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(56) Related Art
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US 6,214,325 B1 (PHILIPPE et al) 10 April 2001
DE 3 402 146 A1 (HENKEL KGaA) 25 July 1985
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WO 2008/004058 A2 (POLYPLUS-TRANSFECTION SA) 10 January 2008
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(54) **Title**: COMPOSITIONS FOR NUCLEIC ACID DELIVERY

(57) **Abstract**: A method for delivering a nucleic acid to a cell can include exposing sample cells to a composition which includes charged lipids.

COMPOSITIONS FOR NUCLEIC ACID DELIVERY

CLAIM OF PRIORITY

This application claims priority to provisional U.S. Patent Application No. 61/267,419, filed December 7, 2009, to provisional U.S. Patent Application No. 61/334,398, filed May 13, 2010, and to provisional U.S. Patent Application No. 61/384,303, filed September 19, 2010, each of which is incorporated by reference in its entirety.

TECHNICAL FIELD

The present invention relates to lipids, lipid particles, compositions including lipid particles, and methods for making and using these.

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)).

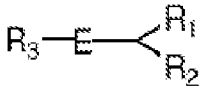
To attempt to improve efficacy, investigators have also employed lipid-based carrier systems to deliver chemically modified or unmodified therapeutic nucleic acids. In Zelphati, O and Szoka, F.C., *J. Contr. Rel.* 41:99-119 (1996), the authors refer to the

use of anionic (conventional) liposomes, pH sensitive liposomes, immunoliposomes, fusogenic liposomes, and charged 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. The present invention provides such compositions, methods of making the compositions, and methods of using the compositions to introduce nucleic acids into cells, including for the treatment of diseases.

SUMMARY

According to a first aspect of the present invention there is provided a compound having the formula:



XXXIII

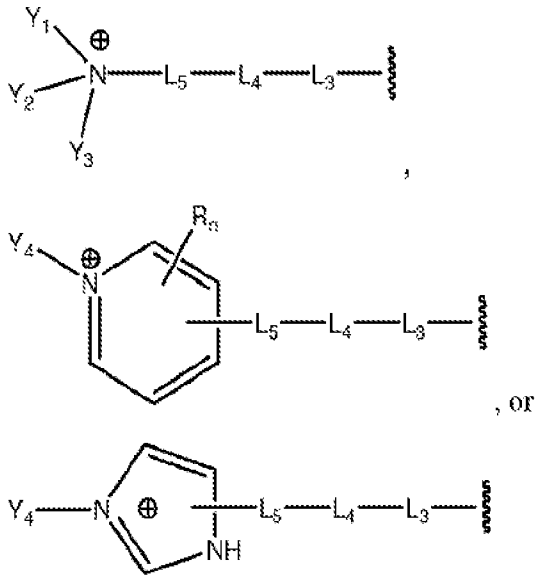
wherein:

R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkoxy, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkenyloxy, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} alkynyloxy, or optionally substituted C_{10}-C_{30} acyl;

E is -OC(O)N(R')-, -N(R')C(O)O-;

R' is H, alkyl, heteroalkyl, aralkyl, cyclic alkyl or heterocyclyl;

R_3 has the formula:



wherein

each of Y_1, Y_2, Y_3, and Y_4, independently, is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl; or

any two of Y_1, Y_2, and Y_3 are taken together with the N atom to which they are attached to form a 3- to 8- member heterocycle; or

Y_1, Y_2, and Y_3 are all be taken together with the N atom to which they are attached to form a bicyclic 5- to 12- member heterocycle;

each R_n, independently, is H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl;

L_3 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

L_4 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

L_5 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

each occurrence of R_5 and R_6 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl; or two R_5 groups on adjacent carbon atoms are taken together to form a double bond between their respective carbon atoms; or two R_5 groups on adjacent carbon atoms and two R6 groups on the same adjacent carbon atoms are taken together to form a triple bond between their respective carbon atoms;

each a, independently, is 0, 1, 2, or 3;

wherein

an R_5 or R_6 substituent from any of L_3, L_4, or L_5 is optionally taken with an R_5 or R_6 substituent from any of L_3, L_4, or L_5 to form a 3- to 8- member cycloalkyl, heterocyclyl, aryl, or heteroaryl group; and

any one of Y_1, Y_2, or Y_3, is optionally taken together with an R_5 or R_6 group from any of L_3, L_4, and L_5, and atoms to which they are attached, to form a 3- to 8- member heterocyclyl group;

each Q, independently, is H, alkyl, acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl or heterocyclyl; and

wherein

"alkyl" means a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms,

"cycloalykl" refers to partially saturated or fully saturated cycloalkyl,

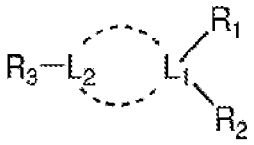
the term "acyl" refers to hydrogen, alkyl, partially saturated or fully saturated cycloalkyl, partially saturated or fully saturated heterocycle, aryl, and heteroaryl substituted carbonyl groups, and

"substituted" refers to the replacement of one or more hydrogen radicals in a given structure with the radical of a specified substituent selected from 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 aliphatic.

According to a second aspect of the present invention there is provided a method for delivering a nucleic acid to a cell comprising contacting the cell with a composition comprising a compound according to the first aspect.

According to a third aspect of the present invention there is provided a use of a compound according to the first aspect in the manufacture of a composition for delivering a nucleic acid to a cell.

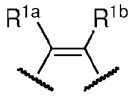
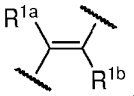
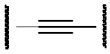
In one aspect, a method for delivering a nucleic acid to a cell can include contacting cells with a composition comprising a neutral lipid and a cationic lipid having the formula:

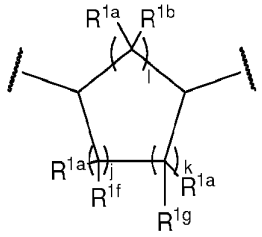


wherein:

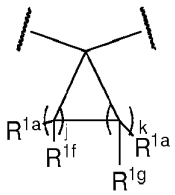
R_1 and R_2 are each independently for each occurrence a C_{10} to C_{30} group having the formula $–L^{1a}-(CR^{1a}R^{1b})_\alpha-[L^{1b}-(CR^{1a}R^{1b})_\beta]_\gamma-L^{1c}-R^{1c}$, wherein: L^{1a} is a bond, $-CR^{1a}R^{1b}-$, -O-, -CO-, $-NR^{1d}-$, -S-, or a combination thereof.

Each R^{1a} and each R^{1b}, independently, is H; halo; hydroxy; cyano; C_1-C_6 alkyl optionally substituted by halo, hydroxy, or alkoxy; C_3-C_8 cycloalkyl optionally substituted by halo, hydroxy, or alkoxy; $-OR^{1c}$; $-NR^{1c}R^{1d}$; aryl; heteroaryl; or heterocyclyl.

Each L^{1b}, independently, is a bond, $-(CR^{1a}R^{1b})_{1-2}-$, -O-, -CO-, $-NR^{1d}-$, -S-, -,  ,  ,  , or a combination thereof; or has the formula

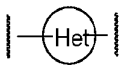


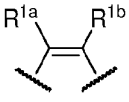
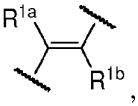
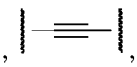
wherein j, k, and l are each independently 0, 1, 2, or 3, provided that the sum of j, k and l is at least 1 and no greater than 8; and R^{1f} and R^{1g} are each independently R^{1b}, or adjacent R^{1f} and R^{1g}, taken together, are optionally a bond; or has the formula



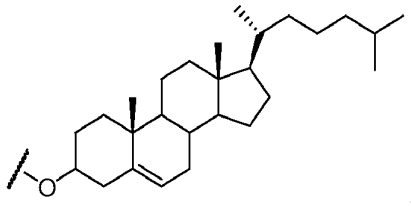
wherein j and k are each independently 0, 1, 2, 3, or 4 provided that the sum of j and k is at least 1; and R^{1f} and R^{1g} are each independently R^{1b}, or adjacent R^{1f} and R^{1g}, taken together, are optionally a bond;

or has the formula:  wherein -Ar- is a 6 to 14 membered arylene group optionally substituted by zero to six R^{1a} groups;

or has the formula:  wherein -Het- is a 3 to 14 membered heterocyclylene or heteroarylene group optionally substituted by zero to six R^{1a} groups.

L^{1c} is $-(CR^{1a}R^{1b})_{1-2}-$, -O-, -CO-, $-NR^{1d}-$, -S-,  ,  ,  , or a combination thereof.

R^{1c} is H; halo; hydroxy; cyano; C_1-C_6 alkyl optionally substituted by halo, hydroxy, or alkoxy; C_3-C_8 cycloalkyl optionally substituted by halo, hydroxy, or alkoxy; aryl; heteroaryl; or heterocyclyl; or R^{1c} has the formula:



R^{1d} is H; halo; hydroxy; cyano; C_1-C_6 alkyl optionally substituted by halo, hydroxy, or alkoxy; C_3-C_8 cycloalkyl optionally substituted by halo, hydroxy, or alkoxy; aryl; heteroaryl; or heterocyclyl.

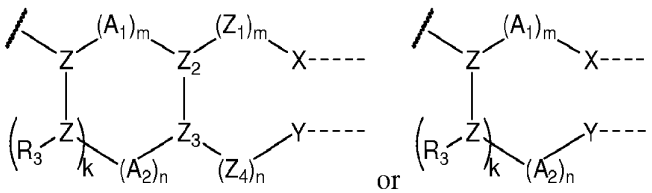
α is 0-6; each β, independently, is 0-6; and γ is 0-6.

represents a connection between L_2 and L_1 which is:

(1) a single bond between one atom of L_2 and one atom of L_1, wherein L_1 is $C(R_a)$, O, S or N(Q); L_2 is $-(CR_5R_6)_x-$, $-C(O)-(CR_5R_6)_x-$, $-(CR_5R_6)_x-CR_5=CR_5-(CR_5R_6)_y-$, $-C(O)-(CR_5R_6)_x-CR_5=CR_5-(CR_5R_6)_y-$, -O-, -S-, -N(Q)-, =N-, $=C(R_5)-$, $-CR_5R_6-O-$, $-CR_5R_6-N(Q)-$, $-CR_5R_6-S-$, -C(O)N(Q)-, -C(O)O-, -N(Q)C(O)-, -OC(O)-, -C(O)-, or $-X-C(R_5)(YR_3)-$; wherein X and Y are each, independently, selected from the group consisting of -O-, -S-, alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O, -OC(O)O-, $-OS(O)(Q_2)O-$, and $-OP(O)(Q_2)O-$. R_a is H, alkyl, alkoxy, -OH, -N(Q)Q, or -SQ.

(2) A double bond between one atom of L_2 and one atom of L_1, wherein L_1 is C; L_2 is $-(CR_5R_6)_x-CR_5=$, $-C(O)-(CR_5R_6)_x-CR_5=$, -N(Q)=, -N=, -O-N=, -N(Q)-N=, or -C(O)N(Q)-N=.

(3) A single bond between a first atom of L_2 and a first atom of L_1, and a single bond between a second atom of L_2 and the first atom of L_1, wherein L_1 is C or $C(R_a)-(CR_5R_6)_x-C(R_a)$; L_2 has the formula



wherein

X is the first atom of L_2, Y is the second atom of L_2, - - - - - represents a single bond to the first atom of L_1, and X and Y are each, independently, selected from the group consisting of -O-, -S-,

alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O,

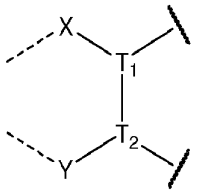
-OC(O)O-, -OS(O)(Q_2)O-, and -OP(O)(Q_2)O-.

Z_1 and Z_4 are each, independently, -O-, -S-, -CH_2-, -CHR^5-, or -CR^5R^5-; Z_2 is CH or N; Z_3 is CH or N; or Z_2 and Z_3, taken together, are a single C atom. A_1 and A_2 are each, independently, -O-, -S-, -CH_2-, -CHR^5-, or -CR^5R^5-. Each Z is N, C(R_5), or C(R_3).

k is 0, 1, or 2; each m, independently, is 0 to 5; and each n, independently, is 0 to 5; where m and n taken together result in a 3, 4, 5, 6, 7 or 8 member ring.

(4) A single bond between a first atom of L_2 and a first atom of L_1, and a single bond between the first atom of L_2 and a second atom of L_1, wherein

(A) L_1 has the formula:



wherein

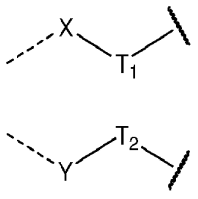
X is the first atom of L_1, Y is the second atom of L_1, - - - - - represents a single bond to the first atom of L_2, and X and Y are each, independently, selected from the group consisting of -O-, -S-,

alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O,

-OC(O)O-, -OS(O)(Q_2)O-, and -OP(O)(Q_2)O-.

T_1 is CH or N; T_2 is CH or N; or T_1 and T_2 taken together are C=C; L_2 is CR_5; or

(B) L_1 has the formula:



wherein

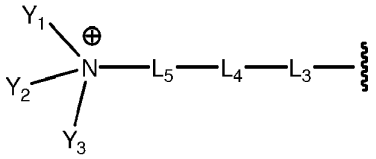
X is the first atom of L_1, Y is the second atom of L_1, - - - - - represents a single bond to the first atom of L_2, and X and Y are each, independently, selected from the group consisting of -O-, -S-,

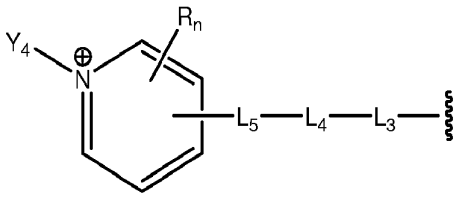
alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O-, -OC(O)O-, -OS(O)(Q_2)O-, and -OP(O)(Q_2)O-.

T_1 is -CR_5R_6-, -N(Q)-, -O-, or -S-; T_2 is -CR_5R_6-, -N(Q)-, -O-, or -S-; L_2 is CR_5 or N.

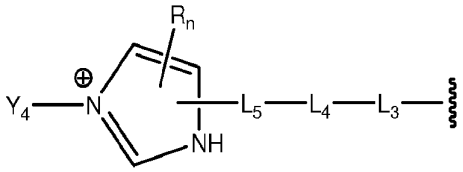
Each of x and y, independently, is 0, 1, 2, 3, 4, or 5.

R_3 has the formula:





, or



wherein

Y_1 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_1 is optionally substituted by 0 to 6 R_n; Y_2 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_2 is optionally substituted by 0 to 6 R_n; Y_3 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_3 is optionally substituted by 0 to 6 R_n; Y_4 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_4 is optionally substituted by 0 to 6 R_n; or any two of Y_1, Y_2, and Y_3 are taken together with the N atom to which they are attached to form a 3- to 8- member heterocycle optionally substituted by 0 to 6 R_n; or Y_1, Y_2, and Y_3 are all be taken together with the N atom to which they are attached to form a bicyclic 5- to 12- member heterocycle optionally substituted by 0 to 6 R_n.

Each R_n, independently, is H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl.

L_3 is a bond, -N(Q)-, -O-, -S-, -$(CR_7R_8)_a$-, -C(O)-, or a combination of any two of these.

L_4 is a bond, -N(Q)-, -O-, -S-, -$(CR_7R_8)_a$-, -C(O)-, or a combination of any two of these.

L_5 is a bond, -N(Q)-, -O-, -S-, -$(CR_7R_8)_a$-, -C(O)-, or a combination of any two of these.

Each occurrence of R_7 and R_8 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl; or two R_7 groups on adjacent carbon atoms are taken together to form a double bond between their respective carbon atoms; or two R_7 groups on adjacent carbon atoms and two R_8 groups on the same adjacent carbon atoms are taken together to form a triple bond between their respective carbon atoms.

Each a, independently, is 0, 1, 2, or 3; wherein an R_7 or R_8 substituent from any of L_3, L_4, or L_5 is optionally taken with an R_7 or R_8 substituent from any of L_3, L_4, or L_5 to form a 3- to 8- member cycloalkyl, heterocyclyl, aryl, or heteroaryl group; and any one of Y_1, Y_2, or Y_3, is optionally taken together with an R_7 or R_8 group from any of L_3, L_4, and L_5, and atoms to which they are attached, to form a 3- to 8- member heterocyclyl group.

Each occurrence of R_5 and R_6 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl. Each Q, independently, is H, alkyl, acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl or heterocyclyl; and each Q_2, independently, is O, S, N(Q)Q, alkyl or alkoxy.

In some embodiments, the composition can further include a lipid capable of reducing aggregation. The composition can further include a nucleic acid. The nucleic acid can include a chemically modified nucleic acid. The nucleic acid can be 10 to 50 nucleotides long. The the nucleic acid can be an oligonucleotide. The oligonucleotide can be 10 to 50 nucleotides long. The oligonucleotide can be double stranded or single stranded. More particularly, in some embodiments, the nucleic acid can be siRNA or mRNA. In other embodiments, the nucleic acid can be an antisense nucleic acid, a microRNA, an antimicro RNA, an antagomir, a microRNA inhibitor or an immune stimulatory nucleic acid.

In some embodiments, the sample cells can be in suspension. In some circumstances, the volume of the sample cells in suspension can be at least 0.050 L, at least 3 L, at least 25 L or at least 40 L.

In some embodiments, a method for delivering a nucleic acid to sample cells can further include culturing untreated control cells that have not been exposed to the composition.

In some embodiments, the cell density of the sample cells can increase after the sample cells have been exposed to the composition. In some circumstances, the cell density of the sample cells can increase exponentially for a period of time after the sample cells have been exposed to the composition. In some embodiments, the cell density of the sample cells can be greater than or equal to the cell density of the untreated control cells as measured three days after the sample cells have been exposed to the composition.

In some embodiments, the sample cell viability can be greater than 90% as measured three days after the sample cells have been exposed to the composition.

In some embodiments, a method for delivering a nucleic acid to sample cells can further include measuring a level of a protein in the sample cells and untreated control cells, the protein can be produced from an mRNA that an siRNA delivered into the sample cells was directed against.

In some embodiments, the protein level in the sample cells can be less than the protein level in the untreated control cells as measured at one day after the sample cells have been exposed to the composition. In some circumstances, the protein level in the sample cells can be less than 60% of the protein level in the untreated control cells as measured at one day after the sample cells have been exposed to the composition or as measured at one sample cell doubling time after the sample cells have been exposed to the composition. In some circumstances, the protein level in the sample cells can be less than 70% of the protein level in the untreated control cells as measured at three days after the sample cells have been exposed to the composition or as measured at three times the sample cell doubling time after the sample cells have been exposed to the composition. In some circumstances, the protein level in the sample cells can be less than 75% of the protein level in the untreated control cells as measured at five days after the sample cells have been exposed to the composition or as measured at five times the sample cell doubling time after the sample cells have been exposed to the composition.

In another aspect, a storage-stable composition can include a cryoprotectant selected from sucrose, trehalose, glucose, 2-hydroxypropyl-α-cyclodextrin, and sorbitol, and a cationic lipid having the formula described above. The composition can further include a neutral lipid; a sterol; and/or a lipid capable of reducing aggregation. The composition can include a nucleic acid. The cryoprotectant can be present at from 5 wt% to 25 wt%, or at from 7 wt% to 15 wt%. The cryoprotectant can include sucrose. The

composition can be lyophilized, i.e., in a lyophilized state.

In another aspect, a method for reconstituting a storage-stable composition can include resuspending the composition in a liquid. It can further include adding a lipid and/or a nucleic acid to the resuspended composition.

Other features, advantages, and embodiments will be apparent from the description, the drawings, and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph depicting relative gene expression in a knockdown experiment with varying concentrations of siRNA.

FIG. 2 is a graph depicting relative gene expression in a knockdown experiment as a function of N/P ratio.

FIG. 3 is a graph depicting relative gene expression in a knockdown experiment with varying concentrations of siRNA.

FIG. 4 is a graph depicting expression knockdown measured using different transfection compositions.

FIG. 5 is a graph depicting particle sizes of liposomes.

FIGS. 6A-6D are graphs depicting expression knockdown measured using different transfection compositions.

FIG. 7 is a graph depicting relative gene expression in a knockdown experiment with varying concentrations of siRNA and varying liposome compositions.

FIG. 8 is a graph depicting expression knockdown measured using different transfection compositions.

FIG. 9 is a graph depicting relative gene expression in a knockdown experiment with varying concentrations of siRNA and varying liposome compositions.

FIG. 10 is a graph depicting expression knockdown measured using different transfection compositions.

FIG. 11 is a graph depicting cell viability as a function of lipid concentration for various lipids.

FIG. 12 is a graph depicting cell viability as a function of lipid concentration for various lipids.

FIG. 13 is a graph depicting expression knockdown measured using different transfection compositions.

FIG. 14 is a graph depicting percent GFP signal remaining after a GFP knockdown experiment using different transfection compositions to deliver siRNA directed against GFP mRNA.

FIG. 15 is a graph depicting relative gene expression in a knockdown experiment using varying concentrations of a transfection reagent, K8.

FIG. 16 is a graph depicting relative gene expression in a knockdown experiment using varying concentrations of two transfection reagents, K8 and P8.

FIG. 17 is a graph depicting cell viability and cell density following exposure to lipid formulation P8.

FIG. 18 is a graph depicting relative LDH activity as a function of time.

FIG. 19 is a graph depicting cell viability and cell density following exposure to lipid formulation P8.

FIG. 20 is a graph depicting relative LDH activity as a function of time.

DETAILED DESCRIPTION

The present invention is based, in part, upon the discovery of charged lipids that provide advantages when used in lipid particles for the *in vivo* delivery of a therapeutic agent. In particular, as illustrated by the accompanying Examples, the present invention provides nucleic acid-lipid particle compositions comprising a charged lipid according to the present invention. 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. Additionally compositions and methods of use are disclosed that can provide for amelioration of the toxicity observed with certain therapeutic nucleic acid-lipid particles.

In certain embodiments, the present invention specifically provides for improved compositions for the delivery of siRNA molecules. It is shown herein that these compositions are effective in down-regulating the protein levels and/or mRNA levels of target proteins. Furthermore, it is shown that the activity of these improved compositions is dependent on the presence of a certain charged lipids and that the molar ratio of charged lipid in the formulation can influence activity.

The lipid particles and compositions of the present invention may be used for a variety of purposes, including the delivery of associated or encapsulated therapeutic

agents to cells, both *in vitro* and *in vivo*. Accordingly, the present invention provides methods of treating diseases or disorders in a subject in need thereof, by contacting the subject with a lipid particle of the present invention associated with a suitable therapeutic agent.

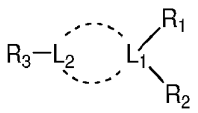
As described herein, the lipid particles of the present invention are particularly useful for the delivery of nucleic acids, including, *e.g.*, siRNA molecules and plasmids. Therefore, the lipid particles and compositions of the present invention may be used to modulate the expression of target genes and proteins both in vitro and in vivo by contacting cells with a lipid particle of the present invention 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 the charged lipids of the present invention, as well as 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.

LIPIDS

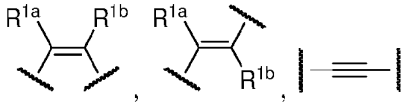
The present invention provides novel lipids having certain design features. As shown in Figure 5, the lipid design features include at least one of the following: a head group with a quaternary amine, and optionally, a varying pKa, a cationic, 1°, 2° and 3°, monoamine, di and triamine, oligoamine/polyamine, a low pKa head groups – imidazoles and pyridine, guanidinium, anionic, zwitterionic and hydrophobic tails can include symmetric and/or unsymmetric chains, long and shorter, saturated and unsaturated chain the back bone includes Backbone glyceride and other acyclic analogs, cyclic, spiro, bicyclic and polycyclic linkages with ethers, esters, phosphate and analogs, sulfonate and analogs, disulfides, pH sensitive linkages like acetals and ketals, imines and hydrazones, and oximes.

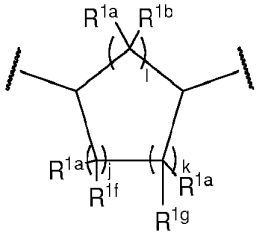
Lipids can be advantageously used in lipid particles for the *in vivo* delivery of therapeutic agents to cells. Among such lipids are those having the formula:



Each of R^1 or R^2 is independently a C_{10} to C_{30} group having the formula $-L^{1a}-(CR^{1a}R^{1b})_\alpha-[L^{1b}-(CR^{1a}R^{1b})_\beta]_\gamma-L^{1c}-R^{1c}$, where: L^{1a} is a bond, $-CR^{1a}R^{1b}-$, -O-, -CO-, $-NR^{1d}-$, -S-, or a combination thereof.

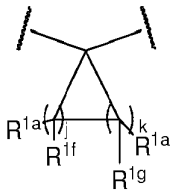
Each R^{1a} and each R^{1b}, independently, is H; halo; hydroxy; cyano; C_1-C_6 alkyl optionally substituted by halo, hydroxy, or alkoxy; C_3-C_8 cycloalkyl optionally substituted by halo, hydroxy, or alkoxy; $-OR^{1c}$; $-NR^{1c}R^{1d}$; aryl; heteroaryl; or heterocyclyl;

Each L^{1b}, independently, is a bond, $-(CR^{1a}R^{1b})_{1-2}-$, -O-, -CO-, $-NR^{1d}-$, -S-,  , or a combination thereof, or has the formula

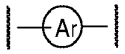


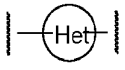
wherein j, k, and l are each independently 0, 1, 2, or 3, provided that the sum of j, k and l is at least 1 and no greater than 8; and R^{1f} and R^{1g} are each independently R^{1b}, or adjacent R^{1f} and R^{1g}, taken together, are optionally a bond;

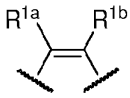
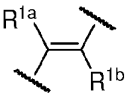
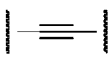
or has the formula



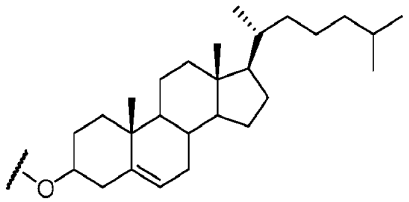
wherein j and k are each independently 0, 1, 2, 3, or 4 provided that the sum of j and k is at least 1; and R^{1f} and R^{1g} are each independently R^{1b}, or adjacent R^{1f} and R^{1g}, taken together, are optionally a bond;

or has the formula:  wherein -Ar- is a 6 to 14 membered arylene group optionally substituted by zero to six R^{1a} groups;

or has the formula:  wherein -Het- is a 3 to 14 membered heterocyclylene or heteroarylene group optionally substituted by zero to six R^{1a} groups.

L^{1c} is $-(CR^{1a}R^{1b})_{1-2}-$, -O-, -CO-, $-NR^{1d}-$, -S-, -,  ,  ,  , or a combination thereof.

R^{1c} is H; halo; hydroxy; cyano; C_1-C_6 alkyl optionally substituted by halo, hydroxy, or alkoxy; C_3-C_8 cycloalkyl optionally substituted by halo, hydroxy, or alkoxy; aryl; heteroaryl; or heterocyclyl; or R^{1c} has the formula:



R^{1d} is H; halo; hydroxy; cyano; C_1-C_6 alkyl optionally substituted by halo, hydroxy, or alkoxy; C_3-C_8 cycloalkyl optionally substituted by halo, hydroxy, or alkoxy; aryl; heteroaryl; or heterocyclyl.

α is 0-6; each β, independently, is 0-6; and γ is 0-6.

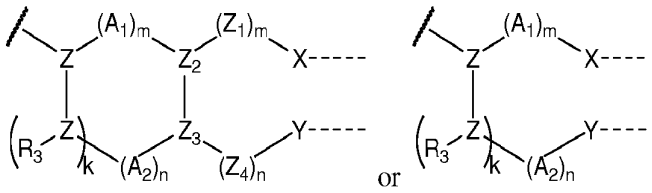
 represents a connection between L_2 and L_1 which is:

(1) a single bond between one atom of L_2 and one atom of L_1, wherein L_1 is $C(R_a)$, O, S or N(Q); L_2 is $-(CR_5R_6)_x-$, $-C(O)-(CR_5R_6)_x-$, $-(CR_5R_6)_x-CR_5=CR_5-(CR_5R_6)_y-$, $-C(O)-(CR_5R_6)_x-CR_5=CR_5-(CR_5R_6)_y-$, -O-, -S-, -N(Q)-, =N-, $=C(R_5)-$, $-CR_5R_6-O-$, $-CR_5R_6-N(Q)-$, $-CR_5R_6-S-$, $-C(O)N(Q)-$, $-C(O)O-$, $-N(Q)C(O)-$, -OC(O)-, -C(O)-, or $-X-C(R_5)(YR_3)-$; wherein X and Y are each, independently, selected from the group consisting of -O-, -S-, alkylene, -N(Q)-, -C(O)-, -O(CO)-, $-OC(O)N(Q)-$, $-N(Q)C(O)O-$, -C(O)O, $-OC(O)O-$, $-OS(O)(Q_2)O-$, and $-OP(O)(Q_2)O-$.

R_a is H, alkyl, alkoxy, -OH, -N(Q)Q, or -SQ.

(2) a double bond between one atom of L_2 and one atom of L_1, wherein L_1 is C; L_2 is $-(CR_5R_6)_x-CR_5=$, $-C(O)-(CR_5R_6)_x-CR_5=$, -N(Q)=, -N-, -O-N=, -N(Q)-N=, or -C(O)N(Q)-N=.

(3) a single bond between a first atom of L_2 and a first atom of L_1, and a single bond between a second atom of L_2 and the first atom of L_1, wherein L_1 is C or $C(R_a)-(CR_5R_6)_x-C(R_a)$; L_2 has the formula



wherein

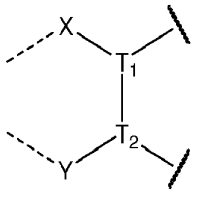
X is the first atom of L_2, Y is the second atom of L_2, - - - - - represents a single bond to the first atom of L_1, and X and Y are each, independently, selected from the group consisting of -O-, -S-, alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O, -OC(O)O-, -OS(O)(Q_2)O-, and -OP(O)(Q_2)O-; Z_1 and Z_4 are each, independently, -O-, -S-, -CH_2-, -CHR^5-, or -CR^5R^5-; Z_2 is CH or N; Z_3 is CH or N; or Z_2 and Z_3, taken together, are a single C atom; A_1 and A_2 are each, independently, -O-, -S-, -CH_2-, -CHR^5-, or -CR^5R^5-.

Each Z is N, C(R_5), or C(R_3).

k is 0, 1, or 2; each m, independently, is 0 to 5; each n, independently, is 0 to 5; where m and n taken together result in a 3, 4, 5, 6, 7 or 8 member ring.

(4) a single bond between a first atom of L_2 and a first atom of L_1, and a single bond between the first atom of L_2 and a second atom of L_1, wherein

(A) L_1 has the formula:

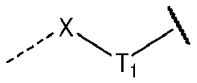


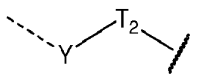
wherein

X is the first atom of L_1, Y is the second atom of L_1, - - - - - represents a single bond to the first atom of L_2, and X and Y are each, independently, selected from the group consisting of -O-, -S-, alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O, -OC(O)O-, -OS(O)(Q_2)O-, and -OP(O)(Q_2)O-; T_1 is CH or N; T_2 is CH or N; or T_1 and T_2 taken together are C=C.

L_2 is CR_5.

(B) L_1 has the formula:





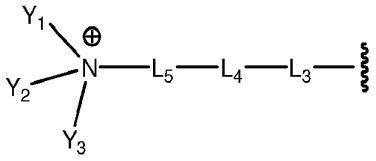
wherein

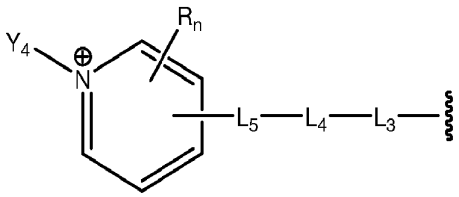
X is the first atom of L_1, Y is the second atom of L_1, - - - - - represents a single bond to the first atom of L_2, and X and Y are each, independently, selected from the group consisting of -O-, -S-,

alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O, -OC(O)O-, -OS(O)(Q_2)O-, and -OP(O)(Q_2)O-; T_1 is -CR_5R_6-, -N(Q)-, -O-, or -S-; T_2 is -CR_5R_6-, -N(Q)-, -O-, or -S-.

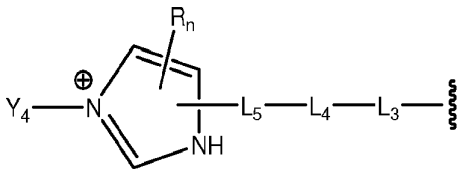
L_2 is CR_5 or N; each of x and y, independently, is 0, 1, 2, 3, 4, or 5.

R_3 has the formula:





, or



Y_1 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_1 is optionally substituted by 0 to 6 R_n. Y_2 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_2 is optionally substituted by 0 to 6 R_n. Y_3 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_3 is optionally substituted by 0 to 6 R_n. Y_4 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_4 is optionally substituted by 0 to 6 R_n; or any two of Y_1, Y_2, and Y_3 are taken together with the N atom to which they are attached to form a 3- to 8- member heterocycle optionally substituted by 0 to 6 R_n; or Y_1, Y_2, and Y_3 are all be taken together with the N atom to which they are attached to form a bicyclic 5- to 12- member heterocycle optionally substituted by 0 to 6 R_n.

Each R_n, independently, is H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl. L_3 is a bond, -N(Q)-, -O-, -S-, -$(CR_7R_8)_a$-, -C(O)-, or a combination of any two of these. L_4 is a bond, -N(Q)-, -O-, -S-, -$(CR_7R_8)_a$-, -C(O)-, or a combination of any two of these. L_5 is a bond, -N(Q)-, -O-, -S-, -$(CR_7R_8)_a$-, -C(O)-, or a combination of any two of these.

Each occurrence of R_7 and R_8 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl; or two R_7 groups on adjacent carbon atoms are taken together to form a double bond between their respective carbon atoms; or two R_7 groups on adjacent carbon atoms and two R_8 groups on the same adjacent carbon atoms are taken together to form a triple bond between their respective carbon atoms.

Each a, independently, is 0, 1, 2, or 3; wherein an R_7 or R_8 substituent from any of L_3, L_4, or L_5 is optionally taken with an R_7 or R_8 substituent from any of L_3, L_4, or L_5 to form a 3- to 8- member cycloalkyl, heterocyclyl, aryl, or heteroaryl group; and any one of Y_1, Y_2, or Y_3, is optionally taken together with an R_7 or R_8 group from any of L_3, L_4, and L_5, and atoms to which they are attached, to form a 3- to 8- member heterocyclyl group.

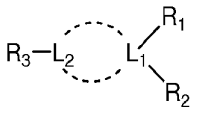
Each occurrence of R_5 and R_6 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl.

Each Q, independently, is H, alkyl, acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl or heterocyclyl.

Each Q_2, independently, is O, S, N(Q)Q, alkyl or alkoxy.

In some embodiments, Y_3 and/or Y_4 is absent, such that the lipid does not include a quaternary nitrogen atom.

In one aspect, a compound can have the formula:



wherein:

R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkoxy, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkenyloxy, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} alkynyloxy, or optionally substituted C_{10}-C_{30} acyl;

represents a connection between L_2 and L_1 which is:

(1) a single bond between one atom of L_2 and one atom of L_1, wherein

L_1 is $C(R_x)$ or N;

L_2 is $-CR_5R_6-$, -O-, -S-, -N(Q)-,

$=C(R_5)-$, -C(O)N(Q)-, -C(O)O-, -N(Q)C(O)-, -OC(O)-, or -C(O)-;

(2) a double bond between one atom of L_2 and one atom of L_1; wherein

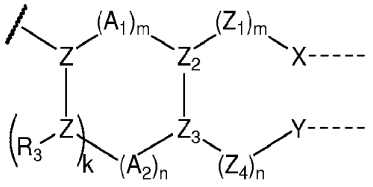
L_1 is C;

L_2 is $-CR_5=$, -N(Q)=, -N-, -O-N=, -N(Q)-N=, or -C(O)N(Q)-N=;

(3) a single bond between a first atom of L_2 and a first atom of L_1, and a single bond between a second atom of L_2 and the first atom of L_1, wherein

L_1 is C;

L_2 has the formula



wherein

X is the first atom of L_2, Y is the second atom of L_2, - - - - - represents a single bond to the first atom of L_1, and X and Y are each, independently, selected from the group consisting of -O-, -S-,

alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O, -OC(O)O-, -O S(O)(Q_2)O-, and -OP(O)(Q_2)O-;

Z_1 and Z_4 are each, independently, -O-, -S-, $-CH_2-$, $-CHR^5-$, or $-CR^5R^5-$;

Z_2 is CH or N;

Z_3 is CH or N;

or Z_2 and Z_3, taken together, are a single C atom;

A_1 and A_2 are each, independently, -O-, -S-, $-CH_2-$, $-CHR^5-$, or $-CR^5R^5-$;

each Z is N, $C(R_5)$, or $C(R_3)$;

k is 0, 1, or 2;

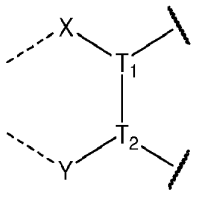
each m, independently, is 0 to 5;

each n, independently, is 0 to 5;

where m and n taken together result in a 3, 4, 5, 6, 7 or 8 member ring;

(4) a single bond between a first atom of L_2 and a first atom of L_1, and a single bond between the first atom of L_2 and a second atom of L_1, wherein

(A) L_1 has the formula:



wherein

X is the first atom of L_1, Y is the second atom of L_1, - - - - - represents a single bond to the first atom of L_2, and X and Y are each, independently, selected from the group consisting of -O-, -S-,

alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O, -OC(O)O-, -OS(O)(Q_2)O-, and -OP(O)(Q_2)O-;

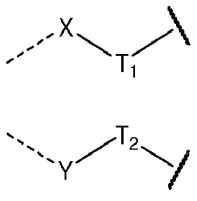
T_1 is CH or N;

T_2 is CH or N;

or T_1 and T_2 taken together are C=C;

L_2 is CR_5; or

(B) L_1 has the formula:



wherein

X is the first atom of L_1, Y is the second atom of L_1, - - - - - represents a single bond to the first atom of L_2, and X and Y are each, independently, selected from the group consisting of -O-, -S-,

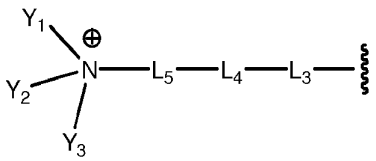
alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O, -OC(O)O-, -OS(O)(Q_2)O-, and -OP(O)(Q_2)O-;

T_1 is -CR_5R_5-, -N(Q)-, -O-, or -S-;

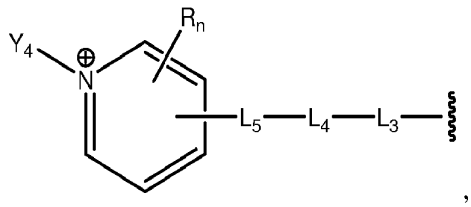
T_2 is -CR_5R_5-, -N(Q)-, -O-, or -S-;

L_2 is CR_5 or N;

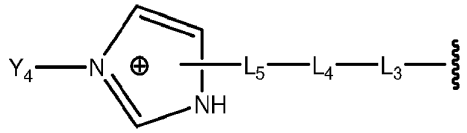
R_3 has the formula:



,



, or



wherein

each of Y_1, Y_2, Y_3, and Y_4, independently, is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl; or

any two of Y_1, Y_2, and Y_3 are taken together with the N atom to which they are attached to form a 3- to 8- member heterocycle; or

Y_1, Y_2, and Y_3 are all be taken together with the N atom to which they are attached to form a bicyclic 5- to 12- member heterocycle;

each R_n, independently, is H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl;

L_3 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

L_4 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

L_5 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

each occurrence of R_5 and R_6 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl; or two R_5 groups on adjacent carbon atoms are taken together to form a double bond between their respective carbon atoms; or two R_5 groups on adjacent carbon atoms and two R_6 groups on the same adjacent carbon atoms are taken together to form a triple bond between their respective carbon atoms;

each a, independently, is 0, 1, 2, or 3;

wherein

an R_5 or R_6 substituent from any of L_3, L_4, or L_5 is optionally taken with an R_5 or R_6 substituent from any of L_3, L_4, or L_5 to form a 3- to 8- member cycloalkyl, heterocyclyl, aryl, or heteroaryl group; and

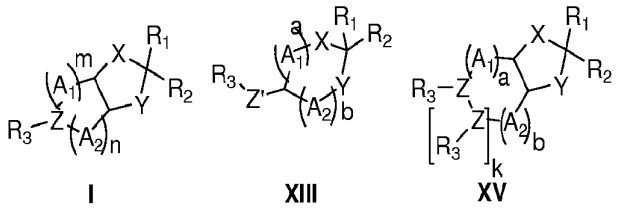
any one of Y_1, Y_2, or Y_3, is optionally taken together with an R_5 or R_6 group from any of L_3, L_4, and L_5, and atoms to which they are attached, to form a 3- to 8- member heterocyclyl group;

each Q, independently, is H, alkyl, acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl or heterocyclyl; and

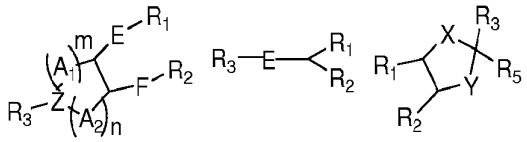
each Q_2, independently, is O, S, N(Q)(Q), alkyl or alkoxy.

In some embodiments, represents a connection between L_2 and L_1 which is a single bond between one atom of L_2 and one atom of L_1, wherein L_1 is $C(R_x)$, O, S or N(Q); and L_2 is $-CR_5R_6-$, -O-, -S-, -N(Q)-, $=C(R_5)-$, -C(O)N(Q)-, -C(O)O-, -N(Q)C(O)-, -OC(O)-, or -C(O)-.

In another aspect, a compound having formula I, XIII, XV, XVII, XXXIII, or XXXV:



I XIII XV



XVII XXXIII XXXV

wherein:

R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkoxy, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkenyloxy, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} alkynyloxy, or optionally substituted C_{10}-C_{30} acyl;

R_3 is independently for each occurrence H, optionally substituted C_1-C_{10} alkyl, optionally substituted C_2-C_{10} alkenyl, optionally substituted C_2-C_{10} alkynyl, optionally substituted alkylheterocycle, optionally substituted heterocyclealkyl, optionally substituted alkylphosphate, optionally substituted phosphoalkyl, optionally substituted alkylphosphorothioate, optionally substituted phosphorothioalkyl, optionally substituted alkylphosphorodithioate, optionally substituted phosphorodithioalkyl, optionally substituted alkylphosphonate, optionally substituted phosphonoalkyl, optionally

substituted amino, optionally substituted alkylamino, optionally substituted di(alkyl)amino, optionally substituted aminoalkyl, optionally substituted alkylaminoalkyl, optionally substituted di(alkyl)aminoalkyl, optionally substituted hydroxyalkyl, optionally substituted polyethylene glycol (PEG, mw 100-40K), optionally substituted mPEG (mw 120-40K), optionally substituted heteroaryl, or optionally substituted heterocycle;

at least one R_3 includes a quaternary amine;

X and Y are each independently -O-, -S-, alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O, -OC(O)O-, -OS(O)(Q_2)O-, or -OP(O)(Q_2)O-;

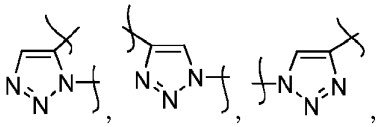
Q is H, alkyl, ω-aminoalkyl, ω-(substituted)aminoalkyl, ω-phosphoalkyl, or ω-thiophosphoalkyl;

Q_2 is independently for each occurrence O, S, N(Q)(Q), alkyl or alkoxy;

A_1, A_2, A_3, A_4, A_5 and A_6 are each independently -O-, -S-, $-CH_2-$, $-CHR^5-$, $-CR^5R^5-$;

A_8 is independently for each occurrence $-CH_2-$, $-CHR^5-$, $-CR^5R^5-$;

E and F are each independently for each occurrence $-CH_2-$, -O-, -S-, -SS-, -CO-, -C(O)O-, -C(O)N(R')-, -OC(O)N(R')-, -N(R')C(O)N(R")-, -C(O)-N(R')-N=C(R"')-; -N(R')-N=C(R")-, -O-N=C(R")-, -C(S)O-, -C(S)N(R')-, -OC(S)N(R')-, -N(R')C(S)N(R")-, -C(S)-N(R')-N=C(R"'); -S-N=C(R"); -C(O)S-, -SC(O)N(R')-, -OC(O)-, -N(R')C(O)-, -N(R')C(O)O-, -C(R"')=N-N(R')-; -C(R"')=N-N(R')- C(O)-, -C(R"')=N-O-, -OC(S)-, -SC(O)-, -N(R')C(S)-, -N(R')C(S)O-, -N(R')C(O)S-, -C(R"')=N-N(R')-C(S)-, -C(R"')=N-S-, C[=N(R')]O, C[=N(R')]N(R"), -OC[=N(R')]-, -N(R")C[=N(R')]N(R"')-, -N(R")C[=N(R')]-,



, arylene, heteroarylene, cycloalkylene, or heterocyclylene;

Z is N or C(R_3);

Z' is -O-, -S-, -N(Q)-, or alkylene;

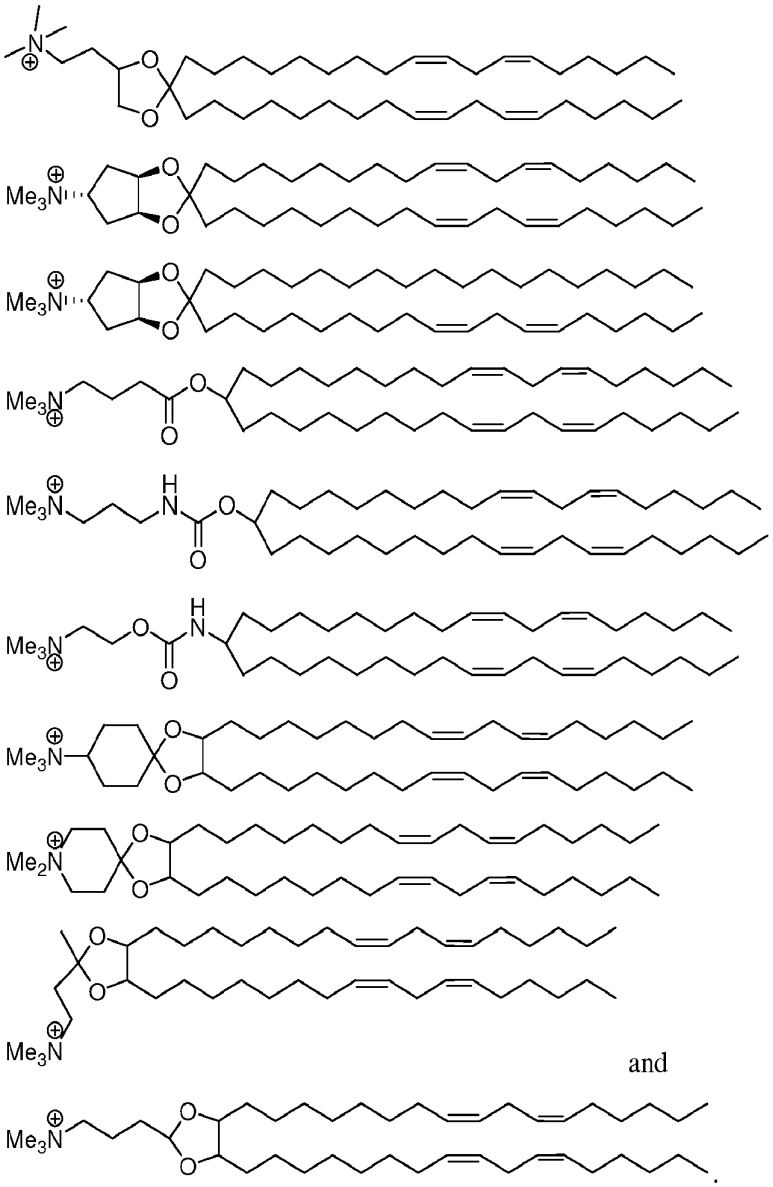
each R', R", and R"', independently, is H, alkyl, alkyl, heteroalkyl, aralkyl, cyclic alkyl, or heterocyclyl;

R^5 is H, halo, cyano, hydroxy, amino, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted cycloalkyl;

i and j are each independently 0-10; and

a and b are each independently 0-2.

In another aspect, a compound can be selected from the group consisting of:

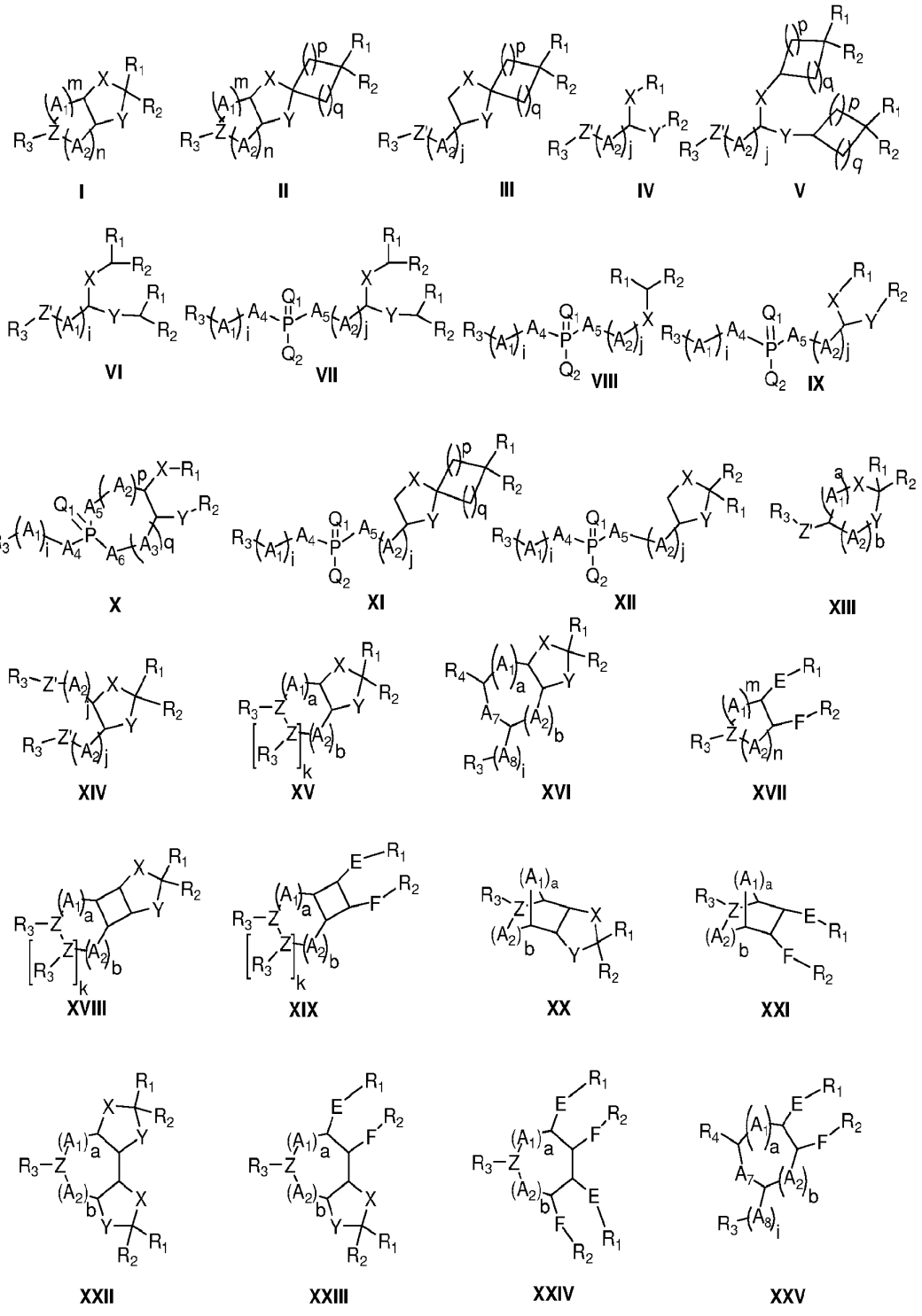


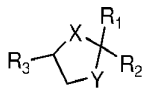
and

In another aspect, a composition can include a compound as described above, a neutral lipid, and a sterol. The composition can further include a nucleic acid. The nucleic acid can be RNA.

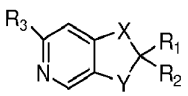
In one embodiment, E is O(CO), (CO)O, OC(O)N(R'), or N(R')C(O)O.

In another aspect, the lipid has one of the following structures, salts or isomers thereof:

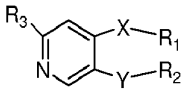




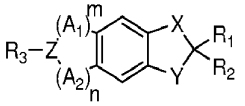
XXVI



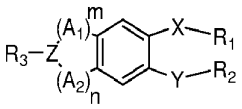
XXVII



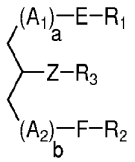
XXVIII



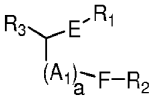
XXIX



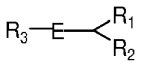
XXX



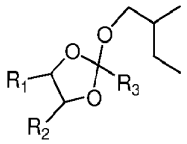
XXXI



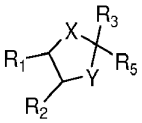
XXXII



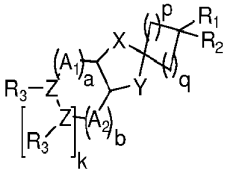
XXXIII



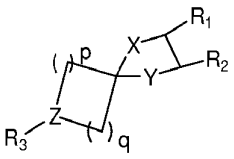
XXXIV



XXXV



XXXVI



XXXVII

R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkoxy, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkenyloxy, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} alkynyloxy, or optionally substituted C_{10}-C_{30} acyl.

R_3 is independently for each occurrence H, optionally substituted C_1-C_{10} alkyl, optionally substituted C_2-C_{10} alkenyl, optionally substituted C_2-C_{10} alkynyl, optionally substituted alkylheterocycle, optionally substituted heterocyclealkyl, optionally substituted alkylphosphate, optionally substituted phosphoalkyl, optionally substituted alkylphosphorothioate, optionally substituted phosphorothioalkyl, optionally substituted alkylphosphorodithioate, optionally substituted phosphorodithioalkyl, optionally substituted alkylphosphonate, optionally substituted phosphonoalkyl, optionally substituted amino, optionally substituted alkylamino, optionally substituted di(alkyl)amino, optionally substituted aminoalkyl, optionally substituted alkylaminoalkyl, optionally substituted di(alkyl)aminoalkyl, optionally substituted hydroxyalkyl, optionally substituted polyethylene glycol (PEG, mw 100-40K), optionally substituted mPEG (mw 120-40K), optionally substituted heteroaryl, or optionally substituted heterocycle.

At least one R_3 includes a quaternary amine.

X and Y are each independently -O-, -S-, alkylene, -N(Q)-, -C(O)-, -O(CO)-, -OC(O)N(Q)-, -N(Q)C(O)O-, -C(O)O, -OC(O)O-, -OS(O)(Q_2)O-, or -OP(O)(Q_2)O-.

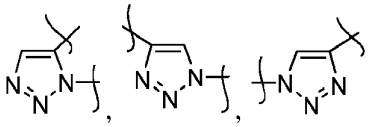
Q is H, alkyl, ω-aminoalkyl, ω-(substituted)aminoalkyl, ω-phosphoalkyl, or ω-thiophosphoalkyl.

Q_2 is independently for each occurrence O, S, N(Q)(Q), alkyl or alkoxy.

A_1, A_2, A_3, A_4, A_5 and A_6 are each independently -O-, -S-, -CH_2-, -CHR^5-, -CR^5R^5-.

A_8 is independently for each occurrence -CH_2-, -CHR^5-, -CR^5R^5-.

E and F are each independently for each occurrence -CH_2-, -O-, -S-, -SS-, -CO-, -C(O)O-, -C(O)N(R')-, -OC(O)N(R')-, -N(R')C(O)N(R")-, -C(O)-N(R')-N=C(R"')-; -N(R')-N=C(R")-, -O-N=C(R")-, -C(S)O-, -C(S)N(R')-, -OC(S)N(R')-, -N(R')C(S)N(R")-, -C(S)-N(R')-N=C(R"'); -S-N=C(R"); -C(O)S-, -SC(O)N(R')-, -OC(O)-, -N(R')C(O)-, -N(R')C(O)O-, -C(R"')=N-N(R')-; -C(R"')=N-N(R')- C(O)-, -C(R"')=N-O-, -OC(S)-, -SC(O)-, -N(R')C(S)-, -N(R')C(S)O-, -N(R')C(O)S-, -C(R"')=N-N(R')-C(S)-, -C(R"')=N-S-, C[=N(R')]O, C[=N(R')]N(R"), -OC[=N(R')]-, -N(R")C[=N(R')]N(R"')-, -N(R")C[=N(R')]-,



, arylene, heteroarylene, cycloalkylene, or heterocyclylene.

Z is N or C(R_3).

Z' is -O-, -S-, -N(Q)-, or alkylene.

Each R', R", and R"', independently, is H, alkyl, alkyl, heteroalkyl, aralkyl, cyclic alkyl, or heterocyclyl.

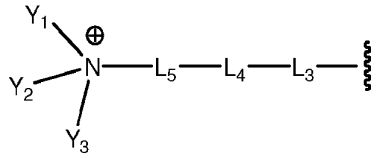
R^5 is H, halo, cyano, hydroxy, amino, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted cycloalkyl.

i and j are each independently 0-10.

a and b are each independently 0-2.

In some circumstances, R_3 is ω-(substituted)aminoalkyl. The ω-amino group can be a quaternary amine. Examples quaternary ω-(substituted)aminoalkyl groups include 2-(trimethylamino)ethyl, 3-(triisopropylamino)propyl, or 3-(N-methyl-N-ethyl-N-isopropylamino)-1-methylpropyl.

In some circumstances, R_3 has the formula:



where each of Y_1, Y_2, and Y_3, is independently, alkyl, cycloalkyl, aryl, aralkyl, or alkynyl. For example, each of Y_1, Y_2, and Y_3, is independently C_1-C_6 alkyl or C_3-C_6 cycloalkyl, C_1-C_4 alkyl, or C_1-C_3 alkyl.

Any two of Y_1, Y_2, and Y_3 can be taken together with the N atom to which they are attached to form a 3- to 8- member heterocycle. For example, Y_1, and Y_2 can be taken together with the N atom to which they are attached to form a pyrrolidine, a pyrrole, an oxazole, an imidazole, a pyridine, a piperidine, or other N-containing heterocycles. In some cases, Y_1, Y_2, and Y_3 can all be taken together with the N atom to which they are attached to form a bicyclic 5- to 12- member heterocycle. For example, Y_1, Y_2, and Y_3 can all be taken together the N atom to which they are attached to form a quinuclidine, a tropane, a 1,4-diazabicyclo[2.2.2]octane, or other bicyclic heterocycles.

Each Y_1, Y_2, Y_3, independently, can be optionally substituted alkyl, optionally substituted cycloalkyl, optionally substituted aryl, optionally substituted aralkyl, or optionally substituted alkynyl. When two or more of Y_1, Y_2, and Y_3 are taken together with the N atom to which they are attached to form a heterocyclic group, the heterocyclic group can be optionally substituted.

L_3 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

L_4 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

L_5 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

Each of R_5 and R_6 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl. Two R_5 groups on adjacent carbon atoms can be taken together to form a double bond between their respective carbon atoms. Two R_5 groups on adjacent carbon atoms and two R_6 groups on the same adjacent carbon atoms can be taken together to form a triple bond between their respective carbon atoms.

Each R_5 and R_6, independently, can be optionally substituted alkyl, optionally substituted alkoxy, optionally substituted cycloalkyl, optionally substituted aryl, optionally substituted heteroaryl, or optionally substituted heterocyclyl.

Each a, independently, is 0, 1, 2, or 3.

In some cases, an R_5 or R_6 substituent from L_3 can be taken with an R_5 or R_6 substituent from L_4 to form a 3- to 8- member cycloalkyl or heterocycle group. Similarly, an R_5 or R_6 substituent from L_3 can be taken with an R_5 or R_6 substituent from L_5 to form a 3- to 8- member cycloalkyl or heterocycle group; or an R_5 or R_6 substituent from L_4 can be taken with an R_5 or R_6 substituent from L_5 to form a 3- to 8- member cycloalkyl or heterocycle group. A cycloalkyl group or heterocycle group formed by R_5 or R_6 substituents from L_3, L_4, or L_5 can be optionally substituted. By way of illustration only, one exemplary R_3 group having this structural feature includes (5-(2-(N,N-diethyl-N-methyl)amino)ethyl-4-methyl-1,3-dioxan-2-yl)methyl.

In some cases, Y_1, Y_2, or Y_3 can be taken together with an R_5 or R_6 group from any of L_3, L_4, and L_5, and atoms to which they are attached, to form a 3- to 8- member heterocycle. By way of illustration only, some exemplary R_3 groups having this structural feature include (N,N-dimethylpyrrolidin-2-yl)methyl, and 2-(N-methyl-N-ethylpiperidin-4-yl)ethyl.

In one embodiment, X and Y can be independently -O-, -S-, alkylene, or -N(Q)-.

It has been found that charged lipids comprising unsaturated alkyl chains are particularly useful for forming lipid nucleic acid particles with increased membrane fluidity. In one embodiment, at least one of R_1 or R_2 comprises at least one, at least two or at least three sites of unsaturation, e.g. double bond or triple bond.

In one embodiment, only one of R_1 or R_2 comprises at least one, at least two or at least three sites of unsaturation.

In one embodiment, R_1 and R_2 both comprise at least one, at least two or at least three sites of unsaturation.

In one embodiment, R_1 and R_2 comprise different numbers of unsaturation, e.g., one of R_1 and R_2 has one site of unsaturation and the other has two or three sites of unsaturation.

In one embodiment, R_1 and R_2 both comprise the same number of unsaturation sites.

In one embodiment, R_1 and R_2 comprise different types of unsaturation, e.g. unsaturation in one of R_1 and R_2 is double bond and in the other unsaturation is triple bond.

In one embodiment, R_1 and R_2 both comprise the same type of unsaturation, e.g. double bond or triple bond.

In one embodiment, at least one of R_1 or R_2 comprises at least one double bond and at least one triple bond.

In one embodiment, only one of R_1 or R_2 comprises at least one double bond and at least one triple bond.

In one embodiment, R_1 and R_2 both comprise at least one double bond and at least one triple bond.

In one embodiment, R_1 and R_2 are both same, e.g. R_1 and R_2 are both linoleyl (C18) or R_1 and R_2 are both heptadeca-9-enyl.

In one embodiment, R_1 and R_2 are different from each other.

In one embodiment, at least one of R_1 and R_2 is cholesterol.

In one embodiment, at least one of R_1 or R_2 comprises at least one methylene group where one or both H atoms are replaced by F, e.g. fluoromethylene or difluoromethylene. In one embodiment, both R_1 and R_2 comprise at least one methylene group with one or two H replaced by F, e.g. . fluoromethylene or difluoromethylene.

In one embodiment, only one of R_1 and R_2 comprises at least one methylene group with one or both H replaced by F.

In one embodiment, at least one of R_1 or R_2 terminates in fluoromethyl, difluormethyl, or trifluoromethyl. In one embodiment, both R_1 and R_2 terminate in fluoromethyl, difluormethyl, or trifluoromethyl.

In one embodiment, at least one of R_1 or R_2 is $-(CF_2)_y$-Z''-$(CH_2)_y$-CH_3, wherein each y is independently 1-10 and Z'' is O, S or N(Q).

In one embodiment, both of R_1 and R_2 are $-(CF_2)_y$-Z''-$(CH_2)_y$-CH_3, wherein each y is independently 1-10 and Z'' is O, S or N(Q).

In one embodiment, at least one of R_1 or R_2 is $-(CH_2)_y$-Z''-$(CF_2)_y$-CF_3, wherein each y is independently 1-10 and Z'' is O, S or N(Q).

In one embodiment, both of R_1 and R_2 are $-(CH_2)_y$-Z''-$(CF_2)_y$-CF_3, wherein each y is independently 1-10 and Z'' is O, S or N(Q).

In one embodiment, at least one of R_1 or R_2 is $-(CF_2)_y-(CF_2)_y-CF_3$, wherein each y is independently 1-10.

In one embodiment, both of R_1 and R_2 are $-(CF_2)_y-(CF_2)_y-CF_3$, wherein each y is independently 1-10.

In some embodiments, R_1 and R_2 are, independently, selected from the group consisting of lineolyl, γ-linoenyl, n-octadecanyl, n-decanyl, n-dodecanyl, and 9-methyloctadecanyl. In some embodiments the lipid can have (R_1, R_2) selected from the group consisting of (lineolyl, lineolyl), (γ-linoenyl, γ-linoenyl), (lineolyl, n-octadecanyl), (lineolyl, n-decanyl), (lineolyl, n-dodecanyl), and (9-methyloctadecanyl, 9-methyloctadecanyl).

In one embodiment, when Z is $C(R_3)$, at least one R_3 is ω-aminoalkyl or ω -(substituted)aminoalkyl.

In one embodiment, when Z' is O, S or alkyl, at least one R_3 is ω-aminoalkyl or ω -(substituted)aminoalkyl.

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.

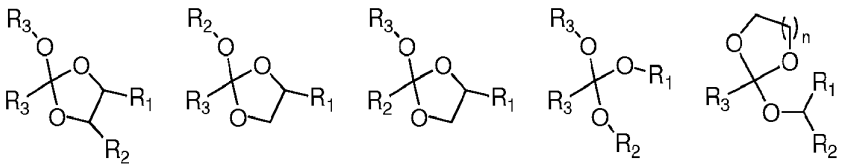
In one embodiment, the lipid is enriched in one enantiomer, e.g. the lipid has at least 95%, at least 90%, at least 80% or at least 70% enantiomer excess.

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.

In another aspect, the invention features a compound of formula XXXIVa, XXXIVb, XXXIVc, XXXIVd, or XXXIVe, salts or isomers thereof:



XXXIVa XXXIVb XXXIVc XXXIVd XXXIVe

wherein:

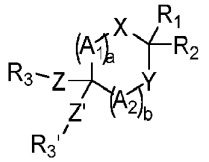
R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkenyl, or optionally substituted C_{10}-C_{30} alkynyl;

R_3 is defined as above.

n is 1, 2, or 3.

In some embodiments, R_3 is optionally substituted heterocyclealkyl, optionally substituted amino, optionally substituted alkylamino, optionally substituted di(alkyl)amino, optionally substituted aminoalkyl, optionally substituted alkylaminoalkyl, optionally substituted di(alkyl)aminoalkyl, or optionally substituted heterocycle.

In one aspect, the lipid is a compound of formula XIIIa:



(XIIIa)

wherein:

R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkenyl, or optionally substituted C_{10}-C_{30} alkynyl.

At least one of R_3 and $R_{3'}$ includes a quaternary amine.

R_3 and $R_{3'}$ are independently for each occurrence defined as R_3 above;

or R_3 and $R_{3'}$ can be taken together with the atoms to which they are attached to form an optionally substituted carbocyclyl, optionally substituted heterocyclyl, optionally substituted aryl or optionally substituted heteroaryl; each of which is substituted with 0-4 occurrences of R_4;

each R_4 is independently selected from optionally substituted C_1-C_{10} alkyl, optionally substituted C_2-C_{10} alkenyl, optionally substituted C_2-C_{10} alkynyl, optionally substituted amino, optionally substituted alkylamino, optionally substituted di(alkyl)amino, optionally substituted aminoalkyl, optionally substituted alkylaminoalkyl,

optionally substituted di(alkyl)aminoalkyl, optionally substituted hydroxyalkyl, optionally substituted aryl, optionally substituted heteroaryl, or optionally substituted heterocycle;

X and Y are each independently -O-, -S-, alkylene, or -N(Q)-;

Q is H, alkyl, ω-aminoalkyl, ω-(substituted)aminoalkyl, ω-phosphoalkyl, or ω-thiophosphoalkyl;

A_1 and A_2 are each independently -O-, -S-, or $-CR^5R^5-$; and

R^5 is H, halo, cyano, hydroxy, amino, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted cycloalkyl; and

Z and Z' are each independently selected from -O-, -S-, -N(Q)-, alkylene or absent; and

a and b are each independently 0-2.

In some embodiments, X and Y are each independently O.

In some embodiments, the sum of a and b is 1, 2, or 3.

In some embodiments, A_1 and A_2 are each independently $-CR^5R^5-$.

In some embodiments, Z and Z' are each a bond.

In some embodiments, R_3 and $R_{3'}$ can be taken together with the atoms to which they are attached to form an optionally substituted carbocyclyl, optionally substituted heterocyclyl, optionally substituted aryl or optionally substituted heteroaryl.

In some embodiments, R_3 and $R_{3'}$ can be taken together with the atoms to which they are attached to form an optionally substituted carbocyclyl (e.g., optionally substituted with amino, alkylamino, or dialkylamino).

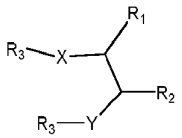
In some embodiments, R_3 and $R_{3'}$ can be taken together with the atoms to which they are attached to form an optionally substituted heterocyclyl (e.g., a nitrogen containing heterocyclyl).

In some embodiments, R_3 and $R_{3'}$ are taken together to form a carbocyclic ring (e.g., cyclohexyl) substituted with 0-3 occurrence of R_4.

In some embodiments, R_3 and $R_{3'}$ are taken together to form a heterocyclic ring (e.g., piperidine) substituted with 0-3 occurrences of R_4.

In some embodiments, each R_4 is independently selected from optionally optionally substituted amino, optionally substituted alkylamino, optionally substituted di(alkyl)amino, optionally substituted aminoalkyl, optionally substituted alkylaminoalkyl, optionally substituted di(alkyl)aminoalkyl, and optionally substituted hydroxyalkyl.

In one aspect, the lipid is a compound of formula XXXIX, salts or isomers thereof:



XXXIX

wherein:

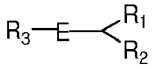
R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} acyl.

R_3 is defined as above.

X and Y are each independently O, C(O)O, S, alkyl or N(Q);

Q is H, alkyl, ω-aminoalkyl, ω-(substituted)aminoalkyl, ω-phosphoalkyl or ω-thiophosphoalkyl.

In one aspect, the lipid is a compound of formula XXXIII, salts or isomers thereof

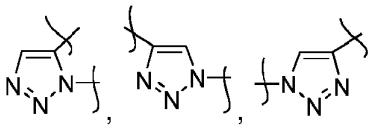


XXXIII

wherein:

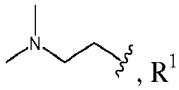
R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} acyl;

Eis $-CH_2-$, -O-, -S-, -SS-, -CO-, -C(O)O-, -C(O)N(R')-, -OC(O)N(R')-, -N(R')C(O)N(R")-, -C(O)-N(R')-N=C(R"')-; -N(R')-N=C(R")-, -O-N=C(R")-, -C(S)O-, -C(S)N(R')-, -OC(S)N(R')-, -N(R')C(S)N(R")-, -C(S)-N(R')-N=C(R"'); -S-N=C(R"); -C(O)S-, -SC(O)N(R')-, -OC(O)-, -N(R')C(O)-, -N(R')C(O)O-, -C(R"')=N-N(R')-; -C(R"')=N-N(R')- C(O)-, -C(R"')=N-O-, -OC(S)-, -SC(O)-, -N(R')C(S)-, -N(R')C(S)O-, -N(R')C(O)S-, -C(R"')=N-N(R')-C(S)-, -C(R"')=N-S-, C[=N(R')]O, C[=N(R')]N(R"), -OC[=N(R')]-, -N(R")C[=N(R')]N(R"')-, -N(R")C[=N(R')]-,

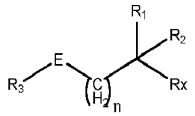

, arylene, heteroarylene, cycloalkylene, or heterocyclylene.

Q is H, alkyl, ω-aminoalkyl, ω-(substituted)aminoalky, ω-phosphoalkyl or ω-thiophosphoalkyl.

In one embodiment, R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkoxy, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkenyloxy, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} alkynyloxy, or optionally substituted C_{10}-C_{30} acyl.

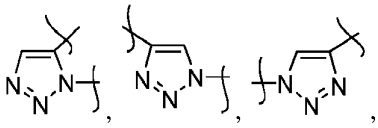
In one embodiment, the lipid is a compound of formula XXXIII, provided that when E is -C(O)O- and R^3 is  , R^1 and R^2 are not both linoleyl.

In one embodiment, the invention features a lipid of formula XXXVIII:

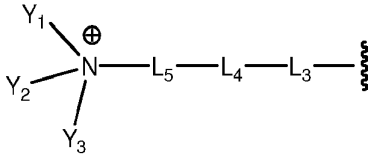


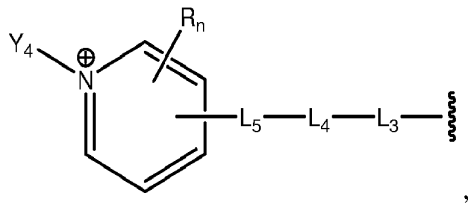
wherein

Eis -CH_2-, -O-, -S-, -SS-, -CO-, -C(O)O-, -C(O)N(R')-, -OC(O)N(R')-, -N(R')C(O)N(R")-, -C(O)-N(R')-N=C(R"')-; -N(R')-N=C(R")-, -O-N=C(R")-, -C(S)O-, -C(S)N(R')-, -OC(S)N(R')-, -N(R')C(S)N(R")-, -C(S)-N(R')-N=C(R"'); -S-N=C(R"); -C(O)S-, -SC(O)N(R')-, -OC(O)-, -N(R')C(O)-, -N(R')C(O)O-, -C(R"')=N-N(R')-; -C(R"')=N-N(R')- C(O)-, -C(R"')=N-O-, -OC(S)-, -SC(O)-, -N(R')C(S)-, -N(R')C(S)O-, -N(R')C(O)S-, -C(R"')=N-N(R')-C(S)-, -C(R"')=N-S-, C[=N(R')]O, C[=N(R')]N(R"), -OC[=N(R')]-, -N(R")C[=N(R')]N(R"')-, -N(R")C[=N(R')]-,

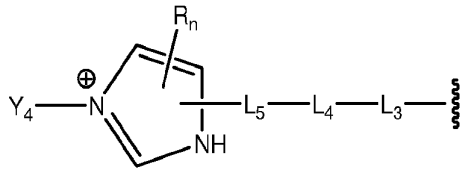

, arylene, heteroarylene, cycloalkylene, or heterocyclylene.

R_3 has the formula:





, or



Y_1 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_1 is optionally substituted by 0 to 6 R_n. Y_2 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_2 is optionally substituted by 0 to 6 R_n. Y_3 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_3 is optionally substituted by 0 to 6 R_n. Y_4 is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl, wherein Y_4 is optionally substituted by 0 to 6 R_n; or any two of Y_1, Y_2, and Y_3 are taken together with the N atom to which they are attached to form a 3- to 8- member heterocycle optionally substituted by 0 to 6 R_n; or Y_1, Y_2, and Y_3 are all be taken together with the N atom to which they are attached to form a bicyclic 5- to 12- member heterocycle optionally substituted by 0 to 6 R_n.

Each R_n, independently, is H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl. L_3 is a bond, -N(Q)-, -O-, -S-, -$(CR_7R_8)_a$-, -C(O)-, or a combination of any two of these. L_4 is a bond, -N(Q)-, -O-, -S-, -$(CR_7R_8)_a$-, -C(O)-, or a combination of any two of these. L_5 is a bond, -N(Q)-, -O-, -S-, -$(CR_7R_8)_a$-, -C(O)-, or a combination of any two of these.

Each occurrence of R_7 and R_8 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl; or two R_7 groups on adjacent carbon atoms are taken together to form a double bond between their respective carbon atoms; or two R_7 groups on adjacent carbon atoms and two R_8 groups on the same adjacent carbon atoms are taken together to form a triple bond between their respective carbon atoms.

Each a, independently, is 0, 1, 2, or 3; wherein an R_7 or R_8 substituent from any of L_3, L_4, or L_5 is optionally taken with an R_7 or R_8 substituent from any of L_3, L_4, or L_5 to form a 3- to 8- member cycloalkyl, heterocyclyl, aryl, or heteroaryl group; and any one of Y_1, Y_2, or Y_3, is optionally taken together with an R_7 or R_8 group from any of L_3, L_4, and L_5, and atoms to which they are attached, to form a 3- to 8- member heterocyclyl group.

Each occurrence of R_5 and R_6 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl.

Each Q, independently, is H, alkyl, acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl or heterocyclyl.

Each Q_2, independently, is O, S, N(Q)Q, alkyl or alkoxy.

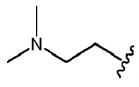
Q is H, alkyl, ω-aminoalkyl, ω-(substituted)aminoalkyl, ω-phosphoalkyl, or ω-thiophosphoalkyl.

R_1 and R_2 and R_x are each independently for each occurrence H, optionally substituted C_1-C_{10} alkyl, optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} acyl. In some embodiments, at least one of R_1, R_2 and R_x is not H.

In some embodiments, at least two of R_1, R_2 and R_x is not H.

R_3 is defined as above.

n is 0, 1, 2, or 3.

In one embodiment, where the lipid is a compound of formula XXXVIII, when E is C(O)O, R^3 is  , and one of R_1, R_2, or R_x is H, then the remaining of R_1, R_2, or R_x are not both linoleyl.

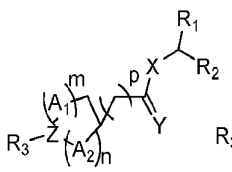
In some embodiments, each of R_1 and R_2 is independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} acyl.

In some embodiments, R_x is H or optionally substituted C_1-C_{10} alkyl.

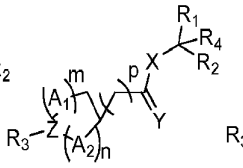
In some embodiments, R_x is optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} acyl.

The present invention comprises of synthesis of lipids described herein in racemic as well as in optically pure form.

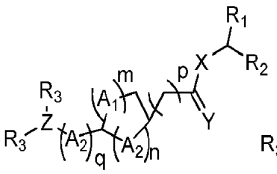
In one aspect, a lipid has formula LX, LXI, LXII, LXIII, LXIV, LXV, LXVI, LXVII, LXVIII, LXIX, LXX, LXXI, LXXII, or LXXIII:



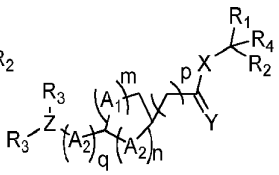
LX



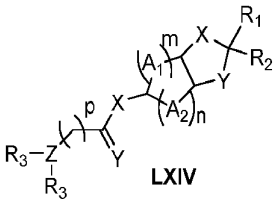
LXI



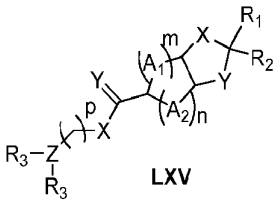
LXII



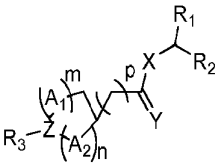
LXIII



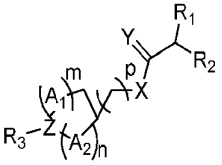
LXIV



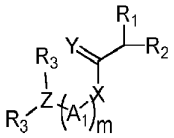
LXV



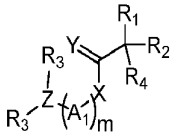
LXVI



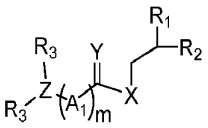
LXVII



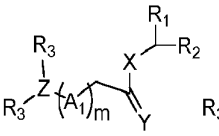
LXVIII



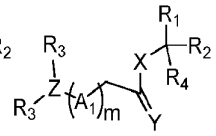
LXIX



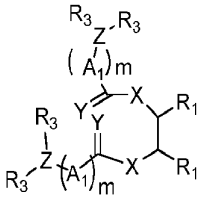
LXX



LXXI



LXXII



LXXIII

wherein:

X and Y are each independently -O-, -S-, $-CH_2-$, or $-N(Q_3)-$; where Q is H, Me, Et, or $-(CH_2)_r-N(Q_3)(Q_4)$;

Z is N, CH, C(Me), C(Et);

Q_1 is O or S;

Q_2 is O or S;

Each of A_1 and A_2, independently, are CH_2, CHF, or CF_2;

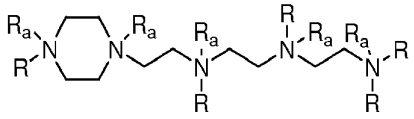
m, n, p and q are each independently 0 to 5.

In formulas LX, LXI, LXII, LXIII, LXIV, LXV, LXVI, LXVII, LXVIII, LXIX, LXX, LXXI, LXXII, and LXXIII, R_1, R_2 and R_4 are each independently selected from the group consisting of alkyl groups having about 10 to 30 carbon atoms, wherein R_1, R_2 and R_4 independently comprises of: fully saturated alkyl chain, at least one double bond, at

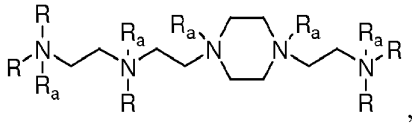
least one triple bond, at least one hetero atom, at least one CF_2, at least one CHF or at least one perfluoroalkylated chain. CF_2/CHF could be on the lipid anchor or on the core.

R_3 is defined as above.

In one embodiment, the lipid can be a compound having the formula:

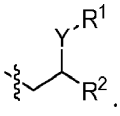


, or having the formula:

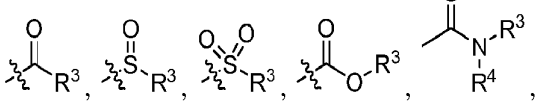
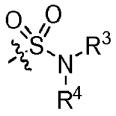


, or a mixture thereof.

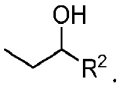
Each R_a, independently, is absent, H, alkyl, or cycloalkyl. In one embodiment, R_a is alkyl or cycloalkyl for no more than two occurrences. In one embodiment, R_a is alkyl or cycloalkyl for no more than one occurrence.

In one embodiment, R, for at least 3 occurrences, is  . In one embodiment, Y is O or NR^4. In one embodiment, Y is O. In one embodiment, Y is O for each occurrence. In one embodiment, R^1 is H. In one embodiment, R^1 is H for each occurrence.

In one embodiment, R^1 is  ,wherein R^3 alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, or heteroalkynyl, each of which is optionally substituted with one or more substituent (e.g., a hydrophilic substituent). In one embodiment, R^1 is  , and R^3 alkyl optionally substituted with one or more substituent (e.g., a hydrophilic substituent). In one embodiment, R^3 is substituted with -OH.

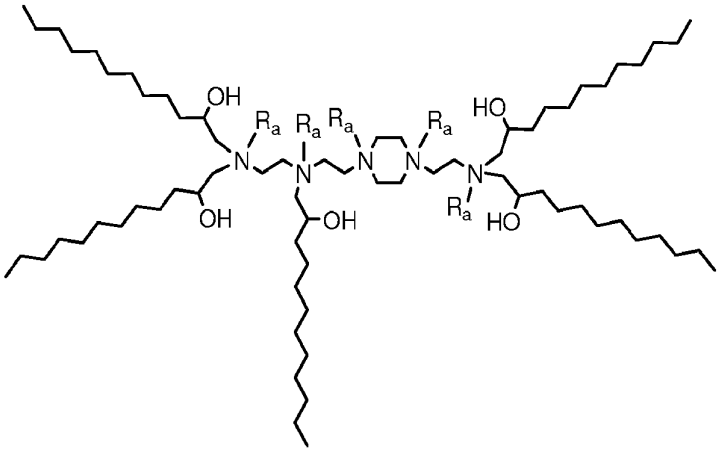
In one embodiment, R^1 is R^3,  , or  ; wherein R^3 alkyl is optionally substituted with one or more substituent. In one embodiment, R^3 is substituted with a hydrophilic substituent. R^4, for each occurrence is independently H alkyl, alkenyl, alkynyl, heteroalkyl, heteroalkenyl, or heteroalkynyl; each of which is optionally substituted with one or more substituent. In one embodiment,

R^3 is substituted with -OH. In one embodiment, R^2 is alkyl, alkenyl, or alkynyl. In one embodiment, R^2 is alkyl (e.g., C_6-C_{18} alkyl, e.g., C_8-C_{12} alkyl, e.g., C_{10} alkyl).

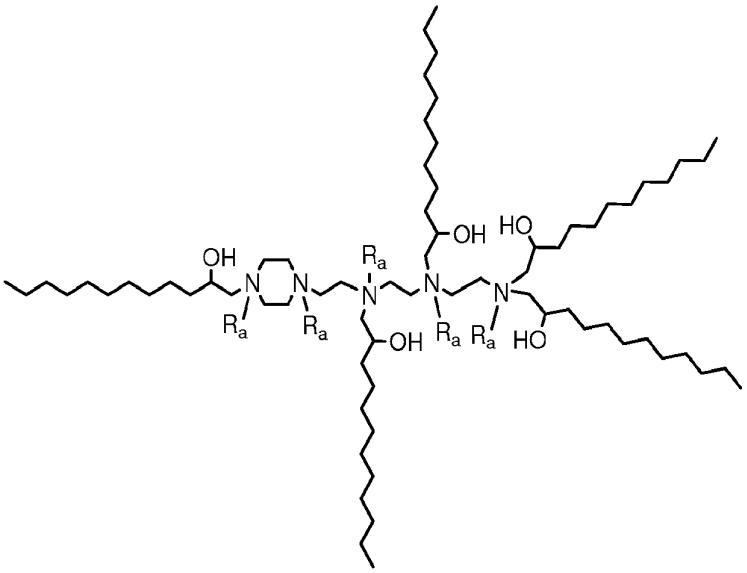
In one embodiment, R for at least 3 (e.g., at least 4 or 5) occurrences is 

. In one embodiment, R^2 is alkyl (e.g., C_6-C_{18} alkyl, e.g., C_8-C_{12} alkyl, e.g., C_{10} alkyl). In one embodiment, R for at least 1 occurrence (e.g., 1 or 2 occurrences) is H.

The lipid can be a compound having the formula:



, or a compound having the formula:

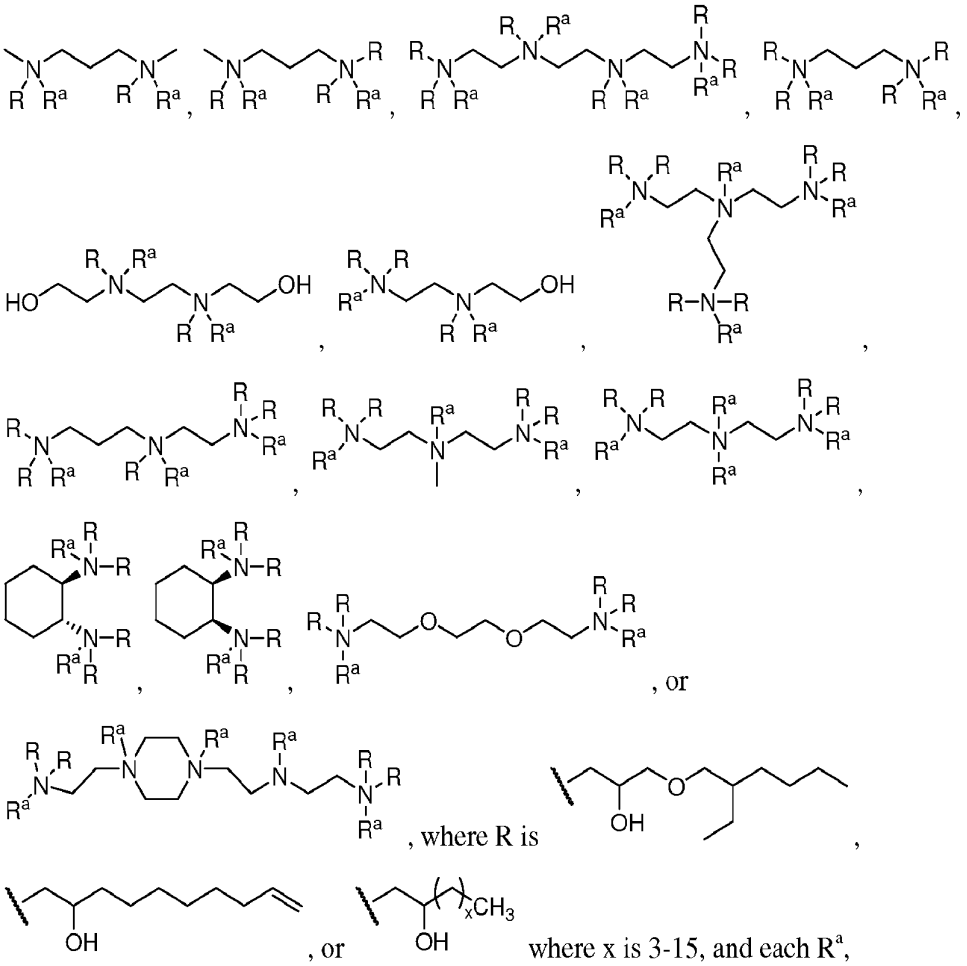


, or a mixture thereof.

In one embodiment, no more than two instances of R_a are alkyl or cycloalkyl. In one embodiment, no more than one instance of R_a are alkyl or cycloalkyl. In one embodiment, one or two instances of R_a are methyl, and the remaining instances of R_a are each absent or H.

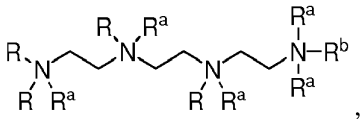
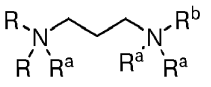
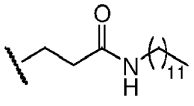
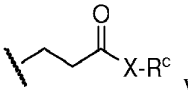
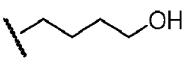
In some embodiments, the lipid can be a quaternary lipid derived from the compounds disclosed in Akinc, A., et al., "Development of lipidoid-siRNA formulations for systemic delivery of RNAi therapeutics," Nat. Biotechnol. 26, (2008), 561-569; Love, K.T., et al., "Lipid-like materials for low-dose, in vivo gene silencing," PNAS 107, 5, (2010), 1864-1869; or Mahon, K.P., et al., "Combinatorial approach to determine functional group effects on lipidoid-mediated siRNA delivery," Bioconjug Chem. 2010 Aug 18;21(8):1448-54; each of which is incorporated by reference in its entirety.

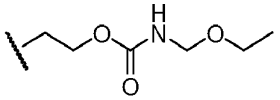
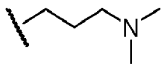
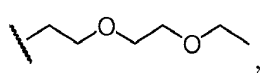
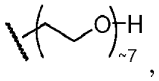
For example, the lipid can have one of the following formulas:

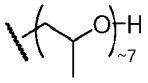
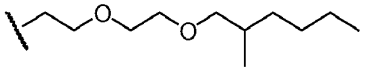
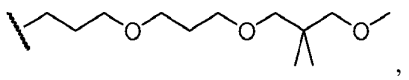


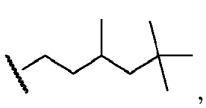
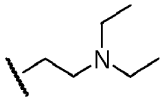
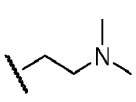
where x is 3-15, and each R^a, independently, is absent, H, alkyl, or cycloalkyl. In some cases, R^a is alkyl or cycloalkyl for no more than two occurrences, or R^a can be alkyl or cycloalkyl for no more than one occurrence. In some cases, R^a is methyl.

In another example, the lipid can have one of the following formulas:

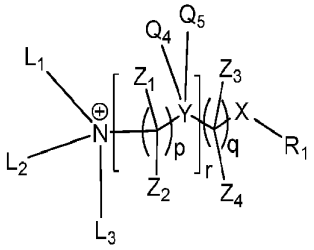

, or  where R is  , R^b is  where X is O or NH and R^c is  ,

 ,  ,  ,  ,

 ,  ,  ,

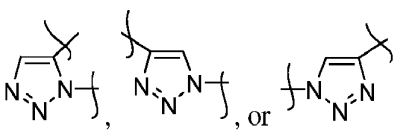
 ,  ,  , or  where x is 7-17, and each R^a, independently, is absent, H, alkyl, or cycloalkyl. In some cases, R^a is alkyl or cycloalkyl for no more than two occurrences, or R^a can be alkyl or cycloalkyl for no more than one occurrence. In some cases, R^a is methyl.

In another aspect, the lipid can have the formula:



wherein X

is $-CH_2-$, -O-, -S-, -SS-, -CO-, -C(O)O-, -C(O)N(R')-, -OC(O)N(R')-, -N(R')C(O)N(R")-, -C(O)-N(R')-N=C(R"')-; -N(R')-N=C(R")-, -O-N=C(R")-, -C(S)O-, -C(S)N(R')-, -OC(S)N(R')-, -N(R')C(S)N(R")-, -C(S)-N(R')-N=C(R"'); -S-N=C(R"); -C(O)S-, -SC(O)N(R')-, -OC(O)-, -N(R')C(O)-, -N(R')C(O)O-, -C(R"')=N-N(R')-; -C(R"')=N-N(R')- C(O)-, -C(R"')=N-O-, -OC(S)-, -SC(O)-, -N(R')C(S)-, -N(R')C(S)O-, -N(R')C(O)S-, -C(R"')=N-N(R')-C(S)-, -C(R"')=N-S-, C[=N(R')]O, C[=N(R')]N(R"), -OC[=N(R')]-, -N(R")C[=N(R')]N(R"')-, -N(R")C[=N(R')]-,

 .

Y is N, O or S; and when Y is O or S, then Q_4 and Q_5 are absent.

When Y is N, Q_4 and Q_5 can independently be H, alkyl (e.g., a primary, secondary or tertiary alkyl, such as, for example, Me, Et, isopropyl, or *tert*-butyl), alkenyl, alkynyl, aryl, aralkyl, cycloalkyl, or heterocyclyl. When Q_4 or Q_5 includes a double or triple bond, the double or triple bond can be anywhere in the chain. When there are two or more double (or triple) bonds or combination of both then the double (or triple) bonds can be separated by at least 1 saturated carbon atom. In some occurrences, two or more multiple bonds can be conjugated. In some occurrences, two double bonds are tied to the same carbon atom. Double bonds can be *cis*, *trans*, or combination of *cis* and *trans*.

L_1, L_2 and L_3 are each independently alkyl (e.g., primary, secondary or tertiary alkyl, such as, for example, Me, Et, isopropyl, or *tert*-butyl), aryl, aralkyl, alkenyl or alkynyl.

R_1 is a C_6 to C_{60} group selected from alkyl, alkenyl, alkynyl, heteroalkyl, aralkyl, cycloalkyl, and heterocyclyl. When Q_4 or Q_5 includes a double or triple bond, the double or triple bond can be anywhere in the chain. When there are two or more double (or triple) bonds or combination of both then the double (or triple) bonds can be separated by at least 1 saturated carbon atom. In some occurrences, two or more multiple bonds can be conjugated. In some occurrences, two double bonds are tied to the same carbon atom. Double bonds can be *cis*, *trans*, or combination of *cis* and *trans*. In some embodiments, R_1 and R_2 are independently branched alkyl. In one example branched alkyl include  wherein R_{100} is independently selected from oleyl, linoleyl, steryl, palmityl and the like.

R', R", and R"' are independently H, or a C_1 to C_{30} group selected from alkyl, alkenyl, alkynyl, heteroalkyl, aralkyl, cycloalkyl and heterocyclyl. When R', R", or R"' includes a double or triple bond, the double or triple bond can be anywhere in the chain. When there are two or more double (or triple) bonds or combination of both then the double (or triple) bonds can be separated by at least 1 saturated carbon atom. In some occurrences, two or more multiple bonds can be conjugated. In some occurrences, two double bonds are tied to the same carbon atom. Double bonds can be *cis*, *trans*, or combination of *cis* and *trans*.

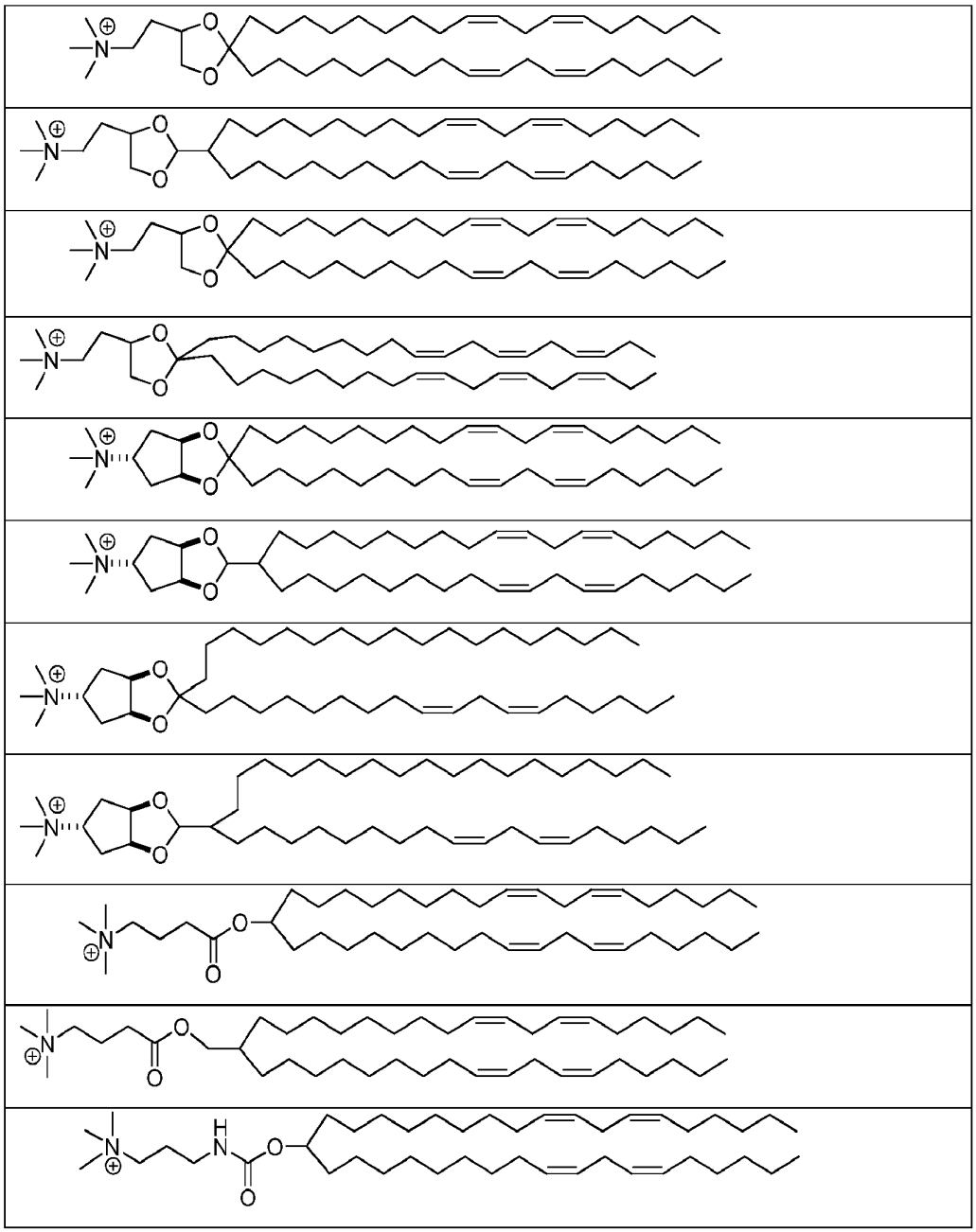
Each of Z_1, Z_2, Z_3, and Z_4, is independently H, F, XR_1, $N(Q_6)(Q_7)$ or -$[Y(Q_4,Q_5)$-$[C(Z_1,Z_2)]_p$-$N(Q_1,Q_2,Q_3)$, or a C_1 to C_{30} group selected from alkyl, substituted alkyl,

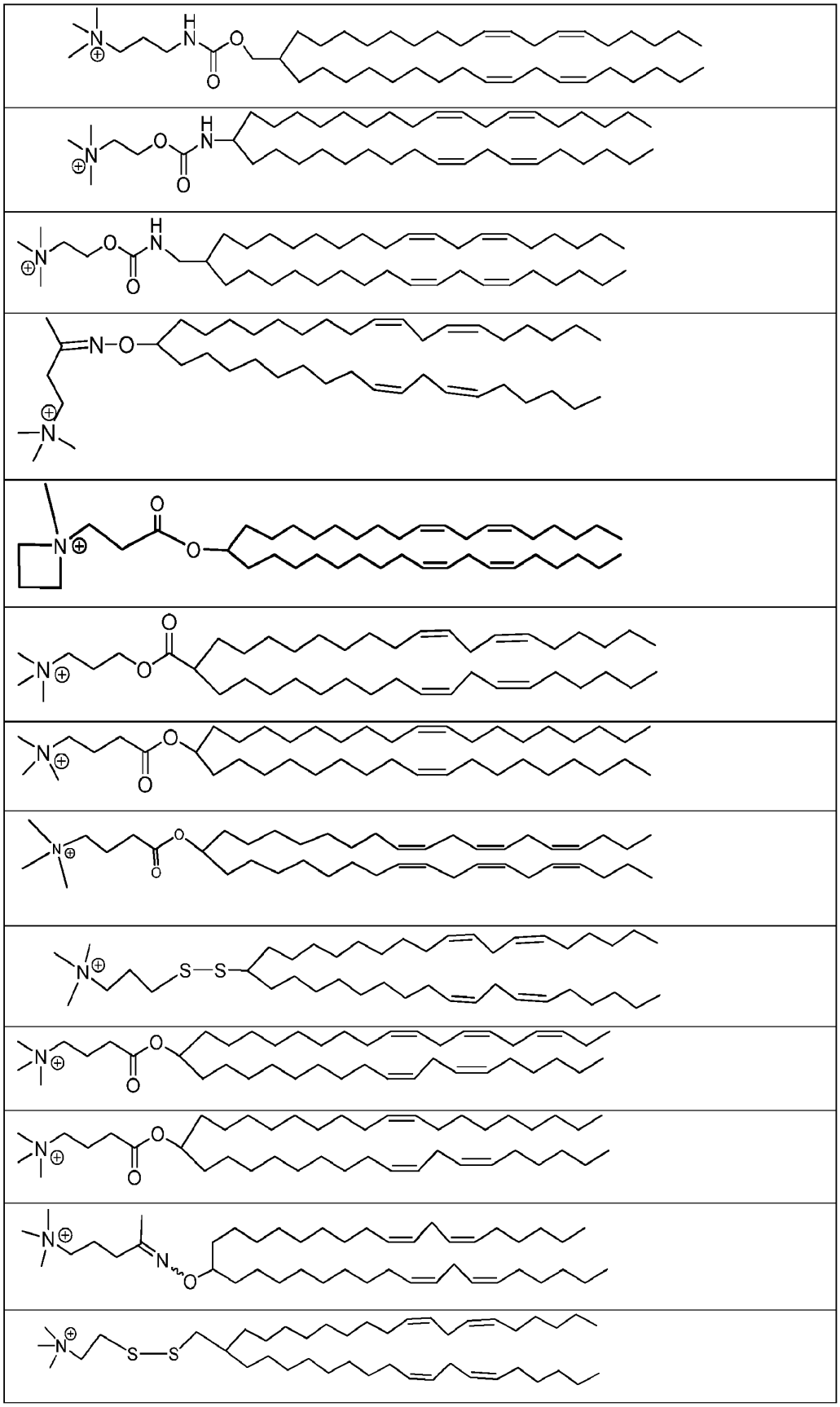
heteroalkyl, aralkyl, cycloalkyl and heterocyclyl. In some occurrence Z_1 is (=O), (=S) or (=NR') and Z_2 is absent.

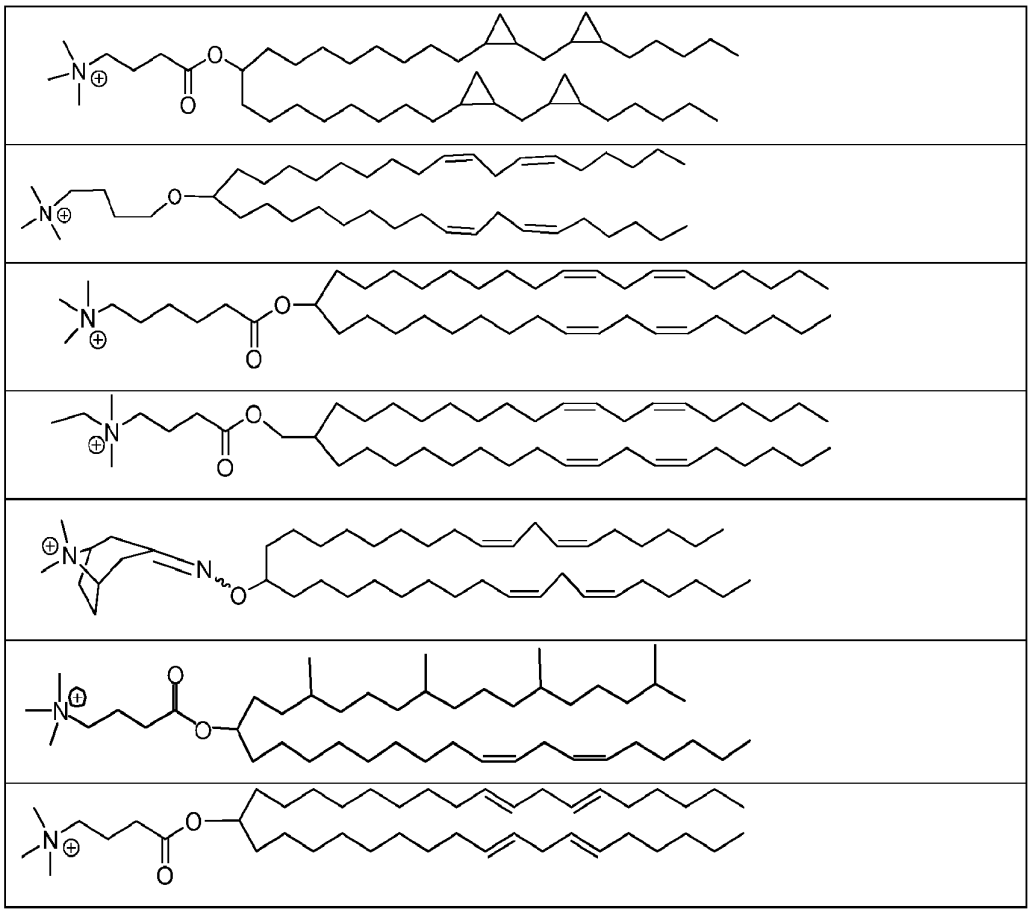
p is 0 to 19. q is 0 to 20. r is 0 to 100.

Additional synthetic techniques for making lipids can be found in provisional U.S. Patent Application No. 61/333,122, filed May 10, 2010, which is incorporated by reference in its entirety.

Table 1. Some exemplary quaternary amine lipids







The lipid including a quaternary amine can be in the form of a salt, i.e. complexed with a counterion. The counterion can be any anion, such as an organic or inorganic anion. Suitable examples of such anions include tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, α-ketoglutarate, and α-glycerophosphate. Inorganic can include chloride, sulfate, nitrate, bicarbonate, and carbonate salts.

Generally, a lipid including a quaternary amine can be prepared from a corresponding lipid that includes a tertiary amine. The tertiary amine is converted to a quaternary amine by, e.g., alkylation with an appropriate alkyl halide. For example, a lipid including a dimethylamino group (i.e., a tertiary amine) can be converted to the corresponding trimethylamino group by reaction with methyl chloride. Methods for making lipids including tertiary amine groups are described in, for example, in application no. PCT/US09/63933, filed November 10, 2009, and applications referred to therein, including 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. 61/185,438, filed June 9,

2009; no. 61/225,898, filed July 15, 2009; and no. 61/234,098, filed August 14, 2009; each of these documents is incorporated by reference in its entirety. In general, the lipids described in these applications are suitable for converting to the corresponding quaternary amine. See, for example, Table 1 of application no. PCT/US09/63933, filed November 10, 2009, at pages 33-50.

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."

Other charged lipids would include those having alternative fatty acid groups and other quaternary 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 or acyl groups, they can be the same or different. In general, lipids (e.g., a charged 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. Charged 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 charged lipid) and the fatty acid or fatty alkyl portion of the charged lipid. Suitable scaffolds are known to those of skill in the art.

In certain embodiments, charged lipids of the present invention 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 charged 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. Lipids that have more than one protonatable or

deprotonatable group, or which are zwiterrionic, are not excluded from use in the invention.

In certain embodiments, protonatable lipids (i.e., charged lipids) according to the invention have a pKa of the protonatable group in the range of about 4 to about 11. Typically, lipids will have a pKa 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 pKa 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. pKa 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.

The compositions described herein can include mixtures of charged lipids. For example, the compositions (e.g., lipoplexes and/or lipid nanoparticles) can include lipids that have quaternary amines and lipids that do not have quaternary amines, but do have a protonatable amine group. Suitable lipids for the compositions (both as quaternary or non-quaternary amines) include those described in WO 2010/054406, WO 2010/054405, WO 2010/054401, WO 2010/054384, U.S. Application No. 61/309,697, filed March 2, 2010; U.S. Application No. 61/321,829, filed April 7, 2010; U.S. Application No. 61/369,530, filed July 30, 2010; U.S. Application No. 61/333,122, filed May 10, 2010; U.S. Application No. 61/369,535, filed July 30, 2010; and U.S. Application No. 61/351,146, filed June 3, 2010, each of which is incorporated by reference in its entirety.

CRYOPROTECTANTS AND FREEZE-DRYING

The formulations can include a cryoprotectant. A formulation can be suspended in a buffer containing a cryprotectant at a volume measured to obtain a final desired lipid concentration. The suspension can be agitated to thoroughly mix the cryoprotectant with the lipid nanoparticles. The suspension can be extruded or filtered to select nanoparticles of a given size. This can result in a final formulation, which can be stored under appropriate conditions until use. After storage of the lipid nanoparticles with a

cryoprotectant, the lipid nanoparticles can be used for delivery of nucleic acids to cells without increased cell death or decreased delivery efficiency.

A cryoprotectant can be a compound used to protect the formulation from damage due to cold, for example, freezing. A cryoprotectant can include a polyol, e.g., a carbohydrate, for example, sucrose, trehalose, glucose or a 2-hydroxypropyl-α-cyclodextrin. A sugar alcohol, such as sorbitol, can also be included in a cryoprotectant. A cryprotectant can include a protein, a peptide or an amino acid. For example, a cryoprotectant can include proline or hydroxyl proline. An organic compound, such as glycerol, ethylene glycol, or propylene glycol, can be included in a cryoprotectant. In some instances, a cryoprotectant can include a polymer, for example, polyvinylpyrrolidone, polyethylene glycol or gelatin or hydroxyethylcellulose.

A formulation can be mixed and solvent can be removed, which can result in a residue. The residue can be resuspended in a buffer including a cryoprotectant, or the residue can be resuspended in a buffer and then a cryoprotectant can be added. The result can be a suspension of formulation in buffer. The formulation can include lipid nanoparticles. The lipid nanoparticles can also include a nucleic acid. The buffer can be a buffer solution or a buffered media. The pH of the buffer can be greater than 5.0, greater than 6.0, greater than 6.5, greater than 7.0, greater than 7.1, greater than 7.2, greater than 7.3, greater than 7.4, greater than 7.5, greater than 7.6, greater than 7.7, greater than 7.8, greater than 7.9, greater than 8.0 or greater than 9.0. The pH of the buffer can be less than 9.0, less than 8.0, less than 7.9, less than 7.8, less than 7.7, less than 7.6, less than 7.5, less than 7.4, less than 7.3, less than 7.2, less than 7.1, less than 7.0, less than 6.5, less than 6.0 or less than 5.0. The buffer can be pH 7.4. The suspension can include less than 20%, less than 15%, less than 12%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5% or less than 3% cryoprotectant by volume. The suspension can include more than 3%, more than 5%, more than 6%, more than 7%, more than 8%, more than 9%, more than 10% more than 12%, more than 15%, or more than 20% cryoprotectant by volume. In particular, the suspension can include 5% or 10% cryoprotectant by volume.

Once the formulation residue is resuspended, the lipid concentration within the suspension can be greater than 0.25 mg/mL, greater than 0.5 mg/mL, greater than 1.0 mg/mL or greater than 1.5 mg/mL. The lipid concentration within the suspension can be

less than 2.0 mg/mL, less than 1.5 mg/mL, less than 1.0 mg/mL, less than 0.5 mg/mL, or less than 0.25 mg/mL. More specifically, the concentration can be 1.0 mg/mL.

The suspension can be mixed or agitated to distribute the cryoprotectant throughout the suspension. The mixing or agitation can occur at 4 ºC, 25 ºC or 37 ºC. The mixing or agitation can occur for greater than 5 minutes, greater than 10 minutes, greater than 15 minutes, greater than 30 minutes or greater than an hour. Mixing or agitation can occur by shaking, pipetting or stirring.

After agitation, the lipid nanoparticles contained in the suspension can be selected for size. For example, the nanoparticles can be filtered or extruded. In some cases, the resuspension can be extruded through a filter, for example a polycarbonate filter. The resuspension can also be syringe filtered. In either case, the filter can allow particles less than 0.5 μm, less than 0.45 μm, less than 0.4 μm, less than 0.35 μm, less than 0.3 μm, less than 0.25 μm, less than 0.2 μm or less than 0.15 μm to pass through. Specifically, a filter can have a pore size of 0.45 μm, 0.4 μm, 0.22 μm or 0.2 μm.

Once the lipid nanoparticles have been filtered or extruded, a final suspension of the formulation can contain only lipid nanoparticles smaller than the pore size of the filter. Lipid nanoparticle sizes can be less than 300 nm, less than 275 nm, less than 250 nm, less than 225 nm, less than 200 nm, less than 175 nm, less than 150 nm, less than 125 nm or less than 100 nm.

The final suspension of the formulation can be stored at cold temperatures, for example, less than or equal to 25 ºC, less than or equal to 4 ºC, less than or equal to 0 ºC, than or equal to -20 ºC or less than or equal to -80 ºC, until the formulation is used. The cold formulation can be prepared for use by warming the formulation (e.g., at room temperature) until the formulation is at room temperature or adequately thawed.

The final suspension of the formulation can also be stored at low moisture. For example, the formulation can be dried, lyophilized or freeze-dried. Freeze-drying can be accomplished by storing the formulation at -80 ºC and then lyophilizing the formulation. The low moisture formulation can be prepared for use by rehydrating the formulation, for instance, by resuspending the formulation in a liquid. The liquid can be water, a buffer solution or cell culture media.

The formulation can be stored at cold temperatures or low moisture for greater than 1hour, greater than 2 hours, greater than 6 hours, greater than 12 hours, greater than 24 hours, greater than 2 days, greater than 3 days, greater than 4, greater than 5 days,

greater than 6 days or greater than 1 week and still remain an effective transfection reagent. The formulation can be used for transfections after being stored at cold temperature or at low moisture.

After storage, the formulation can be reconstituted. For example, a lyophilized formulation can be reconstituted by warming following by resuspension; or simply by resuspension in a cold or warm liquid (e.g., water, buffer or media). A formulation stored as a cold or frozen solution can be reconstituted by warming to a desired temperature, e.g., 4 °C, room temperature, or 37 °C. Reconstitution can also include altering the formulation. Formulations may be stored with or without nucleic acids included; when stored without a nucleic acid present, reconsistitution can include adding a nucleic acid to the formulation. Altering the formulation can also include adding additional or different lipids to the formulation. The order of various steps of reconstitution may be varied.

APOLIPOPROTEINS

In one embodiment, the formulations of the invention 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 ($ApoA\text{-}I_M$) and ApoA-I Paris ($ApoA\text{-}I_P$) 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 within the scope of the invention.

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, the present invention contemplates, 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). 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 of the present invention 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 in the invention also include recombinant, synthetic, semi-synthetic or purified apolipoproteins. Methods for obtaining apolipoproteins or equivalents thereof, utilized by the invention 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 utilized in the invention further include apolipoprotein agonists such as peptides and peptide analogues that mimic the activity of ApoA-I, ApoA-I Milano ($ApoA\text{-}I_M$), ApoA-I Paris ($ApoA\text{-}I_P$), 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 of the present invention 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 of the invention 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 of the invention, the apolipoprotein is derived from the same species as the individual to which the apolipoprotein is administered.

TRANSFECTIONS

In one aspect, a method for delivering a nucleic acid to a cell can include exposing sample cells to a composition containing a charged lipid. The charged lipid can include the charged lipids described herein.

A sample cell can include a eukaryotic cell. The eukaryotic cell can be a stem cell, primary cell or a cell in a cell line. The cell line can be a primary cell line, a secondary cell line or an immortalized cell line. Exemplary cell lines can include Chinese hamster ovary (CHO) cells, HeLa cells, U2OS cells, Caco-2 cells, HT29 cells, NIH3T3 cells, PC12 cells, HepG2 cells, U937 cells, Vero cells, BHK cells, ME-180 cells, A549 cells, HEK-293 cells, MCF-7 cells, Jurkat cells, Mdck cells, 3T3 cells, COS-7 cells or GH3 cells. More specifically, the cell line can include GFP-CHO cells or DG44-CHO cells. The cells can be non-adherent suspension cells, including, but not limited to, suspension CHO cells, suspension BHK cells, suspension NS0 cells, suspension HeLa cells and suspension HEK293 cells.

Exposing sample cells to a composition can include contacting the cells with the composition, adding the composition to the media the cells are cultured in or incubating the cells in a solution containing the composition.

In another aspect, a method for delivering a nucleic acid to sample cells can include forming the composition and exposing the sample cells to the composition.

The composition can be purchased, provided or formed. Charged lipids can be prepared for use in transfection by forming into liposomes and mixing with the macromolecules to be introduced into the cell. Macromolecules that can be delivered to cells with the transfection reagents can be macromolecules having at least one negative charge in the molecule. Such macromolecules can include, but are not limited to, proteins, polypeptides and nucleic acids, such as RNA and DNA.

Methods of forming liposomes can include, but are not limited to, sonication, extrusion, extended vortexing, reverse evaporation, and homogenization, which can include microfulidization. Additional methods of forming liposomes are well known in the art.

Sonication can produce small, unilamellar vesicles (SUV) with diameters in the range of 15-50 nm. Bath sonicators can be instrumentation used for preparation of SUV (Avanti Polar Lipids, Inc., 700 Industrial Park Drive, Alabaster, Ala. 35007). Sonication can be accomplished by placing a test tube containing the suspension in a bath sonicator (or placing the tip of a sonicator in the test tube) and sonicating for 5-10 minutes above the gel-liquid crystal transition temperature of the lipid. Mean size and uniformity can be influenced by lipid composition and concentration, temperature, sonication time, power, volume, and sonicator tuning. Reverse evaporation can be used to form larger liposome vesicles (>1000 nm) known as giant unilamellar vesicles (GUV's).

Another method of forming liposomal compositions can be extrusion. Lipid extrusion can be a technique in which a lipid suspension is forced through a polycarbonate filter with a defined pore size to yield particles having a diameter near the pore size of the filter used. Extrusion through filters with pores having an approximately 100 nm diameter typically can yield large, unilamellar vesicles (LUV) with a mean diameter of 120 nm-140 nm. Mean particle size can also depend on lipid composition and can be reproducible from batch to batch.

In some embodimente, the formed liposomes can be approximately 120 nm to 800 nm in diameter.

In some embodiments, the composition can further include a nucleic acid. The nucleic acid can be deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). The nucleic acid can include a chemically modified nucleic acid. Chemical modifications can include

methylation, acetylation, oxidation, intercalation, thymine dimerization, PEGylation or phosphorylation. The chemical modifications can include using, for example, a phosphorothioate, methyl phosphonate or phosphoramidate linkage at the internucleotide phosphodiester bridge. Additionally, the chemical modifications can include a modification of the nucleotide base, for example, 5-propynyl-pyrimidine, or of the sugar, for example, 2'modified sugars.

The nucleic acid can be 10 to 50 nucleotides long. The the nucleic acid can be an oligonucleotide. The oligonucleotide can be 10 to 50 nucleotides long. The oligonucleotide can be double stranded or single stranded. More particularly, in some embodiments, the nucleic acid can be siRNA or mRNA. The siRNA can be single stranded or double stranded. In other embodiments, the nucleic acid can be a shRNA, an antisense nucleic acid, a microRNA, an antimicro RNA, an antagomir, a microRNA inhibitor or an immune stimulatory nucleic acid.

In some embodiments, the sample cells can be in suspension. In some circumstances, the volume of the sample cells in suspension can be at least 0.050 L, at least 0.1 L, at least 0.5 L, at least 1L, at least 3 L, at least 5 L, at least 10 L, at least 25 L, at least 40 L or more than 40 L. The suspension can be cultured in a bioreactor, a flask, a tube or a tank.

The suspension can be cultured with or without serum.

In some embodiments, a method for delivering a nucleic acid to sample cells can further include culturing untreated control cells that have not been exposed to the composition. In other words, a culture of cells is divided into at least two groups of cells including the sample cells and the untreated control cells. The sample cells are exposed to the composition. The untreated control cells are not exposed to the composition and provide a negative control to compare sample cell results against. The untreated control cells can indicate results that are independent of treatment with the composition.

In some embodiments, a cell density of the sample cells can increase after the sample cells have been exposed to the composition. The cell density can be greater than $0.1x10^{-6}$ cells/mL, greater than $0.5x10^{-6}$ cells/mL, greater than $1.0x10^{-6}$ cells/mL, greater than $1.5x10^{-6}$ cells/mL, greater than $2.0x10^{-6}$ cells/mL, greater than $2.5x10^{-6}$ cells/mL, greater than $3.0x10^{-6}$ cells/mL or greater than $3.5x10^{-6}$ cells/mL. The cell density can increase by greater than $0.1x10^{-6}$ cells/mL per day, greater than $0.5x10^{-6}$ cells/mL per day, greater than $1.0x10^{-6}$ cells/mL per day, greater than $1.5x10^{-6}$ cells/mL per day, greater

than $2.0x10^{-6}$ cells/mL per day or greater than $2.5x10^{-6}$ cells/mL per day. In some circumstances, the cell density of the sample cells can increase exponentially for a period of time after the sample cells have been exposed to the composition.

In some embodiments, the cell density of the sample cells can be greater than or equal to the cell density of the untreated control cells. The cell density measurement can be taken one day, two days, three days, four days, five days, six days, 1 week or greater than 1 week after the sample cells have been exposed to the composition.

In some embodiments, the sample cell viability can be greater than 75%, greater than 80%, greater than 85%, greater than 90% or greater than 95%. The sample cell viability can be measured one day, two days, three days, four days, five days or greater than five days after the sample cells have been exposed to the composition.

In some embodiments, a method for delivering a nucleic acid to sample cells can further include measuring a level of a protein in the sample cells and untreated control cells, the protein can be produced from an mRNA that an siRNA delivered into the sample cells is directed against.

The mRNA that an siRNA molecule is directed against can be determined by the sequence of the siRNA. The siRNA sequence can be complementary to the sequence of its target mRNA. Therefore, when the siRNA is incorporated into the RISC complex, the RISC complex can bind to the mRNA with the sequence complementary to the siRNA and the RISC complex can cleave the mRNA. This decreases the level of that mRNA in the cell, and consequently, it can decrease the level of protein translated from that mRNA.

An siRNA can target RNA other than an mRNA. An siRNA can have a sequence that is directed against more than one mRNA, thereby affecting the levels of more than one mRNA and more than one protein.

Measuring a level of a protein can include measuring the quantity of the protein, measuring an activity of the protein or measuring a downstream effect of the protein. The downstream effect can include activation of another molecule, modification of another molecule or the presence or absence of another molecule. Measuring a level of the protein can be accomplished using techniques well known in the art. Techniques for measuring the quantity of a protein can include an ultraviolet absorption assay, for example 260nm and 280nm absorbance reading, a Bradford assays, a Lowry, a Biuret assay, a bicinchoninic assay, or a quantitative Western blot. Techniques for measuring

the activity of a protein can include a SDS-Page, a Western blot, a BIAcore assay or an enzyme-linked immunosorbent assay (ELISA).

In some embodiments, the protein level in the sample cells can be less than the protein level in the untreated control cells. The protein level in the sample cells can be less than 40%, less than 50%, less than 60%, less than 70%, less than 75% or less than 80% of the protein level in the untreated control cells. The protein level in the sample cells and the protein level in the untreated control cells can be measured one day, two days, three days, four days, five days, six days, 1 week or greater than 1 week after the sample cells have been exposed to the composition. The protein level in the sample cells and the protein level in the untreated control cells can be measured one doubling time, two doubling times, three doubling times, four doubling times, five doubling times or greater than five doubling times after the sample cells have been exposed to the composition. A doubling time can be the period of time required for the quantity of cells to double. For example, if it takes one cell 24 hours to grow and divide to two cells, the doubling time is 24 hours.

In some embodiments, the cell density of the sample cells can increase after the sample cells have been exposed to the composition. The cell density can be greater than $0.1x10^{-6}$ cells/mL, greater than $0.5x10^{-6}$ cells/mL, greater than $1.0x10^{-6}$ cells/mL, greater than $1.5x10^{-6}$ cells/mL, greater than $2.0x10^{-6}$ cells/mL, greater than $2.5x10^{-6}$ cells/mL, greater than $3.0x10^{-6}$ cells/mL or greater than $3.5x10^{-6}$ cells/mL. The cell density can increase by greater than $0.1x10^{-6}$ cells/mL per day, greater than $0.5x10^{-6}$ cells/mL per day, greater than $1.0x10^{-6}$ cells/mL per day, greater than $1.5x10^{-6}$ cells/mL per day, greater than $2.0x10^{-6}$ cells/mL per day or greater than $2.5x10^{-6}$ cells/mL per day. In some circumstances, the cell density of the sample cells can increase exponentially for a period of time after the sample cells have been exposed to the composition. In some circumstances, the cell density of the sample cells can increase exponentially for a period of time after the sample cells have been exposed to the composition.

In some circumstances, the cell density of the sample cells can be greater than or equal to the cell density of the control cells. The cell density measurement can be taken one day, two days, three days, four days, five days, six days, 1 week or greater than 1 week after the sample cells have been exposed to the composition.

In some embodiments, the sample cell viability can be greater than 75%, greater than 80%, greater than 85%, greater than 90% or greater than 95%. The sample cell

viability can be measured one day, two days, three days, four days, five days or greater than five days after the sample cells have been exposed to the composition.

In some embodiments, a method for delivering a nucleic acid to sample cells can further include measuring a level of a protein in the sample cells and the untreated control cells, where the nucleic acid can be an siRNA and the protein can be produced from an mRNA that the siRNA is directed against.

The mRNA that an siRNA molecule is directed against can be determined by the sequence of the siRNA. The siRNA sequence can be complementary to the sequence of its target mRNA. Therefore, when the siRNA is incorporated into the RISC complex, the RISC complex can bind to the mRNA with the sequence complementary to the siRNA and the RISC complex can cleave the mRNA. This decreases the level of that mRNA in the cell, and consequently, it can decrease the level of protein translated from that mRNA.

An siRNA can target RNA other than an mRNA. An siRNA can have a sequence that is directed against more than one mRNA, thereby affecting the levels of more than one mRNA and more than one protein.

Measuring a level of a protein can include measuring the quantity of the protein, measuring an activity of the protein or measuring a downstream effect of the protein. The downstream effect can include activation of another molecule, modification of another molecule or the presence or absence of another molecule. Measuring a level of the protein can be accomplished using techniques well known in the art. Techniques for measuring the quantity of a protein can include an ultraviolet absorption assay, for example 260nm and 280nm absorbance reading, a Bradford assays, a Lowry, a Biuret assay, a bicinchoninic assay, or a quantitative Western blot. Techniques for measuring the activity of a protein can include a SDS-Page, a Western blot, a BIAcore assay or an enzyme-linked immunosorbent assay (ELISA).

In some embodiments, the protein level in the sample cells can be less than the protein level in the control cells. The protein level in the sample cells can be less than 40%, less than 50%, less than 60%, less than 70%, less than 75% or less than 80% of the protein level in the control cells. The protein level in the sample cells and the protein level in the control cells can be measured one day, two days, three days, four days, five days, six days, 1 week or greater than 1 week after the sample cells have been exposed to the composition. The protein level in the sample cells and the protein level in the control cells can be measured one doubling time, two doubling times, three doubling times, four

doubling times, five doubling times or greater than five doubling times after the sample cells have been exposed to the composition. A doubling time can be the period of time required for the quantity of cells to double. For example, if it takes one cell 24 hours to grow and divide to two cells, the doubling time is 24 hours.

The transfection methods can be applied to *in vitro* and *in vivo* transfection of cells, particularly to transfection of eukaryotic cells including animal cells. The methods can be used to generate transfected cells which express useful gene products. The methods can also be employed as a step in the production of transgenic animals. The methods are useful as a step in any therapeutic method requiring introducing of nucleic acids into cells. In particular, these methods are useful in cancer treatment, in in vivo and ex vivo gene therapy, and in diagnostic methods. The transfection compositions can be employed as research reagents in any transfection of cells done for research purposes. Nucleic acids that can be transfected by the methods of include DNA and RNA from any source comprising natural bases or non-natural bases, and include those encoding and capable of expressing therapeutic or otherwise useful proteins in cells, those which inhibit undesired expression of nucleic acids in cells, those which inhibit undesired enzymatic activity or activate desired enzymes, those which catalyze reactions (Ribozymes), and those which function in diagnostic assays.

The reagents and methods provided herein can are also readily adapted to introduce biologically active anionic macromolecules other than nucleic acids including, among others, polyamines, polyamine acids, polypeptides, proteins, biotin, and polysaccharides into cells. Other materials useful, for example as therapeutic agents, diagnostic materials and research reagents, can be complexed by the polycharged lipid aggregates and delivered into cells by the methods of this invention.

The methods and materials are useful in the development and practice of cell based assays and in the screening of libraries of molecules by cell based assays. In such assays, one or more cells are contacted with a test compound after the macromolecule, particularly an expression vector, is introduced into the one or more cells. Preferably, the one or more cells are contacted with the test compound for a selected time, for example within 5 days, after the macromolecule is introduced into the one or more cells.

LIPID PARTICLES

The present invention also provides lipid particles comprising one or more of the charged lipids described above. A complex of nucleic acid and lipid particles can be referred to as an association complex. An association complex of nucleic acid and lipid particle may be a liposome, a nanoparticle, an ion pair, a lipoplex, or a combination thereof. Lipoplexes are composed of charged lipid bilayers sandwiched between DNA layers, as described, *e.g.*, in Felgner, *Scientific American.* 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. The invention contemplates both single-layered liposomes, which are referred to as unilamellar, and multi-layered liposomes, which are referred to as multilamellar.

The lipid particles of the present invention may further comprise one or more additional lipids and/or other components such as cholesterol. Other lipids may be included in the liposome compositions of the present invention 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 of the present invention, 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 of the present invention include bilayer stabilizing components such as polyamide oligomers (*see, e.g.*, U.S. Patent No. 6,320,017), peptides, proteins, detergents, lipid-derivatives, such as PEG coupled to phosphatidylethanolamine and PEG conjugated to ceramides (*see*, U.S. Patent No. 5,885,613).

In particular embodiments, the lipid particles include one or more of a second amino lipid or charged lipid, a neutral lipid, and a sterol.

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 phosphocholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), cardiolipins, 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 in the present invention are DOPE, DSPC, POPC, DPPC or any related phosphatidylcholine. The neutral lipids useful in the present invention 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 protonatable 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 of the present invention. Such protonatable 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 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).

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 of the present invention. "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.

In some cases, the lipid particle can include 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.

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). Other compounds with uncharged, hydrophilic, steric-barrier moieties, which prevent aggregation during formulation, like PEG, Gm1 or ATTA, can also be coupled to lipids for use as in the methods and compositions of the invention. 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. 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 are useful in the present invention can have a variety of "anchoring" lipid portions to secure the PEG portion to the surface of the lipid vesicle. Examples of suitable PEG-modified lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-ceramide conjugates (*e.g.,* PEG-CerC14 or PEG-CerC20) which are described in co-pending USSN 08/486,214, 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 mins. in some assays. As illustrated in U.S. Pat. Application SN 08/486,214, at least three characteristics influence the rate of exchange: length of acyl chain, saturation of acyl chain, and size of the steric-barrier head group. Compounds having suitable variations of these features may be useful for the invention. 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.

Also suitable for inclusion in the lipid particles of the present invention are programmable fusion lipids. 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. In the latter case, 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 is 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 of this invention 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). 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 one exemplary embodiment, the lipid particle comprises a mixture of a charged lipid of the present invention, one or more different neutral lipids, and a sterol (*e.g.*, cholesterol). In certain embodiments, the lipid mixture consists of or consists essentially of a charged lipid as described herein, a neutral lipid, and cholesterol. In further preferred embodiments, the lipid particle consists of or consists essentially of the above lipid mixture in molar ratios of about 50-90% charged lipid, 0-50% neutral lipid, and 0-10% cholesterol. In certain embodiments, the lipid particle can further include a PEG-modified lipid (*e.g.*, a PEG-DMG or PEG-DMA).

In one embodiment, the lipid particle consists of a charged lipid (e.g., a quaternary nitrogen containing lipid) and a protonatable lipid, a neutral lipid or a steroid, or a combination thereof. The particles can be formulated with a nucleic acid therapeutic agent so as to attain a desired N/P ratio.The N/P ratio is the ratio of number of molar equivalent of cationic nitrogen (N) atoms present in the lipid particle to the number of molar equivalent of anionic phosphate (P) of the nucleic acid backbone. For example, the N/P ratio can be in the range of about 1 to about 50. In one example, the range is about 1 to about 20, about 1 to about 10, about 1 to about 5.

In particular embodiments, the lipid particle consists of or consists essentially of a charged lipid, DOPE, and cholesterol. In particular embodiments, the particle includes lipids in the following mole percentages: charged lipid, 45-63 mol %; DOPE, 35-55 mol %; and cholesterol, 0-10 mol %. The particles can be formulated with a nucleic acid therapeutic agent so as to attain a desired N/P ratio. The N/P ratio is the ratio of number of moles cationic nitrogen (N) atoms (i.e., charged lipids) to the number of molar equivalents of anionic phosphate (P) backbone groups of the nucleic acid. For example,

the N to P ratio can be in the range of about 5:1 to about 1:1. In certain embodiments, the charged lipid is chosen from compound 205, 201, or 204 (see Scheme 1 below).

In another group of embodiments, the neutral lipid, DOPE, in these compositions is replaced with POPC, DPPC, DPSC or SM.

Therapeutic agent-Lipid Particle Compositions and Formulations

The present invention includes compositions comprising a lipid particle of the present invention and an active agent, wherein the active agent is associated with the lipid particle. 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 according to the invention 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 according to the invention are ellipticin and ellipticin analogs or derivatives, epothilones, intracellular kinase inhibitors and camptothecins.

Nucleic Acid-Lipid Particles

In certain embodiments, lipid particles of the present invention 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 of the present invention are 15-50 nucleotides in length.

In the context of this invention, the terms "polynucleotide" and "oligonucleotide" refer to a polymer or oligomer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The terms "polynucleotide" and "oligonucleotide" also 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 according to this invention includes any form of nucleic acid that is known. The nucleic acids used herein can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids. Examples of double-stranded DNA 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, siRNA, antimicroRNA, antagomirs, microRNA inhibitor, supermirs, and triplex-forming oligonucleotides. The nucleic acid that is present in a lipid-nucleic acid particle of this invention may include one or more of the oligonucleotide modifications described below.

Nucleic acids of the present invention may be of various lengths, generally dependent upon the particular form of nucleic acid. For example, in particular embodiments, plasmids or genes may be from about 1,000 to 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) of the present invention 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 of the present invention 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).

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 invention includes the use of RNAi molecules comprising any of these different types of double-stranded molecules. 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 within the invention.

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 by through making the 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 embodiment, 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, the invention can be utilized in instances where non-target specific-activities are found with antisense, or where an antisense sequence containing one or more mismatches with the target sequence is the most preferred for a particular use.

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

protein synthesis by a targeted gene. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U. S. Patent 5,739,119 and U. S. Patent 5,759,829). 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). 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).

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 Ser. Nos. 11/502,158 and 11/657,341 (the disclosure of each of which are incorporated herein by reference).

An antagomir can include ligand-conjugated monomer subunits and monomers for oligonucleotide synthesis. Exemplary monomers are described in U.S. Application No. 10/916,185, filed on August 10, 2004. An antagomir can have a ZXY structure, such as is described in PCT Application No. PCT/US2004/07070 filed on March 8, 2004. An antagomir can be complexed with an amphipathic moiety. Exemplary amphipathic moieties for use with oligonucleotide agents are described in PCT Application No. PCT/US2004/07070, filed on March 8, 2004.

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)). 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). 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 of the invention, 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 U S A. 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 □ 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 U S A. 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 according to the invention are that they have a specific substrate binding site which is complementary to one or more of the target gene DNA or RNA regions, and that they have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

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. No. WO 93/23569 and Int. Pat. Appl. Publ. No. 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. No. WO 92/07065; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. 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 of the present invention may be immunostimulatory, including immunostimulatory oligonucleotides (ISS; single-or double-stranded) capable of inducing an immune response when administered to a subject, which may be a mammal 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), or CpG motifs, as well as other known ISS features (such as multi-G domains, see WO 96/11266).

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." According to the present invention, 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' TCCATGA**C**GTTCCTGA**C**GT 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 3. 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 NAME	SEQ ID	ODN SEQUENCE (5'-3') .
ODN 22 murine Phosphokinase C - alpha		5'-CAG CCA TGG TTC CCC CCA AC-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 oligonucleotides (ODNs) suitable for use in the compositions and methods of the invention are described in Raney *et al.*, Journal of Pharmacology and Experimental Therapeutics, 298:1185-1192 (2001). 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. An supermir featured in the invention 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 MoI 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 of the invention 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, $O(CH_2)_n$AMINE, (*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 used in accordance with this invention 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

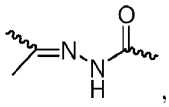
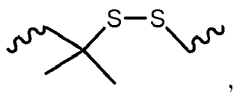
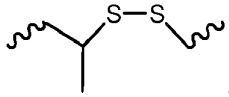
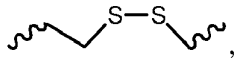
N-2 substitued purine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,459,255. 3-Deaza purine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,457,191. 5,6-Substituted pyrimidine nucleoside amidites can be prepared as is described in U.S. Pat. No. 5,614,617.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

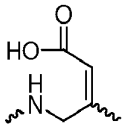
Linkers

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 $-[(P\text{-}Q\text{-}R)_q\text{-}X\text{-}(P'\text{-}Q'\text{-}R')_{q'}]_{q''}\text{-}T\text{-}$, 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 ,  ,  ,  ,  ,

 or 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 charged 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 charged 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 – $NHCHR^ACO)NHCHR^BC(O)$-, 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 of the present invention. 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 of the present invention are targeting ligands.

Some ligands can have endosomolytic properties. The endosomolytic ligands promote the lysis of the endosome and/or transport of the composition of the invention, 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 of the invention, 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 4.

Table 4: 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	GLFGALAEALAEALAEHLAEALAEALEALAAGGSC	6
GALA-INF3	GLFEAIEGFIENGWEGLAEALAEALEALAAGGSC	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, charged 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 5 shows some examples of targeting ligands and their associated receptors.

Table 5: Targeting Ligands and their associated receptors

Liver Cells	Ligand	Receptor
1) Parenchymal Cell (PC) (Hepatocytes)	Galactose	ASGP-R (Asiologlycoprotein receptor)
	Gal NAc (n-acetyl-galactosamine)	ASPG-R Gal NAc Receptor
	Lactose	
	Asialofetuin	ASPG-r
2) 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
	Sugar-Albumin conjugates	
	Mannose-6-phosphate	Mannose-6-phosphate receptor
3) 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 6, for example).

Table 6. 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	GALFLGWLGAAGSTMGAWSQPKKKR KV	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	LLGDFFRKSKEKIGKEFKRIVQRIKDFL RNLVPRTES	
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQG GPR	
α-defensin	ACYCRIPACIAGERRYGTCIYQGRLWA FCC	
b-defensin	DHYNCVSSGGQCLYSACPIFTKIQGTC YRGKAKCCK	
Bactenecin	RKCRIVVIRVCR	
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGF PPRFPPRFPGKR-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 to the present invention as ligands (e.g. as PK modulating ligands).

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

Other ligands amenable to the invention are described in copending applications USSN: 10/916,185, filed August 10, 2004; USSN: 10/946,873, filed September 21, 2004; USSN: 10/833,934, filed August 3, 2007; USSN: 11/115,989 filed April 27, 2005 and USSN: 11/944,227 filed November 21, 2007, 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. Generally, 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; 6,559, 279; each of which is herein incorporated by reference.

Characteristic of Nucleic Acid-Lipid Particles

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

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

The concentration of lipid 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) particels of the invention 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.

The present invention also provides lipid-therapeutic agent compositions 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 of the present invention, 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 of the invention make use of certain charged lipids, the synthesis, preparation and characterization of which is described below and in the accompanying Examples. In addition, the present invention provides methods of

preparing lipid particles, including those associated with a therapeutic agent, *e.g.*, a nucleic acid. 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 protonatable 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 protonatable lipids are termed titratable cationic lipids and can be used in the formulations of the invention 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, the present invention provides methods of preparing lipid/nucleic acid formulations. In the methods described herein, a mixture of lipids is combined with a buffered aqueous solution of nucleic acid to produce an intermediate mixture containing nucleic acid encapsulated in lipid particles, *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 of the present invention that is selected from among lipids which have a pKa such that the lipid is cationic at pH below the pKa and neutral at pH above the pKa, and a second lipid component that is selected from among lipids that prevent particle aggregation during lipid-nucleic acid particle formation. In particular embodiments, the amino lipid is a novel charged lipid of the present invention.

In preparing the nucleic acid-lipid particles of the invention, 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 charged lipids, neutral lipids (other than a charged 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 charged 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% charged 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 lipid chosen from Table 1 or Table 2, DSPC, Chol and PEG-DMG or PEG-DMA, more preferably in a molar ratio of about 20-60% charged 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% charged lipid/DSPC/Chol/PEG-DMG or PEG-DMA), 35/15/40/10 (mol% charged lipid/DSPC/Chol/PEG-DMG or PEG-DMA) or 52/13/30/5 (mol% charged 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.

In accordance with the invention, 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). 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 of the present invention 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 pKa 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 pKa 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 of the present invention 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 a nucleic acid-lipid particles of the present invention. While the following description o various methodsof using the lipid particles and related pharmaceutical compositions of the present invention 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, the present invention provides methods for introducing a nucleic acid into a cell. 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 of the present invention with the cells for a period of time sufficient for intracellular delivery to occur.

The compositions of the present invention 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 with respect to the scope of the invention, 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 of the invention 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 of the present invention include introduction of antisense oligonucleotides in cells (see, Bennett, *et al., Mol. Pharm.* 41:1023-1033 (1992)).

Alternatively, the compositions of the present invention can also be used for 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 of the invention can be used in the treatment of infectious diseases.

For *in vivo* administration, the pharmaceutical compositions are preferably administered parenterally, *i.e.*, intraarticularly, intravenously, intraperitoneally, subcutaneously, or intramuscularly. 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 of the present invention may be practiced in a variety of hosts. Preferred hosts include mammalian species, such as humans, non-human primates, dogs, cats, cattle, horses, sheep, and the like.

Dosages for the lipid-therapeutic agent particles of the present invention 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, the present invention provides a method of modulating the expression of a target polynucleotide or polypeptide. These methods generally comprise contacting a cell with a lipid particle of the present invention 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, the present invention provides a method of modulating the expression of a polypeptide by a cell, comprising providing to a cell a

lipid particle that consists of or consists essentially of a lipid chosen from Table 1 or Table 2, DSPC, Chol and PEG-DMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% charged 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 approximately 40/10/40/10 (mol% charged lipid/DSPC/Chol/PEG-DMG or PEG-DMA), 35/15/40/10 (mol% charged lipid/DSPC/Chol/PEG-DMG or PEG-DMA) or 52/13/30/5 (mol% charged 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 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, the present invention provides a method of treating a disease or disorder characterized by overexpression of a polypeptide in a subject, comprising providing to the subject a pharmaceutical composition of the present invention, 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 lipid chosen from Table 1 or Table 2, DSPC, Chol and PEG-DMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% charged 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% charged lipid/DSPC/Chol/PEG-DMG or PEG-

DMA), 35/15/40/10 (mol% charged lipid/DSPC/Chol/PEG-DMG or PEG-DMA) or 52/13/30/5 (mol% charged 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, the present invention includes a method of treating a disease or disorder characterized by underexpression of a polypeptide in a subject, comprising providing to the subject a pharmaceutical composition of the present invention, 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 lipid chosen from Table 1 or Table 2, DSPC, Chol and PEG-DMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% charged 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% charged lipid/DSPC/Chol/PEG-DMG or PEG-DMA), 35/15/40/10 (mol% charged lipid/DSPC/Chol/PEG-DMG or PEG-DMA) or 52/13/30/5 (mol% charged 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.

The present invention further provides a method of inducing an immune response in a subject, comprising providing to the subject the pharmaceutical composition of the present invention, 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 a lipid chosen from Table 1 or Table 2, DSPC, Chol and PEG-DMG or PEG-DMA, *e.g.*, in a molar ratio of about 20-60% charged 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% charged lipid/DSPC/Chol/PEG-DMG or PEG-DMA), 35/15/40/10 (mol% charged lipid/DSPC/Chol/PEG-DMG or PEG-DMA) or 52/13/30/5 (mol% charged 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 further embodiments, the pharmaceutical composition is provided to the subject in combination with a vaccine or antigen. Thus, the present invention itself provides vaccines comprising a lipid particle of the present invention, 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 antigens suitable for use in the present invention 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 in the present invention.

Tumor-associated antigens suitable for use in the subject invention 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 of the invention 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, MTAI 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

"Alkyl" means a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms. Representative saturated straight chain alkyls include methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, and the like; while saturated branched alkyls include isopropyl, *sec*-butyl, isobutyl, *tert*-butyl, isopentyl, and the like. Representative saturated cyclic alkyls include cyclopropyl,

cyclobutyl, cyclopentyl, cyclohexyl, and the like; while unsaturated cyclic alkyls include cyclopentenyl and cyclohexenyl, and the like.

"Alkenyl" means an alkyl, as defined above, containing at least one double bond between adjacent carbon atoms. Alkenyls include both cis and trans isomers. Representative straight chain and branched alkenyls include ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 2,3-dimethyl-2-butenyl, and the like.

"Alkynyl" means any alkyl or alkenyl, as defined above, which additionally contains at least one triple bond between adjacent carbons. Representative straight chain and branched alkynyls include acetylenyl, propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, 2-pentynyl, 3-methyl-1 butynyl, and the like.

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 includes groups such as (C1-C20)alkanoyl (e.g., formyl, acetyl, propionyl, butyryl, valeryl, caproyl, t- butylacetyl, etc.), (C3-C20)cycloalkylcarbonyl (e.g., cyclopropylcarbonyl, cyclobutylcarbonyl, cyclopentylcarbonyl, cyclohexylcarbonyl, etc.), heterocyclic carbonyl (e.g., pyrrolidinylcarbonyl, pyrrolid-2-one-5 -carbonyl, piperidinylcarbonyl, piperazinylcarbonyl, tetrahydrofuranylcarbonyl, etc.), aroyl (e.g., benzoyl) and heteroaroyl (e.g., thiophenyl-2-carbonyl, thiophenyl-3 -carbonyl, furanyl-2-carbonyl, furanyl-3 -carbonyl, lH-pyrroyl-2-carbonyl, lH-pyrroyl-3 -carbonyl, benzo[b]thiophenyl-2-carbonyl, etc.).

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

"Heterocycle" means a 5- to 7-membered monocyclic, or 7- to 10-membered bicyclic, heterocyclic ring which is either saturated, unsaturated, or aromatic, and which contains from 1 or 2 heteroatoms independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen heteroatom may be optionally quaternized, including bicyclic rings in which any of the above heterocycles are fused to a benzene ring. The heterocycle may be attached via any heteroatom or carbon atom. Heterocycles include heteroaryls as defined below. Heterocycles include morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl,

piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, and the like.

The term "heteroaryl" refers to an aromatic 5-8 membered monocyclic, 8-12 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, said heteroatoms 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), wherein any ring atom can be substituted. The heteroaryl groups herein described may also contain fused rings that share a common carbon-carbon bond. The term "alkylheterocyle" refers to a heteroaryl wherein at least one of the ring atoms is substituted with alkyl, alkenyl or alkynyl

The term "substituted" 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 aliphatic. It is understood that the substituent may be further substituted. Exemplary substituents include amino, alkylamino, dialkylamino, and cyclic amino compounds.

"Halogen" means 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 of the invention 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 within the context of this invention 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 of the present invention may be prepared by known organic synthesis techniques, including the methods described in more detail in the Examples.

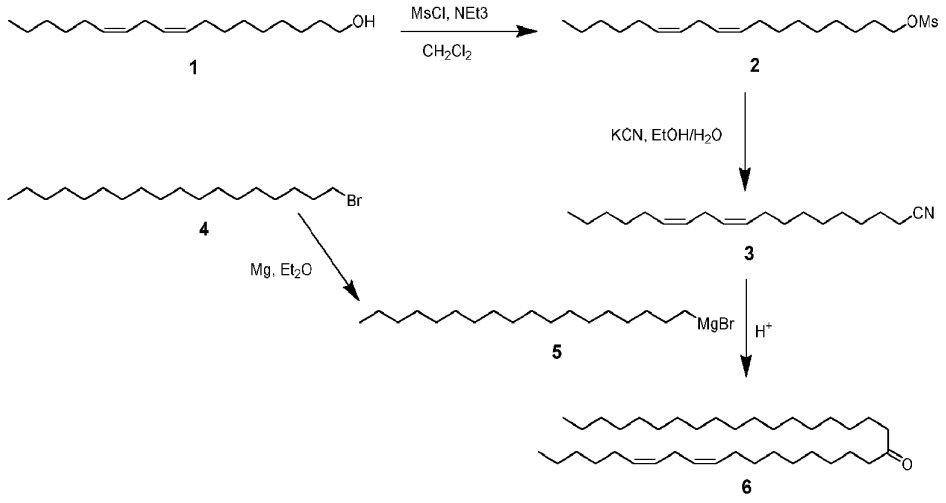
EXAMPLES

Materials/Methods

Synthesis of (3aR,5s,6aS)-N,N,N-trimethyl-2-((9Z,12Z)-octadeca-9,12-dienyl)-2-octadecyl tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-aminium chloride L8

The method for synthesizing (3aR,5s,6aS)-N,N,N-trimethyl-2-((9Z,12Z)-octadeca-9,12-dienyl)-2-octadecyl tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-aminium chloride (L8) is briefly described below.

Scheme 10. Synthesis of Linoleylstearyl ketone or (6Z,9Z)-heptatriaconta-6,9-dien-19-one (LinS-K, 6)



Synthesis of linoleyl mesylate or (9Z,12Z)-octadeca-9,12-dienyl methanesulfonate (2):

Referring to **Scheme 10**, triethylamine (13.13 g, 130 mmol) was added to a solution of the linoleyl alcohol **1** (26.6 g, 100 mmol) in dichloromethane (100 mL) and the solution was cooled in an ice-bath. To this cold solution, a solution of methanesulfonyl chloride (12.6 g, 110 mmol) in dichloromethane (60 mL) was added dropwise; after the completion of the addition, the reaction mixture was allowed to warm to ambient temperature and stirred overnight. Completion of the reaction was confirmed by TLC. The reaction mixture was diluted with dichloromethane (200 mL), washed with water (200 mL), satd. $NaHCO_3$ (200 mL), brine (100 mL) and dried over anhydrous Na_2SO_4. After evaporation of solvent *in vacuo* the crude product was purified by flash silica column chromatography using 0-10% Et_2O in hexane. The pure fractions were combined and concentrated to obtain the mesylate **2** as colorless oil (30.6 g, 89%). ^1H NMR (400 MHz, $CDCl_3$): δ = 5.42-5.21 (m, 4H), 4.20 (t, 2H), 3.06 (s, 3H), 2.79 (t, 2H), 2.19-2.00 (m, 4H), 1.90-1.70 (m, 2H), 1.06-1.18 (m, 18H), 0.88 (t, 3H). ^{13}C NMR ($CDCl_3$): δ = 130.76, 130.54, 128.6, 128.4, 70.67, 37.9, 32.05, 30.12, 29.87, 29.85, 29.68, 29.65, 29.53, 27.72, 27.71, 26.15, 25.94, 23.09, 14.60. MS. MW calc. for $C_{19}H_{36}O_3S$: 344.53; found: 343.52 [M-H].

Synthesis of (10Z,13Z)-10,13-Nonadecadienenitrile or Linoleyl-1-cyanide (3)

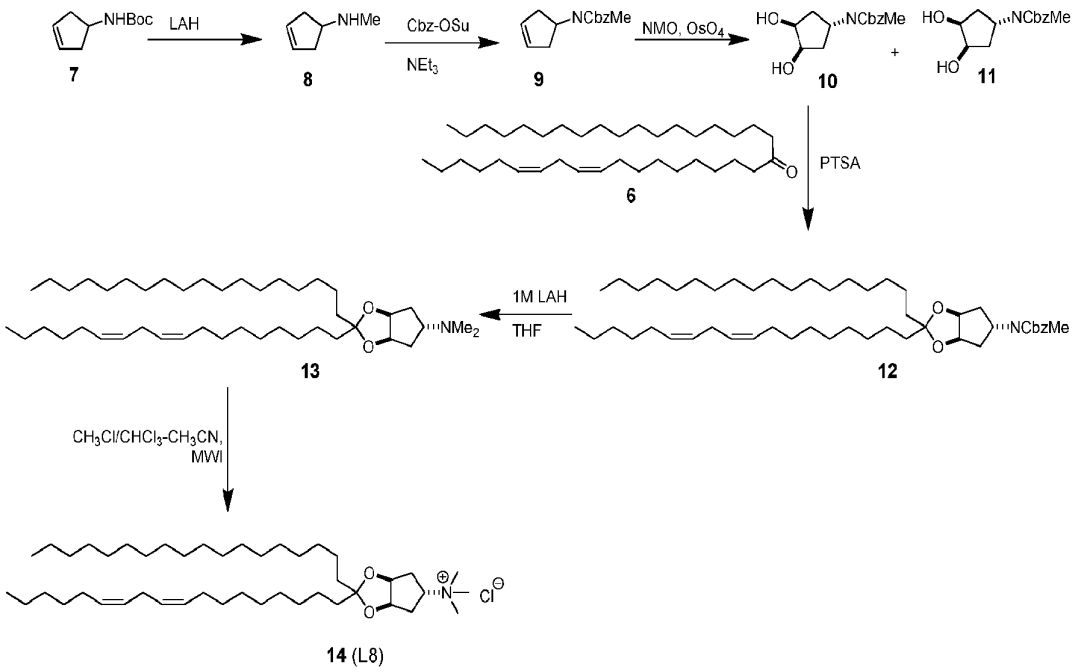
To a solution of the mesylate **2** (3.44 g, 10 mmol) in ethanol (90 mL), a solution of KCN (1.32 g, 20 mmol) in water (10 mL) was added and the mixture was refluxed for

30 minutes. After cooling, completion of reaction was confirmed by TLC. Water was added to the cooled reaction mixture and the product was extracted into ether (2 x 200 mL) followed by standard work-up. The crude product thus obtained was purified by column chromatography (0-10 % Et_2O in hexanes) to obtain compound **3** as colorless oil (2 g, 74%). ^1H NMR (400 MHz, $CDCl_3$): δ = 5.33-5.22 (m, 4H), 2.70 (t, 2H), 2.27-2.23 (m, 2H), 2.00-1.95 (m, 4H), 1.61-1.54 (m, 2H), 1.39-1.20 (m, 18H), 0.82 (t, 3H). ^{13}C NMR ($CDCl_3$): δ = 130.20, 129.96, 128.08, 127.87, 119.78, 70.76, 66.02, 32.52, 29.82, 29.57, 29.33, 29.24, 29.19, 29.12, 28.73, 28.65, 27.20, 27.16, 25.62, 25.37, 22.56, 17.10, 14.06. MS. MW calc. for $C_{19}H_{33}N$: 275.47; found: 276.6 [M+H].

Synthesis of (6Z,9Z)-heptatriaconta-6,9-dien-19-one (6):

Freshly activated Mg turnings (0.144 g, 6 mmol) were charged into a flame dried 500 mL 2NRB flask equipped with a magnetic stir bar and a reflux condenser. This set-up was degassed and flushed with argon and 10 mL of anhydrous ether was added to the flask via syringe. The commercially available stearyl bromide **26** (2.65 g, 5 mmol) dissolved in anhydrous ether (10 mL) was added dropwise via syringe to the flask. After completing the addition, the reaction mixture was kept at 35 °C for 1 hour in a warm water bath and then cooled over an ice bath. The cyanide **3** (1.38 g, 5 mmol) dissolved in anhydrous ether (20 mL) was added drop-wise into the reaction mixture with stirring. An exothermic reaction was observed and the mixture was allowed to stir overnight at ambient temperature. The reaction was quenched by drop-wise addition acetone (10 mL), followed by ice cold water (60 mL). The reaction mixture was subsequently treated with aq. H_2SO_4 (10 % by volume, 200 mL) until the solution became homogeneous and the layers were separated. The product was extracted into ether (2x100 mL), followed by standard work-up. The crude residue thus obtained was purified by column chromatography using 0-0.7% ether in hexane as eluting system to obtain a pure ketone **6** as a colorless oil. ^1H-NMR (400MHz , $CDCl_3$): δ = 5.42-5.30 (m,4H), 2.79-2.78 (t,2H), 2.40-2.37 (t,4H), 2.08-2.03 (m,4H),1.58-1.54 (m,4H), 1.36-1.26 (br m, aliphatic protons), 0.91-0.87 (t, 6H). IR (cm-1): 2924, 2854, 1716, 1465, 1375, 721.

Scheme 11. Synthesis of L8 (14)



Synthesis of N-methylcyclopent-3-enamine 8:

A solution of *t*-butyl 3-cyclopentenylcarbamate (**7**, 10g, 0.0492 mol) in anhydrous THF (70 mL) was added slowly into a stirred suspension of $LiAlH_4$ (3.74 g, 0.09852 mol) in THF (anhydrous, 200 mL) at 0 °C under nitrogen atmosphere. After completing the addition, the reaction mixture was warmed to room temperature and then heated to reflux for 4 hours. Progress of the reaction was monitored by TLC. After completion of reaction (by TLC), the mixture was cooled to 0 °C and quenched with careful addition of saturated Na_2SO_4 solution. Reaction mixture was stirred for 4 hours at room temperature and filtered off. Residue was washed well with THF. The filtrate and washings were mixed and diluted with 400 mL dioxane and 26 mL conc. HCl and stirred for 20 minutes at room temperature. The volatilities were removed *in vacuo* to furnish the hydrochloride salt of **8** as a white solid. Yield: 7.12 g. ^{1}H-NMR (400MHz, DMSO-d_6): δ= 9.34 (broad, 2H), 5.68 (s, 2H), 3.74 (m, 1H), 2.66-2.60 (m, 2H), 2.50-2.45 (m, 5H).

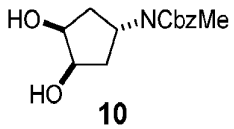
Synthesis of benzyl cyclopent-3-enyl(methyl)carbamate 9:

NEt_3 (37.2 mL, 0.2669 mol) was added to a stirred solution of compound **8** in 100 mL dry DCM in a 250 mL two neck RBF and cooled to 0 °C under nitrogen atmosphere. After a slow addition of N-(benzyloxy-carbonyloxy)-succinimide (20 g, 0.08007 mol) in 50 mL dry DCM, reaction mixture was allowed to warm to room temperature. After completion of the reaction (2-3 h by TLC) mixture was washed successively with 1N HCl

solution (1 x 100 mL) and saturated $NaHCO_3$ solution (1 x 50 mL) followed by standard work-up. The crude was then subjected to silica gel column chromatography to obtain **9** as a sticky mass. Yield: 11 g (89%). ^{1}H-NMR (400MHz, $CDCl_3$): δ = 7.36-7.27(m, 5H), 5.69 (s, 2H), 5.12 (s, 2H), 4.96 (br., 1H) 2.74 (s, 3H), 2.60(m, 2H), 2.30-2.25(m, 2H). MS. MW calc. for $C_{14}H_{17}NO_2$: 231.13; found: 232.3 [M+H].

Synthesis of benzyl (1S,3R,4S)-3,4-dihydroxycyclopentyl(methyl)carbamate (10):

The cyclopentene **9** (5 g, 0.02164 mol) was dissolved in a solution of 220 mL acetone and water (10:1) in a single neck 500 mL RBF and to it was added N-methyl morpholine-N-oxide (7.6 g, 0.06492 mol) followed by 4.2 mL of 7.6% solution of OsO_4 (0.275 g, 0.00108 mol) in *tert*-butanol at room temperature. After completion of the reaction (~ 3 hours), the mixture was quenched with addition of solid Na_2SO_3 and resulting mixture was stirred for 1.5 hours at room temperature. Reaction mixture was diluted with DCM (300 mL) and washed with water (2 x 100 mL) followed by saturated $NaHCO_3$ (1 x 50 mL) solution, water (1 x 30 mL) and finally with brine (1x 50 mL). Organic phase was dried over an Na_2SO_4 and solvent was removed *in vacuo*. Silica gel column chromatographic purification of the crude material afforded a mixture of diastereomers, which were separated by preparative HPLC (Mobile phase A: 0.05% trifluoroacetic acid, mobile phase B: 100% acetonitrile, 0.01 min-22 min 20 mL/min, 80:20 A:B; 22 min-46 min 16 mL/min, 80:20 A:B, 46 min 16 mL/min, 80:20 A:B). The process yielded 6 grams of crude benzyl (1S,3R,4S)-3,4-dihydroxycyclopentyl(methyl)carbamate (10), shown below.



10

Diol 10 - Peak-1:

White solid; 5.13 g (96%). ^{1}H-NMR (400MHz, DMSO-d_6): δ= 7.39-7.31(m, 5H), 5.04(s, 2H), 4.78-4.73 (m, 1H), 4.48-4.47(d, 2H), 3.94-3.93(m, 2H), 2.71(s, 3H), 1.72-1.67(m, 4H). LC-MS. MW calc. for $C_{14}H_{19}NO_4$: 265.13; found: 266.3 [M+H], [M+NH_4 +]-283.5 present, HPLC-97.86%. Stereochemistry was confirmed by X-ray.

Synthesis of Ketone 12:

A mixture of compound **10** (1.85 g, 7.8 mmol), ketone **6** (2.74 g, 5.2 mmol) and *p*-TSA (0.1 eq) was heated under toluene reflux with Dean-Stark apparatus for 3 hours.

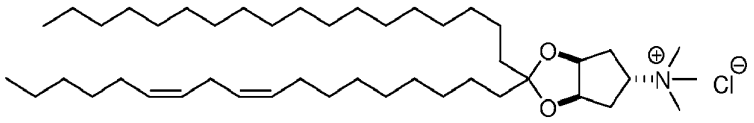
Removal of solvent *in vacuo* followed by column chromatography afforded compound **12** (3.6 g, 93 %) as a colorless oil. ^{1}H-NMR (400MHz, $CDCl_3$): δ = 7.35-7.33(m, 4H), 7.30-7.27(m, 1H), 5.37-5.27(m, 8H), 5.12(s, 2H), 4.75(m,1H), 4.58-4.57(m,2H), 2.78-2.74(m,7H), 2.06-2.00(m,8H), 1.96-1.91(m, 2H), 1.62(m, 4H), 1.48(m, 2H), 1.37-1.25(br m, 36H), 0.87(m, 6H). HPLC-ELSD: 98.65%.

Synthesis of amine 13:

A solution of compound **12** (2 g, 2.68 mmol) in hexane (20 mL) was added in a drop-wise fashion to an ice-cold solution of LAH in THF (1 M, 5.4 mL). After completing the addition, the mixture was heated at 40 °C over 0.5 hour then cooled again on an ice bath. The mixture was carefully hydrolyzed with saturated aqueous Na_2SO_4 then filtered through celite and solvent were removed *in vacuo* to obtain an oily residue. Silica gen column chromatography of the crude thus obtained gave pure amine **13** (1.32 g, 79%) as a colorless oil. ^{1}H-NMR (400MHz, $CDCl_3$): δ = 7.34-7.33 (m, 4H), 7.30-7.28 (m, 1H), 5.36-5.32 (m, 4H), 5.12 (s, 2H), 4.77-4.70 (m, 1H), 4.58-4.57 (m,2H), 2.78-2.75 (m,5H), 2.04-2.00 (m, 4H), 1.95-1.92 (m, 2H), 1.62 (m,4H), 1.56-1.53 (m,2H), 1.35-1.23 (br m, 40H), 0.87-0.84 (m, 6H). HPLC-ELSD: 98.34%.

Synthesis of (3aR,5s,6aS)-N,N,N-trimethyl-2-((9Z,12Z)-octadeca-9,12-dienyl)-2-octadecyltetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-aminium chloride, 14:

Compound **13** (500 mg, 0.778 mmol) was dissolved in chloroform-acetonitrile (1:1) mixture (5mL each) and bubbled with chloromethane (1.263g, 16.14 mmol) for 1.5 minutes at ice bath. The reaction mixture was then heated in a pressure bottle for 3 hours at 100°C. The TLC showed complete disappearance of the starting lipid **13**. Aqueous workup then silica gel column chromatography (0-20% CH_3OH/CH_2Cl_2) gave compound **14** (480 mg, 89%), shown below, as a white waxy solid.



14

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.40 – 5.26 (m, 4H), 4.70 (d, *J* = 3.2, 2H), 3.80 – 3.61 (m, 1H), 3.48 (s, 9H), 2.75 (s, 2H), 2.35 – 2.20 (m, 2H), 2.15 – 2.08 (m, 2H), 2.06 – 1.99 (m, 4H), 1.60 (d, *J* = 8.4, 2H), 1.46 (d, *J* = 7.2, 2H), 1.32 – 1.20 (m, 50H), 0.89 – 0.83 (m, 6H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 129.76, 129.74, 129.64, 129.60, 127.57, 127.52, 127.45, 112.77, 73.59, 52.03, 35.50, 34.25, 34.20, 32.91, 31.46, 31.06, 29.36,

29.28, 29.24, 29.20, 29.11, 29.06, 29.00, 28.90, 28.83, 26.76, 26.74, 25.18, 23.97, 23.13, 22.23, 22.11, 13.66, 13.62. MS (LC-MS) MW calc. for $C_{45}H_{86}NO_2^+$: 672.67; found 672.69.

Formulation of transfection reagents

Cationic lipid and colipids in chloroform were dried by a N_2 stream followed by vacuum-dessication to remove residual organic solvent. The dried lipid film was hydrated using 10 mM HEPES buffer, pH 7.4 at 37 °C. The formed liposomes were extruded to yield an average particle size of ~200 nm.

	L8	DOPE	CHOL	Total
Lipid excipients molar comp. (%)	48	47	5	100
Excipient MW	672	744	386.7	
Lipid weight (mg)	4.66	5.06	0.28	10.00
Lipid (wt %)	46.58	50.62	2.80	
Volume taken from the stock solution (µL)	186	203	11	400
Volume of buffer added to make final formulation (mL)				10.00

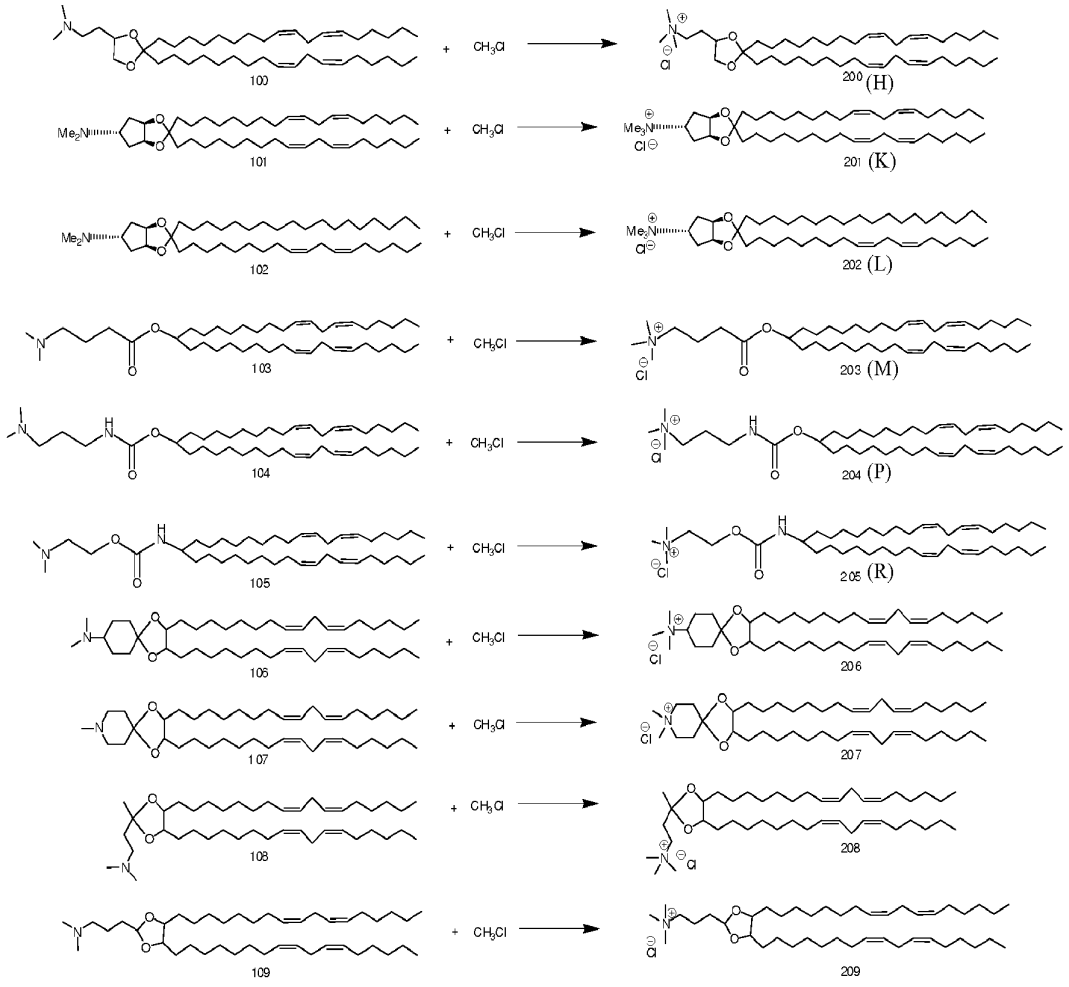
Excipient molar ratio: Cationic lipid (L8), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), Cholesterol – 48:47:5

Each lipid component was dissolved in chloroform to a working concentration (25 mg/mL). Next, the desired amount of each excipients was pipetted into a glass vial and thoroughly mixed. The chloroform was carefully evaporated using an argon stream at ambient temperature inside a fume hood to make a thin film inside. The lipid residue was placed on a vacuum pump for 10-15 minutes to remove any residual organic solvent.

The vial was removed from the vacuum pump and the residue immediately suspended in calculated volume of 10 mM HEPES at pH 7.4 to obtain a final lipid concentration of 1 mg/mL. The mixture was stirred in an agitator at 37 °C for 1 hr. The suspension was extruded (LIPEX™ Extruder) using polycarbonate filters (two 0.2 µm

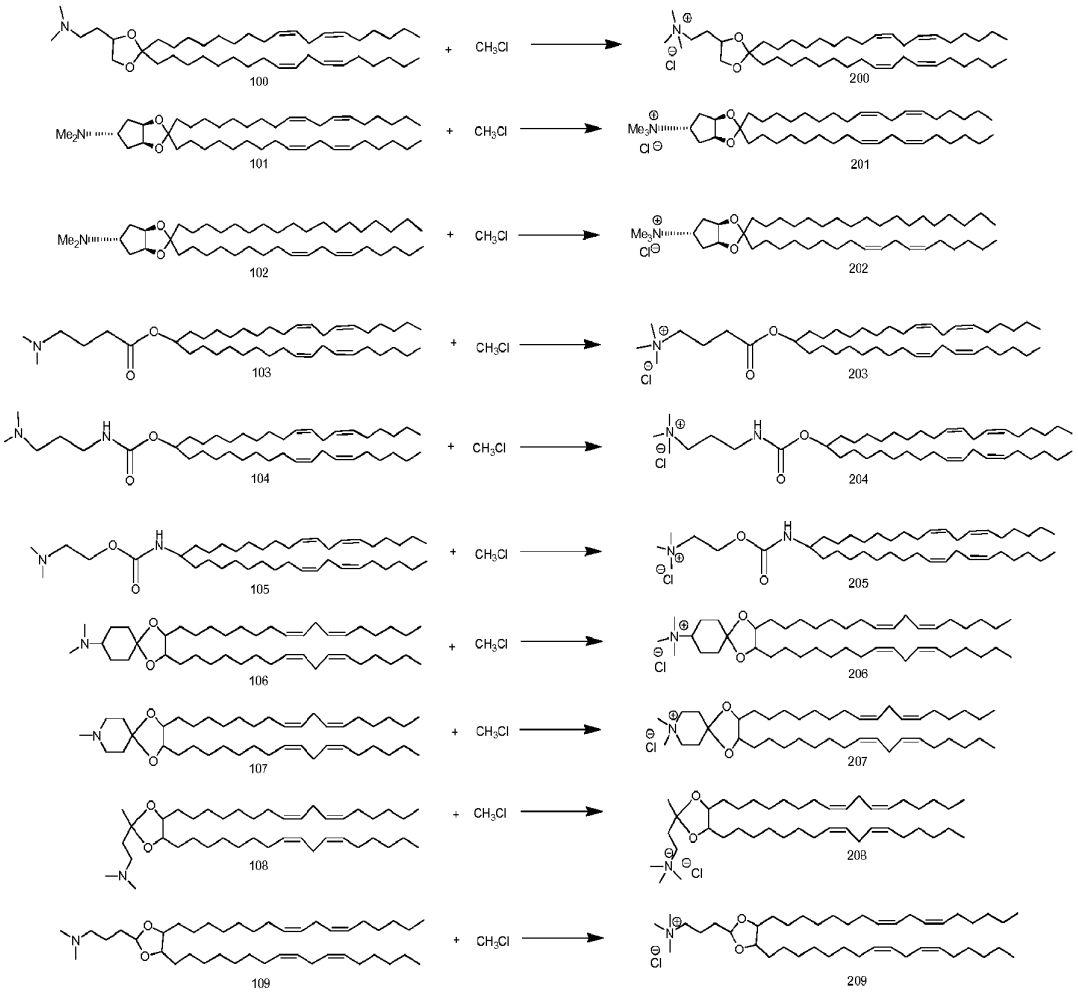
filters and one 0.4 μm prefilter, Whatman Nuclepore Track-Etch Membrane). The final formulation was filtered using a 0.45 μm sterile filter before use. Particle size was measured by dynamic light scattering. 10 μL of the final formulation was diluted to 1 mL in pre-filtered PBS buffer (0.02 micron filter) for measurement.

Scheme 12. Final synthetic step to produce the quartenary amine cationic lipids used to make transfection reagents.



Testing of transfection reagents on plated GFP-CHO cells

Scheme 1



Preparation of Compound 200

Compound 100 (500 mg, 0.778 mmol) was dissolved in chloroform:acetonitrile (1:1) mixture (5 mL each) and bubbled with chloromethane (1.263 g, 16.14 mmol) for 1.5 min while the reaction vessel was submerged in an ice bath. The reaction mixture was then subjected to microwave synthesis for 2 h at 100 °C with 250 W power supply and 247 psi pressure. Thin layer chromatography showed a complete reaction. Aqueous workup and column chromatography (0-20% CH_3OH/CH_2Cl_2) gave compound 200, 480 mg, 89%, as a white waxy solid. ^1H NMR (400 MHz, $CDCl_3$) δ 5.41 – 5.28 (m, 8H), 4.12 (t, J = 6.0, 2H), 3.95 (d, J = 4.7, 1H), 3.61 – 3.49 (m, 2H), 3.47 (s, 9H), 2.76 (t, J = 6.4, 4H), 2.03 (q, J = 6.7, 8H), 1.96 – 1.83 (m, 2H), 1.55 (dd, J = 14.0, 10.2, 4H), 1.37 – 1.22 (m, 36H), 0.87 (t, J = 6.8, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 130.50, 130.48, 130.42, 130.37, 128.30, 128.25, 128.21, 128.19, 113.52, 72.82, 69.64, 64.83, 53.75, 37.78, 37.12, 31.81, 30.18, 29.97, 29.94, 29.87, 29.82, 29.63, 29.59, 28.20, 27.52, 27.49, 25.92, 24.33,

24.16, 22.86, 14.36. MS (LC-MS) m/z : Calcd for $C_{44}H_{82}NO_2$, the ammonium ion, 100%) 656.10. Found 656.66.

Preparation of Compound 201

Compound 201 was prepared from compound 101, using the method same as for preparation of compound 200. Aqueous workup then column chromatography (0-20% CH_3OH/CH_2Cl_2) gave compound 201, 81%, as a white waxy solid. ^1H NMR (400 MHz, $CDCl_3$) δ 5.40 – 5.24 (m, 8H), 4.70 (d, *J* = 3.3, 2H), 3.77 – 3.67 (m, 1H), 3.47 (s, 9H), 2.75 (t, *J* = 6.4, 4H), 2.25 (dd, *J* = 13.1, 5.7, 2H), 2.12 (d, *J* = 11.6, 2H), 2.03 (dd, *J* = 13.6, 6.7, 8H), 1.60 (d, *J* = 8.2, 2H), 1.43 (dd, *J* = 24.3, 6.3, 2H), 1.39 – 1.16 (m, 36H), 0.87 (t, *J* = 6.7, 6H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 129.96, 129.94, 129.84, 129.80, 127.78, 127.73, 127.65, 127.64, 112.96, 73.80, 52.26, 35.72, 34.43, 33.10, 31.27, 29.57, 29.50, 29.43, 29.40, 29.32, 29.27, 29.21, 29.09, 29.03, 26.97, 26.95, 25.38, 24.19, 23.35, 22.31, 13.82. MS (LC-MS) m/z : Calcd for $C_{45}H_{82}NO_2$, the ammonium ion, 100%) 668.10. Found 668.66.

Preparation of Compound 202

Compound 202 was prepared from compound 102, using the method same as for preparation of compound 200. Aqueous workup then column chromatography (0-20% CH_3OH/CH_2Cl_2) gave compound 202, 75.4%, as a white waxy solid. ^1H NMR (400 MHz, $CDCl_3$) δ 5.40 – 5.26 (m, 4H), 4.70 (d, *J* = 3.2, 2H), 3.80 – 3.61 (m, 1H), 3.48 (s, 9H), 2.75 (s, 2H), 2.35 – 2.20 (m, 2H), 2.15 – 2.08 (m, 2H), 2.06 – 1.99 (m, 4H), 1.60 (d, *J* = 8.4, 2H), 1.46 (d, *J* = 7.2, 2H), 1.32 – 1.20 (m, 50H), 0.89 – 0.83 (m, 6H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 129.76, 129.74, 129.64, 129.60, 127.57, 127.52, 127.45, 112.77, 73.59, 52.03, 35.50, 34.25, 34.20, 32.91, 31.46, 31.06, 29.36, 29.28, 29.24, 29.20, 29.11, 29.06, 29.00, 28.90, 28.83, 26.76, 26.74, 25.18, 23.97, 23.13, 22.23, 22.11, 13.66, 13.62. MS (LC-MS) m/z : Calcd for $C_{45}H_{86}NO_2$, the ammonium ion, 100%) 672.13. Found 672.69.

Preparation of Compound 203

Compound 203 was prepared from compound 103, using the method same as for preparation of compound 200. Aqueous workup then column chromatography (0-20% CH_3OH/CH_2Cl_2) gave compound 203, 94%, as a white waxy solid. ^1H NMR (400 MHz, $CDCl_3$) δ 5.44 – 5.23 (m, 8H), 4.89 – 4.77 (m, 1H), 3.69 – 3.63 (m, 2H), 3.45 (s, 9H),

2.75 (t, J = 6.4, 4H), 2.46 (t, J = 6.6, 2H), 2.02 (dd, J = 13.8, 6.9, 10H), 1.48 (d, J = 5.8, 4H), 1.40 – 1.14 (m, 36H), 0.87 (t, J = 6.7, 6H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 171.55, 129.86, 129.76, 127.64, 127.57, 75.31, 65.29, 53.03, 33.64, 31.18, 29.58, 29.33, 29.20, 29.18, 29.00, 28.95, 26.89, 26.86, 25.29, 25.04, 22.22, 18.08, 13.73. MS (LC-MS) m/z : Calcd for $C_{44}H_{82}NO_2$, the ammonium ion, 100%) 656.1. Found 656.65.

Preparation of Compound 204

Compound 204 was prepared from compound 104, using the method same as for preparation of compound 200. Aqueous workup then column chromatography (0-20% CH_3OH/CH_2Cl_2) gave compound 204, 78.3%, as a white waxy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.78 (s, 1H), 5.40 – 5.27 (m, 8H), 4.68 – 4.63 (m, 1H), 3.72 (s, 2H), 3.40 (s, 9H), 3.31 – 3.22 (m, 2H), 2.75 (t, J = 6.4, 4H), 2.03 (dd, J = 13.7, 6.8, 10H), 1.49 – 1.38 (m, 4H), 1.36 – 1.11 (m, 36H), 0.87 (t, J = 6.7, 6H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 157.28, 130.18, 130.10, 127.95, 127.89, 75.28, 64.75, 53.50, 37.95, 34.31, 31.50, 29.67, 29.63, 29.57, 29.55, 29.32, 29.30, 27.22, 27.18, 25.61, 25.37, 23.86, 22.55, 14.06. MS (LC-MS) m/z : Calcd for $C_{44}H_{83}N_2O_2$, the ammonium ion, 100%) 671.1. Found 671.65.

Preparation of Compound 205

Compound 205 was prepared from compound 105, using the method same as for preparation of compound 200. Aqueous workup then column chromatography (0-20% CH_3OH/CH_2Cl_2) gave compound 205, 91%, as a white waxy solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.44 (d, J = 9.1, 1H), 5.40 – 5.26 (m, 8H), 4.51 (s, 2H), 4.02 – 3.98 (m, 2H), 3.52 (s, 10H), 2.75 (t, J = 6.4, 4H), 2.02 (q, J = 6.8, 8H), 1.42 (dd, J = 16.2, 6.6, 4H), 1.38 – 1.20 (m, 36H), 0.87 (t, J = 6.8, 6H). ^{13}C NMR (101 MHz, cdcl$_3$) δ 155.18, 130.50, 130.40, 128.27, 128.20, 65.66, 58.15, 54.64, 52.22, 35.42, 31.81, 29.98, 29.87, 29.64, 29.61, 27.53, 27.49, 26.29, 25.92, 22.86, 14.38. MS (LC-MS) m/z : Calcd for $C_{43}H_{81}N_2O_2$, the ammonium ion, 100%) 657.1. Found 657.64.

Preparation of Compound 206

Compound 206 is prepared from compound 106, using the method same as for preparation of compound 200. Aqueous workup then column chromatography (0-15% MeOH/DCM) gives compound 206.

Preparation of Compound 207

Compound 207 is prepared from compound 107, using the method same as for preparation of compound 200. Aqueous workup then column chromatography (0-15% MeOH/DCM) gives compound 207.

Preparation of Compound 208

Compound 208 is prepared from compound 108, using the method same as for preparation of compound 200. Aqueous workup then column chromatography (0-15% MeOH/DCM) gives compound 208.

Preparation of Compound 209

Compound 209 is prepared from compound 109, using the method same as for preparation of Compound 200. Aqueous workup then column chromatography (0-15% MeOH/DCM) gives compound 209.

siRNA

Double-stranded siRNAs were synthesized by Alnylam Pharmaceuticals with antisense sequence 5'UCGAAGUACUCAGCGUAAGdTdT (target – luciferase) and sense sequence 5'CUUACGCUGAGUACUUCGAdTdT. Purification of the synthesized oligoribonucleotides was achieved by anion exchange HPLC as per our established procedures. Double stranded siRNAs were obtained by annealing equimolar amounts of sense and antisense strands.

Preparation of Liposomes

Liposomes were prepared using a film hydration method whereby the charged lipid and colipids in chloroform were dried into a thin film by N_2 flux followed by vacuum dessication for 10 min to remove residual organic solvent. The dried lipid film was hydrated using 10 mM HEPES buffer, pH 7.4 for 1 h in 37 °C shaker water bath. Formed multilamellar vesicles were vortexed with siRNA for 1 min and then extruded using a LIPEX™ extruder with polycarbonate membranes of sequential sizes (400 nm, 200 nm, 200 nm) at 200-300 psi pressure, to form large unilamellar vesicles. The formed vesicles were sterile filtered using 0.45 μm filter, prior to transfection and cell viability studies. The liposomes were characterized using particle size analyser (Wyatt

Technologies, Dyna ProTitan). All the formulations had uniform particle size of 200-250nm.

Cell culture

HeLa (human cervical cancer cell line) cells stably transfected with luciferase enzymes were used. Cells were cultured in Dulbecco' Modified Eagle's medium (DMEM) with 10% heat inactivated fetal bovine serum (FBS) and antibiotics (Zeocin – 0.5 mg/mL, Puromyin- 0.5 μg/mL) at 37 °C with 5% CO_2 atmosphere.

Transfection Experiment

24 h before the experiment, HeLa cells (80-100% confluent) were trypsinized and resuspended in fresh DMEM media with FBS (without anitibiotics). The cells were plated at density of 10,000 cells/well in white 96 well plates (View plate, 96 TC, Perkin Elmer). The plated cells were incubated at 37 °C in 5% CO_2. On the day of experiment, liposomes and the siRNA were diluted in Opti-MEM I (Invitrogen) and mixed in appropriate proportions followed by incubation for 20 min with shaking to form liposome-siRNA complexes. Negative controls used were (i) untreated cells, and (ii) cells treated with only siRNA (lipid free). Positive controls were cells treated with siRNA complexes with Lipofectamine 2000™ (LF2000), or DOTAP:DOPE (1:1). Other commercially available formulations namely Lipofectamine™, Lipofectin™, were also tested.

In a second experiment HeLa cells are substituted with CHO cells or non-adeherant suspension of CHO cells.

Gene-knockdown assay

Dual Luc Assay (Promega) was performed 22 h post-transfection to determine the silencing efficiency of the liposomal formulations. The procedure was followed as per the manufacturer's protocol.

Liposomes were prepared using compounds 200-209 along with colipids DOPE and cholesterol. Formulation optimization was performed using compound 200. Liposomes were prepared with 50-90 mol% compound 200, 10-50 mol% DOPE, and 0 or 10 mol% cholesterol. See Table 1. Dose response studies were performed with these liposomes using 2 μg/mL lipid with siRNA concentration varying in the range 10 pM to 1 μM.

Table 1.

Code	Compound 200 mol%	DOPE mol%	Chol mol%
NF7	50	50	0
NF8	60	40	0
NF9	70	30	0
NF10	80	20	0
NF11	90	10	0
NF12	50	40	10
NF13	60	30	10
NF14	70	20	10
NF15	80	10	10
NF16	90	0	10

As seen from FIG. 1, formulations NF7 and NF12 had a higher silencing efficiency (60-75%, or expression of 40-25 %) than other formulations. Also from FIG. 2 it was found the N/P ratio of 0.3 to 0.44 provided efficient transfection.

Formulations with the composition (mol%): charged lipid (45-63), DOPE (35-55), cholesterol (0-10) (H1-H11) were prepared (Table 2). As seen from FIG. 3, H1-H4, H8-H11 showed good silencing (70-75 %) with N/P between 0.3 – 0.39 as represented by % maximum knockdown (100 – minimum % expression) (Figure 4). On comparing all the efficient formulations of compound 200, it was found that all the formulations provided higher % efficiency than both Lipofectin™ and Lipofectamine™; and an efficiency between those of DOTAP:DOPE and LF2000™.

Table 2

Code	Compound 200 Mol%	DOPE Mol%	Chol Mol%	N/P
H1	45.6	54.4	0	0.32
H2	48.1	51.9	0	0.33
H3	50.6	49.4	0	0.35
H4	53.1	46.9	0	0.37
H5	58.0	42.0	0	0.41
H6	60.5	39.5	0	0.42
H7	62.9	37.1	0	0.44
H8	52.7	37.3	10	0.39
H9	52.9	42.1	5	0.38
H10	53.0	44.5	2.5	0.37
H11	47.9	47.1	5	0.34

Also to see the effect of particle size, H9 and H11 were formulated in different particle size (FIG. 5). A particle size in the range 200 nm to 250 nm seemed to be optimal; larger size did not further improve the silencing efficacy.

Formulations of other charged lipids (compounds 201-204) with similar compositions were formulated (Table 3). These were designated as follows: compound 201, K; compound 202, L; compound 203, M; compound 204, P. All formulations were tested for transfection and cell viability, along with controls.

Table 3

Series	Charged lipid mol %	DOPE mol %	Chol mol %	Total mol%
1	45.56	54.44	0	100
2	48.08	51.92	0	100
3	50.60	49.40	0	100
4	53.10	46.90	0	100
5	52.73	37.27	10	100
6	52.92	42.08	5	100
7	53.01	44.49	2.5	100
8	47.94	47.06	5	100

From the results in FIG. 6A-CD, all the 32 formulations exceeded Lipofectin™ and Lipofectamine™ in terms of silencing efficacy. The best candidates for each of the charged lipid, were tested for transfection efficiency using keeping LF2000 and DOTAP:DOPE as positive controls. The results (FIG. 7) obtained were the average from three independent experiments in triplicate (n = 9).

As shown in FIG. 8, formulations K2, and P5-P8 had a maximum knockdown higher than LF2000 and much higher than DOTAP:DOPE. P7 was as potent as LF2000 and much more potent than DOTAP:DOPE (Table 4).

Table 4

IC_{50} (nM)	
H1	NA
K1	0.266
K2	0.38
L2	7.07

L8	0.932
M5	0.574
P5	0.108
P6	0.254
P7	0.054
P8	0.106
LF2000	0.0229
DOTAP	0.147

Dose response studies of compound 205 (designated R) formulations were performed with positive controls. The max knockdown of the R-formulations was higher than the controls (FIG. 9). More than 80% knockdown from all the R formulations (except R1) and almost 90% knockdown from formulations R5, R6 and R7 was achieved. These values were higher than LF2000 (75%) and DOTAP:DOPE (63%) at relatively low siRNA concentration of 10nM (FIG. 10). Formulatins R1-R8 were more efficient than LF2000 and DOTAP:DOPE. All the R-formulations were as potent as LF2000 and 100-fold more potent than DOTAP:DOPE (Table 5).

Table 5

IC_{50} (nM)	
R1	0.0998
R2	0.0275
R3	0.0136
R4	0.0265
R5	0.0122
R6	0.0104
R7	0.0203
R8	0.00915
LF2000	0.0148
DOTAP	0.918

Cell Viability studies

Cell-Titer Blue assay (Promega) was used to determine cell viability of the lipoplexes. The assay is based on the ability of live cells to reduce non-fluorescent dye (resazurin, blue color) to its fluorescent metabolite (resorufin, pink color). Cell viability was tested 22 h after transfection, by adding an appropriate volume of Cell Titer Blue™ reagent to the cells and then incubating for 2.5 h at 37 °C. The read-out was obtained using a fluorometer with filter settings as 540 nm (excitation) and 590 nm (emission).

Percentage cell viability was obtained by normalizing to the untreated cells. Cell viability experiments were performed three times in triplicate (n = 9).

Most of the charged lipids used were toxic. Cell viability was also tested over a range of lipid concentrations 0-60 μg/mL. From FIG. 11 and the IC_{50} values (lipid concentration at which 50% cells are viable) shown in Table 6, it was determined that the formulations (except L8 and M5) were safer than LF2000. In particular, compound 205 (R) formulations R1, R2, R3 and R8 were notably safer than LF2000 (FIG. 12 and Table 7).

Table 6

	IC_{50} (μg/mL)
H1	> 100
K1	> 100
K2	> 60
L2	29.16
L8	19.18
M5	13.2
P5	34.16
P6	53.48
P7	> 60
P8	> 100
LF2000	16.82
DOTAP:DOPE	> 100

Table 7

IC_{50} (μg/mL)	
R1	>100
R2	>100
R3	> 60
R4	51.71
R5	17.47
R6	18.17
R7	35.9
R8	>60
LF2000	16.82
DOTAP:DOPE	>100

From the average of obtained results, compound 205 (R) formulations were most efficient, while compound 202 (L) were least efficient. The rank order was as follows (FIG. 13):

R > K~P > H~M > L

Nine different transfection agents (**Table 1 and Scheme 12**) and Lipofectamine RNAiMax (Invitrogen) were used to deliver 1 nM of a potent siRNA against GFP to a GFP-CHO cell line. RNAiMax was tested at 0.4 µL/mL (concentration not available) and the nine transfection agents were used at 0.5, 1, and 2.5 µg/mL. RNAiMax was used as a positive control. Mixtures of transfection reagents and siRNA were made in black optical bottom 96 well plates and then cells were added. After 2 days, the relative GFP intensities were measured using a fluorescent plate reader.

Table 1: Transfection reagents used in current study.

Formulation Number	Cationic Lipid Mol %	DOPE %	Cholesterol %
1	48.08	51.92	-
2	47.94	47.06	5
3	45.56	54.44	-
4 (K8)	47.94	47.06	5
5 (L8)	47.94	47.06	5
6	53.01	44.49	2.5
7	47.94	47.06	5
8 (P8)	47.94	47.06	5
9	47.94	47.06	5

Testing of transfection reagents on suspended DG44 CHO cells

The three active transfection agents, K8, L8 and P8, from the GFP-CHO testing were used to transfect suspended CHO cells. 5 µL of 10 µM LDH-A siRNA was added to a tube and 500 µL CD DG44 media added to it. Transfection reagent was added to the mixture, the tube mixed by pipette aspiration and incubated at room temperature for 15 minutes. Then the mixture was added to 49.5 mL of media containing 200,000 cells/mL. The flask was incubated and shaken at 120 rpm for several days. LDH activity was measured by VetTest 8008 slide analyzer.

40L transfection

DG44 cells were grown in Invitrogen CD DG44 media. To seed the 40L bioreactor, cells were taken from 4-1L disposable bioreactors. The starting cell density in the 40L of culture was 120,000 cells/mL. The bioreactor was allowed to equilibrate, with the cells added for 1 hour prior to transfection. For transfection, 400 µL of LDH-A siRNA (100 uM stock solution) was added to 400 mL of media and mixed. Then 32 mL of 1 mg/mL P8 reagent was added and again mixed. This was allowed to incubate for 15

minutes at room temperature and then added to the 40L bioreactor. Cell density and viability were measured using a Vi-Cell cell counter. The efficiency of transfection was determined by measuring LDH activity using a VetTest 8008 slide analyzer.

Evaluation of nine transfection agents for uptake efficiency in CHO cells in shake flasks

To gauge the effectiveness of the transfection agents at introducing a nucleic acid into CHO cells, the transfection agents were used to introduce a potent GFP siRNA into GFP-CHO cells. Compared to an effective concentration of Lipofectamine RNAiMAx, three of the transfection agents were active (**FIG. 14**). These formulations were designated K8, L8 and P8. No obvious cytotoxicity was observed with any concentration of any formulation.

As K8 was the most active formulation in the GFP-CHO cells, it was tested using DG44 CHO cells in 50 mL of culture in a 250 mL shake flask and a potent LDH siRNA. A range of K8 concentrations was tested along with an effective concentration of Lipofectamine RNAiMAx. After 3 days, LDH activity was lower in cultures where K8 was used (**FIG. 15**). There was also a higher cell density in flasks that had 0.6 or 1.2 µg/mL of K8 conpared to RNAiMAx. It appears that RNAiMAx inhibited growth of CHO cell in suspension compared to K8. The highest concentrations of K8 reduced the cell density, even though the LDH activity was still reduced.

Because some transfection reagents did not seem to have the same activity in shake flasks as in a 96 well plate, the 3 active transfection reagent formulations were tested similarly in 50 mL of DG44 culture in 250 mL shake flasks. Surprisingly, P8 which was only marginally active against GFP-CHO cells, performed the best using suspended DG44 cell culture (**FIG. 16**). After 5 days, 0.8 µg/mL of P8 resulted in the most LDH activity knockdown. Also, it is significant that the cell density in the presence of P8 was greater than or equal to cells without transfection reagent added. P8 at a final concentration of 0.8 µg/mL has been used numerous times in smaller bioreactors and would be tested in a 40L system.

Evaluation of cationic lipid formulation P8 for uptake efficiency in a 40L bioreactor.

After seeding the 40L bioreactor, the cells generally grew with a doubling time of approximately 24 hours and the cell viability was over 98% (**FIG. 17**). The cells reached a peak concentration of 3.1×10^6 cells/mL at day 5 and then began to decline. As expected in this unfed batch culture, by day 6 the cells were in decline.

The LDH activity declined as the cells were growing following seeding and transfection. The LDH activity was reduced ~80%, even as the cells had doubled over 3 times (**FIG. 18**). There was diminished LDH activity through the entire experiment. The diminished LDH activity suggests that the transfection was successful with no detectable toxicity in the CHO cells.

Evaluation of cationic lipid formulation P8 for uptake efficiency in a 3L bioreactor.

The same LDH-directed siRNA formulated with P8 (single dose, 1 nM final concentration) that was used in the 250 mL shake flask study was evaluated in the 3 L bioreactor. After seeding the 3 L bioreactor and dosing with the P8-formulated LDH siRNA, the cells generally grew with a doubling time of approximately 30 h and the cell viability was over 97% (FIG. 19). The DG44 cells reached a peak concentration of 3.0 x 10^6 cells/mL at day 4.

The LDH activity declined during the cell growth phase following seeding and transfection. The LDH activity was reduced >80% even as the cells had doubled 3 times (FIG. 20). The observed decrease in LDH activity following a single siRNA dose suggested high uptake efficiency with no detectable adverse effect on CHO cell growth or viability.

Cryoprotectants and Lyophilization

General protocol for making the formulation

To a glass vial, various amounts of a siRNA complexing lipid, a membrane fusogenic lipid and cholesterol dissolved in chloroform were pipetted out. The lipid solutions were mixed thoroughly and evaporated the chloroform using an argon stream at ambient temperature inside a fume hood to make a thin film inside. The residual organic solvent was removed under vacuo. The filmy residue was suspended in a suitable buffer containing a suitable cryprotectant to obtain a final desired lipid concentration. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded one or more times (LIPEX™ Extruder) through polycarbonate filters with pore sizes corresponding to the desired particle size. The final formulation was filtered through a 0.45 -µm filter and stored under appropriate conditions until use.

General protocol for the storage of formulation at 4 °C

To a glass vial, various amounts of a siRNA complexing lipid, a membrane fusogenic lipid and cholesterol dissolved in chloroform were pipetted out. The lipid solutions were mixed thoroughly and evaporated the chloroform using an argon stream at

ambient temperature inside a fume hood to make a thin film inside. The residual organic solvent was removed under vacuo. The filmy residue was suspended in a suitable buffer containing a suitable cryprotectant to obtain a final desired lipid concentration. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded one or more times (LIPEX™ Extruder) through polycarbonate filters with pore sizes corresponding to the desired particle size. The final formulation was filtered through a 0.45 -µm filter and stored at 4 °C before use.

General protocol for the storage of formulation under frozen conditions

To a glass vial, various amounts of a siRNA complexing lipid, a membrane fusogenic lipid and cholesterol dissolved in chloroform were pipetted out. The lipid solutions were mixed thoroughly and evaporated the chloroform using an argon stream at ambient temperature inside a fume hood to make a thin film inside. The residual organic solvent was removed under vacuo. The filmy residue was suspended in a suitable buffer containing a suitable cryprotectant to obtain a final desired lipid concentration. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded one or more times (LIPEX™ Extruder) through polycarbonate filters with pore sizes corresponding to the desired particle size. The final formulation was filtered through a 0.45 -µm filter and stored frozen at –20 to -80 °C until use.

General protocol for the storage of formulation as freeze dried powder

To a glass vial, various amounts of a siRNA complexing lipid, a membrane fusogenic lipid and cholesterol dissolved in chloroform were pipetted out. The lipid solutions were mixed thoroughly and evaporated the chloroform using an argon stream at ambient temperature inside a fume hood to make a thin film inside. The residual organic solvent was removed under vacuo. The filmy residue was suspended in a suitable buffer containing a suitable cryprotectant to obtain a final desired lipid concentration. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded one or more times (LIPEX™ Extruder) through polycarbonate filters with pore sizes corresponding to the desired particle size. The final formulation was filtered through a 0.45 -µm filter. The formulation was then lyophilized and the powder was stored at –20 to -80 °C until use. Particles were then resuspended in water before complexation with nucleic acid. **General protocol for the preparation and storage of lipid transfection agent complexed with siRNA as freeze dried powder**

To a glass vial, various amounts of a siRNA complexing lipid, a membrane fusogenic lipid and cholesterol dissolved in chloroform were pipetted out. The lipid solutions were mixed thoroughly and evaporated the chloroform using an argon stream at ambient temperature inside a fume hood to make a thin film inside. The residual organic solvent was removed under vacuo. The filmy residue was suspended in a suitable buffer containing a suitable cryprotectant to obtain a final desired lipid concentration. The mixture was agitated at 37 ºC for 1 hr. The suspension was then extruded one or more times (LIPEX™ Extruder) through polycarbonate filters with pore sizes corresponding to the desired particle size. The final formulation was filtered through a 0.45 -µm filter. Complexes of nucleic acid with the lipid particles were made by incubating the nucleic acid with the formulation. The formulation was then lyophilized and the powder was stored at –20 to -80 ºC until use. Particles were then resuspended in water or cell culture media before using for transfection.

Table 2. Lipid excipients required to prepare 10 mL P8 formulation with 1 mg/mL total lipid

	Lipid P	**DOPE**	**CHOL**	**Total**
Lipid excipients molar comp. (%)	48	47	5	100
Excipient MW	672	744	386.7	
Lipid weight (mg)	4.66	5.06	0.28	10.00
Lipid (wt %)	46.58	50.62	2.80	
Volume (µL) taken from the stock solution (25 mg/mL)	186	203	11	400
Volume of buffer added to make final formulation (mL)				10.00

Table 3. Lipid excipients required to prepare 10 mL L8 formulation with 1 mg/mL total lipid

	Lipid L	**DOPE**	**CHOL**	**Total**
Lipid excipients molar comp. (%)	48	47	5	100
Excipient MW	672	744	386.7	

Lipid weight (mg)	4.66	5.06	0.28	10.00
Lipid (wt %)	46.58	50.62	2.80	
Volume (μL) taken from the stock solution (25 mg/mL)	186	203	11	400
Volume of buffer added to make final formulation (mL)				10.00

Example 1. Preparation of P8 formulation with sucrose as cryoprotectent

To a 10-mL glass vial, 120 μL Lipid P, 130 μL 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 10 mM HEPES (pH 7.4) containing 10 wt % sucrose for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) in a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and was stored at 4 °C until use.

Example 2. Preparation of L8 formulation with sucrose as cryoprotectent

To a 10-mL glass vial, 120 μL Lipid L, 130 μL 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 10 mM HEPES (pH 7.4) containing 10 wt % sucrose for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) in a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and was stored at 4 °C until use.

Example 3. Preparation of P8 formulation with glucose as cryoprotectent

To a 10-mL glass vial, 120 μL Lipid P, 130 μL 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed

using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 10 mM HEPES (pH 7.4) containing 5 wt % glucose for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) in a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and was stored at 4 °C until use.

Example 4. Storage of P8 formulation as a freeze-dried powder with sucrose

Forty μL of the formulation prepared by method described in Example 1 was freeze dried and the white powder was stored at -80 °C until use. For the transfection experiment in a 50-mL scale, the powder was suspended in 40 μL of water which was then mixed with 5 μL of 10 μM siRNA solution in PBS buffer diluted with 500 μL CD DG44 media.

Example 5. Storage of P8 formulation in frozen condition with sucrose

Forty μL of the formulation prepared by method described in Example 1 was stored frozen at -80 °C. For the transfection experiment in a 50-mL scale, the mixture was thawed slowly at room temperature and then mixed with 5 μL of 10 μM siRNA solution in PBS buffer diluted with 500 μL CD DG44 media.

Example 6. Storage of P8 formulation as a freeze dried powder with glucose

Forty μL of the formulation containing 5 wt % glucose prepared by method described in Example 2 was freeze dried and the white powder was stored at -80 °C until use. For the transfection experiment in a 50-mL scale, the powder was suspended in 40 μL of water and was then mixed with 5 μL of 10 μM siRNA solution in PBS buffer diluted with 500 μL CD DG44 media.

Example 7. Storage of P8 formulation frozen with glucose

Forty μL of the formulation containing 5 wt % glucose prepared by method described in Example 2 was stored frozen at -80 °C. For the transfection experiment in a 50-mL scale, the mixture was thawed slowly at room temperature and then mixed with 5 μL of 10 μM siRNA solution in PBS buffer diluted with 500 μL CD DG44 media.

Example 8. Preparation of P8 formulation by extrusion through syringe membrane filters

To a 10-mL glass vial, 120 μL Lipid P, 130 μL 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 7 μL cholesterol solution (25 mg/mL in chloroform)

were added. The lipid solutions were mixed thoroughly, and chloroform was removed using an argon stream at ambient temperature inside a fume hood to make a thin film inside. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 10 mM HEPES (pH 7.4) containing 10 wt % of sucrose for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C using a shaker for 1 hr. The dispersion was then extruded through a PVDF membrane filter with 0.22-μm pore size at 60 °C. The final formulation was filtered through a 0.45-μm filter and separated into three fractions. One fraction was stored at 4 °C. The second fraction was frozen at -80 °C. The third fraction was frozen at -80 °C for 2 hours and then lyophilized in a freeze-drier.

Example 9. Preparation of liposome/siRNA complexes and storage as a lyophilized powder

Forty μL of the P8 formulation prepared by high-pressure extrusion or by PDVF syringe membrane filtration methods described in Examples 1 and 8, respectively, were mixed with 5 μL of 10 μM siRNA solution in PBS buffer. The mixture was incubated for 15 minutes at room temperature, frozen at -80 °C for 2 hours and then lyophilized in a freeze-drier. The resulting white powder was stored at -80 °C until use. For the transfection experiment in a 50-mL scale, the powder was suspended in 500 μL of CD DG44 media at room temperature.

Example 10. Preparation of L8/siRNA lipoplex as a freeze dried powder

Forty μL of the L8 formulation prepared by high-pressure extrusion or by PDVF syringe membrane filtration methods described in Examples 1 and 8, respectively, were mixed with 5 μL of 10 μM siRNA solution in PBS buffer. The mixture was incubated for 15 minutes at room temperature, frozen at -80 °C for 2 hours and then lyophilized in a freeze-drier. The resulting white powder was stored at -80 °C until use. For the transfection experiment in a 50-mL scale, the powder was suspended in 500 μL of CD DG44 media at room temperature.

Example 11. Use of Compound 103 (see Scheme 12) (rather than DOPE) as a fusogenic lipid in the L8 formulation

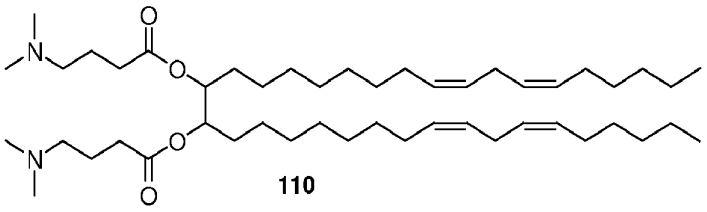
To a 10-mL glass vial, 60 μL Lipid L, 65 μL compound 103 and 3.5 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum.

The filmy residue was suspended in 3.2 mL 10 mM HEPES (pH 7.4) containing 10 wt % of sucrose for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded once through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 12. Use of Compound 103 (see Scheme 12) as a siRNA complexation agent at acidic pH

In this example, a formulation of compound 103 with DOPE and cholesterol at acidic pH was prepared. The compound 103 lipid, which was cationic at this pH, acted as an siRNA condensation agent, whereas DOPE was fusogenic lipid. To a 10-mL glass vial, 60 μL MC-3, 65 μL 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 3.5 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 3.2 mL 25 mM sodium acetate buffer (pH 5.3) for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 13. Use of compound 110 as a siRNA complexation agent at acidic pH



110

In this example a formulation of compound 110 with DOPE and cholesterol at acidic pH was prepared. The compound 110 lipid, which has a two-amine head group, was cationic at acidic pH and acted as a siRNA condensation agent, whereas DOPE was fusogenic lipid. To a 10-mL glass vial, 60 μL compound 110, 65 μL 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 3.5 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in

3.2 mL 25 mM sodium acetate buffer (pH 5.3) for a final lipid concentration of 1 mg/mL. The clear solution was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 13. Use of C12-200 as a siRNA complexation agent at acidic pH

In this example a formulation of C12-200 (see Love, K.T., et al., "Lipid-like materials for low-dose, in vivo gene silencing," PNAS 107, 5, (2010), 1864-1869, which is incorporated by reference in its entirety) with DOPE and cholesterol at acidic pH was prepared. The C120-200 lipid, which has a five-amine head group, was cationic at acidic pH and acted as a siRNA condensation agent, whereas DOPE was fusogenic lipid. To a 10-mL glass vial, 200 μL C12-200, 130 μL 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 8.44 mL of 25 mM sodium acetate buffer (pH 5.3) for a final lipid concentration of 1 mg/mL. The clear solution was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 14. Preparation of L8 formulation with DLPE as the fusogenic lipid

To a 10-mL glass vial, 120 μL Lipid L, 130 μL 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine (DLPE) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 10 mM HEPES containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 15. Storage of P8 formulation as a freeze dried powder with dextran as a cryoprotectant

A 40 μL of the formulation containing 20% w/v dextran in 10 mM HEPES buffer prepared by method described in Example 2 was freeze dried and the white powder was stored at -80 °C until use. For the transfection experiment in a 50 mL scale, the powder was suspended in 40 μL of water which was then mixed with 5 μL of 10 μM siRNA solution in PBS buffer diluted with 500 μL CD DG44 media.

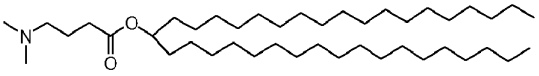
Example 16. Storage of P8 formulation as a freeze dried powder with polyvinyl pyrrollidone as a cryoprotectant

A 40 μL of the formulation containing 20% w/v polyvinyl pyrrollidone in 10 mM HEPES buffer prepared by method described in Example 2 was freeze dried and the white powder was stored at -80 °C until use. For the transfection experiment in a 50 mL scale, the powder was suspended in 40 μL of water which was then mixed with 5 μL of 10 μM siRNA solution in PBS buffer diluted with 500 μL CD DG44 media.

Example 17. Storage of P8 formulation as a freeze dried powder with a mixture of polyvinyl pyrrollidone and sucrose as a cryoprotectant

A 40 μL of the formulation containing 20% w/v polyvinyl pyrrollidone/sucrose mixture in 10 mM HEPES buffer prepared by method described in Example 2 was freeze dried and the white powder was stored at -80 °C until use. For the transfection experiment in a 50 mL scale, the powder was suspended in 40 μL of water which was then mixed with 5 μL of 10 μM siRNA solution in PBS buffer diluted with 500 μL CD DG44 media.

Example 18. Use of novel lipids containing protonatable amine groups at pH 7.4 as a siRNA complexation agent in a formulation



111

In this example, a formulation of compound 111 with DOPE and cholesterol at physiological pH was prepared. The compound 111 lipid, which was cationic at this pH (pKa >7.4), acted as an siRNA condensation agent, whereas DOPE was fusogenic lipid. To a 10-mL glass vial, 120 μL compound 111, 130 μL 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.4 mL 10 mM

HEPES buffer (pH 7.4) containing 10 wt% sucrose for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 19. Preparation of L8 formulation with DLPC

To a 10-mL glass vial, 120 μL lipid L, 130 μL 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DLPC) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 10 mM HEPES containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 20. Preparation of L8 formulation with DLPC and DOPE as helper lipids

To a 10-mL glass vial, 120 μL lipid L, 130 μL of a 1:1 mixture of 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DLPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 10 mM HEPES containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 21. Preparation of L8 formulation with DOPS as helper lipid

To a 10-mL glass vial, 120 μL lipid L, 130 μL of 2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic

solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 10 mM HEPES containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 22. Preparation of L8 formulation with DLPC and DOPS as helper lipids

To a 10-mL glass vial, 120 μL lipid L, 130 μL of a 1:1 mixture of 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DLPC) and 2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 10 mM HEPES containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 23. Preparation of L8 formulation with DOPE and DOPS as helper lipids

To a 10-mL glass vial, 120 μL lipid L, 130 μL of a 1:1 mixture of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 10 mM HEPES containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 24. Preparation of formulation with compound 102 (see Scheme 12) as siRNA complexing agent at acidic pH

To a 10-mL glass vial, 120 μL compound 102, 130 μL 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DLPC) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 25 mM sodium acetate buffer (pH 5.3) containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 25. Preparation of formulation with compound 102 (see Scheme 12) as siRNA complexing agent at acidic pH with DLPC and DOPE as helper lipids

To a 10-mL glass vial, 120 μL compound 102, 130 μL of a 1:1 mixture of 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DLPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 25 mM sodium acetate buffer (pH 5.3) containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 26. Preparation of formulation with compound 102 (see Scheme 12) as siRNA complexing agent at acidic pH with 2-dioleoyl-sn-glycero-3-phosphatidylserine as helper lipid

To a 10-mL glass vial, 120 μL compound 102, 130 μL of 2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 25 mM sodium acetate buffer (pH 5.3) containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension

was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 27. Preparation of formulation with compound 102 (see Scheme 12) as siRNA complexing agent at acidic pH with DLPC and DOPS as helper lipids

To a 10-mL glass vial, 120 μL compound 102, 130 μL of a 1:1 mixture of 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DLPC) and 2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL 25 mM sodium acetate buffer (pH 5.3) containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 28. Preparation of formulation with compound 102 (see Scheme 12) as siRNA complexing agent at acidic pH with DOPE and DOPS as helper lipids

To a 10-mL glass vial, 120 μL compound 102, 130 μL of a 1:1mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 2-dioleoyl-sn-glycero-3-phosphatidylserine (DOPS) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.44 mL25 mM sodium acetate buffer (pH 5.3) containing 10 wt % of sucrose at pH 7.4 for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 29. Preparation of formulations using a mixture of quaternized and nonquaternized lipid

In this example, a new formulation of compound 102 and lipid L with DOPE and cholesterol at physiological pH was prepared. To a 10-mL glass vial, 120 μL of 1:1 a

mixture of compound 102 and lipid L, 130 μL 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 7 μL cholesterol solution (25 mg/mL in chloroform) were added. The lipid solutions were mixed thoroughly, and the chloroform was removed using an argon stream at ambient temperature inside a fume hood. The residual organic solvent was removed under vacuum. The filmy residue was suspended in 6.4 mL 10 mM HEPES buffer (pH 7.4) containing 10 wt% sucrose for a final lipid concentration of 1 mg/mL. The mixture was agitated at 37 °C for 1 hr. The suspension was then extruded through polycarbonate filters (one 0.4-μm prefilter and two 0.2-μm filters, Whatman Nuclepore Track-Etch Membrane) using a LIPEX™ Extruder. The final formulation was filtered through a 0.45-μm filter and stored at 4 °C until use.

Example 30. Storage of formulations prepared as described in examples 19 – 29

The formulations (examples 19 – 29) containing sucrose as a cryoprotective agent were filtered through a 0.45-μm filter and separated into three fractions. One fraction was stored at 4 °C. The second fraction was frozen at -80 °C. The third fraction was frozen at -80 °C for 2 hours and then lyophilized in a freeze-drier and stored at -80 °C until use.

Example 31. Storage of formulations prepared as described in examples 19 – 29

The formulations (examples 19 – 29) containing a mixture of sucrose and polyvinyl pyrrollidone as a cryoprotective agent were filtered through a 0.45-μm filter and separated into three fractions. One fraction is stored at 4 °C. The second fraction is frozen at -80 °C. The third fraction is frozen at -80 °C for 2 hours and then lyophilized in a freeze-drier and stored at -80 °C until use.

Example 32. Preparation formulations by extrusion through syringe membrane filters

The aqueous suspensions containing mixture of lipids in compositions as described in examples 19-29 were extruded through a PVDF membrane filter with 0.22-μm pore size at 60 °C. The final formulation were filtered through a 0.45-μm filter and separated into three fractions. One fraction was stored at 4 °C. The second fraction was frozen at -80 °C. The third fraction was frozen at -80 °C for 2 hours and then lyophilized in a freeze-drier and stored at -80 °C until use.

Example 33. Preparation of siRNA lipoplexes as a freeze dried powder

Forty μL of the formulations described in examples 19 to 29 were mixed with 5 μL of 10 μM siRNA solution in PBS buffer. The mixture was incubated for 15 minutes at room temperature, frozen at -80 °C for 2 hours and then lyophilized in a freeze-drier. The

resulting white powder was stored at -80 °C until use. For the transfection experiment in a 50-mL scale, the powder was suspended in 500 µL of CD DG44 media at room temperature.

Table 4. Transfection Agents – Formulation, storage, in vitro siRNA activity and cell viability

Formulation				+/- siRNA	Storage conditions	Reconstitution	Cell viability	siRNA Activity % LDH KD
Excipient	Cryoprotectant		Preparation					
	Name	+/-						
Lipid P /DOPE/Chol in 10 mM HEPES buffer	Sucrose (10 wt %)	-	Extrusion	-	At 4 °C for 3 days	Solution warmed to 37 °C		25
		+	Extrusion	-	At 4 °C for 3 days	Solution warmed to 37 °C		64
		+	Extrusion	-	Stored frozen at -80 °C for 3 days	Thawed and warmed to 37 °C		72
		+	Extrusion	-	Freeze dried and stored at -80 °C for 3 days	Warmed to RT		63
		-	Filtered through 0.22 µm filter at 60 °C	-	At 4 °C for 3 days	Solution warmed to 37 °C		37
		+	Filtered through 0.22 µm filter at 60 °C	-	At 4 °C for 3 days	Solution warmed to 37 °C		59
		+	Filtered through 0.22 µm filter at 60 °C	-	Stored frozen at -80 °C for 3 days	Thawed and warmed to 37 °C		63
		+	Filtered through 0.22 µm filter at 60 °C	-	Freeze dried and stored at -80 °C for 3 days	Warmed to RT and mixed with siRNA in media		65
		+	Filtered through 0.22 µm filter at 60 °C	+	Freeze dried and stored at -80 °C and tested after 4 hours	Warmed to RT and mixed with media	95	74
		+	Filtered through 0.22 µm filter at 60 °C	+	Freeze dried and stored at -80 °C and tested after 3 weeks	Warmed to RT and mixed with media	86	87

		+	Filtered through 0.22 µm filter at 60 °C	+	Freeze dried and stored at -80 °C and tested after 5 weeks	Warmed to RT and mixed with media	86	71
Lipid L /DOPE/Chol in 10 mM HEPES buffer		+	Filtered through 0.22 µm filter at 60 °C	+	Freeze dried and stored at -80 °C and tested after 4 hours	Warmed to RT and mixed with media	95	89
		+	Filtered through 0.22 µm filter at 60 °C	+	Freeze dried and stored at -80 °C and tested after 3 weeks	Warmed to RT and mixed with media	77	66
		+	Filtered through 0.22 µm filter at 60 °C	+	Freeze dried and stored at -80 °C and tested after 5 weeks	Warmed to RT and mixed with media	63	56

kD – knock down; RT – room temperature

Table 5. Transfection Agents – Formulation, storage, in vitro siRNA activity and cell viability

5

Formulation excipients	Buffer	Preparation	Storage condition	Particle size (nm)	% PD	% LDH reduction (siRNA activity)	% cell viability
compound 110/ DOPE/Chol	25 mM NaAcetate	extrusion	4 °C, 2 days	109	16.8	69.4	98
MC-3/ DOPE/Chol	25 mM NaAcetate	extrusion	4 °C, 2 days	198	16.5	70.3	99
Lipid L/compound 103/Chol	10 mM Hepes/ 10 wt %sucrose	extrusion	4 °C, 2 days	188	22	68.4	98
Lipid L/ DOPE/Chol(L8)+siRNA complex	10 mM Hepes/ 10 wt %sucrose	syringe filtration at 60 °C	freeze dried complex at -80 °C, 2 days	268	15	89	97
Lipid P/ DOPE/Chol(P8)+siRNA complex	10 mM Hepes/ 10 wt %sucrose	syringe filtration at 60 °C	freeze dried complex at -80 °C, 2 days	216	17	40.7	97
Lipid P/ DOPE/Chol(P8)	10 mM Hepes/ 5 wt % glucose	syringe filtration at 60 °C	4 °C, 3 days	150	23	64	95
Lipid P/ DOPE/Chol(P8)	10 mM Hepes/ 5 wt % glucose	extrusion	Freeze dried powder at 80 °C, 3 days	190	70	70	94
Lipid P/ DOPE/Chol(P8)	10 mM Hepes/ 10 wt %sucrose	extrusion	4 °C, 3 days	150	23	64	96
Lipid P/	10 mM Hepes/	extrusion	Frozen at -	187	15	72	93

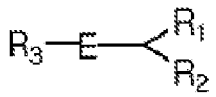
DOPE/Chol(P8)	5 wt % glucose		80 °C, 3 days				
Lipid P/ DOPE/Chol(P8)	10 mM Hepes/ 10 wt %sucrose	extrusion	Freeze dried powder at -80 °C, 3 days	297	86	63	95
Lipid P/ DOPE/Chol(L8)	10 mM Hepes/ 10 wt %sucrose	syriage filtration at 60 °C	4 °C, 2 days	268	15	88.3	97
Lipid P/ DOPE/Chol(P8)	10 mM Hepes/ 10 wt %sucrose	syriage filtration at 60 °C	4 °C, 2 days	216	17	87.3	98
Lipid L/ DOPE/Chol(L8)	10 mM Hepes/ 10 wt %sucrose	extruded	Freeze dried powder at -80 °C, 2 days	231	21	88.3	98
Lipid L/ DOPE/Chol(L8)	10 mM Hepes/ 10 wt %sucrose	syriage filtration at 60 °C	Freeze dried powder at -80 °C, 2 days	268	15	87.3	98
Lipid P/ DOPE/Chol(P8)	10 mM Hepes/ 10 wt %sucrose	syriage filtration at 60 °C	Freeze dried powder at -80 °C, 2 days	216	17	84.7	97
Lipid P/ DOPE/Chol (P8)	10 mM Hepes/ 10 wt %sucrose	extruded	Freeze dried powder at -80 °C, 2 days	220	15	78.3	97
Lipid P/ DOPE/Chol(L8)	10 mM Hepes/ 10 wt %sucrose	syriage filtration at 60 °C	Frozen at -80 °C, 2 days	268	15	73.7	98
C120-200/ DOPE/Chol	25 mM NaAcetate (pH 5.3)	extrusion	4 °C, 2 days	248	15	80.4	94.8

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A compound having the formula:



XXXIII

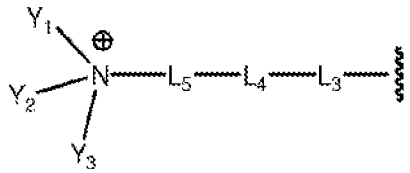
wherein:

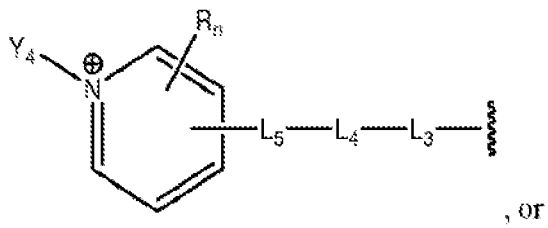
R_1 and R_2 are each independently for each occurrence optionally substituted C_{10}-C_{30} alkyl, optionally substituted C_{10}-C_{30} alkoxy, optionally substituted C_{10}-C_{30} alkenyl, optionally substituted C_{10}-C_{30} alkenyloxy, optionally substituted C_{10}-C_{30} alkynyl, optionally substituted C_{10}-C_{30} alkynyloxy, or optionally substituted C_{10}-C_{30} acyl;

E is -OC(O)N(R')-, -N(R')C(O)O-;

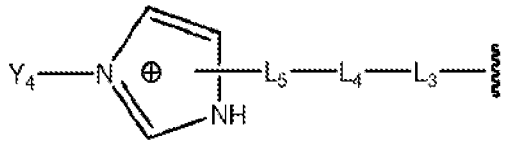
R' is H, alkyl, heteroalkyl, aralkyl, cyclic alkyl or heterocyclyl;

R_3 has the formula:





, or



wherein

each of Y_1, Y_2, Y_3, and Y_4, independently, is alkyl, cycloalkyl, aryl, aralkyl, or alkynyl; or

any two of Y_1, Y_2, and Y_3 are taken together with the N atom to which they are attached to form a 3- to 8- member heterocycle; or

Y_1, Y_2, and Y_3 are all be taken together with the N atom to which they are attached to form a bicyclic 5- to 12- member heterocycle;

each R_n, independently, is H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl;

L_3 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

L_4 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

L_5 is a bond, -N(Q)-, -O-, -S-, -$(CR_5R_6)_a$-, -C(O)-, or a combination of any two of these;

each occurrence of R_5 and R_6 is, independently, H, halo, cyano, hydroxy, amino, alkyl, alkoxy, cycloalkyl, aryl, heteroaryl, or heterocyclyl; or two R_5 groups on adjacent carbon atoms are taken together to form a double bond between their respective carbon atoms; or two R_5 groups on adjacent carbon atoms and two R_6 groups on the same adjacent carbon atoms are taken together to form a triple bond between their respective carbon atoms;

each a, independently, is 0, 1, 2, or 3;

wherein

an R_5 or R_6 substituent from any of L_3, L_4, or L_5 is optionally taken with an R_5 or R_6 substituent from any of L_3, L_4, or L_5 to form a 3- to 8- member cycloalkyl, heterocyclyl, aryl, or heteroaryl group; and

any one of Y_1, Y_2, or Y_3, is optionally taken together with an R_5 or R_6 group from any of L_3, L_4, and L_5, and atoms to which they are attached, to form a 3- to 8- member heterocyclyl group;

each Q, independently, is H, alkyl, acyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl or heterocyclyl; and

wherein

"alkyl" means a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms,

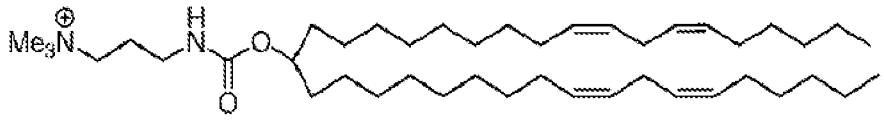
"cycloalykl" refers to partially saturated or fully saturated cycloalkyl,

"acyl" refers to hydrogen, alkyl, partially saturated or fully saturated cycloalkyl, partially saturated or fully saturated heterocycle, aryl, and heteroaryl substituted carbonyl groups, and

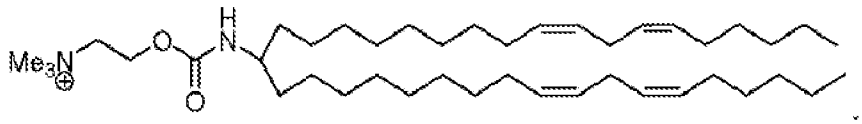
"substituted" refers to the replacement of one or more hydrogen radicals in a given structure with the radical of a specified substituent selected from halo,

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 aliphatic.

2. The compound of claim 1, selected from the group consisting of:



; and



3. A method for delivering a nucleic acid to a cell comprising contacting the cell with a composition comprising a compound according to claim 1 or 2.

4. The method of claim 3, wherein the composition further comprises a lipid capable of reducing aggregation, wherein the lipid is selected from the group consisting of polyethylene glycol (PEG)-modified lipids, monosialoganglioside Gm1, and polyamide oligomers.

5. The method of claim 3 or 4, wherein the composition further comprises the nucleic acid.

6. The method of any one of claims 3 to 5, wherein the cell is in suspension.

7. The method of claim 6, wherein the volume of the suspension is at least 0.050 L, at least 3 L, at least 25 L, or at least 40 L.

8. The method of any one of claims 3 to 7, wherein the nucleic acid includes a chemically modified nucleic acid.

9. The method of any one of claims 3 to 8, wherein the nucleic acid is 10 to 50 nucleotides long.

10. The method of any one of claims 3 to 9, wherein the nucleic acid is a double-stranded or single-stranded oligonucleotide.

11. The method of any one of claims 3 to 10, wherein the nucleic acid is siRNA, mRNA, an antisense nucleic acid, a microRNA, an antimicro RNA, an antagomir, a microRNA inhibitor, or an immune stimulatory nucleic acid.

12. Use of a compound according to claim 1 or 2 in the manufacture of a composition for delivering a nucleic acid to a cell.

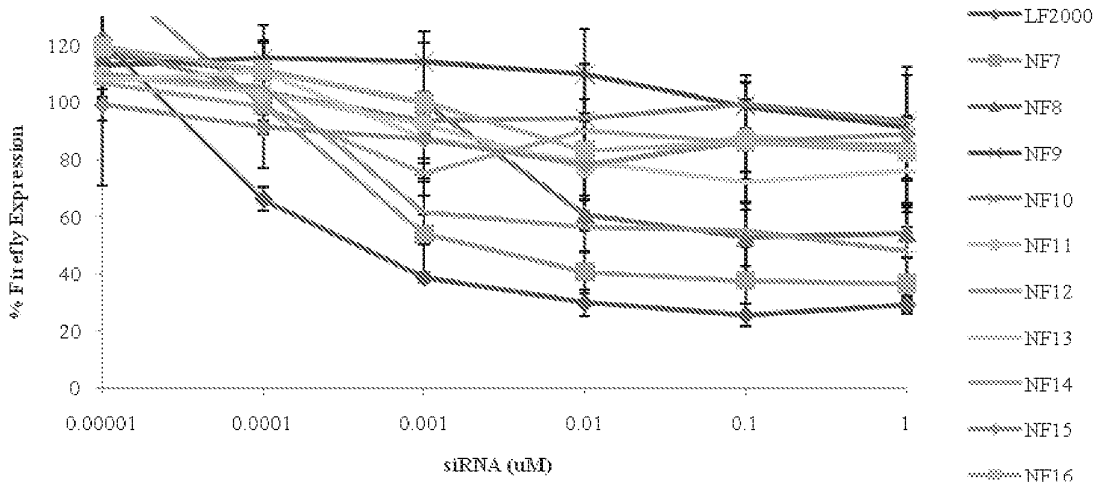

% Firefly Expression
120
100
80
60
40
20
0
0.00001
0.0001
0.001
0.01
0.1
1
siRNA (uM)
LF2000
NF7
NF8
NF9
NF10
NF11
NF12
NF13
NF14
NF15
NF16

FIG. 1

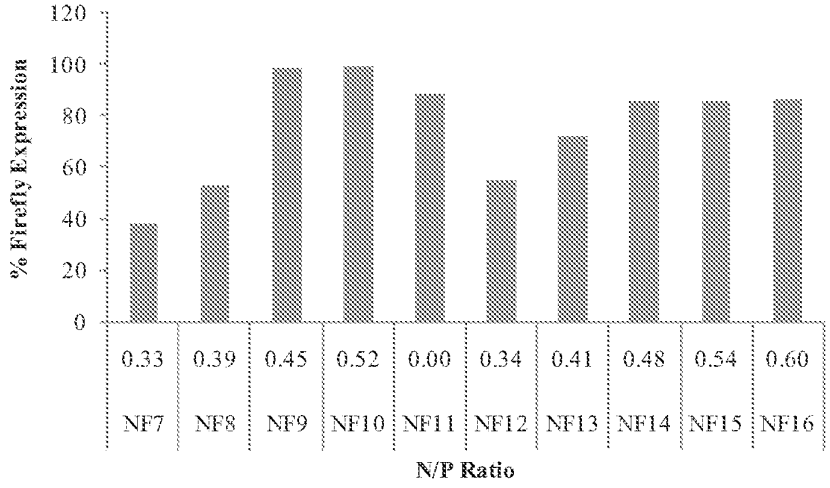

% Firefly Expression
120
100
80
60
40
20
0
0.33
0.39
0.45
0.52
0.00
0.34
0.41
0.48
0.54
0.60
NF7
NF8
NF9
NF10
NF11
NF12
NF13
NF14
NF15
NF16
N/P Ratio

FIG. 2

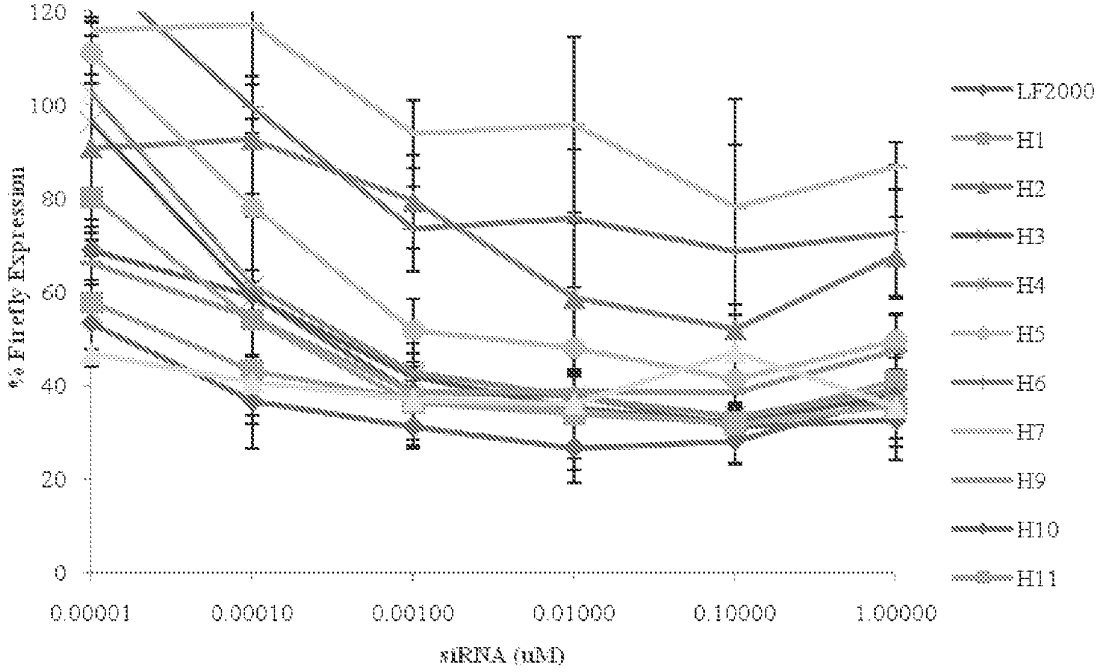

120
100
80
60
40
20
0
% Firefly Expression
0.00001
0.00010
0.00100
0.01000
0.10000
1.00000
siRNA (nM)
LF2000
H1
H2
H3
H4
H5
H6
H7
H9
H10
H11

FIG. 3

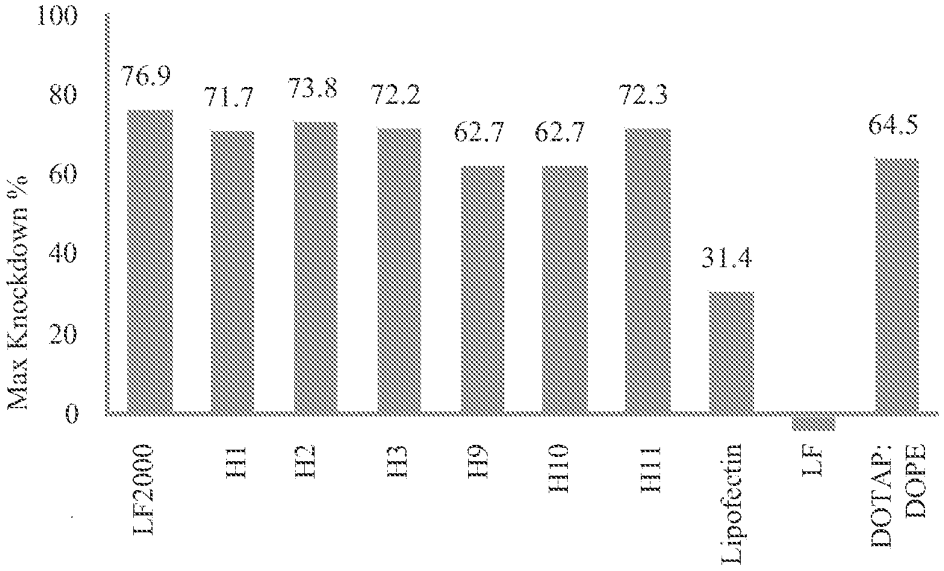

100
80
60
40
20
0
Max Knockdown %
76.9
71.7
73.8
72.2
62.7
62.7
72.3
31.4
64.5
LF2000
H1
H2
H3
H9
H10
H11
Lipofectin
LF
DOTAP: DOPE

FIG. 4

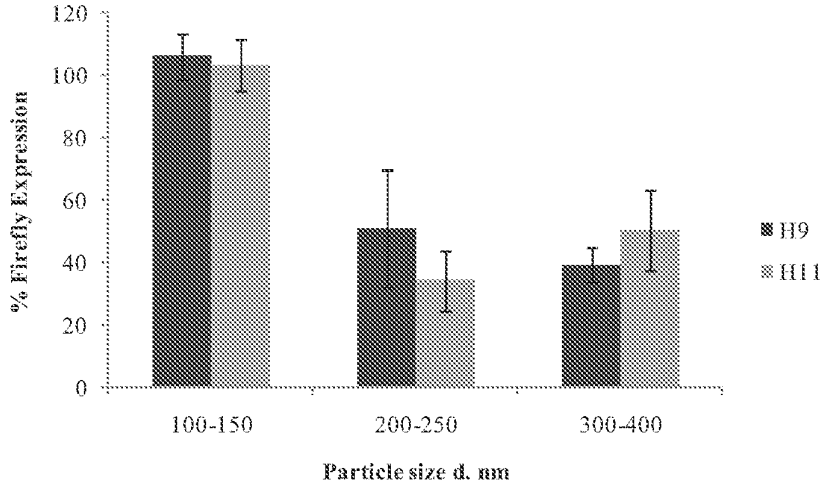

120
100
80
60
40
20
0
% Firefly Expression
H9
H11
100-150
200-250
300-400
Particle size d. nm

FIG. 5

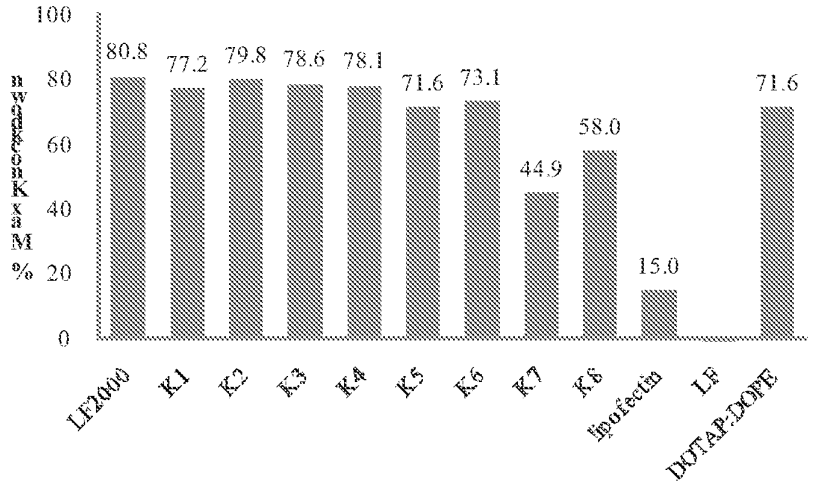

100
80
60
40
20
0
% mRNA Knockdown
80.8
77.2
79.8
78.6
78.1
71.6
73.1
44.9
58.0
15.0
71.6
LF2000
K1
K2
K3
K4
K5
K6
K7
K8
lipofectin
LF
DOTAP:DOPE

FIG. 6A

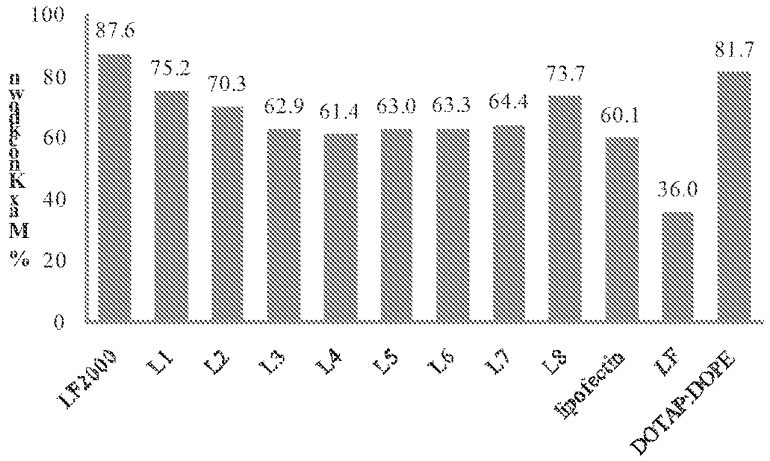

100
80
60
40
20
0
87.6
75.2
70.3
62.9
61.4
63.0
63.3
64.4
73.7
60.1
36.0
81.7
LF2000
L1
L2
L3
L4
L5
L6
L7
L8
lipofectin
LF
DOTAP:DOPE

FIG. 6B

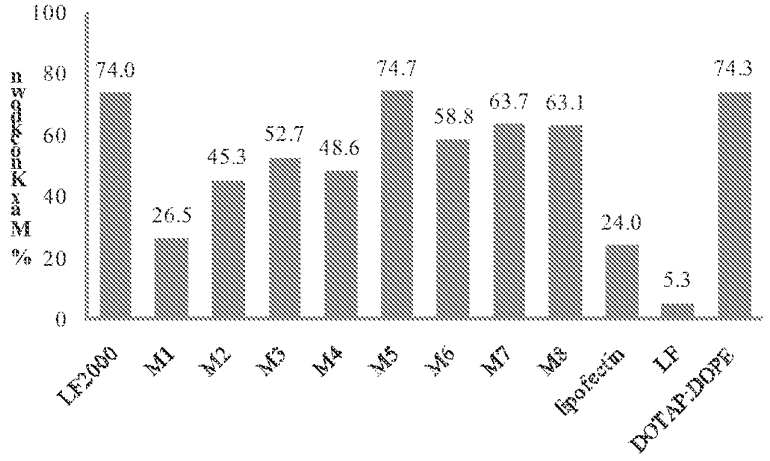

100
80
60
40
20
0
74.0
26.5
45.3
52.7
48.6
74.7
58.8
63.7
63.1
24.0
5.3
74.3
LF2000
M1
M2
M3
M4
M5
M6
M7
M8
lipofectin
LF
DOTAP:DOPE

FIG. 6C

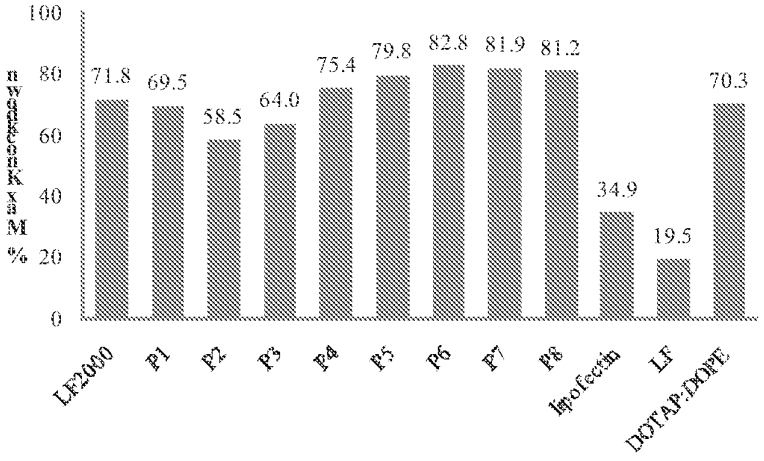

100
80
60
40
20
0
71.8
69.5
58.5
64.0
75.4
79.8
82.8
81.9
81.2
34.9
19.5
70.3
LF2000
P1
P2
P3
P4
P5
P6
P7
P8
lipofectin
LF
DOTAP:DOPE

FIG. 6D

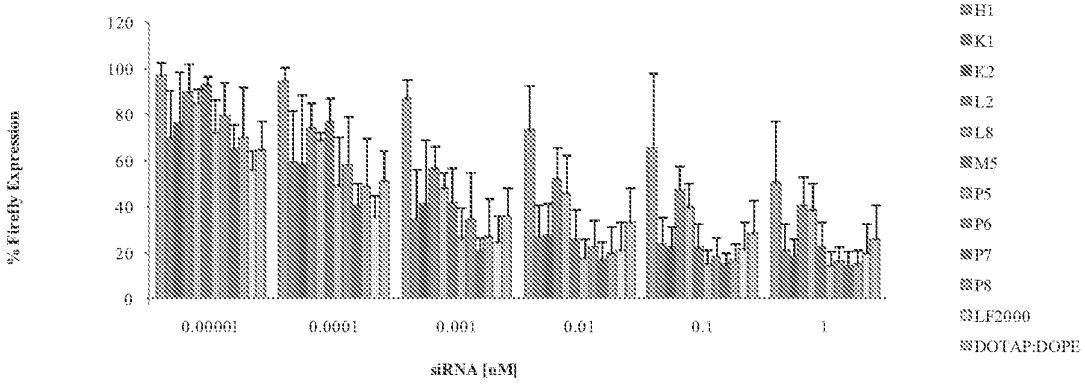

% Firefly Expression
120
100
80
60
40
20
0
0.00001
0.0001
0.001
0.01
0.1
1
siRNA [uM]
H1
K1
E2
L2
L8
M5
P5
P6
P7
P8
LF2000
DOTAP:DOPE

FIG. 7

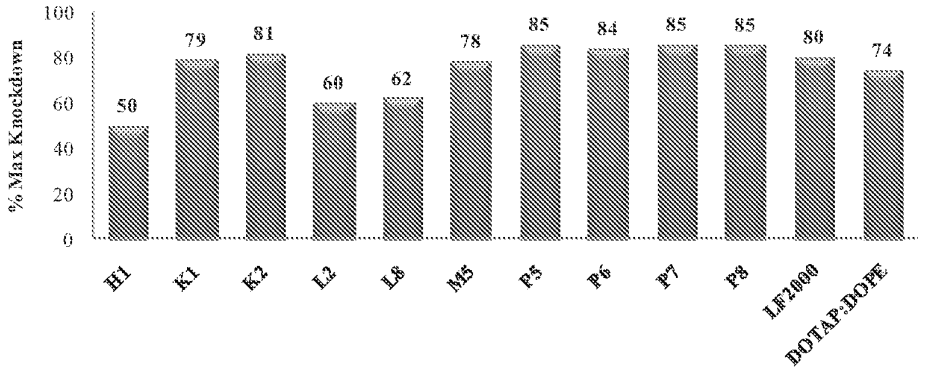

% Max Knockdown
100
80
60
40
20
0
50
79
81
60
62
78
85
84
85
85
80
74
H1
K1
K2
L2
L8
M5
P5
P6
P7
P8
LF2000
DOTAP:DOPE

FIG. 8

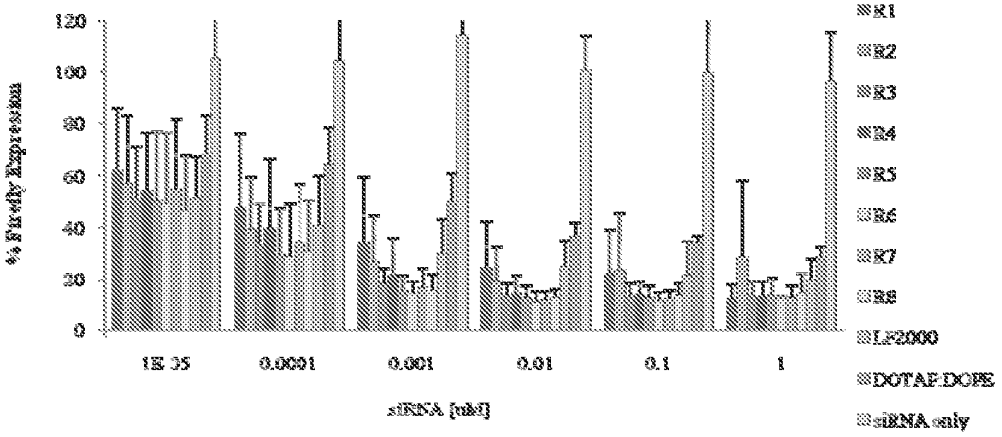

% Firefly Expression
120
100
80
60
40
20
0
1E-05
0.0001
0.001
0.01
0.1
1
siRNA [nM]
R1
R2
R3
R4
R5
R6
R7
R8
LF2000
DOTAP:DOPE
siRNA only

FIG. 9

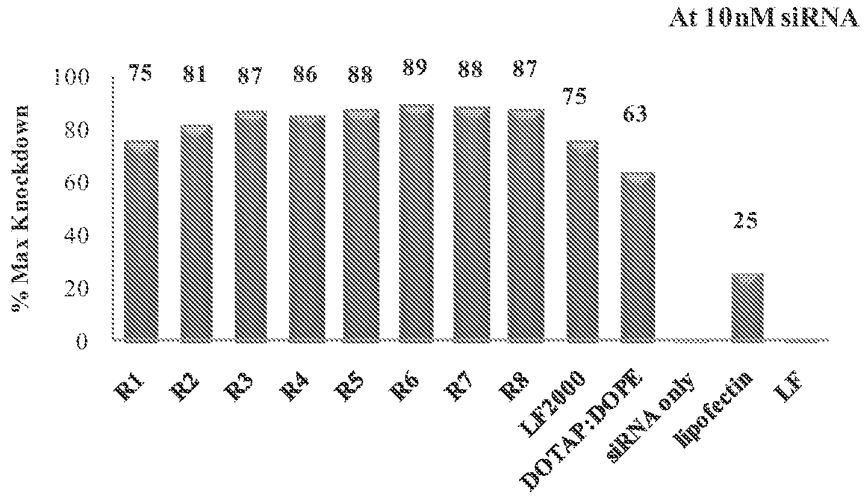

At 10nM siRNA
% Max Knockdown
100
80
60
40
20
0
75
81
87
86
88
89
88
87
75
63
25
R1
R2
R3
R4
R5
R6
R7
R8
LF2000
DOTAP:DOPE
siRNA only
lipofectin
LF

FIG. 10

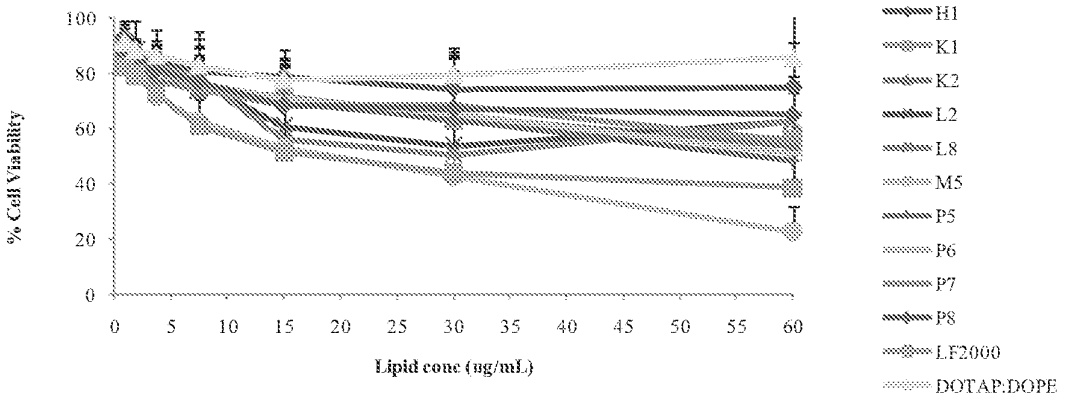

% Cell Viability
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
55
60
Lipid conc (ug/mL)
H1
K1
K2
L2
L8
M5
P5
P6
P7
P8
LF2000
DOTAP:DOPE

FIG. 11

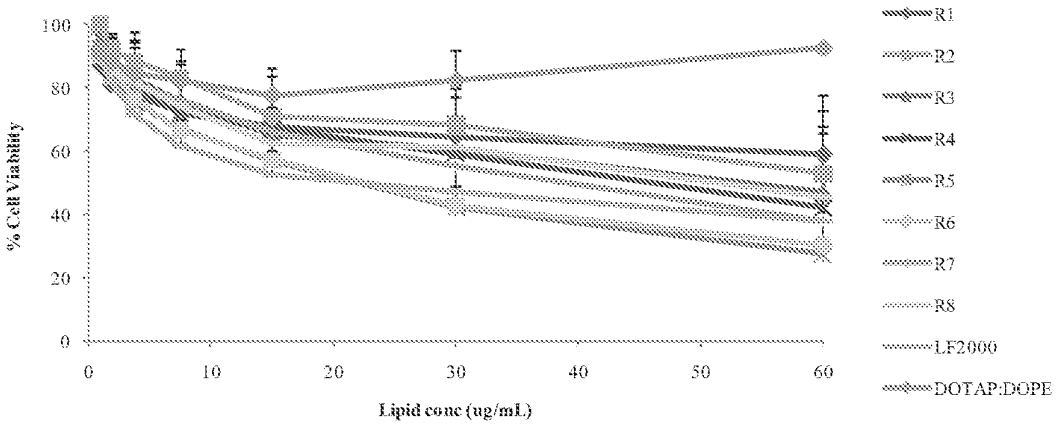

100
80
60
40
20
0
% Cell Viability
0
10
20
30
40
50
60
Lipid conc (ug/mL)
R1
R2
R3
R4
R5
R6
R7
R8
LF2000
DOTAP:DOPE

FIG. 12

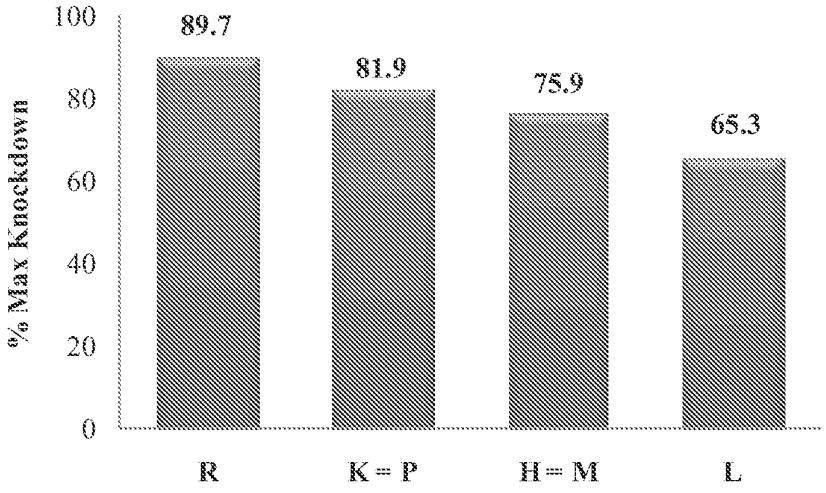

100
80
60
40
20
0
% Max Knockdown
89.7
81.9
75.9
65.3
R
K = P
H = M
L

FIG. 13

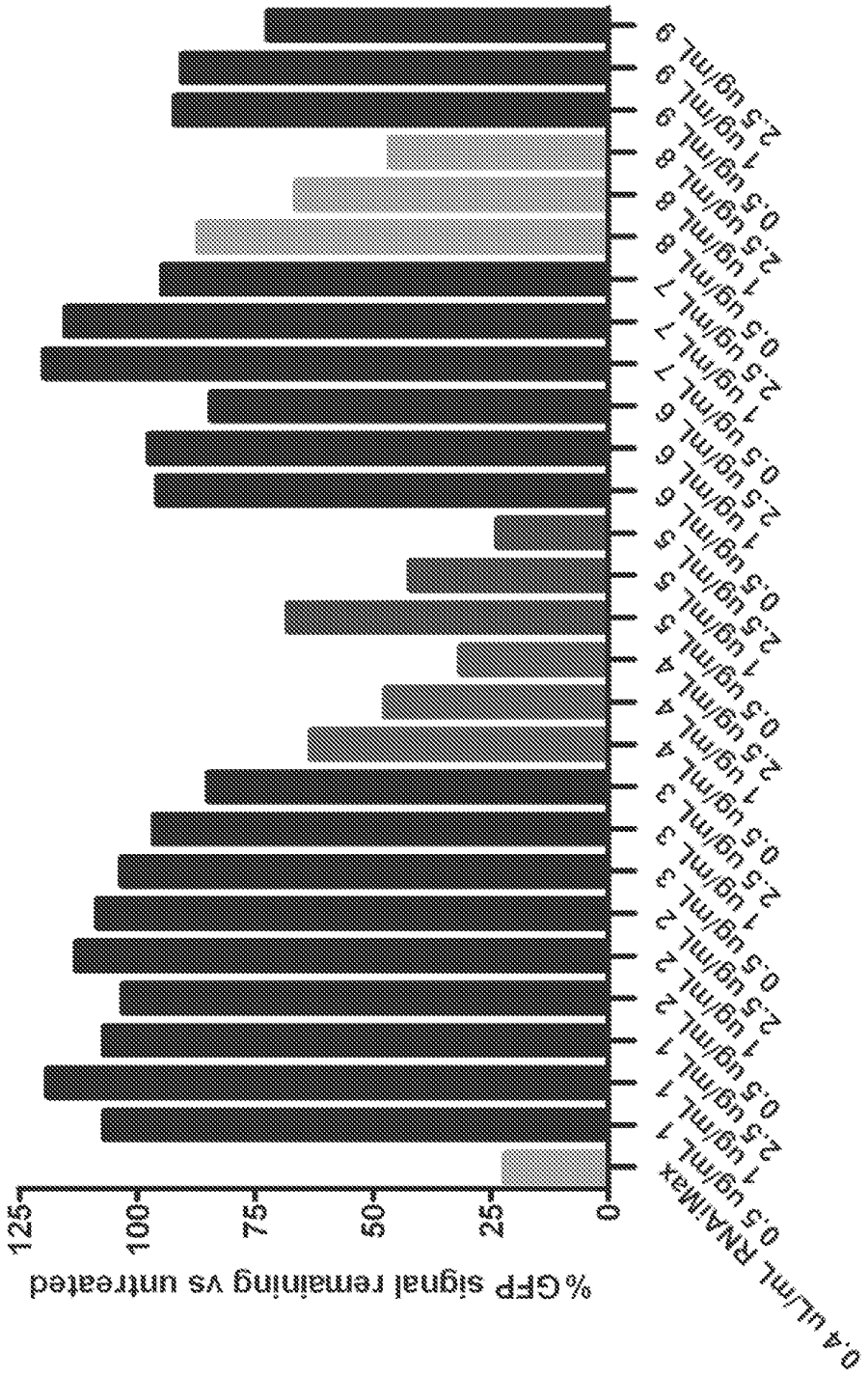

% GFP signal remaining vs untreated
125
100
75
50
25
0
0.4 uL/mL RNAiMax
0.5 ug/mL 1
1 ug/mL 1
2.5 ug/mL 1
0.5 ug/mL 2
1 ug/mL 2
2.5 ug/mL 2
0.5 ug/mL 3
1 ug/mL 3
2.5 ug/mL 3
0.5 ug/mL 4
1 ug/mL 4
2.5 ug/mL 4
0.5 ug/mL 5
1 ug/mL 5
2.5 ug/mL 5
0.5 ug/mL 6
1 ug/mL 6
2.5 ug/mL 6
0.5 ug/mL 7
1 ug/mL 7
2.5 ug/mL 7
0.5 ug/mL 8
1 ug/mL 8
2.5 ug/mL 8
0.5 ug/mL 9
1 ug/mL 9
2.5 ug/mL 9

FIG. 14

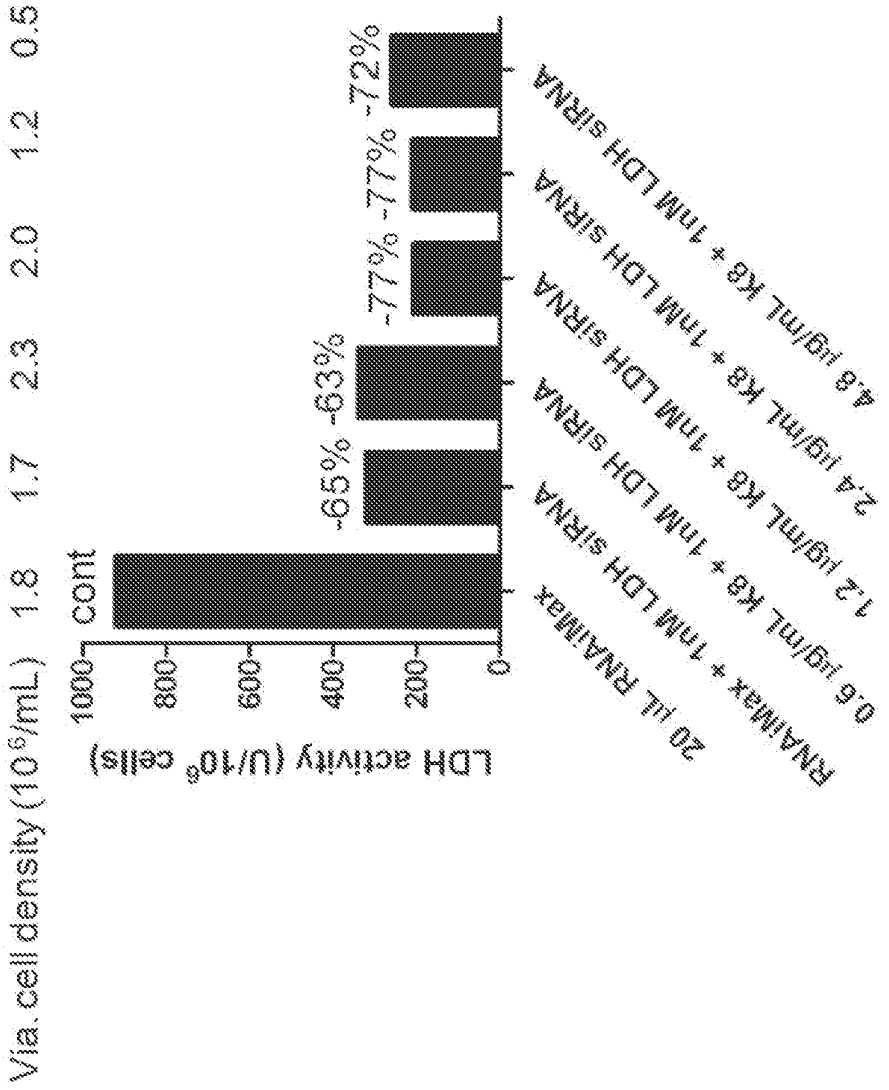

Via. cell density (10^6/mL) 1.8 1.7 2.3 2.0 1.2 0.5
cont
-65% -63% -77% -77% -72%
LDH activity (U/10^6 cells)
1000
800
600
400
200
0
20 µL RNAiMax
RNAiMax + 1nM LDH siRNA
0.6 µg/mL K8 + 1nM LDH siRNA
1.2 µg/mL K8 + 1nM LDH siRNA
2.4 µg/mL K8 + 1nM LDH siRNA
4.8 µg/mL K8 + 1nM LDH siRNA

FIG. 15

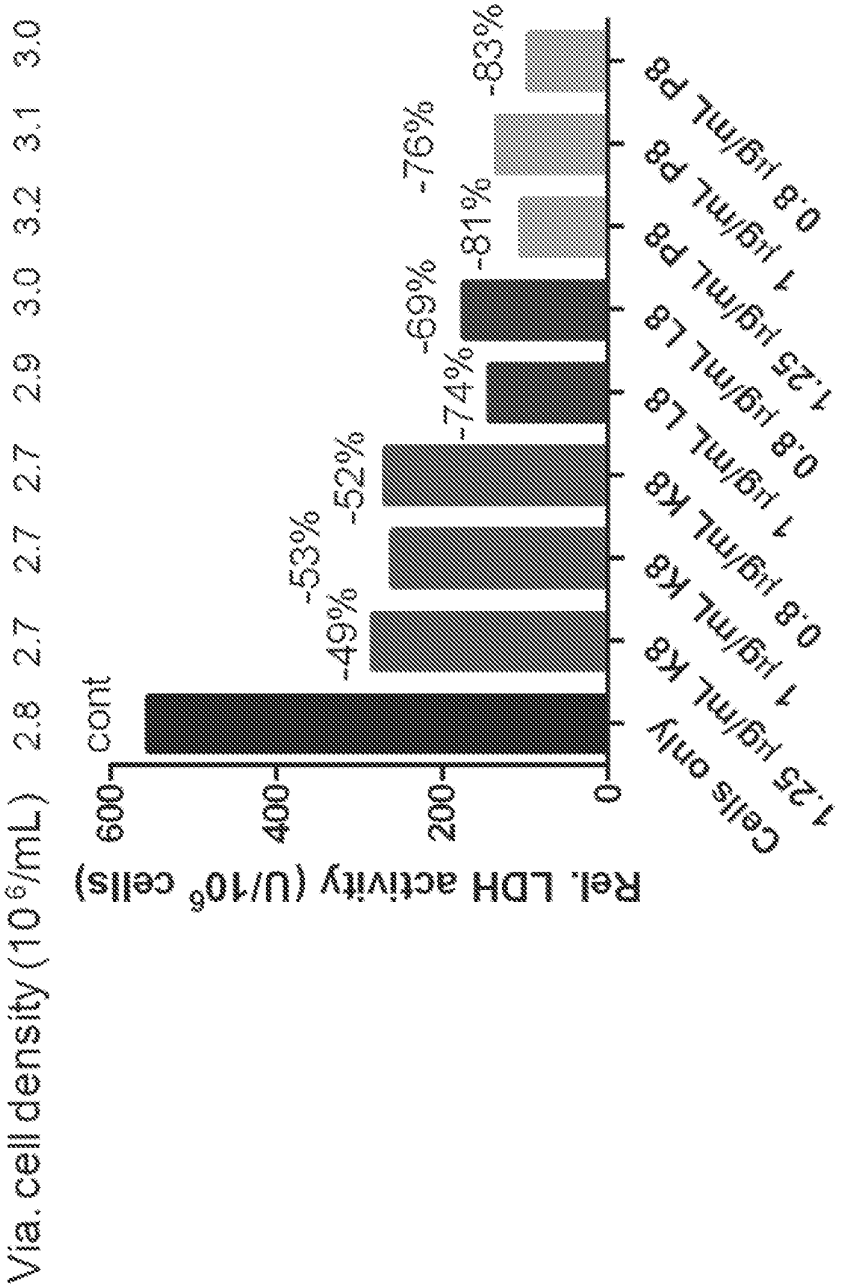

Via. cell density (10^6/mL)
2.8 2.7 2.7 2.7 2.9 3.0 3.2 3.1 3.0
cont
-49% -53% -52% -74% -69% -81% -76% -83%
Rel. LDH activity (U/10^6 cells)
600
400
200
0
Cells only
4.25 μg/mL K8
1 μg/mL K8
0.8 μg/mL K8
1 μg/mL L8
0.8 μg/mL L8
1.25 μg/mL L8
1 μg/mL P8
0.8 μg/mL P8

FIG. 16

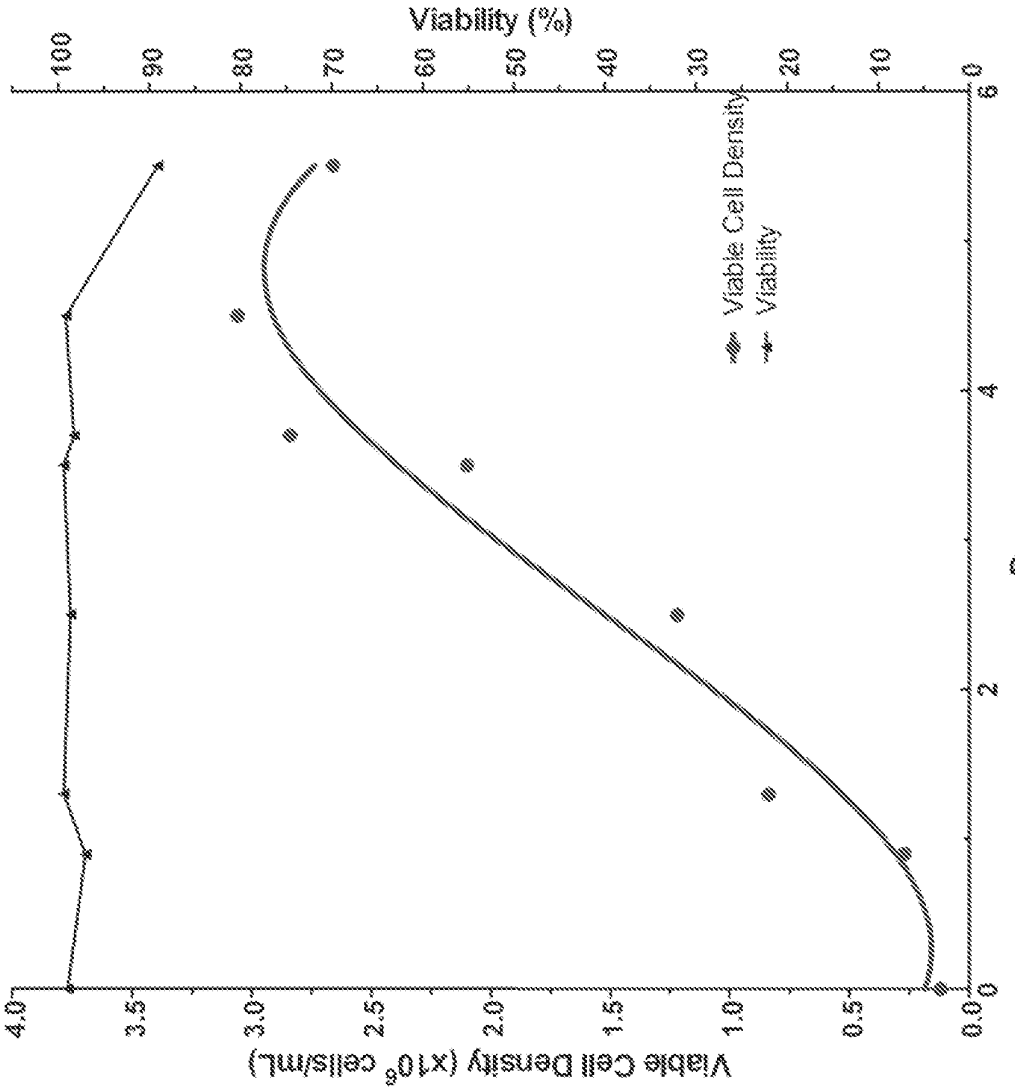

Viability (%)
100
90
80
70
60
50
40
30
20
10
0
Viable Cell Density
Viability
Viable Cell Density (x10^6 cells/mL)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
Days

FIG. 17

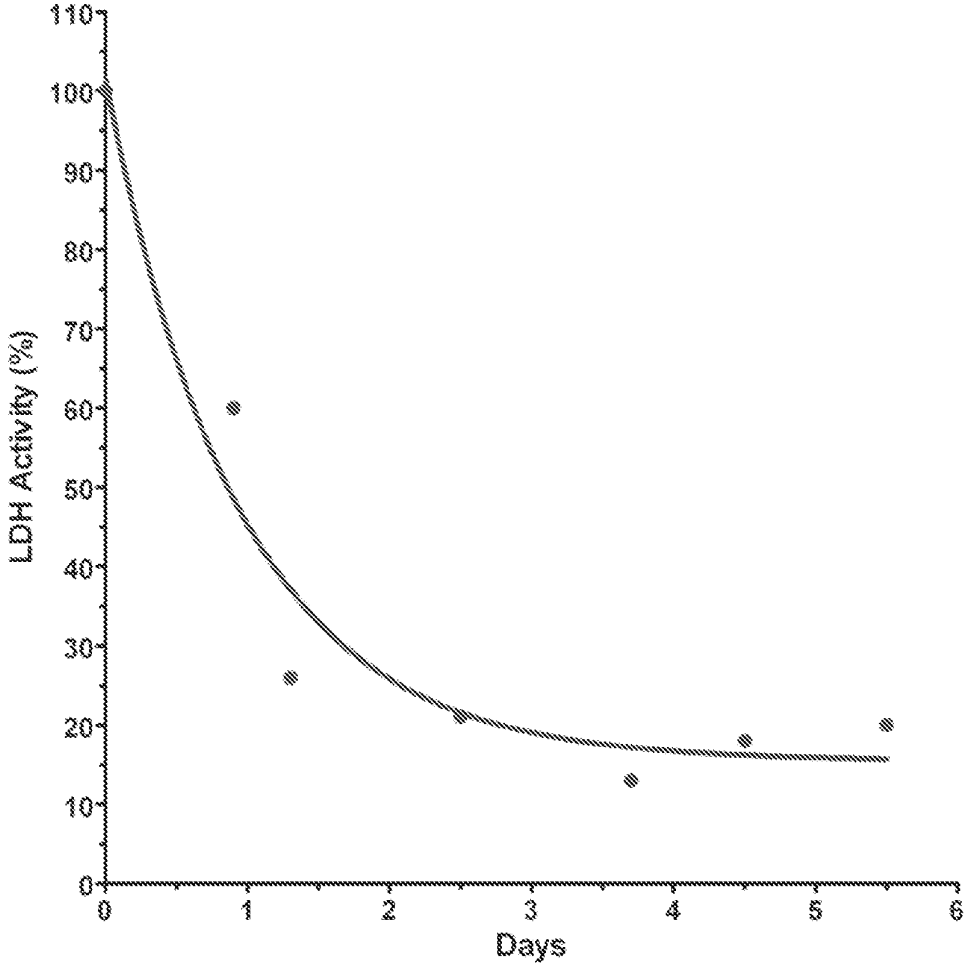


FIG. 18

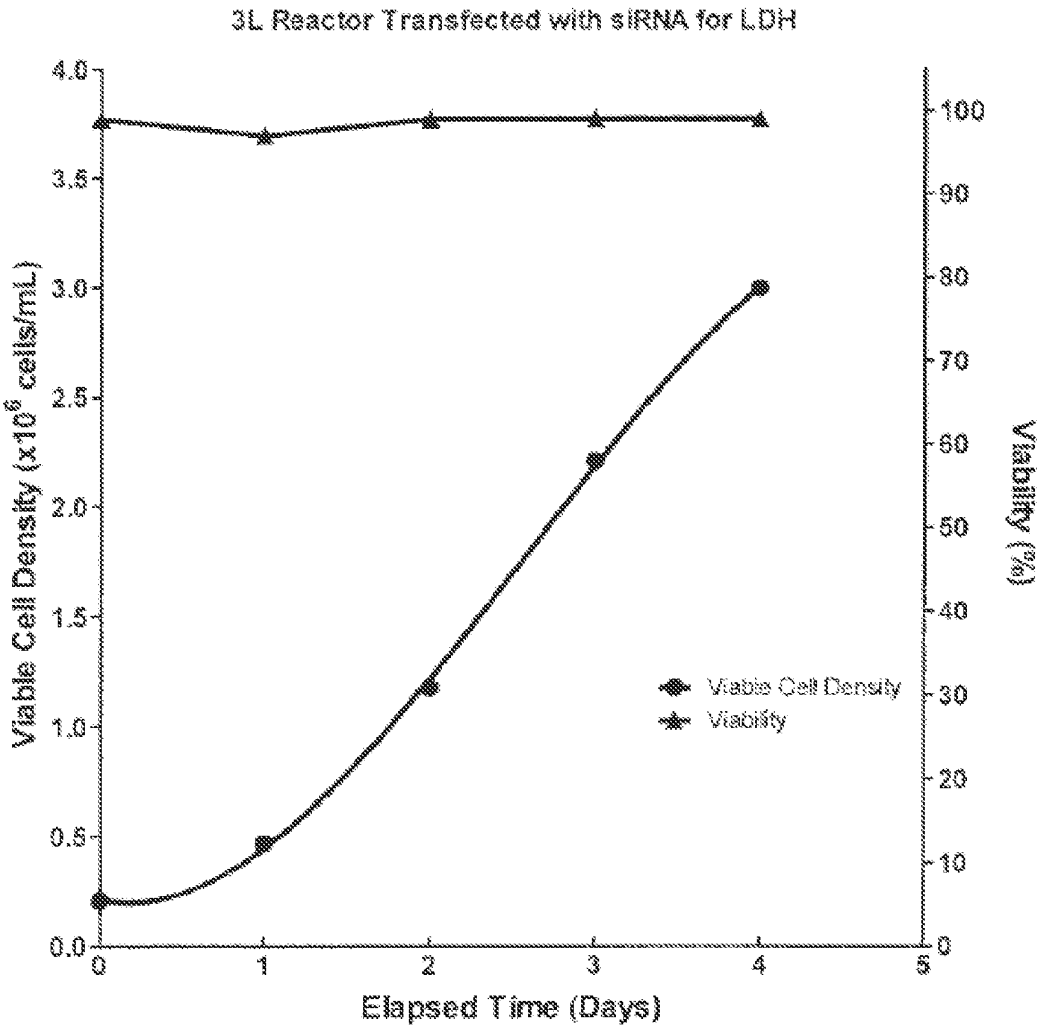

3L Reactor Transfected with siRNA for LDH
Viable Cell Density (x10^6 cells/mL)
Viability (%)
Elapsed Time (Days)
Viable Cell Density
Viability
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
10
20
30
40
50
60
70
80
90
100
0
1
2
3
4
5

FIG. 19

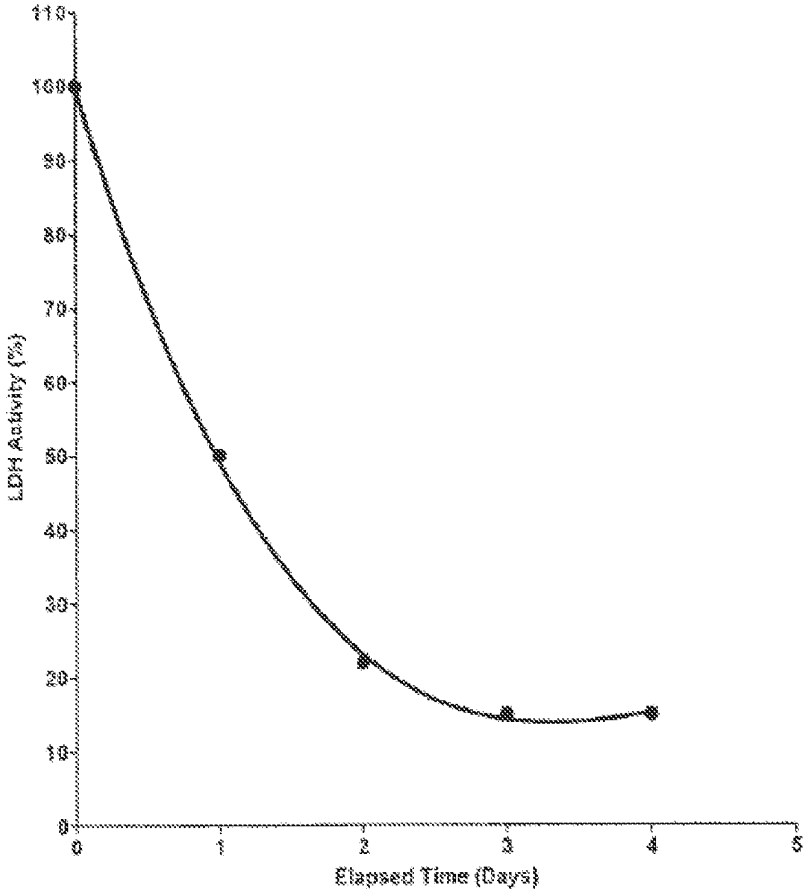

LDH Activity (%)
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Elapsed Time (Days)

FIG. 20