodiments to the cationic lipids of Formula II, R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently selected from the group consisting of:





-continued

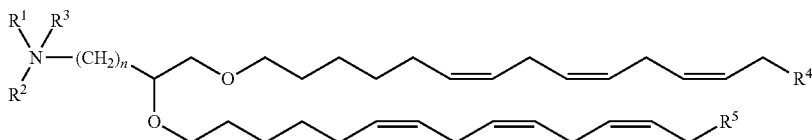


In certain embodiments, cationic lipids falling within the scope of Formula II include, but are not limited to, the following: 2,2-dilinoyleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA; "XTC2" or "C2K"), DLen-C2K-DMA,  $\gamma$ -DLin-C2K-DMA, DPan-C2K-DMA, or mixtures thereof. In preferred embodiments, the cationic lipid of Formula II comprises DLin-K-C2-DMA.

In some embodiments, the cationic lipids of Formula II form a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula II is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

The synthesis of DLin-K-C2-DMA is described herein and in PCT Publication No. WO 2010/042877, the disclosure of which is incorporated herein by reference in its entirety for all purposes.

In a further aspect, cationic lipids of Formula III having the following structure are useful in the present invention:



(III)

or salts thereof, wherein:  $R^1$  and  $R^2$  are either the same or different and are independently an optionally substituted  $C_1$ - $C_6$  alkyl,  $C_2$ - $C_6$  alkenyl, or  $C_2$ - $C_6$  alkynyl, or  $R^1$  and  $R^2$  may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures

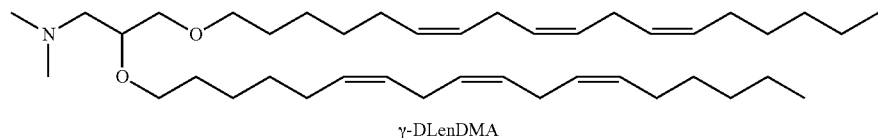
is below the  $pK_a$  of the cationic lipid such that the amino head group is protonated. In an alternative embodiment,  $R^3$  is an optionally substituted  $C_1$ - $C_4$  alkyl to provide a quaternary amine. In further embodiments,  $R^4$  and  $R^5$  are independently an optionally substituted  $C_2$ - $C_6$  or  $C_2$ - $C_4$  alkyl or  $C_2$ - $C_6$  or  $C_2$ - $C_4$  alkenyl.

In an alternative embodiment, the cationic lipid of Formula III comprises ester linkages between the amino head group and one or both of the alkyl chains. In some embodiments, the cationic lipid of Formula III forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula III is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

Although each of the alkyl chains in Formula III contains cis double bonds at positions 6, 9, and 12 (i.e., cis,cis,cis- $\Delta^6$ ,

$\Delta^9$ ,  $\Delta^{12}$ ), in an alternative embodiment, one, two, or three of these double bonds in one or both alkyl chains may be in the trans configuration.

In a particularly preferred embodiment, the cationic lipid of Formula III has the structure:

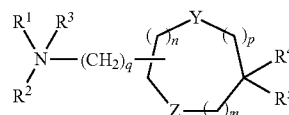
 $\gamma$ -DLenDMA

thereof;  $R^3$  is either absent or is hydrogen (H) or a  $C_1$ - $C_6$  alkyl to provide a quaternary amine;  $R^4$  and  $R^5$  are either absent or present and when present are either the same or different and are independently an optionally substituted  $C_1$ - $C_{10}$  alkyl or  $C_2$ - $C_{10}$  alkenyl; and n is 0, 1, 2, 3, or 4.

In some embodiments,  $R^1$  and  $R^2$  are independently an optionally substituted  $C_1$ - $C_4$  alkyl,  $C_2$ - $C_4$  alkenyl, or  $C_2$ - $C_4$  alkynyl. In a preferred embodiment,  $R^1$  and  $R^2$  are both methyl groups. In another preferred embodiment,  $R^4$  and  $R^5$  are both butyl groups. In yet another preferred embodiment, n is 1. In other embodiments,  $R^3$  is absent when the pH is above the  $pK_a$  of the cationic lipid and  $R^3$  is hydrogen when the pH

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In another aspect, cationic lipids of Formula IV having the following structure are useful in the present invention:



(IV)

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or salts thereof, wherein:  $R^1$  and  $R^2$  are either the same or different and are independently an optionally substituted  $C_1$ - $C_6$  alkyl,  $C_2$ - $C_6$  alkenyl, or  $C_2$ - $C_6$  alkynyl, or  $R^1$  and  $R^2$  may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;  $R^3$  is either absent or is hydrogen (H) or a  $C_1$ - $C_6$  alkyl to provide a quaternary amine;  $R^4$  and  $R^5$  are either the same or different and are independently an optionally substituted  $C_{12}$ - $C_{24}$  alkyl,  $C_{12}$ - $C_{24}$  alkenyl,  $C_{12}$ - $C_{24}$  alkynyl, or  $C_{12}$ - $C_{24}$  acyl, wherein at least one of  $R^4$  and  $R^5$  comprises at least three sites of unsaturation or a substituted  $C_{12}$ - $C_{24}$  alkyl; m, n, and p are either the same or different and are independently either 0, 1, or 2, 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 are independently O, S, or NH.

In some embodiments,  $R^1$  and  $R^2$  are independently an optionally substituted  $C_1$ - $C_4$  alkyl,  $C_2$ - $C_4$  alkenyl, or  $C_2$ - $C_4$  alkynyl. In a preferred embodiment,  $R^1$  and  $R^2$  are both methyl groups. In another preferred embodiment, q is 2. In other embodiments,  $R^3$  is absent when the pH is above the  $pK_a$  of the cationic lipid and  $R^3$  is hydrogen when the pH is below the  $pK_a$  of the cationic lipid such that the amino head group is protonated. In an alternative embodiment,  $R^3$  is an optionally substituted  $C_1$ - $C_4$  alkyl to provide a quaternary amine. In further embodiments,  $R^4$  and  $R^5$  are independently an optionally substituted  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  alkyl,  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  alkenyl,  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  alkynyl, or  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  acyl.

In embodiments where at least one of  $R^4$  and  $R^5$  comprises a branched alkyl group (e.g., a substituted  $C_{12}$ - $C_{24}$  alkyl group), the branched alkyl group may comprise a  $C_{12}$ - $C_{24}$  alkyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more)  $C_1$ - $C_6$  alkyl substituents. In particular embodiments, the branched alkyl group comprises a  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  alkyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6)  $C_1$ - $C_4$  alkyl (e.g., methyl, ethyl, propyl,

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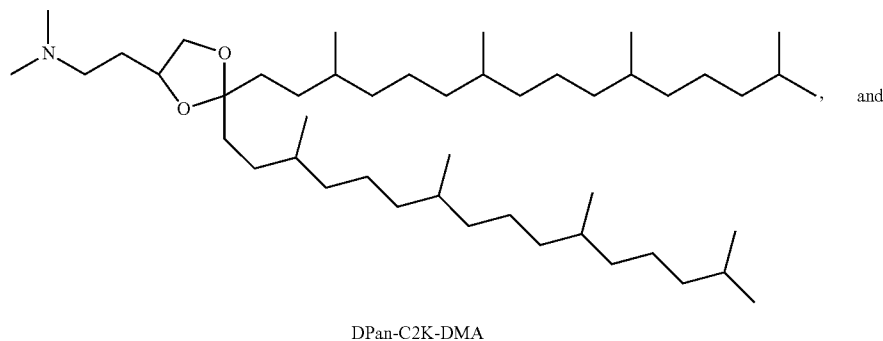
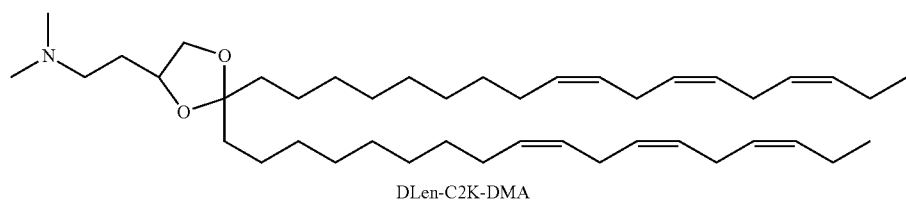
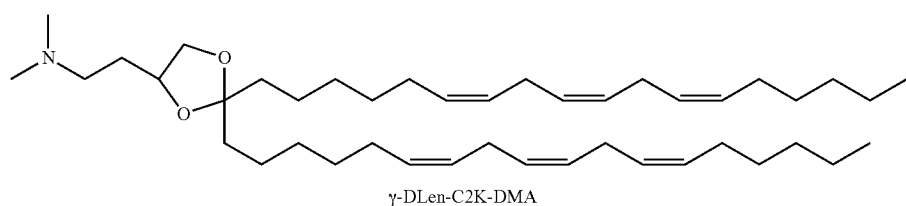
or butyl) substituents. Preferably, the branched alkyl group comprises a phytanyl (3,7,11,15-tetramethyl-hexadecanyl) moiety. In other preferred embodiments,  $R^4$  and  $R^5$  are both phytanyl moieties.

In alternative embodiments, at least one of  $R^4$  and  $R^5$  comprises a branched acyl group (e.g., a substituted  $C_{12}$ - $C_{24}$  acyl group). In certain instances, the branched acyl group may comprise a  $C_{12}$ - $C_{24}$  acyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more)  $C_1$ - $C_6$  alkyl substituents. In particular embodiments, the branched acyl group comprises a  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  acyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6)  $C_1$ - $C_4$  alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. Preferably, the branched acyl group comprises a phytanoyl (3,7,11,15-tetramethyl-hexadecanoyl) moiety.

In embodiments where at least one of  $R^4$  and  $R^5$  comprises at least three sites of unsaturation, the double bonds present in one or both alkyl chains may be in the cis and/or trans configuration. In some embodiments,  $R^4$  and  $R^5$  are independently selected from the group consisting of a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a phytanyl moiety, as well as acyl derivatives thereof (e.g., linolenyl,  $\gamma$ -linolenyl, phytanoyl, etc.). In certain instances, the octadecatrienyl moiety is a linolenyl moiety or a  $\gamma$ -linolenyl moiety. In preferred embodiments,  $R^4$  and  $R^5$  are both linolenyl moieties or  $\gamma$ -linolenyl moieties. In particular embodiments,  $R^4$  and  $R^5$  independently comprise a backbone of from about 16 to about 22 carbon atoms, and one or both of  $R^4$  and  $R^5$  independently comprise at least three, four, five, or six sites of unsaturation.

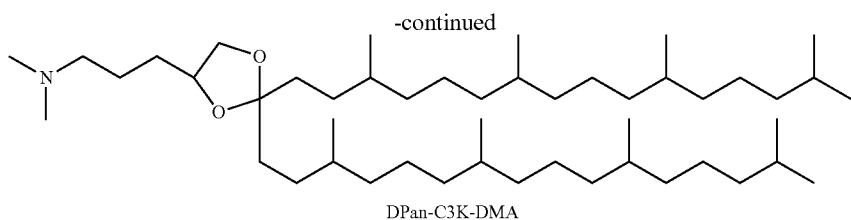
In some embodiments, the cationic lipid of Formula IV forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula IV is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

In a particularly preferred embodiment, the cationic lipid of Formula IV has a structure selected from the group consisting of:

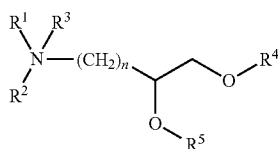


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In yet another aspect, cationic lipids of Formula V having the following structure are useful in the present invention:



or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are joined to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof; R<sup>3</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; R<sup>4</sup> and R<sup>5</sup> are either the same or different and are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, C<sub>12</sub>-C<sub>24</sub> alkenyl, C<sub>12</sub>-C<sub>24</sub> alkynyl, or C<sub>12</sub>-C<sub>24</sub> acyl; and n is 0, 1, 2, 3, or 4.

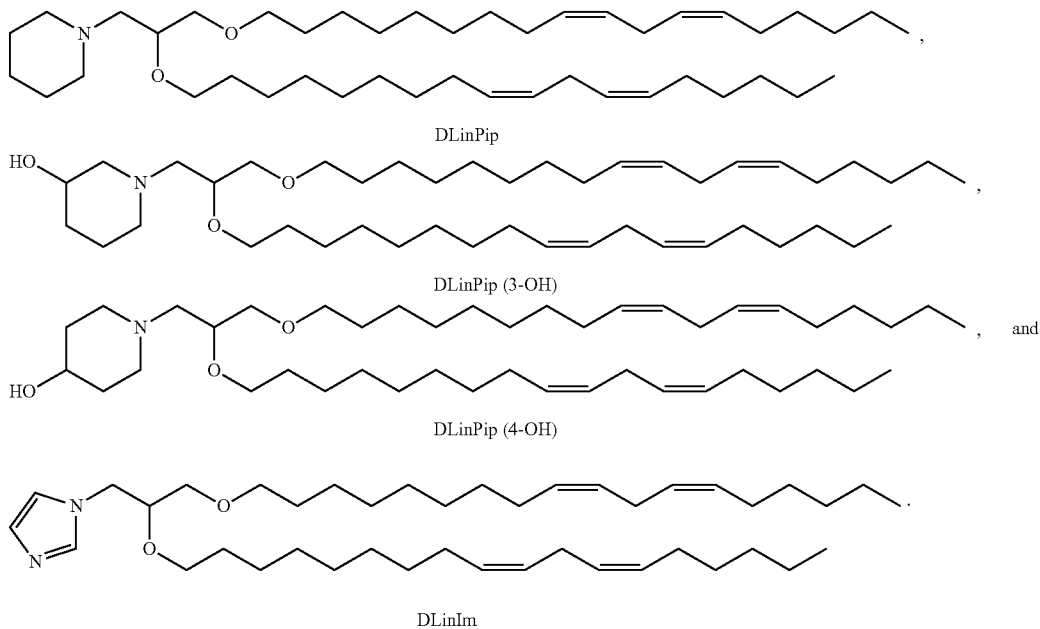
In some embodiments, R<sup>1</sup> and R<sup>2</sup> are joined to form a heterocyclic ring of 5 carbon atoms and 1 nitrogen atom. In certain instances, the heterocyclic ring is substituted with a substituent such as a hydroxyl group at the ortho, meta, and/or para positions. In a preferred embodiment, n is 1. In other embodiments, R<sup>3</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>3</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is

protonated. In an alternative embodiment, R<sup>3</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine. In further embodiments, R<sup>4</sup> and R<sup>5</sup> are independently an optionally substituted C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkenyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkynyl, or C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> acyl.

In certain embodiments, R<sup>4</sup> and R<sup>5</sup> are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (e.g., a phytanyl moiety), as well as acyl derivatives thereof (e.g., linoleoyl, linolenoyl, γ-linolenoyl, phytanoyl, etc.). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a γ-linolenyl moiety. In particular embodiments, R<sup>4</sup> and R<sup>5</sup> are both linoleyl moieties, linolenyl moieties, γ-linolenyl moieties, or phytanyl moieties.

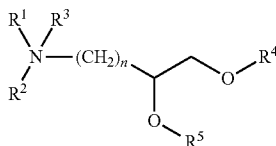
In some embodiments, the cationic lipid of Formula V forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula V is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

In a particularly preferred embodiment, the cationic lipid of Formula V has a structure selected from the group consisting of:



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In still yet another aspect, cationic lipids of Formula VI having the following structure are useful in the present invention:



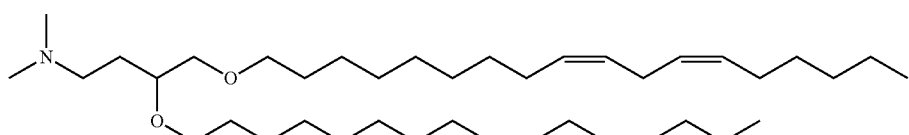
or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently an optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, or C<sub>2</sub>-C<sub>6</sub> alkynyl, or R<sup>1</sup> and R<sup>2</sup> may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof; R<sup>3</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; R<sup>4</sup> and R<sup>5</sup> are either the same

(VI)

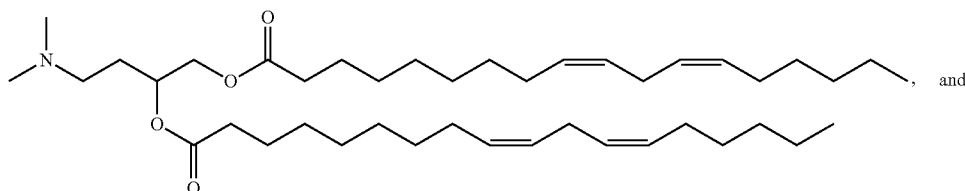
5 a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (e.g., a phytanyl moiety), as well as acyl derivatives thereof (e.g., linoleoyl, linolenoyl,  $\gamma$ -linolenoyl, phytanoyl, etc.). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a  $\gamma$ -linolenyl moiety. In particular embodiments, R<sup>4</sup> and R<sup>5</sup> are both linoleyl moieties, linolenyl moieties,  $\gamma$ -linolenyl moieties, or phytanyl moieties.

15 In some embodiments, the cationic lipid of Formula VI forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula VI is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

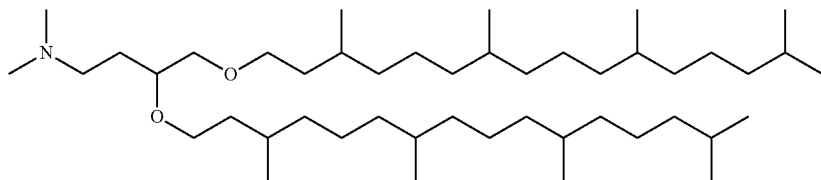
20 In a particularly preferred embodiment, the cationic lipid of Formula VI has a structure selected from the group consisting of:



C2-DLinDMA



C2-DLinDAP



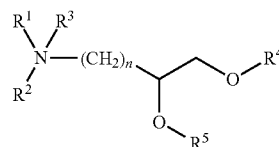
C2-DPanDMA

or different and are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, C<sub>12</sub>-C<sub>24</sub> alkenyl, C<sub>12</sub>-C<sub>24</sub> alkynyl, or C<sub>12</sub>-C<sub>24</sub> acyl; and n is 2, 3, or 4.

In some embodiments, R<sup>1</sup> and R<sup>2</sup> are independently an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>2</sub>-C<sub>4</sub> alkenyl, or C<sub>2</sub>-C<sub>4</sub> alkynyl. In a preferred embodiment, R<sup>1</sup> and R<sup>2</sup> are both methyl groups. In another preferred embodiment, n is 2. In other embodiments, R<sup>3</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>3</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R<sup>3</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine. In further embodiments, R<sup>4</sup> and R<sup>5</sup> are independently an optionally substituted C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkenyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkynyl, or C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> acyl.

In certain embodiments, R<sup>4</sup> and R<sup>5</sup> are independently selected from the group consisting of a dodecadienyl moiety,

50 In another aspect, cationic lipids of Formula VII having the following structure are useful in the present invention:



(VII)

or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently an optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, or C<sub>2</sub>-C<sub>6</sub> alkynyl, or R<sup>1</sup> and R<sup>2</sup> may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures

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thereof;  $R^3$  is either absent or is hydrogen (H) or a  $C_1$ - $C_6$  alkyl to provide a quaternary amine;  $R^4$  and  $R^5$  are different and are independently an optionally substituted  $C_1$ - $C_{24}$  alkyl,  $C_2$ - $C_{24}$  alkenyl,  $C_2$ - $C_{24}$  alkynyl, or  $C_1$ - $C_{24}$  acyl; and  $n$  is 0, 1, 2, 3, or 4.

In some embodiments,  $R^1$  and  $R^2$  are independently an optionally substituted  $C_1$ - $C_4$  alkyl,  $C_2$ - $C_4$  alkenyl, or  $C_2$ - $C_4$  alkynyl. In a preferred embodiment,  $R^1$  and  $R^2$  are both methyl groups. In another preferred embodiment,  $n$  is 1. In other embodiments,  $R^3$  is absent when the pH is above the  $pK_a$  of the cationic lipid and  $R^3$  is hydrogen when the pH is below the  $pK_a$  of the cationic lipid such that the amino head group is protonated. In an alternative embodiment,  $R^3$  is an optionally substituted  $C_1$ - $C_4$  alkyl to provide a quaternary amine. In further embodiments,  $R^4$  and  $R^5$  are different and are independently an optionally substituted  $C_4$ - $C_{20}$  alkyl,  $C_4$ - $C_{20}$  alkenyl,  $C_4$ - $C_{20}$  alkynyl, or  $C_4$ - $C_{20}$  acyl.

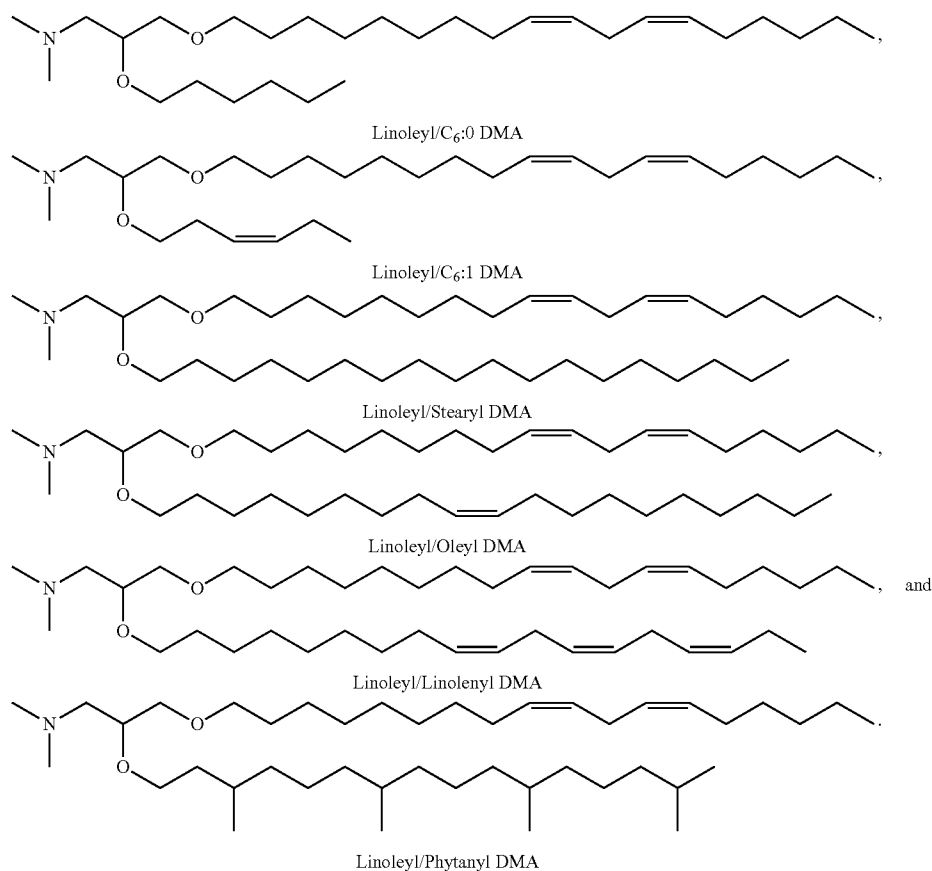
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and  $R^5$  is an optionally substituted  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  alkyl,  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  alkenyl,  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  alkynyl, or  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  acyl.

In particular embodiments,  $R^4$  is a linoleyl moiety, and  $R^5$  is a  $C_6$  alkyl moiety, a  $C_6$  alkenyl moiety, an octadecyl moiety, an oleyl moiety, a linolenyl moiety, a  $\gamma$ -linolenyl moiety, or a phytanyl moiety. In other embodiments, one of  $R^4$  or  $R^5$  is a phytanyl moiety.

In some embodiments, the cationic lipid of Formula VII forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula VII is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

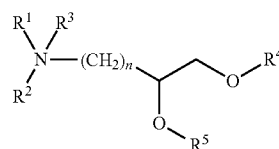
In a particularly preferred embodiment, the cationic lipid of Formula VII is an asymmetric lipid having a structure selected from the group consisting of:



In some embodiments,  $R^4$  is an optionally substituted  $C_{12}$ - $C_{24}$  alkyl,  $C_{12}$ - $C_{24}$  alkenyl,  $C_{12}$ - $C_{24}$  alkynyl, or  $C_{12}$ - $C_{24}$  acyl, and  $R^5$  is an optionally substituted  $C_4$ - $C_{10}$  alkyl,  $C_4$ - $C_{10}$  alkenyl,  $C_4$ - $C_{10}$  alkynyl, or  $C_4$ - $C_{10}$  acyl. In certain instances,  $R^4$  is an optionally substituted  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  alkyl,  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  alkenyl,  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  alkynyl, or  $C_{12}$ - $C_{20}$  or  $C_{14}$ - $C_{22}$  acyl, and  $R^5$  is an optionally substituted  $C_4$ - $C_8$  or  $C_6$  alkyl,  $C_4$ - $C_8$  or  $C_6$  alkenyl,  $C_4$ - $C_8$  or  $C_6$  alkynyl, or  $C_4$ - $C_8$  or  $C_6$  acyl.

In other embodiments,  $R^4$  is an optionally substituted  $C_4$ - $C_{10}$  alkyl,  $C_4$ - $C_{10}$  alkenyl,  $C_4$ - $C_{10}$  alkynyl, or  $C_4$ - $C_{10}$  acyl, and  $R^5$  is an optionally substituted  $C_{12}$ - $C_{24}$  alkyl,  $C_{12}$ - $C_{24}$  alkenyl,  $C_{12}$ - $C_{24}$  alkynyl, or  $C_{12}$ - $C_{24}$  acyl. In certain instances,  $R^4$  is an optionally substituted  $C_4$ - $C_8$  or  $C_6$  alkyl,  $C_4$ - $C_8$  or  $C_6$  alkenyl,  $C_4$ - $C_8$  or  $C_6$  alkynyl, or  $C_4$ - $C_8$  or  $C_6$  acyl,

In yet another aspect, cationic lipids of Formula VIII having the following structure are useful in the present invention:

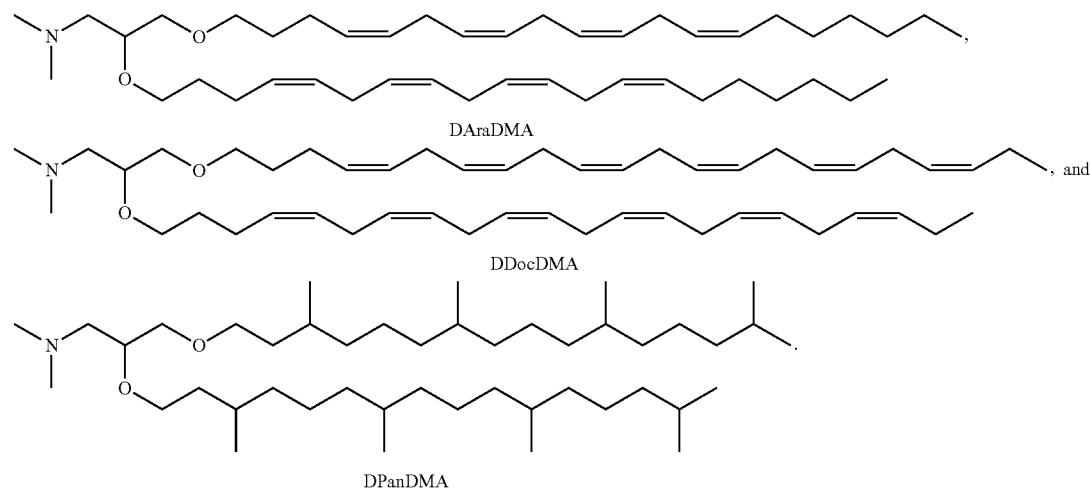


(VIII)

or salts thereof, wherein:  $R^1$  and  $R^2$  are either the same or different and are independently an optionally substituted  $C_1$ - $C_6$  alkyl,  $C_2$ - $C_6$  alkenyl, or  $C_2$ - $C_6$  alkynyl, or  $R^1$  and  $R^2$

may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof; R<sup>3</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; R<sup>4</sup> and R<sup>5</sup> are either the same or different and are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, C<sub>12</sub>-C<sub>24</sub> alkenyl, C<sub>12</sub>-C<sub>24</sub> alkynyl, or C<sub>12</sub>-C<sub>24</sub> acyl, wherein at least one of R<sup>4</sup> and R<sup>5</sup> comprises at least four sites of unsaturation or a substituted C<sub>12</sub>-C<sub>24</sub> alkyl; and n is 0, 1, 2, 3, or 4.

In some embodiments, R<sup>1</sup> and R<sup>2</sup> are independently an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>2</sub>-C<sub>4</sub> alkenyl, or C<sub>2</sub>-C<sub>4</sub> alkynyl. In a preferred embodiment, R<sup>1</sup> and R<sup>2</sup> are both methyl groups. In another preferred embodiment, n is 1. In other embodiments, R<sup>3</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>3</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R<sup>3</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine. In further embodiments, R<sup>4</sup> and R<sup>5</sup> are independently an optionally substituted C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkenyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkynyl, or C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> acyl.



In embodiments where at least one of R<sup>4</sup> and R<sup>5</sup> comprises a branched alkyl group (e.g., a substituted C<sub>12</sub>-C<sub>24</sub> alkyl group), the branched alkyl group may comprise a C<sub>12</sub>-C<sub>24</sub> alkyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C<sub>1</sub>-C<sub>6</sub> alkyl substituents. In particular embodiments, the branched alkyl group comprises a C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C<sub>1</sub>-C<sub>4</sub> alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. Preferably, the branched alkyl group comprises a phytanyl (3,7,11,15-tetramethyl-hexadecanyl) moiety.

In alternative embodiments, at least one of R<sup>4</sup> and R<sup>5</sup> comprises a branched acyl group (e.g., a substituted C<sub>12</sub>-C<sub>24</sub> acyl group). In certain instances, the branched acyl group may comprise a C<sub>12</sub>-C<sub>24</sub> acyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C<sub>1</sub>-C<sub>6</sub> alkyl substituents. In particular embodiments, the branched acyl group comprises a C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> acyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C<sub>1</sub>-C<sub>4</sub> alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. Preferably, the branched acyl group comprises a phytanoyl (3,7,11,15-tetramethyl-hexadecanoyl) moiety.

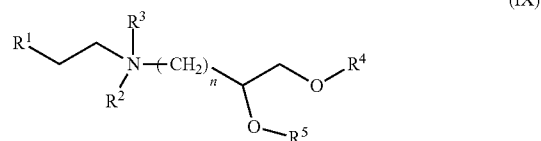
In embodiments where at least one of R<sup>4</sup> and R<sup>5</sup> comprises at least four sites of unsaturation, the double bonds present in

one or both alkyl chains may be in the cis and/or trans configuration. In a particular embodiment, R<sup>4</sup> and R<sup>5</sup> independently comprise four, five, or six sites of unsaturation. In some instances, R<sup>4</sup> comprises four, five, or six sites of unsaturation and R<sup>5</sup> comprises zero, one, two, three, four, five, or six sites of unsaturation. In other instances, R<sup>4</sup> comprises zero, one, two, three, four, five, or six sites of unsaturation and R<sup>5</sup> comprises four, five, or six sites of unsaturation. In a preferred embodiment, both R<sup>4</sup> and R<sup>5</sup> comprise four, five, or six sites of unsaturation. In particular embodiments, R<sup>4</sup> and R<sup>5</sup> independently comprise a backbone of from about 18 to about 24 carbon atoms, and one or both of R<sup>4</sup> and R<sup>5</sup> independently comprise at least four, five, or six sites of unsaturation.

In some embodiments, the cationic lipid of Formula VIII forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula VIII is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

In a particularly preferred embodiment, the cationic lipid of Formula VIII has a structure selected from the group consisting of:

In still yet another aspect, cationic lipids of Formula IX having the following structure are useful in the present invention:



or salts thereof, wherein: R<sup>1</sup> is hydrogen (H) or —(CH<sub>2</sub>)<sub>q</sub>—NR<sup>6</sup>R<sup>7</sup>R<sup>8</sup>, wherein: R<sup>6</sup> and R<sup>7</sup> are either the same or different and are independently an optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, or C<sub>2</sub>-C<sub>6</sub> alkynyl, or R<sup>6</sup> and R<sup>7</sup> may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof; R<sup>8</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; and q is 0, 1, 2, 3, or 4; R<sup>2</sup> is an

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optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, or C<sub>2</sub>-C<sub>6</sub> alkynyl; R<sup>3</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; R<sup>4</sup> and R<sup>5</sup> are either the same or different and are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, C<sub>12</sub>-C<sub>24</sub> alkenyl, C<sub>12</sub>-C<sub>24</sub> alkynyl, or C<sub>12</sub>-C<sub>24</sub> acyl; and n is 0, 1, 2, 3, or 4.

In some embodiments, R<sup>2</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>2</sub>-C<sub>4</sub> alkenyl, or C<sub>2</sub>-C<sub>4</sub> alkynyl. In other embodiments, R<sup>3</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>3</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R<sup>3</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine. In certain embodiments, R<sup>4</sup> and R<sup>5</sup> are independently an optionally substituted C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkenyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkynyl, or C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> acyl.

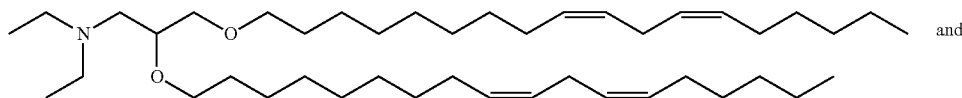
In further embodiments, R<sup>6</sup> and R<sup>7</sup> are independently an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>2</sub>-C<sub>4</sub> alkenyl, or C<sub>2</sub>-C<sub>4</sub> alkynyl. In other embodiments, R<sup>8</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>8</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R<sup>8</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine.

In a preferred embodiment, R<sup>1</sup> is hydrogen and R<sup>2</sup> is an ethyl group. In another preferred embodiment, R<sup>6</sup> and R<sup>7</sup> are both methyl groups. In certain instances, n is 1. In certain other instances, q is 1.

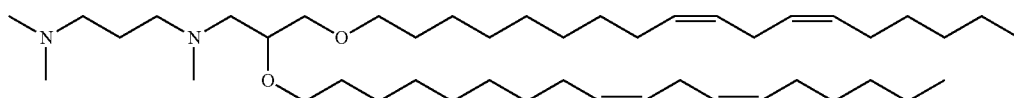
In certain embodiments, R<sup>4</sup> and R<sup>5</sup> are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradecatrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (e.g., a phytanyl moiety), as well as acyl derivatives thereof (e.g., linoleoyl, linolenoyl,  $\gamma$ -linolenoyl, phytanoyl, etc.). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a  $\gamma$ -linolenyl moiety. In particular embodiments, R<sup>4</sup> and R<sup>5</sup> are both linoleyl moieties, linolenyl moieties,  $\gamma$ -linolenyl moieties, or phytanyl moieties.

In some embodiments, the cationic lipid of Formula IX forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula IX is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

In a particularly preferred embodiment, the cationic lipid of Formula IX has a structure selected from the group consisting of:



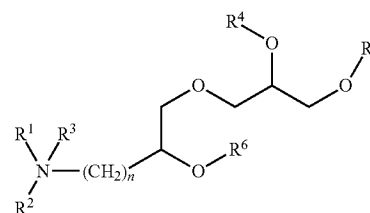
DLinDEA



2N-DLinDMA

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In another aspect, cationic lipids of Formula X having the following structure are useful in the present invention:



(X)

or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently an optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, or C<sub>2</sub>-C<sub>6</sub> alkynyl, or R<sup>1</sup> and R<sup>2</sup> may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof; R<sup>3</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; R<sup>4</sup>, R<sup>5</sup>, and R<sup>6</sup> are either the same or different and are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, C<sub>12</sub>-C<sub>24</sub> alkenyl, C<sub>12</sub>-C<sub>24</sub> alkynyl, or C<sub>12</sub>-C<sub>24</sub> acyl; and n is 0, 1, 2, 3, or 4.

In some embodiments, R<sup>1</sup> and R<sup>2</sup> are independently an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>2</sub>-C<sub>4</sub> alkenyl, or C<sub>2</sub>-C<sub>4</sub> alkynyl. In a preferred embodiment, R<sup>1</sup> and R<sup>2</sup> are both methyl groups. In another preferred embodiment, n is 1. In other embodiments, R<sup>3</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>3</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R<sup>3</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine. In further embodiments, R<sup>4</sup>, R<sup>5</sup>, and R<sup>6</sup> are independently an optionally substituted C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkenyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkynyl, or C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> acyl.

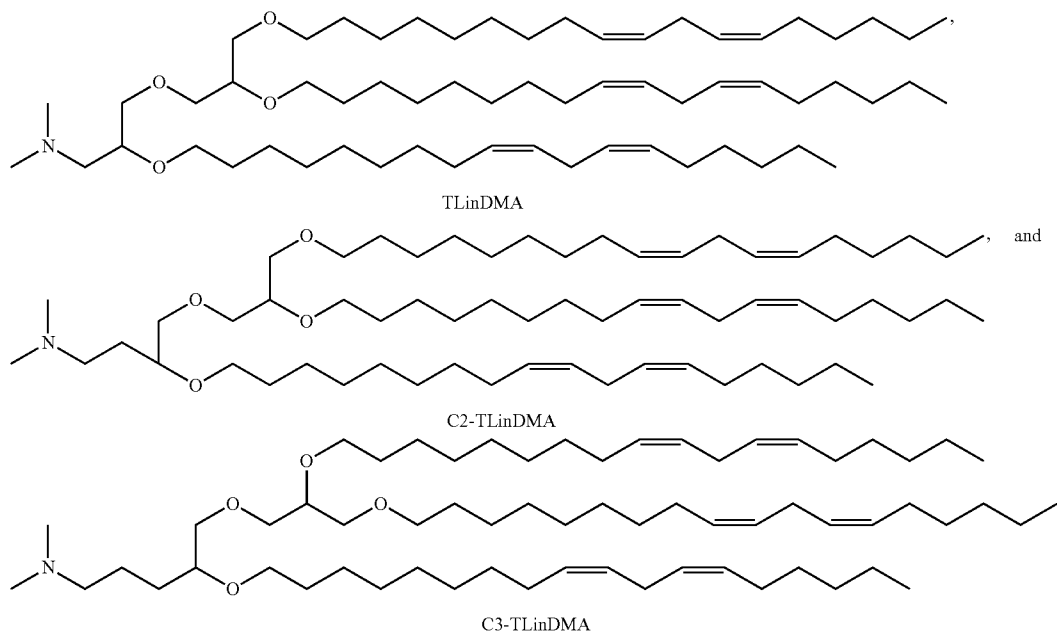
In certain embodiments, R<sup>4</sup>, R<sup>5</sup>, and R<sup>6</sup> are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradecatrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (e.g., a phytanyl moiety), as well as acyl derivatives thereof (e.g., linoleoyl, linolenoyl,  $\gamma$ -linolenoyl, phytanoyl, etc.). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a  $\gamma$ -linolenyl moiety. In particular embodiments, R<sup>4</sup>, R<sup>5</sup>, and

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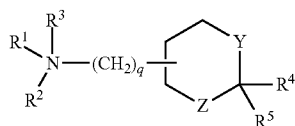
R<sup>6</sup> are all linoleyl moieties, linolenyl moieties,  $\gamma$ -linolenyl moieties, or phytanyl moieties.

In some embodiments, the cationic lipid of Formula X forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula X is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

In a particularly preferred embodiment, the cationic lipid of Formula X has a structure selected from the group consisting of:



In yet another aspect, cationic lipids of Formula XI having the following structure are useful in the present invention:



or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently an optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, or C<sub>2</sub>-C<sub>6</sub> alkynyl, or R<sup>1</sup> and R<sup>2</sup> may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof; R<sup>3</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; R<sup>4</sup> and R<sup>5</sup> are either the same or different and are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, C<sub>12</sub>-C<sub>24</sub> alkenyl, C<sub>12</sub>-C<sub>24</sub> alkynyl, or C<sub>12</sub>-C<sub>24</sub> acyl; q is 0, 1, 2, 3, or 4; and Y and Z are either the same or different and are independently O, S, or NH, wherein if q is 1, R<sup>1</sup> and R<sup>2</sup> are both methyl groups, R<sup>4</sup> and R<sup>5</sup> are both linoleyl moieties, and Y and Z are both O, then the alkylamino group is attached to one of the two carbons adjacent to Y or Z (i.e., at the '4' or '6' position of the 6-membered ring).

In some embodiments, R<sup>1</sup> and R<sup>2</sup> are independently an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>2</sub>-C<sub>4</sub> alkenyl, or C<sub>2</sub>-C<sub>4</sub>

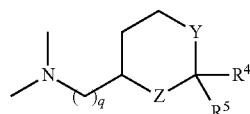
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alkynyl. In a preferred embodiment, R<sup>1</sup> and R<sup>2</sup> are both methyl groups. In another preferred embodiment, q is 2. In a particular embodiment, Y and Z are both oxygen (O). In other embodiments, R<sup>3</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>3</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R<sup>3</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine. In further embodiments, R<sup>4</sup> and R<sup>5</sup> are independently

an optionally substituted C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkenyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkynyl, or C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> acyl.

In other embodiments, R<sup>4</sup> and R<sup>5</sup> are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (e.g., a phytanyl moiety), as well as acyl derivatives thereof (e.g., linoleoyl, linolenyl,  $\gamma$ -linolenyl, phytanoyl, etc.). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecatrienyl moiety is a linolenyl moiety or a  $\gamma$ -linolenyl moiety. In particular embodiments, R<sup>4</sup> and R<sup>5</sup> are both linoleyl moieties, linolenyl moieties,  $\gamma$ -linolenyl moieties, or phytanyl moieties.

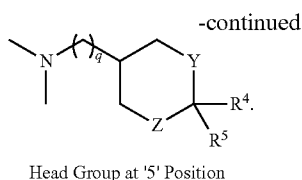
The alkylamino head group of Formula XI may be attached to the '4' or '5' position of the 6-membered ring as shown below in an exemplary embodiment wherein R<sup>1</sup> and R<sup>2</sup> are both methyl groups:



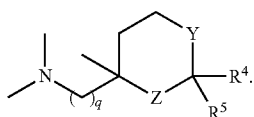
Head Group at '4' Position; or



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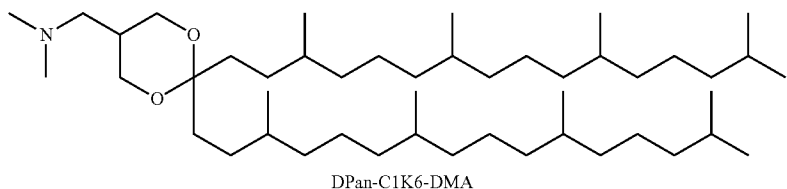
In further embodiments, the 6-membered ring of Formula XI may be substituted with 1, 2, 3, 4, or 5 independently selected C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, C<sub>2</sub>-C<sub>6</sub> alkynyl, C<sub>1</sub>-C<sub>6</sub> alkoxy, or hydroxyl substituents. In one particular embodiment, the 6-membered ring is substituted with 1, 2, 3, 4, or 5 independently selected C<sub>1</sub>-C<sub>4</sub> alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. An exemplary embodiment of a cationic lipid of Formula XI having a substituted 6-membered ring (methyl group attached to the '4' position) and wherein R<sup>1</sup> and R<sup>2</sup> are both methyl groups is shown below:



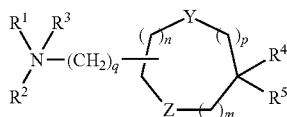
In particular embodiments, the cationic lipids of Formula XI may be synthesized using 2-hydroxymethyl-1,4-butanediol and 1,3,5-pentanetriol (or 3-methyl-1,3,5-pentanetriol) as starting materials.

In some embodiments, the cationic lipid of Formula XI forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula XI is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

In a particularly preferred embodiment, the cationic lipid of Formula XI has the structure:



In still yet another aspect, the present invention provides a cationic lipid of Formula XII having the following structure:



or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently an optionally substituted

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C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, or C<sub>2</sub>-C<sub>6</sub> alkynyl, or R<sup>1</sup> and R<sup>2</sup> may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof; R<sup>3</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; R<sup>4</sup> and R<sup>5</sup> are either the same or different and are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, C<sub>12</sub>-C<sub>24</sub> alkenyl, C<sub>12</sub>-C<sub>24</sub> alkynyl, or C<sub>12</sub>-C<sub>24</sub> acyl, wherein at least one of R<sup>4</sup> and R<sup>5</sup> comprises at least one site of unsaturation in the trans (K) configuration; m, n, and p are either the same or different and are independently either 0, 1, or 2, 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 are independently O, S, or NH.

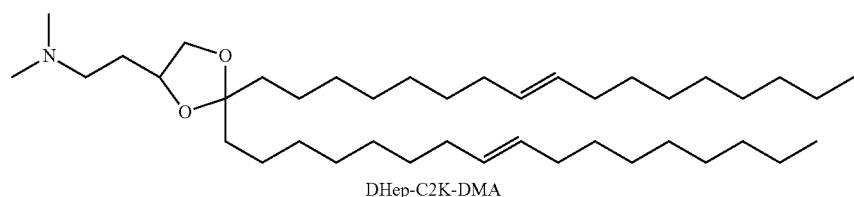
In some embodiments, R<sup>1</sup> and R<sup>2</sup> are independently an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>2</sub>-C<sub>4</sub> alkenyl, or C<sub>2</sub>-C<sub>4</sub> alkynyl. In a preferred embodiment, R<sup>1</sup> and R<sup>2</sup> are both methyl groups. In another preferred embodiment, q is 2. In other embodiments, R<sup>3</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>3</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R<sup>3</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine. In further embodiments, R<sup>4</sup> and R<sup>5</sup> are independently an optionally substituted C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkenyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkynyl, or C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> acyl.

In certain embodiments, at least one of R<sup>4</sup> and R<sup>5</sup> further comprises one, two, three, four, five, six, or more sites of unsaturation in the cis and/or trans configuration. In some instances, R<sup>4</sup> and R<sup>5</sup> are independently selected from any of the substituted or unsubstituted alkyl or acyl groups described herein, wherein at least one or both of R<sup>4</sup> and R<sup>5</sup> comprises at least one, two, three, four, five, or six sites of unsaturation in the trans configuration. In one particular embodiment, R<sup>4</sup> and R<sup>5</sup> independently comprise a backbone of from about 12 to about 22 carbon atoms (e.g., 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22 carbon atoms), and one or both of R<sup>4</sup> and R<sup>5</sup> independently comprise at least one, two, three, four, five, or six

sites of unsaturation in the trans configuration. In some preferred embodiments, at least one of R<sup>4</sup> and R<sup>5</sup> comprises an (E)-heptadecyl moiety. In other preferred embodiments, R<sup>4</sup> and R<sup>5</sup> are both (E)-8-heptadecyl moieties.

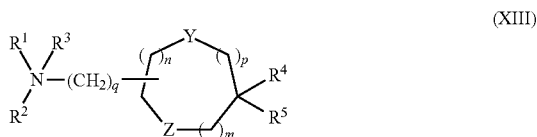
In some embodiments, the cationic lipid of Formula XII forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula XII is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

In a particularly preferred embodiment, the cationic lipid of Formula XII has the structure:



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In another aspect, the present invention provides a cationic lipid of Formula XIII having the following structure:



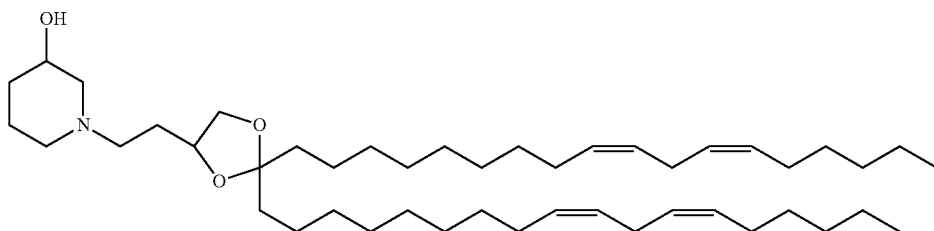
or salts thereof, wherein: R<sup>1</sup> and R<sup>2</sup> are joined to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof; R<sup>3</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine; R<sup>4</sup> and R<sup>5</sup> are either the same or different and are independently an optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, C<sub>12</sub>-C<sub>24</sub> alkenyl, C<sub>12</sub>-C<sub>24</sub> alkynyl, or C<sub>12</sub>-C<sub>24</sub> acyl; m, n, and p are either the same or different and are independently either 0, 1, or 2, 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 are independently O, S, or NH.

In some embodiments, R<sup>1</sup> and R<sup>2</sup> are joined to form a heterocyclic ring of 5 carbon atoms and 1 nitrogen atom. In certain instances, the heterocyclic ring is substituted with a substituent such as a hydroxyl group at the ortho, meta, and/or para positions. In a preferred embodiment, q is 2. In other embodiments, R<sup>3</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>3</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R<sup>3</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine. In further embodiments, R<sup>4</sup> and R<sup>5</sup> are independently an optionally substituted C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkenyl, C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkynyl, or C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> acyl.

In certain embodiments, R<sup>4</sup> and R<sup>5</sup> are independently selected from the group consisting of a dodecadienyl moiety, a tetradecadienyl moiety, a hexadecadienyl moiety, an octadecadienyl moiety, an icosadienyl moiety, a dodecatrienyl moiety, a tetradectrienyl moiety, a hexadecatrienyl moiety, an octadecatrienyl moiety, an icosatrienyl moiety, and a branched alkyl group as described above (e.g., a phytanyl moiety), as well as acyl derivatives thereof (e.g., linoleoyl, linolenoyl, γ-linolenoyl, phytanoyl, etc.). In some instances, the octadecadienyl moiety is a linoleyl moiety. In other instances, the octadecadienyl moiety is a linolenyl moiety or a γ-linolenyl moiety. In particular embodiments, R<sup>4</sup> and R<sup>5</sup> are both linoleyl moieties, linolenyl moieties, γ-linolenyl moieties, or phytanyl moieties.

In some embodiments, the cationic lipid of Formula XIII forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula XIII is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

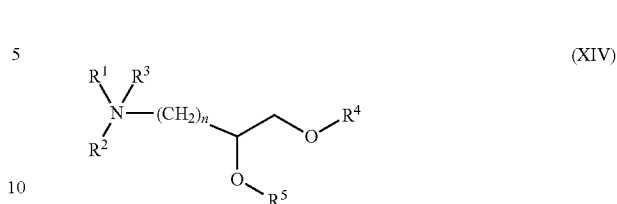
In a particularly preferred embodiment, the cationic lipid of Formula XIII has the structure:



DLin-C2K-Pip-3OH

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In yet another aspect, the present invention provides a cationic lipid of Formula XIV having the following structure:



or salts thereof, wherein:

R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently an optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, C<sub>2</sub>-C<sub>6</sub> alkenyl, or C<sub>2</sub>-C<sub>6</sub> alkynyl, or R<sup>1</sup> and R<sup>2</sup> may join to form an optionally substituted heterocyclic ring of 4 to 6 carbon atoms and 1 or 2 heteroatoms selected from the group consisting of nitrogen (N), oxygen (O), and mixtures thereof;

R<sup>3</sup> is either absent or is hydrogen (H) or a C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine;

R<sup>4</sup> and R<sup>5</sup> are either the same or different and are independently a substituted C<sub>12</sub>-C<sub>24</sub> alkyl; and

n is 0, 1, 2, 3, or 4.

In some embodiments, R<sup>1</sup> and R<sup>2</sup> are independently an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>2</sub>-C<sub>4</sub> alkenyl, or C<sub>2</sub>-C<sub>4</sub> alkynyl. In a preferred embodiment, R<sup>1</sup> and R<sup>2</sup> are both methyl groups. In one particular embodiment, n is 1. In another particular embodiment, n is 2. In other embodiments, R<sup>3</sup> is absent when the pH is above the pK<sub>a</sub> of the cationic lipid and R<sup>3</sup> is hydrogen when the pH is below the pK<sub>a</sub> of the cationic lipid such that the amino head group is protonated. In an alternative embodiment, R<sup>3</sup> is an optionally substituted C<sub>1</sub>-C<sub>4</sub> alkyl to provide a quaternary amine.

In embodiments where at least one of R<sup>4</sup> and R<sup>5</sup> comprises a branched alkyl group (e.g., a substituted C<sub>12</sub>-C<sub>24</sub> alkyl group), the branched alkyl group may comprise a C<sub>12</sub>-C<sub>24</sub> alkyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C<sub>1</sub>-C<sub>6</sub> alkyl substituents. In particular embodiments, the branched alkyl group comprises a C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> alkyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C<sub>1</sub>-C<sub>4</sub> alkyl (e.g., methyl, ethyl, propyl, or butyl) substituents. Preferably, the branched alkyl group comprises a phytanyl (3,7,11,15-tetramethyl-hexadecanyl) moiety. In particular embodiments, R<sup>4</sup> and R<sup>5</sup> are both phytanyl moieties.

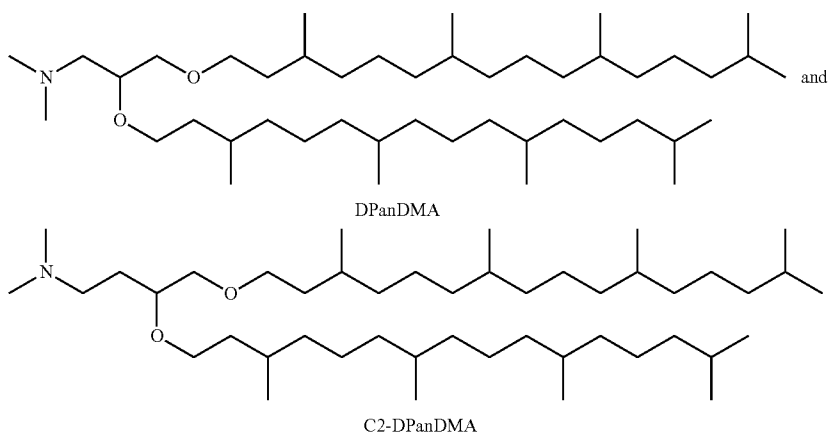
In alternative embodiments, at least one of R<sup>4</sup> and R<sup>5</sup> comprises a branched acyl group (e.g., a substituted C<sub>12</sub>-C<sub>24</sub> acyl group). In certain instances, the branched acyl group may comprise a C<sub>12</sub>-C<sub>24</sub> acyl having at least 1-6 (e.g., 1, 2, 3, 4, 5, 6, or more) C<sub>1</sub>-C<sub>6</sub> alkyl substituents. In particular embodiments, the branched acyl group comprises a C<sub>12</sub>-C<sub>20</sub> or C<sub>14</sub>-C<sub>22</sub> acyl with 1-6 (e.g., 1, 2, 3, 4, 5, 6) C<sub>1</sub>-C<sub>4</sub> alkyl (e.g.,

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methyl, ethyl, propyl, or butyl) substituents. Preferably, the branched acyl group comprises a phytanoyl (3,7,11,15-tetramethyl-hexadecanoyl) moiety. In particular embodiments, R<sup>4</sup> and R<sup>5</sup> are both phytanoyl moieties.

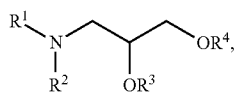
In some embodiments, the cationic lipid of Formula XIV forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula XIV is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

In a particularly preferred embodiment, the cationic lipid of Formula XIV has a structure selected from the group consisting of:



The synthesis of cationic lipids of Formulas III-XIV is described herein and in PCT Application No. PCT/CA2010/001029, entitled "Improved Cationic Lipids and Methods for the Delivery of Therapeutic Agents," filed Jun. 30, 2010, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In some embodiments, a mixture of cationic lipids or salts thereof can be included in the lipid particles of the present invention. In these embodiments, the mixture of cationic lipids includes a cationic lipid of Formulas I-XIV together with one or more additional cationic lipids. Other cationic lipids suitable for use in combination with the cationic lipids of Formulas I-XIV include cationic lipids of Formula XV having the following structure (or salts thereof):



wherein R<sup>1</sup> and R<sup>2</sup> are independently selected and are H or C<sub>1</sub>-C<sub>3</sub> alkyls, R<sup>3</sup> and R<sup>4</sup> are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R<sup>3</sup> and R<sup>4</sup> comprises at least two sites of unsaturation. In some instances, R<sup>1</sup> and R<sup>2</sup> are both methyl groups. In certain instances, R<sup>3</sup> and R<sup>4</sup> are both the same, i.e., R<sup>3</sup> and R<sup>4</sup> are both linoleyl (C<sub>18</sub>), etc. In other instances, R<sup>3</sup> and R<sup>4</sup> are different, i.e., R<sup>3</sup> is tetradecatrienyl (C<sub>14</sub>) and R<sup>4</sup> is linoleyl (C<sub>18</sub>). In a preferred embodiment, the cationic lipid of Formula XV is symmetrical, i.e., R<sup>3</sup> and R<sup>4</sup> are both the same. In another preferred embodiment, both R<sup>3</sup> and R<sup>4</sup> comprise at least two sites of unsaturation. In some embodiments, R<sup>3</sup> and R<sup>4</sup> are independently selected from the group consisting of

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dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R<sup>3</sup> and R<sup>4</sup> are both linoleyl. In some embodiments, R<sup>3</sup> and R<sup>4</sup> comprise at least three sites of unsaturation and are independently selected from, e.g., dodecatrienyl, tetradecatrienyl, hexadecatrienyl, linolenyl, and icosatrienyl. In particular embodiments, the cationic lipid of Formula XV comprises 1,2-dilinolelyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenlyloxy-N,N-dimethylaminopropane (DLenDMA), or mixtures thereof.

In some embodiments, the cationic lipid of Formula XV forms a salt (preferably a crystalline salt) with one or more

anions. In one particular embodiment, the cationic lipid of Formula XV is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

Other cationic lipids suitable for use in combination with the cationic lipids of Formulas I-XIV include cationic lipids of Formula XVI having the following structure (or salts thereof):



wherein R<sup>1</sup> and R<sup>2</sup> are independently selected and are H or C<sub>1</sub>-C<sub>3</sub> alkyls, R<sup>3</sup> and R<sup>4</sup> are independently selected and are alkyl groups having from about 10 to about 20 carbon atoms, and at least one of R<sup>3</sup> and R<sup>4</sup> comprises at least two sites of unsaturation. In certain instances, R<sup>3</sup> and R<sup>4</sup> are both the same, i.e., R<sup>3</sup> and R<sup>4</sup> are both linoleyl (C<sub>18</sub>), etc. In certain other instances, R<sup>3</sup> and R<sup>4</sup> are different, i.e., R<sup>3</sup> is tetradecatrienyl (C<sub>14</sub>) and R<sup>4</sup> is linoleyl (C<sub>18</sub>). In a preferred embodiment, the cationic lipid of Formula XVI is symmetrical, i.e., R<sup>3</sup> and R<sup>4</sup> are both the same. In another preferred embodiment, both R<sup>3</sup> and R<sup>4</sup> comprise at least two sites of unsaturation. In some embodiments, R<sup>3</sup> and R<sup>4</sup> are independently selected from the group consisting of dodecadienyl, tetradecadienyl, hexadecadienyl, linoleyl, and icosadienyl. In a preferred embodiment, R<sup>3</sup> and R<sup>4</sup> are both linoleyl. In some embodiments, R<sup>3</sup> and R<sup>4</sup> comprise at least three sites of unsaturation and are independently selected from, e.g., dodecatrienyl, tetradecatrienyl, hexadecatrienyl, linolenyl, and icosatrienyl.

In some embodiments, the cationic lipid of Formula XVI forms a salt (preferably a crystalline salt) with one or more anions. In one particular embodiment, the cationic lipid of Formula XVI is the oxalate (e.g., hemioxalate) salt thereof, which is preferably a crystalline salt.

The synthesis of cationic lipids such as DLinDMA and DLenDMA, as well as additional cationic lipids falling within the scope of Formulas XV and XVI, is described in U.S. Patent Publication No. 20060083780, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In addition to the cationic lipids of Formulas XV-XVI, other cationic lipids suitable for use in combination with one or more cationic lipids of Formulas I-XIV include, but are not limited to, 1,2-dioleoylcarbamoyloxy-3-dimethylaminopropane (DO-C-DAP), 1,2-dimyristoleoyl-3-dimethylaminopropane (DMDAP), 1,2-dioleoyl-3-trimethylaminopropane chloride (DOTAP.Cl), dilinoleylmethyl-3-dimethylaminopropionate (DLin-M-K-DMA; also known as DLin-M-DMA), N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), 1,2-dioleoyloxy-N,N-dimethylaminopropane (DODMA), 1,2-distearoyloxy-N,N-dimethylaminopropane (DSDMA), N-(1-(2,3-dioleoyloxy)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-dioleoyloxy-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-dioleoyloxybenzylamine (DMOBA), 1,2-N,N'-dioleoylcarbamyl-3-dimethylaminopropane (DOcarbDAP), 1,2-N,N'-dilinoleylcarbamyl-3-dimethylaminopropane (DLincarbDAP), 1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-dilinoleyoxy-3-(dimethylamino)acetoxopropane (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-linoleoyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-dilinoleyoxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-dilinoleyoxy-3-(N-methylpiperazino)propane (DLin-MPZ), 3-(N,N-dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-dioleylamino)-1,2-propanediol (DOAP), 1,2-dilinoleyoxy-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), and mixtures thereof.

Additional cationic lipids suitable for use in combination with one or more cationic lipids of Formulas I-XIV include, without limitation, cationic lipids such as (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (DLin-M-C3-DMA or "MC3") and certain analogs thereof as described in U.S. Provisional Patent Application No. 61/334,104, entitled "Novel Cationic Lipids and Methods of Use Thereof," filed May 12, 2010, and PCT Publication Nos. WO 2010/054401, WO 2010/054405, WO 2010/054406, and WO 2010/054384, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

The synthesis of cationic lipids such as DO-C-DAP, DMDAP, DOTAP.Cl, DLin-M-K-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 2010/042877, the disclosure of which is incorporated herein by reference in its entirety for all purposes.

The synthesis of cationic lipids such as DLin-C-DAP, DLinDAC, DLinMA, DLinDAP, DLin-S-DMA, DLin-2-DMAP, DLinTMA.Cl, DLinTAP.Cl, DLinMPZ, DLinAP, DOAP, and DLin-EG-DMA, as well as additional cationic lipids, is described in PCT Publication No. WO 09/086,558, 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.

The synthesis of a number of other cationic lipids and related analogs has been described in 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 can be used, such as, e.g., LIPOFECTIN® (including DOTMA and DOPE, available from GIBCO/BRL); LIPOFECTAMINE® (including DOSPA and DOPE, available from GIBCO/BRL); and TRANSFECTAM® (including DOGS, available from Promega Corp.).

In some embodiments, the cationic lipid comprises from about 45 mol % to about 90 mol %, from about 45 mol % to about 85 mol %, from about 45 mol % to about 80 mol %, from about 45 mol % to about 75 mol %, from about 45 mol % to about 70 mol %, from about 45 mol % to about 65 mol %, from about 45 mol % to about 60 mol %, from about 45 mol % to about 55 mol %, 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 %, from about 50 mol % to about 60 mol %, from about 55 mol % to about 65 mol % or from about 55 mol % to about 70 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In certain preferred embodiments, the cationic lipid comprises from about 50 mol % to about 58 mol %, from about 51 mol % to about 59 mol %, from about 51 mol % to about 58 mol %, from about 51 mol % to about 57 mol %, from about 52 mol % to about 58 mol %, from about 52 mol % to about 57 mol %, from about 52 mol % to about 56 mol %, or from about 53 mol % to about 55 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In certain other embodiments, the cationic lipid comprises (at least) about 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 additional embodiments, the cationic lipid comprises from about 2 mol % to about 60 mol %, from about 5 mol % to about 50 mol %, from about 10 mol % to about 50 mol %, from about 20 mol % to about 50 mol %, from about 20 mol % to about 40 mol %, from about 30 mol % to about 40 mol

%, or about 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

Additional percentages and ranges of cationic lipids suitable for use in the lipid particles of the present invention are described in PCT Publication No. WO 09/127,060, U.S. application Ser. No. 12/794,701, filed Jun. 4, 2010, PCT Application No. PCT/CA2010/001029, entitled "Improved Cationic Lipids and Methods for the Delivery of Therapeutic Agents," filed Jun. 30, 2010, and U.S. application Ser. No. 12/828,189, entitled "Novel Lipid Formulations for Delivery of Therapeutic Agents to Solid Tumors," filed Jun. 30, 2010, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

It should be understood that the percentage of cationic lipid present in the lipid particles of the invention is a target amount, and that the actual amount of cationic lipid present in the formulation may vary, for example, by  $\pm 5$  mol %. For example, in the 1:57 lipid particle (e.g., SNALP) formulation, the target amount of cationic lipid is 57.1 mol %, but the actual amount of cationic lipid may be  $\pm 5$  mol %,  $\pm 4$  mol %,  $\pm 3$  mol %,  $\pm 2$  mol %,  $\pm 1$  mol %,  $\pm 0.75$  mol %,  $\pm 0.5$  mol %,  $\pm 0.25$  mol %, or  $\pm 0.1$  mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle). Similarly, in the 7:54 lipid particle (e.g., SNALP) formulation, the target amount of cationic lipid is 54.06 mol %, but the actual amount of cationic lipid may be  $\pm 5$  mol %,  $\pm 4$  mol %,  $\pm 3$  mol %,  $\pm 2$  mol %,  $\pm 1$  mol %,  $\pm 0.75$  mol %,  $\pm 0.5$  mol %,  $\pm 0.25$  mol %, or  $\pm 0.1$  mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle).

### C. 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), palmitoyloleoyl-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. Non-limiting examples of cholesterol derivatives include polar analogues such as  $5\alpha$ -cholestanol,  $5\beta$ -coprostanol, cholesteryl-(2'-hydroxy)-ethyl ether, cholesteryl-(4'-hydroxy)-butyl ether, and 6-ketocholestanol; non-polar analogues such as  $5\alpha$ -cholestanane, cholestenone,  $5\alpha$ -cholestanone,  $5\beta$ -cholestanone, and cholesteryl decanoate; and mixtures thereof.

In preferred embodiments, the cholesterol derivative is a polar analogue such as cholesteryl-(4'-hydroxy)-butyl ether. The synthesis of cholesteryl-(2'-hydroxy)-ethyl ether is described in PCT Publication No. WO 09/127,060, the disclosure of which is herein incorporated by reference in its entirety for all purposes.

In some 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. 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 yet other 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.

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 10 mol % to about 60 mol %, from about 20 mol % to about 55 mol %, from about 20 mol % to about 45 mol %, from about 20 mol % to about 40 mol %, from about 25 mol % to about 50 mol %, from about 25 mol % to about 45 mol %, from about 30 mol % to about 50 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 35 mol % to about 45 mol %, from about 37 mol % to about 42 mol %, or about 35 mol %, 36 mol %, 37 mol %, 38 mol %, 39 mol %, 40 mol %, 41 mol %, 42 mol %, 43 mol %, 44 mol %, or 45 mol % (or any fraction thereof or range therein) 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 mol %, 45 mol %, 50 mol %, 55 mol %, or 60 mol % of the total lipid present in the particle.

In some embodiments, the phospholipid component in the mixture may comprise from about 2 mol % to about 20 mol %, from about 2 mol % to about 15 mol %, from about 2 mol % to about 12 mol %, from about 4 mol % to about 15 mol %, or from about 4 mol % to about 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In certain preferred embodiments, the phospholipid component in the mixture comprises from about 5 mol % to about 10 mol %, from about 5 mol % to about 9 mol %, from about 5 mol % to about 8 mol %, from about 6 mol % to about 9 mol %, from about 6 mol % to about 8 mol %, or about 5 mol %, 6 mol %, 7 mol %, 8 mol %, 9 mol %, or 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. As a non-limiting example, a 1:57 lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC at about 7 mol % (or any fraction thereof), e.g., in a mixture with cholesterol or a cholesterol derivative at about 34 mol % (or any fraction thereof) of the total lipid present in the particle. As another non-limiting example, a 7:54 lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise a phospholipid such as DPPC or DSPC

at about 7 mol % (or any fraction thereof), e.g., in a mixture with cholesterol or a cholesterol derivative at about 32 mol % (or any fraction thereof) of the total lipid present in the particle.

In other embodiments, the cholesterol component in the mixture may comprise from about 25 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 27 mol % to about 37 mol %, from about 25 mol % to about 30 mol %, or from about 35 mol % to about 40 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In certain preferred embodiments, the cholesterol component in the mixture comprises from about 25 mol % to about 35 mol %, from about 27 mol % to about 35 mol %, from about 29 mol % to about 35 mol %, from about 30 mol % to about 35 mol %, from about 30 mol % to about 34 mol %, from about 31 mol % to about 33 mol %, or about 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, or 35 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. In other embodiments, the cholesterol component in the mixture comprises about 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. Typically, a 1:57 lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise cholesterol or a cholesterol derivative at about 34 mol % (or any fraction thereof), e.g., in a mixture with a phospholipid such as DPPC or DSPC at about 7 mol % (or any fraction thereof) of the total lipid present in the particle. Typically, a 7:54 lipid particle formulation comprising a mixture of phospholipid and cholesterol may comprise cholesterol or a cholesterol derivative at about 32 mol % (or any fraction thereof), e.g., in a mixture with a phospholipid such as DPPC or DSPC at about 7 mol % (or any fraction thereof) of the total lipid present in the particle.

In embodiments where the lipid particles are phospholipid-free, the cholesterol or derivative thereof may comprise up to about 25 mol %, 30 mol %, 35 mol %, 40 mol %, 45 mol %, 50 mol %, 55 mol %, or 60 mol % of the total lipid present in the particle.

In some embodiments, the cholesterol or derivative thereof in the phospholipid-free lipid particle formulation may comprise from about 25 mol % to about 45 mol %, from about 25 mol % to about 40 mol %, from about 30 mol % to about 45 mol %, from about 30 mol % to about 40 mol %, from about 31 mol % to about 39 mol %, from about 32 mol % to about 38 mol %, from about 33 mol % to about 37 mol %, from about 35 mol % to about 45 mol %, from about 30 mol % to about 35 mol %, from about 35 mol % to about 40 mol %, or about 30 mol %, 31 mol %, 32 mol %, 33 mol %, 34 mol %, 35 mol %, 36 mol %, 37 mol %, 38 mol %, 39 mol %, 40 mol %, 41 mol %, 42 mol %, 43 mol %, 44 mol %, or 45 mol % (or any fraction thereof or range therein) of the total lipid present in the particle. As a non-limiting example, a 1:62 lipid particle formulation may comprise cholesterol at about 37 mol % (or any fraction thereof) of the total lipid present in the particle. As another non-limiting example, a 7:58 lipid particle formulation may comprise cholesterol at about 35 mol % (or any fraction thereof) of the total lipid present in the particle.

In other embodiments, the non-cationic lipid comprises from about 5 mol % to about 90 mol %, from about 10 mol % to about 85 mol %, from about 20 mol % to about 80 mol %, about 10 mol % (e.g., phospholipid only), or about 60 mol % (e.g., phospholipid and cholesterol or derivative thereof) (or any fraction thereof or range therein) of the total lipid present in the particle.

Additional percentages and ranges of non-cationic lipids suitable for use in the lipid particles of the present invention are described in PCT Publication No. WO 09/127,060, U.S. application Ser. No. 12/794,701, filed Jun. 4, 2010, PCT Application No. PCT/CA2010/001029, entitled "Improved Cationic Lipids and Methods for the Delivery of Therapeutic Agents," filed Jun. 30, 2010, and U.S. application Ser. No. 12/828,189, entitled "Novel Lipid Formulations for Delivery of Therapeutic Agents to Solid Tumors," filed Jun. 30, 2010, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

It should be understood that the percentage of non-cationic lipid present in the lipid particles of the invention is a target amount, and that the actual amount of non-cationic lipid present in the formulation may vary, for example, by  $\pm 5$  mol %. For example, in the 1:57 lipid particle (e.g., SNALP) formulation, the target amount of phospholipid is 7.1 mol % and the target amount of cholesterol is 34.3 mol %, but the actual amount of phospholipid may be  $\pm 2$  mol %,  $\pm 1.5$  mol %,  $\pm 1$  mol %,  $\pm 0.75$  mol %,  $\pm 0.5$  mol %,  $\pm 0.25$  mol %, or  $\pm 0.1$  mol % of that target amount, and the actual amount of cholesterol may be  $\pm 3$  mol %,  $\pm 2$  mol %,  $\pm 1$  mol %,  $\pm 0.75$  mol %,  $\pm 0.5$  mol %,  $\pm 0.25$  mol %, or  $\pm 0.1$  mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle). Similarly, in the 7:54 lipid particle (e.g., SNALP) formulation, the target amount of phospholipid is 6.75 mol % and the target amount of cholesterol is 32.43 mol %, but the actual amount of phospholipid may be  $\pm 2$  mol %,  $\pm 1.5$  mol %,  $\pm 1$  mol %,  $\pm 0.75$  mol %,  $\pm 0.5$  mol %,  $\pm 0.25$  mol %, or  $\pm 0.1$  mol % of that target amount, and the actual amount of cholesterol may be  $\pm 3$  mol %,  $\pm 2$  mol %,  $\pm 1$  mol %,  $\pm 0.75$  mol %,  $\pm 0.5$  mol %,  $\pm 0.25$  mol %, or  $\pm 0.1$  mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle).

#### D. Lipid Conjugates

In addition to cationic and non-cationic lipids, the lipid particles of the invention (e.g., SNALP) may further 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, POZ-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 dialkylxypropyls (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 suitable for use in the invention include, without limitation, mPEG2000-1,2-di-O-alkyl-sn3-carbomoylglyceride (PEG-C-DOMG). The synthesis of PEG-C-DOMG is described in PCT Publication No. WO 09/086,558, the disclosure of which is herein incorporated by reference in its entirety for all purposes. Yet additional suitable PEG-lipid conjugates include, without limitation, 1-[8'-(1,2-dimyristoyl-3-propanoxy)-carboxamido-3',6'-dioxoacetylanil]carbamoyl- $\omega$ -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.

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, but are not limited to, the following: monomethoxypolyethylene glycol (MePEG-OH), monomethoxypolyethylene glycol-succinate (MePEG-S), monomethoxypolyethylene glycol-succinimidyl succinate (MePEG-S-NHS), monomethoxypolyethylene glycol-amine (MePEG-NH<sub>2</sub>), monomethoxypolyethylene glycol-tresylate (MePEG-TRES), monomethoxypolyethylene glycol-imidazolyl-carbonyl (MePEG-IM), as well as such compounds containing a terminal hydroxyl group instead of a terminal methoxy group (e.g., HO-PEG-S, HO-PEG-S-NHS, HO-PEG-NH<sub>2</sub>, etc.). 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<sub>2</sub>COOH) 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 other instances, the PEG moiety has an average molecular weight of from about 550 daltons to about 1000 daltons, from about 250 daltons to about 1000 daltons, from about 400 daltons to about 1000 daltons, from about 600 daltons to about 900 daltons, from about 700 daltons to about 800 daltons, or about 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 daltons. In preferred embodiments, the PEG moiety has an average molecular weight of about 2,000 daltons or about 750 daltons.

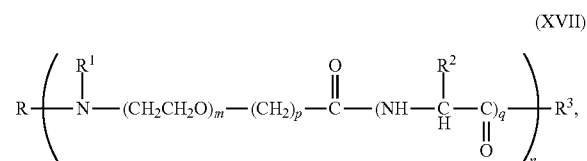
In certain instances, the PEG can be optionally substituted by an alkyl, alkoxy, acyl, or aryl group. The PEG can be conjugated directly to the lipid or may be linked to the lipid via a linker moiety. Any linker moiety suitable for coupling the PEG to a lipid can be used including, e.g., non-ester containing 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<sub>2</sub>CH<sub>2</sub>C(O)—), succinamidyl (—NHC(O)CH<sub>2</sub>CH<sub>2</sub>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—), succinyl, phosphate esters (—O—(O)POH—O—), sulfonate esters, and combinations thereof.

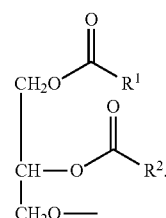
Phosphatidylethanolamines having a variety of acyl chain groups of varying chain lengths and degrees of saturation can be conjugated to PEG to form the lipid conjugate. Such phosphatidylethanolamines are commercially available, or can be isolated or synthesized using conventional techniques known to those of skilled in the art. Phosphatidyl-ethanolamines containing saturated or unsaturated fatty acids with carbon chain lengths in the range of C<sub>10</sub> to C<sub>20</sub> 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" includes, 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:



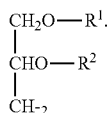
wherein R is a member selected from the group consisting of hydrogen, alkyl and acyl; R<sup>1</sup> is a member selected from the group consisting of hydrogen and alkyl; or optionally, R and R<sup>1</sup> and the nitrogen to which they are bound form an azido moiety; R<sup>2</sup> is a member of the group selected from hydrogen, optionally substituted alkyl, optionally substituted aryl and a side chain of an amino acid; R<sup>3</sup> is a member selected from the group consisting of hydrogen, halogen, hydroxy, alkoxy, mercapto, hydrazino, amino and NR<sup>4</sup>R<sup>5</sup>, wherein R<sup>4</sup> and R<sup>5</sup> 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" or "DAG" includes a compound having 2 fatty acyl chains, R<sup>1</sup> and R<sup>2</sup>, 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, lauroyl (C<sub>12</sub>), myristoyl (C<sub>14</sub>), palmitoyl (C<sub>16</sub>), stearoyl (C<sub>18</sub>), and icosoyl (C<sub>20</sub>). In preferred embodiments, R<sup>1</sup> and R<sup>2</sup> are the same, i.e., R<sup>1</sup> and R<sup>2</sup> are both myristoyl (i.e., dimyristoyl), R<sup>1</sup> and R<sup>2</sup> are both stearoyl (i.e., distearoyl), etc. Diacylglycerols have the following general formula:



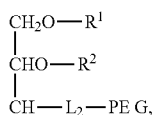
97

The term "dialkylxypropyl" or "DAA" includes a compound having 2 alkyl chains, R<sup>1</sup> and R<sup>2</sup>, both of which have independently between 2 and 30 carbons. The alkyl groups can be saturated or have varying degrees of unsaturation. Dialkylxypropyls have the following general formula:



(XIX)

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



(XX)

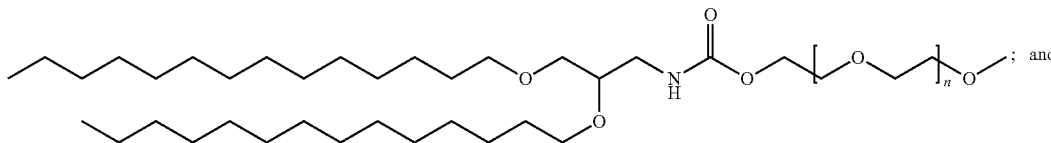
98

daltons to about 900 daltons, from about 700 daltons to about 800 daltons, or about 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 daltons. 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 groups. In certain embodiments, the terminal hydroxyl group is substituted with a methoxy or methyl group.

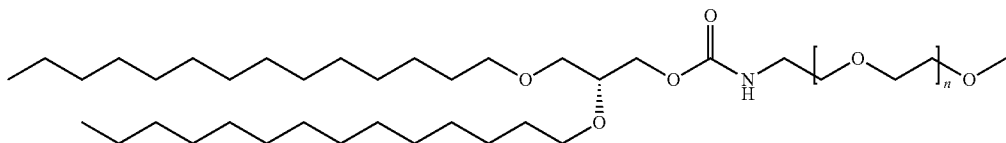
In a preferred embodiment, "L" is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide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



(PEG-C-DOMG)



wherein R<sup>1</sup> and R<sup>2</sup> 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, decyl (C<sub>10</sub>), lauryl (C<sub>12</sub>), myristyl (C<sub>14</sub>), palmityl (C<sub>16</sub>), stearyl (C<sub>18</sub>), and icosyl (C<sub>20</sub>). In preferred embodiments, R<sup>1</sup> and R<sup>2</sup> are the same, i.e., R<sup>1</sup> and R<sup>2</sup> are both myristyl (i.e., dimyristyl), R<sup>1</sup> and R<sup>2</sup> are both stearyl (i.e., distearyl), etc.

In Formula XX 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 other instances, the PEG moiety has an average molecular weight of from about 550 daltons to about 1000 daltons, from about 250 daltons to about 1000 daltons, from about 400 daltons to about 1000 daltons, from about 600

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 PEG-didecylxypropyl (C<sub>10</sub>) conjugate, a PEG-dilauryloxypropyl (C<sub>12</sub>) conjugate, a PEG-dimyristyloxypropyl (C<sub>14</sub>) conjugate, a PEG-dipalmitoxypropyl (C<sub>16</sub>) conjugate, or a PEG-distearyloxypropyl (C<sub>18</sub>) conjugate. In these embodiments, the PEG preferably has an average molecular weight of about 750 or about 2,000 daltons. In one particularly preferred embodi-



ment, the PEG-lipid conjugate comprises PEG2000-C-DMA, wherein the "2000" denotes the average molecular weight of the PEG, the "C" denotes a carbamate linker moiety, and the "DMA" denotes dimyristyloxypropyl. In another particularly preferred embodiment, the PEG-lipid conjugate comprises PEG750-C-DMA, wherein the "750" denotes the average molecular weight of the PEG, the "C" denotes a carbamate linker moiety, and the "DMA" denotes dimyristyloxypropyl. In particular embodiments, the terminal hydroxyl group of the PEG is substituted with a methyl group. Those of skill in the art will readily appreciate that other dialkylxypropyls 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 lipid particles (e.g., SNALP) 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); U.S. Pat. No. 6,852,334; 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 XXI:



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

With reference to Formula XXI, "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.

In some embodiments, the lipid conjugate (e.g., PEG-lipid) 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 %, 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 other embodiments, the lipid conjugate (e.g., PEG-lipid) comprises from about 0 mol % to about 20 mol %, from about 0.5 mol % to about 20 mol %, from about 2 mol % to about 20 mol %, from about 1.5 mol % to about 18 mol %, from about 2 mol % to about 15 mol %, from about 4 mol % to about 15 mol %, from about 2 mol % to about 12 mol %, from about 5 mol % to about 12 mol %, or about 2 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

In further embodiments, the lipid conjugate (e.g., PEG-lipid) comprises from about 4 mol % to about 10 mol %, from about 5 mol % to about 10 mol %, from about 5 mol % to

about 9 mol %, from about 5 mol % to about 8 mol %, from about 6 mol % to about 9 mol %, from about 6 mol % to about 8 mol %, or about 5 mol %, 6 mol %, 7 mol %, 8 mol %, 9 mol %, or 10 mol % (or any fraction thereof or range therein) of the total lipid present in the particle.

Additional examples, percentages, and/or ranges of lipid conjugates suitable for use in the lipid particles of the invention are described in PCT Publication No. WO 09/127,060, U.S. application Ser. No. 12/794,701, filed Jun. 4, 2010, PCT Application No. PCT/CA2010/001029, entitled "Improved Cationic Lipids and Methods for the Delivery of Therapeutic Agents," filed Jun. 30, 2010, U.S. application Ser. No. 12/828,189, entitled "Novel Lipid Formulations for Delivery of Therapeutic Agents to Solid Tumors," filed Jun. 30, 2010, U.S. Provisional Application No. 61/294,828, filed Jan. 13, 2010, U.S. Provisional Application No. 61/295,140, filed Jan. 14, 2010, and PCT Publication No. WO 2010/006282, the disclosures of which are herein incorporated by reference in their entirety for all purposes.

It should be understood that the percentage of lipid conjugate (e.g., PEG-lipid) present in the lipid particles of the invention is a target amount, and that the actual amount of lipid conjugate present in the formulation may vary, for example, by  $\pm 2$  mol %. For example, in the 1:57 lipid particle (e.g., SNALP) formulation, the target amount of lipid conjugate is 1.4 mol %, but the actual amount of lipid conjugate may be  $\pm 0.5$  mol %,  $\pm 0.4$  mol %,  $\pm 0.3$  mol %,  $\pm 0.2$  mol %,  $\pm 0.1$  mol %, or  $\pm 0.05$  mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids present in the particle). Similarly, in the 7:54 lipid particle (e.g., SNALP) formulation, the target amount of lipid conjugate is 6.76 mol %, but the actual amount of lipid conjugate may be  $\pm 2$  mol %,  $\pm 1.5$  mol %,  $\pm 1$  mol %,  $\pm 0.75$  mol %,  $\pm 0.5$  mol %,  $\pm 0.25$  mol %, or  $\pm 0.1$  mol % of that target amount, with the balance of the formulation being made up of other lipid components (adding up to 100 mol % of total lipids 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 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 lipid particle and, in turn, the rate at which the lipid particle becomes fusogenic. For instance, when a PEG-DAA conjugate is used as the lipid conjugate, the rate at which the 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 alkyl groups on the PEG-DAA conjugate. 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 lipid particle becomes fusogenic. Other methods which can be used to control the rate at which the lipid particle becomes fusogenic will become apparent to those of skill in the art upon reading this disclosure. Also, by controlling the composition and concentration of the lipid conjugate, one can control the lipid particle (e.g., SNALP) size.

#### V. 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 (e.g., siRNA) is entrapped within the lipid portion of the particle and is protected from degradation, can be

formed by any method known in the art including, but not limited to, a continuous mixing method, a direct dilution process, and an in-line dilution process.

In particular embodiments, the cationic lipids may comprise lipids of Formulas I-XIV or salts thereof, alone or in combination with other cationic lipids. In other 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-dielaoidyl-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 dialkylloxypropyls), cholesterol, derivatives thereof, or combinations thereof.

In certain embodiments, the present invention provides nucleic acid-lipid particles (e.g., SNALP) produced via a continuous mixing method, e.g., a process that includes providing an aqueous solution comprising a nucleic acid (e.g., interfering RNA) in a first reservoir, providing an organic lipid solution in a second reservoir (wherein the lipids present in the organic lipid solution are solubilized in an organic solvent, e.g., a lower alkanol such as ethanol), 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 lipid vesicle (e.g., liposome) encapsulating the nucleic acid within the lipid vesicle. This process and the apparatus for carrying out 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 lipid vesicle 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 nucleic acid-lipid particles formed using the continuous mixing method typically have a size of from about 30 nm to about 150 nm, 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, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm (or any fraction thereof or

range therein). The particles thus formed do not aggregate and are optionally sized to achieve a uniform particle size.

In another embodiment, the present invention provides nucleic acid-lipid particles (e.g., SNALP) produced via a direct dilution process that includes forming a lipid vesicle (e.g., liposome) solution and immediately and directly introducing the lipid vesicle 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 lipid vesicle solution introduced thereto. As a non-limiting example, a lipid vesicle 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 nucleic acid-lipid particles (e.g., SNALP) produced via an in-line dilution process in which a third reservoir containing dilution buffer is fluidly coupled to a second mixing region. In this embodiment, the lipid vesicle (e.g., 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 lipid vesicle 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° (e.g., about 90°). 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 lipid vesicle solution introduced thereto from the first mixing region. This embodiment advantageously allows for more control of the flow of dilution buffer mixing with the lipid vesicle solution in the second mixing region, and therefore also the concentration of lipid vesicle 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 and in-line 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 nucleic acid-lipid particles formed using the direct dilution and in-line dilution processes typically have a size of from about 30 nm to about 150 nm, 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, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, less than about 120 nm, 110 nm, 100 nm, 90 nm, or 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm (or any fraction thereof or range therein). 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 present in the particles 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 may 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 brand name 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 polyethylenimine. 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 nucleic acid-lipid particle (e.g., SNALP) will range from about 0.01 to about 0.2, from about 0.05 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 (input) also falls within this range. In other embodiments, the particle 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 nucleic acid-lipid particle (e.g., 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), or 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), 15 (15:1), 16 (16:1), 17 (17:1), 18 (18:1), 19 (19:1), 20 (20:1), 21 (21:1), 22 (22:1), 23

(23:1), 24 (24:1), or 25 (25:1), or any fraction thereof or range therein. The ratio of the starting materials (input) 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 the “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-DAAAs and PEG-DAGs). Methods of making SNALP-CPLs 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.

#### VI. Kits

The present invention also provides lipid particles (e.g., SNALP) in kit form. In some embodiments, the kit comprises 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). Preferably, the kit comprises a container (e.g., a vial or ampoule) which holds the lipid particles of the invention (e.g., SNALP), wherein the particles are produced by one of the processes set forth herein. In certain embodiments, the kit may further comprise an endosomal membrane destabilizer (e.g., calcium ions). The kit typically contains the particle compositions of the invention, either as a suspension in a pharmaceutically acceptable carrier or in dehydrated form, with instructions for their rehydration (if lyophilized) and administration.

As explained herein, it has surprisingly been found that the SNALP formulations of the present invention containing at least one cationic lipid of Formulas I-XIV, either alone or in combination with other cationic lipids, show increased potency and/or increased tolerability when targeting a gene of interest in the liver, such as, e.g., APOB, APOC3, PCSK9, DGAT1, and/or DGAT2, when compared to other SNALP formulations. For instance, as set forth in the Examples below, it has been found that a lipid particle (e.g., SNALP) containing, e.g., DLin-K-C2-DMA (“C2K”),  $\gamma$ -DLenDMA, Linoleyl/Linolenyl DMA (“Lin/Len”), C2-DPanDMA, DPan-C2K-DMA, DPan-C3K-DMA,  $\gamma$ -DLen-C2K-DMA, DLen-C2K-DMA, or C2-TLinDMA was unexpectedly more potent in silencing APOB expression in vivo compared to SNALP containing DLinDMA or DLenDMA. In addition, as set forth in the Examples below, it has been found that a lipid particle (e.g., SNALP) comprising an APOB siRNA described herein and containing, e.g., DLin-K-C2-DMA, displayed an unexpectedly more favorable toxicity profile in vivo compared to SNALP formulations containing DLinDMA. As such, in certain preferred embodiments, the kits of the invention comprise a 1:57, 1:62, 7:54, or 7:58 lipid particle (e.g., SNALP) containing one or more cationic lipids of Formulas I-XIV, such as C2K,  $\gamma$ -DLenDMA, Linoleyl/Linolenyl DMA (“Lin/Len”), C2-DPanDMA, DPan-C2K-DMA, DPan-C3K-DMA,  $\gamma$ -DLen-C2K-DMA, DLen-C2K-

DMA, and/or C2-TLinDMA. Those of skill in the art will appreciate that the lipid particles can be present in a container as a suspension or in dehydrated form.

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 particularly useful for introducing an interfering RNA (e.g., an siRNA molecule) targeting a gene of interest (such as APOB, APOC3, PCSK9, DGAT1, DGAT2, or combinations thereof) into the liver. As noted, it has surprisingly been found that the SNALP formulations of the present invention containing a cationic lipid of Formula I-XIV are unexpectedly more potent at silencing APOB expression and/or display increased tolerability in vivo compared to SNALP formulations containing other cationic lipids such as DLinDMA. Accordingly, the present invention also provides methods for introducing an interfering RNA (e.g., an siRNA) into a liver 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 (e.g., cells of the liver, such as hepatocytes) for a period of time sufficient for delivery of the interfering RNA to the liver 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 lipid particle formation. Thus, after the lipid particle (e.g., SNALP) 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 alphanatocopherol, and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

In some embodiments, the lipid particles of the invention (e.g., SNALP) are particularly useful in methods for the therapeutic delivery of one or more nucleic acids comprising an interfering RNA sequence (e.g., siRNA). In particular, it is an object of 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 (such as APOB, APOC3, PCSK9, DGAT1, DGAT2, or combinations thereof). 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 of a mammalian subject for the treatment of metabolic diseases and disorders (e.g., diseases and disorders in which the liver is a target and liver diseases and disorders). 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 may be administered to the mammal. In some instances, an interfering RNA (e.g., siRNA) is formulated into a SNALP containing a cationic lipid of Formula I-XIV, and the particles are administered to patients requiring such treatment. In other instances, cells are removed from a patient, the interfering RNA is delivered in vitro (e.g., using a SNALP described herein), and the cells are re injected into the patient.

#### 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 non-immunogenic, 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 administra-

tion, 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 Straubinger 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.

In embodiments where the lipid particles of the present invention (e.g., SNALP) are administered intravenously, at least about 5%, 10%, 15%, 20%, or 25% of the total injected dose of the particles 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 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 is detectable at least about 1 hour after administration of the particle. In certain embodiments, the presence of a therapeutic agent such as a nucleic acid is detectable liver cells (e.g., hepatocytes) at about 8, 12, 24, 36, 48, 60, 72 or 96 hours after administration. In other embodiments, downregulation of expression of a target sequence, such as APOB, by 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, such as APOB, by an interfering RNA (e.g., siRNA) occurs preferentially in liver cells (e.g., hepatocytes). In further embodiments, the presence or effect of an interfering RNA (e.g., siRNA) in cells at a site proximal or distal to the site of administration or in liver cells (e.g., hepatocytes) 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.

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-glyc-

erol 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 polytetrafluoroethylene 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 in 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 (e.g., liver cells, i.e., hepatocytes).

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  $\mu\text{mol}$  and about 10  $\text{mmol}$ . 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  $2 \times 10^4$  cells/ml. The concentration of the suspension added to the cells is preferably of from about 0.01 to 0.2  $\mu\text{g/ml}$ , more preferably about 0.1  $\mu\text{g/ml}$ .

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.

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 or other lipid particle 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,  $\beta$ -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 down-regulation 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 particularly well suited for treating metabolic diseases and disorders by targeting, e.g., APOB *in vivo*. In preferred embodiments, an interfering RNA (e.g., an siRNA) in a SNALP formulation containing a cationic lipid of Formula I-XIV is delivered to liver cells (e.g., hepatocytes), which surprisingly results in increased silencing of the target gene of interest (e.g., APOB). The methods and compositions can be employed with liver cells (e.g., hepatocytes) 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).

#### 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 as Texas red, tetra-rhodamine isothiocyanate (TRITC), etc., digoxigenin, biotin, phycoerythrin, AMCA, CyDyes™, and the like; radiolabels such as  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{33}\text{P}$ , 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 the 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), Q $\beta$ -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 Appli-

cations (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 Q $\beta$ -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.

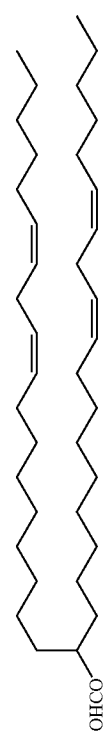
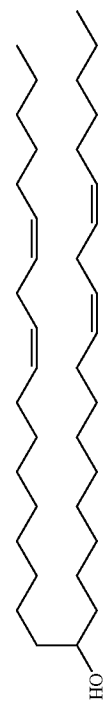
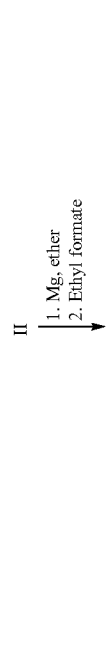
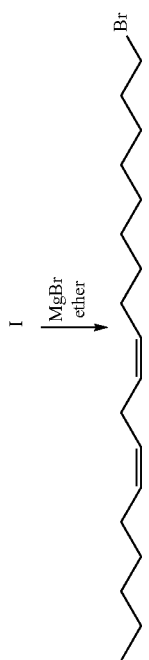
#### Example 1

##### Synthesis of 2,2-Dilinoleyl-4-Dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA)

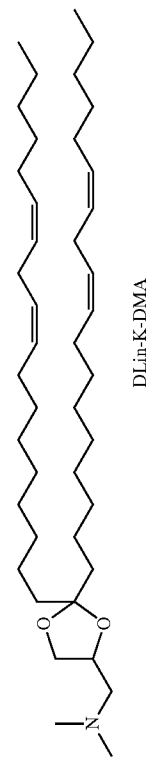
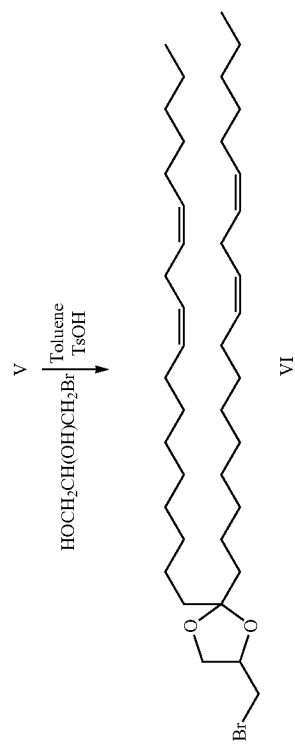
DLin-K-DMA was synthesized as shown in the schematic and described below.



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## Synthesis of Linoleyl Bromide (II)

A mixture of linoleyl methane sulfonate (6.2 g, 18 mmol) and magnesium bromide etherate (17 g, 55 mmol) in anhydrous ether (300 mL) was stirred under argon overnight (21 hours). The resulting suspension was poured into 300 mL of chilled water. Upon shaking, the organic phase was separated. The aqueous phase was extracted with ether (2×150 mL). The combined ether phase was washed with water (2×150 mL), brine (150 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solvent was evaporated to afford 6.5 g of colourless oil. The crude product was purified by column chromatography on silica gel (230-400 mesh, 300 mL) and eluted with hexanes. This gave 6.2 g (approximately 100%) of linoleyl bromide (II). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 5.27-5.45 (4H, m, 2×CH=CH), 3.42 (2H, t, CH<sub>2</sub>Br), 2.79 (2H, t, C=C—CH<sub>2</sub>—C=C), 2.06 (4H, q, 2× allylic CH<sub>2</sub>), 1.87 (2H, quintet, CH<sub>2</sub>), 1.2-1.5 (16H, m), 0.90 (3H, t, CH<sub>3</sub>) ppm.

## Synthesis of Dilinoleyl Methanol (III)

To a suspension of Mg turnings (0.45 g, 18.7 mmol) with one crystal of iodine in 200 mL of anhydrous ether under nitrogen was added a solution of linoleyl bromide (II) in 50 mL of anhydrous ether at room temperature. The resulting mixture was refluxed under nitrogen overnight. The mixture was cooled to room temperature. To the cloudy mixture under nitrogen was added dropwise at room temperature a solution of ethyl formate (0.65 g, 18.7 mmol) in 30 mL of anhydrous ether. Upon addition, the mixture was stirred at room temperature overnight (20 hours). The ether layer was washed with 10% H<sub>2</sub>SO<sub>4</sub> aqueous solution (100 mL), water (2×100 mL), brine (150 mL), and then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Evaporation of the solvent gave 5.0 g of pale oil. Column chromatography on silica gel (230-400 mesh, 300 mL) with 0-7% ether gradient in hexanes as eluent afforded two products, dilinoleyl methanol (2.0 g, III) and dilinoleylmethyl formate (1.4 g, IV). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) for dilinoleylmethyl formate (IV) δ: 8.10 (1H, s, CHO), 5.27-5.45 (8H, m, 4×CH=CH), 4.99 (1'-1, quintet, OCH), 2.78 (4H, t, 2×C=C—CH<sub>2</sub>—C=C), 2.06 (8H, q, 4× allylic CH<sub>2</sub>), 1.5-1.6 (4H, m, 2×CH<sub>2</sub>), 1.2-1.5 (32H, m), 0.90 (6H, t, 2×CH<sub>3</sub>) ppm.

Dilinoleylmethyl formate (IV, 1.4 g) and KOH (0.2 g) were stirred in 85% EtOH at room temperature under nitrogen overnight. Upon completion of the reaction, half of the solvent was evaporated. The resulting mixture was poured into 150 mL of 5% HCL solution. The aqueous phase was extracted with ether (3×100 mL). The combined ether extract was washed with water (2×100 mL), brine (100 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Evaporation of the solvent gave 1.0 g of dilinoleyl methanol (III) as colourless oil. Overall, 3.0 g (60%) of dilinoleyl methanol (III) were afforded. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) for dilinoleyl methanol (III) δ: ppm.

## Synthesis of Dilinoleyl Ketone (V)

To a mixture of dilinoleyl methanol (2.0 g, 3.8 mmol) and anhydrous sodium carbonate (0.2 g) in 100 mL of CH<sub>2</sub>Cl<sub>2</sub> was added pyridinium chlorochromate (PCC, 2.0 g, 9.5 mmol). The resulting suspension was stirred at room temperature for 60 min. Ether (300 mL) was then added into the mixture, and the resulting brown suspension was filtered through a pad of silica gel (300 mL). The silica gel pad was further washed with ether (3×200 mL). The ether filtrate and

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washes were combined. Evaporation of the solvent gave 3.0 g of an oily residual as a crude product. The crude product was purified by column chromatography on silica gel (230-400 mesh, 250 mL) eluted with 0-3% ether in hexanes. This gave 1.8 g (90%) of dilinoleyl ketone (V). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 5.25-5.45 (8H, m, 4×CH=CH), 2.78 (4H, t, 2×C=C—CH<sub>2</sub>—C=C), 2.39 (4H, t, 2×COCH<sub>2</sub>), 2.05 (8H, q, 4× allylic CH<sub>2</sub>), 1.45-1.7 (4H, m), 1.2-1.45 (32H, m), 0.90 (6H, t, 2×CH<sub>3</sub>) ppm.

## Synthesis of

## 2,2-Dilinoleyl-4-bromomethyl-[1,3]-dioxolane (VI)

A mixture of dilinoleyl methanol (V, 1.3 g, 2.5 mmol), 3-bromo-1,2-propanediol (1.5 g, 9.7 mmol) and p-toluene sulfonic acid hydrate (0.16 g, 0.84 mmol) in 200 mL of toluene was refluxed under nitrogen for 3 days with a Dean-Stark tube to remove water. The resulting mixture was cooled to room temperature. The organic phase was washed with water (2×50 mL), brine (50 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Evaporation of the solvent resulted in a yellowish oily residue. Column chromatography on silica gel (230-400 mesh, 100 mL) with 0-6% ether gradient in hexanes as eluent afforded 0.1 g of pure VI and 1.3 g of a mixture of VI and the starting material. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 5.27-5.45 (8H, m, 4×CH=CH), 4.28-4.38 (1H, m, OCH), 4.15 (1H, dd, OCH), 3.80 (1H, dd, OCH), 3.47 (1H, dd, CHBr), 3.30 (1H, dd, CHBr), 2.78 (4H, t, 2×C=C—CH<sub>2</sub>—C=C), 2.06 (8H, q, 4× allylic CH<sub>2</sub>), 1.52-1.68 (4H, m, 2×CH<sub>2</sub>), 1.22-1.45 (32H, m), 0.86-0.94 (6H, m, 2×CH<sub>3</sub>) ppm.

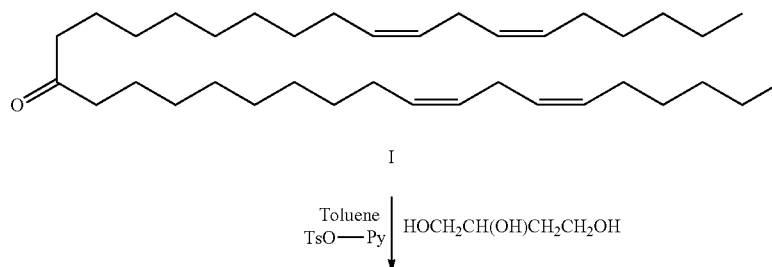
## Synthesis of 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA)

Anhydrous dimethyl amine was bubbled into an anhydrous THF solution (100 mL) containing 1.3 g of a mixture of 2,2-dilinoleyl-4-bromomethyl-[1,3]-dioxolane (VI) and dilinoleyl ketone (V) at 0° C. for 10 min. The reaction flask was then sealed and the mixture stirred at room temperature for 6 days. Evaporation of the solvent left 1.5 g of a residual. The crude product was purified by column chromatography on silica gel (230-400 mesh, 100 mL) and eluted with 0-5% methanol gradient in dichloromethane. This gave 0.8 g of the desired product DLin-K-DMA. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 5.25-5.45 (8, m, 4×CH=CH), 4.28-4.4 (1H, m, OCH), 4.1 (1H, dd, OCH), 3.53 (1H, t OCH), 2.78 (4H, t, 2×C=C—CH<sub>2</sub>—C=C), 2.5-2.65 (2H, m, NCH<sub>2</sub>), 2.41 (6H, s, 2×NCH<sub>3</sub>), 2.06 (8H, q, 4× allylic CH<sub>2</sub>), 1.56-1.68 (4H, m, 2×CH<sub>2</sub>), 1.22-1.45 (32H, m), 0.90 (6H, t, 2×CH<sub>3</sub>) ppm.

## Example 2

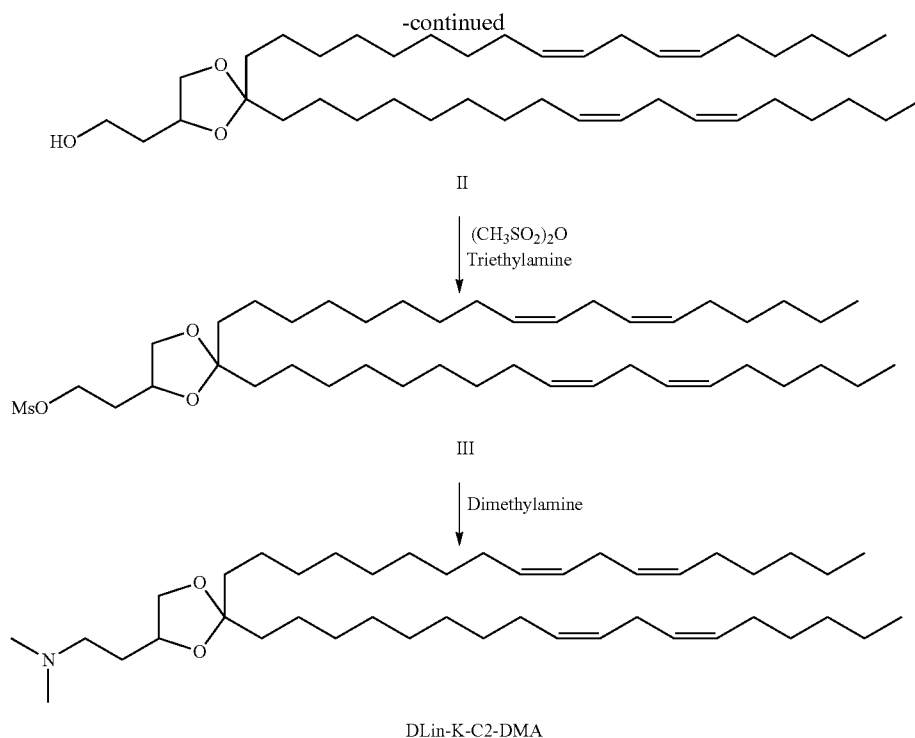
## Synthesis of 2,2-Dilinoleyl-4-(2-Dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA)

DLin-K-C2-DMA was synthesized as shown in the schematic diagram and description below.



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Synthesis of  
2,2-Dilinoleyl-4-(2-hydroxyethyl)-[1,3]-dioxolane  
(II)

A mixture of dilinoleyl ketone (I, previously prepared as described in Example 1, 527 mg, 1.0 mmol), 1,3,4-butanetriol (technical grade, ca. 90%, 236 mg, 2 mmol) and pyridinium p-toluenesulfonate (50 mg, 0.2 mmol) in 50 mL of toluene was refluxed under nitrogen overnight with a Dean-Stark tube to remove water. The resulting mixture was cooled to room temperature. The organic phase was washed with water (2×30 mL), brine (50 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. Evaporation of the solvent resulted in a yellowish oily residual (0.6 g). The crude product was purified by column chromatography on silica gel (230-400 mesh, 100 mL) with dichloromethane as eluent. This afforded 0.5 g of pure II as colourless oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 5.25-5.48 (8H, m, 4×CH=CH), 4.18-4.22 (1H, m, OCH), 4.08 (1H, dd, OCH), 3.82 (2H, t, OCH<sub>2</sub>), 3.53 (1H, t, OCH), 2.78 (4H, t, 2×C=C-CH<sub>2</sub>-C=C), 2.06 (8H, q, 4× allylic CH<sub>2</sub>), 1.77-1.93 (2H, m, CH<sub>2</sub>), 1.52-1.68 (4H, m, 2×CH<sub>2</sub>), 1.22-1.45 (32H, m), 0.86-0.94 (6H, t, 2×CH<sub>3</sub>) ppm.

Synthesis of 2,2-Dilinoleyl-4-(2-methanesulfonyl-ethyl)-[1,3]-dioxolane (III)

To a solution of 2,2-dilinoleyl-4-(2-hydroxyethyl)-[1,3]-dioxolane (II, 500 mg, 0.81 mmol) and dry triethylamine (218 mg, 2.8 mmol) in 50 mL of anhydrous CH<sub>2</sub>Cl<sub>2</sub> was added methanesulfonyl anhydride (290 mg, 1.6 mmol) under nitrogen. The resulting mixture was stirred at room temperature

overnight. The mixture was diluted with 25 mL of CH<sub>2</sub>Cl<sub>2</sub>. The organic phase was washed with water (2×30 mL), brine (50 mL), and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solvent was evaporated to afford 510 mg of yellowish oil. The crude product was used in the following step without further purification.

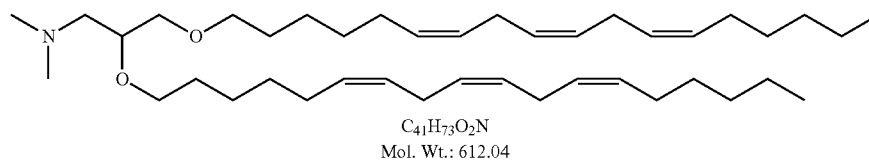
Synthesis of 2,2-Dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-K-C2-DMA)

To the above crude material (III) under nitrogen was added 20 mL of dimethylamine in THF (2.0 M). The resulting mixture was stirred at room temperature for 6 days. An oily residual was obtained upon evaporation of the solvent. Column chromatography on silica gel (230-400 mesh, 100 mL) with 0-5% methanol gradient in dichloromethane as eluent resulted in 380 mg of the product DLin-K-C2-DMA as pale oil. <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ: 5.27-5.49 (8, m, 4×CH=CH), 4.01-4.15 (2H, m, 2×OCH), 3.49 (1H, t OCH), 2.78 (4H, t, 2×C=C-CH<sub>2</sub>-C=C), 2.34-2.54 (2H, m, NCH<sub>2</sub>), 2.30 (6H, s, 2×NCH<sub>3</sub>), 2.06 (8H, q, 4× allylic CH<sub>2</sub>), 1.67-1.95 (2H, m, CH<sub>2</sub>), 1.54-1.65 (4H, m, 2×CH<sub>2</sub>), 1.22-1.45 (32H, m), 0.90 (6H, t, 2×CH<sub>3</sub>) ppm.

Example 3

Synthesis of  
1,2-Di-γ-linolenyloxy-N,N-dimethylaminopropane  
(γ-DLenDMA)

γ-DLenDMA having the structure shown below was synthesized as described below.



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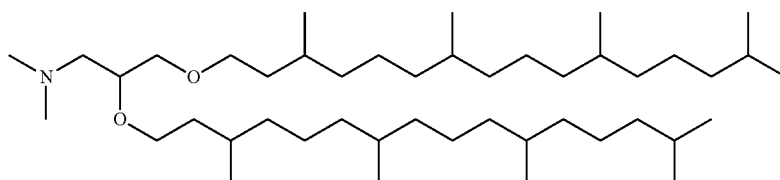
A 250 mL round bottom flask was charged with 3-(dimethylamino)-1,2-propanediol (0.8 g, 6.7 mmol), tetrabutylammonium hydrogen sulphate (1 g), gamma linolenyl mesylate (cis-6,9,12-octadecatriene sulphonic acid) (5 g, 14.6 mmol), and 30 mL toluene. After stirring for 15 minutes, the reaction was cooled to 0-5° C. A solution of 40% sodium hydroxide (15 mL) was added slowly. The reaction was left to stir for approximately 48 hours. An additional 15 mL of toluene was then added to the reaction vessel, along with 40% sodium hydroxide (15 mL). After the reaction was stirred for an additional 12 hours, water (50 mL) and isopropyl acetate (50 mL) were added and stirred for 15 minutes. The mixture was then transferred to a 500 mL separatory funnel and allowed to separate. The lower aqueous phase was run off and the organic phase was washed with saturated sodium chloride (2x50 mL). Since the aqueous and organic phases resulting from the saturated sodium chloride washes could not be completely separated after 20 minutes, the lower aqueous phase (slightly yellow) was run off and back extracted with chloroform (~45 mL). The organic phase was dried with MgSO<sub>4</sub>, filtered, and the solvent evaporated.

The crude product, an orange liquid, was purified on column chromatography using silica gel (60 g) with 0-3% methanol gradient in dichloromethane to yield 3.19 g. The product was further purified via column chromatography on silica gel (50 g) with 10-30% ethyl acetate gradient in hexanes to yield 1.26 g pure product.

## Example 4

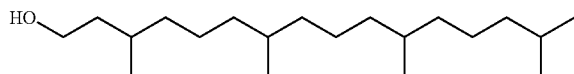
Synthesis of  
1,2-Diphytanyloxy-3-(N,N-dimethyl)-propylamine  
(DPanDMA)

DPanDMA having the structure shown below was synthesized as described below.



$C_{45}H_{93}NO_2$   
Exact Mass: 679.72  
Mol. Wt.: 680.23  
C, 79.46; H, 13.78; N, 2.06; O, 4.70

## Step 1: Synthesis of Phytanol:



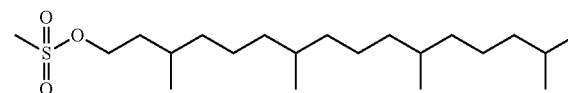
$C_{20}H_{42}O_2$   
Exact Mass: 298.32  
Mol. Wt.: 298.55  
C, 80.46; H, 14.18; O, 5.36

Phytol (21.0 g, 70.8 mmol), ethanol (180 mL) and a stir bar were added to a 500 mL round bottom flask. Raney Nickel 2800 (as purchased, a 50% by weight solution in water if used as purchased, Nickel >89% metal present) (6.8 g, 51.5 mmol)

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was added, and the flask sealed and flushed with hydrogen. A 12" needle was used to bubble hydrogen through the solution for 10 minutes. The reaction was stirred for 5 days, using a balloon as a hydrogen reservoir. Hydrogen was also bubbled through the reaction mixture at 24 h and 48 h, 5 minutes each time. The metal catalyst was then removed by filtering through Celite. The ethanolic solution was concentrated, and 200 mL of DCM added to the resulting oil. The solution was washed with water (2x100 mL), dried over MgSO<sub>4</sub>, and concentrated. TLC indicated formation of the phytanol product, yield 20.0 g.

## Step 2: Synthesis of Phytanyl Mesylate:



$C_{21}H_{44}O_3S$   
Exact Mass: 376.30  
Mol. Wt.: 376.64  
C, 66.97; H, 11.78; O, 12.74; S, 8.51

Phytanyl (20.0 g, 66.7 mmol), triethylamine (18.6 mL, 133 mmol), and a stir bar were added to a 1000 mL round bottom flask. The flask was sealed and flushed with nitrogen. Anhydrous DCM (250 mL) was added, and the mixture cooled to -15° C. (ice and NaCl). Mesyl Chloride (10.4 mL, 133 mmol) was added slowly via syringe over a 30 minute period, and the reaction stirred at -15° C. for a further 1.5 hours. At this point TLC showed that the starting material had been used up. The solution was diluted with DCM (250 mL) and washed with saturated NaHCO<sub>3</sub> (2x200 mL). The organic phase was then dried (MgSO<sub>4</sub>), filtered, and concentrated (rotovap). The crude product was purified by column chromatography. Yield: 21.5 g, 85.7%.

## Step 3: Synthesis of DPanDMA:

Sodium hydride (2.5 g, 100 mmol) was added to a 250 mL round bottom flask, along with benzene (40 mL) and a stir bar. In a 50 mL beaker, a solution was made from the N,N-Dimethyl-3-aminopropane-1,2-diol (1.42 g, 12 mmol) and benzene (60 mL). This was added to the reaction vessel and the reaction stirred for 10 minutes (effervescence). Phytanyl Mesylate (10.52 g, 28 mmol) was added and the flask fitted with a condenser, flushed with nitrogen, and heated to reflux. After 18 hours, the flask was removed from the heat and allowed to cool. The volume was made up to 200 mL with benzene. EtOH was added slowly to quench unreacted sodium hydride. Once quenching was complete, the reaction mixture was washed twice with EtOH/H<sub>2</sub>O, in a ratio to the benzene of 1:1:0.6 benzene:water:ethanol. The aqueous phases were combined and extracted with CHCl<sub>3</sub> (2x100

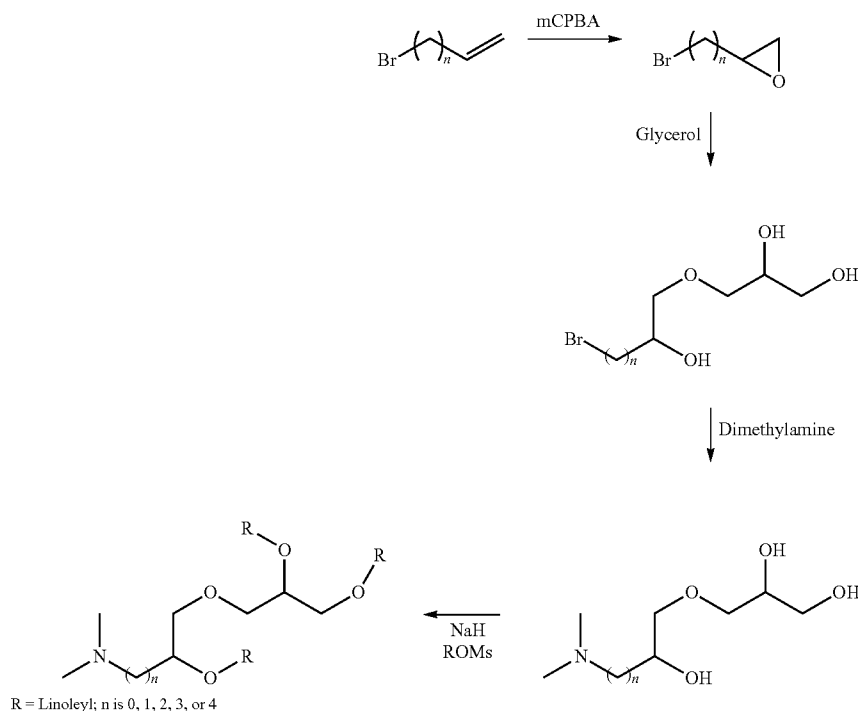
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mL). Finally, the organic phase was dried ( $\text{MgSO}_4$ ), filtered, and concentrated (rotovap). Purification by column chromatography yielded DPanDMA as a pale yellow oil (6.1 g, 8.97 mmol, 74.7%).

## Example 5

## Synthesis of Cationic Lipids of the TLinDMA Family

The following diagram provides a general scheme for synthesizing members of the C(n)-TLinDMA family of cationic lipids:



TLinDMA (1-(2,3-linoleloxypropoxy)-2-(linoleloxy)-(N,N-dimethyl)-propyl-3-amine) (Compound III) was synthesized as follows:

## Synthesis of Compound I:

A 1000 ml round bottom flask was charged with epibromohydrin (5 g, 37 mmol), glycerol (10 g, 110 mmol), a stir bar and then flushed with nitrogen. Anhydrous chloroform (350 mL) was added via cannula, followed by  $\text{BF}_3 \cdot \text{Et}_2\text{O}$  (0.5 mL, 3.7 mmol) and refluxed for 3 hours under nitrogen. The reaction mixture was cooled and subsequently stirred at room temperature overnight. Upon completion of the reaction, the reaction mixture was concentrated and the crude product (15 g) was purified via column chromatography using silica gel (150 g).

## Synthesis of Compound II:

A 500 mL round bottom flask was charged with Compound I (3.8 g, 17 mmol) and a stir bar. After flushing with nitrogen, dimethylamine in a 2.0 M methyl alcohol solution (170 mL) was added via cannula. The resulting mixture was stirred at room temperature for 48 hours. The progress of the reaction was monitored using TLC. The crude product was used without further purification.

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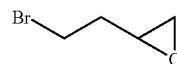
## Synthesis of TLinDMA (Compound III):

A 100 mL round bottom flask was charged with a stir bar, NaH (0.6 g, 24 mmol), and 25 mL benzene. Subsequently, Compound II (0.4 g, 2 mmol) was added followed immediately by linoleyl methane sulfonate (2.8 g, 8 mmol). The reaction was flushed with nitrogen and refluxed overnight. Progress of the reaction was monitored via TLC. The reaction mixture was transferred to a 250 mL separatory funnel and diluted with benzene to a final volume of 50 mL. The reaction was quenched with ethanol (30 mL) and then washed with water (50 mL). The lower aqueous phase was run off and the reaction mixture was washed again with ethanol (30 mL) and

water (50 mL). The organic phase was dried with  $\text{MgSO}_4$ , filtered, and solvent removed. The crude product (2.3 g) was purified via column chromatography on silica gel (60 g) with 0-3% methanol gradient in dichloromethane.

C2-TLinDMA (Compound VII) was synthesized as follows:

## Synthesis of Compound IV:



Chemical Formula:  $\text{C}_4\text{H}_7\text{BrO}$

Exact Mass: 150.0

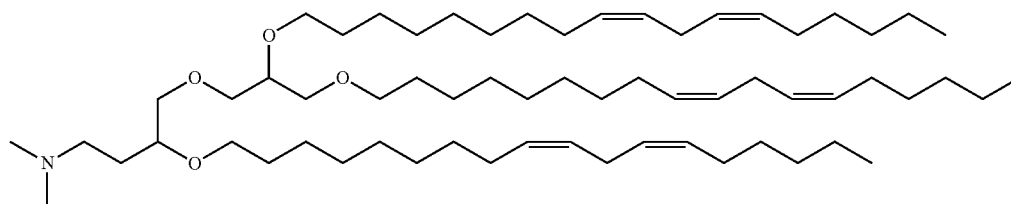
Molecular Weight: 151.0

Elemental Analysis: C, 31.82; H, 4.67; Br, 52.92; O, 10.60

A solution of 4-bromo-1-butene (11.5 g, 85 mmol) in  $\text{CH}_2\text{Cl}_2$  (anh., 120 ml) was prepared under nitrogen in a 1000 ml RBF with a magnetic stirrer. In a separate flask, a solution of 3-chloroperbenzoic acid (77%, MW 173, 44.05 g, 196 mmol) in  $\text{CH}_2\text{Cl}_2$  (anh., 250 ml) prepared and added to the

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reaction mixture by cannula. The reaction was stirred for 3 days, and then concentrated. The product (oil/white solid mixture) was re-dissolved in THF (300 mL) and a solution of

Chemical Formula: C<sub>63</sub>H<sub>117</sub>NO<sub>4</sub>

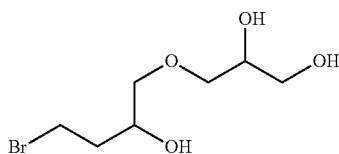
Exact Mass: 951.9

Molecular Weight: 952.6

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was monitored using t.l.c. The reaction mixture was concentrated and the crude product used without further purification. Synthesis of C2-TLinDMA (Compound VII):

4% sodium dithionite (180 mL) added to remove excess peracid. The mixture (now cloudy) was stirred for 20 minutes and then EtOAc (750 mL) added. The mixture was transferred to a separating funnel and the organic was washed with water (100 mL), sat. NaHCO<sub>3</sub> (2×300 mL, EFFERVESCENCE), water again (300 mL) and brine (300 mL). The solution was concentrated and the product purified by chromatography. Synthesis of Compound V:

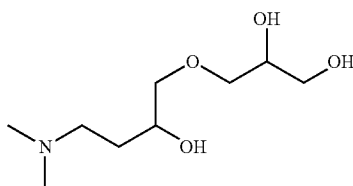
Chemical Formula: C<sub>7</sub>H<sub>15</sub>BrO<sub>4</sub>

Exact Mass: 242.0

Molecular Weight: 243.1

A 250 ml round bottom flask was charged with Compound IV (1.3 g, 9 mmol), glycerol (2.5 g, 27 mmol), a stir bar and then flushed with nitrogen. Anhydrous chloroform (100 mL) was added via cannula, followed by BF<sub>3</sub>·Et<sub>2</sub>O (0.15 mL, 1.1 mmol) and refluxed for 3 hours under nitrogen. The reaction mixture was subsequently stirred at room temperature overnight.

Synthesis of Compound VI:

Chemical Formula: C<sub>9</sub>H<sub>21</sub>NO<sub>4</sub>

Exact Mass: 207.1

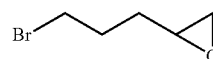
Molecular Weight: 207.3

A 50 mL round bottom flask was charged with Compound V (0.3 g, 1.2 mmol) and a stir bar. After flushing with nitrogen, dimethylamine in a 2.0 M methyl alcohol solution (25 mL) was added via syringe. The resulting mixture was stirred at room temperature for 48 hours. The progress of the reaction

A 100 mL round bottom flask was charged with a stir bar, NaH (0.6 g, 24 mmol), and 25 mL benzene. Compound VI (0.37 g, 1.8 mmol) was added followed immediately by linoleyl methane sulfonate (2.8 g, 8 mmol). The reaction was refluxed overnight and progress of the reaction was monitored via t.l.c. The reaction mixture was transferred to a 250 mL separatory funnel and diluted with benzene to a final volume of 50 mL. The reaction was quenched with ethanol (30 mL) and then washed with water (50 mL). The lower aqueous phase was run off and the reaction mixture washed again with ethanol (30 mL) and water (50 mL). The organic phase was dried with MgSO<sub>4</sub>, filtered, and solvent removed. The crude product, 2.5 g, was purified using column chromatography on silica gel (60 g), eluted with 0-3% methanol gradient in DCM.

C3-TLinDMA (Compound XI) was synthesized as follows:

Synthesis of Compound VIII:

Chemical Formula: C<sub>5</sub>H<sub>9</sub>BrO

Exact Mass: 164.0

Molecular Weight: 165.0

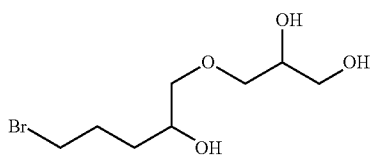
Elemental Analysis: C, 36.39; H, 5.50; Br, 48.42; O, 9.69

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A solution of 5-bromo-1-pentene (85 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (anh., 120 ml) is prepared under nitrogen in a 1000 ml RBF with a magnetic stirrer. In a separate flask, a solution of 3-chloroperbenzoic acid (77%, MW 173, 44.05 g, 196 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (anh., 250 ml) is prepared and added to the reaction mixture by cannula. The reaction is stirred for 3 days, and then concentrated. The product (oil/white solid mixture) is re-dissolved in THF (300 mL) and a solution of 4% sodium dithionite (180 mL) added to remove excess peracid. The mixture (now cloudy) is stirred for 20 minutes and then EtOAc (750 mL) added. The mixture is transferred to a separating funnel and the organic is washed with water (100 mL), sat. NaHCO<sub>3</sub> (2×300 mL, EFFERVESCENCE), water again (300 mL) and brine (300 mL). The solution is concentrated and the product purified by chromatography.

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Synthesis of Compound IX:

Chemical Formula:  $C_8H_{17}BrO_4$ 

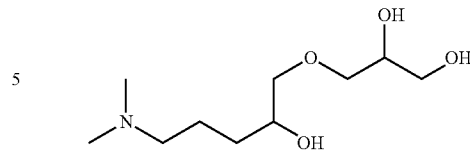
Exact Mass: 256.0

Molecular Weight: 257.1

A 250 ml round bottom flask is charged with Compound VIII (1.3 g, 9 mmol), glycerol (2.5 g, 27 mmol), a stir bar and then flushed with nitrogen. Anhydrous chloroform (100 mL) is added via cannula, followed by  $BF_3 \cdot Et_2O$  (0.15 mL, 1.1 mmol) and refluxed for 3 hours under nitrogen. The reaction mixture is subsequently stirred at room temperature overnight.

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Synthesis of Compound X:

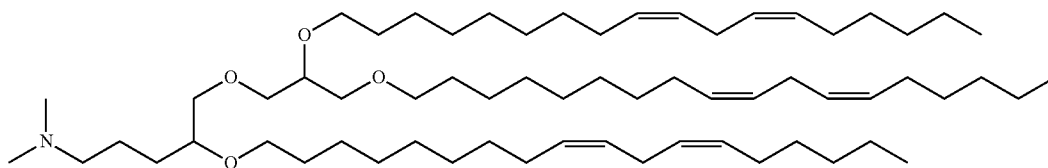
Chemical Formula:  $C_{10}H_{23}NO_4$ 

Exact Mass: 221.2

Molecular Weight: 221.3

A 50 mL round bottom flask is charged with Compound IX (0.3 g, 1.2 mmol) and a stir bar. After flushing with nitrogen, dimethylamine in a 2.0 M methyl alcohol solution (25 mL) is added via syringe. The resulting mixture is stirred at room temperature for 48 hours. The progress of the reaction is monitored using t.l.c. The reaction mixture is concentrated and the crude product used without further purification.

Synthesis of Compound XI:

Chemical Formula:  $C_{64}H_{119}NO_4$ 

Exact Mass: 965.9

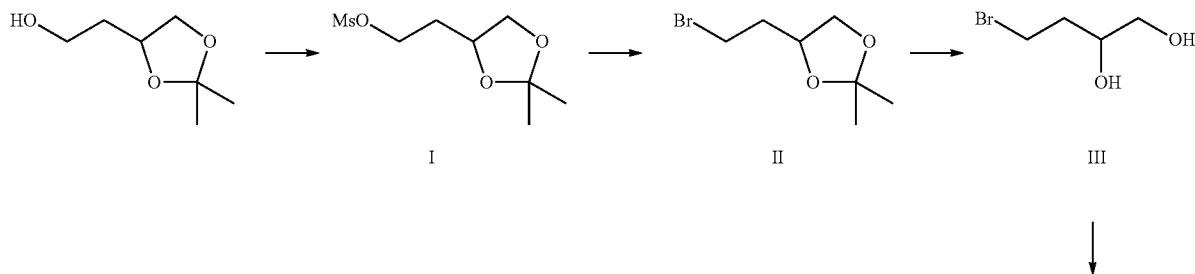
Molecular Weight: 966.6

A 100 mL round bottom flask is charged with a stir bar, NaH (0.6 g, 24 mmol), and 25 mL benzene. Compound X (0.37 g, 1.8 mmol) is added followed immediately by linoleyl methane sulfonate (2.8 g, 8 mmol). The reaction is refluxed overnight and progress of the reaction monitored via t.l.c. The reaction mixture is transferred to a 250 mL separatory funnel and diluted with benzene to a final volume of 50 mL. The reaction is quenched with ethanol (30 mL) and then washed with water (50 mL). The lower aqueous phase is run off and the reaction mixture washed again with ethanol (30 mL) and water (50 mL). The organic phase is dried with  $MgSO_4$ , filtered, and solvent removed. The crude product, 2.5 g, is purified using column chromatography on silica gel (60 g), eluted with 0-3% methanol gradient in DCM.

## Example 6

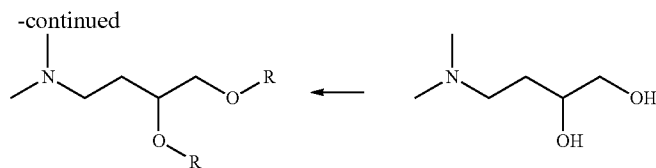
## Synthesis of Novel C2 Lipids

Novel C2 lipids (Compounds V-VII) having the structures shown below were synthesized as shown in the following schematic diagram.

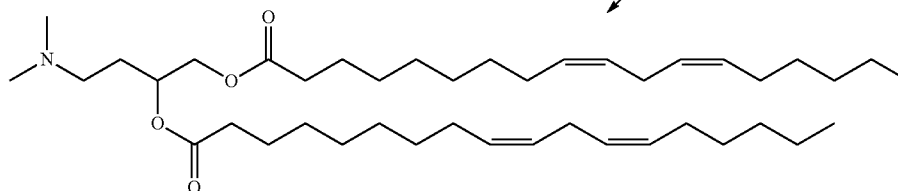


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130

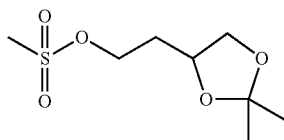


V: C2-DLinDMA: R = Linoleyl  
VI: C2-DPanDMA: R = Phytanyl



VII: C2-DLinDAP

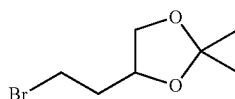
Step 1: Synthesis of 4-(2-Methanesulfonylethyl)-2,2-dimethyl-1,3-dioxolane (Compound I)



$C_8H_{16}O_5S$   
Exact Mass: 224.07  
Mol. Wt.: 224.28  
C, 42.84; H, 7.19; O, 35.67; S, 14.30

4-(2-Hydroxyethyl)-2,2-dimethyl-1,3-dioxolane (25 g, 170 mmol), triethylamine (55.9 mL, 400 mmol), and a stir bar were added to a 1000 mL round bottom flask. The flask was sealed and flushed with nitrogen. Anhydrous DCM (600 mL) was added, and the mixture cooled to approx  $-5^\circ C$ . (ice and NaCl). Mesyl chloride (19.9 mL, 255 mmol, 1.5 eq) was added slowly via syringe over a 60 minute period, and the reaction stirred at  $-5^\circ C$ . for a further 1.5 hours. At this point TLC showed that the starting material had been consumed. The solution was diluted with DCM (350 mL), divided into two (~500 mL) portions, and each portion worked up as follows: the solution was transferred to a 1000-mL separating funnel and washed with saturated  $NaHCO_3$  (2x200 mL). The organic phase was then dried ( $MgSO_4$ ), filtered, and concentrated (rotovap). The crude product was purified by column chromatography. Final yield: 32.0 g, 84.1%.

Step 2: Synthesis of 4-(2-Bromoethyl)-2,2-dimethyl-1,3-dioxolane (Compound II)

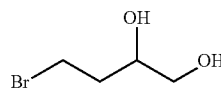


$C_7H_{13}BrO_2$   
Exact Mass: 208.01  
Mol. Wt.: 209.08  
C, 40.21; H, 6.27; Br, 38.22; O, 15.30

Magnesium bromide etherate (40 g, 130 mmol) and a stir bar were added to a 2000 mL round bottom flask and flushed with nitrogen. A solution of 4-(2-methanesulfonylethyl)-2,2-dimethyl-1,3-dioxolane (I) (17.5 g, 78 mmol) in anhydrous diethyl ether (900 mL) was added via cannula, and the sus-

pension stirred overnight. The ether was first decanted into a beaker. Water (200 mL) and ether (300 mL) were added to the precipitate and stirred for 5 minutes. The precipitate was dissolved, and the ether phase was then collected and added to the ether solution from the reaction. The organic phase was then washed, concentrated to about 500 mL, washed with water, dried over anhydrous  $Mg_2SO_4$ , filtered, and concentrated to yield a yellow oil (16.0 g). This was purified by flash chromatography to yield 10.6 g of product (50.7 mmol, 65%).

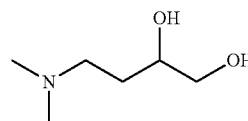
Step 3: Synthesis of 4-Bromobutane-1,2-diol (Compound III)



$C_4H_9BrO_2$   
Exact Mass: 167.98  
Mol. Wt.: 169.02  
C, 28.42; H, 5.37; Br, 47.28; O, 18.93

4-(2-Bromoethyl)-2,2-dimethyl-1,3-dioxolane (II) (9 g, 43 mmol) was added to a 500 mL RBF with a stirbar. 100 mL of  $MeOH:H_2O:HCl$  in a ratio of (60:20:5) were added. After 30 minutes, sat.  $NaHCO_3$  (~75 mL) was added (effervescence), until pH paper indicated solution was basic. At this point the mixture was slightly cloudy. Ether (300 mL) was added (while stirring) and the cloudiness disappeared. The reaction mixture was transferred to a 1000 mL sep funnel and the 2 phases separated. The extraction of the aqueous phase was repeated two more times (2x300 mL ether). Organics were combined, dried over  $MgSO_4$  and concentrated to yield a colorless oil (7.0 g), which was purified by column chromatography.

Step 4: Synthesis of 4-(Dimethylamino)-1,2-butanediol (Compound IV)



Chemical Formula:  $C_6H_{15}NO_2$   
Exact Mass: 133.1  
Molecular Weight: 133.2  
Elemental Analysis: C, 54.11; H, 11.35;  
N, 10.52; O, 24.03



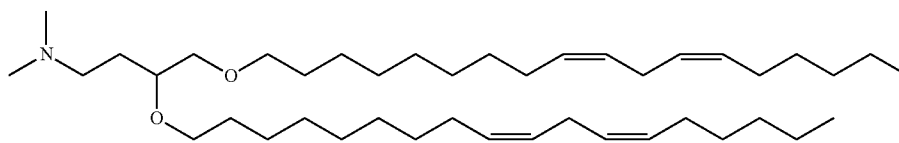
## 131

4-Bromobutane-1,2-diol (III) (1 g, 6.0 mmol) was added to a 50 mL RBF with a stir bar, sealed, and flushed with nitrogen. 30 mL of Dimethylamine (2.0M solution in MeOH) was delivered by cannula and the reaction stirred overnight. TLC indicated all the starting material had disappeared. The sol-

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vent (and DMA) were removed by evaporation and the crude product used without further purification.

Synthesis of 1,2-Dilinoleyloxy-(N,N-dimethyl)-butyl-4-amine (C2-DLinDMA) (Compound V)



Chemical Formula:  $C_{43}H_{79}NO_3$

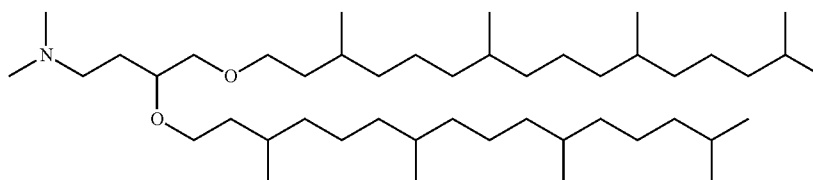
Exact Mass: 657.61

Mol. Wt.: 658.09

C, 78.48; H, 12.10; N, 2.13; O, 7.29

- 20 4-(Dimethylamino)-1,2-butanediol (IV) (1.3 g, 3.4 mmol), linoleyl mesylate (2.0 g, 5.8 mmol), tetrabutylammonium hydrogen sulphate (0.5 g, 1.5 mmol), toluene (30 mL), and a stir bar were added to a 100 mL RBF. 30 mL of 40% NaOH was made and added to the reaction mixture. The resulting mixture was stirred at room temperature, under nitrogen for 25 60 hours. Deionized water (50 mL) and isopropyl acetate (50 mL) were added and the mixture stirred vigorously for a further 10-15 min. The mixture was transferred to a 250 mL separating funnel and allowed to separate and the aqueous phase removed. The organic phase was washed twice with water (2x30 mL) using MeOH to aid the separation, and the 30 organic phase was dried ( $MgSO_4$ ), filtered, and concentrated to obtain a dark yellow oil. The oil was purified by column chromatography.

Synthesis of 1,2-Diphytanyloxy-(N,N-dimethyl)-butyl-4-amine (C2-DPanDMA) (Compound VI)



Chemical Formula:  $C_{46}H_{95}NO_2$

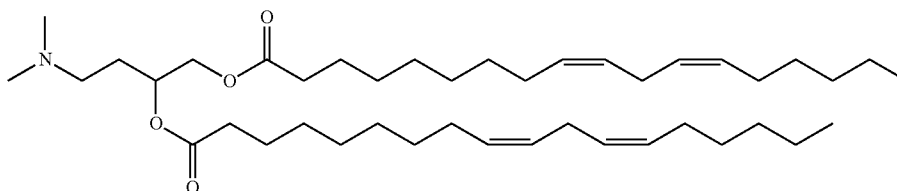
Exact Mass: 693.7

Molecular Weight: 694.3

Elemental Analysis: C, 79.58; H, 13.79; N, 2.02; O, 4.61

- 50 Sodium hydride (360 mg, 15 mmol), benzene (40 mL), and a stir bar were added to a 50 mL round bottom flask. 4-(Dimethylamino)-1,2-butanediol (IV) (200 mg, 1.5 mmol) was added and the reaction stirred for 10 minutes (effervescence). 55 Phytanyl Mesylate (1.07 g, 2.92 mmol) was then added and the flask fitted with a condenser, flushed with nitrogen, and heated to reflux. After 18 hours, the flask was allowed to cool to room temperature. The volume was made up to 40 mL with benzene. EtOH was added slowly to quench unreacted sodium hydride. Once quenching was complete, the reaction 60 mixture was washed twice with an EtOH/ $H_2O$ , in a ratio of 1:1:0.6 benzene:water:ethanol. The aqueous washes were combined and extracted with  $CHCl_3$  (2x20 mL). Finally, the organics were combined, dried ( $MgSO_4$ ), filtered, and concentrated (rotovap). Purification by column chromatography yielded a pale yellow oil (250 mg, 0.145 mmol, 25%). 65

Synthesis of 1,2-Dilinoleoyloxy-(N,N-dimethyl)-butyl-4-amine (C2-DLInDAP) (Compound VII)



Chemical Formula: C<sub>42</sub>H<sub>75</sub>NO<sub>4</sub>

Exact Mass: 657.6

Molecular Weight: 658.0

Elemental Analysis: C, 76.66; H, 11.49; N, 2.13; O, 9.73

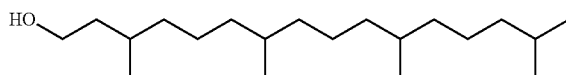
A flask containing 4-(Dimethylamino)-1,2-butanediol (IV) (crude, 266 mg, 2 mmol (max)), TEA (0.84 mL, 6 mmol), and DMAP (24 mg, 0.2 mmol) was flushed with nitrogen before the addition of anhydrous CH<sub>2</sub>Cl<sub>2</sub> (50 mL). Linoleoyl chloride (1.2 g, 4 mmol) was added and the solution stirred overnight. The solution was rinsed into a 250 mL separatory funnel with DCM (~70 mL) and washed with water (2×50 mL). The organic was dried (MgSO<sub>4</sub>), concentrated, and purified by chromatography.

Example 7

Synthesis of Novel Phytanyl Cationic Lipids

DPan-C2K-DMA, DPan-C1K6-DMA, and DPan-C3K-DMA having the structures shown below were synthesized as shown in the following schematic diagram.

Synthesis of Phytanol



C<sub>20</sub>H<sub>42</sub>O

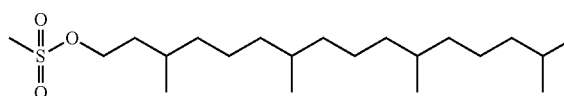
Exact Mass: 298.32

Mol. Wt.: 298.55

C, 80.46; H, 14.18; O, 5.36

Phytol (21.0 g, 70.8 mmol), ethanol (180 mL) and a stir bar were added to a 500 mL round bottom flask. Raney Nickel 2800 (as purchased, a 50% by weight solution in water if used as purchased, Nickel >89% metal present) (6.8 g, 51.5 mmol) was added, and the flask sealed and flushed with hydrogen. A 12" needle was used to bubble hydrogen through the solution for 10 minutes. The reaction was stirred for 5 days, using a balloon as a hydrogen reservoir. Hydrogen was also bubbled through the reaction mixture at 24 h and 48 h, 5 minutes each time. The metal catalyst was then removed by filtering through Celite. The ethanolic solution was concentrated, and 200 mL of DCM added to the resulting oil. The solution was washed with water (2×100 mL), dried over MgSO<sub>4</sub>, and concentrated. TLC indicated formation of the phytanol product, yield 20.0 g.

Synthesis of Phytanyl Mesylate:



C<sub>21</sub>H<sub>44</sub>O<sub>3</sub>S

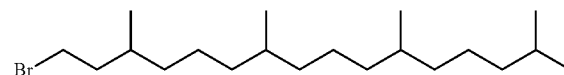
Exact Mass: 376.30

Mol. Wt.: 376.64

C, 66.97; H, 11.78; O, 12.74; S, 8.51

Phytanol (20.0 g, 66.7 mmol), triethylamine (18.6 mL, 133 mmol) and a stir bar were added to a 1000 mL round bottom flask. The flask was sealed and flushed with nitrogen. Anhydrous DCM (250 mL) was added, and the mixture cooled to -15° C. (Ice and NaCl). Mesyl Chloride (10.4 mL, 133 mmol) was added slowly via syringe over a 30 minute period, and the reaction stirred at -15° C. for a further 1.5 hours. At this point TLC showed that the starting material had been used up. The solution was diluted with DCM (250 mL) and washed with saturated NaHCO<sub>3</sub> (2×200 mL). The organic phase was then dried (MgSO<sub>4</sub>), filtered and concentrated (rotovap). The crude product was purified by column chromatography. Yield 21.5 g, 85.7%.

Synthesis of Phytanyl Bromide:



Chemical Formula: C<sub>20</sub>H<sub>41</sub>Br

Exact Mass: 360.2

Molecular Weight: 361.4

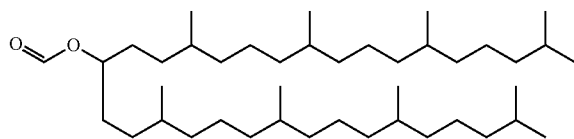
Elemental Analysis: C, 66.46; H, 11.43; Br, 22.11

Magnesium bromide etherate (17 g, 55 mmol) and a stir bar were added to a 500 mL round bottom flask. The flask was sealed and flushed with nitrogen and anhydrous diethyl ether (200 mL) added via cannula. A solution of phytanyl mesylate (10.9 g, 28.9 mmol (FW=377)) in anhydrous ether (50 mL) was also added via cannula, and the suspension stirred overnight. The following morning a precipitate had formed on the side of the flask. Chilled water (200 mL) was added (ppt dissolved) and the mixture transferred to a 1000-mL separating funnel. After shaking, the organic phase was separated. The aqueous phase was then extracted with ether (2×150 mL) and all ether phases combined. The ether phase was washed

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with water (2×150 mL), brine (150 mL) and dried over anhydrous  $Mg_2SO_4$ . The solution was filtered, concentrated, and purified by flash chromatography. Final yield 9.5 g (26.3 mmol, 91.1%).

Synthesis of Compound A:



Chemical Formula:  $C_{42}H_{84}O_2$

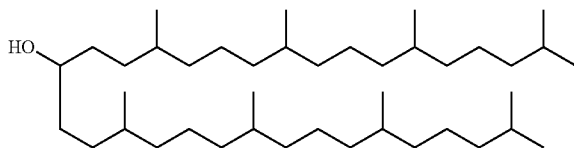
Exact Mass: 620.65

Molecular Weight: 621.12

Elemental Analysis: C, 81.22; H, 13.63; O, 5.15

Magnesium turnings (720 mg, 30 mmol), a crystal of iodine, and a stirbar were added to a 500 mL round-bottom flask. The flask was flushed with nitrogen and anhydrous diethyl ether (200 mL) added via cannula. A solution of phytanyl bromide (9.5 g, 26.3 mmol) in anhydrous ether (20 mL) was added and the resulting cloudy mixture refluxed overnight. The mixture was cooled to RT and, without removing the subaseal or condenser, ethyl formate (2.2 g, 2.41 mL, 30 mmol) added via syringe and 12" needle. The addition was made dropwise, directly into the reaction mixture, and the cloudy suspension again stirred overnight. R.M. was transferred to a 500-mL sep. funnel with ether (50 mL), and washed with 10%  $H_2SO_4$  (100 mL—the cloudy R.M. now clarified upon shaking), water (2×100 mL) and brine. The organic was dried over anhydrous  $Mg_2SO_4$ , filtered, and concentrated. Yield (crude) was 8 g. TLC indicated that the majority of product was the diphytanylmethyl formate, which was purified by chromatography (0-6% ether in hexane).

Synthesis of Compound B:



Chemical Formula:  $C_{41}H_{84}O$

Exact Mass: 592.65

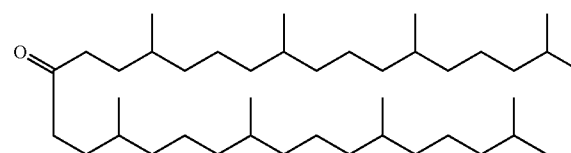
Molecular Weight: 593.11

Elemental Analysis: C, 83.03; H, 14.28; O, 2.70

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The purified formate (A) (5.5 g, 8.86 mmol) was then transferred to a 1000 mL round bottom flask with stirbar and 90% EtOH (500 mL) and KOH (2.0 g, 35.7 mmol) added. The reaction mixture was clear, and was stirred overnight. The following day the mixture was concentrated by rotovap to 50% of its volume and then poured into 200 mL of 5% HCl. The aqueous phase was extracted with ether (3×100 mL). The combined ether extracts were washed with water (3×200 mL), dried ( $MgSO_4$ ), and concentrated. TLC (DCM) revealed reaction to have gone cleanly to completion, and the product (5.5 g, 100%) was used without further purification.

Synthesis of Compound C:



Chemical Formula:  $C_{41}H_{82}O$

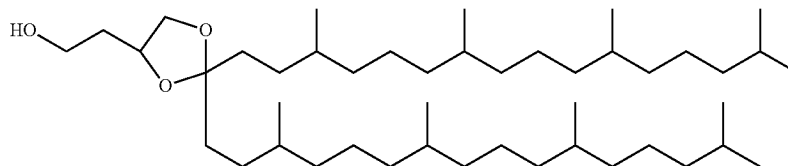
Exact Mass: 590.6

Molecular Weight: 591.1

Elemental Analysis: C, 83.31; H, 13.98; O, 2.71

To a mixture of Compound B (5.5 g, 9.3 mmol), pyridinium chlorochromate (PCC) (5.5 g, 25.5 mmol) and anhydrous sodium carbonate (0.6 g, 5.66 mmol) in DCM were added. The resulting suspension was stirred for 1 h, but TLC indicated still some starting material (SM) remaining. The suspension was stirred another hour, and appeared to have progressed slightly, but not to completion. Further PCC (1.0 g) and sodium carbonate (0.2 g) were added and the reaction stirred overnight. Reaction had now gone to completion. Ether (300 mL) was then added to the mixture and the resulting brown suspension filtered through a pad of silica (300 mL), washing the pad with ether (3×100 mL). The ether phases were combined, concentrated, and purified to yield 5.0 g (90%) of ketone.

Synthesis of Compound D:



Chemical Formula:  $C_{45}H_{90}O_3$

Exact Mass: 678.69

Molecular Weight: 679.19

Elemental Analysis: C, 79.58; H, 13.36; O, 7.07

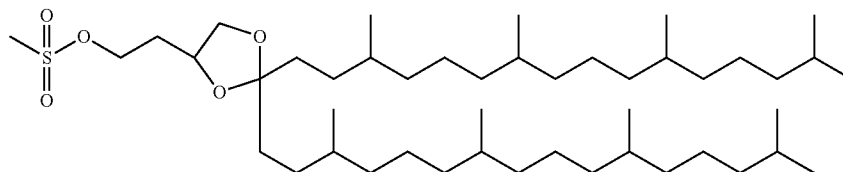
## 137

A 100 mL round bottom flask was charged with Compound C (1.4 g, 2.4 mmol), 1,2,4-butanetriol (0.51 g, 4.8 mmol), pyridinium p-toluenesulfonate (0.06 g, 0.24 mmol), and a stir bar. The reaction vessel was flushed with nitrogen and anhydrous toluene (30 mL) added via cannula. The flask was equipped with a Dean-Stark tube and condenser and flushed with nitrogen. The reaction was refluxed under nitrogen overnight and progress of the reaction monitored via TLC. After refluxing for three hours, reaction solution deposited in the Dean-Stark tube was removed via syringe (20 mL) and the reaction vessel immediately replenished with fresh toluene

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(20 mL). This was repeated every hour, for a total of three times, and then left to reflux mildly overnight. After cooling to room temperature, the reaction mixture was transferred to a 250 mL separatory funnel with toluene (2×5 mL), washed with 5% aqueous Na<sub>2</sub>CO<sub>3</sub> (2×50 mL), water (50 mL), and dried over MgSO<sub>4</sub>. Evaporation of the solvent gave 1.67 g of crude product which was purified via column chromatography on silica gel (50 g) using dichloromethane as eluent. Yield: 1.4 g, 2.06 mmol, 86%.

Synthesis of Compound E:



Chemical Formula: C<sub>46</sub>H<sub>92</sub>O<sub>5</sub>S

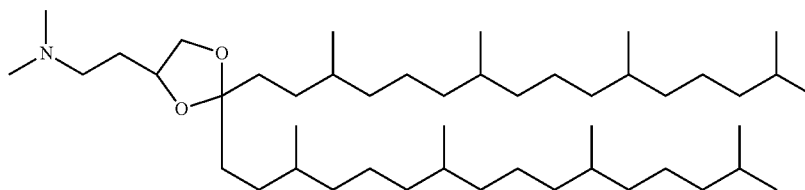
Exact Mass: 756.67

Molecular Weight: 757.28

Elemental Analysis: C, 72.96; H, 12.25; O, 10.56; S, 4.23

A 100 mL round bottom flask was charged with Compound D (1.4 g, 2.06 mmol) and a stir bar. The vessel was flushed with nitrogen and DCM (25 mL) added. Subsequently, triethylamine (0.72 g, 7.1 mmol, 0.99 mL) was added via syringe and the resulting solution cooled to -15° C. (NaCl, ice). In a separate 50 mL round bottom flask, a solution of methanesulfonic anhydride (0.74 g, 4.1 mmol) and DCM (20 mL) was prepared. This solution was added drop wise to the above solution over a 30 minute period. The reaction vessel was maintained at -15° C. The reaction mixture was stirred at room temperature overnight and monitored via TLC. The reaction mixture was then diluted with DCM (25 mL), and washed with NaHCO<sub>3</sub> (2×30 mL), then dried over anhydrous MgSO<sub>4</sub>. The crude product (1.7 g) was used in the next step without further purification.

Synthesis of DPan-C2K-DMA:



Chemical Formula: C<sub>47</sub>H<sub>95</sub>NO<sub>2</sub>

Exact Mass: 705.74

Molecular Weight: 706.26

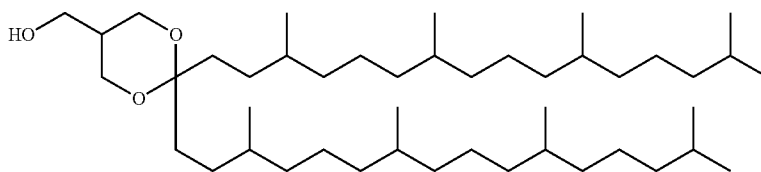
Elemental Analysis: C, 79.93; H, 13.56; N, 1.98; O, 4.53

A 500 mL round bottom flask was charged with crude Compound E (1.7 g, 2.5 mmol) and a stir bar. The reaction vessel was flushed with nitrogen and dimethylamine in THF (2.0 M, 65 mL) subsequently added via syringe. The resulting mixture was stirred for three days at room temperature. The reaction was concentrated and the crude product purified by column chromatography using silica gel (40 g) with a gradient of 0-5% methanol in dichloromethane.

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Synthesis of Compound F:

Chemical Formula: C<sub>45</sub>H<sub>90</sub>O<sub>3</sub>

Exact Mass: 678.69

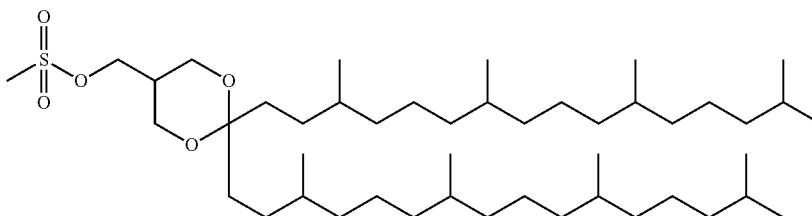
Molecular Weight: 679.19

Elemental Analysis: C, 79.58; H, 13.36; O, 7.07

A 100 mL round bottom flask was charged with Compound C (1.2 g, 2.1 mmol), 2-hydroxymethyl-1,3-propanediol (0.45 g, 4.2 mmol), pyridinium p-toluenesulfonate (0.05 g, 0.21 mmol), and a stir bar. The reaction vessel was flushed with nitrogen and anhydrous toluene (45 mL) subsequently added via cannula. The flask was equipped with a Dean-Stark tube and condenser and flushed with nitrogen. The reaction was refluxed under nitrogen overnight and progress of the reaction monitored via TLC. After refluxing for three hours, reaction solution deposited in the Dean-Stark tube was removed via syringe (20 mL) and the reaction vessel immediately replen-

15 ished with fresh toluene (20 mL). This was repeated every hour, for a total of three times, and then left to reflux mildly overnight. After cooling to room temperature, the reaction mixture was transferred to a 250 mL separatory funnel with toluene (2x5 mL), washed with 5% aqueous Na<sub>2</sub>CO<sub>3</sub> (2x50 mL), water (50 mL), and dried over MgSO<sub>4</sub>. Evaporation of the solvent gave 1.44 g of crude product which was then purified via column chromatography on silica gel (35 g) with 0-3% methanol gradient in dichloromethane.

Synthesis of Compound G:

Chemical Formula: C<sub>46</sub>H<sub>92</sub>O<sub>3</sub>S

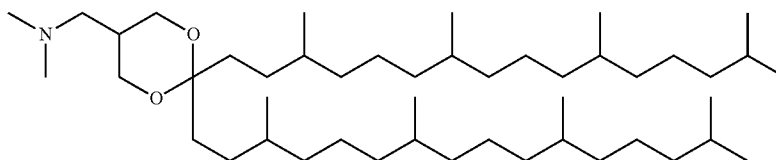
Exact Mass: 756.67

Molecular Weight: 757.28

Elemental Analysis: C, 72.96; H, 12.25; O, 10.56; S, 4.23

40 A 250 mL round bottom flask was charged with Compound F (1.2 g, 1.8 mmol) and a stir bar. The vessel was flushed with nitrogen and DCM (25 mL) added. Subsequently, triethylamine (0.62 g, 6.1 mmol, 0.85 mL) was added via syringe and the resulting solution cooled to -15° C. (NaCl, ice). In a separate 50 mL round bottom flask, a solution of methanesulfonyl anhydride (0.67 g, 3.7 mmol) and DCM (20 mL) was prepared. This solution was added drop wise to the above solution over a 30 minute period. The reaction vessel was maintained at -15° C. during the addition. The reaction mixture was stirred at room temperature overnight and monitored via TLC. The reaction mixture was then diluted with DCM (25 mL) and washed with NaHCO<sub>3</sub> (2x30 mL), then dried over anhydrous MgSO<sub>4</sub>. The crude product (1.6 g) was used in the following step without further purification.

Synthesis of DPan-C1K6-DMA:

Chemical Formula: C<sub>47</sub>H<sub>95</sub>NO<sub>2</sub>

Exact Mass: 705.74

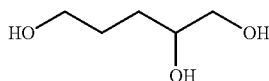
Molecular Weight: 706.26

Elemental Analysis: C, 79.93; H, 13.56; N, 1.98; O, 4.53

## 141

A 250 mL round bottom flask was charged with crude Compound G (1.6 g, 2.1 mmol) and a stir bar. The reaction vessel was flushed with nitrogen and dimethylamine in THF (2.0 M, 60 mL) subsequently added via syringe. The resulting mixture was stirred for six days at room temperature. After solvent was evaporated, the crude product was purified using column chromatography on silica gel (30 g) with 0-30% ethyl acetate gradient in hexanes.

Synthesis of Compound H:

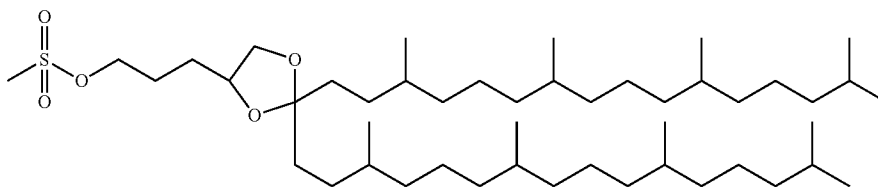


Chemical Formula:  $C_4H_{12}O_3$   
Exact Mass: 120.08  
Molecular Weight: 120.15  
Elemental Analysis: C, 49.98; H, 10.07; O, 39.95

## 142

A 100 mL round bottom flask was charged with Compound C (1.0 g, 1.8 mmol), Compound H (crude, 0.450 g, 3.6 mmol), pyridinium p-toluenesulfonate (0.05 g, 0.24 mmol), and a stir bar. The reaction vessel was flushed with nitrogen and anhydrous toluene (45 mL) subsequently added via cannula. The flask was equipped with a Dean-Stark tube and condenser and flushed with nitrogen. The reaction was refluxed under nitrogen overnight and progress of reaction monitored via TLC. After refluxing for three hours, reaction solution deposited in the Dean-Stark tube was removed via syringe (20 mL) and the reaction vessel immediately replenished with fresh toluene (20 mL). This was repeated every hour, for a total of five times, and then left to reflux mildly overnight. After cooling to room temperature, the reaction mixture was transferred to a 250 mL separatory funnel with toluene (2x5 mL), washed with 5% aqueous  $Na_2CO_3$  (2x50 mL), water (50 mL), and dried over  $MgSO_4$ . Evaporation of the solvent gave 1.13 g of crude product which was then purified via column chromatography on silica gel (30 g) using dichloromethane as eluent. Yield, 1.0 g.

Synthesis of Compound J:

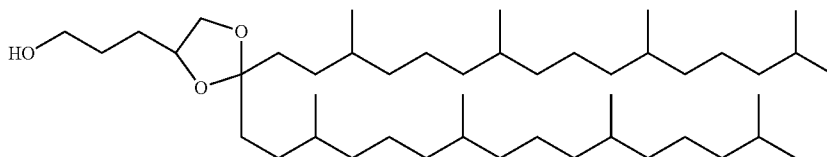


Chemical Formula:  $C_{47}H_{94}O_3S$   
Exact Mass: 770.68  
Molecular Weight: 771.31  
Elemental Analysis: C, 73.19; H, 12.28; O, 10.37; S, 4.16

35

A 50 mL round bottom flask was charged with (R)- $\gamma$ -hydroxymethyl- $\gamma$ -butyrolactone (1.0 g, 8.6 mmol), flushed with nitrogen, and sealed with a rubber septum. Anhydrous THF (40 mL) was subsequently added via syringe. The (R)- $\gamma$ -hydroxymethyl- $\gamma$ -butyrolactone solution was then added drop wise under nitrogen to a prepared solution containing  $LiAlH_4$  (3.5 g, 92 mmol) in 160 mL anhydrous THF. During the addition, the reaction vessel was maintained at  $0^\circ C$ . The resulting suspension was stirred at room temperature overnight. The reaction mixture was cooled to  $0^\circ C$  and brine (10-22 mL) added very slowly using a Pasteur pipette. The mixture was stirred under nitrogen at room temperature overnight. The white solid was filtered and washed with THF (3x25 mL). The organics were combined and concentrated. After solvent was removed, the crude product seemed to contain water along with an oily residue; therefore, the crude product was azeotroped within ethanol (100 mL) resulting in a yellow oil. The crude product (0.45 g) was used in the next step without further purification.

Synthesis of Compound I:



Chemical Formula:  $C_{46}H_{92}O_3$   
Exact Mass: 692.70  
Molecular Weight: 693.22  
Elemental Analysis: C, 79.70; H, 13.38; O, 6.92

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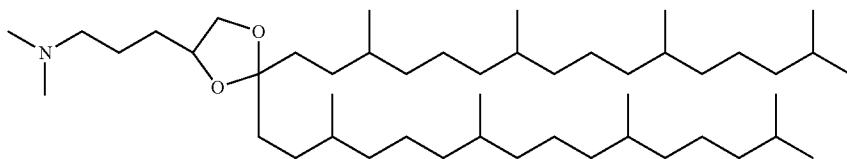
50

A 250 mL round bottom flask was charged with Compound I (1.0 g, 1.44 mmol) and a stir bar. The vessel was flushed with nitrogen and DCM (25 mL) added. Subsequently, triethylamine (0.51 g, 5 mmol, and 0.7 mL) was added via syringe and the resulting solution cooled to  $-15^\circ C$ . (NaCl, ice). In a separate 50 mL round bottom flask, a solution of methanesulfonic anhydride (0.54 g, 3.0 mmol) and anhydrous DCM (20 mL) was prepared. This solution was added drop wise to the above solution over a 30 minute period. The reaction vessel was maintained at  $-15^\circ C$ . The reaction mixture was stirred at room temperature overnight and monitored via TLC. The reaction mixture was then diluted with DCM (25 mL) and washed with  $NaHCO_3$  (2x30 mL), then dried over anhydrous  $MgSO_4$ . The crude product (1.2 g) was used in the next step without further purification.

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Synthesis of DPan-C3K-DMA:

Chemical Formula:  $C_{48}H_{97}NO_2$ 

Exact Mass: 719.75

Molecular Weight: 720.29

Elemental Analysis: C, 80.04; H, 13.57; N, 1.94; O, 4.44

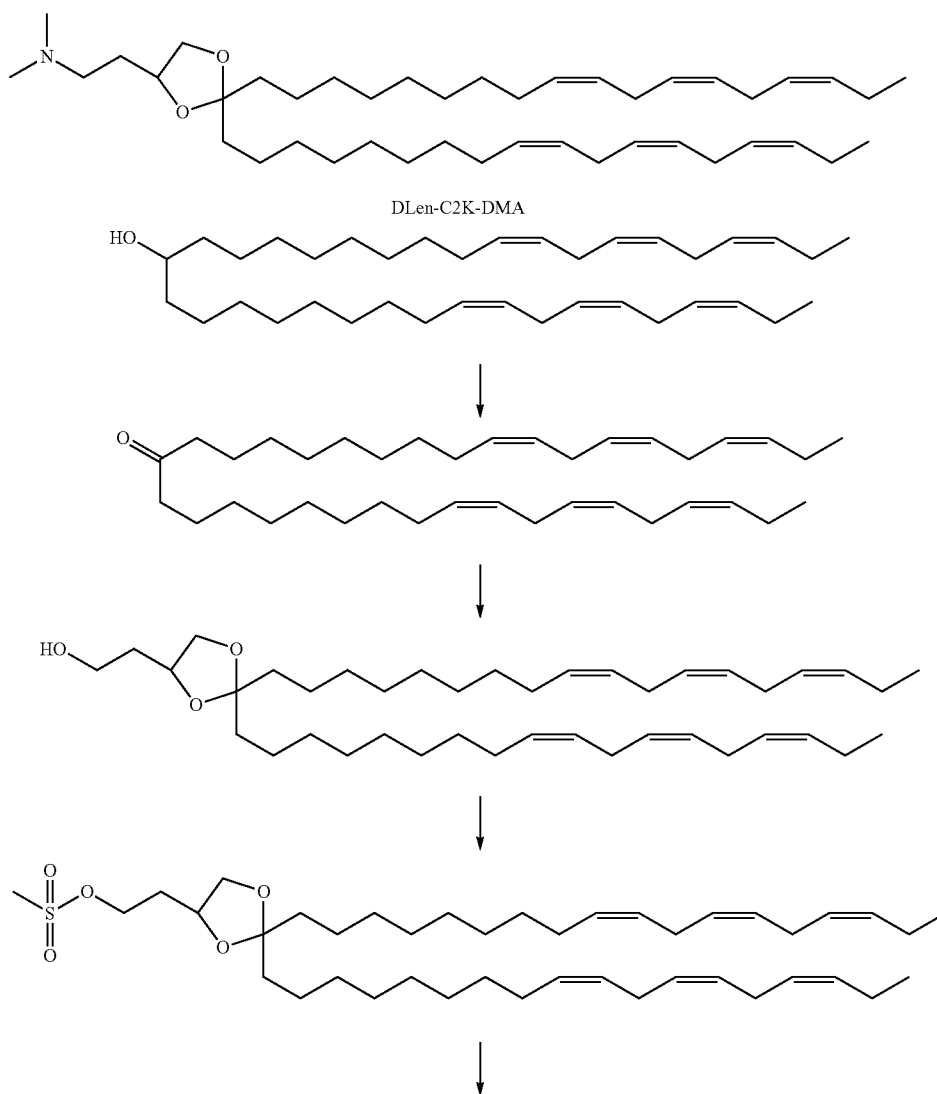
15

A 100 mL round bottom flask was charged with crude Compound J (1.2 g, 1.6 mmol) and a stir bar. The reaction vessel was flushed with nitrogen and dimethylamine in THF (2.0 M, 45 mL) subsequently added via syringe. The resulting mixture was stirred for four days at room temperature. After solvent was evaporated, the crude product was purified using column chromatography on silica gel (30 g) with 0-30% ethyl acetate gradient in hexanes.

Example 8

Synthesis of DLen-C2K-DMA

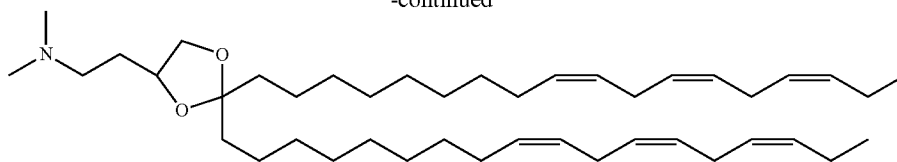
DLen-C2K-DMA having the structure shown below was synthesized as shown in the following schematic diagram.



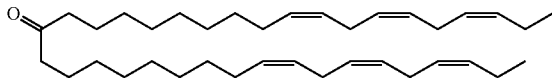
145

146

-continued



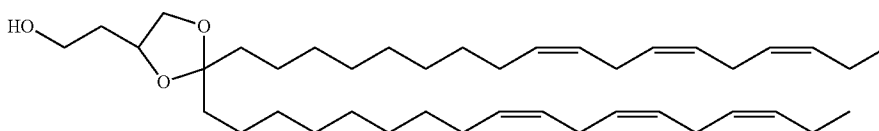
Synthesis of Dilinolenyl Ketone:



To a 1000 mL RBF containing a solution of dilinolenyl methanol (6.0 g, 11.4 mmol) in anh. DCM (200 mL) was

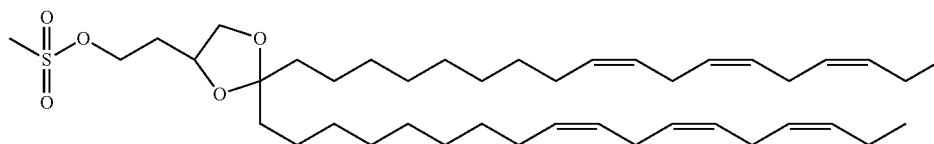
10 added pyridinium chlorochromate (7.39 g, 34.2 mmol), anh. sodium carbonate (1.0 g, 5.66 mmol) and a stirbar. The resulting suspension was stirred under nitrogen at RT for 3 h, after which time TLC indicated all SM to have been consumed. Ether (300 mL) was then added to the mixture and the resulting brown suspension filtered through a pad of silica (300 mL), washing the pad with ether (3×100 mL). The ether phases were combined, concentrated and purified to yield 4.2 g (8.0 mmol, 70%) of the ketone.

Synthesis of Linolenyl Ketal:



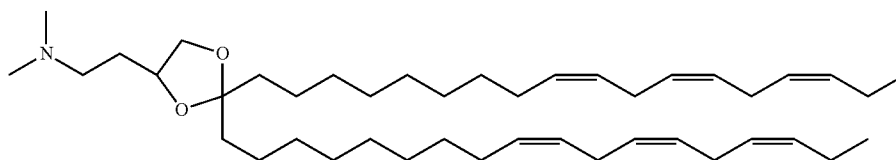
A 100 mL RBF was charged with dilinolenyl ketone (4.2 g, 8.2 mmol), 1,2,4-butanetriol (3.4 g, 32 mmol), PPTS (200 mg, 0.8 mmol) and a stir bar. The flask was flushed with nitrogen and anhydrous toluene (60 mL) added. The reaction vessel was fitted with a Dean Stark tube and condenser and brought to reflux and the reaction was left overnight. After cooling to room temperature, the reaction mixture diluted with toluene (50 mL), and washed with 5% aq. Na<sub>2</sub>CO<sub>3</sub> (2×50 mL), water (50 mL), dried (MgSO<sub>4</sub>) and purified by chromatography to yield 3.0 g (4.9 mmol, 59%) of the ketal.

Mesylate Formation:



A 250 mL RBF was charged with the linolenyl ketal (3.0 g, 4.9 mmol), TEA (2.2 mL, 15.6 mmol) and a stir bar. The flask was flushed with nitrogen, anh. DCM (20 mL) added, and the solution cooled to -15° C. In a separate 50 mL flask, a solution of MsCl (9.7 mmol, 2 eqv.) in anhydrous DCM (30 mL) was prepared, then transferred to the reaction vessel by syringe over 20 minutes. The reaction was stirred for 90 minutes at -15° C., at which point starting material had been consumed. The reaction mixture was diluted with a further 50 mL of DCM, washed with NaHCO<sub>3</sub> (2×50 mL), dried (MgSO<sub>4</sub>) and purified by chromatography. Final yield 3.1 g, 4.5 mmol, 92%.

Synthesis of DLen-C2K-DMA:





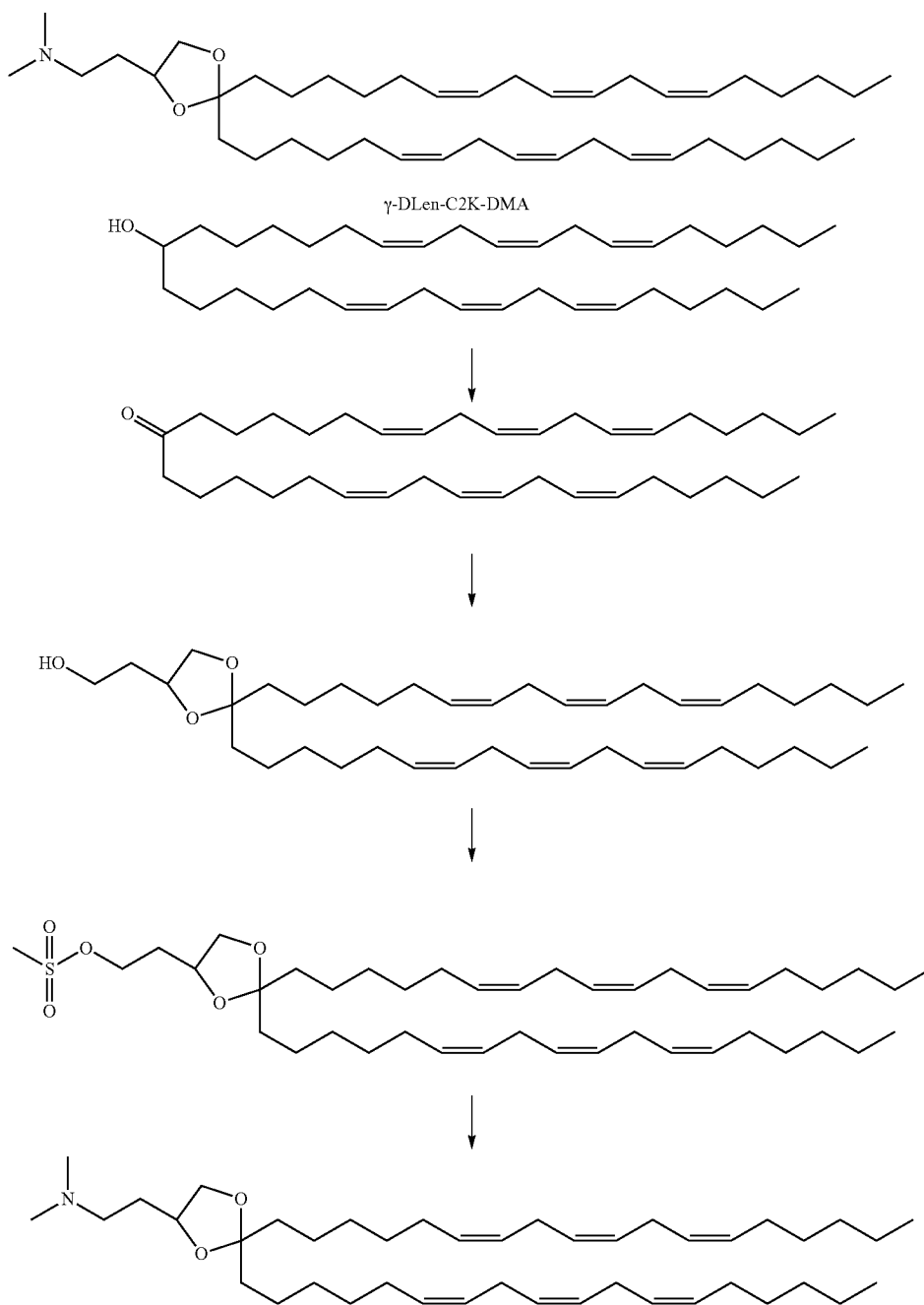
## 147

A 250 mL RBF was charged with the mesylate (3.0 g, 4.35 mmol), isopropanol (25 mL) and a stir bar. The flask was flushed with nitrogen, sealed, and a 2.0 M solution of dimethylamine in methanol (120 mL) added via cannula. The reaction was stirred at room temperature for 3 days. The solution was concentrated and purified by chromatography. Final yield 2.49 g, 3.9 mmol, 90%.

## Example 9

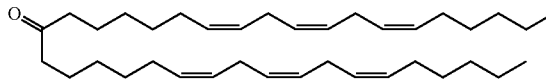
Synthesis of  $\gamma$ -DLen-C2K-DMA

$\gamma$ -DLen-C2K-DMA having the structure shown below was synthesized as shown in the following schematic diagram.



## 148

Synthesis of di- $\gamma$ -linolenyl ketone:

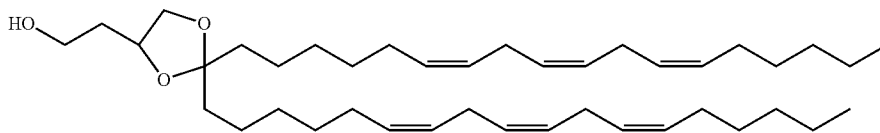


To a 1000 mL RBF containing a solution of di- $\gamma$ -linolenyl methanol (6.0 g, 11.4 mmol) in anh. DCM (200 mL) was added pyridinium chlorochromate (7.39 g, 34.2 mmol), anh. sodium carbonate (1.0 g, 5.66 mmol) and a stirbar. The resulting suspension was stirred under nitrogen at RT for 3 h, after which time TLC indicated all SM to have been consumed.

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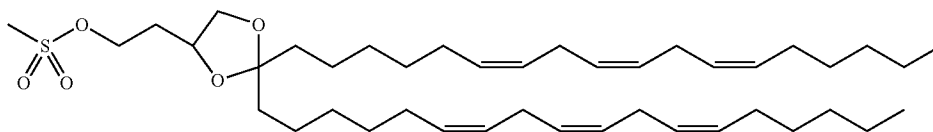
Ether (300 mL) was then added to the mixture and the resulting brown suspension filtered through a pad of silica (300 mL), washing the pad with ether (3×100 mL). The ether phases were combined, concentrated and purified to yield 5.5 g (10.5 mmol, 92%) of ketone.

Synthesis of  $\gamma$ -linolenyl ketal:



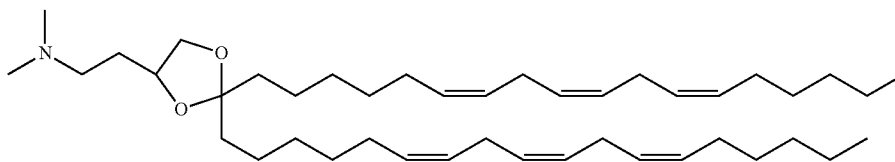
A 100 mL RBF was charged with di- $\gamma$ -linolenyl ketone (2.14 g, 4.1 mmol), 1,2,4-butanetriol (1.7 g, 16.0 mmol), PPTS (100 mg, 0.4 mmol) and a stir bar. The flask was flushed with nitrogen and anhydrous toluene (30 mL) added. The reaction vessel was fitted with a Dean Stark tube and condenser and brought to reflux and the reaction was left overnight. After cooling to room temperature, the reaction mixture was washed with 5% aq.  $\text{Na}_2\text{CO}_3$  (2×50 mL), water (50 mL), dried ( $\text{MgSO}_4$ ) and purified by chromatography to yield 1.34 g (2.2 mmol, 53%) of the ketal.

Mesylyate Formation:



A 250 mL RBF was charged with the  $\gamma$ -linolenyl ketal (1.34 g, 2.19 mmol), TEA (1 mL, 7.1 mmol) and a stir bar. The flask was flushed with nitrogen, anhyd. DCM (10 mL) added, and the solution cooled to  $-15^\circ\text{C}$ . In a separate 50 mL flask, a solution of MsCl (342  $\mu\text{L}$ , 4.4 mmol, 2 eqv.) in anhydrous DCM (15 mL) was prepared, then transferred to the reaction vessel by syringe over 20 minutes. The reaction was stirred for 90 minutes at  $-15^\circ\text{C}$ ., at which point starting material had been consumed. The reaction mixture was diluted with a further 50 mL of DCM, washed with  $\text{NaHCO}_3$  (2×50 mL), dried ( $\text{MgSO}_4$ ) and purified by chromatography. Final yield 1.31 g, 1.90 mmol, 87%.

Synthesis of  $\gamma$ -DLen-C2K-DMA:



A 250 mL RBF was charged with the mesylyate (1.31 g, 1.9 mmol), isopropanol (10 mL) and a stir bar. The flask was flushed with nitrogen, sealed, and a 2.0 M solution of dimethylamine in methanol (60 mL) added via cannula. The reaction was stirred at room temperature for 3 days. The solution was concentrated and purified by chromatography. Final yield 1.1 g, 1.72 mmol, 91%.

150

Example 10

Lipid Encapsulation of siRNA

5 All siRNA molecules used in these studies were chemically synthesized and annealed using standard procedures.

15 In some embodiments, siRNA molecules were encapsulated into serum-stable nucleic acid-lipid particles (SNALP) composed of the following lipids: (1) the lipid conjugate PEG2000-C-DMA (3-N-[(methoxypoly(ethylene glycol) 2000)carbamoyl]-1,2-dimyristyloxypropylamine); (2) one or more cationic lipids or salts thereof (e.g., cationic lipids of Formula I-XIV and/or other cationic lipids described herein); (3) the phospholipid DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) (Avanti Polar Lipids; Alabaster, Ala.); and (4) synthetic cholesterol (Sigma-Aldrich Corp.; St. Louis, Mo.) in the molar ratio 1.4:57.1:7.1:34.3, respectively. In other words, siRNA molecules were encapsulated into

SNALP of the following "1:57" formulation: 1.4% PEG2000-C-DMA; 57.1% cationic lipid; 7.1% DPPC; and 34.3% cholesterol. It should be understood that the 1:57 formulation is a target formulation, 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 57.1 mol % $\pm$ 5 mol %, and the amount of lipid conjugate will be 1.4 mol % $\pm$ 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).

For vehicle controls, empty particles with identical lipid composition may be formed in the absence of siRNA.

Example 11

Characterization of Novel ApoB SNALP Formulations Containing Various Cationic Lipids

65 This example demonstrates the efficacy of novel SNALP formulations containing cationic lipids described herein with

an siRNA targeting APOB in a mouse liver model. The APOB siRNA sequence used in this study is provided in Table 1.

TABLE 1

siRNA	APOB siRNA Sequence	% 2'OMe-Modified	% Modified in DS Region
ApoB-10164	5'-AGUG <u>CAUCACACUGAAUACC</u> -3' (SEQ ID NO: 1) 3'- <u>GUUCACAGUAGUGACUUAU</u> -5' (SEQ ID NO: 2)	7/42 = 16.7%	7/38 = 18.4%

Column 1: The number after "ApoB" refers to the nucleotide position of the 5' base of the sense strand relative to the human APOB mRNA sequence NM\_000384.

Column 2: 2'OMe nucleotides are indicated in bold and underlined. The 3'-overhangs on one or both strands of the siRNA molecule may alternatively comprise 1-4 deoxythymidine (dT) nucleotides, 1-4 modified and/or unmodified uridine (U) ribonucleotides, or 1-2 additional ribonucleotides having complementarity to the target sequence or the complementary strand thereof.

Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA molecule are provided.

Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA molecule are provided.

1:57 SNALP formulations containing encapsulated APOB siRNA were prepared as described in Section V above with the following cationic lipids: (1) DLinDMA; (2) DLin-K-C2-DMA ("C2K"); (3) DLin-K-C3-DMA ("C3K"); (4) DLin-K-C4-DMA ("C4K"); (5) DLin-K6-DMA; (6) DLin-C2-DMA; (7) DLenDMA; (8)  $\gamma$ -DLenDMA (" $\gamma$ -DLenDMA"); (9) DLin-K-DMA; (10) DLinMorph; (11) Linoleyl/Oleyl DMA ("Lin/Ol"); (12) Linoleyl/Linolenyl DMA ("Lin/Len"); (13) Linoleyl/Phytanyl DMA ("Lin/Pan"); (14) Linoleyl/Stearyl DMA ("Lin/Str"); and (15) Linoleyl/C6:1 DMA ("Lin/C6:1").

Each SNALP formulation was administered by intravenous (IV) injection at 0.1 mg/kg into female BALB/c mice (n=3 per group). Plasma total cholesterol and/or liver ApoB mRNA levels were evaluated at 48 hours after SNALP administration. For dose response studies, SNALP formulations were administered by IV injection at 0.01 mg/kg, 0.03 mg/kg, or 0.1 mg/kg into female Balb/c mice (n=3 per group). Liver ApoB mRNA levels were evaluated at 48 hours after SNALP administration.

FIGS. 1-3 show a comparison of the plasma total cholesterol knockdown efficacy and/or the liver ApoB mRNA knockdown activity of each of these SNALP formulations (Error bars=SD). FIG. 4 shows a dose response evaluation of three different doses of SNALP formulations containing either DLinDMA, DLin-K-C2-DMA ("C2K"), DLenDMA, or  $\gamma$ -DLenDMA on liver ApoB mRNA knockdown activity (Error bars=SD).

These figures illustrate that SNALP formulations containing either DLin-K-C2-DMA ("C2K") or  $\gamma$ -DLenDMA were unexpectedly more potent in silencing ApoB expression in vivo compared to SNALP formulations containing either DLinDMA or DLenDMA. These figures also illustrate that a SNALP formulation containing an asymmetric cationic lipid such as Linoleyl/Linolenyl DMA ("Lin/Len") displayed greater ApoB silencing activity compared to a SNALP formulation containing DLinDMA.

#### Example 12

##### Characterization of Additional Novel ApoB SNALP Formulations Containing Various Cationic Lipids

This example demonstrates the efficacy of additional novel SNALP formulations containing cationic lipids described herein with an siRNA targeting APOB in a mouse liver model. The APOB siRNA sequence used in these studies is provided in Table 1.

1:57 SNALP formulations containing encapsulated APOB siRNA at a 6:1 lipid:drug (L:D) ratio were prepared with the

following cationic lipids: (1) DLinDMA; (2) Linoleyl/Linolenyl DMA ("Lin/LenDMA"); (3) DPanDMA; (4)

TLinDMA; (5) Linoleyl/C6:0 DMA ("Lin/6:0"); (6) C2-DPanDMA; (7) DLin-C2K-Pip (3OH); and (8) DHep-C2K-DMA.

Each SNALP formulation was administered by intravenous (IV) injection at 0.1 mg/kg into female Balb/c mice (n=3 per group). Livers were collected at 48 hours after SNALP administration and liver ApoB mRNA levels were evaluated by performing an ApoB/GAPDH QG assay. Table 2 provides a characterization of the SNALP formulations used in this in vivo study.

TABLE 2

SNALP	Size (nm)	Poly	Encapsulation %
1:57 DLinDMA	74.96	0.023	79
	77.88	0.054	
1:57 Lin/LenDMA	81.12	0.023	63
	98.21	0.010	
1:57 DPanDMA	64.84	0.063	78
	65.44	0.033	
1:57 TLinDMA	71.98	0.037	51
	73.12	0.069	
1:57 Lin/6:0	101.7	0.095	78
	97.64	0.119	
1:57 C2-DPanDMA	95.09	0.058	90
	98.26	0.052	
1:57 DLin-C2K-Pip (3OH)	68.92	0.093	73
	73.27	0.067	
1:57 DHep-C2K-DMA	75.29	0.034	89
	79.89	0.037	
1:57 DLinDMA (TFU)	82.23	0.039	89

Columns 2 & 3: The bottom value in each entry corresponds to the particle size and polydispersity observed 10 days after the formulation was prepared.

FIG. 5 illustrates, inter alia, that: (1) DHep-C2K-DMA, which contains double bonds in the trans configuration thought to reduce potency, was unexpectedly comparable to DLinDMA with respect to silencing activity; (2) C2-DPanDMA, which contains saturated fatty alkyl chains thought to decrease potency, was unexpectedly more potent compared to DLinDMA and substantially more potent compared to DPanDMA with respect to silencing activity; (3) TLinDMA displayed silencing activity that was comparable to DLinDMA; and (4) Lin/LenDMA had more potent silencing activity than DLinDMA. A similar study with 1:57 SNALP containing C2-TLinDMA showed that C2-TLinDMA (49% knockdown) was more potent than DLinDMA (25% knockdown) with respect to ApoB silencing activity.

In another study, 1:57 SNALP formulations containing encapsulated APOB siRNA at a 6:1 L:D ratio were prepared with the following cationic lipids: (1) DLinDMA; (2) C2-DPanDMA; (3) DPan-C2K-DMA; (4) DPan-C3K-DMA; and (5) DPan-C1K6-DMA.

Each SNALP formulation was administered by intravenous (IV) injection at 0.1 mg/kg into female Balb/c mice (n=3 per group). Livers were collected at 48 hours after SNALP administration and liver ApoB mRNA levels were evaluated by performing an ApoB/GAPDH QG assay. Table 3 provides a characterization of the SNALP formulations used in this *in vivo* study.

TABLE 3

	Size (nm)	Poly	Encapsulation %
1:57 DLinDMA	76.63	0.033	81
1:57 C2-DPanDMA	85.80	0.003	88
1:57 DPan-C2K-DMA	79.06	0.020	87
1:57 DPan-C3K-DMA	93.46	0.002	90
1:57 DPan-C1K6-DMA	72.78	0.031	77

FIG. 6 illustrates that C2-DPanDMA, DPan-C2K-DMA, and DPan-C3K-DMA, which contains saturated fatty alkyl chains thought to decrease potency, were unexpectedly more potent compared to DLinDMA with respect to silencing activity. C2-DPanDMA had the greatest activity of all the phytanyl-containing cationic lipids tested.

## Example 13

## Characterization of Additional Novel ApoB SNALP Formulations Containing Various Cationic Lipids

This example demonstrates the efficacy of additional novel SNALP formulations containing cationic lipids described herein with an siRNA targeting APOB in a mouse liver model. The APOB siRNA sequence used in these studies is provided in Table 1.

1:57 SNALP formulations containing encapsulated APOB siRNA were prepared as described in Section V above with the following cationic lipids: (1) DLin-C2K-DMA (“C2K”); (2)  $\gamma$ -DLen-C2K-DMA (“g-DLen-C2K-DMA”); and (3) DLen-C2K-DMA.

For dose response studies, SNALP formulations were administered by IV injection at 0.01 mg/kg, 0.033 mg/kg, or 0.1 mg/kg into female Balb/c mice (n=3 per group). Liver ApoB mRNA levels were evaluated at 48 hours after SNALP administration by a branched DNA assay (QuantiGene assay) to assess ApoB mRNA relative to the housekeeping gene GAPDH.

FIG. 7 shows a comparison of the liver ApoB mRNA knockdown activity of each of these SNALP formulations at three different doses (Error bars=SD), as well as the KD<sub>50</sub> values obtained for each of these formulations. In particular, FIG. 7 shows that a SNALP formulation containing g-DLen-C2K-DMA displayed similar ApoB silencing activity at all three doses and an identical KD<sub>50</sub> value compared to a SNALP formulation containing C2K. Furthermore, FIG. 7 shows that a SNALP formulation containing DLen-C2K-DMA displayed considerable potency in silencing ApoB mRNA expression.

## Example 14

## Increased Potency of Novel C2K SNALP Formulation at Silencing ApoB Expression

This example further demonstrates the surprising increase in potency observed for the DLin-K-C2-DMA (“C2K”) SNALP formulation at silencing ApoB expression in both mouse and rat liver models. The APOB siRNA sequence used in these studies is provided in Table 1 above.

1:57 SNALP formulations containing encapsulated APOB siRNA were prepared as described in Section V above with

either C2K or DLinDMA as the cationic lipid component. APOB C2K SNALP formulations were administered by intravenous (IV) injection at 0.01 mg/kg, 0.05 mg/kg, or 0.25 mg/kg into female Balb/c mice (n=4 per group), while APOB DLinDMA SNALP formulations were administered by IV injection at 0.05 mg/kg, 0.10 mg/kg, or 0.25 mg/kg into female Balb/c mice (n=4 per group). For the rat study, C2K or DLinDMA SNALP formulations were administered by IV injection at 0.25 mg/kg into Sprague Dawley rats. Liver ApoB mRNA levels were evaluated at 48 hours after SNALP administration.

FIG. 8 shows a dose response evaluation of three different doses of SNALP formulations containing either DLinDMA or C2K on liver ApoB mRNA knockdown activity in mice (Error bars=SD). FIG. 9 shows the reproducibility of the dose response study in mice using two independent SNALP batches. FIG. 10 shows that the improved liver ApoB mRNA knockdown activity of C2K SNALP versus DLinDMA SNALP is preserved in rats.

Importantly, these figures illustrate that the C2K SNALP formulation was about 5 times more potent than a corresponding DLinDMA SNALP formulation based on the KD<sub>50</sub> for liver ApoB mRNA silencing in mice. These figures also illustrate that the degree of ApoB mRNA silencing at 0.25 mg/kg for both formulations is comparable between rat and mouse.

## Example 15

## Characterization of Inflammatory Response to APOB DLinDMA and C2K SNALP Formulations in Human Whole Blood

Inflammatory response to DLinDMA or C2K SNALPs containing APOB siRNA was evaluated by measuring cytokine induction *ex vivo* in whole blood samples taken from human subjects. For both the DLinDMA and C2K formulations, the SNALPs contained either no siRNA payload (“empty”) or APOB siRNA payload. The APOB siRNAs tested included “ApoB-8” (the APOB siRNA exemplified in Examples 12-15 and Table 1), “2/5” (described in Table 4), “3/5” (described in Table 4), and “6/5” (described in Table 4). Briefly, fresh blood was isolated, immediately diluted 1:1 with 0.9% saline solution, and plated 0.45 mL/well into 48 well tissue culture treated plates. SNALPs were diluted in formulation PBS and added to the plated blood samples at a concentration of either 300 nM or 1200 nM. After 24 hours, the plates were centrifuged at 1200 rpm for 20 minutes and the supernatant (plasma) collected. Cytokine induction was measured by ELISA and/or Cytometric Bead Array.

FIGS. 11 and 12 show the results of cytokine induction assays for three donors at increasing SNALP concentrations. FIG. 11 shows that inflammatory response to APOB SNALP formulations, as measured by the concentration of the cytokine TNF, was significantly higher for SNALP DLinDMA formulations than for SNALP C2K formulations for two of the three donors. Additionally, all three donors exhibited significantly less inflammatory response to the APOB siRNAs 2/5, 3/5, and 6/5 as compared to the APOB siRNA ApoB-8. Similarly, FIG. 12 shows that the DLinDMA formulation induces a stronger IL-8 cytokine response than the C2K formulation, as measured by ELISA. Moreover, the APOB siRNAs 3/5 and 6/5 in C2K SNALPs generally induced less of an immunostimulatory response than did the ApoB-8 siRNA in C2K SNALPs. These figures demonstrate that the C2K SNALP formulation is less immunostimulatory than the DLinDMA SNALP formulation. Additionally, these figures demonstrate that increasing the number of selective 2'OMe modifications to the siRNA sequence (e.g., 2'OMe modifications at G's and/or U's in the double-stranded and/or 3' overhang regions of the siRNA sequence) can decrease the immunostimulatory response to the siRNA.

TABLE 4

siRNA	APOB siRNA Sequence	% 2'OMe-Modified	% Modified in DS Region
2/5	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 3) 3'- <u>GUUCACAGUAGUGUGACU</u> UAU-5' (SEQ ID NO: 11)	12/42 = 28.6%	10/38 = 26.3%
3/5	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 4) 3'- <u>GUUCACAGUAGUGUGACU</u> UAU-5' (SEQ ID NO: 11)	13/42 = 31.0%	11/38 = 28.9%
6/5	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 7) 3'- <u>GUUCACAGUAGUGUGACU</u> UAU-5' (SEQ ID NO: 11)	14/42 = 33.3%	12/38 = 31.6%

Column 1: "2/5," "3/5," and "6/5" refer to APOB sense strand annealed to antisense strand (e.g., sense strand 2 annealed to antisense strand 5 = 2/5).

Column 2: 2'OMe nucleotides are indicated in bold and underlined. The 3'-overhangs on one or both strands of the siRNA molecule may alternatively comprise 1-4 deoxythymidine (dT) nucleotides, 1-4 modified and/or unmodified uridine (U) ribonucleotides, or 1-2 additional ribonucleotides having complementarity to the target sequence or the complementary strand thereof.

Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA molecule are provided.

Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA molecule are provided.

### Example 16

#### In Vitro and In Vivo Activity Screen of Modified APOB siRNAs in C2K SNALPs

As shown in FIGS. 11 and 12, APOB siRNAs which have the same nucleotide sequence as ApoB-8 but which have an increased number of modified nucleotides are less immunostimulatory than ApoB-8. This example demonstrates that APOB siRNAs which have the same nucleotide sequence as ApoB-8 but which have an increased number of modified nucleotides are at least as effective as ApoB-8 in knocking down ApoB mRNA expression.

APOB siRNAs of the same nucleotide sequence as ApoB-8 (exemplified in Examples 12-15 and Table 1, and also called "ApoB-10164") were modified to incorporate an increasing number and alternate patterns of 2'OMe nucleotides. Six different sense strands (S-1 to S-6) and six different antisense

strands (AS-1 to AS-6) were designed. Sense strand 1 (S-1) is the same pattern of modification as the ApoB-8 sense strand (SEQ ID NO:1), and antisense strand 1 (AS-1) is the same pattern of modification as the ApoB-8 antisense strand (SEQ ID NO:2), and were generated as synthesis controls. APOB double-stranded siRNAs were generated by mix and match annealing of sense strands 2-6 (S-2 to S-6) and antisense strands 2-6 (AS-2 to AS-6). Compared to siApoB-8 (also referred to in this example as "1/1"), the number of modifications for double-stranded APOB siRNAs increased from 7 to about 9-12 in the double-stranded region. Additionally, some of the patterns of modification include 2'OMe-modified nucleotides in the 3' overhang of one or both strands of the siRNA, such that the number of modifications are further increased to about 10-14 in the entire siRNA molecule. Table 5 shows modified APOB sense strands 1-6 (S-1 to S-6), modified ApoB antisense strands 1-6 (AS-1 to AS-6), and the double-stranded APOB siRNAs that resulted from the mix and match annealing of S-2 to S-6 with AS-2 to AS-6.

TABLE 5

siRNA	APOB siRNA Sequence	% 2'-OMe-Modified	% Modified in DS Region
S-1	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 1)	3/21 = 14.3%	3/19 = 15.8%
S-2	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 3)	5/21 = 23.8%	5/19 = 26.3%
S-3	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 4)	6/21 = 28.6%	6/19 = 31.6%
S-4	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 5)	5/21 = 23.8%	5/19 = 26.3%
S-5	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 6)	7/21 = 33.3%	7/19 = 36.8%
S-6	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 7)	7/21 = 33.3%	7/19 = 36.8%
AS-1	5'-UAU <u>UCAGUGUGAUGACACU</u> UG-3' (SEQ ID NO: 2)	4/21 = 19.0%	4/19 = 21.1%
AS-2	5'-UAU <u>UCAGUGUGAUGACACU</u> UG-3' (SEQ ID NO: 8)	5/21 = 23.8%	5/19 = 26.3%
AS-3	5'-UAU <u>UCAGUGUGAUGACACU</u> UG-3' (SEQ ID NO: 9)	5/21 = 23.8%	5/19 = 26.3%
AS-4	5'-UAU <u>UCAGUGUGAUGACACU</u> UG-3' (SEQ ID NO: 10)	6/21 = 28.6%	4/19 = 21.1%
AS-5	5'-UAU <u>UCAGUGUGAUGACACU</u> UG-3' (SEQ ID NO: 11)	7/21 = 33.3%	5/19 = 26.3%
AS-6	5'-UAU <u>UCAGUGUGAUGACACU</u> UG-3' (SEQ ID NO: 12)	7/21 = 33.3%	5/19 = 26.3%
1/1	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 1) 3'- <u>GUUCACAGUAGUGUGACU</u> UAU-5' (SEQ ID NO: 2)	7/42 = 16.7%	7/38 = 18.4%
2/2	5'-AGUG <u>UCAUCACACUGAAU</u> ACC-3' (SEQ ID NO: 3) 3'- <u>GUUCACAGUAGUGUGACU</u> UAU-5' (SEQ ID NO: 8)	10/42 = 23.8%	10/38 = 26.3%

TABLE 5-continued

siRNA	APOB siRNA Sequence	% 2'-OMe-Modified	% Modified in DS Region
2/3	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 3) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 9)	10/42 = 23.8%	10/38 = 26.3%
3/2	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 4) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 8)	11/42 = 26.2%	11/38 = 28.9%
3/3	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 4) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 9)	11/42 = 26.2%	11/38 = 28.9%
4/2	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 5) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 8)	10/42 = 23.8%	10/38 = 26.3%
4/3	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 5) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 9)	10/42 = 23.8%	10/38 = 26.3%
5/2	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 6) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 8)	12/42 = 28.6%	12/38 = 31.6%
5/3	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 6) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 9)	12/42 = 28.6%	12/38 = 31.6%
6/2	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 7) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 8)	12/42 = 28.6%	12/38 = 31.6%
6/3	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 7) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 9)	12/42 = 28.6%	12/38 = 31.6%
2/4	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 3) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 10)	11/42 = 26.2%	9.38 = 23.7%
2/5	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 3) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 11)	12/42 = 28.6%	10/38 = 26.3%
2/6	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 3) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 12)	12/42 = 28.6%	10/38 = 26.3%
3/4	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 4) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 10)	12/42 = 28.6%	10/38 = 26.3%
3/5	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 4) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 11)	13/42 = 31.0%	11/38 = 28.9%
3/6	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 4) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 12)	13/42 = 31.0%	11/38 = 28.9%
4/4	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 5) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 10)	11/42 = 26.2%	9.38 = 23.7%
4/5	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 5) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 11)	12/42 = 28.6%	10/38 = 26.3%
4/6	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 5) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 12)	12/42 = 28.6%	10/38 = 26.3%
5/4	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 6) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 10)	13/42 = 31.0%	11/38 = 28.9%
5/5	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 6) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 11)	14/42 = 33.3%	12/38 = 31.6%
5/6	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 6) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 12)	14/42 = 33.3%	12/38 = 31.6%
6/4	5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 7) 3' -GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO: 10)	13/42 = 31.0%	11/38 = 28.9%

TABLE 5-continued

siRNA	APOB siRNA Sequence	% 2'-OMe-Modified	% Modified in DS Region
6/5	5'- <b>AGUGUCAUCACACUGAAU</b> ACC-3' (SEQ ID NO: 7) 3'- <b>GUUCACAGUAGUGACUUAU</b> -5' (SEQ ID NO: 11)	14/42 = 33.3%	12/38 = 31.6%
6/6	5'- <b>AGUGUCAUCACACUGAAU</b> ACC-3' (SEQ ID NO: 7) 3'- <b>GUUCACAGUAGUGACUUAU</b> -5' (SEQ ID NO: 12)	14/42 = 33.3%	12/38 = 31.6%

Column 1: Sense strand, antisense strand, or sense strand/antisense strand. APOB sense strands 1-6 and antisense strands 1-6 were designed with alternate patterns of modification. APOB sense strands 2-6 were mix and match annealed to APOB antisense strands 2-6 (e.g., sense strand 2 annealed to antisense strand 5 = 2/5). 1/1, which is the same as ApoB-10164 in Example 5, was a synthesis control.

Column 2: 2'OMe nucleotides are indicated in bold and underlined. The 3'-overhangs on one or both strands of the siRNA molecule may alternatively comprise 1-4 deoxythymidine (dT) nucleotides, 1-4 modified and/or unmodified uridine (U) ribonucleotides, or 1-2 additional ribonucleotides having complementarity to the target sequence or the complementary strand thereof.

Column 3: The number and percentage of 2'OMe-modified nucleotides in the siRNA molecule are provided.

Column 4: The number and percentage of modified nucleotides in the double-stranded (DS) region of the siRNA molecule are provided.

1:57 SNALP formulations containing encapsulated APOB duplexes as described in Table 5 were prepared at 3 mg scale with the cationic lipid DLin-C2K-DMA. For the in vitro assays, transfections of human primary hepatocytes were performed on Primaria plates according to standard protocols using a SNALP dose range of 0.125-0.00781 µg/mL. Cells were plated at 50,000 cells/well and incubated overnight at 37° C. At transfection, SNALP was diluted to the desired dose and pre-incubated with serum at 37° C. for 1 hour, then the cell media was replaced with 80 µL fresh media and 20 µL pre-incubated SNALP. The cells were incubated with SNALP for 24 hours, then the media was removed and the cells lysed for QuantiGene Analysis. Quantitation of mRNA levels was accomplished using individual standard curves for 1/1-C2K and 2/5-C2K. The remaining SNALPs were quantitated against the 1/1-C2K curve, which resulted in differences of up to 12% in the actual dose that was administered; therefore, FIGS. 13 and 14 also depict the actual dose administered for a SNALP where the dose varied from the 0.125 µg/mL intended dose.

FIG. 13 shows the knockdown efficiency in human primary hepatocytes from C2K SNALPs comprising the modified APOB siRNA sequences of Table 5 (n=2). Surprisingly, the APOB mRNA knockdown activity of exemplary 2'OMe-modified APOB SNALP formulations containing C2K was similar to or greater than the silencing activity observed with the 1/1-C2K SNALP formulation (i.e., the ApoB-8 SNALP). The results show that increasing the number of modifications, from 7 in ApoB-8 to up to 14 in some of the modified APOB siRNAs, does not decrease activity, and in some cases increases silencing activity.

FIG. 14 shows a comparison of in vitro silencing activity of selected modified APOB siRNAs for different SNALP formulations (DLinDMA vs. DLin-C2K-DMA as the cationic lipid). Silencing activity is measured as the percentage of ApoB mRNA expression relative to transfection with PBS control. Overall, there was increased silencing activity with C2K SNALPs than with DLinDMA SNALPs. In particular, 2/5-C2K and 3/4-C2K exhibited greater silencing activity than 1/1-C2K (i.e., the ApoB-8 SNALP).

Next, 1:57 SNALP formulations comprising the APOB siRNAs 1/1, 2/2, 2/5, 3/2, 3/5, 4/2, 4/5, or 6/5 and DLin-C2K-DMA were utilized to assess silencing activity in vivo in mice. Each SNALP formulation was administered by intravenous (IV) bolus injection at 0.01, 0.02, or 0.05 mg/kg into female BALB/c mice (n=4 per group). Liver ApoB mRNA levels were evaluated at 48 hours after SNALP administration by QuantiGene Analysis. Quantitation of mRNA levels was

accomplished using individual standard curves for 1/1-C2K and 2/5-C2K. The remaining SNALPs were quantitated against the 1/1-C2K curve, which resulted in differences of up to 18% in the actual dose that was administered; therefore, FIGS. 15 and 16 also depict the percentage difference in the actual dose administered for a SNALP where the dose varied from the intended dose.

FIG. 15 shows the silencing activity of 0.02 mg/kg of 1:57 DLin-C2K-DMA formulated modified APOB SNALPs. All of the modified APOB SNALPs are generally as effective as 1/1-C2K (i.e., the ApoB-8 SNALP) in silencing ApoB expression. In particular, 3/5-C2K showed the greatest silencing activity of all the modified SNALPs tested.

FIG. 16 shows a comparison of in vivo silencing activity for selected modified APOB siRNAs in different SNALP formulations (DLinDMA vs. DLin-C2K-DMA as the cationic lipid). Surprisingly, although the modified APOB siRNA sequences all generally were at least as effective as 1/1 (i.e., ApoB-8) in both the DLinDMA and DLin-C2K-DMA formulations, different modified APOB siRNA sequences were most effective for the different cationic lipid compositions. In particular, siRNA sequence 2/5 showed the greatest silencing activity in DLinDMA, but siRNA sequence 3/5 showed the greatest silencing activity in DLin-C2K-DMA.

FIG. 17 shows the silencing activity of APOB siRNA 1/1 ("siApoB-8") or APOB siRNA 3/5 ("siApoB-10") formulated in 1:57 SNALP containing DLinDMA or DLin-C2K-DMA ("C2K"). For these dose response studies, SNALP formulations were administered by IV injection at 0.05, 0.10, or 0.25 mg/kg for DLinDMA and at 0.01, 0.025, or 0.05 mg/kg for C2K into female Balb/c mice (n=4 per group). Liver ApoB mRNA levels were evaluated at 48 hours after SNALP administration (Error bars=SD). In particular, FIG. 17 shows that DLinDMA SNALP formulations containing either siApoB-8 or siApoB-10 displayed similar silencing activities based on the KD<sub>50</sub> for liver ApoB mRNA silencing in mice. Similarly, FIG. 17 shows that C2K SNALP formulations containing either siApoB-8 or siApoB-10 displayed similar silencing activities based on the KD<sub>50</sub> for liver ApoB mRNA silencing in mice. Notably, C2K SNALP formulations containing either siApoB-8 or siApoB-10 were significantly more potent than the corresponding DLinDMA SNALP formulations based on a comparison of their KD<sub>50</sub> values.

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 refer-

ences, 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:

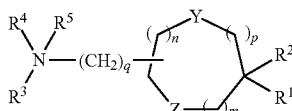
1. A nucleic acid-lipid particle comprising:

(a) an siRNA that silences Apolipoprotein B (APOB) expression, wherein the siRNA consists of the following sense and antisense strand sequences:

5'-AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO:4)

3'-GUUCACAGUAGUGUGACUUAU-5' (SEQ ID NO:11), wherein the bolded nucleotides are 2'OME nucleotides;

(b) a cationic lipid having the following structure:



or salts thereof, wherein:

R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkenyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkynyl, or optionally substituted C<sub>12</sub>-C<sub>24</sub> acyl;

R<sup>3</sup> and R<sup>4</sup> are either the same or different and are independently optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, optionally substituted C<sub>2</sub>-C<sub>6</sub> alkenyl, or optionally substituted C<sub>2</sub>-C<sub>6</sub> alkynyl or R<sup>3</sup> and R<sup>4</sup> 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<sup>5</sup> is either absent or hydrogen or C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine;

m, n and p are either the same or different and are independently either 0, 1 or 2, with the proviso that m, n, and p are not simultaneously 0;

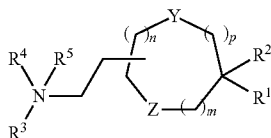
q is 0, 1, 2, 3, or 4;

Y and Z are either the same or different and are independently O, S, or NH; and

(c) a non-cationic lipid.

2. The nucleic acid-lipid particle in accordance with claim 1, wherein at least one of R<sup>1</sup> and R<sup>2</sup> has at least two sites of unsaturation.

3. The nucleic acid-lipid particle in accordance with claim 1, wherein the cationic lipid has the following structure:



or salts thereof, wherein:

R<sup>1</sup> and R<sup>2</sup> are either the same or different and are independently optionally substituted C<sub>12</sub>-C<sub>24</sub> alkyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkenyl, optionally substituted C<sub>12</sub>-C<sub>24</sub> alkynyl, or optionally substituted C<sub>12</sub>-C<sub>24</sub> acyl;

R<sup>3</sup> and R<sup>4</sup> are either the same or different and are independently optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, optionally substituted C<sub>2</sub>-C<sub>6</sub> alkenyl, or optionally substituted C<sub>2</sub>-C<sub>6</sub> alkynyl or R<sup>3</sup> and R<sup>4</sup> 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;

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R<sup>5</sup> is either absent or is hydrogen or C<sub>1</sub>-C<sub>6</sub> alkyl to provide a quaternary amine;

m, n, and p are either the same or different and are independently either 0, 1 or 2, with the proviso that m, n, and p are not simultaneously 0;

Y and Z are either the same or different and are independently O, S, or NH.

4. The nucleic acid-lipid particle in accordance with claim 3, wherein the cationic lipid is 2,2-dilinoleyl-4-(2-dimethylaminoethyl)[1,3]-dioxolane (DLin-K-C2-DMA).

5. The nucleic acid-lipid particle in accordance with claim 1, further comprising a conjugated lipid that inhibits aggregation of particles.

6. The nucleic acid-lipid particle in accordance with claim 5, wherein the conjugated lipid that inhibits aggregation of particles is a polyethyleneglycol (PEG)-lipid conjugate.

7. The nucleic acid-lipid particle in accordance with claim 6, 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.

8. A method for introducing an interfering RNA that silences APOB expression into a cell, the method comprising: contacting the cell with a nucleic acid-lipid particle in accordance with claim 1.

9. A method for the in vivo delivery of an interfering RNA that silences APOB expression, the method comprising: administering to a mammal a nucleic acid-lipid particle in accordance with claim 1.

10. The nucleic acid-lipid particle in accordance with claim 1, wherein R<sup>3</sup> and R<sup>4</sup> are both methyl groups.

11. The nucleic acid-lipid particle in accordance with claim 1, wherein the cationic lipid is 2,2-dilinoleyl-4-dimethylaminomethyl-1-[1,3]-dioxolane (DLin-K-DMA).

12. The nucleic acid-lipid particle in accordance with claim 1, wherein the non-cationic lipid is a mixture of a phospholipid and cholesterol or a cholesterol derivative.

13. The nucleic acid-lipid particle in accordance with claim 6, wherein the PEG-lipid conjugate is member selected from the group consisting of: a PEG-diacylglycerol (PEG-DAG) conjugate, a PEG dialkylxypropyl (PEG-DAA) conjugate, a PEG-phospholipid conjugate, a PEG-ceramide (PEG-Cer) conjugate, and a mixture thereof.

14. The nucleic acid-lipid particle in accordance with claim 1, wherein the cationic lipid comprises from about 50 mol % to about 80 mol % of the total lipid present in the particle.

15. The nucleic acid-lipid particle in accordance with claim 1, wherein the non-cationic lipid comprises a mixture of a phospholipid and cholesterol or a derivative thereof comprising from about 25 mol % to about 50 mol % of the total lipid present in the particle.

16. The nucleic acid-lipid particle in accordance with claim 5, wherein the conjugated lipid that inhibits aggregation of particles comprises from about 0.5 mol % to about 2 mol % of the total lipid present in the particle.

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

18. The method in accordance with claim 8, wherein the cell is in a human.

19. The method in accordance with claim 18, wherein the human has a disease or disorder associated with APOB expression or overexpression and wherein APOB expression is silenced by the interfering RNA.

20. The method in accordance with claim 18, wherein the human has a disease or disorder selected from the group

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consisting of atherosclerosis, angina pectoris, high blood pressure, diabetes, and hypothyroidism.

21. The method in accordance with claim 18, wherein the human has a disease or disorder which involves hypercholesterolemia and wherein serum cholesterol levels are lowered when APOB expression is silenced by the interfering RNA.

22. The method in accordance with claim 9, wherein the mammal is a human.

23. The method in accordance with claim 22, wherein the human has a disease or disorder associated with APOB expression or overexpression and wherein APOB expression is silenced by the interfering RNA.

24. The method in accordance with claim 22, wherein the human has a disease or disorder selected from the group consisting of atherosclerosis, angina pectoris, high blood pressure, diabetes, and hypothyroidism.

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25. The method in accordance with claim 22, wherein the human has a disease or disorder which involves hypercholesterolemia and wherein serum cholesterol levels are lowered when APOB expression is silenced by the interfering RNA.

26. An siRNA that silences Apolipoprotein B (APOB) expression consisting of the following sense and antisense strand sequences:

10 5' -AGUGUCAUCACACUGAAUACC-3' (SEQ ID NO: 4)

3' -GUUCACAGUAGUGACUUAAU-5', (SEQ ID NO: 11)

wherein the bolded nucleotides are 2'OMe nucleotides.

\* \* \* \* \*