(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 November 2011 (17.11.2011)

PCT

(10) International Publication Number
WO 2011/143230 A1

(51) International Patent Classification:
A61K 9/127 (2006.01) *A61K 48/00* (2006.01)

(21) International Application Number:
PCT/US2011/035939

(22) International Filing Date:
10 May 2011 (10.05.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/333,122 10 May 2010 (10.05.2010) US

(71) Applicant *(for all designated States except US)*: **ALNYLAM PHARMACEUTICALS** [US/US]; 300 Third Street, Cambridge, MA 02142 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **MANOHARAN, Muthiah** [US/US]; 300 Third Street, Cambridge, MA 02142 (US). **RAJEEV, Kallanthottathil G.** [IN/US]; 300 Third Street, Cambridge, MA 02142 (US).

(74) Agent: **LESSLER, Jay, P.**; Blank Rome LLP, The Chrysler Building, 405 Lexington Avenue, New York, New York 10174 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2011/143230 A1

(54) Title: METHODS AND COMPOSITIONS FOR DELIVERY OF ACTIVE AGENTS

(57) Abstract: A lipid particle can include a cationic lipid. Synthesis of the cationic lipid can include a ylide-based reaction, such as a Wittig reaction or sulfur ylide reaction. In some cases, the synthesis can also include a Michael addition or a related addition reaction.

METHODS AND COMPOSITIONS FOR DELIVERY OF ACTIVE AGENTS

Claim of Priority

This application claims the benefit of U.S. Patent Application No. 61/333,122, filed May 10, 2010, which is incorporated by reference in its entirety.

Technical Field

The present invention relates to methods and compositions for delivery of nucleic acids.

Background

Therapeutic nucleic acids include, *e.g.*, small interfering RNA (siRNA), micro RNA (miRNA), antisense oligonucleotides, ribozymes, plasmids, immune stimulating nucleic acids, antisense, antagomir, antimir, microRNA mimic, supermir, U1 adaptor, and aptamer. These nucleic acids act via a variety of mechanisms. In the case of siRNA or miRNA, these nucleic acids can down-regulate intracellular levels of specific proteins through a process termed RNA interference (RNAi). Following introduction of siRNA or miRNA into the cell cytoplasm, these double-stranded RNA constructs can bind to a protein termed RISC. The sense strand of the siRNA or miRNA 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 siRNA or miRNA. Having bound the complementary mRNA the RISC complex cleaves the mRNA and releases the cleaved strands. RNAi can provide down-regulation of specific proteins by targeting specific destruction of the corresponding mRNA that encodes for protein synthesis.

The therapeutic applications of RNAi are extremely broad, since siRNA and miRNA constructs can be synthesized with any nucleotide sequence directed against a target protein. To date, siRNA constructs have shown the ability to specifically down-regulate target proteins in both *in vitro* and *in vivo* models. In addition, siRNA constructs are currently being evaluated in clinical studies.

However, two problems currently faced by siRNA or miRNA 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 the free siRNA or miRNA. These double-stranded constructs can be stabilized by incorporation of chemically modified nucleotide linkers within the molecule, for example, phosphothioate groups.

However, these chemical modifications provide only limited protection from nuclease digestion and may decrease the activity of the construct. Intracellular delivery of siRNA or miRNA can be facilitated by use of carrier systems such as polymers, cationic liposomes or by chemical modification of the construct, for example by the covalent attachment of cholesterol molecules. However, improved delivery systems are required to increase the potency of siRNA and miRNA molecules and reduce or eliminate the requirement for chemical modification.

Antisense oligonucleotides and ribozymes can also inhibit mRNA translation into protein. In the case of antisense constructs, these single stranded deoxynucleic acids have a complementary sequence to that of the target protein mRNA and can bind to the mRNA by Watson-Crick base pairing. This binding either prevents translation of the target mRNA and/or triggers RNase H degradation of the mRNA transcripts. Consequently, antisense oligonucleotides have tremendous potential for specificity of action (*i.e.*, down-regulation of a specific disease-related protein). To date, these compounds have shown promise in several *in vitro* and *in vivo* models, including models of inflammatory disease, cancer, and HIV (reviewed in Agrawal, *Trends in Biotech.* 14:376-387 (1996)). Antisense can also affect cellular activity by hybridizing specifically with chromosomal DNA. Advanced human clinical assessments of several antisense drugs are currently underway. Targets for these drugs include the bcl2 and apolipoprotein B genes and mRNA products.

Immune-stimulating nucleic acids include deoxyribonucleic acids and ribonucleic acids. In the case of deoxyribonucleic acids, certain sequences or motifs have been shown to illicit immune stimulation in mammals. These sequences or motifs include the CpG motif, pyrimidine-rich sequences and palindromic sequences. It is believed that the CpG motif in deoxyribonucleic acids is specifically recognized by an endosomal receptor, toll-like receptor 9 (TLR-9), which then triggers both the innate and acquired immune stimulation pathway. Certain immune stimulating ribonucleic acid sequences have also been reported. It is believed that these RNA sequences trigger immune activation by binding to toll-like receptors 6 and 7 (TLR-6 and TLR-7). In addition, double-stranded RNA is also reported to be immune stimulating and is believe to activate via binding to TLR-3.

One well known problem with the use of therapeutic nucleic acids relates to the stability of the phosphodiester internucleotide linkage and the susceptibility of this linker to nucleases. The presence of exonucleases and endonucleases in serum results in the rapid digestion of

nucleic acids possessing phosphodiester linkers and, hence, therapeutic nucleic acids can have very short half-lives in the presence of serum or within cells. (Zelphati, O., *et al.*, *Antisense. Res. Dev.* 3:323-338 (1993); and Thierry, A.R., *et al.*, pp147-161 in Gene Regulation: Biology of Antisense RNA and DNA (Eds. Erickson, RP and Izant, JG; Raven Press, NY (1992)). Therapeutic nucleic acid being currently being developed do not employ the basic phosphodiester chemistry found in natural nucleic acids, because of these and other known problems.

This problem has been partially overcome by chemical modifications that reduce serum or intracellular degradation. Modifications have been tested at the internucleotide phosphodiester bridge (*e.g.*, using phosphorothioate, methylphosphonate or phosphoramidate linkages), at the nucleotide base (*e.g.*, 5-propynyl-pyrimidines), or at the sugar (*e.g.*, 2'-modified sugars) (Uhlmann E., *et al.* Antisense: Chemical Modifications. Encyclopedia of Cancer, Vol. X., pp 64-81 Academic Press Inc. (1997)). Others have attempted to improve stability using 2'-5' sugar linkages (*see, e.g.*, U.S. Pat. No. 5,532,130). Other changes have been attempted. However, none of these solutions have proven entirely satisfactory, and *in vivo* free therapeutic nucleic acids still have only limited efficacy.

In addition, as noted above relating to siRNA and miRNA, problems remain with the limited ability of therapeutic nucleic acids to cross cellular membranes (see, Vlassov, *et al.*, *Biochim. Biophys. Acta* 1197:95-1082 (1994)) and in the problems associated with systemic toxicity, such as complement-mediated anaphylaxis, altered coagulatory properties, and cytopenia (Galbraith, *et al., Antisense Nucl. Acid Drug Des.* 4:201-206 (1994)).

To attempt to improve efficacy, investigators have also employed lipid-based carrier systems to deliver chemically modified or unmodified therapeutic nucleic acids. In Zelphati, O and Szoka, F.C., *J. Contr. Rel.* 41:99-119 (1996), the authors refer to the use of anionic (conventional) liposomes, pH sensitive liposomes, immunoliposomes, fusogenic liposomes, and cationic lipid/antisense aggregates. Similarly siRNA has been administered systemically in cationic liposomes, and these nucleic acid-lipid particles have been reported to provide improved down-regulation of target proteins in mammals including non-human primates (Zimmermann et al., *Nature* 441: 111-114 (2006)).

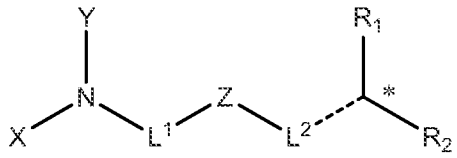
In spite of this progress, there remains a need in the art for improved lipid-therapeutic nucleic acid compositions that are suitable for general therapeutic use. Preferably, these

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 lipid-nucleic acid 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. Compositions, methods of making the compositions, and methods of using the compositions to introduce nucleic acids into cells, including for the treatment of diseases are provided.

Summary

In one aspect, the present invention relates to a lipid particle that includes a neutral lipid, a lipid capable of reducing aggregation, and a cationic lipid.

In one embodiment, the cationic lipid is a compound of formula IA:



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

X and Y are each, independently, alkyl or aryl;

L^1 and L^2 are each, independently, an alkylene linking unit, which may optionally contain one or more double bonds and further may optionally be interrupted by one or more heteroatoms and/or one or more cycloalkylene groups;

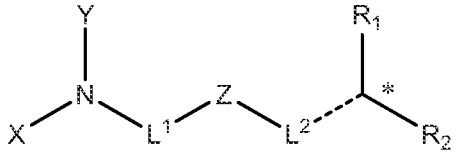
R_1 and R_2 are each, independently, a C_{10}-C_{30} aliphatic group, which may optionally contain one or more double bonds, and may optionally be interrupted by one or more heteroatoms and/or one or more cyclalkylene groups;

Z is -C(O)O-, -OC(O)-, -C(O)N(R^3)-, -N(R^3)C(O)-, -O(CO)N(R^3), -N(R^3)C(O)O-, -C(O)N(R^3)C(O)O-, -OC(O)N(R^3)C(O)- or -N(R^3)C(O)N(R^3)-;

each occurrence of R^3 is independently H, alkyl or aryl, and

---- represents a single bond or a double bond, wherein when ---- represents a single bond, C* is further substituted by hydrogen or alkyl.

In another embodiment, the cationic lipid is a compound of formula IB:



or a salt thereof (e.g., a pharmaceutically acceptable salt thereof), wherein

X and Y are each, independently, C_1-C_4 alkyl (e.g., methyl);

L^1 and L^2 are each, independently, an C_2-C_{10} linear or branched alkylene linking unit, which may optinally contain one or more double bonds and further may optionally be interrupted by one or more heteroatoms and/or one or more cycloalkylene groups (e.g., cyclopropyl groups);

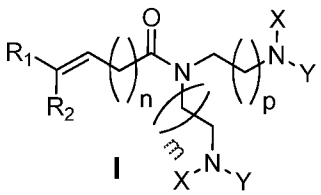
R_1 and R_2 are each, independently, a C_{10}-C_{30} aliphatic group, which may optionally contain one or more double bonds, and may optionally be interrupted by one or more heteroatoms and/or one or more cyclalkylene groups;

Z is -C(O)O-, -OC(O)-, -C(O)N(R^3)-, -N(R^3)C(O)-, -O(CO)N(R^3), -N(R^3)C(O)O-, -C(O)N(R^3)C(O)O-, -OC(O)N(R^3)C(O)- or -N(R^3)C(O)N(R^3)-;

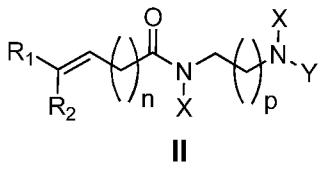
each occurrence of R^3 is independently H or C_1-C_4 alkyl (e.g., methyl); and

---- represents a single bond or a double bond, wherein when ---- represents a single bond, C* is further substituted by hydrogen or alkyl.

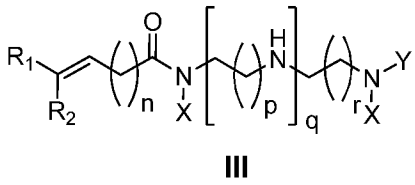
In another embodiment, the cationic lipid is a compound selected from compounds of formulas I-XL:



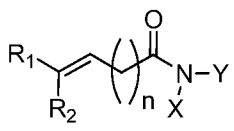
I



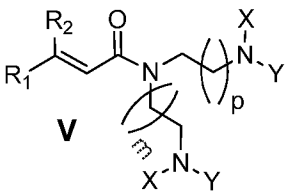
II



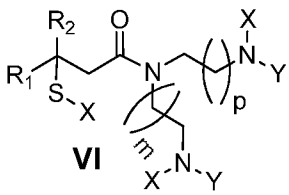
III



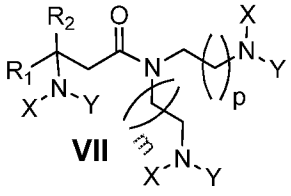
IV



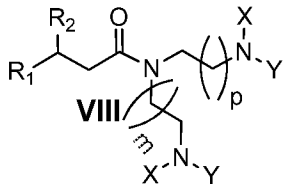
V



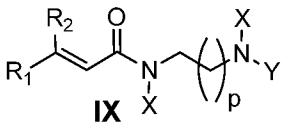
VI



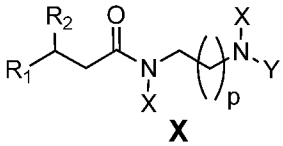
VII



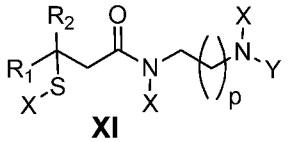
VIII



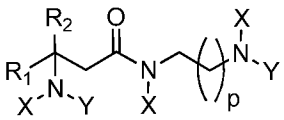
IX



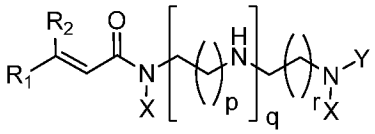
X



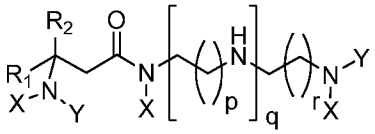
XI



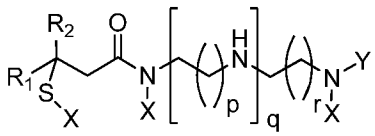
XII



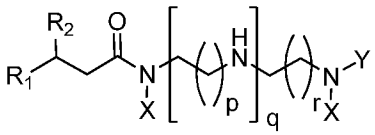
XIII



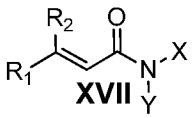
XIV



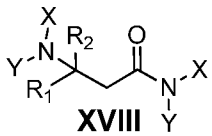
XV



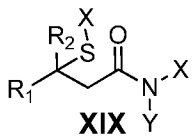
XVI



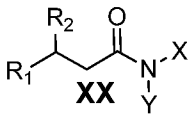
XVII



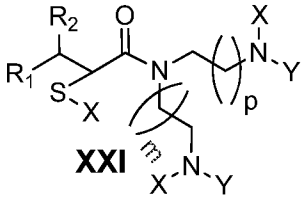
XVIII



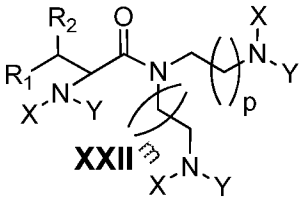
XIX



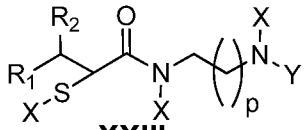
XX



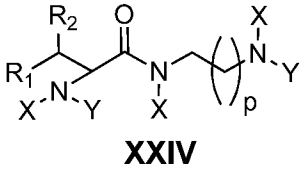
XXI



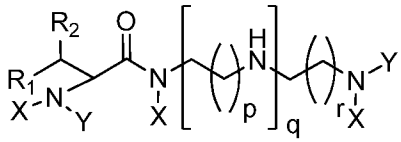
XXII



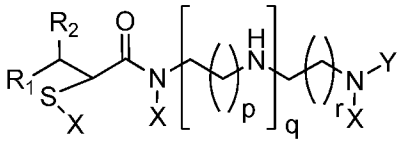
XXIII



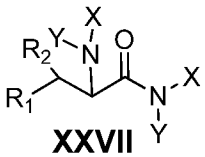
XXIV



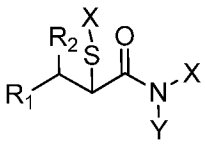
XXV



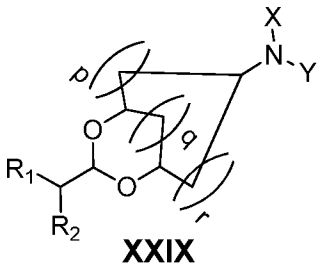
XXVI



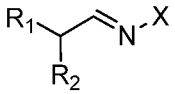
XXVII



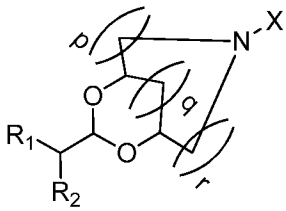
XXVIII



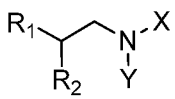
XXIX



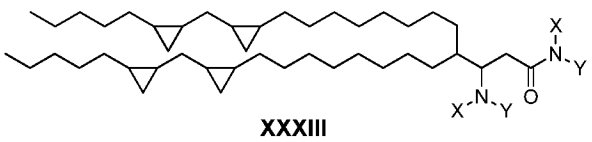
XXX



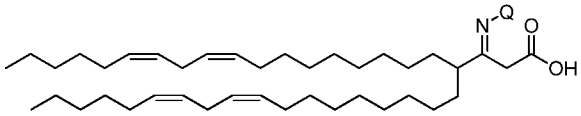
XXXI



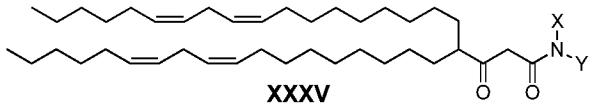
XXXII



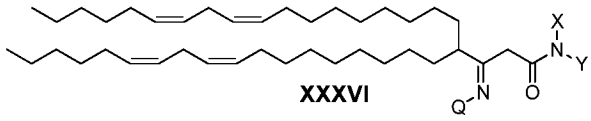
XXXIII



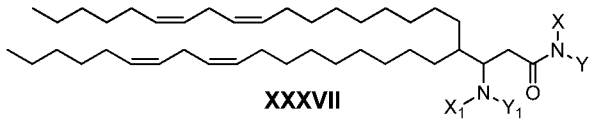
XXXIV



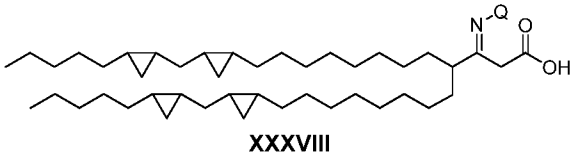
XXXV



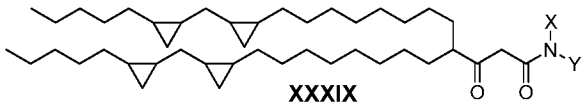
XXXVI



XXXVII

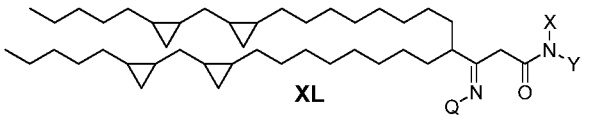


XXXVIII



XXXIX

and



XL

and salts thereof (e.g., pharmaceutically acceptable salts thereof), wherein

each occurrence of X and Y are each, independently, alkyl or aryl;

R_1 and R_2 are each, independently, a C_{10}-C_{30} aliphatic group, which may optionally contain one or more double bonds, and may optionally be interrupted by one or more heteroatoms and/or one or more cyclalkylene groups;

Q is H, alkyl, OH, alkoxy, acyl, or aryl; and

m, n, p, q, and r are each, independently, 0-20 (e.g., 0, 1, 2, 3 or 4).

The neutral lipid can be selected from DSPC, DPPC, POPC, DOPE, or SM. The lipid capable of reducing aggregation can be a PEG lipid. The lipid particle may further comprise a sterol.

In another aspect, the present invention relates to a lipid particle that includes a compound of any of formulas I-XL as described herein. For instance, the lipid particle can contain from about 20 to about 60% mole percentage of the compound of any of formulas I-XL (a cationic lipid). The lipid particle can further include one or more of a PEG lipid, a neutral lipid, and a sterol. In one embodiment, the lipid particle includes a cationic lipid of the present invention, a neutral lipid and a sterol. The neutral lipid can be selected from DSPC, DPPC, POPC, DOPE, and SM. The lipid particle may further include a PEG lipid, such as PEG-DMA, PEG-DMG, or a combination thereof. For instance, the lipid particle can contain a cationic lipid of the invention present at a mole percentage of about 20% and about 60%; a neutral lipid present at a mole percentage of about 5% to about 25%; a sterol present at a mole percentage of about 25% to about 55%; and a PEG lipid at a mole percentage of about 0.5% to about 15%.

The lipid particle can further include an active agent. The active agent can be a nucleic acid selected from a plasmid, an immunostimulatory oligonucleotide, an siRNA, an antisense oligonucleotide, a microRNA, an antagomir, an aptamer, and a ribozyme.

Another aspect of the invention is a pharmaceutical composition which includes a lipid particle of the present invention and a pharmaceutically acceptable carrier.

Yet another aspect is a method of modulating the expression of a target gene in a cell by providing to the cell a lipid particle of the present invention. The active agent can be a nucleic acid selected from a plasmid, an immunostimulatory oligonucleotide, an siRNA, an antisense oligonucleotide, a microRNA, an antagomir, an aptamer, and a ribozyme.

Yet another aspect is a method of treating a disease or disorder characterized by the overexpression of a polypeptide in a subject by providing to the subject a pharmaceutical composition of the present invention, wherein the active agent is a nucleic acid selected from an siRNA, a microRNA, and an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense oligonucleotide includes a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof.

Yet another aspect is a method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject by providing to the subject a pharmaceutical composition of the present invention, wherein the active agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof.

Yet another aspect is a method of inducing an immune response in a subject by providing

to the subject a pharmaceutical composition wherein the active agent is an immunostimulatory oligonucleotide.

The target gene can be selected from Factor VII, Eg5, PCSK9, TPX2, apoB, SAA, TTR, RSV, PDGF beta gene, Erb-B gene, Src gene, CRK gene, GRB2 gene, RAS gene, MEKK gene, JNK gene, RAF gene, Erk1/2 gene, PCNA(p21) gene, MYB gene, JUN gene, FOS gene, BCL-2 gene, Cyclin D gene, VEGF gene, EGFR gene, Cyclin A gene, Cyclin E gene, WNT-1 gene, beta-catenin gene, c-MET gene, PKC gene, NFKB gene, STAT3 gene, survivin gene, Her2/Neu gene, SORT1 gene, XBP1 gene, topoisomerase I gene, topoisomerase II alpha gene, p73 gene, p21(WAF1/CIP1) gene, p27(KIP1) gene, PPM1D gene, RAS gene, caveolin I gene, MIB I gene, MTAI gene, M68 gene, tumor suppressor genes, and p53 tumor suppressor gene. The target gene can contain one or more mutations.

The active agent can be a nucleic acid. The nucleic acid can be an oligonucleotide of between 10 and 50 nucleotides in length.The nucleic acid agent can be double stranded or single stranded. The nucleic acid can be a deoxyribonucleic acid. In another embodiment, the nucleic acid can be a ribonucleic acid. The nucleic acid can be a double stranded siRNA, or can be a single stranded siRNA.

The nucleic acid can be an antisense nucleic acid, a microRNA, an antimicroRNA, an antagomir, a microRNA inhibitor, or an immune stimulatory nucleic acid.

Yet another aspect is a transfection agent that includes the composition or lipid particles described above. The agent, when contacted with cells, can efficiently deliver nucleic acids to the cells. Yet another aspect is a method of delivering a nucleic acid to the interior of a cell, by obtaining or forming a composition or lipid particles described above, and contacting the composition or lipid particles with a cell.

The cells can be mammalian cells. The mammalian cells can be selected from CHO, CHO GFP, CHO DG44, NIH3T3, HEK293-MSR, HeLa, A549, PC12, HepG2, Jurkat, U937, COS-7, Vero, BHK and ME-180 cell lines, and corresponding non-adhereent suspension cells.

Other features and aspects will be apparent from the description and the claims.

Detailed Description

Nucleic acid-lipid particle compositions can include a compound of formula IA-XL. In one embodiment, the cationic lipid is a compound of formula IA. In one embodiment, the

cationic lipid is a compound of formula IB. In another embodiemnt, the cationic lipid is a compound of one of formulas I-XL.

In one embodiment, X are Y are independently alkyl (e.g., methyl, ethyl, isopropyl, n-butyl, t-butyl). In another embodiment, X and Y are both methyl.

In one embodiment, L^1 is a linear alkylene unit. For example, in one embodiment, L^1 is $-(CH_2)_3-$. In another embodiment, L^1 is $-(CH_2)_4-$.

In one embodiment, L^2 is a linear alkylene unit. For example, in one embodiment, L^2 is $-(CH_2)_7-$. In another embodiment, L^2 is $-(CH_2)_4-$. In another embodiment, L^2 is $-(CH_2)_5-$. In one embodiment, L^2 contains one double bond. In yet another embodiment, L^2 contains one or more cycloalkylene (e.g., cyclopropylene) groups. In yet another embodiment, L^2 contains one double bond and one or more cycloalkylene groups (e.g., cyclopropylene).

In one embodiment, R_1 and R_2 are each, independently, C_{10}-C_{30} alkyl or C_{10}-C_{30} alkenyl. In one embodiment, R_1 and R_2 are each, independently, C_{10}-C_{20} alkyl or C_{10}-C_{20} alkenyl. In another embodiment, R_1 and R_2 are each, independently contains one or more double bonds. In yet another embodiment, one of R_1 and R_2 is a linear C_{10}-C_{30} alkyl or C_{10}-C_{30} alkenyl group and the other of R_1 and R_2 is a branched C_{10}-C_{30} alkyl or C_{10}-C_{30} alkenyl group. In yet another embodiment, R_1 and R_2 are independently a linear C_{10}-C_{30} alkyl or C_{10}-C_{30} alkenyl group.

In one embodiment, Z is -C(O)O-, -C(O)N(R^3)-, -O(CO)N(R^3), -C(O)N(R^3)C(O)O-, or -N(R^3)C(O)N(R^3)-.

In one embodiment, R^3 is H or alkyl (e.g., methyl).

In one embodiment, the dashed line ----- represents a single bond. In another embodiment, the dashed line ---- represents a double bond. In one embodiment, ---- represents a single bond and C* is further substituted with hydrogen.

In one preferred embodiment, R_1 and R_2 in formula IA or IB are uninterrupted.

In another embodiment of a compound of formula IA or IB, L^1 and L^2 in formula IA or IB are independently saturated and the dashed line represents a single bond. For example, L^1 and L^2 can be independently saturated and uninterrupted, linear or branched C_2-C_{10} alkylene. In another embodiment, L^1 is a saturated and uninterrupted, linear or branched C_2-C_{10} alkylene, and L^2 is a saturated linear or branched C_2-C_{10} alkylene which interrupted by one or two cyclopropyl groups.

In yet another embodiment, L^1 and L^2 in formula IA or IB are saturated and the dashed line represents a double bond. For example, L^1 and L^2 can be independently saturated and uninterrupted, linear or branched C_2-C_{10} alkylene. In another embodiment, L^1 is a saturated and uninterrupted, linear or branched C_2-C_{10} alkylene, and L^2 is a saturated linear or branched C_2-C_{10} alkylene which interrupted by one or two cyclopropyl groups.

In some embodiments, a composition described herein provides increased activity of the nucleic acid and/or improved tolerability of the compositions *in vivo*, which can result in a significant increase in therapeutic index as compared to lipid-nucleic acid particle compositions previously described.

In certain embodiments, compositions for the delivery of siRNA molecules are described. These compositions are effective in down-regulating the protein levels and/or mRNA levels of target proteins. The activity of these compositions can be influenced by the presence of cationic lipids and the molar ratio of cationic lipid in the formulation.

The lipid particles and compositions may be used for a variety of purposes, including the delivery of associated or encapsulated therapeutic agents to cells, both *in vitro* and *in vivo*. Accordingly, methods of treating diseases or disorders in a subject in need thereof can include contacting the subject with a lipid particle associated with a suitable therapeutic agent.

As described herein, the lipid particles are particularly useful for the delivery of nucleic acids, including, *e.g.*, siRNA molecules and plasmids. Therefore, the lipid particles and compositions may be used to modulate the expression of target genes and proteins both in vitro and in vivo by contacting cells with a lipid particle associated with a nucleic acid that reduces target gene expression (e.g., an siRNA) or a nucleic acid that may be used to increase expression of a desired protein (*e.g.*, a plasmid encoding the desired protein).

Various exemplary embodiments of lipids, lipid particles and compositions comprising the same, and their use to deliver therapeutic agents and modulate gene and protein expression are described in further detail below.

Cationic Lipids

Cationic lipids can have certain design features including a head group, one or more hydrophobic tails, and a liker between the head group and the one or more tails. The head group can include an amine; for example an amine having a desired pK_a. The pK_a can be influenced by

the structure of the lipid, particularly the nature of head group; e.g., the presence, absence, and location of functional groups such as anionic functional groups, hydrogen bond donor functional groups, hydrogen bond acceptor groups, hydrophobic groups (e.g., aliphatic groups), hydrophilic groups (e.g., hydroxyl or methoxy), or aryl groups. The head group amine can be a cationic amine; a primary, secondary, or tertiary amine; the head group can include one amine group (monoamine), two amine groups (diamine), three amine groups (triamine), or a larger number of amine groups, as in an oligoamine or polyamine. The head group can include a functional group that is less strongly basic than an amine, such as, for example, an imidazole, a pyridine, or a guanidinium group. The head group can be zwitterionic. Other head groups are suitable as well.

The one or more hydrophobic tails can include two hydrophobic chains, which may be the same or different. The tails can be aliphatic; for example, they can be composed of carbon and hydrogen, either saturated or unsaturated but without aromatic rings. The tails can be fatty acid tails; some such groups include octanyl, nonanyl, decyl, lauryl, myristyl, palmityl, stearyl, α-linoleyl, stearidonyl, linoleyl, γ-linolenyl, arachadonyl, oleyl, and others. Other hydrophobic tails are suitable as well.

The linker can include, for example, a glyceride linker, an acyclic glyceride analog linker, or a cyclic linker (including a spiro linker, a bicyclic linker, and a polycyclic linker). The linker can include functional groups such as an ether, an ester, a phosphate, a phosphonate, a phosphorothioate, a sulfonate, a disulfide, an acetal, a ketal, an imine, a hydrazone, or an oxime. Other linkers and functional groups are suitable as well.

In one embodiment, the lipid is a racemic mixture.

In one embodiment, the lipid is enriched in one diastereomer, e.g. the lipid has at least 95%, at least 90%, at least 80% or at least 70% diastereomeric excess.

In one embodiment, the lipid is enriched in one enantiomer, e.g. the lipid has at least 95%, at least 90%, at least 80% or at least 70% enantiomer excess.

In one embodiment, the lipid is chirally pure, e.g. is a single optical isomer.

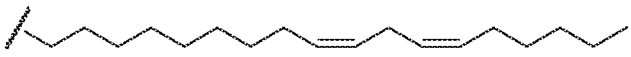
In one embodiment, the lipid is enriched for one optical isomer.

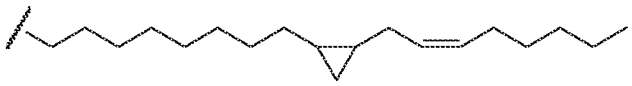
Where a double bond is present (e.g., a carbon-carbon double bond or carbon-nitrogen double bond), there can be isomerism in the configuration about the double bond (i.e. cis/trans or E/Z isomerism). Where the configuration of a double bond is illustrated in a chemical structure, it is understood that the corresponding isomer can also be present. The amount of isomer present

can vary, depending on the relative stabilities of the isomers and the energy required to convert between the isomers. Accordingly, some double bonds are, for practical purposes, present in only a single configuration, whereas others (e.g., where the relative stabilities are similar and the energy of conversion low) may be present as inseparable equilibrium mixture of configurations.

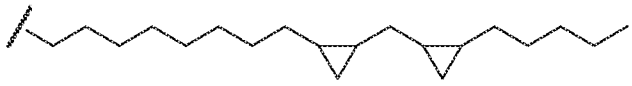
R_1 and/or R_2 can include an unsaturation, such as a double bond, triple bond, or ring. In some cases, R_1 and/or R_2 can include more than one unsaturation. The unsaturation can introduce a degree of structural rigidity compared to a similar but saturated structure. For example, a double bond can be more rigid than a single bond. However, in some cases, an unsaturation can be a site of reactivity. In particular, carbon-carbon double bonds can be more reactive, e.g., to oxidation, than carbon-carbon single bonds. It can therefore, in some cases, be preferable to have single-bonded unsaturations, for example, cyclic structures.

In some cases, a double-bonded unsaturation can be satisfactorily replaced by a cyclic unsaturation. The cyclic unsaturation can be a cycloaliphatic unsaturation; e.g., a cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, or cyclooctyl group. In some cases, the cyclic group can be a polycyclic group, e.g., a bicyclic group or tricyclic group. A bicyclic group can be bridged, fused, or have a spiro structure.

The cyclic unsaturation can provide rigidity. Smaller rings, e.g. 3, 4 or 5 membered rings; can be more rigid than larger ones, e.g., 6, 7 or 8 membered rings. In some cases, a double bond moiety can be replaced by a cyclopropyl moiety, e.g.,  can be replaced by . In just one example, a lineolyl moiety has two carbon-carbon double bonds, each of which can independently be replaced by a cyclic moiety, e.g., a cyclopropyl moiety. Thus, suitable substitutes for lineolyl:  lineolyl can include:

,

, and

.

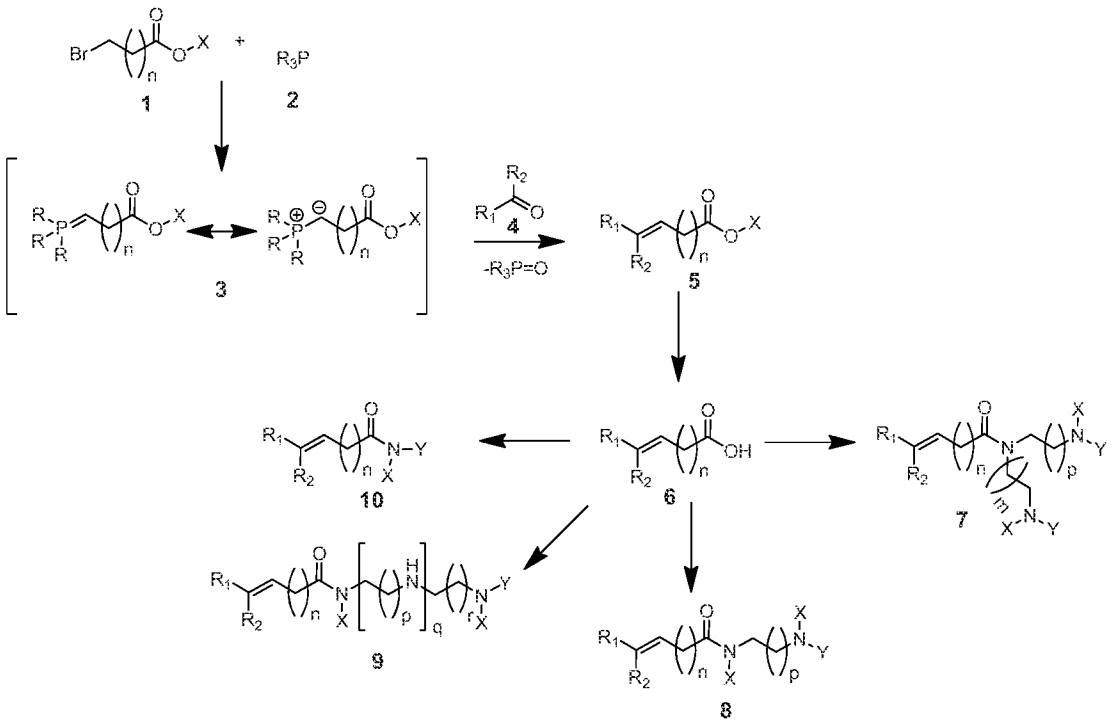
The following schemes provide exemplary synthetic procedures for preparing cationic lipids according to the present invention.

Ketones (or aldehydes) are substrates for the Wittig reaction, described, for example in March "Advanced Organic Chemistry: Reactions, Mechanisms, and Structure," Fourth Edition, Wiley & Sons 1992, which is incorporated by reference in its entirety. Wittig reagents **3** can be prepared from bromoalkyl esters **1**. Wittig reagents share the ylid functionality in common. Other precursors give rise to other Wittig reagents. Upon reaction of ylid **3** with ketone **4**, products **5** result. Typically X can be an alkyl or aryl moiety, such that XO^- is a good leaving group, and esters **5** are readily transformed to the corresponding acids **6**.

The lipids of the present invention can be prepared by the reaction schemes shown below. The variables in the formulas below are as defined as in the formulas above.

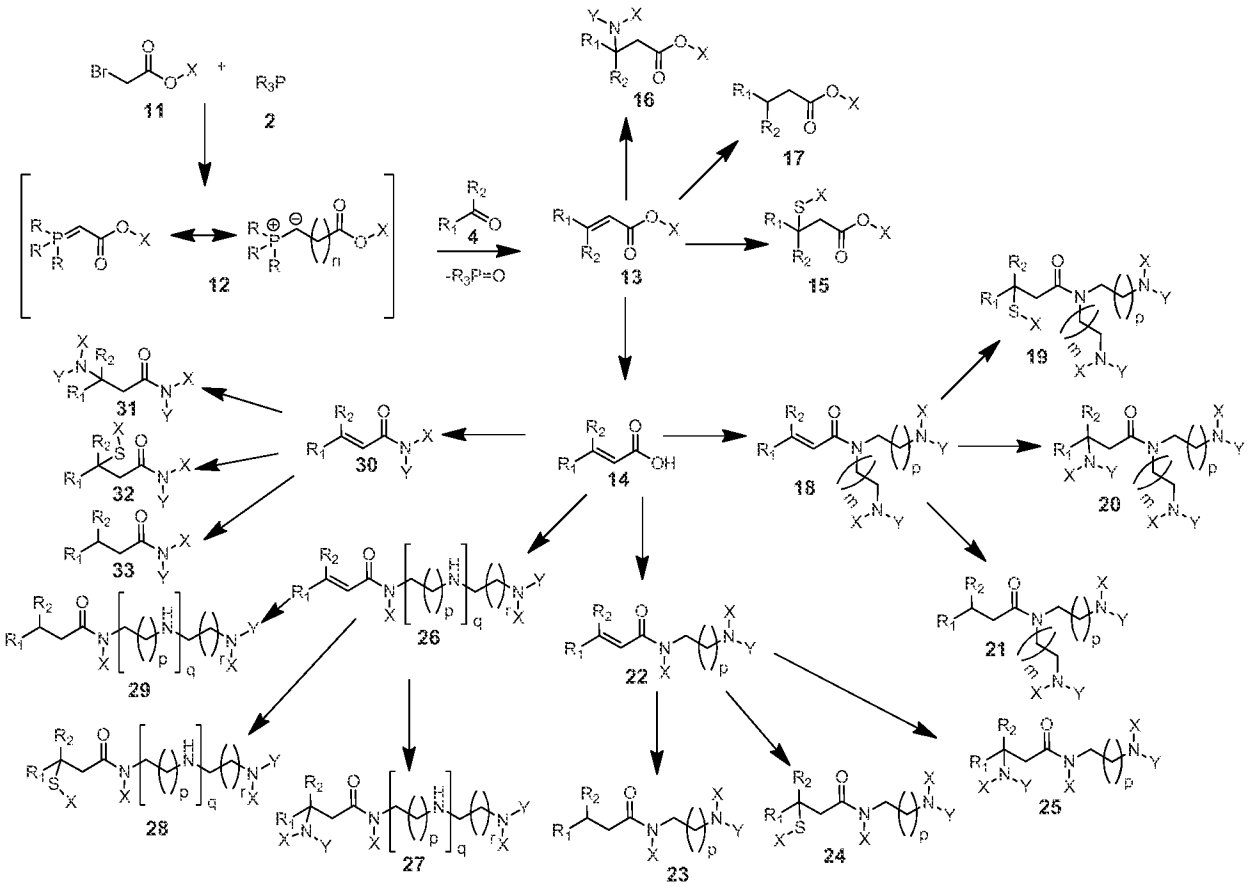
In scheme 1, a Wittig reaction is performed to obtain the compound of formula 5. This product may then be converted into a cationic lipid (e.g., of formulas 7-10), for example, through intermediate compound 6.

Scheme 1 (Wittig Reaction)

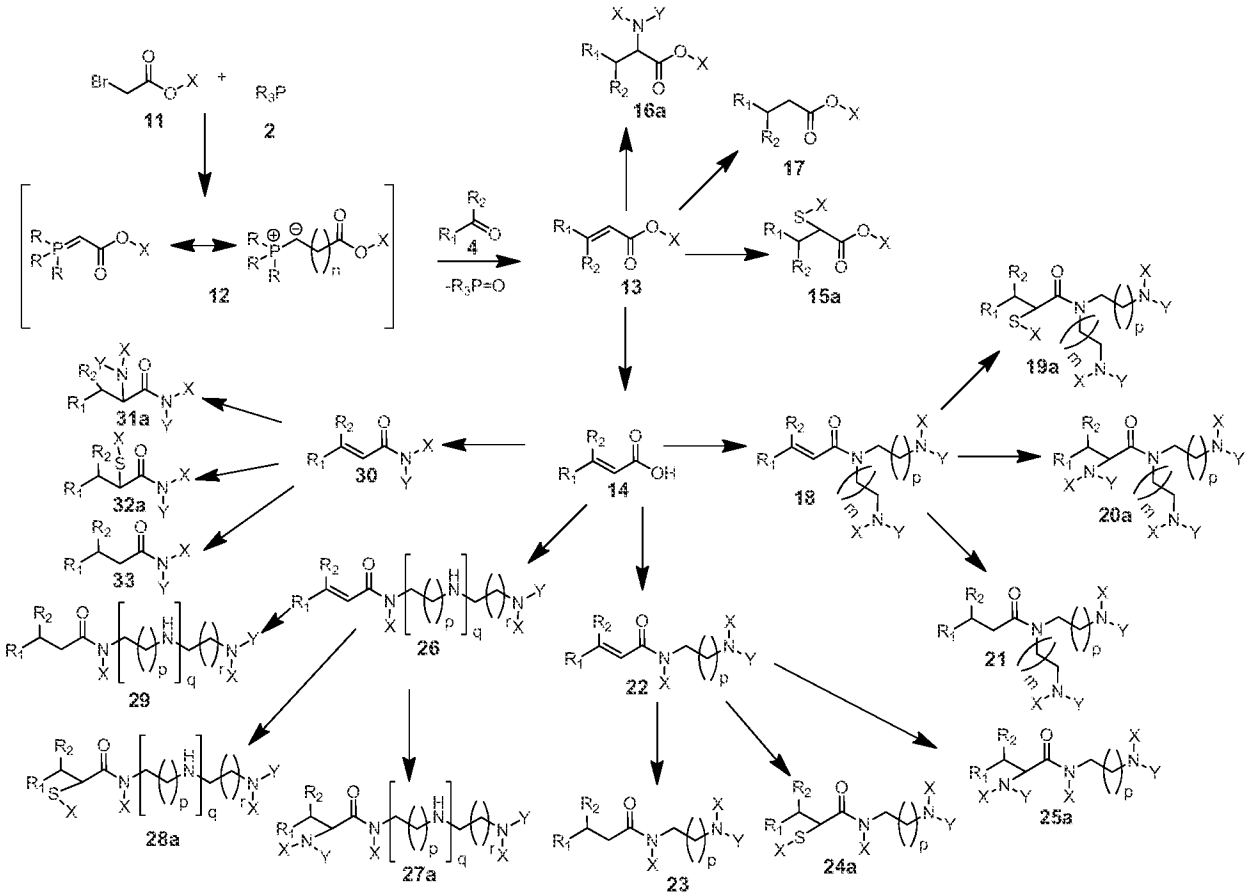


In schemes 2a, 2b and 3, a Wittig reaction is performed to obtain the compound of formula 13. This product may then be converted into a cationic lipid, for example, through intermediate compound 14. The subsequent addition of an amine group, for example, can be performed by a Michael addition.

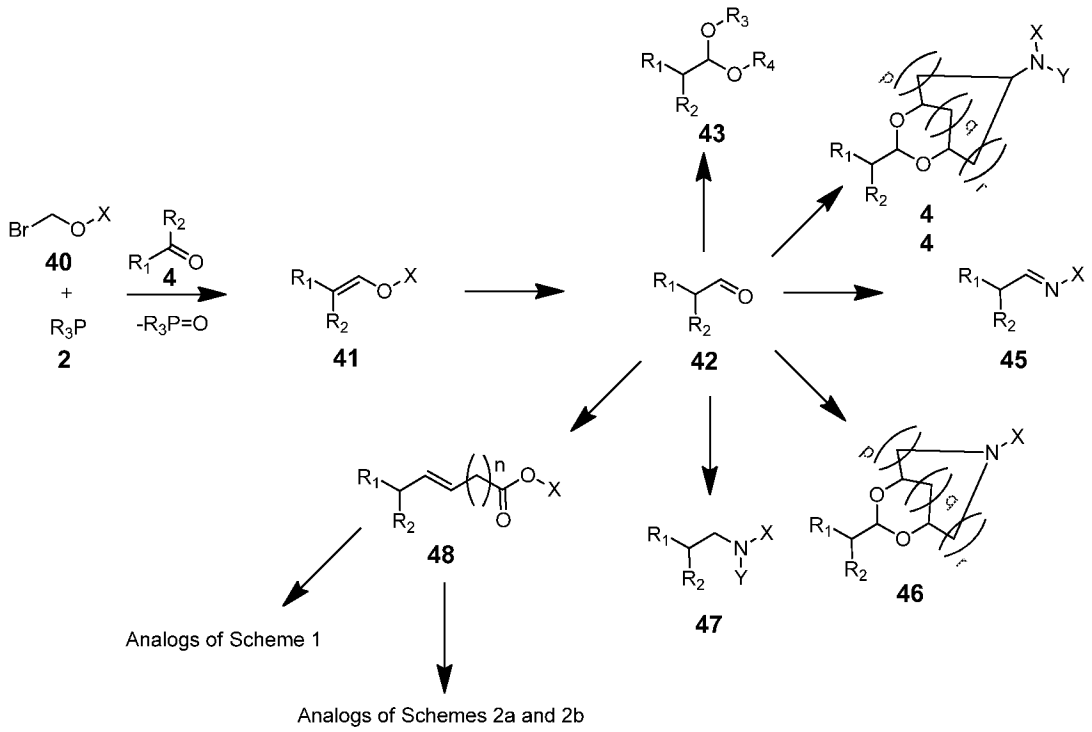
Scheme 2a (Wittig Reaction Followed By 1,4-Michael Addition)



Scheme 2b (Wittig Reaction Followed By 1,2-Michael Addition)

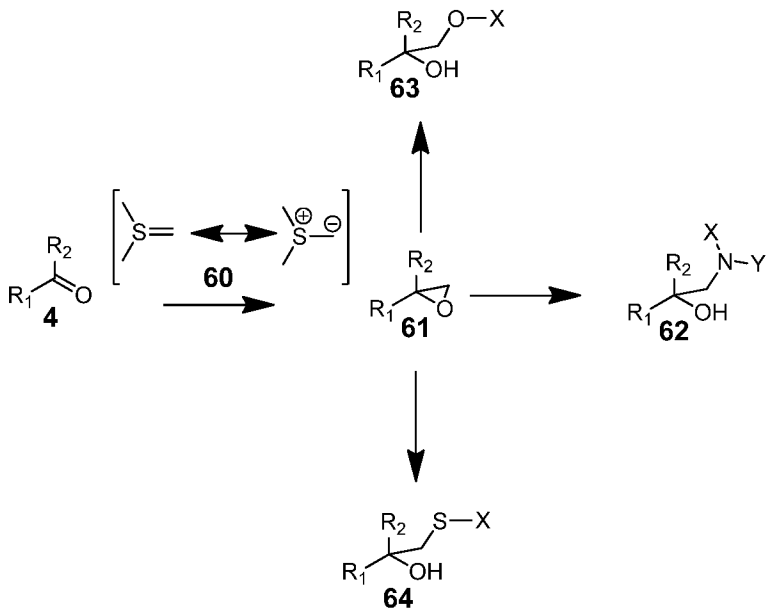


Scheme 3 (Wittig Reaction Followed By Michael Addition)

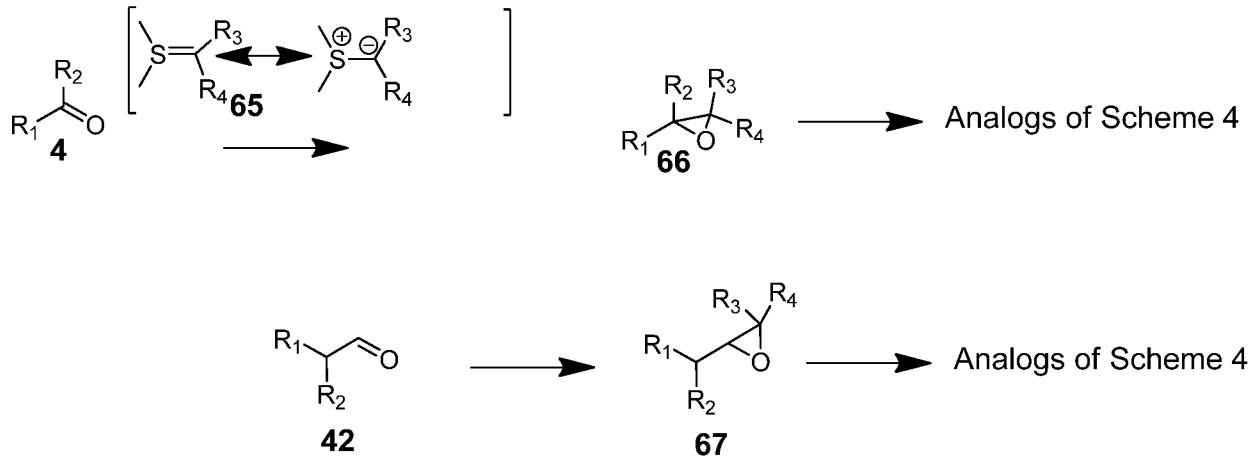


The synthesis of sulfur ylides and other compounds is shown below.

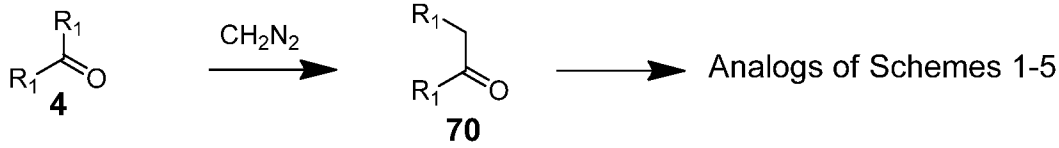
Scheme 4 (Sulfur Ylides)



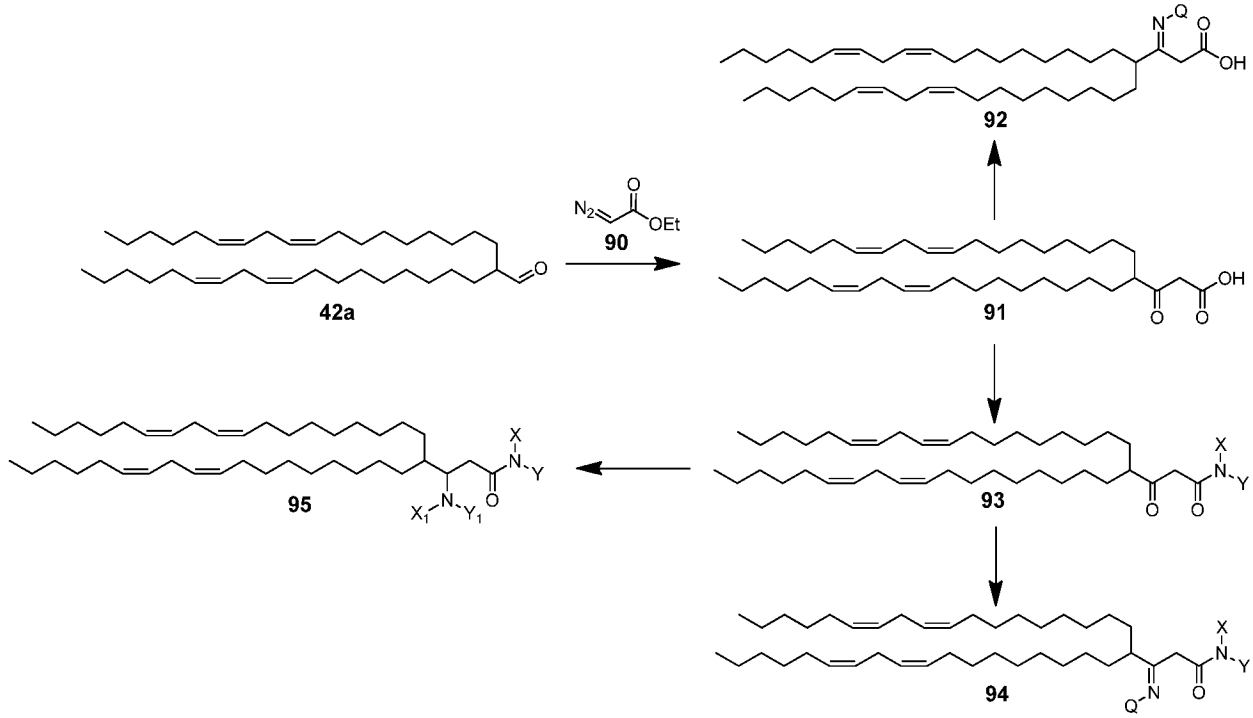
Scheme 5 (Sulfur Ylides)



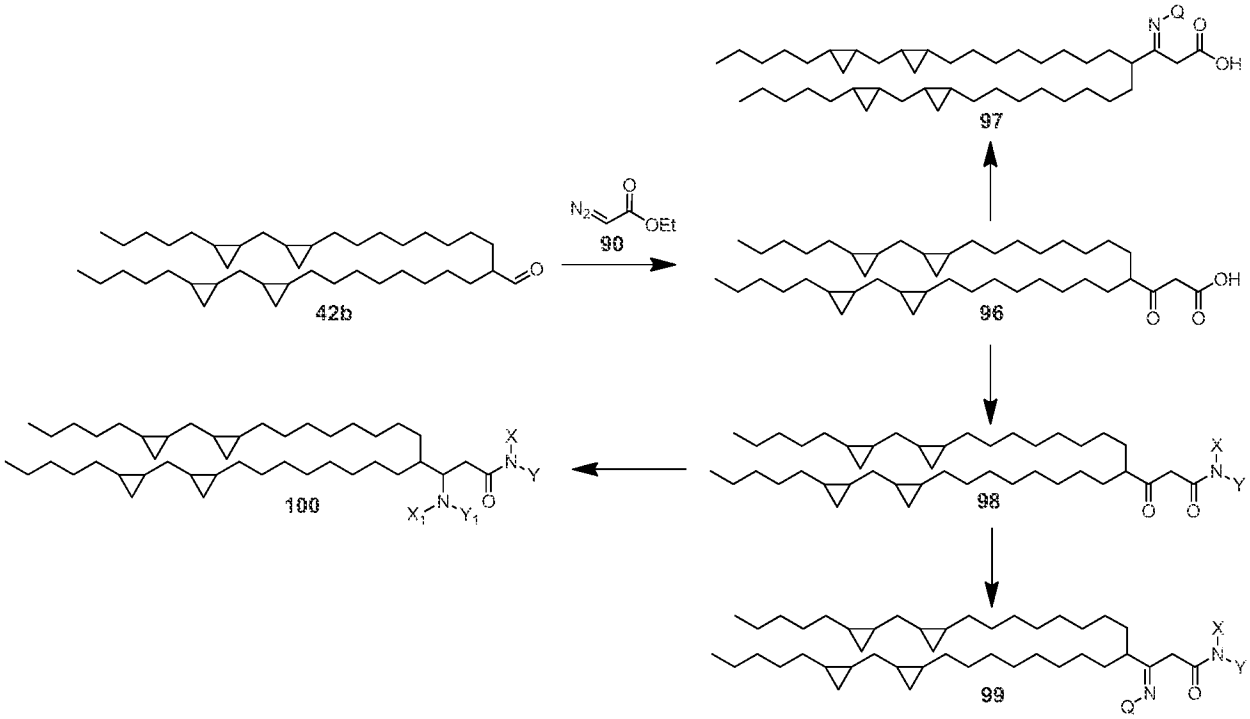
Scheme 6 (CH_2 Insertion Using Diazomethane)



Scheme 7a (β-Keto Acid Analogs) (*See, e.g., J. Org. Chem.*, 54(14), 1989, 3259 (Table 1))

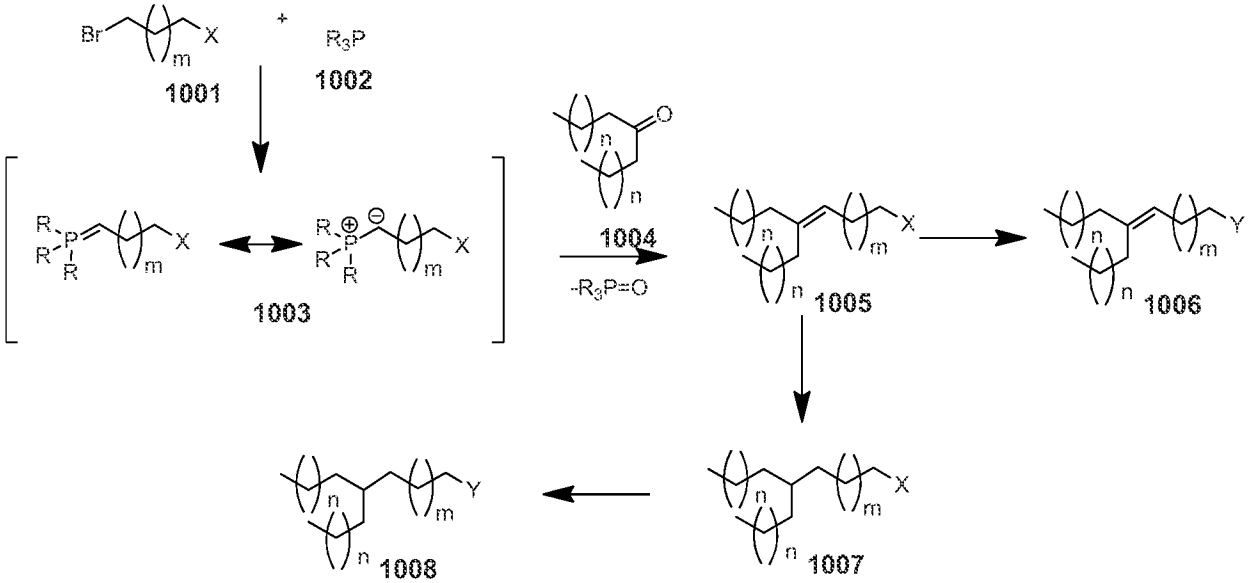


Scheme 7b (β-Keto Acid Analogs)

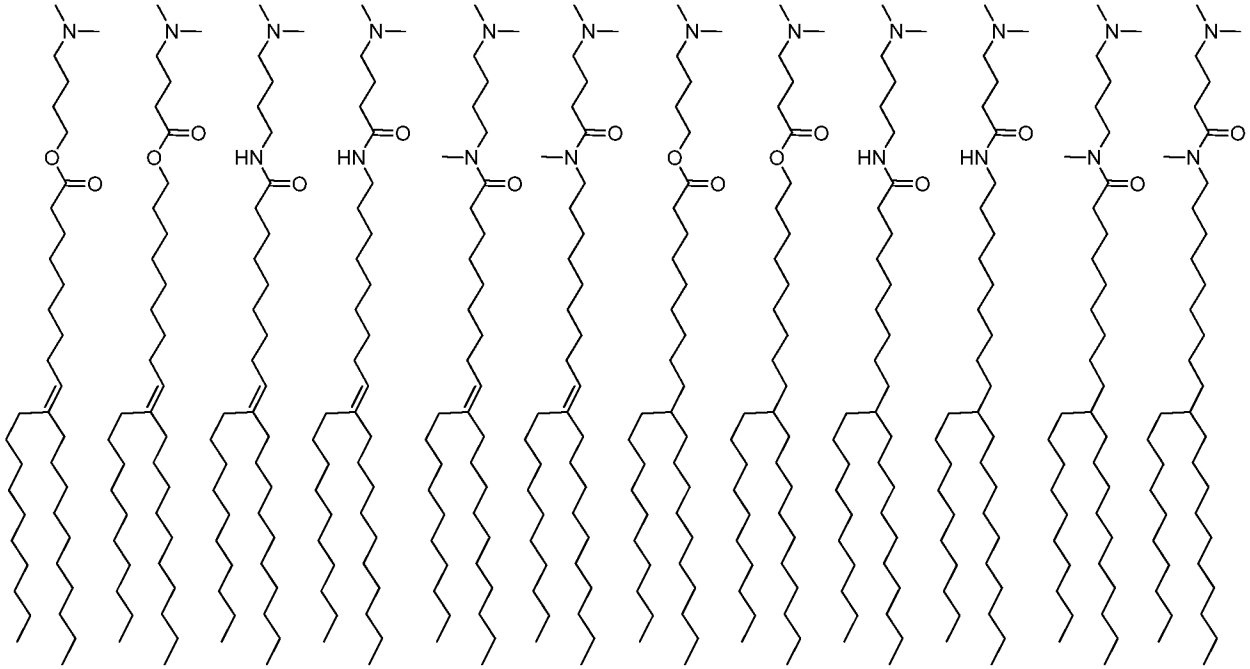


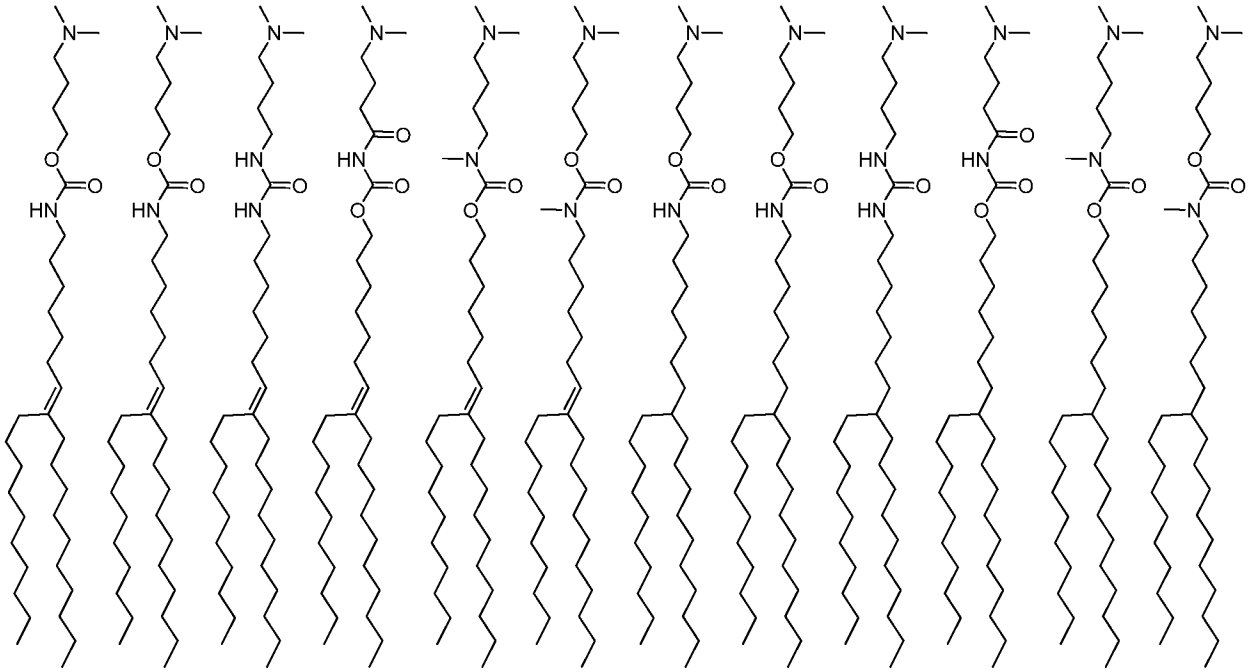
Scheme 8 (Wittig Reaction)

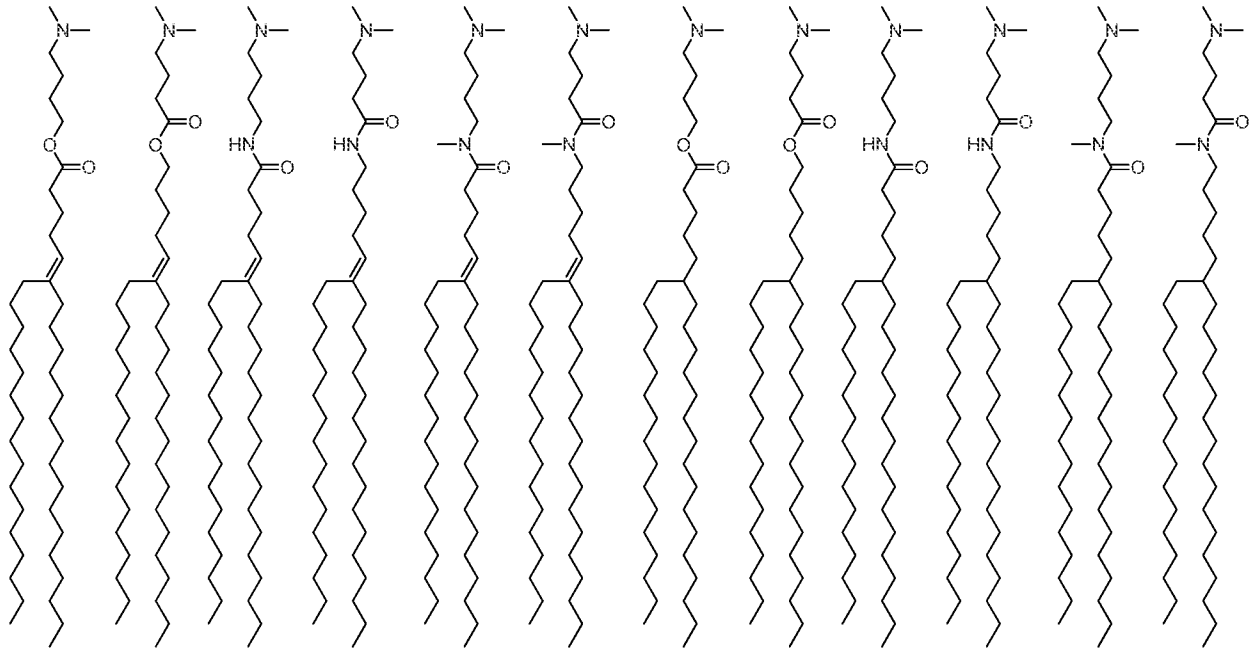
In Scheme 8, a Wittig reaction is performed to obtain the compound of formula 1005. This product may then be converted into a cationic lipid, for example, through an intermediate compound.

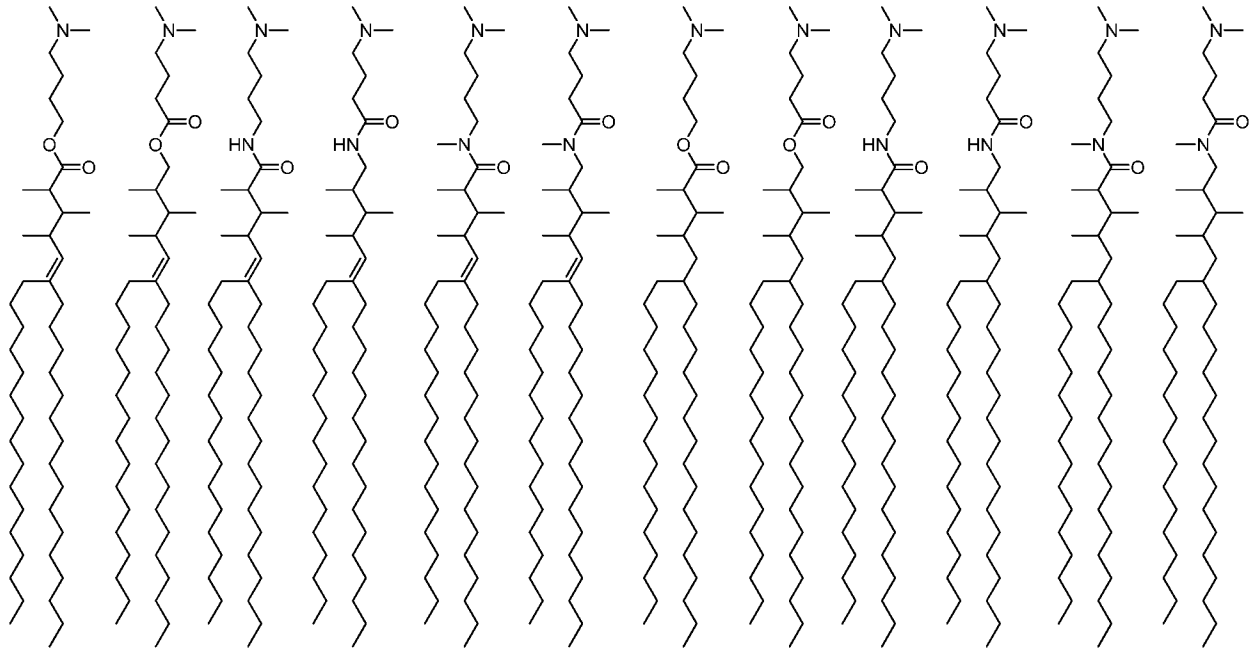


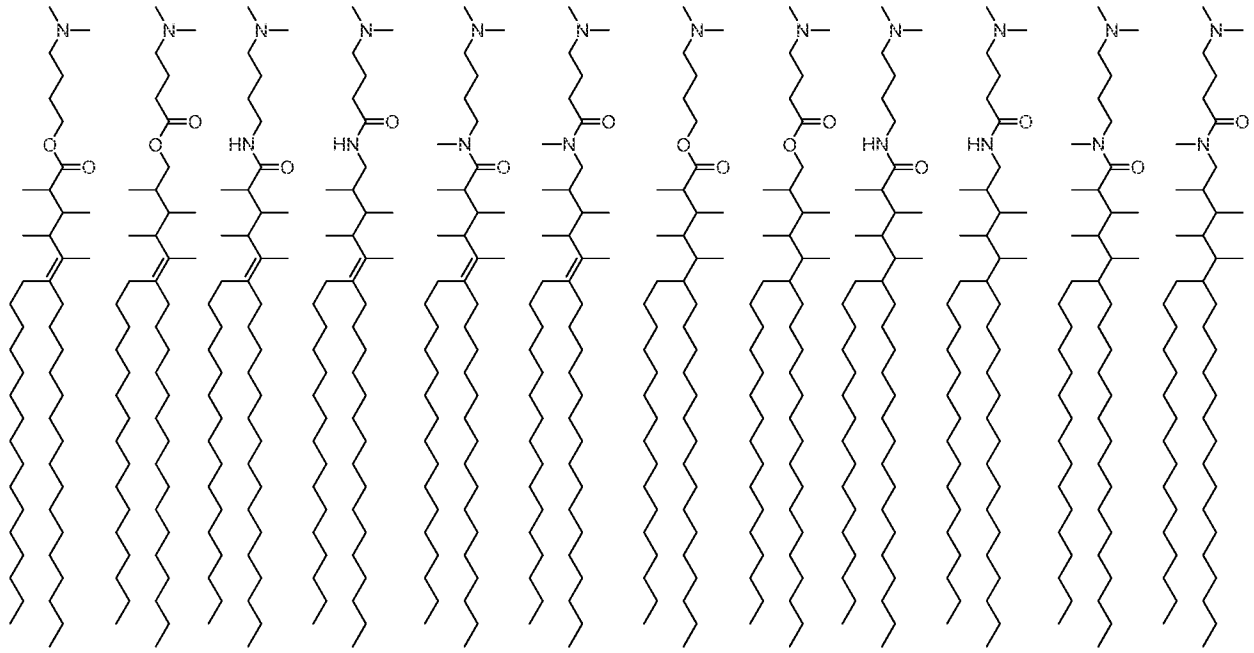
Examples of cationic lipids of the present invention include those shown below and salts thereof (including pharmaceutically acceptable salts thereof).

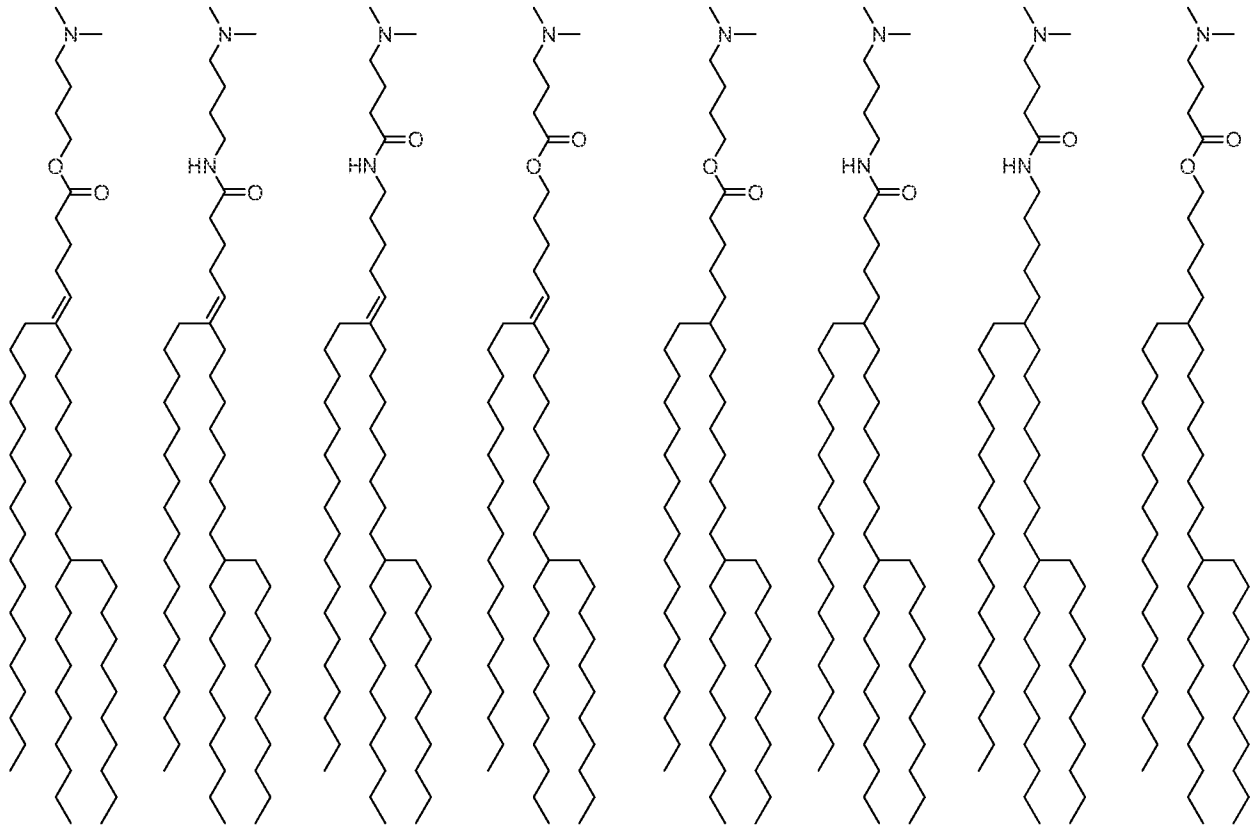


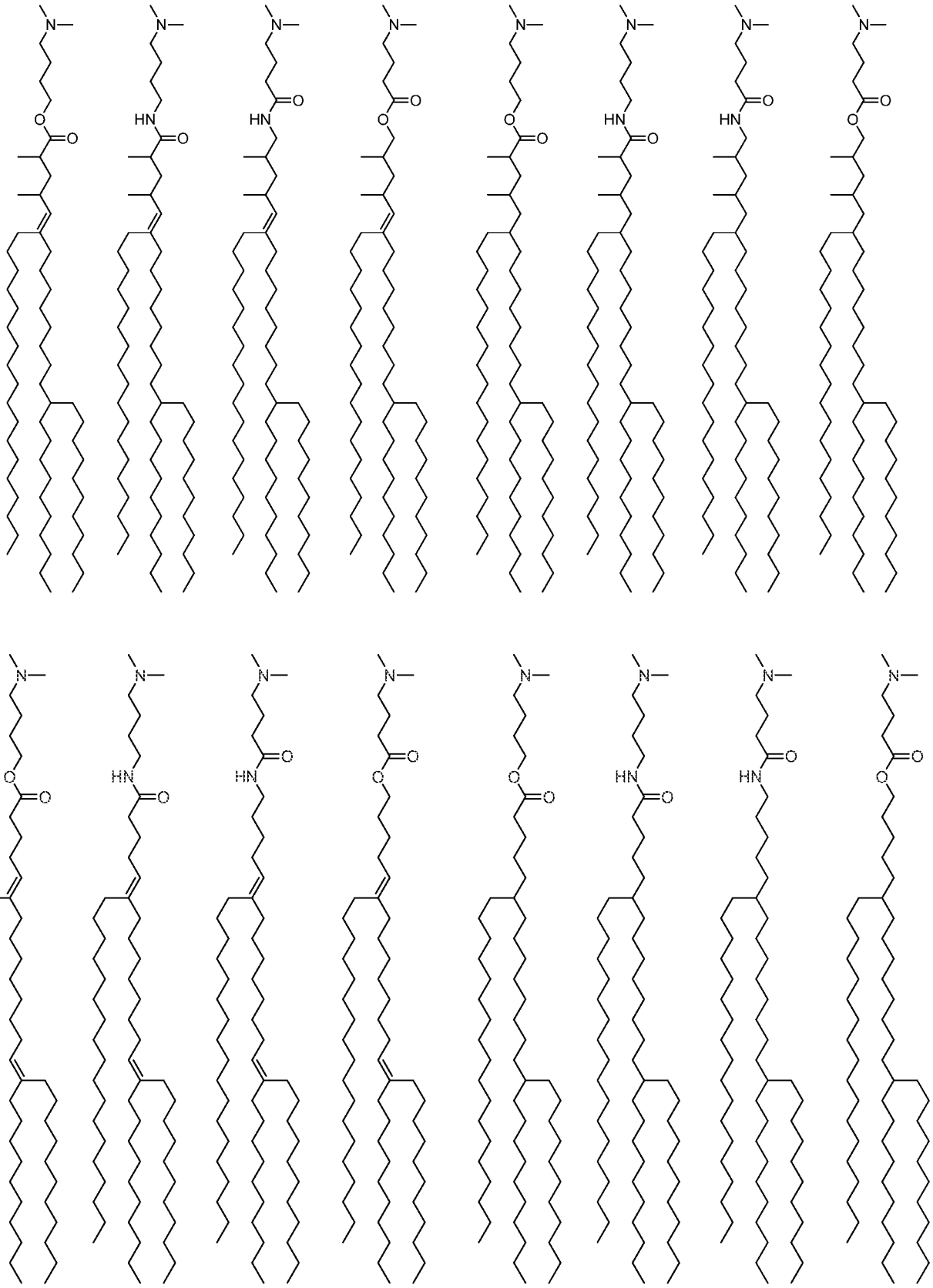


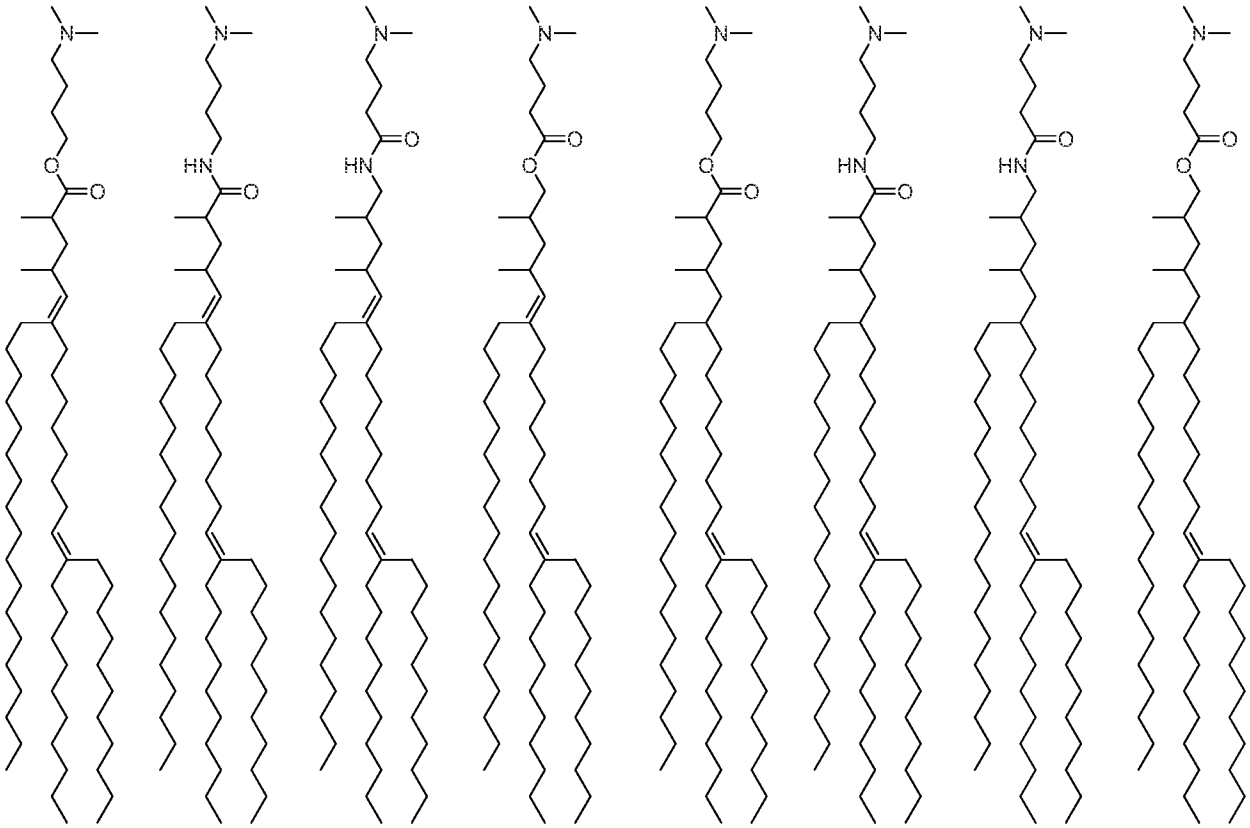


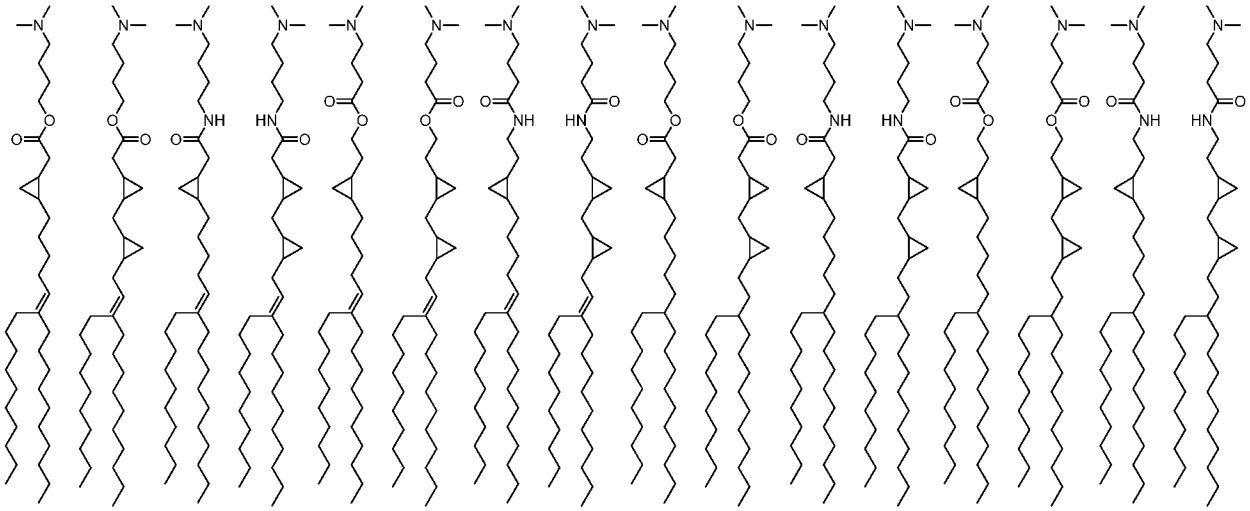


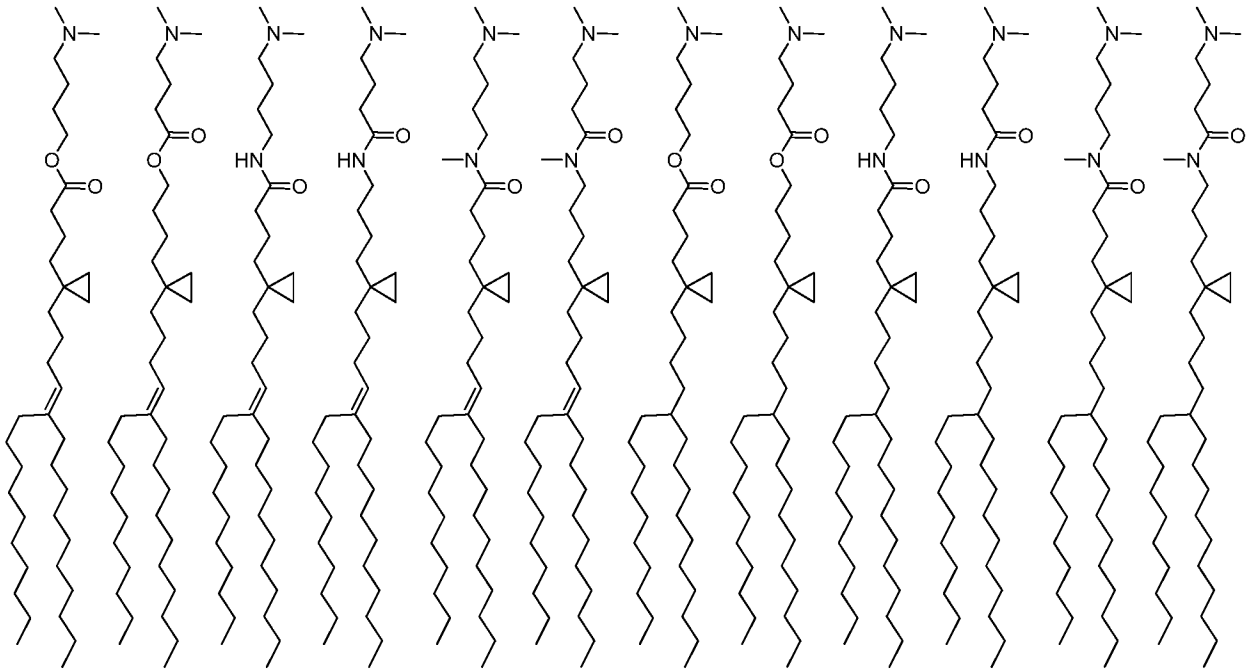












A number of cationic lipids, and methods for making them, are described in, for example, in application nos. PCT/US09/63933, PCT/US09/63927, PCT/US09/63931, and PCT/US09/63897, each filed November 10, 2009, and applications referred to therein, including nos. 61/104,219, filed October 9, 2008; no. 61/113,179, filed November 10, 2008; no. 61/154,350, filed February 20, 2009; no. 61/171,439, filed April 21, 2009; no. 61/175,770, filed May 5, 2009; no. 61/185,438, filed June 9, 2009; no. 61/225,898, filed July 15, 2009; and no. 61/234,098, filed August 14, 2009; WO 2009/086558; and WO 2008/042973. Each of these documents is incorporated by reference in its entirety. See, for example, Tables 1 and 2 of application no. PCT/US09/63933, filed November 10, 2009, at pages 33-51.

In particular embodiments, the lipids are cationic lipids. As used herein, the term "cationic lipid" is meant to include those lipids having one or two fatty acid or fatty aliphatic chains and an amino head group (including an alkylamino or dialkylamino group) that may be protonated to form a cationic lipid at physiological pH. In some embodiments, a cationic lipid is referred to as an "amino lipid."

Other cationic lipids would include those having alternative fatty acid groups and other dialkylamino groups, including those in which the alkyl substituents are different (*e.g.*,

N-ethyl-N-methylamino-, N-propyl-N-ethylamino- and the like). For those embodiments in which R_1 and R_2 are both long chain alkyl, alkenyl, alkynyl, or acyl groups, they can be the same or different. In general, lipids (e.g., a cationic lipid) having less-saturated acyl chains are more easily sized, particularly when the complexes are sized below about 0.3 microns, for purposes of filter sterilization. Cationic lipids containing unsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are typical. Other scaffolds can also be used to separate the amino group (e.g., the amino group of the cationic lipid) and the fatty acid or fatty alkyl portion of the cationic lipid. Suitable scaffolds are known to those of skill in the art.

In certain embodiments, cationic lipids have at least one protonatable or deprotonatable group, such that the lipid is positively charged at a pH at or below physiological pH (e.g. pH 7.4), and neutral at a second pH, preferably at or above physiological pH. Such lipids are also referred to as cationic lipids. It will, of course, be understood that the addition or removal of protons as a function of pH is an equilibrium process, and that the reference to a charged or a neutral lipid refers to the nature of the predominant species and does not require that all of the lipid be present in the charged or neutral form. The lipids can have more than one protonatable or deprotonatable group, or can be zwiterrionic.

In certain embodiments, protonatable lipids (i.e., cationic lipids) have a pK_a of the protonatable group in the range of about 4 to about 11. Typically, lipids will have a pK_a of about 4 to about 7, e.g., between about 5 and 7, such as between about 5.5 and 6.8, when incorporated into lipid particles. Such lipids will be cationic at a lower pH formulation stage, while particles will be largely (though not completely) surface neutralized at physiological pH around pH 7.4. One of the benefits of a pK_a in the range of between about 4 and 7 is that at least some nucleic acid associated with the outside surface of the particle will lose its electrostatic interaction at physiological pH and be removed by simple dialysis; thus greatly reducing the particle's susceptibility to clearance. pK_a measurements of lipids within lipid particles can be performed, for example, by using the fluorescent probe 2-(p-toluidino)-6-napthalene sulfonic acid (TNS), using methods described in Cullis et al., (1986) *Chem Phys Lipids* 40, 127-144, which is incorporated by reference in its entirety.

In particular embodiments, the lipids are charged lipids. As used herein, the term "charged lipid" is meant to include those lipids having one or two fatty acyl or fatty alkyl chains and a quaternary amino head group. The quaternary amine carries a permanent positive charge.

The head group can optionally include a ionizable group, such as a primary, secondary, or tertiary amine that may be protonated at physiological pH. The presence of the quaternary amine can alter the pKa of the ionizable group relative to the pKa of the group in a structurally similar compound that lacks the quaternary amine (e.g., the quaternary amine is replaced by a tertiary amine) In some embodiments, a charged lipid is referred to as an "amino lipid." See, for example, provisional U.S. patent application 61/267,419, filed December 7, 2009, which is incorporated by reference in its entirety.

Apolipoproteins

The formulations can further comprise an apolipoprotein. As used herein, the term "apolipoprotein" or "lipoprotein" refers to apolipoproteins known to those of skill in the art and variants and fragments thereof and to apolipoprotein agonists, analogues or fragments thereof described below.

Suitable apolipoproteins include, but are not limited to, ApoA-I, ApoA-II, ApoA-IV, ApoA-V and ApoE, and active polymorphic forms, isoforms, variants and mutants as well as fragments or truncated forms thereof. In certain embodiments, the apolipoprotein is a thiol containing apolipoprotein. "Thiol containing apolipoprotein" refers to an apolipoprotein, variant, fragment or isoform that contains at least one cysteine residue. The most common thiol containing apolipoproteins are ApoA-I Milano (ApoA-I_M) and ApoA-I Paris (ApoA-I_P) which contain one cysteine residue (Jia et al., 2002, Biochem. Biophys. Res. Comm. 297: 206-13; Bielicki and Oda, 2002, Biochemistry 41: 2089-96). ApoA-II, ApoE2 and ApoE3 are also thiol containing apolipoproteins. Isolated ApoE and/or active fragments and polypeptide analogues thereof, including recombinantly produced forms thereof, are described in U.S. Pat. Nos. 5,672,685; 5,525,472; 5,473,039; 5,182,364; 5,177,189; 5,168,045; 5,116,739; the disclosures of which are herein incorporated by reference. ApoE3 is disclosed in Weisgraber, et al., "Human E apoprotein heterogeneity: cysteine-arginine interchanges in the amino acid sequence of the apo-E isoforms," J. Biol. Chem. (1981) 256: 9077-9083; and Rall, et al., "Structural basis for receptor binding heterogeneity of apolipoprotein E from type III hyperlipoproteinemic subjects," Proc. Nat. Acad. Sci. (1982) 79: 4696-4700. See also GenBank accession number K00396.

In certain embodiments, the apolipoprotein can be in its mature form, in its preproapolipoprotein form or in its proapolipoprotein form. Homo- and heterodimers (where

feasible) of pro- and mature ApoA-I (Duverger et al., 1996, Arterioscler. Thromb. Vasc. Biol. 16(12):1424-29), ApoA-I Milano (Klon et al., 2000, Biophys. J. 79:(3)1679-87; Franceschini et al., 1985, J. Biol. Chem. 260: 1632-35), ApoA-I Paris (Daum et al., 1999, J. Mol. Med. 77:614-22), ApoA-II (Shelness et al., 1985, J. Biol. Chem. 260(14):8637-46; Shelness et al., 1984, J. Biol. Chem. 259(15):9929-35), ApoA-IV (Duverger et al., 1991, Euro. J. Biochem. 201(2):373-83), and ApoE (McLean et al., 1983, J. Biol. Chem. 258(14):8993-9000) can also be utilized.

In certain embodiments, the apolipoprotein can be a fragment, variant or isoform of the apolipoprotein. The term "fragment" refers to any apolipoprotein having an amino acid sequence shorter than that of a native apolipoprotein and which fragment retains the activity of native apolipoprotein, including lipid binding properties. By "variant" is meant substitutions or alterations in the amino acid sequences of the apolipoprotein, which substitutions or alterations, e.g., additions and deletions of amino acid residues, do not abolish the activity of native apolipoprotein, including lipid binding properties. Thus, a variant can comprise a protein or peptide having a substantially identical amino acid sequence to a native apolipoprotein provided herein in which one or more amino acid residues have been conservatively substituted with chemically similar amino acids. Examples of conservative substitutions include the substitution of at least one hydrophobic residue such as isoleucine, valine, leucine or methionine for another. Likewise, for example, the substitution of at least one hydrophilic residue such as, for example, between arginine and lysine, between glutamine and asparagine, and between glycine and serine (see U.S. Pat. Nos. 6,004,925, 6,037,323 and 6,046,166) are conservative substitutions. The term "isoform" refers to a protein having the same, greater or partial function and similar, identical or partial sequence, and may or may not be the product of the same gene and usually tissue specific (see Weisgraber 1990, J. Lipid Res. 31(8):1503-11; Hixson and Powers 1991, J. Lipid Res. 32(9):1529-35; Lackner et al., 1985, J. Biol. Chem. 260(2):703-6; Hoeg et al., 1986, J. Biol. Chem. 261(9):3911-4; Gordon et al., 1984, J. Biol. Chem. 259(1):468-74; Powell et al., 1987, Cell 50(6):831-40; Aviram et al., 1998, Arterioscler. Thromb. Vase. Biol. 18(10):1617-24; Aviram et al., 1998, J. Clin. Invest. 101(8):1581-90; Billecke et al., 2000, Drug Metab. Dispos. 28(11):1335-42; Draganov et al., 2000, J. Biol. Chem. 275(43):33435-42; Steinmetz and Utermann 1985, J. Biol. Chem. 260(4):2258-64; Widler et al., 1980, J. Biol. Chem. 255(21):10464-71; Dyer et al., 1995, J. Lipid Res. 36(1):80-8; Sacre et al., 2003, FEBS Lett.

540(1-3):181-7; Weers, et al., 2003, Biophys. Chem. 100(1-3):481-92; Gong et al., 2002, J. Biol. Chem. 277(33):29919-26; Ohta et al., 1984, J. Biol. Chem. 259(23):14888-93 and U.S. Pat. No. 6,372,886).

In certain embodiments, the methods and compositions include the use of a chimeric construction of an apolipoprotein. For example, a chimeric construction of an apolipoprotein can be comprised of an apolipoprotein domain with high lipid binding capacity associated with an apolipoprotein domain containing ischemia reperfusion protective properties. A chimeric construction of an apolipoprotein can be a construction that includes separate regions within an apolipoprotein (i.e., homologous construction) or a chimeric construction can be a construction that includes separate regions between different apolipoproteins (i.e., heterologous constructions). Compositions comprising a chimeric construction can also include segments that are apolipoprotein variants or segments designed to have a specific character (e.g., lipid binding, receptor binding, enzymatic, enzyme activating, antioxidant or reduction-oxidation property) (see Weisgraber 1990, J. Lipid Res. 31(8):1503-11; Hixson and Powers 1991, J. Lipid Res. 32(9):1529-35; Lackner et al., 1985, J. Biol. Chem. 260(2):703-6; Hoeg et al, 1986, J. Biol. Chem. 261(9):3911-4; Gordon et al., 1984, J. Biol. Chem. 259(1):468-74; Powell et al., 1987, Cell 50(6):831-40; Aviram et al., 1998, Arterioscler. Thromb. Vasc. Biol. 18(10):1617-24; Aviram et al., 1998, J. Clin. Invest. 101(8):1581-90; Billecke et al., 2000, Drug Metab. Dispos. 28(11):1335-42; Draganov et al., 2000, J. Biol. Chem. 275(43):33435-42; Steinmetz and Utermann 1985, J. Biol. Chem. 260(4):2258-64; Widler et al., 1980, J. Biol. Chem. 255(21):10464-71; Dyer et al., 1995, J. Lipid Res. 36(1):80-8; Sorenson et al., 1999, Arterioscler. Thromb. Vasc. Biol. 19(9):2214-25; Palgunachari 1996, Arterioscler. Throb. Vasc. Biol. 16(2):328-38: Thurberg et al., J. Biol. Chem. 271(11):6062-70; Dyer 1991, J. Biol. Chem. 266(23):150009-15; Hill 1998, J. Biol. Chem. 273(47):30979-84).

Apolipoproteins utilized also include recombinant, synthetic, semi-synthetic or purified apolipoproteins. Methods for obtaining apolipoproteins or equivalents thereof are well-known in the art. For example, apolipoproteins can be separated from plasma or natural products by, for example, density gradient centrifugation or immunoaffinity chromatography, or produced synthetically, semi-synthetically or using recombinant DNA techniques known to those of the art (see, e.g., Mulugeta et al., 1998, J. Chromatogr. 798(1-2): 83-90; Chung et al., 1980, J. Lipid Res. 21(3):284-91; Cheung et al., 1987, J. Lipid Res. 28(8):913-29; Persson, et al., 1998, J.

Chromatogr. 711:97-109; U.S. Pat. Nos. 5,059,528, 5,834,596, 5,876,968 and 5,721,114; and PCT Publications WO 86/04920 and WO 87/02062).

Apolipoproteins further include apolipoprotein agonists such as peptides and peptide analogues that mimic the activity of ApoA-I, ApoA-I Milano (ApoA-I_M), ApoA-I Paris (ApoA-I_P), ApoA-II, ApoA-IV, and ApoE. For example, the apolipoprotein can be any of those described in U.S. Pat. Nos. 6,004,925, 6,037,323, 6,046,166, and 5,840,688, the contents of which are incorporated herein by reference in their entireties.

Apolipoprotein agonist peptides or peptide analogues can be synthesized or manufactured using any technique for peptide synthesis known in the art including, e.g., the techniques described in U.S. Pat. Nos. 6,004,925, 6,037,323 and 6,046,166. For example, the peptides may be prepared using the solid-phase synthetic technique initially described by Merrifield (1963, J. Am. Chem. Soc. 85:2149-2154). Other peptide synthesis techniques may be found in Bodanszky et al., Peptide Synthesis, John Wiley & Sons, 2d Ed., (1976) and other references readily available to those skilled in the art. A summary of polypeptide synthesis techniques can be found in Stuart and Young, Solid Phase Peptide. Synthesis, Pierce Chemical Company, Rockford, Ill., (1984). Peptides may also be synthesized by solution methods as described in The Proteins, Vol. II, 3d Ed., Neurath et. al., Eds., p. 105-237, Academic Press, New York, N.Y. (1976). Appropriate protective groups for use in different peptide syntheses are described in the above-mentioned texts as well as in McOmie, Protective Groups in Organic Chemistry, Plenum Press, New York, N.Y. (1973). The peptides might also be prepared by chemical or enzymatic cleavage from larger portions of, for example, apolipoprotein A-I.

In certain embodiments, the apolipoprotein can be a mixture of apolipoproteins. In one embodiment, the apolipoprotein can be a homogeneous mixture, that is, a single type of apolipoprotein. In another embodiment, the apolipoprotein can be a heterogeneous mixture of apolipoproteins, that is, a mixture of two or more different apolipoproteins. Embodiments of heterogenous mixtures of apolipoproteins can comprise, for example, a mixture of an apolipoprotein from an animal source and an apolipoprotein from a semi-synthetic source. In certain embodiments, a heterogenous mixture can comprise, for example, a mixture of ApoA-I and ApoA-I Milano. In certain embodiments, a heterogeneous mixture can comprise, for example, a mixture of ApoA-I Milano and ApoA-I Paris. Suitable mixtures for use in the methods and compositions descreibed herein will be apparent to one of skill in the art.

If the apolipoprotein is obtained from natural sources, it can be obtained from a plant or animal source. If the apolipoprotein is obtained from an animal source, the apolipoprotein can be from any species. In certain embodiments, the apolipoprotien can be obtained from an animal source. In certain embodiments, the apolipoprotein can be obtained from a human source. In preferred embodiments, the apolipoprotein is derived from the same species as the individual to which the apolipoprotein is administered.

Lipid particles

Lipid particles can include one or more of the cationic lipids described above. Lipid particles include, but are not limited to, liposomes. As used herein, a liposome is a structure having lipid-containing membranes enclosing an aqueous interior. Liposomes may have one or more lipid membranes. Liposomes can be single-layered, referred to as unilamellar, or multi-layered, referred to as multilamellar. When complexed with nucleic acids, lipid particles may also be lipoplexes, which are composed of cationic lipid bilayers sandwiched between DNA layers, as described, *e.g.*, in Felgner, *Scientific American*.

The lipid particles may further comprise one or more additional lipids and/or other components such as cholesterol. Other lipids may be included in the liposome compositions for a variety of purposes, such as to prevent lipid oxidation or to attach ligands onto the liposome surface. Any of a number of lipids may be present in liposomes, including amphipathic, neutral, cationic, and anionic lipids. Such lipids can be used alone or in combination. Specific examples of additional lipid components that may be present are described below.

Additional components that may be present in a lipid particle include bilayer stabilizing components such as polyamide oligomers (*see, e.g.*, U.S. Patent No. 6,320,017, which is incorporated by reference in its entirety), peptides, proteins, detergents, lipid-derivatives, such as PEG coupled to phosphatidylethanolamine and PEG conjugated to ceramides (*see*, U.S. Patent No. 5,885,613, which is incorporated by reference in its entirety).

In particular embodiments, the lipid particles include one or more of a second amino lipid or cationic lipid, a neutral lipid, a sterol, and a lipid selected to reduce aggregation of lipid particles during formation, which may result from steric stabilization of particles which prevents charge-induced aggregation during formation.

Lipid particles can include two or more cationic lipids. The lipids can be selected to contribute different advantageous properties. For example, cationic lipids that differ in properties such as amine pK_a, chemical stability, half-life in circulation, half-life in tissue, net accumulation in tissue, or toxicity can be used in a lipid particle. In particular, the cationic lipids can be chosen so that the properties of the mixed-lipid particle are more desireable than the properties of a single-lipid particle of individual lipids.

Net tissue accumulation and long term toxicity (if any) from the cationic lipids can be modulated in a favorable way by choosing mixtures of cationic lipids instead of selecting a single cationic lipid in a given formulation. Such mixtures can also provide better encapsulation and/or release of the drug. A combination of cationic lipids also can affect the systemic stability when compared to single entity in a formulation.

In one example, a series of structurally similar compounds can have varying pK_a values that span a range, e.g. of less than 1 pK_a unit, from 1 to 2 pK_a units, or a range of more than 2 pK_a units. Within the series, it may be found that a pK_a in the middle of the range is associated with an enhancement of advantageous properties (greater effectiveness) or a decrease in disadvantageous properties (e.g., reduced toxicity), compared to compounds having pK_a values toward the ends of the range. In such a case, two (or more) different compounds having pK_a values toward opposing ends of the range can be selected for use together in a lipid particle. In this way, the net properties of the lipid particle (for instance, charge as a function of local pH) can be closer to that of a particle including a single lipid from the middle of the range. Cationic lipids that are structurally dissimilar (for example, not part of the series of structurally similar compounds mentioned above) can also be used in a mixed-lipid particle.

In some cases, two or more different cationic lipids may have widely differing pK_a values, e.g., differing by 3 or more pK_a units. In this case, the net behavior of a mixed lipid particle will not necessarily mimic that of a single-lipid particle having an intermediate pK_a. Rather, the net behavior may be that of a particle having two distinct protonatable (or deprotonatable, as the case may be) site with different pK_a values. In the case of a single lipid, the fraction of protonatable sites that are in fact protonated varies sharply as the pH moves from below the pK_a to above the pK_a (when the pH is equal to the pK_a value, 50% of the sites are protonated). When two or more different cationic lipids may have widely differing pK_a values

(e.g., differing by 3 or more pK_a units) are combined in a lipid particle, the lipid particle can show a more gradual transition from non-protonated to protonated as the pH is varied.

In other examples, two or more lipids may be selected based on other considerations. For example, if one lipid by itself is highly effective but moderately toxic, it might be combined with a lipid that is less effective but non-toxic. In some cases, the combination can remain highly effective but have a greatly reduced toxicity, even where it might be predicted that the combination would be only moderately effective and only slightly less toxic.

The selection may be guided by a measured value of an experimentally determinable characteristic, e.g., a characteristic tha can be assigned a numerical value from the results of an experiment. Experimentally determinable characteristics can include a measure of safety, a measure of efficacy, a measure of interaction with a predetermined biomolecule, or pK_a.

A measure of safety might include a survival rate, an LD_{50}, or a level of a biomarker (such as a serum biomarker) associated with tissue damage (e.g., liver enzymes for liver; CPK for muscle; ionic balance for kidney). A measure of efficacy can be any measurement that indicates whether a therapeutic agent is producing an effect; particularly, whether and/or to what degree it is producing a desired effect, such as treating, preventing, ameliorating, or otherwise improving a disease, disorder, or other clinical condition. The measure of efficacy can be an indirect measure; for example, if a therapeutic agent is intended to produce a particular effect at a cellular level, measurements of that effect on cell cultures can be a measure of efficacy. A measure of interaction with predetermined biomolecules can include a K_d for binding to a particular protein or a measure of the character, degree or extent of interaction with other lipids, including cellular substructures such as cell membranes, endosomal membranes, nuclear membranes, and the like.

The cationic lipids can be selected on the basis of mechanism of action, e.g., whether, under what conditions, or to what extent the lipids interact with predetermined biomolecules. For example, a first cationic lipid can be chosen, in part, because it is associated with an ApoE-dependent mechanism; a second cationic lipid can be chosen, in part, because it is associated with an ApoE-independent mechanism.

For example, a lipid particle can contain a mixture of the cationic lipids described in, e.g., WO 2009/086558, and provisional U.S. Application No. 61/104,219, filed October 9, 2008 (each of which is incorporated by reference in its entirety), and ester analogs thereof. In another

example, a lipid particle can contain a mixture of a lipid, for example, Lipid A, described in PCT/US10/22614, filed January 29, 2010 and a lipid, for example, the lipid of formula V or formula VI, described in US Provisional Application 61/175,770, filed May 5, 2009..

Examples of lipids that reduce aggregation of particles during formation include polyethylene glycol (PEG)-modified lipids, monosialoganglioside Gm1, and polyamide oligomers ("PAO") such as (described in U.S. Pat. No. 6,320,017, which is incorporated by reference in its entirety). Other compounds with uncharged, hydrophilic, steric-barrier moieties, which prevent aggregation during formulation, like PEG, Gm1 or ATTA, can also be coupled to lipids. ATTA-lipids are described, *e.g.*, in U.S. Patent No. 6,320,017, and PEG-lipid conjugates are described, *e.g.*, in U.S. Patent Nos. 5,820,873, 5,534,499 and 5,885,613, each of which is incorporated by reference in its entirety. Typically, the concentration of the lipid component selected to reduce aggregation is about 1 to 15% (by mole percent of lipids).

Specific examples of PEG-modified lipids (or lipid-polyoxyethylene conjugates) that can have a variety of "anchoring" lipid portions to secure the PEG portion to the surface of the lipid vesicle include PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-ceramide conjugates (*e.g.*, PEG-CerC14 or PEG-CerC20) which are described in U.S. Patent No. 5,820,873, incorporated herein by reference, PEG-modified dialkylamines and PEG-modified 1,2-diacyloxypropan-3-amines. Particularly preferred are PEG-modified diacylglycerols and dialkylglycerols.

In embodiments where a sterically-large moiety such as PEG or ATTA are conjugated to a lipid anchor, the selection of the lipid anchor depends on what type of association the conjugate is to have with the lipid particle. It is well known that mPEG (mw2000)-diastearoylphosphatidylethanolamine (PEG-DSPE) will remain associated with a liposome until the particle is cleared from the circulation, possibly a matter of days. Other conjugates, such as PEG-CerC20 have similar staying capacity. PEG-CerC14, however, rapidly exchanges out of the formulation upon exposure to serum, with a $T_{1/2}$ less than 60 min in some assays. As illustrated in U.S. Patent No. 5,820,873, at least three characteristics influence the rate of exchange: length of acyl chain, saturation of acyl chain, and size of the steric-barrier head group. Compounds having suitable variations of these features may be useful. For some therapeutic applications it may be preferable for the PEG-modified lipid to be rapidly lost from the nucleic acid-lipid particle *in vivo* and hence the PEG-modified lipid will possess relatively

short lipid anchors. In other therapeutic applications it may be preferable for the nucleic acid-lipid particle to exhibit a longer plasma circulation lifetime and hence the PEG-modified lipid will possess relatively longer lipid anchors.

It should be noted that aggregation preventing compounds do not necessarily require lipid conjugation to function properly. Free PEG or free ATTA in solution may be sufficient to prevent aggregation. If the particles are stable after formulation, the PEG or ATTA can be dialyzed away before administration to a subject.

Neutral lipids, when present in the lipid particle, can be any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form at physiological pH. Such lipids include, for example diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, dihydrosphingomyelin, cephalin, and cerebrosides. The selection of neutral lipids for use in the particles described herein is generally guided by consideration of, *e.g.*, liposome size and stability of the liposomes in the bloodstream. Preferably, the neutral lipid component is a lipid having two acyl groups, (*i.e.,* diacylphosphatidylcholine and diacylphosphatidylethanolamine). Lipids having a variety of acyl chain groups of varying chain length and degree of saturation are available or may be isolated or synthesized by well-known techniques. In one group of embodiments, lipids containing saturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are preferred. In another group of embodiments, lipids with mono or diunsaturated fatty acids with carbon chain lengths in the range of C_{10} to C_{20} are used. Additionally, lipids having mixtures of saturated and unsaturated fatty acid chains can be used. Preferably, the neutral lipids used are DOPE, DSPC, POPC, DPPC or any related phosphatidylcholine. The neutral lipids may also be composed of sphingomyelin, dihydrosphingomyeline, or phospholipids with other head groups, such as serine and inositol.

The sterol component of the lipid mixture, when present, can be any of those sterols conventionally used in the field of liposome, lipid vesicle or lipid particle preparation. A preferred sterol is cholesterol.

Other cationic lipids, which carry a net positive charge at about physiological pH, in addition to those specifically described above, may also be included in lipid particles. Such cationic lipids include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleyloxy)propyl-N,N-N-triethylammonium chloride ("DOTMA"); N,N-distearyl-N,N-dimethylammonium bromide ("DDAB");

N-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTAP"); 1,2-Dioleyloxy-3-trimethylaminopropane chloride salt ("DOTAP.Cl"); 3β-(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol ("DC-Chol"), N-(1-(2,3-dioleyloxy)propyl)-N-2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoracetate ("DOSPA"), dioctadecylamidoglycyl carboxyspermine ("DOGS"), 1,2-dileoyl-sn-3-phosphoethanolamine ("DOPE"), 1,2-dioleoyl-3-dimethylammonium propane ("DODAP"), N, N-dimethyl-2,3-dioleyloxy)propylamine ("DODMA"), and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE"). 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), and LIPOFECTAMINE (comprising DOSPA and DOPE, available from GIBCO/BRL). In particular embodiments, a cationic lipid is an amino lipid.

Anionic lipids suitable for use in lipid particles include, but are not limited to, phosphatidylglycerol, cardiolipin, diacylphosphatidylserine, diacylphosphatidic acid, N-dodecanoyl phosphatidylethanoloamine, N-succinyl phosphatidylethanolamine, N-glutaryl phosphatidylethanolamine, lysylphosphatidylglycerol, and other anionic modifying groups joined to neutral lipids.

In numerous embodiments, amphipathic lipids are included in lipid particles. "Amphipathic lipids" refer 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. Such compounds include, but are not limited to, phospholipids, aminolipids, and sphingolipids. Representative phospholipids include sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid, palmitoyloleoyl phosphatdylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, distearoylphosphatidylcholine, or dilinoleoylphosphatidylcholine. Other phosphorus-lacking compounds, such as sphingolipids, glycosphingolipid families, diacylglycerols, and β-acyloxyacids, can also be used. Additionally, such amphipathic lipids can be readily mixed with other lipids, such as triglycerides and sterols.

Also suitable for inclusion in the lipid particles are programmable fusion lipids or fusion-promoting lipid. Such lipid particles have little tendency to fuse with cell membranes and deliver their payload until a given signal event occurs. This allows the lipid particle to distribute

more evenly after injection into an organism or disease site before it starts fusing with cells. The signal event can be, for example, a change in pH, temperature, ionic environment, or time. The fusion promoting-lipids can be, for example, compounds of formula (I) as described above. In some cases, the signal event can be a change in pH, for example, such as the difference in pH between an extracelluar environment and an intracellular environment, or between an intracellular environment and an endosomal environment.

When time is the signal event, a fusion delaying or "cloaking" component, such as an ATTA-lipid conjugate or a PEG-lipid conjugate, can simply exchange out of the lipid particle membrane over time. By the time the lipid particle is suitably distributed in the body, it has lost sufficient cloaking agent so as to be fusogenic. With other signal events, it can be desirable to choose a signal that is associated with the disease site or target cell, such as increased temperature at a site of inflammation.

In certain embodiments, it is desirable to target the lipid particles using targeting moieties that are specific to a cell type or tissue. Targeting of lipid particles using a variety of targeting moieties, such as ligands, cell surface receptors, glycoproteins, vitamins (*e.g.,* riboflavin) and monoclonal antibodies, has been previously described (*see, e.g.*, U.S. Patent Nos. 4,957,773 and 4,603,044, each of which is incorporated by reference in its entirety). The targeting moieties can comprise the entire protein or fragments thereof. Targeting mechanisms generally require that the targeting agents be positioned on the surface of the lipid particle in such a manner that the target moiety is available for interaction with the target, for example, a cell surface receptor. A variety of different targeting agents and methods are known and available in the art, including those described, *e.g.*, in Sapra, P. and Allen, TM, *Prog. Lipid Res.* 42(5):439-62 (2003); and Abra, RM *et al.*, *J. Liposome Res.* 12:1-3, (2002).

The use of lipid particles, *i.e.*, liposomes, with a surface coating of hydrophilic polymer chains, such as polyethylene glycol (PEG) chains, for targeting has been proposed (Allen, *et al.*, *Biochimica et Biophysica Acta* 1237: 99-108 (1995); DeFrees, *et al.*, *Journal of the American Chemistry Society* 118: 6101-6104 (1996); Blume, *et al.*, *Biochimica et Biophysica Acta* 1149: 180-184 (1993); Klibanov, *et al.*, *Journal of Liposome Research* 2: 321-334 (1992); U.S. Patent No. 5,013556; Zalipsky, *Bioconjugate Chemistry* 4: 296-299 (1993); Zalipsky, *FEBS Letters* 353: 71-74 (1994); Zalipsky, in Stealth Liposomes Chapter 9 (Lasic and Martin, Eds) CRC Press, Boca Raton Fl (1995). In one approach, a ligand, such as an antibody, for targeting the

lipid particle is linked to the polar head group of lipids forming the lipid particle. In another approach, the targeting ligand is attached to the distal ends of the PEG chains forming the hydrophilic polymer coating (Klibanov, *et al.*, *Journal of Liposome Research* 2: 321-334 (1992); Kirpotin *et al.*, *FEBS Letters* 388: 115-118 (1996)).

Standard methods for coupling the target agents can be used. For example, phosphatidylethanolamine, which can be activated for attachment of target agents, or derivatized lipophilic compounds, such as lipid-derivatized bleomycin, can be used. Antibody-targeted liposomes can be constructed using, for instance, liposomes that incorporate protein A (*see*, Renneisen, *et al.*, *J. Bio. Chem.*, 265:16337-16342 (1990) and Leonetti, *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 87:2448-2451 (1990). Other examples of antibody conjugation are disclosed in U.S. Patent No. 6,027,726, the teachings of which are incorporated herein by reference. Examples of targeting moieties can also include other proteins, specific to cellular components, including antigens associated with neoplasms or tumors. Proteins used as targeting moieties can be attached to the liposomes via covalent bonds (*see*, Heath, *Covalent Attachment of Proteins to Liposomes, 149 Methods in Enzymology* 111-119 (Academic Press, Inc. 1987)). Other targeting methods include the biotin-avidin system.

In some embodiments, the lipid particle includes a mixture of a cationic lipid and a fusion-promoting lipid. The lipid particle can further include a neutral lipid, a sterol, a PEG-modified lipid, or a combination of these. For example, the lipid particle can include a cationic lipid, a fusion-promoting lipid, and a neutral lipid, but no sterol or PEG-modified lipid. The lipid particle can include a cationic lipid, a fusion-promoting lipid, and a neutral lipid, but no sterol or PEG-modified lipid. The lipid particle can include a cationic lipid, a fusion-promoting lipid, and a PEG-modified lipid, but no sterol or neutral lipid. The lipid particle can include a cationic lipid, a fusion-promoting lipid, a sterol, and a neutral lipid, but no PEG-modified lipid. The lipid particle can include a cationic lipid, a fusion-promoting lipid, a sterol, and a PEG-modified lipid, but no neutral lipid. The lipid particle can include a cationic lipid, a fusion-promoting lipid, a neutral lipid, and a PEG-modified lipid, but no sterol. The lipid particle can include a cationic lipid, a fusion-promoting lipid, a sterol, neutral lipid, and a PEG-modified lipid.

In one exemplary embodiment, the lipid particle comprises a mixture of a cationic lipid, a fusion-promoting lipid, neutral lipids (other than a cationic lipid), a sterol (*e.g.*, cholesterol) and a PEG-modified lipid (*e.g.*, a PEG-DMG or PEG-DMA). In certain embodiments, the lipid

mixture consists of or consists essentially of a cationic lipid, a fusion-promoting lipid, a neutral lipid, cholesterol, and a PEG-modified lipid. In further preferred embodiments, the lipid particle includes the above lipid mixture in molar ratios of about 20-70% cationic lipid: 0.1-50% fusion promoting lipid: 5-45% neutral lipid: 20-55% cholesterol: 0.5-15% PEG-modified lipid. In some embodiments, the fusion-promoting lipid can be present in a molar ratio of 0.1-50%, 0.5-50%, 1-50%, 5%-45%, 10%-40%, or 15%-35%. In some embodiments, the fusion-promoting lipid can be present in a molar ratio of 0.1-50%, 0.5-50%, 1-50%, 5%-45%, 10%-40%, or 15%-35%. In some embodiments, the fusion-promoting lipid can be present in a molar ratio of 0.1-50%, 10-50%, 20-50%, or 30-50%. In some embodiments, the fusion-promoting lipid can be present in a molar ratio of 0.1-50%, 0.5-45%, 1-40%, 1%-35%, 1%-30%, or 1%-20%.

In further preferred embodiments, the lipid particle consists of or consists essentially of the above lipid mixture in molar ratios of about 20-70% cationic lipid: 0.1-50% fusion promoting lipid: 5-45% neutral lipid: 20-55% cholesterol: 0.5-15% PEG-modified lipid.

In particular embodiments, the lipid particle comprises, consists of, or consists essentially of a mixture of cationic lipids chosen from, for example, those described in application nos. PCT/US09/63933, PCT/US09/63927, PCT/US09/63931, and PCT/US09/63897, each filed November 10, 2009, and applications referred to therein, including nos. 61/104,219, filed October 9, 2008; no. 61/113,179, filed November 10, 2008; no. 61/154,350, filed February 20, 2009; no. 61/171,439, filed April 21, 2009; no. 61/175,770, filed May 5, 2009; no. 61/185,438, filed June 9, 2009; no. 61/225,898, filed July 15, 2009; no. 61/234,098, filed August 14, 2009; and 61/287,995, filed December 18, 2009; WO 2009/086558; and WO 2008/042973 (each of these documents is incorporated by reference in its entirety. See, for example, Tables 1 and 2 of application no. PCT/US09/63933, filed November 10, 2009, at pages 33-51, and Tables 1-4 and 9 of 61/287,995, at pages 28-53 and 135-141), DSPC, Chol, and either PEG-DMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% cationic lipid: 0.1-50% fusion-promoting lipid: 5-25% DSPC :25-55% Chol:0.5-15% PEG-DMG or PEG-DMA. In particular embodiments, the molar lipid ratio, with regard to mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA) is approximately 40/10/40/10, 35/15/40/10 or 52/13/30/5; this mixture is further combined with a fusion-promoting lipid in a molar ratio of 0.1-50%, 0.1-50%, 0.5-50%, 1-50%, 5%-45%, 10%-40%, or 15%-35%; in other words, when a 40/10/40/10 mixture of lipid/DSPC/Chol/PEG-DMG or PEG-DMA is combined with a fusion-promoting peptide in a

molar ratio of 50%, the resulting lipid particles can have a total molar ratio of (mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA/fusion-promoting peptide) 20/5/20/5/50. In another group of embodiments, the neutral lipid, DSPC, in these compositions is replaced with POPC, DPPC, DOPE or SM.

Therapeutic Agent-Lipid Particle Compositions and Formulations

Compositions that include a lipid particle and an active agent, where the active agent is associated with the lipid particle, are provided. In particular embodiments, the active agent is a therapeutic agent. In particular embodiments, the active agent is encapsulated within an aqueous interior of the lipid particle. In other embodiments, the active agent is present within one or more lipid layers of the lipid particle. In other embodiments, the active agent is bound to the exterior or interior lipid surface of a lipid particle.

"Fully encapsulated" as used herein indicates that the nucleic acid in the particles is not significantly degraded after exposure to serum or a nuclease assay that would significantly degrade free nucleic acids. In a fully encapsulated system, preferably less than 25% of particle nucleic acid is degraded in a treatment that would normally degrade 100% of free nucleic acid, more preferably less than 10% and most preferably less than 5% of the particle nucleic acid is degraded. Alternatively, full encapsulation may be determined by an Oligreen® assay. Oligreen® is an ultra-sensitive fluorescent nucleic acid stain for quantitating oligonucleotides and single-stranded DNA in solution (available from Invitrogen Corporation, Carlsbad, CA). Fully encapsulated also suggests that the particles are serum stable, that is, that they do not rapidly decompose into their component parts upon *in vivo* administration.

Active agents, as used herein, include any molecule or compound capable of exerting a desired effect on a cell, tissue, organ, or subject. Such effects may be biological, physiological, or cosmetic, for example. Active agents may be any type of molecule or compound, including *e.g.*, nucleic acids, peptides and polypeptides, including, *e.g.*, antibodies, such as, *e.g.*, polyclonal antibodies, monoclonal antibodies, antibody fragments; humanized antibodies, recombinant antibodies, recombinant human antibodies, and Primatized™ antibodies, cytokines, growth factors, apoptotic factors, differentiation-inducing factors, cell surface receptors and their ligands; hormones; and small molecules, including small organic molecules or compounds.

In one embodiment, the active agent is a therapeutic agent, or a salt or derivative thereof. Therapeutic agent derivatives may be therapeutically active themselves or they may be prodrugs, which become active upon further modification. Thus, in one embodiment, a therapeutic agent derivative retains some or all of the therapeutic activity as compared to the unmodified agent, while in another embodiment, a therapeutic agent derivative lacks therapeutic activity.

In various embodiments, therapeutic agents include any therapeutically effective agent or drug, such as anti-inflammatory compounds, anti-depressants, stimulants, analgesics, antibiotics, birth control medication, antipyretics, vasodilators, anti-angiogenics, cytovascular agents, signal transduction inhibitors, cardiovascular drugs, *e.g.*, anti-arrhythmic agents, vasoconstrictors, hormones, and steroids.

In certain embodiments, the therapeutic agent is an oncology drug, which may also be referred to as an anti-tumor drug, an anti-cancer drug, a tumor drug, an antineoplastic agent, or the like. Examples of oncology drugs that may be used include, but are not limited to, adriamycin, alkeran, allopurinol, altretamine, amifostine, anastrozole, araC, arsenic trioxide, azathioprine, bexarotene, biCNU, bleomycin, busulfan intravenous, busulfan oral, capecitabine (Xeloda), carboplatin, carmustine, CCNU, celecoxib, chlorambucil, cisplatin, cladribine, cyclosporin A, cytarabine, cytosine arabinoside, daunorubicin, cytoxan, daunorubicin, dexamethasone, dexrazoxane, dodetaxel, doxorubicin, doxorubicin, DTIC, epirubicin, estramustine, etoposide phosphate, etoposide and VP-16, exemestane, FK506, fludarabine, fluorouracil, 5-FU, gemcitabine (Gemzar), gemtuzumab-ozogamicin, goserelin acetate, hydrea, hydroxyurea, idarubicin, ifosfamide, imatinib mesylate, interferon, irinotecan (Camptostar, CPT-111), letrozole, leucovorin, leustatin, leuprolide, levamisole, litretinoin, megastrol, melphalan, L-PAM, mesna, methotrexate, methoxsalen, mithramycin, mitomycin, mitoxantrone, nitrogen mustard, paclitaxel, pamidronate, Pegademase, pentostatin, porfimer sodium, prednisone, rituxan, streptozocin, STI-571, tamoxifen, taxotere, temozolamide, teniposide, VM-26, topotecan (Hycamtin), toremifene, tretinoin, ATRA, valrubicin, velban, vinblastine, vincristine, VP16, and vinorelbine. Other examples of oncology drugs that may be used are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors and camptothecins.

Nucleic Acid-Lipid Particles

In certain embodiments, lipid particles are associated with a nucleic acid, resulting in a nucleic acid-lipid particle. In particular embodiments, the nucleic acid is fully encapsulated in the lipid particle. As used herein, the term "nucleic acid" is meant to include any oligonucleotide or polynucleotide. Fragments containing up to 50 nucleotides are generally termed oligonucleotides, and longer fragments are called polynucleotides. In particular embodiments, oligonucletoides are 15-50 nucleotides in length.

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 includes 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 and increased stability in the presence of nucleases.

The nucleic acid that is present in a lipid-nucleic acid particle includes any form of nucleic acid that is known. The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Examples of double-stranded DNA include structural genes, genes including control and termination regions, and self-replicating systems such as viral or plasmid DNA. Examples of double-stranded RNA include siRNA and other RNA interference reagents. Single-stranded nucleic acids include, *e.g.*, antisense oligonucleotides, ribozymes, microRNA, and triplex-forming oligonucleotides. The nucleic acid that is present in a lipid-nucleic acid particle may include one or more of the oligonucleotide modifications described below.

Nucleic acids may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to 100,000 nucleotide residues in length. In particular embodiments, oligonucleotides may range from about 10 to 100 nucleotides in length. In various related embodiments, oligonucleotides, single-stranded, double-stranded, and triple-stranded, may range in length from about 10 to about 50 nucleotides, from about 20 o about 50 nucleotides, from about 15 to about 30 nucleotides, from about 20 to about 30 nucleotides in length.

In particular embodiments, the oligonucleotide (or a strand thereof) specifically hybridizes to or is complementary to a target polynucleotide. "Specifically hybridizable" and "complementary" are terms which are used to 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. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule 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, in other embodiments, this oligonucleotide includes 1, 2, or 3 base substitutions, e.g. mismatches, as compared to the region of a gene or mRNA sequence that it is targeting or to which it specifically hybridizes.

RNA Interference Nucleic Acids

In particular embodiments, nucleic acid-lipid particles are associated with RNA interference (RNAi) molecules. RNA interference methods using RNAi molecules may be used to disrupt the expression of a gene or polynucleotide of interest. Small interfering RNA (siRNA) has essentially replaced antisense ODN and ribozymes as the next generation of targeted oligonucleotide drugs under development.

SiRNAs are RNA duplexes normally 16-30 nucleotides long that can associate with a cytoplasmic multi-protein complex known as RNAi-induced silencing complex (RISC). RISC loaded with siRNA mediates the degradation of homologous mRNA transcripts, therefore siRNA can be designed to knock down protein expression with high specificity. Unlike other antisense technologies, siRNA function through a natural mechanism evolved to control gene expression through non-coding RNA. This is generally considered to be the reason why their activity is more potent *in vitro* and *in vivo* than either antisense ODN or ribozymes. A variety of RNAi reagents, including siRNAs targeting clinically relevant targets, are currently under pharmaceutical development, as described, *e.g.*, in de Fougerolles, A. *et al.*, Nature Reviews 6:443-453 (2007), which is incorporated by reference in its entirety.

While the first described RNAi molecules were RNA:RNA hybrids comprising both an RNA sense and an RNA antisense strand, it has now been demonstrated that DNA sense:RNA antisense hybrids, RNA sense:DNA antisense hybrids, and DNA:DNA hybrids are capable of mediating RNAi (Lamberton, J.S. and Christian, A.T., (2003) Molecular Biotechnology 24:111-119). Thus, the use of RNAi molecules comprising any of these different types of double-stranded molecules is contemplated. In addition, it is understood that RNAi molecules may be used and introduced to cells in a variety of forms. Accordingly, as used herein, RNAi molecules encompasses any and all molecules capable of inducing an RNAi response in cells, including, but not limited to, double-stranded oligonucleotides comprising two separate strands, *i.e.* a sense strand and an antisense strand, *e.g.*, small interfering RNA (siRNA); double-stranded oligonucleotide comprising two separate strands that are linked together by non-nucleotidyl linker; oligonucleotides comprising a hairpin loop of complementary sequences, which forms a double-stranded region, *e.g.*, shRNAi molecules, and expression vectors that express one or more polynucleotides capable of forming a double-stranded polynucleotide alone or in combination with another polynucleotide.

A "single strand siRNA compound" as used herein, is an siRNA compound which is made up of a single molecule. It may include a duplexed region, formed by intra-strand pairing, *e.g.*, it may be, or include, a hairpin or pan-handle structure. Single strand siRNA compounds may be antisense with regard to the target molecule

A single strand siRNA compound may be sufficiently long that it can enter the RISC and participate in RISC mediated cleavage of a target mRNA. A single strand siRNA compound is at least 14, and in other embodiments at least 15, 20, 25, 29, 35, 40, or 50 nucleotides in length. In certain embodiments, it is less than 200, 100, or 60 nucleotides in length.

Hairpin siRNA compounds will have a duplex region equal to or at least 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs. The duplex region will may be equal to or less than 200, 100, or 50, in length. In certain embodiments, ranges for the duplex region are 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length. The hairpin may have a single strand overhang or terminal unpaired region. In certain embodiments, the overhangs are 2-3 nucleotides in length. In some embodiments, the overhang is at the sense side of the hairpin and in some embodiments on the antisense side of the hairpin.

A "double stranded siRNA compound" as used herein, is an siRNA compound which includes more than one, and in some cases two, strands in which interchain hybridization can form a region of duplex structure.

The antisense strand of a double stranded siRNA compound may be equal to or at least, 14, 15, 16 17, 18, 19, 25, 29, 40, or 60 nucleotides in length. It may be equal to or less than 200, 100, or 50, nucleotides in length. Ranges may be 17 to 25, 19 to 23, and 19 to21 nucleotides in length. As used herein, term "antisense strand" means the strand of an siRNA compound that is sufficiently complementary to a target molecule, e.g. a target RNA.

The sense strand of a double stranded siRNA compound may be equal to or at least 14, 15, 16 17, 18, 19, 25, 29, 40, or 60 nucleotides in length. It may be equal to or less than 200, 100, or 50, nucleotides in length. Ranges may be 17 to 25, 19 to 23, and 19 to 21 nucleotides in length.

The double strand portion of a double stranded siRNA compound may be equal to or at least, 14, 15, 16 17, 18, 19, 20, 21, 22, 23, 24, 25, 29, 40, or 60 nucleotide pairs in length. It may be equal to or less than 200, 100, or 50, nucleotides pairs in length. Ranges may be 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length.

In many embodiments, the siRNA compound is sufficiently large that it can be cleaved by an endogenous molecule, *e.g.*, by Dicer, to produce smaller siRNA compounds, *e.g.*, siRNAs agents

The sense and antisense strands may be chosen such that the double-stranded siRNA compound includes a single strand or unpaired region at one or both ends of the molecule. Thus, a double-stranded siRNA compound may contain sense and antisense strands, paired to contain an overhang, *e.g.*, one or two 5' or 3' overhangs, or a 3' overhang of 1 - 3 nucleotides. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. Some embodiments will have at least one 3' overhang. In one embodiment, both ends of an siRNA molecule will have a 3' overhang. In some embodiments, the overhang is 2 nucleotides.

In certain embodiments, the length for the duplexed region is between 15 and 30, or 18, 19, 20, 21, 22, and 23 nucleotides in length, *e.g.*, in the ssiRNA compound range discussed above. ssiRNA compounds can resemble in length and structure the natural Dicer processed products from long dsiRNAs. Embodiments in which the two strands of the ssiRNA compound

are linked, *e.g.*, covalently linked are also included. Hairpin, or other single strand structures which provide the required double stranded region, and a 3' overhang are also contemplated.

The siRNA compounds described herein, including double-stranded siRNA compounds and single-stranded siRNA compounds can mediate silencing of a target RNA, *e.g.*, mRNA, *e.g.*, a transcript of a gene that encodes a protein. For convenience, such mRNA is also referred to herein as mRNA to be silenced. Such a gene is also referred to as a target gene. In general, the RNA to be silenced is an endogenous gene or a pathogen gene. In addition, RNAs other than mRNA, *e.g.*, tRNAs, and viral RNAs, can also be targeted.

As used herein, the phrase "mediates RNAi" refers to the ability to silence, in a sequence specific manner, a target RNA. While not wishing to be bound by theory, it is believed that silencing uses the RNAi machinery or process and a guide RNA, *e.g.*, an ssiRNA compound of 21 to 23 nucleotides.

In one embodiment, an siRNA compound is "sufficiently complementary" to a target RNA, *e.g.*, a target mRNA, such that the siRNA compound silences production of protein encoded by the target mRNA. In another embodiment, the siRNA compound is "exactly complementary" to a target RNA, *e.g.*, the target RNA and the siRNA compound anneal, for example to form a hybrid made exclusively of Watson-Crick base pairs in the region of exact complementarity. A "sufficiently complementary" target RNA can include an internal region (*e.g.*, of at least 10 nucleotides) that is exactly complementary to a target RNA. Moreover, in certain embodiments, the siRNA compound specifically discriminates a single-nucleotide difference. In this case, the siRNA compound only mediates RNAi if exact complementary is found in the region (*e.g.*, within 7 nucleotides of) the single-nucleotide difference.

MicroRNAs

Micro RNAs (miRNAs) are a highly conserved class of small RNA molecules that are transcribed from DNA in the genomes of plants and animals, but are not translated into protein. Processed miRNAs are single stranded ~17-25 nucleotide (nt) RNA molecules that become incorporated into the RNA-induced silencing complex (RISC) and have been identified as key regulators of development, cell proliferation, apoptosis and differentiation. They are believed to play a role in regulation of gene expression by binding to the 3'-untranslated region of specific mRNAs. RISC mediates down-regulation of gene expression through translational inhibition,

transcript cleavage, or both. RISC is also implicated in transcriptional silencing in the nucleus of a wide range of eukaryotes.

The number of miRNA sequences identified to date is large and growing, illustrative examples of which can be found, for example, in: "*miRBase: microRNA sequences, targets and gene nomenclature*" Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. NAR, 2006, 34, Database Issue, D140-D144; "*The microRNA Registry*" Griffiths-Jones S. NAR, 2004, 32, Database Issue, D109-D111; and also at http://microrna.sanger.ac.uk/sequences/.

Antisense Oligonucleotides

In one embodiment, a nucleic acid is an antisense oligonucleotide directed to a target polynucleotide. The term "antisense oligonucleotide" or simply "antisense" is meant to include oligonucleotides that are complementary to a targeted polynucleotide sequence. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence, e.g. a target gene mRNA. Antisense oligonucleotides are thought to inhibit gene expression by binding to a complementary mRNA. Binding to the target mRNA can lead to inhibition of gene expression either by preventing translation of complementary mRNA strands by binding to it, or by leading to degradation of the target mRNA. Antisense DNA can be used to target a specific, complementary (coding or non-coding) RNA. If binding takes places this DNA/RNA hybrid can be degraded by the enzyme RNase H. In particular embodiments, antisense oligonucleotides contain from about 10 to about 50 nucleotides, more preferably about 15 to about 30 nucleotides. The term also encompasses antisense oligonucleotides that may not be exactly complementary to the desired target gene. Thus, instances where non-target specific-activities are found with antisense, or where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use, are contemplated.

Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, can be used to specifically inhibit protein synthesis by a targeted gene. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U. S. Patent 5,739,119 and U. S. Patent 5,759,829 each of which is

incorporated by reference). Further, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDG1), ICAM-1, E-selectin, STK-1, striatal $GABA_A$ receptor and human EGF (Jaskulski *et al.*, Science. 1988 Jun 10;240(4858):1544-6; Vasanthakumar and Ahmed, Cancer Commun. 1989;1(4):225-32; Peris *et al.*, Brain Res Mol Brain Res. 1998 Jun 15;57(2):310-20; U. S. Patent 5,801,154; U.S. Patent 5,789,573; U. S. Patent 5,718,709 and U.S. Patent 5,610,288, each of which is incorporated by reference). Furthermore, antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.* cancer (U. S. Patent 5,747,470; U. S. Patent 5,591,317 and U. S. Patent 5,783,683, each of which is incorporated by reference).

Methods of producing antisense oligonucleotides are known in the art and can be readily adapted to produce an antisense oligonucleotide that targets any polynucleotide sequence. Selection of antisense oligonucleotide sequences specific for a given target sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense oligonucleotides may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.*, Nucleic Acids Res. 1997, 25(17):3389-402).

Antagomirs

Antagomirs are RNA-like oligonucleotides that harbor various modifications for RNAse protection and pharmacologic properties, such as enhanced tissue and cellular uptake. They differ from normal RNA by, for example, complete 2'-*O*-methylation of sugar, phosphorothioate backbone and, for example, a cholesterol-moiety at 3'-end. Antagomirs may be used to efficiently silence endogenous miRNAs by forming duplexes comprising the antagomir and endogenous miRNA, thereby preventing miRNA-induced gene silencing. An example of antagomir-mediated miRNA silencing is the silencing of miR-122, described in Krutzfeldt *et al*, Nature, 2005, 438: 685-689, which is expressly incorporated by reference herein in its entirety.

Antagomir RNAs may be synthesized using standard solid phase oligonucleotide synthesis protocols. See U.S. Patent Application Publication Nos. 2007/0123482 and 2007/0213292 (each of which is incorporated herein by reference).

An antagomir can include ligand-conjugated monomer subunits and monomers for oligonucleotide synthesis. Exemplary monomers are described in U.S. Patent Application Publication No. 2005/0107325, which is incorporated by reference in its entirety. An antagomir can have a ZXY structure, such as is described in WO 2004/080406, which is incorporated by reference in its entirety. An antagomir can be complexed with an amphipathic moiety. Exemplary amphipathic moieties for use with oligonucleotide agents are described in WO 2004/080406, which is incorporated by reference in its entirety.

Aptamers

Aptamers are nucleic acid or peptide molecules that bind to a particular molecule of interest with high affinity and specificity (Tuerk and Gold, Science **249**:505 (1990); Ellington and Szostak, Nature **346**:818 (1990), each of which is incorporated by reference in its entirety). DNA or RNA aptamers have been successfully produced which bind many different entities from large proteins to small organic molecules. See Eaton, Curr. Opin. Chem. Biol. **1**:10-16 (1997), Famulok, Curr. Opin. Struct. Biol. **9**:324-9(1999), and Hermann and Patel, Science **287**:820-5 (2000), each of which is incorporated by reference in its entirety. Aptamers may be RNA or DNA based, and may include a riboswitch. A riboswitch is a part of an mRNA molecule that can directly bind a small target molecule, and whose binding of the target affects the gene's activity. Thus, an mRNA that contains a riboswitch is directly involved in regulating its own activity, depending on the presence or absence of its target molecule. Generally, aptamers are engineered through repeated rounds of *in vitro* selection or equivalently, SELEX (systematic evolution of ligands by exponential enrichment) to bind to various molecular targets such as small molecules, proteins, nucleic acids, and even cells, tissues and organisms. The aptamer may be prepared by any known method, including synthetic, recombinant, and purification methods, and may be used alone or in combination with other aptamers specific for the same target. Further, as described more fully herein, the term "aptamer" specifically includes "secondary aptamers" containing a consensus sequence derived from comparing two or more known aptamers to a given target.

Ribozymes

According to another embodiment, nucleic acid-lipid particles are associated with ribozymes. Ribozymes are RNA molecules complexes having specific catalytic domains that possess endonuclease activity (Kim and Cech, Proc Natl Acad Sci USA. 1987 Dec;84(24):8788-92; Forster and Symons, Cell. 1987 Apr 24;49(2):211-20). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, Cell. 1981 Dec;27(3 Pt 2):487-96; Michel and Westhof, J Mol Biol. 1990 Dec 5;216(3):585-610; Reinhold-Hurek and Shub, Nature. 1992 May 14;357(6374):173-6). This specificity has been attributed to the requirement that the substrate bind via specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

At least six basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif, for example. Specific examples of hammerhead motifs are described by Rossi *et al.* Nucleic Acids Res. 1992 Sep 11;20(17):4559-65. Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz, Biochemistry 1989 Jun 13;28(12):4929-33; Hampel *et al.*, Nucleic Acids Res. 1990 Jan 25;18(2):299-304 and U. S. Patent 5,631,359. An example of the hepatitis δ virus motif is described by Perrotta and Been, Biochemistry. 1992 Dec 1;31(47):11843-52; an example of the

RNaseP motif is described by Guerrier-Takada *et al.*, Cell. 1983 Dec;35(3 Pt 2):849-57; Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, Cell. 1990 May 18;61(4):685-96; Saville and Collins, Proc Natl Acad Sci USA. 1991 Oct 1;88(19):8826-30; Collins and Olive, Biochemistry. 1993 Mar 23;32(11):2795-9); and an example of the Group I intron is described in U. S. Patent 4,987,071. Important characteristics of enzymatic nucleic acid molecules used are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

Methods of producing a ribozyme targeted to any polynucleotide sequence are known in the art. Ribozymes may be designed as described in Int. Pat. Appl. Publ. Nos. WO 93/23569 and WO 94/02595, each specifically incorporated herein by reference, and synthesized to be tested *in vitro* and *in vivo*, as described therein.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Int. Pat. Appl. Publ. Nos. WO 92/07065, WO 93/15187, and WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U.S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

Immunostimulatory Oligonucleotides

Nucleic acids associated with lipid particles may be immunostimulatory, including immunostimulatory oligonucleotides (ISS; single-or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal or other patient. ISS include, *e.g.*, certain palindromes leading to hairpin secondary structures (see Yamamoto S., et al. (1992) J. Immunol. 148: 4072-4076, which is incorporated by reference in its entirety), or CpG motifs, as well as other known ISS features (such as multi-G domains, see WO 96/11266, which is incorporated by reference in its entirety).

The immune response may be an innate or an adaptive immune response. The immune system is divided into a more innate immune system, and acquired adaptive immune system of

vertebrates, the latter of which is further divided into humoral cellular components. In particular embodiments, the immune response may be mucosal.

In particular embodiments, an immunostimulatory nucleic acid is only immunostimulatory when administered in combination with a lipid particle, and is not immunostimulatory when administered in its "free form." Such an oligonucleotide is considered to be immunostimulatory.

Immunostimulatory nucleic acids are considered to be non-sequence specific when it is not required that they specifically bind to and reduce the expression of a target polynucleotide in order to provoke an immune response. Thus, certain immunostimulatory nucleic acids may comprise a seuqence correspondign to a region of a naturally occurring gene or mRNA, but they may still be considered non-sequence specific immunostimulatory nucleic acids.

In one embodiment, the immunostimulatory nucleic acid or oligonucleotide comprises at least one CpG dinucleotide. The oligonucleotide or CpG dinucleotide may be unmethylated or methylated. In another embodiment, the immunostimulatory nucleic acid comprises at least one CpG dinucleotide having a methylated cytosine. In one embodiment, the nucleic acid comprises a single CpG dinucleotide, wherein the cytosine in said CpG dinucleotide is methylated. In a specific embodiment, the nucleic acid comprises the sequence 5' TAACGTTGAGGGGCAT 3'. In an alternative embodiment, the nucleic acid comprises at least two CpG dinucleotides, wherein at least one cytosine in the CpG dinucleotides is methylated. In a further embodiment, each cytosine in the CpG dinucleotides present in the sequence is methylated. In another embodiment, the nucleic acid comprises a plurality of CpG dinucleotides, wherein at least one of said CpG dinucleotides comprises a methylated cytosine.

In one specific embodiment, the nucleic acid comprises the sequence 5' TTCCATGACGTTCCTGACGT 3'. In another specific embodiment, the nucleic acid sequence comprises the sequence 5' TCCATGACGTTCCTGACGT 3', wherein the two cytosines indicated in bold are methylated. In particular embodiments, the ODN is selected from a group of ODNs consisting of ODN #1, ODN #2, ODN #3, ODN #4, ODN #5, ODN #6, ODN #7, ODN #8, and ODN #9, as shown below.

Table 5. Exemplary Immunostimulatory Oligonucleotides (ODNs)

ODN NAME	SEQ ID	ODN SEQUENCE (5'-3')
ODN 1 human c-myc		5'-TAACGTTGAGGGGCAT-3
* ODN 1m		5'-TAAZGTTGAGGGGCAT-3
ODN 2		5'-TCCATGACGTTCCTGACGTT-3
* ODN 2m		5'-TCCATGAZGTTCCTGAZGTT-3
ODN 3		5'-TAAGCATACGGGGTGT-3
ODN 5		5'-AACGTT-3
ODN 6		5'-GATGCTGTGTCGGGGTCTCCGGGC-3'
ODN 7		5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'
ODN 7m		5'-TZGTZGTTTTGTZGTTTTGTZGTT-3'
ODN 8		5'-TCCAGGACTTCTCTCAGGTT-3'
ODN 9		5'-TCTCCCAGCGTGCGCCAT-3'
ODN 10 murine Intracellular Adhesion Molecule-1		5'-TGCATCCCCCAGGCCACCAT-3
ODN 11 human Intracellular Adhesion Molecule-1		5'-GCCCAAGCTGGCATCCGTCA-3'
ODN 12 human Intracellular Adhesion Molecule-1		5'-GCCCAAGCTGGCATCCGTCA-3'
ODN 13 human erb-B-2		5'-GGT GCTCACTGC GGC-3'
ODN 14 human c-myc		5'-AACC GTT GAG GGG CAT-3'
ODN 15 human c-myc		5'-TAT GCT GTG CCG GGG TCT TCG GGC-3'
ODN 16		5'-GTGCCG GGGTCTTCGGGC-3'
ODN 17 human Insulin Growth Factor 1 - Receptor		5'-GGACCCTCCTCCGGAGCC-3'
ODN 18 human Insulin Growth Factor 1 - Receptor		5'-TCC TCC GGA GCC AGA CTT-3'
ODN 19 human Epidermal Growth Factor - Receptor		5'-AAC GTT GAG GGG CAT-3'
ODN 20 Epidermal Growth Factor - Receptor		5'-CCGTGGTCA TGCTCC-3'
ODN 21 human Vascular Endothelial Growth Factor		5'-CAG CCTGGCTCACCG CCTTGG-3'
ODN 22 murine Phosphokinase C - alpha		5'-CAG CCA TGG TTC CCC CCA AC-3'

ODN NAME	SEQ ID	ODN SEQUENCE (5'-3')
ODN 23		5'-GTT CTC GCT GGT GAG TTT CA-3'
ODN 24 human Bcl-2		5'-TCT CCCAGCGTGCGCCAT-3'
ODN 25 human C-Raf-s		5'-GTG CTC CAT TGA TGC-3'
ODN #26 human Vascular Endothelial Growth Factor Receptor-1		5'-GAGUUCUGAUGAGGCCGAAAGG-CCGAAAGUCUG-3'
ODN #27		5'-RRCGYY-3'
ODN # 28		5'-AACGTTGAGGGGCAT-3'
ODN #29		5'-CAACGTTATGGGGAGA-3'
ODN #30 human c-myc		5'-TAACGTTGAGGGGCAT-3'

"Z" represents a methylated cytosine residue. ODN 14 is a 15-mer oligonucleotide and ODN 1 is the same oligonucleotide having a thymidine added onto the 5' end making ODN 1 into a 16-mer. No difference in biological activity between ODN 14 and ODN 1 has been detected and both exhibit similar immunostimulatory activity (Mui *et al.*, 2001)

Additional specific nucleic acid sequences of suitable oligonucleotides (ODNs) are described in Raney *et al.*, Journal of Pharmacology and Experimental Therapeutics, 298:1185-1192 (2001), incorporated by reference in its entirety. In certain embodiments, ODNs used in the compositions and methods of the present invention have a phosphodiester ("PO") backbone or a phosphorothioate ("PS") backbone, and/or at least one methylated cytosine residue in a CpG motif.

Decoy Oligonucleotides

Because transcription factors recognize their relatively short binding sequences, even in the absence of surrounding genomic DNA, short oligonucleotides bearing the consensus binding sequence of a specific transcription factor can be used as tools for manipulating gene expression in living cells. This strategy involves the intracellular delivery of such "decoy oligonucleotides", which are then recognized and bound by the target factor. Occupation of the transcription factor's DNA-binding site by the decoy renders the transcription factor incapable of subsequently binding to the promoter regions of target genes. Decoys can be used as therapeutic agents, either to inhibit the expression of genes that are activated by a transcription factor, or to upregulate genes that are suppressed by the binding of a transcription factor. Examples of the utilization of

decoy oligonucleotides may be found in Mann et al., J. Clin. Invest., 2000, 106: 1071-1075, which is expressly incorporated by reference herein, in its entirety.

Supermir

A supermir refers to a single stranded, double stranded or partially double stranded oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or both or modifications thereof, which has a nucleotide sequence that is substantially identical to an miRNA and that is antisense with respect to its target. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages and which contain at least one non-naturally-occurring portion which functions similarly. Such modified or substituted oligonucleotides are preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases. In a preferred embodiment, the supermir does not include a sense strand, and in another preferred embodiment, the supermir does not self-hybridize to a significant extent. A supermir can have secondary structure, but it is substantially single-stranded under physiological conditions. An supermir that is substantially single-stranded is single-stranded to the extent that less than about 50% (*e.g.*, less than about 40%, 30%, 20%, 10%, or 5%) of the supermir is duplexed with itself. The supermir can include a hairpin segment, e.g., sequence, preferably at the 3' end can self hybridize and form a duplex region, e.g., a duplex region of at least 1, 2, 3, or 4 and preferably less than 8, 7, 6, or n nucleotides, e.g., 5 nuclotides. The duplexed region can be connected by a linker, e.g., a nucleotide linker, e.g., 3, 4, 5, or 6 dTs, e.g., modified dTs. In another embodiment the supermir is duplexed with a shorter oligo, e.g., of 5, 6, 7, 8, 9, or 10 nucleotides in length, e.g., at one or both of the 3' and 5' end or at one end and in the non-terminal or middle of the supermir.

miRNA mimics

miRNA mimics represent a class of molecules that can be used to imitate the gene silencing ability of one or more miRNAs. Thus, the term "microRNA mimic" refers to synthetic non-coding RNAs (i.e. the miRNA is not obtained by purification from a source of the endogenous miRNA) that are capable of entering the RNAi pathway and regulating gene expression. miRNA mimics can be designed as mature molecules (e.g. single stranded) or mimic

precursors (e.g., pri- or pre-miRNAs). miRNA mimics can be comprised of nucleic acid (modified or modified nucleic acids) including oligonucleotides comprising, without limitation, RNA, modified RNA, DNA, modified DNA, locked nucleic acids, or 2'-O,4'-C-ethylene-bridged nucleic acids (ENA), or any combination of the above (including DNA-RNA hybrids). In addition, miRNA mimics can comprise conjugates that can affect delivery, intracellular compartmentalization, stability, specificity, functionality, strand usage, and/or potency. In one design, miRNA mimics are double stranded molecules (e.g., with a duplex region of between about 16 and about 31 nucleotides in length) and contain one or more sequences that have identity with the mature strand of a given miRNA. Modifications can comprise 2' modifications (including 2'-O methyl modifications and 2' F modifications) on one or both strands of the molecule and internucleotide modifications (e.g. phorphorthioate modifications) that enhance nucleic acid stability and/or specificity. In addition, miRNA mimics can include overhangs. The overhangs can consist of 1-6 nucleotides on either the 3' or 5' end of either strand and can be modified to enhance stability or functionality. In one embodiment, a miRNA mimic comprises a duplex region of between 16 and 31 nucleotides and one or more of the following chemical modification patterns: the sense strand contains 2'-O-methyl modifications of nucleotides 1 and 2 (counting from the 5' end of the sense oligonucleotide), and all of the Cs and Us; the antisense strand modifications can comprise 2' F modification of all of the Cs and Us, phosphorylation of the 5' end of the oligonucleotide, and stabilized internucleotide linkages associated with a 2 nucleotide 3' overhang.

Antimir or miRNA inhibitor

The terms "antimir," "microRNA inhibitor," "miR inhibitor," or "inhibitor," are synonymous and refer to oligonucleotides or modified oligonucleotides that interfere with the ability of specific miRNAs. In general, the inhibitors are nucleic acid or modified nucleic acids in nature including oligonucleotides comprising RNA, modified RNA, DNA, modified DNA, locked nucleic acids (LNAs), or any combination of the above. Modifications include 2' modifications (including 2'-0 alkyl modifications and 2' F modifications) and internucleotide modifications (e.g. phosphorothioate modifications) that can affect delivery, stability, specificity, intracellular compartmentalization, or potency. In addition, miRNA inhibitors can comprise conjugates that can affect delivery, intracellular compartmentalization, stability, and/or potency.

Inhibitors can adopt a variety of configurations including single stranded, double stranded (RNA/RNA or RNA/DNA duplexes), and hairpin designs, in general, microRNA inhibitors comprise contain one or more sequences or portions of sequences that are complementary or partially complementary with the mature strand (or strands) of the miRNA to be targeted, in addition, the miRNA inhibitor may also comprise additional sequences located 5' and 3' to the sequence that is the reverse complement of the mature miRNA. The additional sequences may be the reverse complements of the sequences that are adjacent to the mature miRNA in the pri-miRNA from which the mature miRNA is derived, or the additional sequences may be arbitrary sequences (having a mixture of A, G, C, or U). In some embodiments, one or both of the additional sequences are arbitrary sequences capable of forming hairpins. Thus, in some embodiments, the sequence that is the reverse complement of the miRNA is flanked on the 5' side and on the 3' side by hairpin structures. Micro-RNA inhibitors, when double stranded, may include mismatches between nucleotides on opposite strands. Furthermore, micro-RNA inhibitors may be linked to conjugate moieties in order to facilitate uptake of the inhibitor into a cell. For example, a micro-RNA inhibitor may be linked to cholesteryl 5-(bis(4-methoxyphenyl)(phenyl)methoxy)-3 hydroxypentylcarbamate) which allows passive uptake of a micro-RNA inhibitor into a cell. Micro-RNA inhibitors, including hairpin miRNA inhibitors, are described in detail in Vermeulen et al., "Double-Stranded Regions Are Essential Design Components Of Potent Inhibitors of RISC Function," RNA 13: 723-730 (2007) and in WO2007/095387 and WO 2008/036825 each of which is incorporated herein by reference in its entirety. A person of ordinary skill in the art can select a sequence from the database for a desired miRNA and design an inhibitor useful for the methods disclosed herein.

U1 adaptor

U1 adaptor inhibit polyA sites and are bifunctional oligonucleotides with a target domain complementary to a site in the target gene's terminal exon and a 'U1 domain' that binds to the U1 smaller nuclear RNA component of the U1 snRNP (Goraczniak, et al., 2008, Nature Biotechnology, 27(3), 257-263, which is expressly incorporated by reference herein, in its entirety). U1 snRNP is a ribonucleoprotein complex that functions primarily to direct early steps in spliceosome formation by binding to the pre-mRNA exon- intron boundary (Brown and Simpson, 1998, Annu Rev Plant Physiol Plant Mol Biol 49:77-95). Nucleotides 2-11 of the 5'end

of U1 snRNA base pair bind with the 5'ss of the pre mRNA. In one embodiment, oligonucleotides are U1 adaptors. In one embodiment, the U1 adaptor can be administered in combination with at least one other iRNA agent.

Oligonucleotide modifications

Unmodified oligonucleotides may be less than optimal in some applications, *e.g.*, unmodified oligonucleotides can be prone to degradation by *e.g.*, cellular nucleases. Nucleases can hydrolyze nucleic acid phosphodiester bonds. However, chemical modifications of oligonucleotides can confer improved properties, and, *e.g.*, can render oligonucleotides more stable to nucleases.

As oligonucleotides are polymers of subunits or monomers, many of the modifications described below occur at a position which is repeated within an oligonucleotide, *e.g.*, a modification of a base, a sugar, a phosphate moiety, or the non-bridging oxygen of a phosphate moiety. It is not necessary for all positions in a given oligonucleotide to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even at a single nucleoside within an oligonucleotide.

In some cases the modification will occur at all of the subject positions in the oligonucleotide but in many, and in fact in most cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in the internal region, may only occur in a terminal regions, *e.g.* at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of an oligonucleotide. A modification may occur in a double strand region, a single strand region, or in both. A modification may occur only in the double strand region of a double-stranded oligonucleotide or may only occur in a single strand region of a double-stranded oligonucleotide. *E.g.*, a phosphorothioate modification at a non-bridging oxygen position may only occur at one or both termini, may only occur in a terminal regions, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. The 5' end or ends can be phosphorylated.

A modification described herein may be the sole modification, or the sole type of modification included on multiple nucleotides, or a modification can be combined with one or more other modifications described herein. The modifications described herein can also be

combined onto an oligonucleotide, e.g. different nucleotides of an oligonucleotide have different modifications described herein.

In some embodiments it is particularly preferred, *e.g.*, to enhance stability, to include particular nucleobases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, *e.g.*, in a 5' or 3' overhang, or in both. *E.g.*, it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang will be modified, *e.g.*, with a modification described herein. Modifications can include, *e.g.*, the use of modifications at the 2' OH group of the ribose sugar, *e.g.*, the use of deoxyribonucleotides, *e.g.*, deoxythymidine, instead of ribonucleotides, and modifications in the phosphate group, *e.g.*, phosphothioate modifications. Overhangs need not be homologous with the target sequence.

Specific modifications are discussed in more detail below.

The Phosphate Group

The phosphate group is a negatively charged species. The charge is distributed equally over the two non-bridging oxygen atoms. However, the phosphate group can be modified by replacing one of the oxygens with a different substituent. One result of this modification to RNA phosphate backbones can be increased resistance of the oligoribonucleotide to nucleolytic breakdown. Thus while not wishing to be bound by theory, it can be desirable in some embodiments to introduce alterations which result in either an uncharged linker or a charged linker with unsymmetrical charge distribution.

Examples of modified phosphate groups include phosphorothioate, phosphoroselenates, borano phosphates, borano phosphate esters, hydrogen phosphonates, phosphoroamidates, alkyl or aryl phosphonates and phosphotriesters. In certain embodiments, one of the non-bridging phosphate oxygen atoms in the phosphate backbone moiety can be replaced by any of the following: S, Se, BR_3 (R is hydrogen, alkyl, aryl), C (i.e. an alkyl group, an aryl group, etc…), H, NR_2 (R is hydrogen, alkyl, aryl), or OR (R is alkyl or aryl). The phosphorous atom in an unmodified phosphate group is achiral. However, replacement of one of the non-bridging oxygens with one of the above atoms or groups of atoms renders the phosphorous atom chiral; in other words a phosphorous atom in a phosphate group modified in this way is a stereogenic

center. The stereogenic phosphorous atom can possess either the "R" configuration (herein Rp) or the "S" configuration (herein Sp).

Phosphorodithioates have both non-bridging oxygens replaced by sulfur. The phosphorus center in the phosphorodithioates is achiral which precludes the formation of oligoribonucleotides diastereomers. Thus, while not wishing to be bound by theory, modifications to both non-bridging oxygens, which eliminate the chiral center, *e.g.* phosphorodithioate formation, may be desirable in that they cannot produce diastereomer mixtures. Thus, the non-bridging oxygens can be independently any one of S, Se, B, C, H, N, or OR (R is alkyl or aryl).

The phosphate linker can also be modified by replacement of bridging oxygen, (i.e. oxgen that links the phosphate to the nucleoside), with nitrogen (bridged phosphoroamidates), sulfur (bridged phosphorothioates) and carbon (bridged methylenephosphonates). The replacement can occur at the either linking oxygen or at both the linking oxygens. When the bridging oxygen is the 3'-oxygen of a nucleoside, replcament with carbobn is preferred. When the bridging oxygen is the 5'-oxygen of a nucleoside, replcament with nitrogen is preferred.

Replacement of the Phosphate Group

The phosphate group can be replaced by non-phosphorus containing connectors. While not wishing to be bound by theory, it is believed that since the charged phosphodiester group is the reaction center in nucleolytic degradation, its replacement with neutral structural mimics should impart enhanced nuclease stability. Again, while not wishing to be bound by theory, it can be desirable, in some embodiment, to introduce alterations in which the charged phosphate group is replaced by a neutral moiety.

Examples of moieties which can replace the phosphate group include methyl phosphonate, hydroxylamino, siloxane, carbonate, carboxymethyl, carbamate, amide, thioether, ethylene oxide linker, sulfonate, sulfonamide, thioformacetal, formacetal, oxime, methyleneimino, methylenemethylimino, methylenehydrazo, methylenedimethylhydrazo and methyleneoxymethylimino. Preferred replacements include the methylenecarbonylamino and methylenemethylimino groups.

Modified phosphate linkages where at least one of the oxygens linked to the phosphate has been replaced or the phosphate group has been replaced by a non-phosphorous group, are also referred to as "non phosphodiester backbone linkage."

Replacement of Ribophosphate Backbone

Oligonucleotide- mimicking scaffolds can also be constructed wherein the phosphate linker and ribose sugar are replaced by nuclease resistant nucleoside or nucleotide surrogates. While not wishing to be bound by theory, it is believed that the absence of a repetitively charged backbone diminishes binding to proteins that recognize polyanions (*e.g.* nucleases). Again, while not wishing to be bound by theory, it can be desirable in some embodiment, to introduce alterations in which the bases are tethered by a neutral surrogate backbone. Examples include the mophilino, cyclobutyl, pyrrolidine and peptide nucleic acid (PNA) nucleoside surrogates. A preferred surrogate is a PNA surrogate.

Sugar modifications

A modified RNA can include modification of all or some of the sugar groups of the ribonucleic acid. *E.g.*, the 2′ hydroxyl group (OH) can be modified or replaced with a number of different "oxy" or "deoxy" substituents. While not being bound by theory, enhanced stability is expected since the hydroxyl can no longer be deprotonated to form a 2′-alkoxide ion. The 2′-alkoxide can catalyze degradation by intramolecular nucleophilic attack on the linker phosphorus atom. Again, while not wishing to be bound by theory, it can be desirable to some embodiments to introduce alterations in which alkoxide formation at the 2′ position is not possible.

Examples of "oxy"-2′ hydroxyl group modifications include alkoxy or aryloxy (OR, *e.g.*, R = H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar); polyethyleneglycols (PEG), $O(CH_2CH_2O)_nCH_2CH_2OR$; "locked" nucleic acids (LNA) in which the 2′ hydroxyl is connected, *e.g.*, by a methylene bridge, to the 4′ carbon of the same ribose sugar; O-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino) and aminoalkoxy, $O(CH_2)_nAMINE$, (*e.g.*, AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl

amino, or diheteroaryl amino, ethylene diamine, polyamino). It is noteworthy that oligonucleotides containing only the methoxyethyl group (MOE), ($OCH_2CH_2OCH_3$, a PEG derivative), exhibit nuclease stabilities comparable to those modified with the robust phosphorothioate modification.

"Deoxy" modifications include hydrogen (*i.e.* deoxyribose sugars, which are of particular relevance to the overhang portions of partially ds RNA); halo (*e.g.*, fluoro); amino (*e.g.* NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); $NH(CH_2CH_2NH)_nCH_2CH_2$-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino,or diheteroaryl amino), -NHC(O)R (R = alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar), cyano; mercapto; alkyl-thio-alkyl; thioalkoxy; and alkyl, cycloalkyl, aryl, alkenyl and alkynyl, which may be optionally substituted with *e.g.*, an amino functionality. Preferred substitutents are 2′-methoxyethyl, 2′-OCH3, 2′-O-allyl, 2′-C- allyl, and 2′-fluoro.

The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, an oligonucleotide can include nucleotides containing *e.g.*, arabinose, as the sugar. The monomer can have an alpha linkage at the 1' position on the sugar, e.g., alpha-nucleosides. Oligonucleotides can also include "abasic" sugars, which lack a nucleobase at C-1′. These abasic sugars can also be further containing modifications at one or more of the constituent sugar atoms. Oligonucleotides can also contain one or more sugars that are in the L form, e.g. L-nucleosides.

Terminal Modifications

The 3′ and 5′ ends of an oligonucleotide can be modified. Such modifications can be at the 3′ end, 5′ end or both ends of the molecule. They can include modification or replacement of an entire terminal phosphate or of one or more of the atoms of the phosphate group. *E.g.*, the 3′ and 5′ ends of an oligonucleotide can be conjugated to other functional molecular entities such as labeling moieties, *e.g.*, fluorophores (*e.g.*, pyrene, TAMRA, fluorescein, Cy3 or Cy5 dyes) or protecting groups (based *e.g.*, on sulfur, silicon, boron or ester). The functional molecular entities can be attached to the sugar through a phosphate group and/or a linker. The terminal atom of the linker can connect to or replace the linking atom of the phosphate group or the C-3′

or C-5′ O, N, S or C group of the sugar. Alternatively, the linker can connect to or replace the terminal atom of a nucleotide surrogate (*e.g.*, PNAs).

When a linker/phosphate-functional molecular entity-linker/phosphate array is interposed between two strands of a dsRNA, this array can substitute for a hairpin RNA loop in a hairpin-type RNA agent.

Terminal modifications useful for modulating activity include modification of the 5' end with phosphate or phosphate analogs. *E.g.*, in preferred embodiments antisense strands of dsRNAs, are 5' phosphorylated or include a phosphoryl analog at the 5' prime terminus. 5'-phosphate modifications include those which are compatible with RISC mediated gene silencing. Suitable modifications include: 5'-monophosphate ((HO)2(O)P-O-5'); 5'-diphosphate ((HO)2(O)P-O-P(HO)(O)-O-5'); 5'-triphosphate ((HO)2(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-guanosine cap (7-methylated or non-methylated) (7m-G-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-adenosine cap (Appp), and any modified or unmodified nucleotide cap structure (N-O-5'-(HO)(O)P-O-(HO)(O)P-O-P(HO)(O)-O-5'); 5'-monothiophosphate (phosphorothioate; (HO)2(S)P-O-5'); 5'-monodithiophosphate (phosphorodithioate; (HO)(HS)(S)P-O-5'), 5'-phosphorothiolate ((HO)2(O)P-S-5'); any additional combination of oxgen/sulfur replaced monophosphate, diphosphate and triphosphates (*e.g.* 5'-alpha-thiotriphosphate, 5'-gamma-thiotriphosphate, etc.), 5'-phosphoramidates ((HO)2(O)P-NH-5', (HO)(NH2)(O)P-O-5'), 5'-alkylphosphonates (R=alkyl=methyl, ethyl, isopropyl, propyl, etc., *e.g.* RP(OH)(O)-O-5'-, (OH)2(O)P-5'-CH2-), 5'-alkyletherphosphonates (R=alkylether=methoxymethyl (MeOCH2-), ethoxymethyl, etc., *e.g.* RP(OH)(O)-O-5'-).

Terminal modifications can also be useful for monitoring distribution, and in such cases the preferred groups to be added include fluorophores, *e.g.*, fluorscein or an Alexa dye, *e.g.*, Alexa 488. Terminal modifications can also be useful for enhancing uptake, useful modifications for this include cholesterol. Terminal modifications can also be useful for cross-linking an RNA agent to another moiety; modifications useful for this include mitomycin C.

Nucleobases

Adenine, guanine, cytosine and uracil are the most common bases found in RNA. These bases can be modified or replaced to provide RNA's having improved properties. *E.g.*, nuclease resistant oligoribonucleotides can be prepared with these bases or with synthetic and natural nucleobases (*e.g.*, inosine, thymine, xanthine, hypoxanthine, nubularine, isoguanisine, or tubercidine) and any one of the above modifications. Alternatively, substituted or modified analogs of any of the above bases, e.g., "unusual bases", "modified bases", "non-natual bases" and "universal bases" described herein, can be employed. Examples include without limitation 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 5-halouracil, 5-(2-aminopropyl)uracil, 5-amino allyl uracil, 8-halo, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine, dihydrouracil, 3-deaza-5-azacytosine, 2-aminopurine, 5-alkyluracil, 7-alkylguanine, 5-alkyl cytosine,7-deazaadenine, N6, N6-dimethyladenine, 2,6-diaminopurine, 5-amino-allyl-uracil, N3-methyluracil, substituted 1,2,4-triazoles, 2-pyridinone, 5-nitroindole, 3-nitropyrrole, 5-methoxyuracil, uracil-5-oxyacetic acid, 5-methoxycarbonylmethyluracil, 5-methyl-2-thiouracil, 5-methoxycarbonylmethyl-2-thiouracil, 5-methylaminomethyl-2-thiouracil, 3-(3-amino-3carboxypropyl)uracil, 3-methylcytosine, 5-methylcytosine, N^4-acetyl cytosine, 2-thiocytosine, N6-methyladenine, N6-isopentyladenine, 2-methylthio-N6-isopentenyladenine, N-methylguanines, or O-alkylated bases. Further purines and pyrimidines include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in the Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, and those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613.

Cationic Groups

Modifications to oligonucleotides can also include attachment of one or more cationic groups to the sugar, base, and/or the phosphorus atom of a phosphate or modified phosphate

backbone moiety. A cationic group can be attached to any atom capable of substitution on a natural, unusual or universal base. A preferred position is one that does not interfere with hybridization, i.e., does not interfere with the hydrogen bonding interactions needed for base pairing. A cationic group can be attached e.g., through the C2' position of a sugar or analogous position in a cyclic or acyclic sugar surrogate. Cationic groups can include e.g., protonated amino groups, derived from e.g., O-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); aminoalkoxy, e.g., $O(CH_2)_nAMINE$, (*e.g.*, AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, or diheteroaryl amino, ethylene diamine, polyamino); amino (*e.g.* NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino, diheteroaryl amino, or amino acid); or $NH(CH_2CH_2NH)_nCH_2CH_2$-AMINE (AMINE = NH_2; alkylamino, dialkylamino, heterocyclyl, arylamino, diaryl amino, heteroaryl amino,or diheteroaryl amino).

Placement within an oligonucleotide

Some modifications may preferably be included on an oligonucleotide at a particular location, e.g., at an internal position of a strand, or on the 5' or 3' end of an oligonucleotide. A preferred location of a modification on an oligonucleotide, may confer preferred properties on the agent. For example, preferred locations of particular modifications may confer optimum gene silencing properties, or increased resistance to endonuclease or exonuclease activity.

One or more nucleotides of an oligonucleotide may have a 2'-5' linkage. One or more nucleotides of an oligonucleotide may have inverted linkages, e.g. 3'-3', 5'-5', 2'-2' or 2'-3' linkages.

A double-stranded oligonucleotide may include at least one 5'-uridine-adenine-3' (5'-UA-3') dinucleotide wherein the uridine is a 2'-modified nucleotide, or a terminal 5'-uridine-guanine-3' (5'-UG-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide, or a terminal 5'-cytidine-adenine-3' (5'-CA-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a terminal 5'-uridine-uridine-3' (5'-UU-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide, or a terminal 5'-cytidine-cytidine-3' (5'-CC-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide, or a terminal 5'-cytidine-uridine-3' (5'-CU-3') dinucleotide, wherein the 5'-cytidine is a 2'-modified nucleotide,

or a terminal 5'-uridine-cytidine-3' (5'-UC-3') dinucleotide, wherein the 5'-uridine is a 2'-modified nucleotide. Double-stranded oligonucleotides including these modifications are particularly stabilized against endonuclease activity.

General References

The oligoribonucleotides and oligoribonucleosides may be synthesized with solid phase synthesis, see for example "Oligonucleotide synthesis, a practical approach", Ed. M. J. Gait, IRL Press, 1984; "Oligonucleotides and Analogues, A Practical Approach", Ed. F. Eckstein, IRL Press, 1991 (especially Chapter 1, Modern machine-aided methods of oligodeoxyribonucleotide synthesis, Chapter 2, Oligoribonucleotide synthesis, Chapter 3, 2'-O--Methyloligoribonucleotide-s: synthesis and applications, Chapter 4, Phosphorothioate oligonucleotides, Chapter 5, Synthesis of oligonucleotide phosphorodithioates, Chapter 6, Synthesis of oligo-2'-deoxyribonucleoside methylphosphonates, and. Chapter 7, Oligodeoxynucleotides containing modified bases. Other particularly useful synthetic procedures, reagents, blocking groups and reaction conditions are described in Martin, P., *Helv. Chim. Acta*, **1995**, *78*, 486-504; Beaucage, S. L. and Iyer, R. P., *Tetrahedron*, **1992**, *48*, 2223-2311 and Beaucage, S. L. and Iyer, R. P., *Tetrahedron*, **1993**, *49*, 6123-6194, or references referred to therein. Modification described in WO 00/44895, WO01/75164, or WO02/44321 can be used herein. The disclosure of all publications, patents, and published patent applications listed herein are hereby incorporated by reference.

Phosphate Group References

The preparation of phosphinate oligoribonucleotides is described in U.S. Pat. No. 5,508,270. The preparation of alkyl phosphonate oligoribonucleotides is described in U.S. Pat. No. 4,469,863. The preparation of phosphoramidite oligoribonucleotides is described in U.S. Pat. No. 5,256,775 or U.S. Pat. No. 5,366,878. The preparation of phosphotriester oligoribonucleotides is described in U.S. Pat. No. 5,023,243. The preparation of borano phosphate oligoribonucleotide is described in U.S. Pat. Nos. 5,130,302 and 5,177,198. The preparation of 3'-Deoxy-3'-amino phosphoramidate oligoribonucleotides is described in U.S. Pat. No. 5,476,925. 3'-Deoxy-3'-methylenephosphonate oligoribonucleotides is described in An, H, *et al. J. Org. Chem.* **2001**, *66*, 2789-2801. Preparation of sulfur bridged nucleotides is described

in Sproat *et al. Nucleosides Nucleotides* **1988**, *7*,651 and Crosstick *et al. Tetrahedron Lett.* **1989**, *30*, 4693.

Sugar Group References

Modifications to the 2' modifications can be found in Verma, S. *et al. Annu. Rev. Biochem.* **1998**, *67*, 99-134 and all references therein. Specific modifications to the ribose can be found in the following references: 2'-fluoro (Kawasaki et. al., *J. Med. Chem.*, **1993**, *36*, 831-841), 2'-MOE (Martin, P. *Helv. Chim. Acta* **1996**, *79*, 1930-1938), "LNA" (Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301-310).

Replacement of the Phosphate Group References

Methylenemethylimino linked oligoribonucleosides, also identified herein as MMI linked oligoribonucleosides, methylenedimethylhydrazo linked oligoribonucleosides, also identified herein as MDH linked oligoribonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified herein as amide-3 linked oligoribonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified herein as amide-4 linked oligoribonucleosides as well as mixed backbone compounds having, as for instance, alternating MMI and PO or PS linkages can be prepared as is described in U.S. Pat. Nos. 5,378,825, 5,386,023, 5,489,677 and in published PCT applications PCT/US92/04294 and PCT/US92/04305 (published as WO 92/20822 WO and 92/20823, respectively). Formacetal and thioformacetal linked oligoribonucleosides can be prepared as is described in U.S. Pat. Nos. 5,264,562 and 5,264,564. Ethylene oxide linked oligoribonucleosides can be prepared as is described in U.S. Pat. No. 5,223,618. Siloxane replacements are described in Cormier,J.F. *et al. Nucleic Acids Res.* **1988**, *16*, 4583. Carbonate replacements are described in Tittensor, J.R. *J. Chem. Soc. C* **1971**, 1933. Carboxymethyl replacements are described in Edge, M.D. *et al. J. Chem. Soc. Perkin Trans. 1* **1972**, 1991. Carbamate replacements are described in Stirchak, E.P. Nucleic Acids Res. 1989, 17, 6129.

Replacement of the Phosphate-Ribose Backbone References

Cyclobutyl sugar surrogate compounds can be prepared as is described in U.S. Pat. No. 5,359,044. Pyrrolidine sugar surrogate can be prepared as is described in U.S. Pat. No.

5,519,134. Morpholino sugar surrogates can be prepared as is described in U.S. Pat. Nos. 5,142,047 and 5,235,033, and other related patent disclosures. Peptide Nucleic Acids (PNAs) are known per se and can be prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, Bioorganic & Medicinal Chemistry, 1996, 4, 5-23. They may also be prepared in accordance with U.S. Pat. No. 5,539,083.

Terminal Modification References

Terminal modifications are described in Manoharan, M. *et al. Antisense and Nucleic Acid Drug Development 12*, 103-128 (2002) and references therein.

Nucleobases References

N-2 substitued purine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,459,255. 3-Deaza purine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,457,191. 5,6-Substituted pyrimidine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,614,617. 5-Propynyl pyrimidine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,484,908.

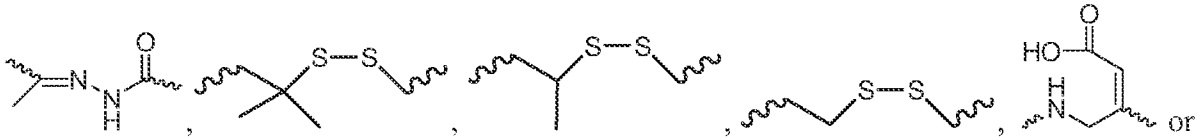
Linkers

The term "linker" means an organic moiety that connects two parts of a compound. Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR^1, C(O), C(O)NH, SO, SO_2, SO_2NH or a chain of atoms, such as substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, arylalkyl, arylalkenyl, arylalkynyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, heterocyclylalkyl, heterocyclylalkenyl, heterocyclylalkynyl, aryl, heteroaryl, heterocyclyl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkynylarylalkynyl, alkylheteroarylalkyl, alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclylalkyl, alkylheterocyclylalkenyl, alkylhererocyclylalkynyl, alkenylheterocyclylalkyl,

alkenylheterocyclylalkenyl, alkenylheterocyclylalkynyl, alkynylheterocyclylalkyl, alkynylheterocyclylalkenyl, alkynylheterocyclylalkynyl, alkylaryl, alkenylaryl, alkynylaryl, alkylheteroaryl, alkenylheteroaryl, alkynylhereroaryl, where one or more methylenes can be interrupted or terminated by O, S, S(O), SO_2, $N(R^1)_2$, C(O), cleavable linking group, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R^1 is hydrogen, acyl, aliphatic or substituted aliphatic.

In one embodiment, the linker is $-[(P\text{-}Q\text{-}R)_q\text{-}X\text{-}(P'\text{-}Q'\text{-}R')_{q'}]_{q''}\text{-}T$-, wherein:

P, R, T, P', R' and T are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH_2, CH_2NH, CH_2O; $NHCH(R^a)C(O)$, -C(O)-$CH(R^a)$-NH-, CH=N-O ,



heterocyclyl;

Q and Q' are each independently for each occurrence absent, $-(CH_2)_n$-, $-C(R^1)(R^2)(CH_2)_n$-, $-(CH_2)_nC(R^1)(R^2)$-, $-(CH_2CH_2O)_mCH_2CH_2$-, or $-(CH_2CH_2O)_mCH_2CH_2NH$-;

X is absent or a cleavable linking group;

R^a is H or an amino acid side chain;

R^1 and R^2 are each independently for each occurrence H, CH_3, OH, SH or $N(R^N)_2$;

R^N is independently for each occurrence H, methyl, ethyl, propyl, isopropyl, butyl or benzyl;

q, q' and q" are each independently for each occurrence 0-20 and wherein the repeating unit can be the same or different;

n is independently for each occurrence 1-20; and

m is independently for each occurrence 0-50.

In one embodiment, the linker comprises at least one cleavable linking group.

In certain embodiments, the linker is a branched linker. The branchpoint of the branched linker may be at least trivalent, but may be a tetravalent, pentavalent or hexavalent atom, or a group presenting such multiple valencies. In certain embodiments, the branchpoint is , -N, -N(Q)-C, -O-C, -S-C, -SS-C, -C(O)N(Q)-C, -OC(O)N(Q)-C, -N(Q)C(O)-C, or -N(Q)C(O)O-C; wherein Q is independently for each occurrence H or optionally substituted alkyl. In other embodiment, the branchpoint is glycerol or glycerol derivative.

Cleavable Linking Groups

A cleavable linking group is one which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In a preferred embodiment, the cleavable linking group is cleaved at least 10 times or more, preferably at least 100 times faster in the target cell or under a first reference condition (which can, e.g., be selected to mimic or represent intracellular conditions) than in the blood of a subject, or under a second reference condition (which can, e.g., be selected to mimic or represent conditions found in the blood or serum).

Cleavable linking groups are susceptible to cleavage agents, e.g., pH, redox potential or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, e.g., oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linking group by reduction; esterases; endosomes or agents that can create an acidic environment, e.g., those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linking group by acting as a general acid, peptidases (which can be substrate specific), and phosphatases.

A cleavable linkage group, such as a disulfide bond can be susceptible to pH. The pH of human serum is 7.4, while the average intracellular pH is slightly lower, ranging from about 7.1-7.3. Endosomes have a more acidic pH, in the range of 5.5-6.0, and lysosomes have an even more acidic pH at around 5.0. Some linkers will have a cleavable linking group that is cleaved at a preferred pH, thereby releasing the cationic lipid from the ligand inside the cell, or into the desired compartment of the cell.

A linker can include a cleavable linking group that is cleavable by a particular enzyme. The type of cleavable linking group incorporated into a linker can depend on the cell to be targeted. For example, liver targeting ligands can be linked to the cationic lipids through a linker that includes an ester group. Liver cells are rich in esterases, and therefore the linker will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

Linkers that contain peptide bonds can be used when targeting cell types rich in peptidases, such as liver cells and synoviocytes.

In general, the suitability of a candidate cleavable linking group can be evaluated by testing the ability of a degradative agent (or condition) to cleave the candidate linking group. It will also be desirable to also test the candidate cleavable linking group for the ability to resist cleavage in the blood or when in contact with other non-target tissue. Thus one can determine the relative susceptibility to cleavage between a first and a second condition, where the first is selected to be indicative of cleavage in a target cell and the second is selected to be indicative of cleavage in other tissues or biological fluids, e.g., blood or serum. The evaluations can be carried out in cell free systems, in cells, in cell culture, in organ or tissue culture, or in whole animals. It may be useful to make initial evaluations in cell-free or culture conditions and to confirm by further evaluations in whole animals. In preferred embodiments, useful candidate compounds are cleaved at least 2, 4, 10 or 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood or serum (or under in vitro conditions selected to mimic extracellular conditions).

Redox cleavable linking groups

One class of cleavable linking groups are redox cleavable linking groups that are cleaved upon reduction or oxidation. An example of reductively cleavable linking group is a disulphide linking group (-S-S-). To determine if a candidate cleavable linking group is a suitable "reductively cleavable linking group," or for example is suitable for use with a particular iRNA moiety and particular targeting agent one can look to methods described herein. For example, a candidate can be evaluated by incubation with dithiothreitol (DTT), or other reducing agent using reagents know in the art, which mimic the rate of cleavage which would be observed in a cell, e.g., a target cell. The candidates can also be evaluated under conditions which are selected to mimic blood or serum conditions. In a preferred embodiment, candidate compounds are cleaved by at most 10% in the blood. In preferred embodiments, useful candidate compounds are degraded at least 2, 4, 10 or 100 times faster in the cell (or under in vitro conditions selected to mimic intracellular conditions) as compared to blood (or under in vitro conditions selected to mimic extracellular conditions). The rate of cleavage of candidate compounds can be

determined using standard enzyme kinetics assays under conditions chosen to mimic intracellular media and compared to conditions chosen to mimic extracellular media.

Phosphate-based cleavable linking groups

Phosphate-based cleavable linking groups are cleaved by agents that degrade or hydrolyze the phosphate group. An example of an agent that cleaves phosphate groups in cells are enzymes such as phosphatases in cells. Examples of phosphate-based linking groups are -O-P(O)(ORk)-O-, -O-P(S)(ORk)-O-, -O-P(S)(SRk)-O-, -S-P(O)(ORk)-O-, -O-P(O)(ORk)-S-, -S-P(O)(ORk)-S-, -O-P(S)(ORk)-S-, -S-P(S)(ORk)-O-, -O-P(O)(Rk)-O-, -O-P(S)(Rk)-O-, -S-P(O)(Rk)-O-, -S-P(S)(Rk)-O-, -S-P(O)(Rk)-S-, -O-P(S)(Rk)-S-. Preferred embodiments are -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O-, -S-P(S)(H)-O-, -S-P(O)(H)-S-, -O-P(S)(H)-S-. A preferred embodiment is -O-P(O)(OH)-O-. These candidates can be evaluated using methods analogous to those described above.

Acid cleavable linking groups

Acid cleavable linking groups are linking groups that are cleaved under acidic conditions. In preferred embodiments acid cleavable linking groups are cleaved in an acidic environment with a pH of about 6.5 or lower (e.g., about 6.0, 5.5, 5.0, or lower), or by agents such as enzymes that can act as a general acid. In a cell, specific low pH organelles, such as endosomes and lysosomes can provide a cleaving environment for acid cleavable linking groups. Examples of acid cleavable linking groups include but are not limited to hydrazones, esters, and esters of amino acids. Acid cleavable groups can have the general formula -C=NN-, C(O)O, or -OC(O). A preferred embodiment is when the carbon attached to the oxygen of the ester (the alkoxy group) is an aryl group, substituted alkyl group, or tertiary alkyl group such as dimethyl pentyl or t-butyl. These candidates can be evaluated using methods analogous to those described above.

Ester-based linking groups

Ester-based cleavable linking groups are cleaved by enzymes such as esterases and amidases in cells. Examples of ester-based cleavable linking groups include but are not limited

to esters of alkylene, alkenylene and alkynylene groups. Ester cleavable linking groups have the general formula -C(O)O-, or -OC(O)-. These candidates can be evaluated using methods analogous to those described above.

Peptide-based cleaving groups

Peptide-based cleavable linking groups are cleaved by enzymes such as peptidases and proteases in cells. Peptide-based cleavable linking groups are peptide bonds formed between amino acids to yield oligopeptides (e.g., dipeptides, tripeptides etc.) and polypeptides. Peptide-based cleavable groups do not include the amide group (-C(O)NH-). The amide group can be formed between any alkylene, alkenylene or alkynelene. A peptide bond is a special type of amide bond formed between amino acids to yield peptides and proteins. The peptide based cleavage group is generally limited to the peptide bond (i.e., the amide bond) formed between amino acids yielding peptides and proteins and does not include the entire amide functional group. Peptide-based cleavable linking groups have the general formula $-NHCHR^{A}C(O)NHCHR^{B}C(O)-$, where R^{A} and R^{B} are the R groups of the two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.

Ligands

A wide variety of entities can be coupled to the oligonucleotides and lipids. Preferred moieties are ligands, which are coupled, preferably covalently, either directly or indirectly via an intervening tether.

In preferred embodiments, a ligand alters the distribution, targeting or lifetime of the molecule into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type, compartment, *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, e.g., compared to a species absent such a ligand. Ligands providing enhanced affinity for a selected target are also termed targeting ligands. Preferred ligands for conjugation to the lipids are targeting ligands.

Some ligands can have endosomolytic properties. The endosomolytic ligands promote the lysis of the endosome and/or transport of the composition, or its components, from the endosome to the cytoplasm of the cell. The endosomolytic ligand may be a polyanionic peptide or peptidomimetic which shows pH-dependent membrane activity and fusogenicity. In certain

embodiments, the endosomolytic ligand assumes its active conformation at endosomal pH. The "active" conformation is that conformation in which the endosomolytic ligand promotes lysis of the endosome and/or transport of the composition, or its components, from the endosome to the cytoplasm of the cell. Exemplary endosomolytic ligands include the GALA peptide (Subbarao et al., Biochemistry, 1987, 26: 2964-2972), the EALA peptide (Vogel et al., J. Am. Chem. Soc., 1996, 118: 1581-1586), and their derivatives (Turk et al., Biochem. Biophys. Acta, 2002, 1559: 56-68). In certain embodiments, the endosomolytic component may contain a chemical group (e.g., an amino acid) which will undergo a change in charge or protonation in response to a change in pH. The endosomolytic component may be linear or branched. Exemplary primary sequences of peptide based endosomolytic ligands are shown in Table 6.

Table 6: List of peptides with endosomolytic activity.

Name	Sequence (N to C)	Ref.
GALA	AALEALAEALEALAEALEALAEAAAAGGC	1
EALA	AALAEALAEALAEALAEALAEALAAAAGGC	2
	ALEALAEALEALAEA	3
INF-7	GLFEAIEGFIENGWEGMIWDYG	4
Inf HA-2	GLFGAIAGFIENGWEGMIDGWYG	5
diINF-7	GLF EAI EGFI ENGW EGMI DGWYGC GLF EAI EGFI ENGW EGMI DGWYGC	5
diINF3	GLF EAI EGFI ENGW EGMI DGGC GLF EAI EGFI ENGW EGMI DGGC	6
GLF	GLFGALAEALAEALAEHLAEALAEALEALA AGGSC	6
GALA-INF3	GLFEAIEGFIENGWEGLAEALAEALEALAAG GSC	6
INF-5	GLF EAI EGFI ENGW EGnI DG K GLF EAI EGFI ENGW EGnI DG	4

n, norleucine

References

1. Subbarao et al., Biochemistry, 1987, 26: 2964-2972.
2. Vogel et al., J. Am. Chem. Soc., 1996, 118: 1581-1586
3. Turk, M. J., Reddy, J. A. et al. (2002). Characterization of a novel pH-sensitive peptide that enhances drug release from folate-targeted liposomes at endosomal pHs. *Biochim. Biophys. Acta* 1559, 56-68.

4. Plank, C. Oberhauser, B. Mechtler, K. Koch, C. Wagner, E. (1994). The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems, J. Biol. Chem. 269 12918–12924.
5. Mastrobattista, E., Koning, G. A. et al. (2002). Functional characterization of an endosome-disruptive peptide and its application in cytosolic delivery of immunoliposome-entrapped proteins. *J. Biol. Chem.* 277, 27135-43.
6. Oberhauser, B., Plank, C. et al. (1995). Enhancing endosomal exit of nucleic acids using pH-sensitive viral fusion peptides. Deliv. Strategies Antisense Oligonucleotide Ther. 247-66.

Preferred ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified oligoribonucleotide, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides.

Ligands in general can include therapeutic modifiers, e.g., for enhancing uptake; diagnostic compounds or reporter groups e.g., for monitoring distribution; cross-linking agents; and nuclease-resistance conferring moieties. General examples include lipids, steroids, vitamins, sugars, proteins, peptides, polyamines, and peptide mimics.

Ligands can include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin); an carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid, an oligonucleotide (e.g. an aptamer). Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

Ligands can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a

kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic or an aptamer. Table 7 shows some examples of targeting ligands and their associated receptors.

Table 7: Targeting Ligands and their associated receptors

Liver cells	Ligand	Receptor
Parenchymal Cell (PC) hepatocytes	Galactose	ASGP-R (Asiologlycoprotein receptor)
	Gal NAc (N-acetyl galactosamine)	ASPG-R Gal NAc Receptor
	Lactose	
	Asialofetuin	ASPG-r
Sinusoidal Endothelial Cell (SEC)	Hyaluronan	Hyaluronan receptor
	Procollagen	Procollagen receptor
	Negatively charged molecules	Scavenger receptors
	Mannose	Mannose receptors
	N-acetyl Glucosamine	Scavenger receptors
	Immunoglobulins	Fc Receptor
	LPS	CD14 Receptor
	Insulin	Receptor mediated transcytosis
	Transferrin	Receptor mediated transcytosis
	Albumins	Non-specific
	Mannose-6-phosphate	Mannose-6-phosphate receptor
Kupffer Cell (KC)	Mannose	Mannose receptors
	Fucose	Fucose receptors
	Albumins	Non-specific
	Mannose-albumin conjugates	

Other examples of ligands include dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic

hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), lipophilic molecules, e.g, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid,O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine)and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[MPEG]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

Ligands can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, or aptamers. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

The ligand can be a substance, e.g, a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

The ligand can increase the uptake of the iRNA agent into the cell by activating an inflammatory response, for example. Exemplary ligands that would have such an effect include tumor necrosis factor alpha (TNFalpha), interleukin-1 beta, or gamma interferon.

In one aspect, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, e.g., human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., a non-kidney target tissue of the body. For example, the target tissue can be the liver, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or

aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA.

A lipid based ligand can be used to modulate, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

In another preferred embodiment, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid based ligand.

In another aspect, the ligand is a moiety, e.g., a vitamin, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include are B vitamin, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells. Also included are HAS, low density lipoprotein (LDL) and high-density lipoprotein (HDL).

In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The peptide or peptidomimetic moiety

can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long (see Table 8, for example).

Table 8. Exemplary Cell Permeation Peptides.

Cell Permeation Peptide	**Amino acid Sequence**	**Reference**
Penetratin	RQIKIWFQNRRMKWKK	Derossi *et al.*, J. Biol. Chem. 269:10444, 1994
Tat fragment (48-60)	GRKKRRQRRRPPQC	Vives *et al.*, J. Biol. Chem., 272:16010, 1997
Signal Sequence-based peptide	GALFLGWLGAAGSTMGAWSQPKKKRKV	Chaloin *et al.*, Biochem. Biophys. Res. Commun., 243:601, 1998
PVEC	LLIILRRRIRKQAHAHSK	Elmquist *et al.*, Exp. Cell Res., 269:237, 2001
Transportan	GWTLNSAGYLLKINLKALAALAKKIL	Pooga *et al.*, FASEB J., 12:67, 1998
Amphiphilic model peptide	KLALKLALKALKAALKLA	Oehlke *et al.*, Mol. Ther., 2:339, 2000
Arg_9	RRRRRRRRR	Mitchell *et al.*, J. Pept. Res., 56:318, 2000
Bacterial cell wall permeating	KFFKFFKFFK	
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRN LVPRTES	
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGP R	
α-defensin	ACYCRIPACIAGERRYGTCIYQGRLWAFC C	
b-defensin	DHYNCVSSGGQCLYSACPIFTKIQGTCYR GKAKCCK	
Bactenecin	RKCRIVVIRVCR	
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPP RFPPRFPGKR-NH2	
Indolicidin	ILPWKWPWWPWRR-NH2	

A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (*e.g.*, consisting primarily of Tyr, Trp or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. In another alternative, the peptide moiety can include a hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF

having the amino acid sequence AAVALLPAVLLALLAP. An RFGF analogue (*e.g.*, amino acid sequence AALLPVLLAAP) containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a "delivery" peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein (GRKKRRQRRRPPQ) and the Drosophila Antennapedia protein (RQIKIWFQNRRMKWKK) have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam *et al.*, Nature, 354:82-84, 1991). Preferably the peptide or peptidomimetic tethered to an iRNA agent via an incorporated monomer unit is a cell targeting peptide such as an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

An RGD peptide moiety can be used to target a tumor cell, such as an endothelial tumor cell or a breast cancer tumor cell (Zitzmann *et al.*, Cancer Res., 62:5139-43, 2002). An RGD peptide can facilitate targeting of an iRNA agent to tumors of a variety of other tissues, including the lung, kidney, spleen, or liver (Aoki *et al.*, Cancer Gene Therapy 8:783-787, 2001). Preferably, the RGD peptide will facilitate targeting of an iRNA agent to the kidney. The RGD peptide can be linear or cyclic, and can be modified, *e.g.*, glycosylated or methylated to facilitate targeting to specific tissues. For example, a glycosylated RGD peptide can deliver an iRNA agent to a tumor cell expressing $\alpha_V\beta_3$ (Haubner *et al.*, Jour. Nucl. Med., 42:326-336, 2001).

Peptides that target markers enriched in proliferating cells can be used. *E.g.*, RGD containing peptides and peptidomimetics can target cancer cells, in particular cells that exhibit an αvβ3 integrin. Thus, one could use RGD peptides, cyclic peptides containing RGD, RGD peptides that include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the αvβ3 integrin ligand. Generally, such ligands can be used to control proliferating cells and angiogeneis. Preferred conjugates of this type lignads that targets PECAM-1, VEGF, or other cancer gene, e.g., a cancer gene described herein.

A "cell permeation peptide" is capable of permeating a cell, *e.g.*, a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial

cell-permeating peptide can be, for example, an α-helical linear peptide (*e.g.*, LL-37 or Ceropin P1), a disulfide bond-containing peptide (*e.g.*, α -defensin, β-defensin or bactenecin), or a peptide containing only one or two dominating amino acids (*e.g.*, PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni *et al.*, Nucl. Acids Res. 31:2717-2724, 2003).

In one embodiment, a targeting peptide tethered to an iRNA agent and/or the carrier oligomer can be an amphipathic α-helical peptide. Exemplary amphipathic α-helical peptides include, but are not limited to, cecropins, lycotoxins, paradaxins, buforin, CPF, bombinin-like peptide (BLP), cathelicidins, ceratotoxins, *S. clava* peptides, hagfish intestinal antimicrobial peptides (HFIAPs), magainines, brevinins-2, dermaseptins, melittins, pleurocidin, H_2A peptides, Xenopus peptides, esculentinis-1, and caerins. A number of factors will preferably be considered to maintain the integrity of helix stability. For example, a maximum number of helix stabilization residues will be utilized (*e.g.*, leu, ala, or lys), and a minimum number helix destabilization residues will be utilized (*e.g.,* proline, or cyclic monomeric units. The capping residue will be considered (for example Gly is an exemplary N-capping residue and/or C-terminal amidation can be used to provide an extra H-bond to stabilize the helix. Formation of salt bridges between residues with opposite charges, separated by $i \pm 3$, or $i \pm 4$ positions can provide stability. For example, cationic residues such as lysine, arginine, homo-arginine, ornithine or histidine can form salt bridges with the anionic residues glutamate or aspartate.

Peptide and peptidomimetic ligands include those having naturally occurring or modified peptides, e.g., D or L peptides; α, β, or γ peptides; N-methyl peptides; azapeptides; peptides having one or more amide, i.e., peptide, linkages replaced with one or more urea, thiourea, carbamate, or sulfonyl urea linkages; or cyclic peptides.

The targeting ligand can be any ligand that is capable of targeting a specific receptor. Examples are: folate, GalNAc, galactose, mannose, mannose-6P, clusters of sugars such as GalNAc cluster, mannose cluster, galactose cluster, or an apatamer. A cluster is a combination of two or more sugar units. The targeting ligands also include integrin receptor ligands, Chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL and HDL ligands. The ligands can also be based on nucleic acid, e.g.,

an aptamer. The aptamer can be unmodified or have any combination of modifications disclosed herein.

Endosomal release agents include imidazoles, poly or oligoimidazoles, PEIs, peptides, fusogenic peptides, polycaboxylates, polyacations, masked oligo or poly cations or anions, acetals, polyacetals, ketals/polyketyals, orthoesters, polymers with masked or unmasked cationic or anionic charges, dendrimers with masked or unmasked cationic or anionic charges.

PK modulator stands for pharmacokinetic modulator. PK modulator include lipophiles, bile acids, steroids, phospholipid analogues, peptides, protein binding agents, PEG, vitamins etc. Examplary PK modulator include, but are not limited to, cholesterol, fatty acids, cholic acid, lithocholic acid, dialkylglycerides, diacylglyceride, phospholipids, sphingolipids, naproxen, ibuprofen, vitamin E, biotin etc. Oligonucleotides that comprise a number of phosphorothioate linkages are also known to bind to serum protein, thus short oligonucleotides, e.g. oligonucleotides of about 5 bases, 10 bases, 15 bases or 20 bases, comprising multiple of phosphorothioate linkages in the backbaone are also amenable as ligands (e.g. as PK modulating ligands).

In addition, aptamers that bind serum components (e.g. serum proteins) are also amenable as PK modulating ligands.

Other amenable ligands are described in U.S. Patent Application Nos. 2005/0107325, 2005/0164235, and 2008-0255345, and U.S. Patent Nos. 7,021,394, and 7,626,014, which are incorporated by reference in their entireties for all purposes.

When two or more ligands are present, the ligands can all have same properties, all have different properties or some ligands have the same properties while others have different properties. For example, a ligand can have targeting properties, have endosomolytic activity or have PK modulating properties. In a preferred embodiment, all the ligands have different properties.

Ligands can be coupled to the oligonucleotides various places, for example, 3'-end, 5'-end, and/or at an internal position. In preferred embodiments, the ligand is attached to the oligonucleotides *via* an intervening tether. The ligand or tethered ligand may be present on a monomer when said monomer is incorporated into the growing strand. In some embodiments, the ligand may be incorporated via coupling to a "precursor" monomer after said "precursor" monomer has been incorporated into the growing strand. For example, a monomer having, e.g.,

an amino-terminated tether (i.e., having no associated ligand), e.g., TAP-$(CH_2)_nNH_2$ may be incorporated into a growing sense or antisense strand. In a subsequent operation, i.e., after incorporation of the precursor monomer into the strand, a ligand having an electrophilic group, e.g., a pentafluorophenyl ester or aldehyde group, can subsequently be attached to the precursor monomer by coupling the electrophilic group of the ligand with the terminal nucleophilic group of the precursor monomer's tether.

For double- stranded oligonucleotides, ligands can be attached to one or both strands. In some embodiments, a double-stranded iRNA agent contains a ligand conjugated to the sense strand. In other embodiments, a double-stranded iRNA agent contains a ligand conjugated to the antisense strand.

In some embodiments, lignad can be conjugated to nucleobases, sugar moieties, or internucleosidic linkages of nucleic acid molecules. Conjugation to purine nucleobases or derivatives thereof can occur at any position including, endocyclic and exocyclic atoms. In some embodiments, the 2-, 6-, 7-, or 8-positions of a purine nucleobase are attached to a conjugate moiety. Conjugation to pyrimidine nucleobases or derivatives thereof can also occur at any position. In some embodiments, the 2-, 5-, and 6-positions of a pyrimidine nucleobase can be substituted with a conjugate moiety. Conjugation to sugar moieties of nucleosides can occur at any carbon atom. Example carbon atoms of a sugar moiety that can be attached to a conjugate moiety include the 2', 3', and 5' carbon atoms. The 1' position can also be attached to a conjugate moiety, such as in an abasic residue. Internucleosidic linkages can also bear conjugate moieties. For phosphorus-containing linkages (e.g., phosphodiester, phosphorothioate, phosphorodithiotate, phosphoroamidate, and the like), the conjugate moiety can be attached directly to the phosphorus atom or to an O, N, or S atom bound to the phosphorus atom. For amine- or amide-containing internucleosidic linkages (e.g., PNA), the conjugate moiety can be attached to the nitrogen atom of the amine or amide or to an adjacent carbon atom.

There are numerous methods for preparing conjugates of oligomeric compounds. In general, an oligomeric compound is attached to a conjugate moiety by contacting a reactive group (e.g., OH, SH, amine, carboxyl, aldehyde, and the like) on the oligomeric compound with a reactive group on the conjugate moiety. In some embodiments, one reactive group is electrophilic and the other is nucleophilic.

For example, an electrophilic group can be a carbonyl-containing functionality and a

nucleophilic group can be an amine or thiol. Methods for conjugation of nucleic acids and related oligomeric compounds with and without linking groups are well described in the literature such as, for example, in Manoharan in Antisense Research and Applications, Crooke and LeBleu, eds., CRC Press, Boca Raton, Fla., 1993, Chapter 17, which is incorporated herein by reference in its entirety.

Representative United States patents that teach the preparation of oligonucleotide conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218, 105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578, 717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118, 802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578, 718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762, 779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904, 582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082, 830; 5,112,963; 5,149,782; 5,214,136; 5,245,022; 5,254, 469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317, 098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510, 475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574, 142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599, 923; 5,599,928; 5,672,662; 5,688,941; 5,714,166; 6,153, 737; 6,172,208; 6,300,319; 6,335,434; 6,335,437; 6,395, 437; 6,444,806; 6,486,308; 6,525,031; 6,528,631; and 6,559, 279; each of which is herein incorporated by reference.

Characteristics of Nucleic Acid-Lipid Particles

Methods and compositions for producing lipid-encapsulated nucleic acid particles in which nucleic acids are encapsulated within a lipid layer are provided. Such nucleic acid-lipid particles, incorporating siRNA oligonucleotides, are characterized using a variety of biophysical parameters including: (1) drug to lipid ratio; (2) encapsulation efficiency; and (3) particle size. High drug to lipid rations, high encapsulation efficiency, good nuclease resistance and serum stability and controllable particle size, generally less than 200 nm in diameter are desirable. In addition, the nature of the nucleic acid polymer is of significance, since the modification of nucleic acids in an effort to impart nuclease resistance adds to the cost of therapeutics while in many cases providing only limited resistance. Unless stated otherwise, these criteria are calculated in this specification as follows:

Nucleic acid to lipid ratio is the amount of nucleic acid in a defined volume of preparation divided by the amount of lipid in the same volume. This may be on a mole per mole

basis or on a weight per weight basis, or on a weight per mole basis. For final, administration-ready formulations, the nucleic acid:lipid ratio is calculated after dialysis, chromatography and/or enzyme (*e.g.*, nuclease) digestion has been employed to remove as much of the external nucleic acid as possible.

Encapsulation efficiency refers to the drug to lipid ratio of the starting mixture divided by the drug to lipid ratio of the final, administration competent formulation. This is a measure of relative efficiency. For a measure of absolute efficiency, the total amount of nucleic acid added to the starting mixture that ends up in the administration competent formulation, can also be calculated. The amount of lipid lost during the formulation process may also be calculated. Efficiency is a measure of the wastage and expense of the formulation; and

Size indicates the size (diameter) of the particles formed. Size distribution may be determined using quasi-elastic light scattering (QELS) on a Nicomp Model 370 sub-micron particle sizer. Particles under 200 nm are preferred for distribution to neo-vascularized (leaky) tissues, such as neoplasms and sites of inflammation.

Pharmaceutical Compositions

The lipid particles, particularly when associated with a therapeutic agent, may beformulated as a pharmaceutical composition, e.g., which further comprises a pharmaceutically acceptable diluent, excipient, or carrier, such as physiological saline or phosphate buffer, selected in accordance with the route of administration and standard pharmaceutical practice.

In particular embodiments, pharmaceutical compositions comprising the lipid-nucleic acid particles are prepared according to standard techniques and further comprise a pharmaceutically acceptable carrier. Generally, normal saline will be employed as the pharmaceutically acceptable carrier. Other suitable carriers include, *e.g.*, water, buffered water, 0.9% saline, 0.3% glycine, and the like, including glycoproteins for enhanced stability, such as albumin, lipoprotein, globulin, *etc*. In compositions comprising saline or other salt containing carriers, the carrier is preferably added following lipid particle formation. Thus, after the lipid-nucleic acid compositions are formed, the compositions can be diluted into pharmaceutically acceptable carriers such as normal saline.

The resulting pharmaceutical preparations may be sterilized by conventional, well known sterilization techniques. The aqueous solutions can then be packaged for use or filtered under

aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, *etc.* Additionally, the lipidic suspension may include lipid-protective agents which protect lipids against free-radical and lipid-peroxidative damages on storage. Lipophilic free-radical quenchers, such as α-tocopherol and water-soluble iron-specific chelators, such as ferrioxamine, are suitable.

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

As noted above, the lipid-therapeutic agent (*e.g.*, nucleic acid) particles may include polyethylene glycol (PEG)-modified phospholipids, PEG-ceramide, or ganglioside G_{M1}-modified lipids or other lipids effective to prevent or limit aggregation. Addition of such components does not merely prevent complex aggregation. Rather, it may also provide a means for increasing circulation lifetime and increasing the delivery of the lipid-nucleic acid composition to the target tissues.

Lipid-therapeutic agent compositions can also be provided in kit form. The kit will typically be comprised of a container that is compartmentalized for holding the various elements

of the kit. The kit will contain the particles or pharmaceutical compositions, preferably in dehydrated or concentrated form, with instructions for their rehydration or dilution and administration. In certain embodiments, the particles comprise the active agent, while in other embodiments, they do not.

Methods of Manufacture

The methods and compositions described make use of certain cationic lipids, the synthesis, preparation and characterization of which is described in, for example, in application nos. PCT/US09/63933, PCT/US09/63927, PCT/US09/63931, and PCT/US09/63897, each filed November 10, 2009, and applications referred to therein, including nos. 61/104,219, filed October 9, 2008; no. 61/113,179, filed November 10, 2008; no. 61/154,350, filed February 20, 2009; no. 61/171,439, filed April 21, 2009; no. 61/175,770, filed May 5, 2009; no. 61/185,438, filed June 9, 2009; no. 61/225,898, filed July 15, 2009; no. 61/234,098, filed August 14, 2009; and 61/287,995, filed December 18, 2009; WO 2009/086558; and WO 2008/042973. Each of these documents is incorporated by reference in its entirety. See, for example, Tables 1 and 2 of application no. PCT/US09/63933, filed November 10, 2009, at pages 33-51, and Tables 1-4 and 9 of 61/287,995, at pages 28-53 and 135-141. In addition, methods of preparing lipid particles, including those associated with a therapeutic agent, *e.g.*, a nucleic acid are described. In the methods described herein, a mixture of lipids is combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid encapsulated in lipid particles wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 3 wt% to about 25 wt%, preferably 5 to 15 wt%. The intermediate mixture may optionally be sized to obtain lipid-encapsulated nucleic acid particles wherein the lipid portions are unilamellar vesicles, preferably having a diameter of 30 to 150 nm, more preferably about 40 to 90 nm. The pH is then raised to neutralize at least a portion of the surface charges on the lipid-nucleic acid particles, thus providing an at least partially surface-neutralized lipid-encapsulated nucleic acid composition.

As described above, several of these cationic lipids are amino lipids that are charged at a pH below the pK_a of the amino group and substantially neutral at a pH above the pK_a. These cationic lipids are termed titratable cationic lipids and can be used in the formulations using a two-step process. First, lipid vesicles can be formed at the lower pH with titratable cationic

lipids and other vesicle components in the presence of nucleic acids. In this manner, the vesicles will encapsulate and entrap the nucleic acids. Second, the surface charge of the newly formed vesicles can be neutralized by increasing the pH of the medium to a level above the pK_a of the titratable cationic lipids present, *i.e.*, to physiological pH or higher. Particularly advantageous aspects of this process include both the facile removal of any surface adsorbed nucleic acid and a resultant nucleic acid delivery vehicle which has a neutral surface. Liposomes or lipid particles having a neutral surface are expected to avoid rapid clearance from circulation and to avoid certain toxicities which are associated with cationic liposome preparations. Additional details concerning these uses of such titratable cationic lipids in the formulation of nucleic acid-lipid particles are provided in U.S. Patent 6,287,591 and U.S. Patent 6,858,225, incorporated herein by reference.

It is further noted that the vesicles formed in this manner provide formulations of uniform vesicle size with high content of nucleic acids. Additionally, the vesicles have a size range of from about 30 to about 150 nm, more preferably about 30 to about 90 nm.

Without intending to be bound by any particular theory, it is believed that the very high efficiency of nucleic acid encapsulation is a result of electrostatic interaction at low pH. At acidic pH (e.g. pH 4.0) the vesicle surface is charged and binds a portion of the nucleic acids through electrostatic interactions. When the external acidic buffer is exchanged for a more neutral buffer (*e.g.*, pH 7.5) the surface of the lipid particle or liposome is neutralized, allowing any external nucleic acid to be removed. More detailed information on the formulation process is provided in various publications (*e.g.*, U.S. Patent 6,287,591 and U.S. Patent 6,858,225).

In view of the above, methods of preparing lipid/nucleic acid formulations are described. In the methods described herein, a mixture of lipids is combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid encapsulated in lipid particles, *e.g.*, wherein the encapsulated nucleic acids are present in a nucleic acid/lipid ratio of about 10 wt% to about 20 wt%. The intermediate mixture may optionally be sized to obtain lipid-encapsulated nucleic acid particles wherein the lipid portions are unilamellar vesicles, preferably having a diameter of 30 to 150 nm, more preferably about 40 to 90 nm. The pH is then raised to neutralize at least a portion of the surface charges on the lipid-nucleic acid particles, thus providing an at least partially surface-neutralized lipid-encapsulated nucleic acid composition.

In certain embodiments, the mixture of lipids includes at least two lipid components: a first lipid component that is selected from among lipids which have a pK_a such that the lipid is cationic at pH below the pK_a and neutral at pH above the pK_a, and a second lipid component that is selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation. In particular embodiments, the amino lipid is a cationic lipid.

In preparing the nucleic acid-lipid particles, the mixture of lipids is typically a solution of lipids in an organic solvent. This mixture of lipids can then be dried to form a thin film or lyophilized to form a powder before being hydrated with an aqueous buffer to form liposomes. Alternatively, in a preferred method, the lipid mixture can be solubilized in a water miscible alcohol, such as ethanol, and this ethanolic solution added to an aqueous buffer resulting in spontaneous liposome formation. In most embodiments, the alcohol is used in the form in which it is commercially available. For example, ethanol can be used as absolute ethanol (100%), or as 95% ethanol, the remainder being water. This method is described in more detail in U.S. Patent 5,976,567).

In one exemplary embodiment, the mixture of lipids is a mixture of cationic lipids, neutral lipids (other than a cationic lipid), a sterol (*e.g.,* cholesterol) and a PEG-modified lipid (*e.g.,* a PEG-DMG or PEG-DMA) in an alcohol solvent. In preferred embodiments, the lipid mixture consists essentially of a cationic lipid, a neutral lipid, cholesterol and a PEG-modified lipid in alcohol, more preferably ethanol. In further preferred embodiments, the first solution consists of the above lipid mixture in molar ratios of about 20-70% cationic lipid: 5-45% neutral lipid:20-55% cholesterol:0.5-15% PEG-modified lipid. In still further preferred embodiments, the first solution consists essentially of a mixture of cationic lipids chosen from lipids described in Tables 1-4 and Table 9, DSPC, Chol and PEG-DMG or PEG-DMA, more preferably in a molar ratio of about 20-60% cationic lipid: 5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA), 35/15/40/10 (mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA) or 52/13/30/5 (mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA). In another group of preferred embodiments, the neutral lipid in these compositions is replaced with POPC, DPPC, DOPE or SM.

The lipid mixture is combined with a buffered aqueous solution that may contain the nucleic acids. The buffered aqueous solution of is typically a solution in which the buffer has a

pH of less than the pK_a of the protonatable lipid in the lipid mixture. Examples of suitable buffers include citrate, phosphate, acetate, and MES. A particularly preferred buffer is citrate buffer. Preferred buffers will be in the range of 1-1000 mM of the anion, depending on the chemistry of the nucleic acid being encapsulated, and optimization of buffer concentration may be significant to achieving high loading levels (*see, e.g.,* U.S. Patent 6,287,591 and U.S. Patent 6,858,225, each of which is incorporated by reference in its entirety). Alternatively, pure water acidified to pH 5-6 with chloride, sulfate or the like may be useful. In this case, it may be suitable to add 5% glucose, or another non-ionic solute which will balance the osmotic potential across the particle membrane when the particles are dialyzed to remove ethanol, increase the pH, or mixed with a pharmaceutically acceptable carrier such as normal saline. The amount of nucleic acid in buffer can vary, but will typically be from about 0.01 mg/mL to about 200 mg/mL, more preferably from about 0.5 mg/mL to about 50 mg/mL.

The mixture of lipids and the buffered aqueous solution of therapeutic nucleic acids is combined to provide an intermediate mixture. The intermediate mixture is typically a mixture of lipid particles having encapsulated nucleic acids. Additionally, the intermediate mixture may also contain some portion of nucleic acids which are attached to the surface of the lipid particles (liposomes or lipid vesicles) due to the ionic attraction of the negatively-charged nucleic acids and positively-charged lipids on the lipid particle surface (the amino lipids or other lipid making up the protonatable first lipid component are positively charged in a buffer having a pH of less than the pK_a of the protonatable group on the lipid). In one group of preferred embodiments, the mixture of lipids is an alcohol solution of lipids and the volumes of each of the solutions is adjusted so that upon combination, the resulting alcohol content is from about 20% by volume to about 45% by volume. The method of combining the mixtures can include any of a variety of processes, often depending upon the scale of formulation produced. For example, when the total volume is about 10-20 mL or less, the solutions can be combined in a test tube and stirred together using a vortex mixer. Large-scale processes can be carried out in suitable production scale glassware.

Optionally, the lipid-encapsulated therapeutic agent (*e.g.*, nucleic acid) complexes which are produced by combining the lipid mixture and the buffered aqueous solution of therapeutic agents (nucleic acids) can be sized to achieve a desired size range and relatively narrow distribution of lipid particle sizes. Preferably, the compositions provided herein will be sized to

a mean diameter of from about 70 to about 200 nm, more preferably about 90 to about 130 nm. Several techniques are available for sizing liposomes to a desired size. One sizing method is described in U.S. Pat. No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles (SUVs) less than about 0.05 microns in size. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. In both methods, the particle size distribution can be monitored by conventional laser-beam particle size determination. For certain methods herein, extrusion is used to obtain a uniform vesicle size.

Extrusion of liposome compositions through a small-pore polycarbonate membrane or an asymmetric ceramic membrane results in a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome complex size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size. In some instances, the lipid-nucleic acid compositions which are formed can be used without any sizing.

In particular embodiments, methods further comprise a step of neutralizing at least some of the surface charges on the lipid portions of the lipid-nucleic acid compositions. By at least partially neutralizing the surface charges, unencapsulated nucleic acid is freed from the lipid particle surface and can be removed from the composition using conventional techniques. Preferably, unencapsulated and surface adsorbed nucleic acids are removed from the resulting compositions through exchange of buffer solutions. For example, replacement of a citrate buffer (pH about 4.0, used for forming the compositions) with a HEPES-buffered saline (HBS pH about 7.5) solution, results in the neutralization of liposome surface and nucleic acid release from the surface. The released nucleic acid can then be removed via chromatography using standard methods, and then switched into a buffer with a pH above the pK_a of the lipid used.

Optionally the lipid vesicles (*i.e.*, lipid particles) can be formed by hydration in an aqueous buffer and sized using any of the methods described above prior to addition of the nucleic acid. As described above, the aqueous buffer should be of a pH below the pK_a of the amino lipid. A solution of the nucleic acids can then be added to these sized, preformed vesicles.

To allow encapsulation of nucleic acids into such "pre-formed" vesicles the mixture should contain an alcohol, such as ethanol. In the case of ethanol, it should be present at a concentration of about 20% (w/w) to about 45% (w/w). In addition, it may be necessary to warm the mixture of pre-formed vesicles and nucleic acid in the aqueous buffer-ethanol mixture to a temperature of about 25° C to about 50° C depending on the composition of the lipid vesicles and the nature of the nucleic acid. It will be apparent to one of ordinary skill in the art that optimization of the encapsulation process to achieve a desired level of nucleic acid in the lipid vesicles will require manipulation of variable such as ethanol concentration and temperature. Examples of suitable conditions for nucleic acid encapsulation are provided in the Examples. Once the nucleic acids are encapsulated within the prefromed vesicles, the external pH can be increased to at least partially neutralize the surface charge. Unencapsulated and surface adsorbed nucleic acids can then be removed as described above.

Method of Use

The lipid particles may be used to deliver a therapeutic agent to a cell, *in vitro* or *in vivo*. In particular embodiments, the therapeutic agent is a nucleic acid, which is delivered to a cell using nucleic acid-lipid particles. While the following description of various methods of using the lipid particles and related pharmaceutical compositions are exemplified by description related to nucleic acid-lipid particles, it is understood that these methods and compositions may be readily adapted for the delivery of any therapeutic agent for the treatment of any disease or disorder that would benefit from such treatment.

In certain embodiments, methods for introducing a nucleic acid into a cell are described. Preferred nucleic acids for introduction into cells are siRNA, immune-stimulating oligonucleotides, plasmids, antisense and ribozymes. These methods may be carried out by contacting the particles or compositions with the cells for a period of time sufficient for intracellular delivery to occur.

The compositions can be adsorbed to almost any cell type. Once adsorbed, the nucleic acid-lipid particles can either be endocytosed by a portion of the cells, exchange lipids with cell membranes, or fuse with the cells. Transfer or incorporation of the nucleic acid portion of the complex can take place via any one of these pathways. Without intending to be limited, it is believed that in the case of particles taken up into the cell by endocytosis the particles then

interact with the endosomal membrane, resulting in destabilization of the endosomal membrane, possibly by the formation of non-bilayer phases, resulting in introduction of the encapsulated nucleic acid into the cell cytoplasm. Similarly in the case of direct fusion of the particles with the cell plasma membrane, when fusion takes place, the liposome membrane is integrated into the cell membrane and the contents of the liposome combine with the intracellular fluid. Contact between the cells and the lipid-nucleic acid compositions, when carried out *in vitro*, will take place in a biologically compatible medium. The concentration of compositions can vary widely depending on the particular application, but is generally between about 1 μmol and about 10 mmol. In certain embodiments, treatment of the cells with the lipid-nucleic acid compositions will generally be carried out at physiological temperatures (about 37 °C) for periods of time from about 1 to 24 hours, preferably from about 2 to 8 hours. For *in vitro* applications, the delivery of nucleic acids can be to any cell grown in culture, whether of plant or animal origin, vertebrate or invertebrate, and of any tissue or type. In preferred embodiments, the cells will be animal cells, more preferably mammalian cells, and most preferably human cells.

In one group of embodiments, a lipid-nucleic acid particle suspension is added to 60-80% confluent plated cells having a cell density of from about 10^3 to about 10^5 cells/mL, more preferably about 2×10^4 cells/mL. The concentration of the suspension added to the cells is preferably of from about 0.01 to 20 μg/mL, more preferably about 1 μg/mL.

In another embodiment, the lipid particles can be may be used to deliver a nucleic acid to a cell or cell line (for example, a tumor cell line). Non-limiting examples of such cell lines include: HELA (ATCC Cat N: CCL-2), KB (ATCC Cat N: CCL-17), HEP3B (ATCC Cat N: HB-8064), SKOV-3 (ATCC Cat N: HTB-77), HCT-116 (ATCC Cat N: CCL-247), HT-29 (ATCC Cat N: HTB-38), PC-3 (ATCC Cat N: CRL-1435), A549 (ATCC Cat N: CCL-185), MDA-MB-231 (ATCC Cat N: HTB-26).

Typical applications include using well known procedures to provide intracellular delivery of siRNA to knock down or silence specific cellular targets. Alternatively applications include delivery of DNA or mRNA sequences that code for therapeutically useful polypeptides. In this manner, therapy is provided for genetic diseases by supplying deficient or absent gene products (*i.e.*, for Duchenne's dystrophy, see Kunkel, *et al., Brit. Med. Bull.* 45(3):630-643 (1989), and for cystic fibrosis, see Goodfellow, *Nature* 341:102-103 (1989)). Other uses for the compositions include introduction of antisense oligonucleotides in cells (see, Bennett, *et al., Mol.*

Pharm. 41:1023-1033 (1992)).

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

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

In other methods, the pharmaceutical preparations may be contacted with the target tissue by direct application of the preparation to the tissue. The application may be made by topical, "open" or "closed" procedures. By "topical," it is meant the direct application of the pharmaceutical preparation to a tissue exposed to the environment, such as the skin, oropharynx, external auditory canal, and the like. "Open" procedures are those procedures which include incising the skin of a patient and directly visualizing the underlying tissue to which the

pharmaceutical preparations are applied. This is generally accomplished by a surgical procedure, such as a thoracotomy to access the lungs, abdominal laparotomy to access abdominal viscera, or other direct surgical approach to the target tissue. "Closed" procedures are invasive procedures in which the internal target tissues are not directly visualized, but accessed via inserting instruments through small wounds in the skin. For example, the preparations may be administered to the peritoneum by needle lavage. Likewise, the pharmaceutical preparations may be administered to the meninges or spinal cord by infusion during a lumbar puncture followed by appropriate positioning of the patient as commonly practiced for spinal anesthesia or metrazamide imaging of the spinal cord. Alternatively, the preparations may be administered through endoscopic devices.

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

The methods may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

Dosages for the lipid-therapeutic agent particles will depend on the ratio of therapeutic agent to lipid and the administrating physician's opinion based on age, weight, and condition of the patient.

In one embodiment, a method of modulating the expression of a target polynucleotide or polypeptide is described. These methods generally comprise contacting a cell with a lipid particle that is associated with a nucleic acid capable of modulating the expression of a target polynucleotide or polypeptide. As used herein, the term "modulating" refers to altering the expression of a target polynucleotide or polypeptide. In different embodiments, modulating can mean increasing or enhancing, or it can mean decreasing or reducing. Methods of measuring the level of expression of a target polynucleotide or polypeptide are known and available in the arts and include, e.g., methods employing reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemical techniques. In particular embodiments, the level of expression of a target polynucleotide or polypeptide is increased or reduced by at least 10%, 20%, 30%, 40%, 50%, or greater than 50% as compared to an appropriate control value.

For example, if increased expression of a polypeptide desired, the nucleic acid may be an

expression vector that includes a polynucleotide that encodes the desired polypeptide. On the other hand, if reduced expression of a polynucleotide or polypeptide is desired, then the nucleic acid may be, *e.g.*, an antisense oligonucleotide, siRNA, or microRNA that comprises a polynucleotide sequence that specifically hybridizes to a polnucleotide that encodes the target polypeptide, thereby disrupting expression of the target polynucleotide or polypeptide. Alternatively, the nucleic acid may be a plasmid that expresses such an antisense oligonucletoide, siRNA, or microRNA.

In one particular embodiment, a method of modulating the expression of a polypeptide by a cell, includes providing to a cell a lipid particle that consists of or consists essentially of a mixture of cationic lipids chosen from lipids described in application nos. PCT/US09/63933, PCT/US09/63927, PCT/US09/63931, and PCT/US09/63897, each filed November 10, 2009, and applications referred to therein, including nos. 61/104,219, filed October 9, 2008; no. 61/113,179, filed November 10, 2008; no. 61/154,350, filed February 20, 2009; no. 61/171,439, filed April 21, 2009; no. 61/175,770, filed May 5, 2009; no. 61/185,438, filed June 9, 2009; no. 61/225,898, filed July 15, 2009; no. 61/234,098, filed August 14, 2009; and 61/287,995, filed December 18, 2009; WO 2009/086558; and WO 2008/042973 (each of these documents is incorporated by reference in its entirety. See, for example, Tables 1 and 2 of application no. PCT/US09/63933, filed November 10, 2009, at pages 33-51, and Tables 1-4 and 9 of 61/287,995, at pages 28-53 and 135-141), DSPC, Chol and PEG-DMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% cationic lipid: 0.1-50% fusion-promoting lipid:5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA, wherein the lipid particle is assocated with a nucleic acid capable of modulating the expression of the polypeptide. In particular embodiments, the molar lipid ratio is 0.1-50% fusion promoting lipid, with the remaining components present in a relative molar lipid ratio (mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA) of approximately 40/10/40/10, 35/15/40/10, or 52/13/30/5. In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DPPC, DOPE or SM.

In particular embodiments, the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the

polypeptide, or a complement thereof, such that the expression of the polypeptide is reduced.

In other embodiments, the nucleic acid is a plasmid that encodes the polypeptide or a functional variant or fragment thereof, such that expression of the polypeptide or the functional variant or fragment thereof is increased.

In related embodiments, a method of treating a disease or disorder characterized by overexpression of a polypeptide in a subject, includes providing to the subject a pharmaceutical composition, wherein the therapeutic agent is selected from an siRNA, a microRNA, an antisense oligonucleotide, and a plasmid capable of expressing an siRNA, a microRNA, or an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense RNA comprises a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof.

In one embodiment, the pharmaceutical composition comprises a lipid particle that consists of or consists essentially of a mixture of cationic lipids chosen from lipids described in Tables 1-4 and Table 9, DSPC, Chol and PEG-DMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% cationic lipid: 5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA, wherein the lipid particle is assocated with the therapeutic nucleic acid. In particular embodiments, the molar lipid ratio is approximately 40/10/40/10 (mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA), 35/15/40/10 (mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA) or 52/13/30/5 (mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA). In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DPPC, DOPE or SM.

In another related embodiment, a method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject, includes providing to the subject a pharmaceutical composition, wherein the therapeutic agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof.

In one embodiment, the pharmaceutical composition comprises a lipid particle that consists of or consists essentially of a mixture of cationic lipids chosen from lipids described in application nos. PCT/US09/63933, PCT/US09/63927, PCT/US09/63931, and PCT/US09/63897, each filed November 10, 2009, and applications referred to therein, including nos. 61/104,219, filed October 9, 2008; no. 61/113,179, filed November 10, 2008; no. 61/154,350, filed February 20, 2009; no. 61/171,439, filed April 21, 2009; no. 61/175,770, filed May 5, 2009; no.

61/185,438, filed June 9, 2009; no. 61/225,898, filed July 15, 2009; no. 61/234,098, filed August 14, 2009; and 61/287,995, filed December 18, 2009; WO 2009/086558; and WO 2008/042973 (each of these documents is incorporated by reference in its entirety. See, for example, Tables 1 and 2 of application no. PCT/US09/63933, filed November 10, 2009, at pages 33-51, and Tables 1-4 and 9 of 61/287,995, at pages 28-53 and 135-141), DSPC, Chol and PEG-DMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% cationic lipid: 0.1-50% fusion-promoting lipid:5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA, wherein the lipid particle is assocated with the therapeutic nucleic acid. In particular embodiments, the molar lipid ratio is 0.1-50% fusion promoting lipid, with the remaining components present in a relative molar lipid ratio (mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA) of approximately 40/10/40/10, 35/15/40/10, or 52/13/30/5. In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DPPC, DOPE or SM.

A method of inducing an immune response in a subject, can include providing to the subject the pharmaceutical composition, wherein the therapeutic agent is an immunostimulatory oligonucleotide. In certain embodiments, the immune response is a humoral or mucosal immune response. In one embodiment, the pharmaceutical composition comprises a lipid particle that consists of or consists essentially of mixture of cationic lipids chosen from lipids described in application nos. PCT/US09/63933, PCT/US09/63927, PCT/US09/63931, and PCT/US09/63897, each filed November 10, 2009, and applications referred to therein, including nos. 61/104,219, filed October 9, 2008; no. 61/113,179, filed November 10, 2008; no. 61/154,350, filed February 20, 2009; no. 61/171,439, filed April 21, 2009; no. 61/175,770, filed May 5, 2009; no. 61/185,438, filed June 9, 2009; no. 61/225,898, filed July 15, 2009; no. 61/234,098, filed August 14, 2009; and 61/287,995, filed December 18, 2009; WO 2009/086558; and WO 2008/042973 (each of these documents is incorporated by reference in its entirety. See, for example, Tables 1 and 2 of application no. PCT/US09/63933, filed November 10, 2009, at pages 33-51, and Tables 1-4 and 9 of 61/287,995, at pages 28-53 and 135-141), DSPC, Chol and PEG-DMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% cationic lipid: 0.1-50% fusion-promoting lipid:5-25% DSPC:25-55% Chol:0.5-15% PEG-DMG or PEG-DMA, wherein the lipid particle is assocated with the therapeutic nucleic acid. In particular embodiments, the molar lipid ratio is 0.1-50% fusion promoting lipid, with the remaining components present in a relative molar lipid ratio (mol% cationic lipid/DSPC/Chol/PEG-DMG or PEG-DMA) of approximately 40/10/40/10,

35/15/40/10, or 52/13/30/5. In another group of embodiments, the neutral lipid in these compositions is replaced with POPC, DPPC, DOPE or SM.

In further embodiments, the pharmaceutical composition is provided to the subject in combination with a vaccine or antigen. Thus, vaccines can include a lipid particle, which comprises an immunostimulatory oligonucleotide, and is also associated with an antigen to which an immune response is desired. In particular embodiments, the antigen is a tumor antigen or is associated with an infective agent, such as, *e.g.*, a virus, bacteria, or parasiste.

A variety of tumor antigens, infections agent antigens, and antigens associated with other disease are well known in the art and examples of these are described in references cited herein. Examples of suitable antigens include, but are not limited to, polypeptide antigens and DNA antigens. Specific examples of antigens are Hepatitis A, Hepatitis B, small pox, polio, anthrax, influenza, typhus, tetanus, measles, rotavirus, diphtheria, pertussis, tuberculosis, and rubella antigens. In a preferred embodiment, the antigen is a Hepatitis B recombinant antigen. In other aspects, the antigen is a Hepatitis A recombinant antigen. In another aspect, the antigen is a tumor antigen. Examples of such tumor-associated antigens are MUC-1, EBV antigen and antigens associated with Burkitt's lymphoma. In a further aspect, the antigen is a tyrosinase-related protein tumor antigen recombinant antigen. Those of skill in the art will know of other antigens suitable for use.

Tumor-associated antigens suitable for use include both mutated and non-mutated molecules that may be indicative of single tumor type, shared among several types of tumors, and/or exclusively expressed or overexpressed in tumor cells in comparison with normal cells. In addition to proteins and glycoproteins, tumor-specific patterns of expression of carbohydrates, gangliosides, glycolipids and mucins have also been documented. Exemplary tumor-associated antigens for use in the subject cancer vaccines include protein products of oncogenes, tumor suppressor genes and other genes with mutations or rearrangements unique to tumor cells, reactivated embryonic gene products, oncofetal antigens, tissue-specific (but not tumor-specific) differentiation antigens, growth factor receptors, cell surface carbohydrate residues, foreign viral proteins and a number of other self proteins.

Specific embodiments of tumor-associated antigens include, *e.g.*, mutated antigens such as the protein products of the Ras p21 protooncogenes, tumor suppressor p53 and BCR-abl oncogenes, as well as CDK4, MUM1, Caspase 8, and Beta catenin; overexpressed antigens such

as galectin 4, galectin 9, carbonic anhydrase, Aldolase A, PRAME, Her2/neu, ErbB-2 and KSA, oncofetal antigens such as alpha fetoprotein (AFP), human chorionic gonadotropin (hCG); self antigens such as carcinoembryonic antigen (CEA) and melanocyte differentiation antigens such as Mart 1/Melan A, gp100, gp75, Tyrosinase, TRP1 and TRP2; prostate associated antigens such as PSA, PAP, PSMA, PSM-P1 and PSM-P2; reactivated embryonic gene products such as MAGE 1, MAGE 3, MAGE 4, GAGE 1, GAGE 2, BAGE, RAGE, and other cancer testis antigens such as NY-ESO1, SSX2 and SCP1; mucins such as Muc-1 and Muc-2; gangliosides such as GM2, GD2 and GD3, neutral glycolipids and glycoproteins such as Lewis (y) and globo-H; and glycoproteins such as Tn, Thompson-Freidenreich antigen (TF) and sTn. Also included as tumor-associated antigens herein are whole cell and tumor cell lysates as well as immunogenic portions thereof, as well as immunoglobulin idiotypes expressed on monoclonal proliferations of B lymphocytes for use against B cell lymphomas.

Pathogens include, but are not limited to, infectious agents, *e.g.*, viruses, that infect mammals, and more particularly humans. Examples of infectious virus include, but are not limited to: Retroviridae (*e.g.*, human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; Picornaviridae (*e.g.*, polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); Calciviridae (*e.g.*, strains that cause gastroenteritis); Togaviridae (*e.g.*, equine encephalitis viruses, rubella viruses); Flaviridae (*e.g.*, dengue viruses, encephalitis viruses, yellow fever viruses); Coronoviridae (*e.g.*, coronaviruses); Rhabdoviradae (*e.g.*, vesicular stomatitis viruses, rabies viruses); Coronaviridae (*e.g.*, coronaviruses); Rhabdoviridae (*e.g.*, vesicular stomatitis viruses, rabies viruses); Filoviridae (*e.g.*, ebola viruses); Paramyxoviridae (*e.g.*, parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); Orthomyxoviridae (*e.g.*,influenza viruses); Bungaviridae (*e.g.*, Hantaan viruses, bunga viruses, phleboviruses and Nairo viruses); Arena viridae (hemorrhagic fever viruses); Reoviridae (*e.g.*, reoviruses, orbiviurses and rotaviruses); Birnaviridae; Hepadnaviridae (Hepatitis B virus); Parvovirida (parvoviruses); Papovaviridae (papilloma viruses, polyoma viruses); Adenoviridae (most adenoviruses); Herpesviridae herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; Poxviridae (variola viruses, vaccinia viruses, pox viruses); and Iridoviridae (*e.g.*, African swine fever virus); and unclassified viruses (*e.g.*, the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a

defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (*i.e.*, Hepatitis C); Norwalk and related viruses, and astroviruses).

Also, gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to Pasteurella species, Staphylococci species, and Streptococcus species. Gram negative bacteria include, but are not limited to, Escherichia coli, Pseudomonas species, and Salmonella species. Specific examples of infectious bacteria include but are not limited to: Helicobacterpyloris, Borelia burgdorferi, Legionella pneumophilia, Mycobacteria sps (*e.g.*, M. tuberculosis, M. avium, M. intracellulare, M. kansaii, M. gordonae), Staphylococcus aureus, Neisseria gonorrhoeae, Neisseria meningitidis, Listeria monocytogenes, Streptococcus pyogenes (Group A Streptococcus), Streptococcus agalactiae (Group B Streptococcus), Streptococcus (viridans group), Streptococcusfaecalis, Streptococcus bovis, Streptococcus (anaerobic sps.), Streptococcus pneumoniae, pathogenic Campylobacter sp., Enterococcus sp., Haemophilus infuenzae, Bacillus antracis, corynebacterium diphtheriae, corynebacterium sp., Erysipelothrix rhusiopathiae, Clostridium perfringers, Clostridium tetani, Enterobacter aerogenes, Klebsiella pneumoniae, Pasturella multocida, Bacteroides sp., Fusobacterium nucleatum, Streptobacillus moniliformis, Treponema pallidium, Treponema pertenue, Leptospira, Rickettsia, and Actinomyces israelli.

Additional examples of pathogens include, but are not limited to, infectious fungi that infect mammals, and more particularly humans. Examples of infectious fingi include, but are not limited to: Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Chlamydia trachomatis, Candida albicans. Examples of infectious parasites include Plasmodium such as Plasmodium falciparum, Plasmodium malariae, Plasmodium ovale, and Plasmodium vivax. Other infectious organisms (*i.e.*, protists) include Toxoplasma gondii.

In one embodiment, the formulations can be used to silence or modulate a target gene such as but not limited to FVII, Eg5, PCSK9, TPX2, apoB, SAA, TTR, RSV, PDGF beta gene, Erb-B gene, Src gene, CRK gene, GRB2 gene, RAS gene, MEKK gene, JNK gene, RAF gene, Erk1/2 gene, PCNA(p21) gene, MYB gene, JUN gene, FOS gene, BCL-2 gene, Cyclin D gene, VEGF gene, EGFR gene, Cyclin A gene, Cyclin E gene, WNT-1 gene, beta-catenin gene, c-MET gene, PKC gene, NFKB gene, STAT3 gene, survivin gene, Her2/Neu gene, SORT1 gene,

XBP1 gene, topoisomerase I gene, topoisomerase II alpha gene, p73 gene, p21(WAF1/CIP1) gene, p27(KIP1) gene, PPM1D gene, RAS gene, caveolin I gene, MIB I gene, MTA1 gene, M68 gene, tumor suppressor genes, p53 tumor suppressor gene, p53 family member DN-p63, pRb tumor suppressor gene, APC1 tumor suppressor gene, BRCA1 tumor suppressor gene, PTEN tumor suppressor gene, mLL fusion gene, BCR/ABL fusion gene, TEL/AML1 fusion gene, EWS/FLI1 fusion gene, TLS/FUS1 fusion gene, PAX3/FKHR fusion gene, AML1/ETO fusion gene, alpha v-integrin gene, Flt-1 receptor gene, tubulin gene, Human Papilloma Virus gene, a gene required for Human Papilloma Virus replication, Human Immunodeficiency Virus gene, a gene required for Human Immunodeficiency Virus replication, Hepatitis A Virus gene, a gene required for Hepatitis A Virus replication, Hepatitis B Virus gene, a gene required for Hepatitis B Virus replication, Hepatitis C Virus gene, a gene required for Hepatitis C Virus replication, Hepatitis D Virus gene, a gene required for Hepatitis D Virus replication, Hepatitis E Virus gene, a gene required for Hepatitis E Virus replication, Hepatitis F Virus gene, a gene required for Hepatitis F Virus replication, Hepatitis G Virus gene, a gene required for Hepatitis G Virus replication, Hepatitis H Virus gene, a gene required for Hepatitis H Virus replication, Respiratory Syncytial Virus gene, a gene that is required for Respiratory Syncytial Virus replication, Herpes Simplex Virus gene, a gene that is required for Herpes Simplex Virus replication, herpes Cytomegalovirus gene, a gene that is required for herpes Cytomegalovirus replication, herpes Epstein Barr Virus gene, a gene that is required for herpes Epstein Barr Virus replication, Kaposi's Sarcoma-associated Herpes Virus gene, a gene that is required for Kaposi's Sarcoma-associated Herpes Virus replication, JC Virus gene, human gene that is required for JC Virus replication, myxovirus gene, a gene that is required for myxovirus gene replication, rhinovirus gene, a gene that is required for rhinovirus replication, coronavirus gene, a gene that is required for coronavirus replication, West Nile Virus gene, a gene that is required for West Nile Virus replication, St. Louis Encephalitis gene, a gene that is required for St. Louis Encephalitis replication, Tick-borne encephalitis virus gene, a gene that is required for Tick-borne encephalitis virus replication, Murray Valley encephalitis virus gene, a gene that is required for Murray Valley encephalitis virus replication, dengue virus gene, a gene that is required for dengue virus gene replication, Simian Virus 40 gene, a gene that is required for Simian Virus 40 replication, Human T Cell Lymphotropic Virus gene, a gene that is required for Human T Cell Lymphotropic Virus replication, Moloney-Murine Leukemia Virus gene, a gene that is required

for Moloney-Murine Leukemia Virus replication, encephalomyocarditis virus gene, a gene that is required for encephalomyocarditis virus replication, measles virus gene, a gene that is required for measles virus replication, Vericella zoster virus gene, a gene that is required for Vericella zoster virus replication, adenovirus gene, a gene that is required for adenovirus replication, yellow fever virus gene, a gene that is required for yellow fever virus replication, poliovirus gene, a gene that is required for poliovirus replication, poxvirus gene, a gene that is required for poxvirus replication, plasmodium gene, a gene that is required for plasmodium gene replication, Mycobacterium ulcerans gene, a gene that is required for Mycobacterium ulcerans replication, Mycobacterium tuberculosis gene, a gene that is required for Mycobacterium tuberculosis replication, Mycobacterium leprae gene, a gene that is required for Mycobacterium leprae replication, Staphylococcus aureus gene, a gene that is required for Staphylococcus aureus replication, Streptococcus pneumoniae gene, a gene that is required for Streptococcus pneumoniae replication, Streptococcus pyogenes gene, a gene that is required for Streptococcus pyogenes replication, Chlamydia pneumoniae gene, a gene that is required for Chlamydia pneumoniae replication, Mycoplasma pneumoniae gene, a gene that is required for Mycoplasma pneumoniae replication, an integrin gene, a selectin gene, complement system gene, chemokine gene, chemokine receptor gene, GCSF gene, Gro1 gene, Gro2 gene, Gro3 gene, PF4 gene, MIG gene, Pro-Platelet Basic Protein gene, MIP-1I gene, MIP-1J gene, RANTES gene, MCP-1 gene, MCP-2 gene, MCP-3 gene, CMBKR1 gene, CMBKR2 gene, CMBKR3 gene, CMBKR5v, AIF-1 gene, I-309 gene, a gene to a component of an ion channel, a gene to a neurotransmitter receptor, a gene to a neurotransmitter ligand, amyloid-family gene, presenilin gene, HD gene, DRPLA gene, SCA1 gene, SCA2 gene, MJD1 gene, CACNL1A4 gene, SCA7 gene, SCA8 gene, allele gene found in LOH cells, or one allele gene of a polymorphic gene.

Definitions

A subject or patient in whom administration of the complex is an effective therapeutic regimen for a disease or disorder is preferably a human, but can be any animal, including a laboratory animal in the context of a clinical trial or screening or activity experiment. Thus, as can be readily appreciated by one of ordinary skill in the art, the methods, compounds and compositions of the present invention are particularly suited to administration to any animal, particularly a mammal, and including, but by no means limited to, humans, domestic animals,

such as feline or canine subjects, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine subjects, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, and cats, avian species, such as chickens, turkeys, and songbirds, i.e., for veterinary medical use.

Many of the chemical groups recited in the generic formulas above are written in a particular order (for example, -OC(O)-). It is intended that the chemical group is to be incorporated into the generic formula in the order presented unless indicated otherwise. For example, a generic formula of the form X-Y-Z where Y is –OC(O)- refers to X-OC(O)-Y unless specified otherwise. It is to be understood that when a chemical group is written in a particular order, the reverse order is also contemplated unless otherwise specified. For example, in a generic formula X-Y-Z where Y is defined as –C(O)NH- (i.e., X-C(O)-NH-Z), the compound where Y is –NHC(O)- (i.e., X-NH-C(O)-Z) is also contemplated unless otherwise specified.

As used herein, an "aliphatic" group is a non-aromatic group in which carbon atoms are linked into chains, and is either saturated or unsaturated.

The term "alkyl" refers to a straight or branched chain saturated hydrocarbon moiety. In one embodiment, the alkyl group is a straight chain saturated hydrocarbon. Unless otherwise specified, the "alkyl" group contains from 1 to 24 carbon atoms. Representative saturated straight chain alkyl groups include methyl, ethyl, n-propyl, n-butyl, n-pentyl, and n-hexyl; while saturated branched alkyl groups include isopropyl, *sec*-butyl, isobutyl, *tert*-butyl, and isopentyl.

The term "alkenyl" refers to a straight or branched chain hydrocarbon moiety having one or more carbon-carbon double bonds. In one embodiment, the alkenyl group contains 1, 2, or 3 double bonds and is otherwise saturated. Unless otherwise specified, the "alkenyl" group contains from 2 to 24 carbon atoms. Alkenyl groups include both cis and trans isomers. Representative straight chain and branched alkenyl groups include ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, and 2,3-dimethyl-2-butenyl.

The term "alkynyl" refers to a straight or branched chain hydrocarbon moiety having one or more carbon-carbon triple bonds. Unless otherwise specified, the "alkynyl" group contains from 2 to 24 carbon atoms. Representative straight chain and branched alkynyl groups include acetylenyl, propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, 2-pentynyl, and 3-methyl-1-butynyl.

The term "acyl" refers to hydrogen, alkyl, partially saturated or fully saturated cycloalkyl,

partially saturated or fully saturated heterocycle, aryl, and heteroaryl substituted carbonyl groups. For example, acyl groups include groups such as (C_1-C_{20})alkanoyl (e.g., formyl, acetyl, propionyl, butyryl, valeryl, caproyl, and t-butylacetyl), (C_3-C_{20})cycloalkylcarbonyl (e.g., cyclopropylcarbonyl, cyclobutylcarbonyl, cyclopentylcarbonyl, and cyclohexylcarbonyl), heterocyclic carbonyl (e.g., pyrrolidinylcarbonyl, pyrrolid-2-one-5-carbonyl, piperidinylcarbonyl, piperazinylcarbonyl, and tetrahydrofuranylcarbonyl), aroyl (e.g., benzoyl) and heteroaroyl (e.g., thiophenyl-2-carbonyl, thiophenyl-3-carbonyl, furanyl-2-carbonyl, furanyl-3-carbonyl, lH-pyrroyl-2-carbonyl, 1H-pyrroyl-3-carbonyl, and benzo[b]thiophenyl-2-carbonyl).

The term "aryl" refers to an aromatic monocyclic, bicyclic, or tricyclic hydrocarbon ring system. Examples of aryl moieties include, but are not limited to, phenyl, naphthyl, anthracenyl, and pyrenyl.

The terms “cycloalkyl” and “cyclalkylene” refer to a saturated monocyclic or bicyclic hydrocarbon moiety such as cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl.

The term "heterocycle" (or “heterocyclyl”) refers to a non-aromatic 5- to 8-membered monocyclic, or 7- to 12-membered bicyclic, or 11- to 14-membered tricyclic ring system which is either saturated or unsaturated, and which contains from 1 to 3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, 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. For instance, the heterocycle may be a cycloalkoxy group. The heterocycle may be attached to the rest of the molecule via any heteroatom or carbon atom in the heterocycle. Heterocycles include, but are not limited to, morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, and tetrahydrothiopyranyl.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 7-12 membered bicyclic, or 11-14 membered tricyclic ring system having 1-3 heteroatoms if monocyclic, 1-6 heteroatoms if bicyclic, or 1-9 heteroatoms if tricyclic, where the heteroatoms are selected from O, N, or S (e.g., carbon atoms and 1-3, 1-6, or 1-9 heteroatoms of N, O, or S if monocyclic, bicyclic, or tricyclic, respectively). The heteroaryl groups herein described may also contain

fused rings that share a common carbon-carbon bond.

The term "substituted", unless otherwise indicated, refers to the replacement of one or more hydrogen radicals in a given structure with the radical of a specified substituent including, but not limited to: halo, alkyl, alkenyl, alkynyl, aryl, heterocyclyl, thiol, alkylthio, oxo, thioxy, arylthio, alkylthioalkyl, arylthioalkyl, alkylsulfonyl, alkylsulfonylalkyl, arylsulfonylalkyl, alkoxy, aryloxy, aralkoxy, aminocarbonyl, alkylaminocarbonyl, arylaminocarbonyl, alkoxycarbonyl, aryloxycarbonyl, haloalkyl, amino, trifluoromethyl, cyano, nitro, alkylamino, arylamino, alkylaminoalkyl, arylaminoalkyl, aminoalkylamino, hydroxy, alkoxyalkyl, carboxyalkyl, alkoxycarbonylalkyl, aminocarbonylalkyl, acyl, aralkoxycarbonyl, carboxylic acid, sulfonic acid, sulfonyl, phosphonic acid, aryl, heteroaryl, heterocyclic, and an aliphatic group. It is understood that the substituent may be further substituted. Exemplary substituents include amino, alkylamino, dialkylamino, and cyclic amino compounds.

The term "halogen" or "halo" refers to fluoro, chloro, bromo and iodo.

The terms "alkylamine" and "dialkylamine" refer to -NH(alkyl) and -N(alkyl)$_2$ radicals respectively.

The term "alkylphosphate" refers to -O-P(Q')(Q")-O-R, wherein Q' and Q" are each independently O, S, N(R)$_2$, optionally substituted alkyl or alkoxy; and R is optionally substituted alkyl, ω-aminoalkyl or ω-(substituted)aminoalkyl.

The term "alkylphosphorothioate" refers to an alkylphosphate wherein at least one of Q' or Q" is S.

The term "alkylphosphonate" refers to an alkylphosphate wherein at least one of Q' or Q" is alkyl.

The terem "hydroxyalkyl" means -O-alkyl radical.

The term "alkylheterocycle" refers to an alkyl where at least one methylene has been replaced by a heterocycle.

The term "ω-aminoalkyl" refers to -alkyl-NH$_2$ radical. And the term "ω-(substituted)aminoalkyl refers to an ω-aminoalkyl wherein at least one of the H on N has been replaced with alkyl.

The term "ω-phosphoalkyl" refers to -alkyl-O-P(Q')(Q")-O-R, wherein Q' and Q" are each independently O or S and R optionally substituted alkyl.

The term "ω-thiophosphoalkyl refers to ω-phosphoalkyl wherein at least one of Q' or Q"

is S.

In some embodiments, the methods may require the use of protecting groups. Protecting group methodology is well known to those skilled in the art (*see, for example*, Protective Groups in Organic Synthesis, Green, T.W. et. al., Wiley-Interscience, New York City, 1999). Briefly, protecting groups are any group that reduces or eliminates unwanted reactivity of a functional group. A protecting group can be added to a functional group to mask its reactivity during certain reactions and then removed to reveal the original functional group. In some embodiments an "alcohol protecting group" is used. An "alcohol protecting group" is any group which decreases or eliminates unwanted reactivity of an alcohol functional group. Protecting groups can be added and removed using techniques well known in the art.

The compounds may be prepared by at least one of the techniques described herein or known organic synthesis techniques.

Examples

Example 1: FVII *in vivo* evaluation using the cationic lipid derived liposomes

C57BL/6 mice (Charles River Labs, MA) receive either saline or siRNA in desired formulations via tail vein injection at a volume of 0.01 mL/g. At various time points post-administration, animals are anesthesized by isofluorane inhalation and blood is collected into serum separator tubes by retro orbital bleed. Serum levels of Factor VII protein are determined in samples using a chromogenic assay (Coaset Factor VII, DiaPharma Group, OH or Biophen FVII, Aniara Corporation, OH) according to manufacturer protocols. A standard curve is generated using serum collected from saline treated animals. In experiments where liver mRNA levels are assessed, at various time points post-administration, animals are sacrificed and livers are harvested and snap frozen in liquid nitrogen. Frozen liver tissue is ground into powder. Tissue lysates are prepared and liver mRNA levels of Factor VII and *apoB* are determined using a branched DNA assay (QuantiGene Assay, Panomics, CA).

Example 2: Determination of efficacy of lipid particle formulations containing various cationic lipids using an *in vivo* rodent Factor VII silencing model.

Factor VII (FVII), a prominent protein in the coagulation cascade, is synthesized in the liver (hepatocytes) and secreted into the plasma. FVII levels in plasma can be determined by a

simple, plate-based colorimetric assay. As such, FVII represents a convenient model for determining sirna-mediated downregulation of hepatocyte-derived proteins, as well as monitoring plasma concentrations and tissue distribution of the nucleic acid lipid particles and siRNA.

Duplex	Sequence 5'-3'	SEQ ID NO:	Target
AD-1661	GGAfUfCAfUfCfUfCAAGfUfCfUfUAfCdTsdT		FVII
	GfUAAGAfCfUfUGAGAfUGAfUfCfCdTsdT		

Lower case is 2'OMe modification and Nf is a 2'F modified nucleobase, dT is deoxythymidine, s is phosphothioate

The cationic lipids shown above are used to formulate liposomes containing the AD-1661duplex using an in-line mixing method, as described in U.S. provisional patent application 61/228,373, which is incorporated by reference in its entirety. Lipid particles are formulated using the following molar ratio: 50% Cationic lipid/ 10% distearoylphosphatidylcholine (DSPC) / 38.5% Cholesterol/ 1.5% PEG-DMG (1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol, with an average PEG molecular weight of 2000).

C57BL/6 mice (Charles River Labs, MA) receive either saline or formulated siRNA via tail vein injection. At various time points after administration, serum samples are collected by retroorbital bleed. Serum levels of Factor VII protein are determined in samples using a chromogenic assay (Biophen FVII, Aniara Corporation, OH). To determine liver mRNA levels of Factor VII, animals are sacrificed and livers were harvested and snap frozen in liquid nitrogen. Tissue lysates are prepared from the frozen tissues and liver mRNA levels of Factor VII are quantified using a branched DNA assay (QuantiGene Assay, Panomics, CA).

FVII activity is evaluated in FVII siRNA-treated animals at 48 hours after intravenous (bolus) injection in C57BL/6 mice. FVII is measured using a commercially available kit for determining protein levels in serum or tissue, following the manufacturer's instructions at a microplate scale. FVII reduction is determined against untreated control mice, and the results are expressed as % Residual FVII. Two dose levels (0.05 and 0.005 mg/kg FVII siRNA) are used in the screen of each novel liposome composition.

Example 3: siRNA formulation using preformed vesicles

Cationic lipid containing particles are made using the preformed vesicle method. Cationic lipid, DSPC, cholesterol and PEG-lipid were solubilized in ethanol at a molar ratio of 40/10/40/10, respectively. The lipid mixture is added to an aqueous buffer (50 mM citrate, pH 4) with mixing to a final ethanol and lipid concentration of 30% (vol/vol) and 6.1 mg/mL respectively and allowed to equilibrate at room temperature for 2 min before extrusion. The hydrated lipids are extruded through two stacked 80 nm pore-sized filters (Nuclepore) at 22°C using a Lipex Extruder (Northern Lipids, Vancouver, BC) until a vesicle diameter of 70-90 nm, as determined by Nicomp analysis, is obtained. This generally requires 1-3 passes. For some cationic lipid mixtures which did not form small vesicles hydrating the lipid mixture with a lower pH buffer (50mM citrate, pH 3) to protonate the phosphate group on the DSPC headgroup helps form stable 70-90 nm vesicles.

The FVII siRNA (solubilised in a 50mM citrate, pH 4 aqueous solution containing 30% ethanol) is added to the vesicles, pre-equilibrated to 35°C, at a rate of ~5mL/min with mixing. After a final target siRNA/lipid ratio of 0.06 (wt/wt) is achieved, the mixture is incubated for a further 30 min at 35°C to allow vesicle re-organization and encapsulation of the FVII siRNA. The ethanol is then removed and the external buffer replaced with PBS (155mM NaCl, 3mM Na2HPO4, 1mM KH2PO4, pH 7.5) by either dialysis or tangential flow diafiltration. The final encapsulated siRNA-to-lipid ratio is determined after removal of unencapsulated siRNA using size-exclusion spin columns or ion exchange spin columns.

Example 4: In vivo determination of efficacy of lipid formulations

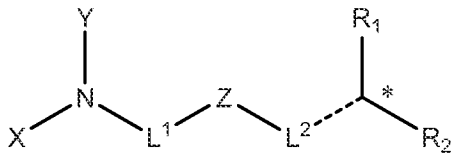
Test formulations are initially assessed for their FVII knockdown in female 7-9 week old, 15-25g, female C57Bl/6 mice at 0.1, 0.3, 1.0 and 5.0 mg/kg with 3 mice per treatment group. All studies include animals receiving either phosphate-buffered saline (PBS, Control group) or a benchmark formulation. Formulations are diluted to the appropriate concentration in PBS immediately prior to testing. Mice are weighed and the appropriate dosing volumes calculated (10 µl/g body weight). Test and benchmark formulations as well as PBS (for Control animals) are administered intravenously via the lateral tail vein. Animals are anesthetised 24 h later with an intraperitoneal injection of Ketamine/Xylazine and 500-700 µl of blood is collected by cardiac puncture into serum separator tubes (BD Microtainer). Blood is centrifuged at 2,000 x g for 10

min at 15°C and serum is collected and stored at -70 °C until analysis. Serum samples are thawed at 37°C for 30 min, diluted in PBS and aliquoted into 96-well assay plates. Factor VII levels are assessed using a chromogenic assay (Biophen FVII kit, Hyphen BioMed) according to manufacturer's instructions and absorbance measured in microplate reader equipped with a 405 nm wavelength filter. Plasma FVII levels are quantified and ED_{50}s (dose resulting in a 50% reduction in plasma FVII levels compared to control animals) calculated using a standard curve generated from a pooled sample of serum from Control animals. Those formulations of interest showing high levels of FVII knockdown (ED_{50} << 0.1 mg/kg) are re-tested in independent studies at a lower dose range to confirm potency and establish ED_{50}.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

WHAT IS CLAIMED IS:

1. A compound of formula IA:



or a salt thereof, wherein

X and Y are each, independently, alkyl or aryl;

L^1 and L^2 are each, independently, alkylene linking units, which may optinally contain one or more double bonds and further may optionally be interrupted by one or more heteroatoms and/or one or more cyclalkylene groups;

R_1 and R_2 are each, independently, C_{10}-C_{30} aliphatic group, each of which may optionally contain one or more double bonds, and may optionally be interrupted by one or more heteroatoms and/or one or more cyclalkylene groups;

Z is -C(O)O-, -OC(O)-, -C(O)N(R^3)-, -N(R^3)C(O)-, -O(CO)N(R^3), -N(R^3)C(O)O-, -C(O)N(R^3)C(O)O-, -OC(O)N(R^3)C(O)- or -N(R^3)C(O)N(R^3)-;

R^3 is H, alkyl or aryl, and

---- represents a single bond or a double bond, wherein when ---- represents a single bond, C* is further substituted by hydrogen or alkyl.

2. The compound of claim 1, wherein X are Y are alkyl

3. The compound of claim 3, wherein X and Y are both methyl.

4. The compound of any of claims 1-3, wherein L^1 is a linear alkylene unit.

5. The compound of any of claims 1-4, wherein L^2 is a linear alkylene unit.

6. The compound of claim 5, wherein L^2 contains one or more double bonds and one or more cycloalkylene groups.

7. The compound of any of claims 1-6, wherein R_1 and R_2 are each, independently, C_{10}-C_{30} alkyl or C_{10}-C_{30} alkenyl.

8. The compound of claim 7, wherein one of R_1 and R_2 is a linear C_{10}-C_{30} alkyl or C_{10}-C_{30} alkenyl group and the other of R_1 and R_2 is a branched C_{10}-C_{30} alkyl or C_{10}-C_{30} alkenyl group.

9. The compound of any of claims 1-8,wherein Z is -C(O)O-, -C(O)N(R^3)-, -O(CO)N(R^3), -C(O)N(R^3)C(O)O-, or -N(R^3)C(O)N(R^3)-.

10. The compound of any of claims 1-9, wherein R^3 is H or alkyl (e.g., methyl).

11. The compound of claim 1, wherein

X and Y are each, independently, C_1-C_4 alkyl (e.g., methyl);

L^1 and L^2 are each, independently, an C_2-C_{10} linear or branched alkylene linking unit, which may optinally contain one or more double bonds and further may optionally be interrupted by one or more heteroatoms and/or one or more cycloalkylene groups;

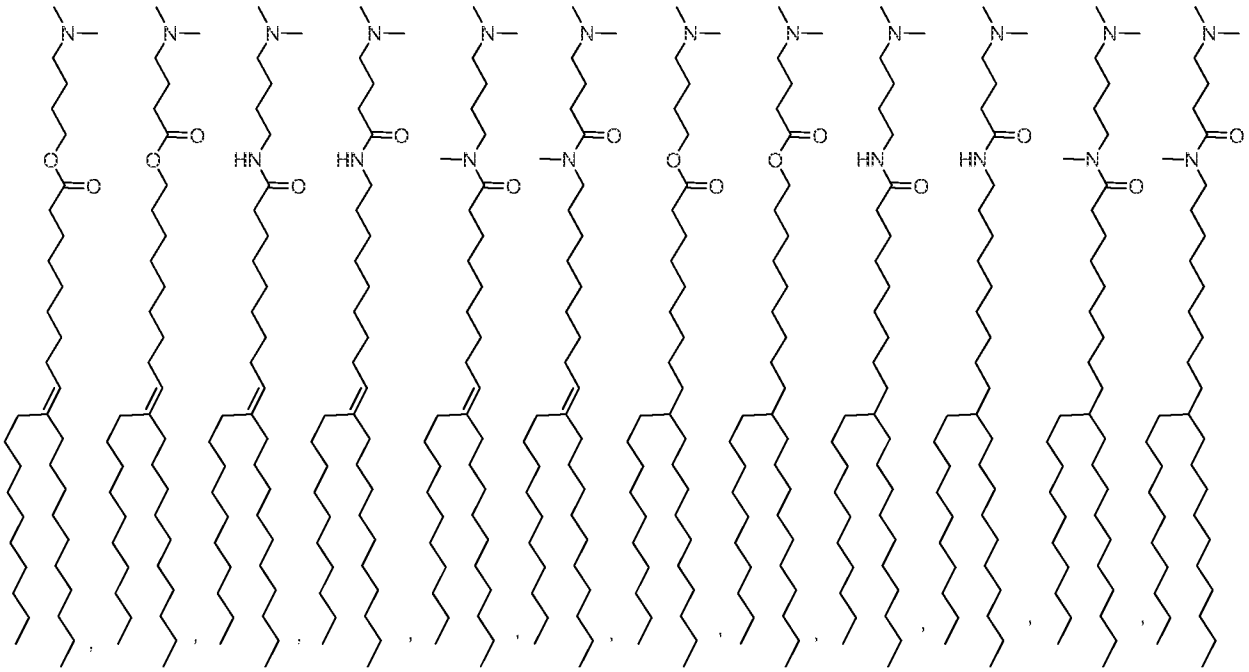
R_1 and R_2 are each, independently, a C_{10}-C_{30} aliphatic group, which may optionally contain one or more double bonds, and may optionally be interrupted by one or more heteroatoms and/or one or more cyclalkylene groups;

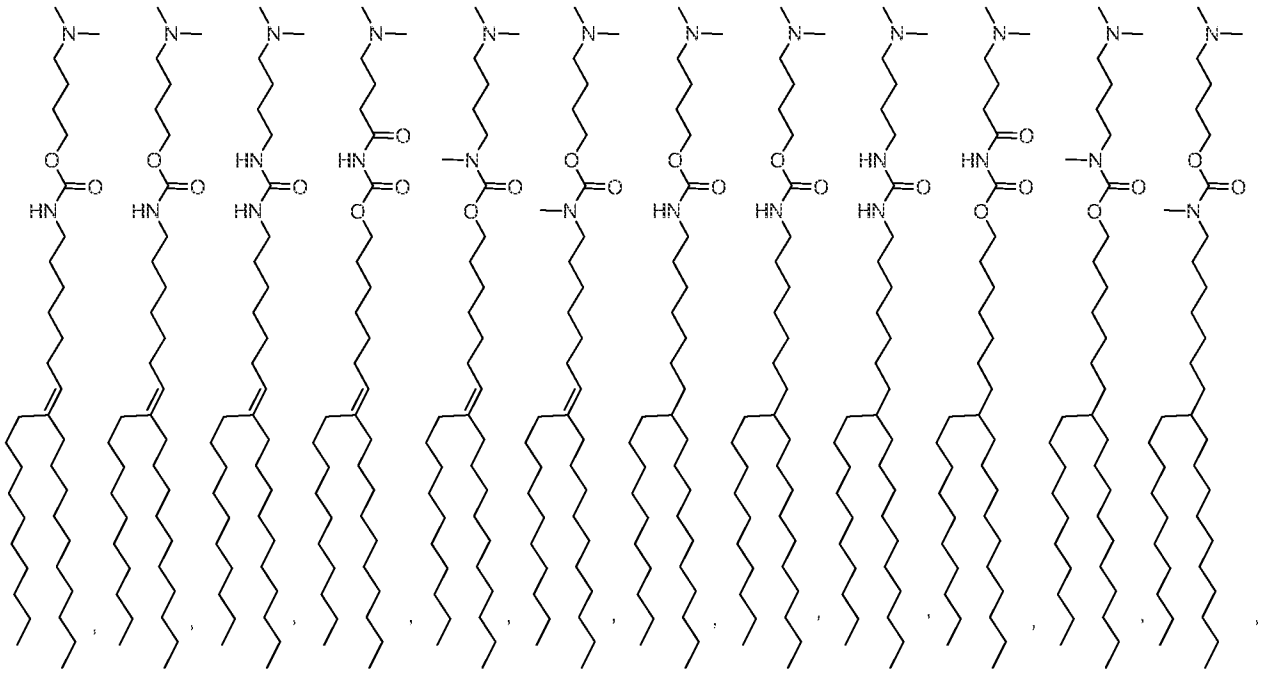
Z is -C(O)O-, -OC(O)-, -C(O)N(R^3)-, -N(R^3)C(O)-, -O(CO)N(R^3), -N(R^3)C(O)O-, -C(O)N(R^3)C(O)O-, -OC(O)N(R^3)C(O)- or -N(R^3)C(O)N(R^3)-;

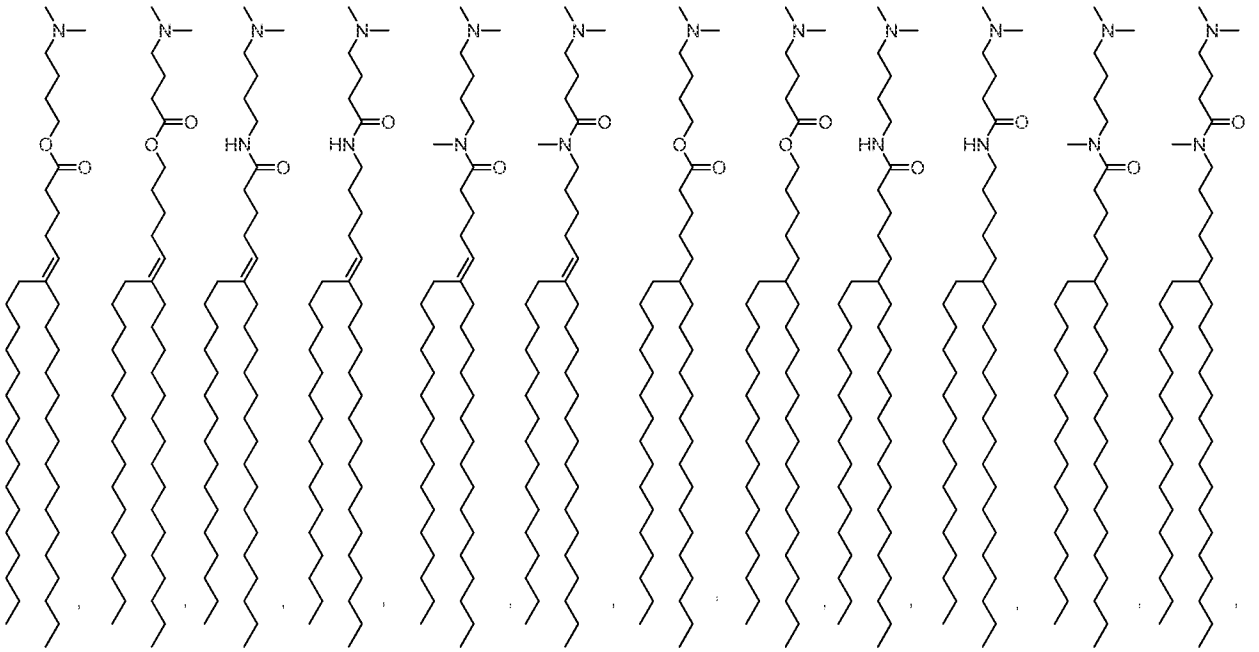
each occurrence of R^3 is independently H or C_1-C_4 alkyl; and

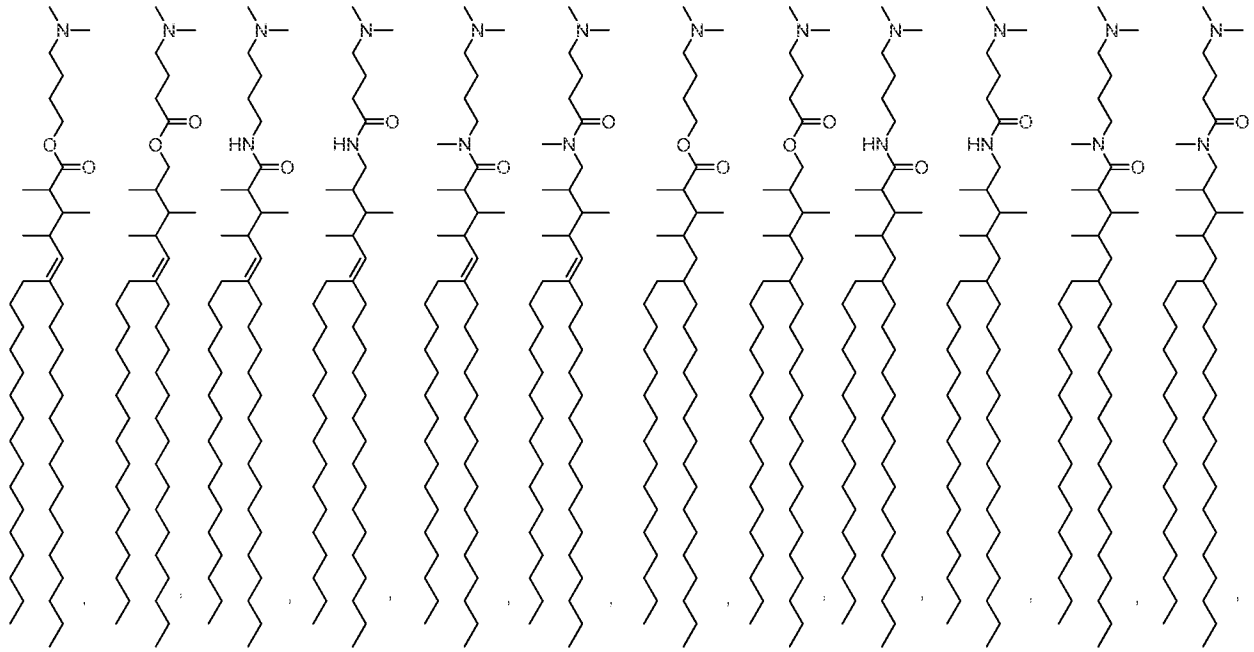
----- represents a single bond or a double bond, wherein when ----- represents a single bond, C* is further substituted by hydrogen or alkyl.

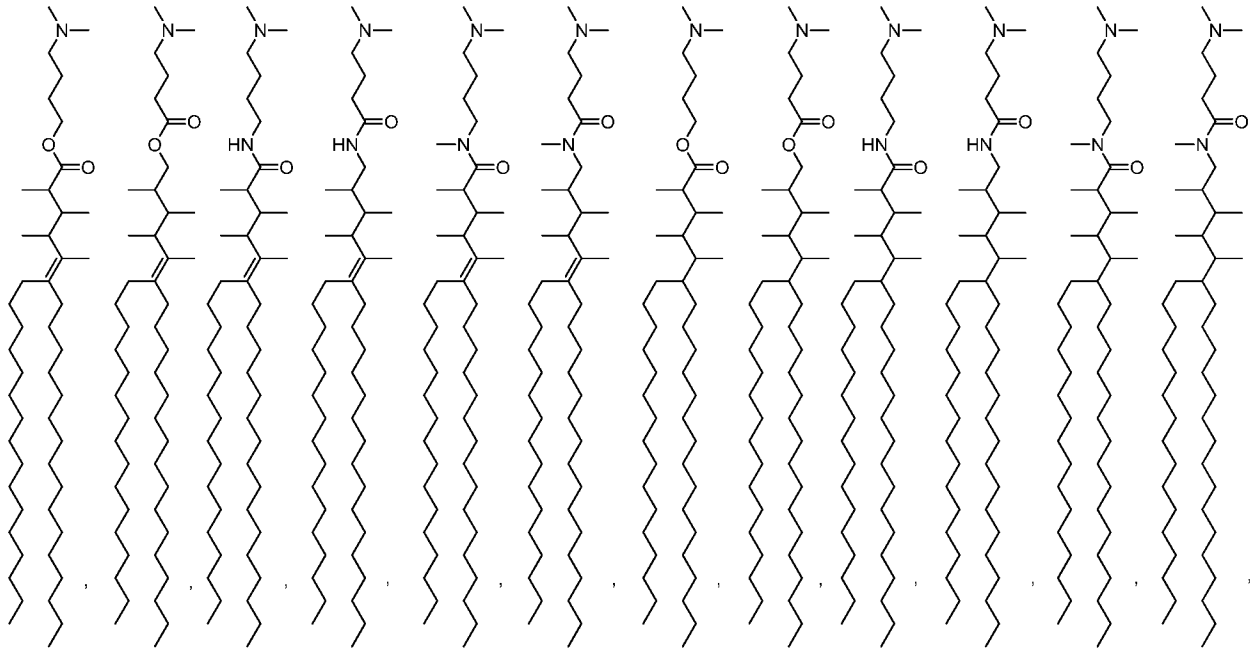
12. The compound of claim 1, selected from:

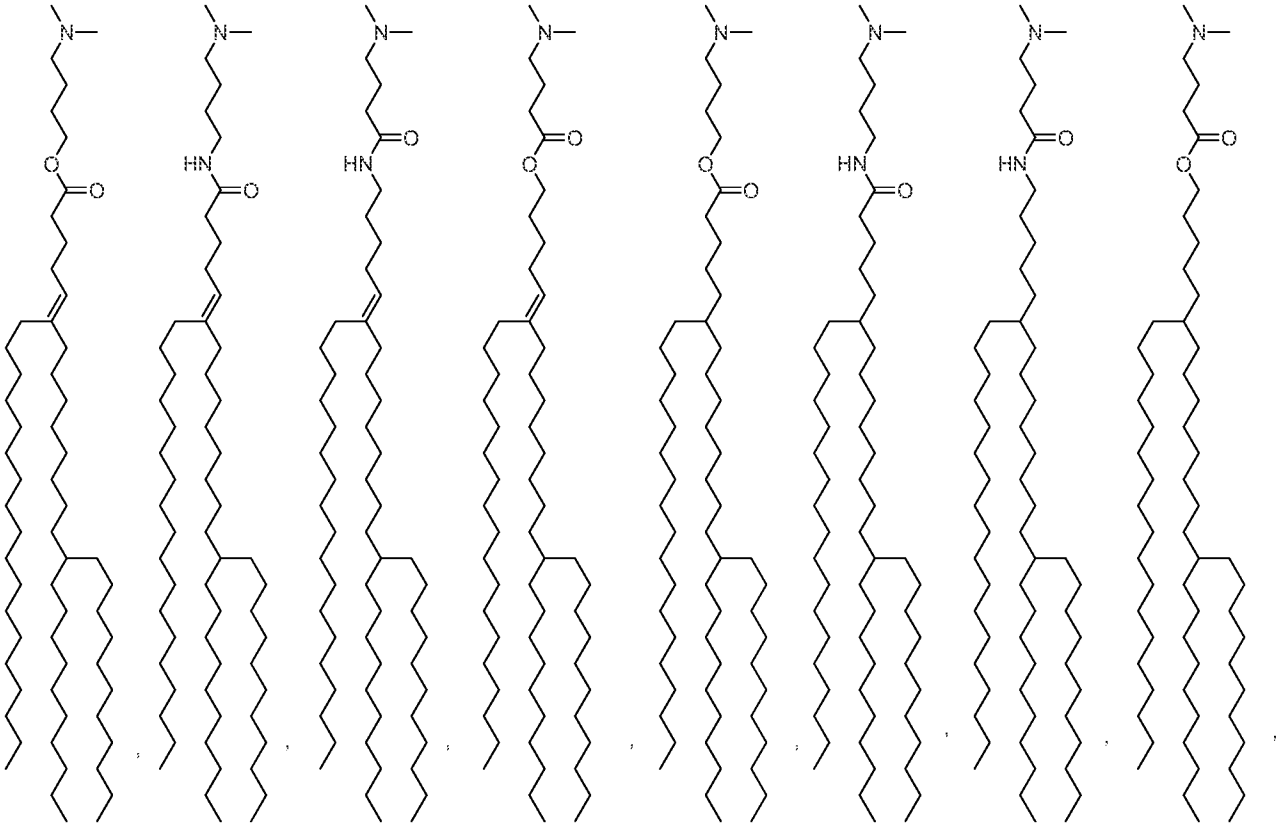


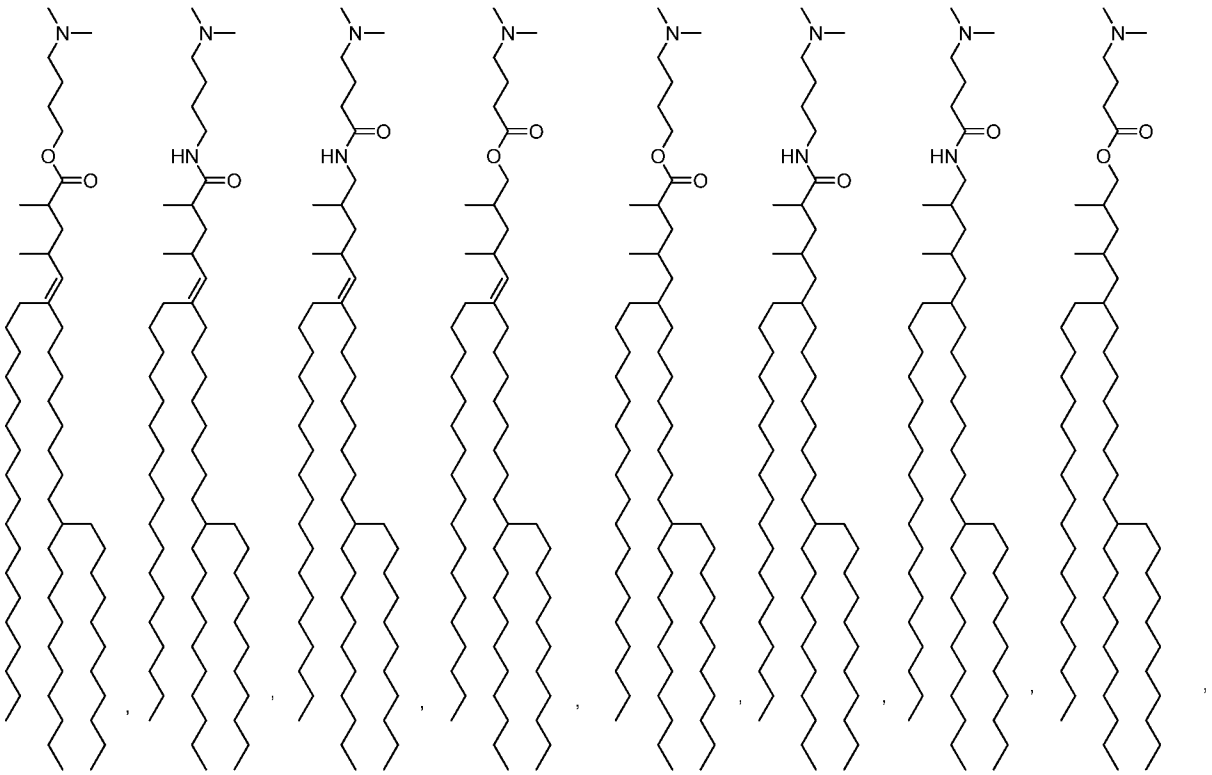


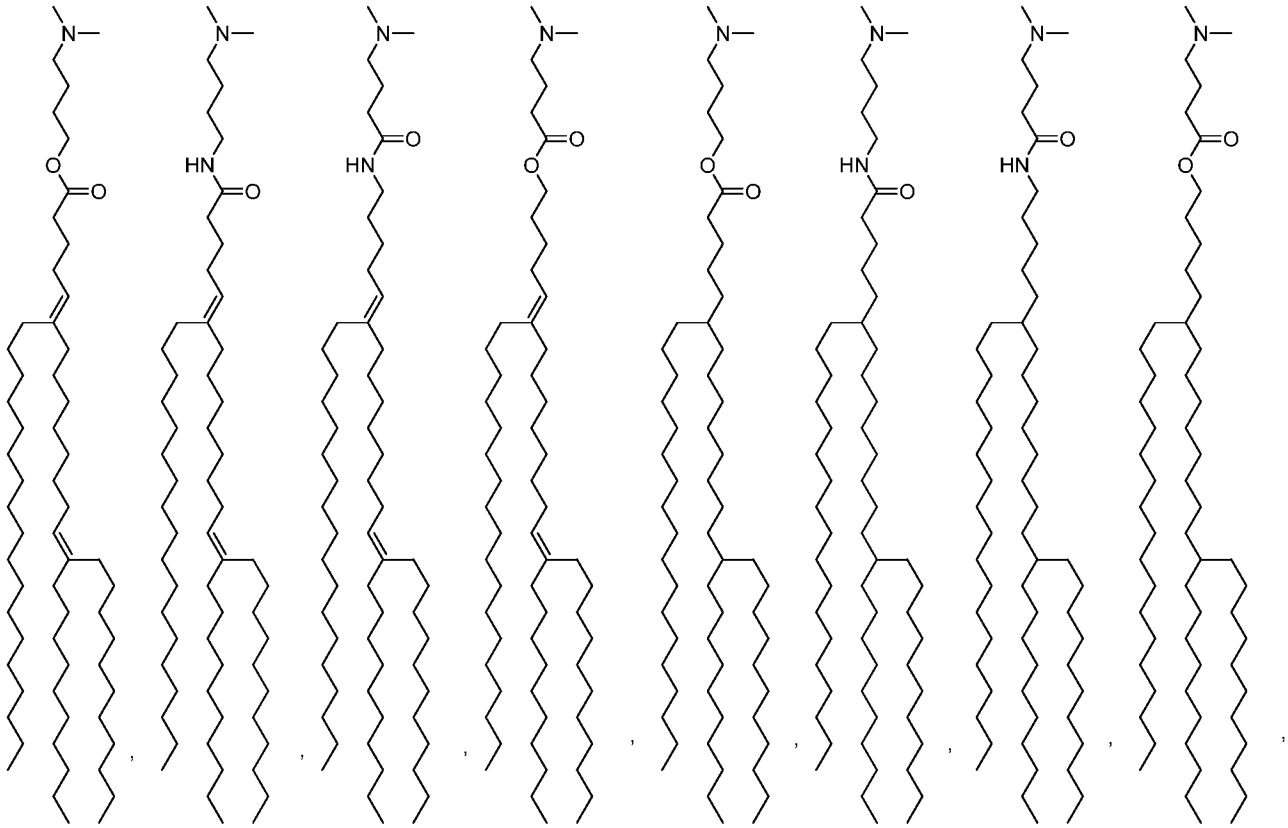


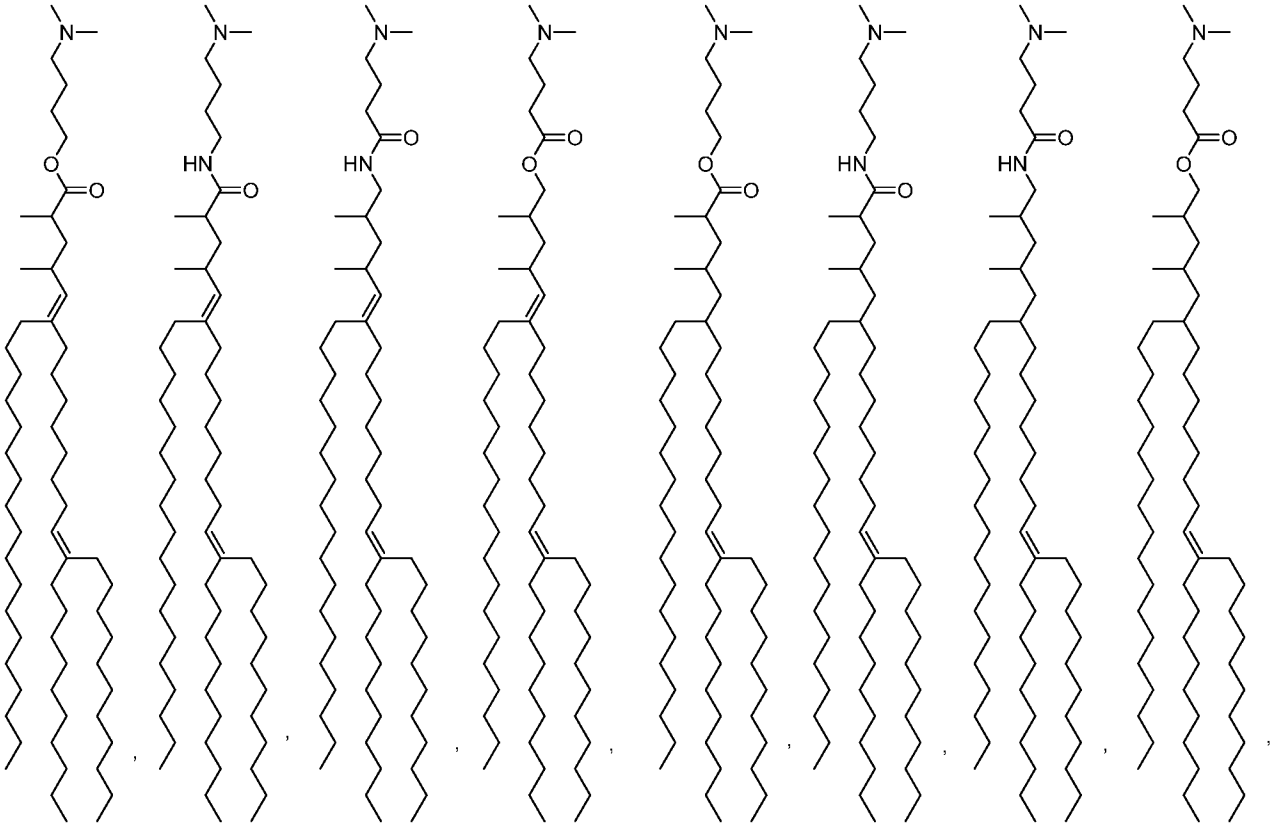


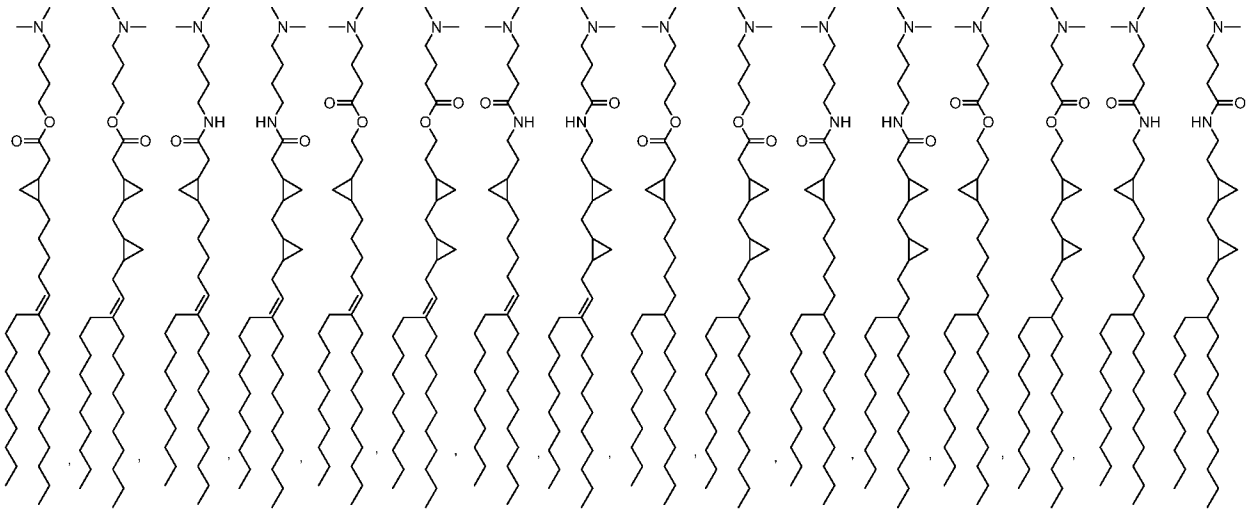


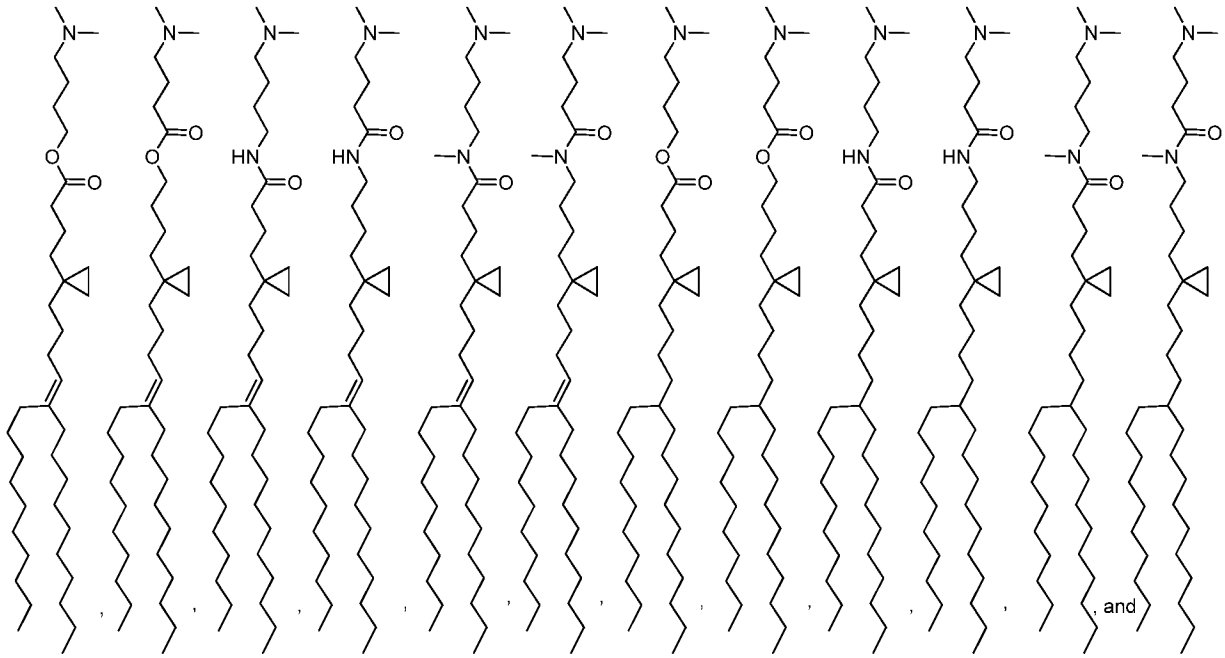






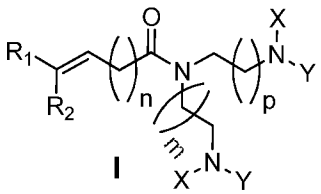


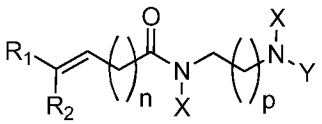


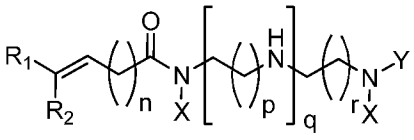


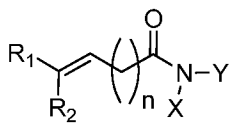
or a salt thereof.

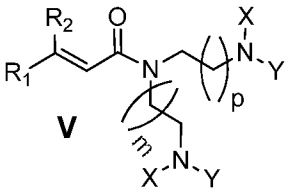
13. A compound selected from a compound of formulas I-XL:

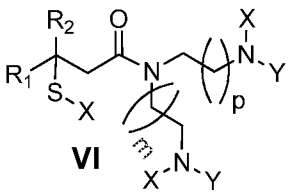


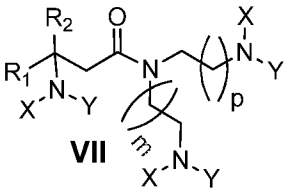


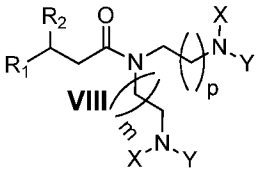




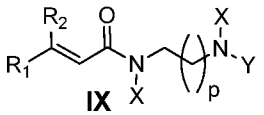




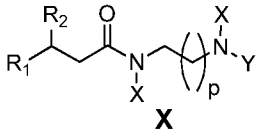




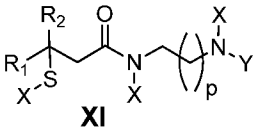
VIII



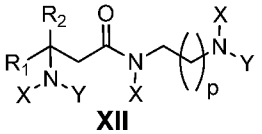
IX



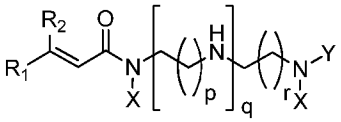
X



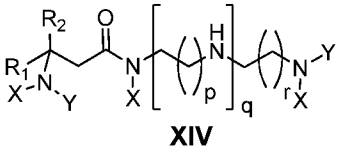
XI



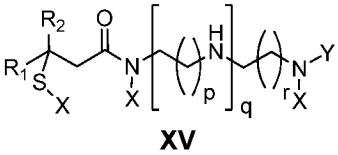
XII



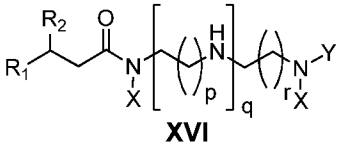
XIII



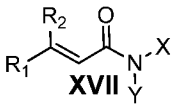
XIV



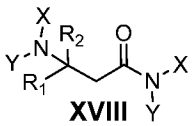
XV



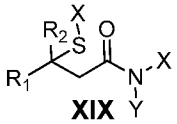
XVI



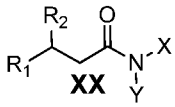
XVII



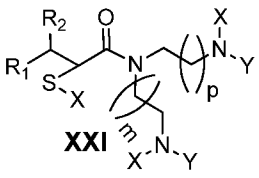
XVIII



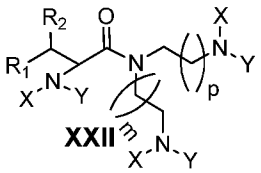
XIX



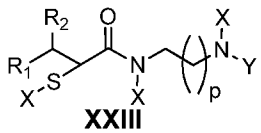
XX



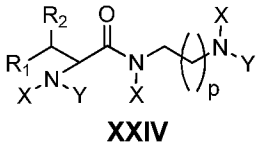
XXI



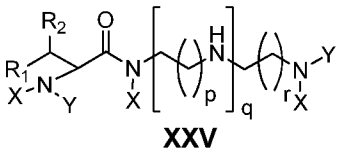
XXII



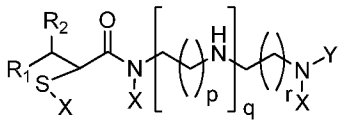
XXIII



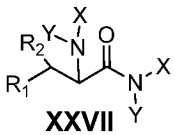
XXIV



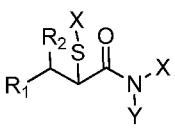
XXV



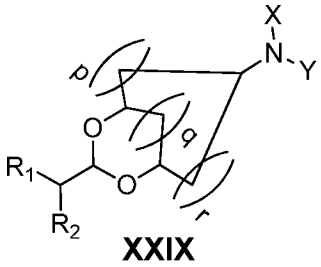
XXVI



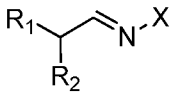
XXVII



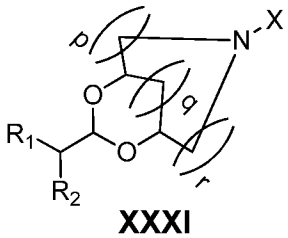
XXVIII



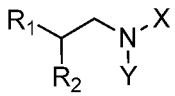
XXIX



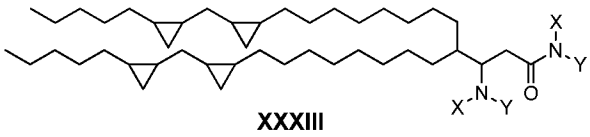
XXX



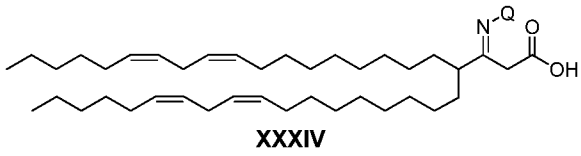
XXXI



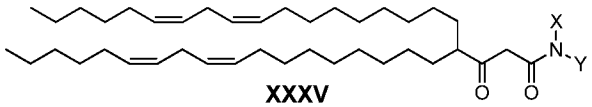
XXXII



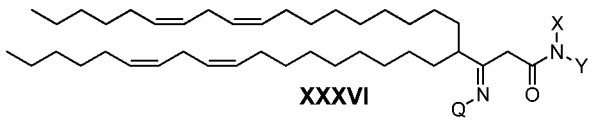
XXXIII



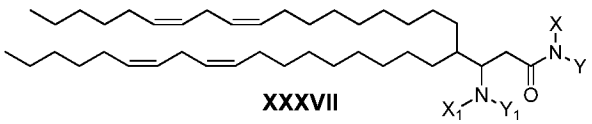
XXXIV



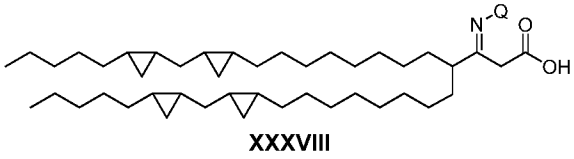
XXXV



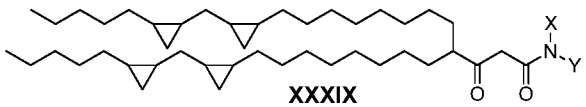
XXXVI



XXXVII

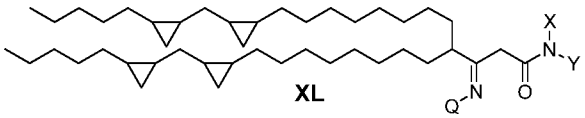


XXXVIII



XXXIX

and



XL

or a salt thereof, wherein

each occurrence of X and Y are each, independently, alkyl or aryl;

R_1 and R_2 are each, independently, a C_{10}-C_{30} aliphatic group, which may optionally contain one or more double bonds, and may optionally be interrupted by one or more heteroatoms and/or one or more cyclalkylene groups;

Q is H, alkyl, OH, alkoxy, acyl, or aryl; and

m, n, p, q, and r are each, independently, 0-20.

14. The compound of any of the preceding claims, wherein the compound is in the form of a pharmaceutically acceptable salt.

15. The compound of any of the preceding claims, wherein the compound is in the form of a cationic lipid.

16. A lipid particle comprising a neutral lipid, a lipid capable of reducing aggregation, and a cationic lipid of claim 15.

17. The lipid particle of claim 16, wherein the neutral lipid is selected from DSPC, DPPC, POPC, DOPE, or SM; the lipid capable of reducing aggregation is a PEG lipid; and the lipid particle further comprises a sterol.

18. The lipid particle of any of claims 16-17, wherein the cationic lipid is present in a mole percentage of about 20% and about 60%; the neutral lipid is present in a mole percentage of about 5% to about 25%; the sterol is present in a mole percentage of about 25% to about 55%; and the PEG lipid is PEG-DMA, PEG-DMG, or a combination thereof, and is present in a mole percentage of about 0.5% to about 15%.

19. The lipid particle of any of claims 15-18, further comprising an active agent.

20. The lipid particle of claim 19, wherein the active agent is a nucleic acid selected from a plasmid, an immunostimulatory oligonucleotide, an siRNA, an antisense oligonucleotide, a microRNA, an antagomir, an aptamer, and a ribozyme.

21. A pharmaceutical composition comprising a lipid particle of any of claims 19-20 and a pharmaceutically acceptable carrier.

22. A method of modulating the expression of a target gene in a cell, comprising providing to the cell a lipid particle of any of claims 19-20.

23. The method of claim 22, wherein the active agent is a nucleic acid selected from a plasmid, an immunostimulatory oligonucleotide, an siRNA, an antisense oligonucleotide, a microRNA, an antagomir, an aptamer, and a ribozyme.

24. A method of treating a disease or disorder characterized by the overexpression of a polypeptide in a subject, comprising providing to the subject the pharmaceutical composition of claim 21, wherein the active agent is a nucleic acid selected from the group consisting of an siRNA, a microRNA, and an antisense oligonucleotide, and wherein the siRNA, microRNA, or antisense oligonucleotide includes a polynucleotide that specifically binds to a polynucleotide that encodes the polypeptide, or a complement thereof.

25. A method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject, comprising providing to the subject the pharmaceutical composition of claim 21, wherein the active agent is a plasmid that encodes the polypeptide or a functional variant or fragment thereof.

26. A method of inducing an immune response in a subject, comprising providing to the subject the pharmaceutical composition of claim 21, wherein the active agent is an immunostimulatory oligonucleotide.

27. The method of claim 26, wherein the target gene is selected from the group consisting of Factor VII, Eg5, PCSK9, TPX2, apoB, SAA, TTR, RSV, PDGF beta gene, Erb-B gene, Src gene, CRK gene, GRB2 gene, RAS gene, MEKK gene, JNK gene, RAF gene, Erk1/2 gene, PCNA(p21) gene, MYB gene, JUN gene, FOS gene, BCL-2 gene, Cyclin D gene, VEGF gene, EGFR gene, Cyclin A gene, Cyclin E gene, WNT-1 gene, beta-catenin gene, c-MET gene, PKC gene, NFKB gene, STAT3 gene, survivin gene, Her2/Neu gene, SORT1 gene, XBP1 gene, topoisomerase I gene, topoisomerase II alpha gene, p73 gene, p21(WAF1/CIP1) gene, p27(KIP1) gene, PPM1D gene, RAS gene, caveolin I gene, MIB I gene, MTAI gene, M68 gene, tumor suppressor genes, and p53 tumor suppressor gene.

28. The method of claim 27, wherein the target gene contains one or more mutations.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/35939

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 9/127; A61K 48/00 (2011.01)
USPC - 424/450; 514/44R
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 424/450; 514/44R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (USPT, PGPB, EPAB, JPAB), Google Patents/Scholar: dimethylamine cationic lipid, lipid P, linker, non-glycerol based cationic lipid, dipalmitoyl, aliphatic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,251,939 B1 (Schwartz et al.) 26 June 2001 (26.06.2001) col 2, ln 41-65, col 4, ln 28-67, col 7, ln 30-38, col 12, ln 66 to col 13, ln 15	1-4, 11-13
Y	US 2010/0055169 A1 (Dande et al.) 04 March 2010 (04.03.2010) para [0004], [0007]-[0011], [0013]-[0014], [0016], [0018], [0034]-[0035]	1-4, 11-13

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 September 2011 (25.09.2011)	05 OCT 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/35939

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-10 and 14-28
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.