According to some ~~embodiment~~examples, the LNPs described by the present disclosure range in mean diameter from about 20 nm to about 75 nm, about 20 nm to about 70 nm, from about 20 nm to about 60 nm, from about 30 nm to about 75 nm, about 30 nm to about 70 nm, from about 30 nm to about 60 nm, from about 40 nm to 75 nm, or from about 40 nm to 70 nm. Smaller size LNPs provide more efficient tissue diffusion, and more efficient uptake and/or targeting. Particularly in the liver, LNPs of a smaller size are needed to pass liver sinusoidal endothelial cells (LSEC) fenestrae (< 100 nm) and to undergo ASGPR-mediated endocytosis (≤ 70 nm). Such smaller size is also advantageous is in targeting and circumventing unwanted immune responses as they can readily evade immune cells. The formulation process and methods described by the present disclosure can encapsulate considerably more therapeutic nucleic acid (*e.g.,* rigid double stranded DNA including ceDNA) than has been previously reported. The LNPs described herein can encapsulate greater than about 60% to about 90% of rigid double stranded DNA, like ceDNA. According to some ~~embodiment~~examples, the LNPs described herein can encapsulate greater than about 60% of rigid double stranded DNA, like ceDNA, greater than about 65% of rigid double stranded DNA, like ceDNA, greater than about 70% of rigid double stranded DNA, like ceDNA, greater than about 75% of rigid double stranded DNA, like ceDNA, greater than about 80% of rigid double stranded DNA, like ceDNA, greater than about 85% of rigid double stranded DNA, like ceDNA, or greater than about 90% of rigid double stranded DNA, like ceDNA.

The formulation process described herein takes advantage of the finding that ceDNA compaction occurs in solvents with 80% to 100% of low molecular weight (LMW) alcohol. The LMW alcohol that can be used for compaction includes, but is not limited to, methanol, ethanol, propanol, isopropanol, butanol or other organic solvent like acetone. Preferably, the compaction of a rigid DNA like ceDNA can be prepared using an ethanolic solution or ethanol-methanol mixture (*e.g.*, EtOH-MeOH 1:1 mixture) at the final concentration of about 80% to about 98%. According to some ~~embodiment~~examples, the final concentration of the low molecular weight alcohol in the solution is between about 80% to about 98%, about 80% to about 95%, about 80% to about 92%, about 80% to about 90%, about 80% to about 85%, about 85% to about 98%, about 85% to about 95%, about 85% to about 92%, about 85% to about 90%, about 90% to about 98%, about 87% to about 97%, about , about 87% to about 95%, about 87% to about 92%, about 87% to about 90%, about 90% to about 95%, about 90% to about 92%, about 95% to about 98%, or about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, or about 98%. For example, when ceDNA in aqueous 90% EtOH is added to or mixed with another ethanolic solution (*e.g.,* 90% EtOH) of lipids in a ratio such that the resulting solution is, for example, 90-92% ethanol and 8-10% water or aqueous buffer, the ceDNA is observed to exist in a highly compacted or denatured state by dynamic light scattering. In such a solvent (*e.g.,* 90-92% ethanol, 8-10% water), both the lipids and

ceDNA are solubilized with no detectable precipitation of either component, leading to successful and more efficient encapsulation of a rigid double stranded DNA like ceDNA into smaller sizes of LNP.

Accordingly, the formulation process described herein reduces the LNP diameter, while maintaining similar or better encapsulation efficiency for rigid TNA like ceDNA relative to the standard process. Without wishing to be bound by theory, this change may likely be attributed to compaction of rigid TNA like ceDNA preferably in 90-92% or up to 95% in an LMW alcohol solution such as ethanol solvent *prior to* formation of LNPs. When LNP formation is then initiated by mixing with the acidic aqueous buffer solution, the lipids are able to nucleate around a smaller and compact DNA (*e.g.,* ceDNA) core as opposed to the standard aqueous process, resulting in significantly smaller particles. Using the process described herein, a rigid TNA like ceDNA can be efficiently encapsulated at a higher number, resulting in TNA-LNPs with much smaller diameters that are beneficial attributes of LNPs to target various tissues that pose size constraints.

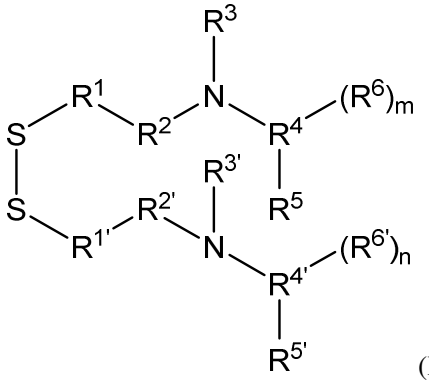
In some ~~embodiment~~examples, a formulation comprises TNA (*e.g.,* ceDNA) encapsulated in LNPs having a mean diameter of about 75 nm (± 3 nm). In some ~~embodiment~~examples, a formulation comprises TNA (*e.g.,* ceDNA) encapsulated in LNPs having a mean diameter of about 72 nm (±3 nm). In some ~~embodiment~~examples, a formulation comprises TNA (*e.g.,* ceDNA) encapsulated in LNPs having a mean diameter of about 70 nm (±4 nm). In some ~~embodiment~~examples, a formulation comprises TNA (*e.g.,* ceDNA) encapsulated in LNPs having a mean diameter of about 68 nm (±4 nm). In some ~~embodiment~~examples, a formulation comprises TNA (*e.g.,* ceDNA) encapsulated in LNPs having a mean diameter of about 65 nm (±4 nm). In some ~~embodiment~~examples, a formulation comprises TNA (*e.g.,* ceDNA) encapsulated in LNPs having a mean diameter of about 60 nm (±4 nm). In some ~~embodiment~~examples, a formulation comprises TNA (*e.g.,* ceDNA) encapsulated in LNPs having a mean diameter of about 55 nm (±4 nm). In some ~~embodiment~~examples, a formulation comprises TNA (*e.g.,* ceDNA) encapsulated in LNPs having a mean diameter of about 50 nm (±4 nm).

According to a first aspect, the disclosure provides a pharmaceutical composition comprising lipid nanoparticle (LNP), wherein the LNP comprises a lipid and a rigid nucleic acid therapeutic (rTNA), wherein the mean diameter of the LNP is between about 20 nm and about 75 nm. According to another aspect, the present disclosure provides a pharmaceutical composition comprising a lipid nanoparticle (LNP), wherein the LNP comprises a lipid and denatured therapeutic nucleic acid (TNA), wherein the LNP is prepared by a method comprising: adding aqueous TNA to one or more low molecular weight alcohol solution comprising ionizable lipids to form a TNA/lipid solution, wherein the final concentration of the low molecular weight alcohol in the solution is between about 80% to about 98%; mixing the TNA/lipid solution with an acidic aqueous buffer; and buffer exchanging with a neutral-pH aqueous buffer, thereby producing the LNP formulation.

According to some ~~embodiment~~examples, the rigid nucleic acid therapeutic is a double stranded nucleic acid. According to some ~~embodiment~~examples, the rigid nucleic acid therapeutic is a closed ended DNA.

According to some ~~embodiment~~examples, the lipid is selected from an ionizable lipid, a non-cationic lipid, a sterol or a derivative thereof, a conjugated lipid, or any combination thereof. According to some ~~embodiment~~examples, the ionizable lipid is a cationic lipid. According to some ~~embodiment~~examples, the cationic lipid is an SS-cleavable lipid.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the ionizable lipid is represented by Formula (I):



(I),

or a pharmaceutically acceptable salt thereof, wherein:

R^1 and $R^{1'}$ are each independently optionally substituted linear or branched C_{1-3} alkylene;

R^2 and $R^{2'}$ are each independently optionally substituted linear or branched C_{1-6} alkylene;

R^3 and $R^{3'}$ are each independently optionally substituted linear or branched C_{1-6} alkyl;

or alternatively, when R^2 is optionally substituted branched C_{1-6} alkylene, R^2 and R^3, taken together with their intervening N atom, form a 4- to 8-membered heterocyclyl;

or alternatively, when $R^{2'}$ is optionally substituted branched C_{1-6} alkylene, $R^{2'}$ and $R^{3'}$, taken together with their intervening N atom, form a 4- to 8-membered heterocyclyl;

R^4 and $R^{4'}$ are each independently $–CR^a$, $–C(R^a)_2CR^a$, or $–[C(R^a)_2]_2CR^a$;

R^a, for each occurrence, is independently H or C_{1-3} alkyl;

or alternatively, when R^4 is $–C(R^a)_2CR^a$, or $–[C(R^a)_2]_2CR^a$ and when R^a is C_{1-3} alkyl, R^3 and R^4, taken together with their intervening N atom, form a 4- to 8-membered heterocyclyl;

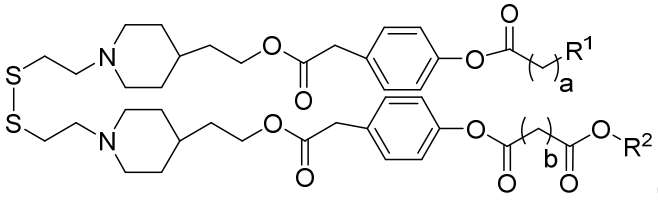
or alternatively, when $R^{4'}$ is $–C(R^a)_2CR^a$, or $–[C(R^a)_2]_2CR^a$ and when R^a is C_{1-3} alkyl, $R^{3'}$ and $R^{4'}$, taken together with their intervening N atom, form a 4- to 8-membered heterocyclyl;

R^5 and $R^{5'}$ are each independently C_{1-20} alkylene or C_{2-20} alkenylene;

R^6 and $R^{6'}$, for each occurrence, are independently C_{1-20} alkylene, C_{3-20} cycloalkylene, or C_{2-20} alkenylene; and

m and n are each independently an integer selected from 1, 2, 3, 4, and 5.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the ionizable lipid is represented by Formula (II):



(II);

or a pharmaceutically acceptable salt thereof, wherein:

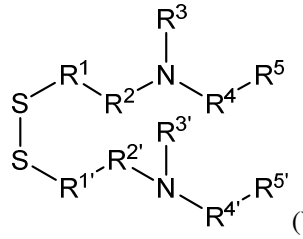
a is an integer ranging from 1 to 20;

b is an integer ranging from 2 to 10;

R^1 is absent or is selected from $(C_2\text{-}C_{20})$alkenyl, $-C(O)O(C_2\text{-}C_{20})$alkyl, and cyclopropyl substituted with $(C_2\text{-}C_{20})$alkyl; and

R^2 is $(C_2\text{-}C_{20})$alkyl.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the ionizable lipid is represented by the Formula (V):



(V);

or a pharmaceutically acceptable salt thereof, wherein:

R^1 and $R^{1'}$ are each independently $(C_1\text{-}C_6)$alkylene optionally substituted with one or more groups selected from R^a;

R^2 and $R^{2'}$ are each independently $(C_1\text{-}C_2)$alkylene;

R^3 and $R^{3'}$ are each independently $(C_1\text{-}C_6)$alkyl optionally substituted with one or more groups selected from R^b;

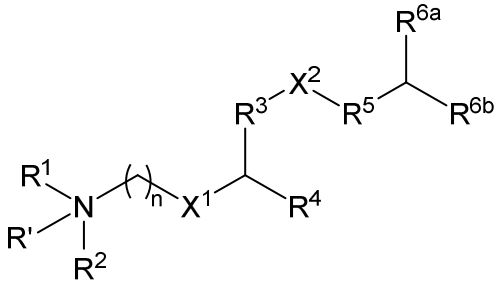
or alternatively, R^2 and R^3 and/or $R^{2'}$ and $R^{3'}$ are taken together with their intervening N atom to form a 4- to 7-membered heterocyclyl;

R^4 and $R^{4'}$ are each a $(C_2\text{-}C_6)$alkylene interrupted by –C(O)O-;

R^5 and $R^{5'}$ are each independently a $(C_2\text{-}C_{30})$alkyl or $(C_2\text{-}C_{30})$alkenyl, each of which are optionally interrupted with –C(O)O- or $(C_3\text{-}C_6)$cycloalkyl; and

R^a and R^b are each halo or cyano.

According to some ~~embodiment~~examples, the ionizable lipid is represented by Formula (XV):



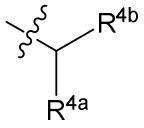
(XV)

or a pharmaceutically acceptable salt thereof, wherein:

R' is absent, hydrogen, or $C_1\text{-}C_6$ alkyl; provided that when R' is hydrogen or $C_1\text{-}C_6$ alkyl, the nitrogen atom to which R', R^1, and R^2 are all attached is protonated;

R^1 and R^2 are each independently hydrogen, C_1-C_6 alkyl, or C_2-C_6 alkenyl;

R^3 is C_1-C_{12} alkylene or C_2-C_{12} alkenylene;

R^4 is C_1-C_{16} unbranched alkyl, C_2-C_{16} unbranched alkenyl, or  ; wherein:

R^{4a} and R^{4b} are each independently C_1-C_{16} unbranched alkyl or C_2-C_{16} unbranched alkenyl;

R^5 is absent, C_1-C_8 alkylene, or C_2-C_8 alkenylene;

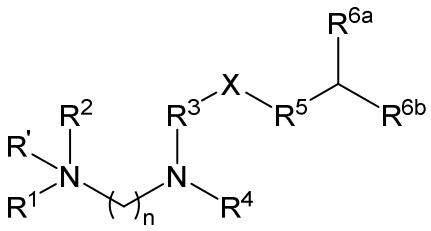
R^{6a} and R^{6b} are each independently C_7-C_{16} alkyl or C_7-C_{16} alkenyl; provided that the total number of carbon atoms in R^{6a} and R^{6b} as combined is greater than 15;

X^1 and X^2 are each independently -OC(=O)-, -SC(=O)-, -OC(=S)-, -C(=O)O-, -C(=O)S-, -S-S-, -C(R^a)=N-, -N=C(R^a)-, -C(R^a)=NO-, -O-N=C(R^a)-, -C(=O)NR^a-, -NR^aC(=O)-, -NR^aC(=O)NR^a-, -OC(=O)O-, -OSi(R^a)$_2$O-, -C(=O)(CR^a_2)C(=O)O-, or OC(=O)(CR^a_2)C(=O)-; wherein:

R^a, for each occurrence, is independently hydrogen or C_1-C_6 alkyl; and

n is an integer selected from 1, 2, 3, 4, 5, and 6.

According to some ~~embodiment~~examples, the ionizable lipid is represented by Formula (XX):



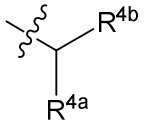
(XX)

or a pharmaceutically acceptable salt thereof, wherein:

R' is absent, hydrogen, or C_1-C_3 alkyl; provided that when R' is hydrogen or C_1-C_3 alkyl, the nitrogen atom to which R', R^1, and R^2 are all attached is protonated;

R^1 and R^2 are each independently hydrogen or C_1-C_3 alkyl;

R^3 is C_3-C_{10} alkylene or C_3-C_{10} alkenylene;

R^4 is C_1-C_{16} unbranched alkyl, C_2-C_{16} unbranched alkenyl, or  ; wherein:

R^{4a} and R^{4b} are each independently C_1-C_{16} unbranched alkyl or C_2-C_{16} unbranched alkenyl;

R^5 is absent, C_1-C_6 alkylene, or C_2-C_6 alkenylene;

R^{6a} and R^{6b} are each independently C_7-C_{14} alkyl or C_7-C_{14} alkenyl;

X is -OC(=O)-, -SC(=O)-, -OC(=S)-, -C(=O)O-, -C(=O)S-, -S-S-, -C(R^a)=N-,

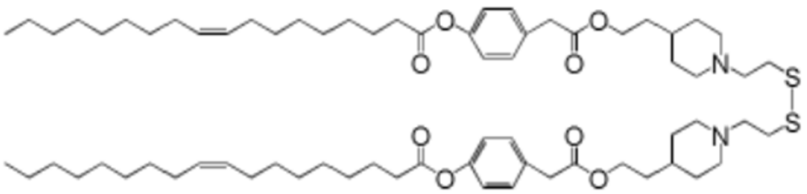
-N=C(R^a)-, -C(R^a)=NO-, -O-N=C(R^a)-, -C(=O)NR^a-, -NR^aC(=O)-, -NR^aC(=O)NR^a-, -OC(=O)O-, -OSi(R^a)$_2$O-, -C(=O)(CR^a_2)C(=O)O-, or OC(=O)(CR^a_2)C(=O)-; wherein:

R^a, for each occurrence, is independently hydrogen or C_1-C_6 alkyl; and

n is an integer selected from 1, 2, 3, 4, 5, and 6.

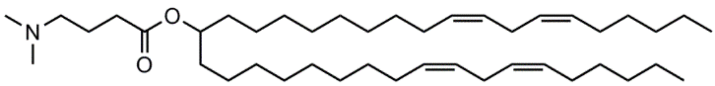
According to some ~~embodiment~~examples, the ionizable lipid is selected from any lipid in Table 2, Table 5, Table 6, Table 7, or Table 8.

According to some ~~embodiment~~examples, the ionizable lipid is a lipid having the structure:



or a pharmaceutically acceptable salt thereof.

According to some ~~embodiment~~examples, the cationic lipid is MC3 (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate (DLin-MC3-DMA or MC3) having the following structure:



DLin-M-C3-DMA ("MC3")

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the LNP further comprises a sterol. According to some ~~embodiment~~examples, the sterol is a cholesterol.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the LNP further comprises a polyethylene glycol (PEG). According to some ~~embodiment~~examples, the PEG is l-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG).

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the LNP further comprises a non-cationic lipid. According to some ~~embodiment~~examples, the non-cationic lipid is selected from the group consisting of distearoyl-sn-glycero-phosphoethanolamine, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE),

distearoyl-phosphatidyl-ethanolamine (DSPE), monomethyl-phosphatidylethanolamine (such as 16-O-monomethyl PE), dimethyl-phosphatidylethanolamine (such as 16-O-dimethyl PE), 18-1-trans PE, 1-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), hydrogenated soy phosphatidylcholine (HSPC), egg phosphatidylcholine (EPC), dioleoylphosphatidylserine (DOPS), sphingomyelin (SM), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), distearoylphosphatidylglycerol (DSPG), dierucoylphosphatidylcholine (DEPC), palmitoyloleyolphosphatidylglycerol (POPG), dielaidoyl-phosphatidylethanolamine (DEPE), 1,2-dilauroyl-sn-glycero-3 -pho sphoethanolamine (DLPE); 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (DPHyPE); lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidicacid,cerebrosides, dicetylphosphate, lysophosphatidylcholine, dilinoleoylphosphatidylcholine, or mixtures thereof.

According to some ~~embodiment~~examples, the non-cationic lipid is selected from the group consisting of dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), and dioleoyl-phosphatidylethanolamine (DOPE).

According to some ~~embodiment~~examples, the PEG or PEG-lipid conjugate is present at about 1.5% to about 3%.

According to some ~~embodiment~~examples, the cholesterol is present at a molar percentage of about 20% to about 40%, and wherein the lipid is present at a molar percentage of about 80% to about 60%.

According to some ~~embodiment~~examples, the cholesterol is present at a molar percentage of about 40%, and wherein the lipid is present at a molar percentage of about 50%.

According to some ~~embodiment~~examples, the composition further comprises a cholesterol, a PEG or PEG-lipid conjugate, and a non-cationic lipid.

According to some ~~embodiment~~examples, the PEG or PEG-lipid conjugate is present at about 1.5% to about 3%, about 1.5% to about 2.75%, about 1.5% to about 2.5%, about 1.5% to about 2%, about 2% to about 3%, about 2% to about 2.75%, about 2% to about 2.5%, about 2.5% to about 3% about 2.5% to about 2.75%, or about 2.5% to about 3%.

According to some ~~embodiment~~examples, the cholesterol is present at a molar percentage of about 30% to about 50%, about 30% to about 45%, about 30% to about 40%, about 30% to about 35%, about 35% to about 40%, about 35% to about 45%, about 35% to about 50%, about 40% to about 45%, about 40% to about 50%, or about 45% to about 50%.

According to some ~~embodiment~~examples, the lipid is present at a molar percentage of about 42.5% to about 62.5%, about 42.5% to about 57.5%, about 42.5% to about 52.5%, about 42.5% to about 47.5%, about 47.5% to about 62.5%, about 47.5% to about 57.5%, about 47.5% to about 52.5%, about 52.5% to about 62.5%, about 52.5% to about 57.5%, or about 57.5% to about 62.5%.

According to some ~~embodiment~~examples, the non-cationic lipid is present at a molar percentage of about 2.5% to about 12.5%, about 2.5% to about 10.5%, about 2.5% to about 8.5%, about 2.5% to

about 6.5%, about 2.5% to about 4.5%, about 4.5% to about 12.5%, about 4.5% to about 10.5%, about 4.5% to about 8.5%, about 4.5% to about 6.5%, about 6.5% to about 12.5%, about 6.5% to about 10.5%, about 6.5% to about 8.5%, about 8.5% to about 12.5%, about 8.5% to about 10.5%, or about 10.5% to about 12.5%.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples herein, the cholesterol is present at a molar percentage of about 40%, the lipid is present at a molar percentage of about 52.5%, the non-cationic lipid is present at a molar percentage of about 7.5%, and wherein the PEG is present at about 3%.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition further comprises dexamethasone palmitate.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the LNP is less than about 75 nm in size. According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the LNP is less than about 70 nm in size, for example less than about 65 nm, less than about 60 nm, less than about 55 nm, less than about 50 nm, less than about 45 nm, less than about 40 nm, less than about 35 nm, less than about 30 nm, less than about 25 nm, less than about 20 nm, less than about 15 nm, or less than about 10 nm in size. According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the LNP is less than about 70 nm, 69 nm, 68 nm, 67 nm, 66 nm, 65 nm, 64 nm, 63 nm, 62 nm, 61 nm, 60 nm, 59 nm, 58 nm, 57 nm, 56 nm, 55 nm, 54 nm, 53 nm, 52 nm, 51 nm, or 50 nm in size.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of about 15:1.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of about 30:1.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of about 40:1.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of about 50:1.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of between about 15:1 to about 30: 1. According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of between about 15:1 to about 40: 1. According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of between about 15:1 to about 50: 1. According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of between about 30:1 to about 40: 1. According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of between about 30:1 to about 50: 1.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of between about 40:1 to about 50: 1.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the composition further comprises N-Acetylgalactosamine (GalNAc). According to some ~~embodiment~~examples, the GalNAc is present in the LNP at a molar percentage of 0.5% of the total lipid. According to some ~~embodiment~~examples, the GalNAc is present in the LNP at a molar percentage of between about 0.3% to about 0.9%, between about 0.4% to about 0.8%, between about 0.5% to about 0.6% of the total lipid.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the rigid therapeutic nucleic acid (rTNA)is closed-ended DNA (ceDNA).

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the rigid therapeutic nucleic acid (rTNA)comprises an expression cassette comprising a promoter sequence and a transgene.

According to some ~~embodiment~~examples, the rigid therapeutic nucleic acid (rTNA)comprises an expression cassette comprising a polyadenylation sequence.

According to some ~~embodiment~~examples, the rigid therapeutic nucleic acid (rTNA) comprises at least one inverted terminal repeat (ITR) flanking either the 5’ or the 3’ end of said expression cassette.

According to some ~~embodiment~~examples, the expression cassette is flanked by two ITRs, wherein the two ITRs comprise one 5’ ITR and one 3’ ITR.

According to some ~~embodiment~~examples, the expression cassette is connected to an ITR at a 3’ end (3’ ITR). According to some ~~embodiment~~examples, the expression cassette is connected to an ITR at a 5’ end (5’ ITR).

According to some ~~embodiment~~examples, at least one of the 5’ ITR or the 3’ ITR is a wild-type AAV ITR. According to some ~~embodiment~~examples, at least one of a 5’ ITR and a 3’ ITR is a modified ITR.

According to some ~~embodiment~~examples, the rigid therapeutic nucleic acid (rTNA) further comprises a spacer sequence between a 5’ ITR and the expression cassette.

According to some ~~embodiment~~examples, the rigid therapeutic nucleic acid (rTNA) further comprises a spacer sequence between a 3’ ITR and the expression cassette.

According to some ~~embodiment~~examples, the spacer sequence is at least 5 base pairs long. According to some ~~embodiment~~examples, the spacer sequence is 5 to 100 base pairs long. According to some ~~embodiment~~examples, the spacer sequence is 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 base pairs long. According to some ~~embodiment~~examples, the spacer sequence is 5 to 500 base pairs long. According to some ~~embodiment~~examples, the spacer sequence is 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, or 500 base pairs long.

According to some ~~embodiment~~examples, the rigid therapeutic nucleic acid (rTNA) has a nick or a gap.

According to some ~~embodiment~~examples, the ITR is an ITR selected from an ITR derived from an AAV serotype, an ITR derived from an ITR of goose virus, an ITR derived from a B19 virus ITR, or a wild-type ITR from a parvovirus.

According to some ~~embodiment~~examples, said AAV serotype is selected from the group consisting of: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 and AAV12.

According to some ~~embodiment~~examples, the ITR is a mutant ITR, and the ceDNA optionally comprises an additional ITR which differs from the first ITR.

According to some ~~embodiment~~examples, the ceDNA comprises two mutant ITRs in both 5' and 3' ends of the expression cassette, optionally wherein the two mutant ITRs are symmetric mutants.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the rigid therapeutic nucleic acid (rTNA) is selected from the group consisting of minigenes, plasmids, minicircles, small interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotides (ASO), ribozymes, ceDNA, ministring, doggybone™, protelomere closed ended DNA, or dumbbell linear DNA, dicer-substrate dsRNA, small hairpin RNA (shRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, DNA viral vectors, viral RNA vector, non-viral vector and any combination thereof.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the rigid therapeutic nucleic acid is a plasmid.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the pharmaceutical composition further comprises a pharmaceutically acceptable excipient.

According to another aspect, the disclosure provides a method of producing a lipid nanoparticle (LNP) formulation, wherein the LNP comprises an ionizable lipid and a closed-ended DNA (ceDNA), the method comprising: adding aqueous ceDNA to one or more low molecular weight alcohols solution comprising ionizable lipids, wherein the final concentration of alcohol in the solution is between about 80% to about 98% form a ceDNA/lipid solution; mixing the ceDNA/lipid solution with an acidic aqueous buffer; and buffer exchanging with a neutral-pH aqueous buffer, thereby producing an LNP formulation. In some examples, the disclosure provides a method of producing a lipid nanoparticle (LNP) formulation, wherein the LNP comprises an ionizable lipid and a closed-ended DNA (ceDNA), the method comprising adding aqueous ceDNA to one or more low molecular weight alcohols (*e.g.,* ethanol, methanol, propanol, or isopropanol) solution comprising cationic or ionizable lipids, wherein the final concentration of alcohol in the solution is between about 80% to about 98% form a ceDNA/lipid solution; mixing the ceDNA/lipid solution with an acidic aqueous buffer; and buffer exchanging with a neutral-pH aqueous buffer, thereby producing an LNP formulation. According to some ~~embodiment~~examples, the final concentration of the low molecular weight alcohol in the solution is between about 80% to about 98%, about 80% to about 95%, about 80% to about 92%, about 80% to about 90%, about 80% to about 85%, about 85% to about 98%, about 85% to about 95%, about 85% to about 92%, about 85% to about 90%, about 90% to about 98%, about 87% to about 97%, about , about 87% to about 95%, about 87% to about 92%, about 87% to about 90%, about 90% to about 95%, about 90% to about 92%, about 95% to about 98%, or about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, or about 98%.

According to another aspect, the disclosure provides a method of producing a lipid nanoparticle (LNP) formulation comprising an ionizable lipid and a closed-ended DNA (ceDNA), the method comprising: adding ceDNA to one or more low molecular weight alcohol solution, wherein the alcohol content of the resulting solution is greater than 80%, adding said ceDNA in >80% alcohol content to ionizable lipids in low molecular weight alcohol to form a ceDNA/lipid solution, wherein the final concentration of the low molecular weight alcohol in the ceDNA-lipid solution is between about 80% to about 95%; mixing the ceDNA/lipid solution with an acidic aqueous buffer; and buffer exchanging with a neutral-pH aqueous buffer, thereby producing the LNP formulation. In some examples, the disclosure provides a method of producing a lipid nanoparticle (LNP) formulation comprising an ionizable lipid and a closed-ended DNA (ceDNA), the method comprising adding ceDNA to one or more low molecular weight alcohols (*e.g.,* ethanol, methanol, propanol, or isopropanol) solution, wherein the alcohol content of the resulting solution is greater than 80%, adding said ceDNA in >80% alcohol content to cationic or ionizable lipids in 80% alcohol, wherein the concentration of the low molecular weight alcohol in the ceDNA-lipid solution is between about 80% to about 95% (*e.g.*, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, or 95%) to form a ceDNA/lipid solution; mixing the ceDNA/lipid solution with an acidic aqueous buffer; and buffer exchanging with a neutral-pH aqueous buffer, thereby producing an LNP formulation. According to some ~~embodiment~~examples, the final concentration of the low molecular weight alcohol in the solution is between about 80% to about 98%, about 80% to about 95%, about 80% to about 92%, about 80% to about 90%, about 80% to about 85%, about 85% to about 98%, about 85% to about 95%, about 85% to about 92%, about 85% to about 90%, about 90% to about 98%, about 87% to about 97%, about , about 87% to about 95%, about 87% to about 92%, about 87% to about 90%, about 90% to about 95%, about 90% to about 92%, about 95% to about 98%, or about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, or about 98%.

According to some ~~embodiment~~examples, the method further comprises a step of diluting the mixed ceDNA/lipid solution with an acidic aqueous buffer.

According to some ~~embodiment~~examples, the one or more low molecular weight alcohol is selected from the group consisting of methanol, ethanol, propanol and isopropanol. According to some ~~embodiment~~examples, the one or more low molecular weight alcohol is ethanol. According to some ~~embodiment~~examples, the one or more low molecular weight alcohol is propanol. According to some ~~embodiment~~examples, the one or more low molecular weight alcohol is methanol. According to some ~~embodiment~~examples, the one or more low molecular weight alcohol is a mixture of ethanol and methanol.

According to some ~~embodiment~~examples, the acid aqueous buffer is selected from malic acid/sodium malate or acetic acid/sodium acetate. According to some ~~embodiment~~examples, the acidic aqueous buffer is at a concentration of between about 10 to 40 millimolar (mM), for example about about 10 mM to about 20 mM, about 10 mM to about 30 mM, about 20 mM to about 30 mM, about 20 mM to about 40 mM, about 30 mM to about 40 mM, or about 10 mM to about 15 mM. According to some ~~embodiment~~examples, the acidic aqueous buffer is at a pH of between about 3 to 5.

According to some ~~embodiment~~examples, the neutral-pH aqueous buffer is Dulbecco's phosphate buffered saline, pH 7.4.

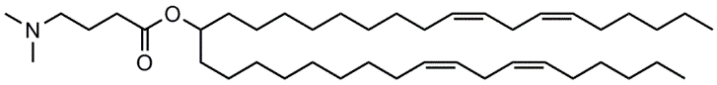
According to some ~~embodiment~~examples, the ceDNA/lipid solution is mixed with the acidic aqueous buffer using microfluidic mixing.

According to some ~~embodiment~~examples, the final alcohol content following the diluting step is between about 4% to about 15% (*e.g.*, about 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, or 15%).

According to some ~~embodiment~~examples, the flow rate ratio between the acidic aqueous buffer and the ceDNA/lipid solution is 2:1, 3:2, 3:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1 or 20:1.

According to some ~~embodiment~~examples, the LNP has a mean diameter of between about 20 nm and about 70 nm, for example about 20 nm, about 25 nm, about 30 nm, about 35 nm, about 40 nm, about 45 nm, about 50 nm, about 55 nm, about 60 nm, about 65 nm or about 70 nm.

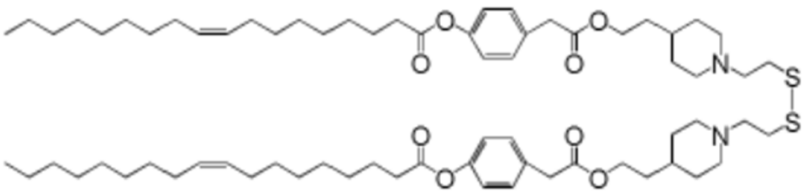
According to some ~~embodiment~~examples, the cationic lipid is MC3 (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate (DLin-MC3-DMA or MC3) having the following structure:



DLin-M-C3-DMA ("MC3")

According to some ~~embodiment~~examples, the ionizable lipid is a SS-cleavable lipid comprising a disulfide bond and a tertiary amine.

According to some ~~embodiment~~examples, the SS-cleavable lipid comprises an ss-OP lipid of the formula:



or a pharmaceutically acceptable salt thereof.

According to another ~~some embodiment~~aspects~~s~~, the disclosure provides an LNP formulation produced by the methods described in the aspects and ~~embodiment~~examples herein.

According to another aspect, the disclosure provides the use of the pharmaceutical composition as disclosed herein in the manufacture of a medicament for treating a genetic disorder in a subject. In another example, the present disclosure provides a method of treating a genetic disorder in a subject, the method comprising administering to the subject an effective amount of the pharmaceutical composition according to any of the previous claims.

According to some ~~embodiment~~examples, the subject is a human.

According to some ~~embodiment~~examples, the genetic disorder is selected from the group consisting of sickle-cell anemia, melanoma, hemophilia A (clotting factor VIII (FVIII) deficiency) and hemophilia B (clotting factor IX (FIX) deficiency), cystic fibrosis (CFTR), familial hypercholesterolemia (LDL receptor defect), hepatoblastoma, Wilson's disease, phenylketonuria (PKU), congenital hepatic

porphyria, inherited disorders of hepatic metabolism, Lesch Nyhan syndrome, sickle cell anemia, thalassaemias, xeroderma pigmentosum, Fanconi's anemia, retinitis pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, mucopolysaccharide storage diseases (*e.g.,* Hurler syndrome (MPS Type I), Scheie syndrome (MPS Type I S), Hurler-Scheie syndrome (MPS Type I H-S), Hunter syndrome (MPS Type II), Sanfilippo Types A, B, C, and D (MPS Types III A, B, C, and D), Morquio Types A and B (MPS IVA and MPS IVB), Maroteaux-Lamy syndrome (MPS Type VI), Sly syndrome (MPS Type VII), hyaluronidase deficiency (MPS Type IX)), Niemann-Pick Disease Types A/B, C1 and C2, Fabry disease, Schindler disease, GM2-gangliosidosis Type II (Sandhoff Disease), Tay-Sachs disease, Metachromatic Leukodystrophy, Krabbe disease, Mucolipidosis Type I, II/III and IV, Sialidosis Types I and II, Glycogen Storage disease Types I and II (Pompe disease), Gaucher disease Types I, II and III, cystinosis, Batten disease, Aspartylglucosaminuria, Salla disease, Danon disease (LAMP-2 deficiency), Lysosomal Acid Lipase (LAL) deficiency, neuronal ceroid lipofuscinoses (CLN1-8, INCL, and LINCL), sphingolipidoses, galactosialidosis, amyotrophic lateral sclerosis (ALS), Parkinson's disease, Alzheimer's disease, Huntington's disease, spinocerebellar ataxia, spinal muscular atrophy, Friedreich's ataxia, Duchenne muscular dystrophy (DMD), Becker muscular dystrophies (BMD), dystrophic epidermolysis bullosa (DEB), ectonucleotide pyrophosphatase 1 deficiency, generalized arterial calcification of infancy (GACI), Leber Congenital Amaurosis, Stargardt macular dystrophy (ABCA4), ornithine transcarbamylase (OTC) deficiency, Usher syndrome, alpha-1 antitrypsin deficiency, and Cathepsin A deficiency. According to some ~~embodiment~~examples, the genetic disorder is Leber congenital amaurosis (LCA).

According to some ~~embodiment~~examples, the LCA is LCA10.

According to some ~~embodiment~~examples, the genetic disorder is Niemann-Pick disease. According to some ~~embodiment~~examples, the genetic disorder is Stargardt macular dystrophy. According to some ~~embodiment~~examples, the genetic disorder is glucose-6-phosphatase (G6Pase) deficiency (glycogen storage disease type I) or Pompe disease (glycogen storage disease type II). According to some ~~embodiment~~examples, the genetic disorder is hemophilia A (Factor VIII deficiency). According to some ~~embodiment~~examples, the genetic disorder is hemophilia B (Factor IX deficiency). According to some ~~embodiment~~examples, the genetic disorder is hunter syndrome (Mucopolysaccharidosis II). According to some ~~embodiment~~examples, the genetic disorder is cystic fibrosis. According to some ~~embodiment~~examples, the genetic disorder is dystrophic epidermolysis bullosa (DEB). According to some ~~embodiment~~examples, the genetic disorder is phenylketonuria (PKU). According to some ~~embodiment~~examples, wherein the genetic disorder is hyaluronidase deficiency.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the method further comprises administering an immunosuppressant.

According to some ~~embodiment~~examples, the immunosuppressant is dexamethasone.

According to some ~~embodiment~~examples of the aspects and ~~embodiment~~examples disclosed herein, the subject exhibits a diminished immune response level against the pharmaceutical composition, as

compared to an immune response level observed with an LNP comprising MC3 as a main cationic lipid, wherein the immune response level against the pharmaceutical composition is at least 50% lower than the level observed with the LNP comprising MC3.

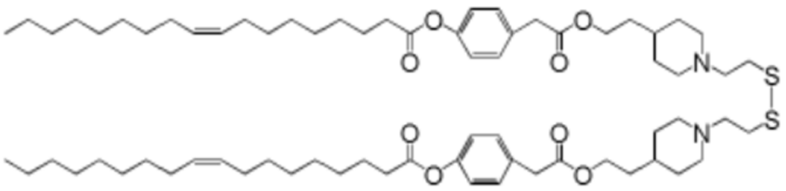
According to some ~~embodiment~~examples, the immune response is measured by detecting the levels of a pro-inflammatory cytokine or chemokine.

According to some ~~embodiment~~examples, the pro-inflammatory cytokine or chemokine is selected from the group consisting of IL-6, IFNα, IFNγ, IL-18, TNFα, IP-10, MCP-1, MIP1α, MIP1β, and RANTES.

According to some ~~embodiment~~examples, at least one of the pro-inflammatory cytokines is under a detectable level in serum of the subject at 6 hours after the administration of the pharmaceutical composition.

According to some ~~embodiment~~examples, the LNP comprising the SS-cleavable lipid and the closed-ended DNA (ceDNA) is not phagocytosed; or exhibits diminished phagocytic levels by at least 50% as compared to phagocytic levels of LNPs comprising MC3 as a main cationic lipid administered at a similar condition.

According to some ~~embodiment~~examples, the SS-cleavable lipid comprises an ssOP lipid of the formula:



or a pharmaceutically acceptable salt thereof.

According to some ~~embodiment~~examples, the LNP further comprises cholesterol and a PEG-lipid conjugate.

According to some ~~embodiment~~examples, the LNP further comprises a noncationic lipid.

According to some ~~embodiment~~examples, the noncationic lipid is selected from the group consisting of dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), and dioleoyl-phosphatidylethanolamine (DOPE).

According to some ~~embodiment~~examples, the LNP further comprises N-Acetylgalactosamine (GalNAc).

According to some ~~embodiment~~examples, the GalNAc is present in the LNP at a molar percentage of 0.5% of the total lipid.

According to another ~~aspect~~example, the disclosure provides a method of increasing therapeutic nucleic acid targeting to the liver of a subject in need of treatment, the method comprising administering to the subject an effective amount of the pharmaceutical composition according to any

of the previous claims, wherein the LNP comprises a therapeutic nucleic acid, ss-cleavable lipid, sterol, and polyethylene glycol (PEG) and N-Acetylgalactosamine (GalNAc).

According to some ~~embodiment~~examples, the PEG is l-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG).

According to some ~~embodiment~~examples, the LNP further comprises a non-cationic lipid.

According to some ~~embodiment~~examples, the non-cationic lipid is selected from the group consisting of dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), and dioleoyl-phosphatidylethanolamine (DOPE).

According to some ~~embodiment~~examples, the GalNAc is present in the LNP at a molar percentage of 0.5% of the total lipid.

According to some ~~embodiment~~examples, the subject is suffering from a genetic disorder.

According to some ~~embodiment~~examples, the genetic disorder is hemophilia A (Factor VIII deficiency). According to some ~~embodiment~~examples, the genetic disorder is hemophilia B (Factor IX deficiency). According to some ~~embodiment~~examples, the genetic disorder is phenylketonuria (PKU).

According to some ~~embodiment~~examples, the therapeutic nucleic acid is selected from the group consisting of minigenes, plasmids, minicircles, small interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotides (ASO), ribozymes, ceDNA, ministring, doggybone™, protelomere closed ended DNA, or dumbbell linear DNA, dicer-substrate dsRNA, small hairpin RNA (shRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, DNA viral vectors, viral RNA vector, non-viral vector and any combination thereof.

According to some ~~embodiment~~examples, the therapeutic nucleic acid is ceDNA.

According to some ~~embodiment~~examples, the ceDNA comprises an expression cassette comprising a promoter sequence and a transgene.

According to some ~~embodiment~~examples, the ceDNA comprises at least one inverted terminal repeat (ITR) flanking either 5’ or 3’ end of said expression cassette.

According to some ~~embodiment~~examples, the ceDNA is selected from the group consisting of a CELiD, a MIDGE, a ministering DNA, a dumbbell shaped linear duplex closed-ended DNA comprising two hairpin structures of ITRs in the 5’ and 3’ ends of an expression cassette, or a doggybone™ DNA, wherein the ceDNA is capsid free and linear duplex DNA.

According to some ~~aspects~~examples, the disclosure provides a method of mitigating a complement response in a subject in need of treatment with a therapeutic nucleic acid (TNA), the method comprising administering to the subject an effective amount of the pharmaceutical composition according to any of the previous claims, wherein the LNP comprises the TNA, a ss-cleavable lipid, a sterol, polyethylene glycol (PEG), and N-Acetylgalactosamine (GalNAc).

According to some ~~embodiment~~examples, the subject is suffering from a genetic disorder.

According to some ~~embodiment~~examples, the genetic disorder is selected from the group consisting of sickle-cell anemia, melanoma, hemophilia A (clotting factor VIII (FVIII) deficiency) and hemophilia

B (clotting factor IX (FIX) deficiency), cystic fibrosis (CFTR), familial hypercholesterolemia (LDL receptor defect), hepatoblastoma, Wilson's disease, phenylketonuria (PKU), congenital hepatic porphyria, inherited disorders of hepatic metabolism, Lesch Nyhan syndrome, sickle cell anemia, thalassaemias, xeroderma pigmentosum, Fanconi's anemia, retinitis pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, mucopolysaccharide storage diseases (*e.g.,* Hurler syndrome (MPS Type I), Scheie syndrome (MPS Type I S), Hurler-Scheie syndrome (MPS Type I H-S), Hunter syndrome (MPS Type II), Sanfilippo Types A, B, C, and D (MPS Types III A, B, C, and D), Morquio Types A and B (MPS IVA and MPS IVB), Maroteaux-Lamy syndrome (MPS Type VI), Sly syndrome (MPS Type VII), hyaluronidase deficiency (MPS Type IX)), Niemann-Pick Disease Types A/B, C1 and C2, Fabry disease, Schindler disease, GM2-gangliosidosis Type II (Sandhoff Disease), Tay-Sachs disease, Metachromatic Leukodystrophy, Krabbe disease, Mucolipidosis Type I, II/III and IV, Sialidosis Types I and II, Glycogen Storage disease Types I and II (Pompe disease), Gaucher disease Types I, II and III, cystinosis, Batten disease, Aspartylglucosaminuria, Salla disease, Danon disease (LAMP-2 deficiency), Lysosomal Acid Lipase (LAL) deficiency, neuronal ceroid lipofuscinoses (CLN1-8, INCL, and LINCL), sphingolipidoses, galactosialidosis, amyotrophic lateral sclerosis (ALS), Parkinson's disease, Alzheimer's disease, Huntington's disease, spinocerebellar ataxia, spinal muscular atrophy, Friedreich's ataxia, Duchenne muscular dystrophy (DMD), Becker muscular dystrophies (BMD), dystrophic epidermolysis bullosa (DEB), ectonucleotide pyrophosphatase 1 deficiency, generalized arterial calcification of infancy (GACI), Leber Congenital Amaurosis, Stargardt macular dystrophy (ABCA4), ornithine transcarbamylase (OTC) deficiency, Usher syndrome, alpha-1 antitrypsin deficiency, and Cathepsin A deficiency.

According to some ~~embodiment~~examples, the rigid therapeutic nucleic acid is selected from the group consisting of minigenes, plasmids, minicircles, small interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotides (ASO), ribozymes, ceDNA, ministring, doggybone™, protelomere closed ended DNA, or dumbbell linear DNA, dicer-substrate dsRNA, small hairpin RNA (shRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, DNA viral vectors, viral vector, non-viral vector and any combination thereof.

According to some ~~embodiment~~examples, the ceDNA is selected from the group consisting of a CELiD, a MIDGE, a ministering DNA, a dumbbell shaped linear duplex closed-ended DNA comprising two hairpin structures of ITRs in the 5' and 3' ends of an expression cassette, or a doggybone™ DNA, wherein the ceDNA is capsid free and linear duplex DNA.

According to some ~~embodiment~~examples, the PEG is l-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG).

According to some ~~embodiment~~examples, the PEG is present in the LNP at a molecular percentage of about 2 to 4%. According to some ~~embodiment~~examples, the PEG is present in the LNP at a molecular percentage of about 3%.

According to some ~~embodiment~~examples, the LNP further comprises a non-cationic lipid. According to some ~~embodiment~~examples, the non-cationic lipid is selected from the group consisting of dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), and dioleoyl-phosphatidylethanolamine (DOPE).

According to some ~~embodiment~~examples, the GalNAc is present in the LNP at a molar percentage of about 0.3 to 1% of the total lipid. According to some ~~embodiment~~examples, the GalNAc is present in the LNP at a molar percentage of about 0.5% of the total lipid.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the present disclosure, briefly summarized above and discussed in greater detail below, can be understood by reference to the illustrative embodiments of the disclosure depicted in the appended drawings. However, the appended drawings illustrate only typical embodiments of the disclosure and are therefore not to be considered limiting of scope, for the disclosure may admit to other equally effective embodiments.

FIG. 1A is a graph that shows condensation of ceDNA as determined by dynamic light scattering. Dynamic light scattering correlation functions show condensation of ceDNA as ethanol content increases. **FIG. 1B** is a graph that shows compaction is reversible upon rehydration.

FIG. 2 is a graph that shows a comparison of diameter of ceDNA LNPs produced by the standard formulation process and the new formulation process described herein.

FIGS. 3A and **3B** are transmission electron microscopy (TEM) images of a ceDNA sample and a plasmid DNA (pDNA) sample, respectively, stored in deionized (DI) water. **FIG. 3A** depics a TEM image of ceDNA stored in deionized water. **FIG. 3B** depics a TEM image of a plasimid stored in deionized water.

FIGS. 4A and 4**B** are TEM images of a ceDNA sample and a pDNA sample, respectively, stored in a low molecular weight alcohol/water solution of 90.9% 1:1 ethanol:methanol in deionized water. **FIG. 4A** depics a TEM image of ceDNA stored in 90.9% 1:1 ethanol:methanol in deionized water. **FIG. 4B** depics a TEM image of a plasimid stored in 90.9% 1:1 ethanol:methanol in deionized water.

FIG. 5 is a TEM image of a ceDNA sample stored in 100% low molecular weight alcohol (*i.e.*, 1:1 ethanol:methanol with no water).

FIGS. 6A and **6B** are TEM images of ceDNA and pDNA, respectively, stored in a basic denaturing condition of 100 mM sodium hydroxide (NaOH) aqueous solution.

DETAILED DESCRIPTION

The immunogenicity associated with viral vector-based gene therapies has limited the number of patients who could be treated due to pre-existing background immunity, as well as prevented the re-dosing of patients either to titrate to effective levels in each patient, or to maintain effects over the

CLAIMS

What is Claimed is:

1. A pharmaceutical composition comprising a lipid nanoparticle (LNP), wherein the LNP comprises a lipid and a rigid therapeutic nucleic acid (rTNA), wherein the mean diameter of the LNP is between about 20 nm and about 75 nm.

2. The pharmaceutical composition of claim 1, wherein the rigid therapeutic nucleic acid is a closed-ended DNA (ceDNA).

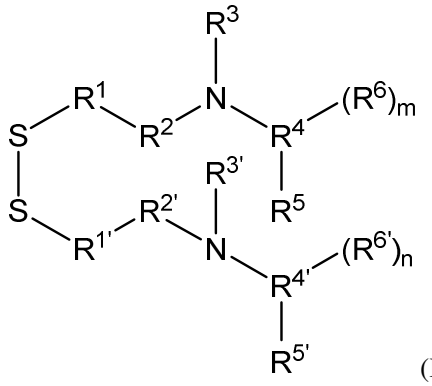
3. The pharmaceutical composition of claim 1, wherein the rigid therapeutic nucleic acid is a double stranded nucleic acid.

4. The pharmaceutical composition of any one of claims 1-3, wherein the lipid is selected from the group consisting of: an ionizable lipid, a non-cationic lipid, a sterol or a derivative thereof, a PEGylated lipid, ~~or~~ and any combination thereof.

5. The pharmaceutical composition of claim 4, wherein the ionizable lipid is a cationic lipid.

6. The pharmaceutical composition of claim ~~4~~5, wherein the cationic lipid is an SS-cleavable lipid.

7. The pharmaceutical composition of claim 5 or claim 6, wherein the cationic lipid is represented by Formula (I):



(I),

or a pharmaceutically acceptable salt thereof, wherein:

R^1 and $R^{1'}$ are each independently optionally substituted linear or branched C_{1-3} alkylene;

R^2 and $R^{2'}$ are each independently optionally substituted linear or branched C_{1-6} alkylene;

R^3 and $R^{3'}$ are each independently optionally substituted linear or branched C_{1-6} alkyl;

or alternatively, when R^2 is optionally substituted branched C_{1-6} alkylene, R^2 and R^3, taken together with their intervening N atom, form a 4- to 8-membered heterocyclyl;

or alternatively, when $R^{2'}$ is optionally substituted branched C_{1-6} alkylene, $R^{2'}$ and $R^{3'}$, taken together with their intervening N atom, form a 4- to 8-membered heterocyclyl;

R^4 and $R^{4'}$ are each independently $–CR^a$, $–C(R^a)_2CR^a$, or $–[C(R^a)_2]_2CR^a$;

R^a, for each occurrence, is independently H or C_{1-3} alkyl;

or alternatively, when R^4 is $–C(R^a)_2CR^a$, or $–[C(R^a)_2]_2CR^a$ and when R^a is C_{1-3} alkyl, R^3 and R^4, taken together with their intervening N atom, form a 4- to 8-membered heterocyclyl;

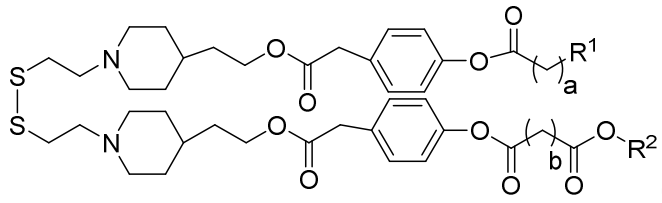
or alternatively, when $R^{4'}$ is $–C(R^a)_2CR^a$, or $–[C(R^a)_2]_2CR^a$ and when R^a is C_{1-3} alkyl, $R^{3'}$ and $R^{4'}$, taken together with their intervening N atom, form a 4- to 8-membered heterocyclyl;

R^5 and $R^{5'}$ are each independently C_{1-20} alkylene or C_{2-20} alkenylene;

R^6 and $R^{6'}$, for each occurrence, are independently C_{1-20} alkylene, C_{3-20} cycloalkylene, or C_{2-20} alkenylene; and

m and n are each independently an integer selected from the group consisting of 1, 2, 3, 4, and 5.

8. The pharmaceutical composition of claim 5 or claim 6, wherein the cationic lipid is represented by Formula (II):



(II);

or a pharmaceutically acceptable salt thereof, wherein:

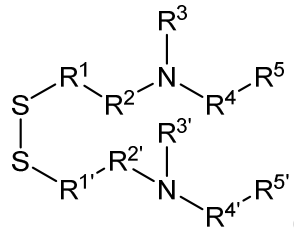
a is an integer ranging from 1 to 20;

b is an integer ranging from 2 to 10;

R^1 is absent or is selected from the group consisting of $(C_2\text{-}C_{20})$alkenyl, $\text{-}C(O)O(C_2\text{-}C_{20})$alkyl, and cyclopropyl substituted with $(C_2\text{-}C_{20})$alkyl; and

R^2 is $(C_2\text{-}C_{20})$alkyl.

9. The pharmaceutical composition of ~~claim 5 or claim 6~~claim 4, wherein the ionizable lipid is represented by the Formula (V):



(V);

or a pharmaceutically acceptable salt thereof, wherein:

R^1 and $R^{1'}$ are each independently (C_1-C_6)alkylene optionally substituted with one or more groups selected from R^a;

R^2 and $R^{2'}$ are each independently (C_1-C_2)alkylene;

R^3 and $R^{3'}$ are each independently (C_1-C_6)alkyl optionally substituted with one or more groups selected from R^b;

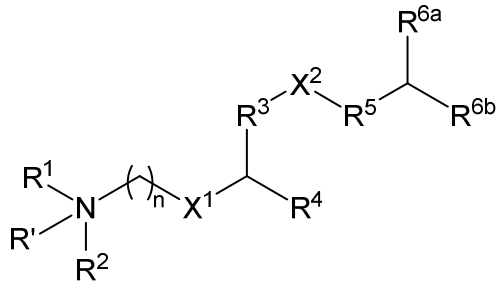
or alternatively, R^2 and R^3 and/or $R^{2'}$ and $R^{3'}$ are taken together with their intervening N atom to form a 4- to 7-membered heterocyclyl;

R^4 and $R^{4'}$ are each a (C_2-C_6)alkylene interrupted by –C(O)O-;

R^5 and $R^{5'}$ are each independently a (C_2-C_{30})alkyl or (C_2-C_{30})alkenyl, each of which are optionally interrupted with –C(O)O- or (C_3-C_6)cycloalkyl; and

R^a and R^b are each halo or cyano.

10. The pharmaceutical composition of claim 5, wherein the cationic lipid is represented by Formula (XV):



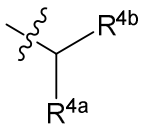
(XV)

or a pharmaceutically acceptable salt thereof, wherein:

R' is absent, hydrogen, or C_1-C_6 alkyl; provided that when R' is hydrogen or C_1-C_6 alkyl, the nitrogen atom to which R', R^1, and R^2 are all attached is protonated;

R^1 and R^2 are each independently hydrogen, C_1-C_6 alkyl, or C_2-C_6 alkenyl;

R^3 is C_1-C_{12} alkylene or C_2-C_{12} alkenylene;

R^4 is C_1-C_{16} unbranched alkyl, C_2-C_{16} unbranched alkenyl, or  ; wherein:

R^{4a} and R^{4b} are each independently C_1-C_{16} unbranched alkyl or C_2-C_{16} unbranched alkenyl;

R^5 is absent, C_1-C_8 alkylene, or C_2-C_8 alkenylene;

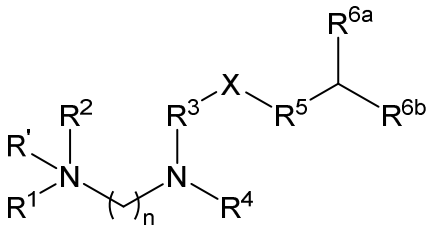
R^{6a} and R^{6b} are each independently C_7-C_{16} alkyl or C_7-C_{16} alkenyl; provided that the total number of carbon atoms in R^{6a} and R^{6b} as combined is greater than 15;

X^1 and X^2 are each independently -OC(=O)-, -SC(=O)-, -OC(=S)-, -C(=O)O-, -C(=O)S-, -S-S-, -C(R^a)=N-, -N=C(R^a)-, -C(R^a)=NO-, -O-N=C(R^a)-, -C(=O)NR^a-, -NR^aC(=O)-, -NR^aC(=O)NR^a-, -OC(=O)O-, -OSi(R^a)$_2$O-, -C(=O)(CR^a_2)C(=O)O-, or OC(=O)(CR^a_2)C(=O)-; wherein:

R^a, for each occurrence, is independently hydrogen or C_1-C_6 alkyl; and

n is an integer selected from the group consisting of 1, 2, 3, 4, 5, and 6.

11. The pharmaceutical composition of claim 5, wherein the cationic lipid is represented by Formula (XX):



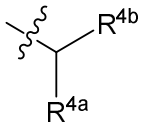
(XX)

or a pharmaceutically acceptable salt thereof, wherein:

R' is absent, hydrogen, or C_1-C_3 alkyl; provided that when R' is hydrogen or C_1-C_3 alkyl, the nitrogen atom to which R', R^1, and R^2 are all attached is protonated;

R^1 and R^2 are each independently hydrogen or C_1-C_3 alkyl;

R^3 is C_3-C_{10} alkylene or C_3-C_{10} alkenylene;

R^4 is C_1-C_{16} unbranched alkyl, C_2-C_{16} unbranched alkenyl, or  ; wherein:

R^{4a} and R^{4b} are each independently C_1-C_{16} unbranched alkyl or C_2-C_{16} unbranched alkenyl;

R^5 is absent, C_1-C_6 alkylene, or C_2-C_6 alkenylene;

R^{6a} and R^{6b} are each independently C_7-C_{14} alkyl or C_7-C_{14} alkenyl;

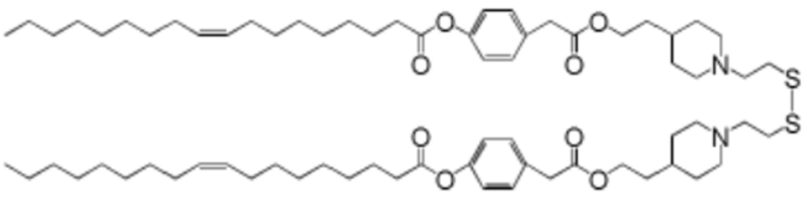
X is -OC(=O)-, -SC(=O)-, -OC(=S)-, -C(=O)O-, -C(=O)S-, -S-S-, -C(R^a)=N-, -N=C(R^a)-, -C(R^a)=NO-, -O-N=C(R^a)-, -C(=O)NR^a-, -NR^aC(=O)-, -NR^aC(=O)NR^a-, -OC(=O)O-, -OSi(R^a)$_2$O-, -C(=O)(CR^a_2)C(=O)O-, or OC(=O)(CR^a_2)C(=O)-; wherein:

R^a, for each occurrence, is independently hydrogen or C_1-C_6 alkyl; and

n is an integer selected from the group consisting of 1, 2, 3, 4, 5, and 6.

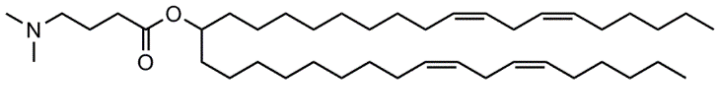
12. The pharmaceutical composition of claim 5 or claim 6, wherein the cationic lipid is selected from the group consisting of any lipid in Table 2, Table 5, Table 6, Table 7, ~~or~~ and Table 8.

13. The pharmaceutical composition of claim 5 or claim 6, wherein the cationic lipid is a lipid having the structure:



or a pharmaceutically acceptable salt thereof.

14. The pharmaceutical composition of claim 5 or claim 6, wherein the cationic lipid is MC3 (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate (DLin-MC3-DMA or MC3) having the following structure:



DLin-M-C3-DMA ("MC3")

~~15. The pharmaceutical composition of any one of claims 1-14, wherein the LNP further comprises a sterol.~~

~~16. The pharmaceutical composition of claim 14, wherein the sterol is cholesterol.~~

~~17. The pharmaceutical composition of claim 14, wherein the sterol is b-sitosterol.~~

~~18. The pharmaceutical composition of any one of claims 1-17, wherein the LNP further comprises a PEGylated lipid.~~

~~19. The pharmaceutical composition of claim 18, wherein the PEGylated lipid is selected from 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol) (PEG-DSPE), or both.~~

~~20. The pharmaceutical composition of any one of claims 1-19, wherein the LNP further comprises a non-cationic lipid.~~

~~21. The pharmaceutical composition of claim 20, wherein the non-cationic lipid is selected from the group consisting of distearoyl-sn-glycero-phosphoethanolamine, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), monomethyl-phosphatidylethanolamine (such as 16-O-monomethyl PE), dimethyl-phosphatidylethanolamine (such as 16-O-dimethyl PE), 18-1-trans PE, 1-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), hydrogenated soy phosphatidylcholine (HSPC), egg phosphatidylcholine (EPC), dioleoylphosphatidylserine (DOPS), sphingomyelin (SM), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), distearoylphosphatidylglycerol (DSPG), dierucoylphosphatidylcholine (DEPC), palmitoyloleyolphosphatidylglycerol (POPG), dielaidoyl-phosphatidylethanolamine (DEPE), 1,2-dilauroyl-sn-glycero-3-pho sphoethanolamine (DLPE); 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (DPHyPE); lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidicacid,cerebrosides, dicetylphosphate, lysophosphatidylcholine, dilinoleoylphosphatidylcholine, or mixtures thereof.~~

~~22. The pharmaceutical composition of claim 21, wherein the non-cationic lipid is selected from the group consisting of dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), and dioleoyl-phosphatidylethanolamine (DOPE).~~

~~23. The pharmaceutical composition of any one of claims 1-22, wherein the LNP further comprises a tissue-specific targeting ligand.~~

~~24. The pharmaceutical composition of claim 23, wherein the tissue-specific targeting ligand is selected from mono-antennary-GalNAc, tri-antennary-GalNAc, and tetra-antennary-GalNAc.~~

~~25. The pharmaceutical composition of claim 23 or claim 24, wherein the tissue-specific targeting ligand is conjugated to the PEGylated lipid.~~

~~26. The pharmaceutical composition of claim 25, wherein the PEGylated lipid is 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-(PEG-DSPE).~~

~~27. The pharmaceutical composition of claim 25 or claim 26, wherein the PEGylated lipid conjugated to the tissue-specific targeting ligand is present at a molar percentage of about 0.5%.~~

~~28. The pharmaceutical composition of any one of claims 18-27, wherein the PEGylated lipid is present at a molar percentage of about 1.5% to about 3%.~~

~~29. The pharmaceutical composition of any one of claims 15-28, wherein the sterol is present at a molar percentage of about 20% to about 40%, and wherein the cationic lipid is present at a molar percentage of about 80% to about 60%.~~

~~30. The pharmaceutical composition of claim 29, wherein the sterol is present at a molar percentage of about 40%, and wherein the cationic lipid is present at a molar percentage of about 50%.~~

~~31~~15. The pharmaceutical composition of any one of claims 1-~~30~~14, wherein the composition further comprises a cholesterol, a PEGylated lipid, and a non-cationic lipid.

~~32. The pharmaceutical composition of claim 25, wherein the PEGylated lipid is present at a molar percentage of about 1.5% to about 3%.~~

~~33. The pharmaceutical composition of claim 31 or claim 32, wherein the cholesterol is present at a molar percentage of about 30% to about 50%.~~

~~34. The pharmaceutical composition of any one of claims 31-33, wherein the lipid is present at a molar percentage of about 42.5% to about 62.5%.~~

~~35. The pharmaceutical composition of any one of claims 31-34, wherein the non-cationic lipid is present at a molar percentage of about 2.5% to about 12.5%.~~

~~36. The pharmaceutical composition of any one of claims 31-35, wherein the cholesterol is present at a molar percentage of about 40%, the lipid is present at a molar percentage of about 52.5%, the non-cationic lipid is present at a molar percentage of about 7.5%, and wherein the PEG is present at about 3%.~~

~~37~~16. The pharmaceutical composition of any one of claims ~~31-36~~1-15, wherein the LNP further comprises a tissue-specific targeting ligand.

~~38~~17. The pharmaceutical composition of claim 16~~37~~, wherein the tissue-specific targeting ligand is selected from the group consisting of: mono-antennary GalNAc, tri-antennary GalNAc, and tetra-antennary GalNAc.

~~39. The pharmaceutical composition of claim 37 or claim 38, wherein the tissue-specific targeting ligand is conjugated to the PEGylated lipid.~~

~~40. The pharmaceutical composition of claim 39, wherein the PEGylated lipid conjugated to the tissue-specific targeting ligand is present at a molar percentage of about 0.5%.~~

~~41. The pharmaceutical composition of any of claims 1-40, wherein the composition further comprises dexamethasone palmitate.~~

~~42~~18. The pharmaceutical composition of any one of claims 1-~~41~~17, wherein the LNP has a mean diameter of less than about 75 nm.

~~43. The pharmaceutical composition of any one of claims 1-42, wherein the LNP has a mean diameter of less than about 70 nm.~~

~~44~~19. The pharmaceutical composition of any one of claims 1-~~43~~18, wherein the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of about 15:1, about 30:1, about 40:1, or about 50:1.

~~45. The pharmaceutical composition of any one of claims 1-43, wherein the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of about 30:1.~~

~~46. The pharmaceutical composition of any one of claims 1-43, wherein the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of about 40:1.~~

~~47. The pharmaceutical composition of any one of claims 1-46, wherein the composition has a total lipid to rigid therapeutic nucleic acid (rTNA) ratio of about 50:1.~~

~~48~~20. The pharmaceutical composition of any one of claims 1-~~47~~19, wherein the rTNA is selected from the group consisting of minigenes, plasmids, minicircles, small interfering RNA

(siRNA), microRNA (miRNA), antisense oligonucleotides (ASO), ribozymes, ceDNA, ministring, doggybone™, protelomere closed ended DNA, or dumbbell linear DNA, dicer-substrate dsRNA, small hairpin RNA (shRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, DNA viral vectors, viral RNA vector, non-viral vector and any combination thereof.

~~49~~21. The pharmaceutical composition of any one of claims 1-~~48~~20, wherein the rigid therapeutic nucleic acid (rTNA) comprises an expression cassette comprising a promoter sequence and a transgene.

~~50. The pharmaceutical composition of any one of claims 1-49, wherein the rigid therapeutic nucleic acid (rTNA) comprises an expression cassette comprising a polyadenylation sequence.~~

~~51. The pharmaceutical composition of claim 49 or claim 50, wherein the rigid therapeutic nucleic acid (rTNA) comprises at least one inverted terminal repeat (ITR) flanking either a 5' end or a 3' end of said expression cassette.~~

~~52. The pharmaceutical composition of claim 51, wherein said expression cassette is flanked by two ITRs, wherein the two ITRs comprise one 5' ITR and one 3' ITR.~~

~~53. The pharmaceutical composition of claim 52, wherein the expression cassette is connected to an ITR at the 3' end (3' ITR).~~

~~54. The pharmaceutical composition of claim 52, wherein the expression cassette is connected to an ITR at the 5' end (5' ITR).~~

~~55. The pharmaceutical composition of claim 52, wherein at least one of the 5' ITR or the 3' ITR is a wild-type AAV ITR.~~

~~56. The pharmaceutical composition of claim 52, wherein at least one of the 5' ITR and the 3' ITR is a modified ITR.~~

~~57. The pharmaceutical composition of claim 52, wherein the rigid therapeutic nucleic acid (rTNA) further comprises a spacer sequence between the 5' ITR and the expression cassette.~~

~~58. The pharmaceutical composition of claim 52, wherein the rigid therapeutic nucleic acid (rTNA) further comprises a spacer sequence between the 3' ITR and the expression cassette.~~

~~59. The pharmaceutical composition of claim 57 or claim 58, wherein the spacer sequence is at least 5 base pairs long.~~

~~60. The pharmaceutical composition of claim 59, wherein the spacer sequence is between about 5 to about 100 base pairs long.~~

~~61. The pharmaceutical composition of claim 59, wherein the spacer sequence is between about 5 to about 500 base pairs long.~~

~~62. The pharmaceutical composition of any one of claims 1-61, wherein the rigid therapeutic nucleic acid (rTNA) comprises a nick or a gap.~~

~~63. The pharmaceutical composition of claim 52, wherein the ITRs are selected from an ITR derived from an AAV serotype, an ITR derived from an ITR of goose virus, an ITR derived from a B19 virus ITR, or a wild type ITR from a parvovirus.~~

~~64. The pharmaceutical composition of claim 63, wherein said AAV serotype is selected from the group consisting of: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 and AAV12.~~

~~65. The pharmaceutical composition of claim 51, wherein the rTNA comprises a first and a second ITR, wherein the first ITR is a mutant ITR, and the second ITR is different from the first ITR.~~

~~66. The pharmaceutical composition of claim 51, wherein the rTNA comprises two mutant ITRs in both 5' and 3' ends of the expression cassette, optionally wherein the two mutant ITRs are symmetric mutants with respect to each other.~~

~~67. The pharmaceutical composition of claim 1, wherein the rTNA is ceDNA.~~

~~68~~22. The pharmaceutical composition of claim ~~67~~2, wherein the ceDNA is selected from the group consisting of a CELiD, DNA-based minicircle, a MIDGE, a ~~ministering~~ ministring DNA, a dumbbell shaped linear duplex closed-ended DNA (ceDNA) comprising two hairpin structures of ITRs in the 5' and 3' ends of an expression cassette, or a doggybone™ DNA.

~~69. The pharmaceutical composition of claim 1, where the rigid therapeutic nucleic acid is a plasmid.~~

~~70. A pharmaceutical composition comprising lipid nanoparticle (LNP), wherein the LNP comprises a lipid and a denatured therapeutic nucleic acid (TNA), wherein the mean diameter of the LNP is between about 20 nm and about 75 nm.~~

~~71. The pharmaceutical composition of claim 70, wherein the denatured TNA has a P-form structure.~~

~~72. The pharmaceutical composition of claim 70 or claim 71, wherein the denatured TNA is prepared by contacting the denatured TNA in a low molecular weight alcohol/aqueous solution or a non-aqueous solvent system comprising one or more low molecular weight alcohols.~~

~~73. The pharmaceutical composition of claim 72, wherein the low molecular weight alcohol/aqueous solution or the non-aqueous solvent system comprises one or more alcohols selected from the group consisting of: ethanol, methanol, and isopropanol.~~

~~74. The pharmaceutical composition of any one of claims 70-73, wherein the LNP has a mean diameter of less than about 75 nm.~~

~~75. The pharmaceutical composition of any one of claims 70-74, wherein the LNP has a mean diameter of less than about 70 nm.~~

~~76. The pharmaceutical composition of any one of claims 70-75, wherein the denatured nucleic acid therapeutic is a double stranded nucleic acid.~~

~~77. The pharmaceutical composition of any one of claims 70-76, wherein the denatured nucleic acid therapeutic is a closed-ended DNA (ceDNA).~~

~~78~~23. A pharmaceutical composition comprising a lipid nanoparticle (LNP), wherein the LNP comprises a lipid and denatured therapeutic nucleic acid (TNA), wherein the LNP is prepared by a method comprising:

adding aqueous TNA to one or more low molecular weight alcohol solution comprising ~~cationic or~~ ionizable lipids to form a TNA/lipid solution, wherein the final concentration of the low molecular weight alcohol in the solution is between about 80% to about 98%;

mixing the TNA/lipid solution with an acidic aqueous buffer; and

buffer exchanging with a neutral-pH aqueous buffer,

thereby producing the LNP formulation.

~~79. The pharmaceutical composition of claim 78, wherein the final concentration of the low molecular weight alcohol in the solution is between about 87% to about 97%.~~

~~80. The pharmaceutical composition of claim 79, wherein the final concentration of the low molecular weight alcohol in the solution is between about 90% to about 95%.~~

~~81. The pharmaceutical composition of claim 79, wherein the final concentration of the low molecular weight alcohol in the solution is between about 92% to about 95%.~~

~~82. The pharmaceutical composition of claim 79, wherein the mean diameter of the LNP is between about 20 nm and about 75 nm.~~

~~83. The pharmaceutical composition of any one of claims 78-82, wherein the LNP has a mean diameter of less than about 75 nm.~~

~~84. The pharmaceutical composition of any one of claims 78-83, wherein the LNP has a mean diameter of less than about 70 nm.~~

~~85. The pharmaceutical composition of any one of claims 78-84, wherein the rigid or denatured nucleic acid therapeutic is a double stranded nucleic acid.~~

~~86. The pharmaceutical composition of any one of claims 78-85, wherein the rigid or denatured nucleic acid therapeutic is a closed-ended DNA (ceDNA).~~

~~87. The pharmaceutical composition of any one of claims 1-86, further comprising a pharmaceutically acceptable excipient.~~

~~88~~24. A method of producing a lipid nanoparticle (LNP) formulation, wherein the LNP comprises an ionizable lipid and a closed-ended DNA (ceDNA), the method comprising:

adding aqueous ceDNA to one or more low molecular weight alcohols solution comprising ~~cationic or~~ ionizable lipids, wherein the final concentration of alcohol in the solution is between about 80% to about 98% form a ceDNA/lipid solution;

mixing the ceDNA/lipid solution with an acidic aqueous buffer; and

buffer exchanging with a neutral-pH aqueous buffer,

thereby producing an LNP formulation.

~~89~~25. A method of producing a lipid nanoparticle (LNP) formulation comprising an ionizable lipid and a closed-ended DNA (ceDNA), the method comprising:

adding ceDNA to one or more low molecular weight alcohol solution, wherein the alcohol content of the resulting solution is greater than 80%,

adding said ceDNA in >80% alcohol content to ~~cationic or~~ ionizable lipids in low molecular weight alcohol to form a ceDNA/lipid solution, wherein the final concentration of the low molecular weight alcohol in the ceDNA-lipid solution is between about 80% to about 95%;

mixing the ceDNA/lipid solution with an acidic aqueous buffer; and

buffer exchanging with a neutral-pH aqueous buffer,

thereby producing the LNP formulation.

~~90. The pharmaceutical composition of claim 89, wherein the final concentration of the low molecular weight alcohol in the solution is between about 87% to about 97%.~~

~~91. The pharmaceutical composition of claim 90, wherein the final concentration of the low molecular weight alcohol in the solution is between about 90% to about 95%.~~

~~92. The pharmaceutical composition of claim 90, wherein the final concentration of the low molecular weight alcohol in the solution is between about 92% to about 95%.~~

~~93. The method of claim 89, further comprising a step of diluting the mixed ceDNA/lipid solution with an acidic aqueous buffer.~~

~~94. The method of any one of claims 89-93, wherein the one or more low molecular weight alcohol is selected from the group consisting of methanol, ethanol, propanol and/or isopropanol.~~

~~95. The method of claim 94, wherein the one or more low molecular weight alcohol is ethanol.~~

~~96. The method of claim 94, wherein the one or more low molecular weight alcohol is propanol.~~

~~97. The method of claim 94, wherein the one or more low molecular weight alcohol is methanol.~~

98. The method of calim 94, wherein the one or more low molecular weight alcohol is a mixture of ethanol and methanol.

99. The method of any one of claims 88-98, wherein the acid aqueous buffer is selected from malic acid/sodium malate or acetic acid/sodium acetate.

100. The method of any one of claims 88-99, wherein the acidic aqueous buffer is at a concentration of between about 10 to 40 millimolar (mM).

101. The method of any one of claims 88-100, wherein the acidic aqueous buffer is at a pH of between about 3 to 5.

102. The method of any one of claims 88-101, wherein the neutral pH aqueous buffer is Dulbecco's phosphate buffered saline, pH 7.4.

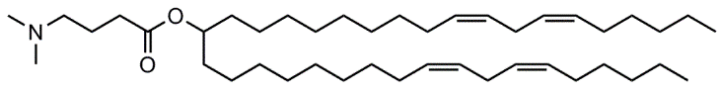
103. The method of claim 88, wherein the ceDNA/lipid solution is mixed with the acidic aqueous buffer using microfluidic mixing.

104. The method of claim 88, wherein the final alcohol content following the diluting step is between about 4% to about 15%.

105. The method of claim 88, wherein the flow rate ratio between the acidic aqueous buffer and the ceDNA/lipid solution is 2:1, 3:2, 3:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 15:1 or 20:1.

106. The method of any one of claims 88-105, wherein the LNP has a mean diameter of between about 20 nm and about 75 nm.

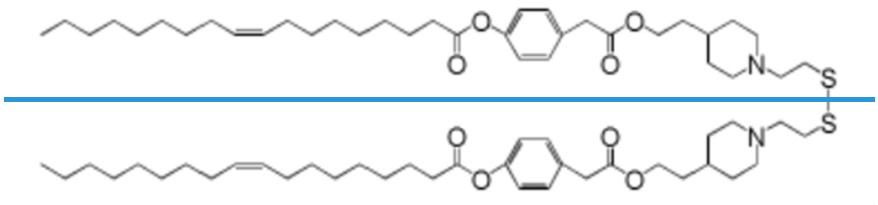
107. The method of any one of claims 88-106, wherein the cationic lipid is MC3 (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate (DLin-MC3-DMA or MC3) having the following structure:



DLin-M-C3-DMA ("MC3")

~~108. The method of any one of claims 88-107, wherein the ionizable lipid is a SS-cleavable lipid compring a disulfide bond and a tertiary amine.~~

~~109. The method of claim 108, wherein the SS-cleavable lipid is a lipid having the structure:~~



~~or a pharmaceutically acceptable salt thereof.~~

~~110~~26. An LNP formulation produced by the method of any one of claims ~~88-109~~24-25.

~~111~~27. Use of the pharmaceutical composition according to any one of claims 1-23 in the manufacture of a medicament for ~~A method of~~ treating a genetic disorder in a subject~~, the method comprising administering to the subject an effective amount of the pharmaceutical composition according to any one of claims 1-110~~.

~~112. The method of claim 111, wherein the subject is a human.~~

~~113~~28. The ~~method~~ use of claim ~~111 or claim 112~~27, wherein the genetic disorder is selected from the group consisting of sickle ~~-~~cell anemia, melanoma, hemophilia A (clotting factor VIII (FVIII) deficiency) and hemophilia B (clotting factor IX (FIX) deficiency), cystic fibrosis (CFTR), familial hypercholesterolemia (LDL receptor defect), hepatoblastoma, Wilson's disease, phenylketonuria (PKU), congenital hepatic porphyria, inherited disorders of hepatic metabolism, Lesch Nyhan syndrome, ~~sickle cell anemia~~, thalassaemias, xeroderma pigmentosum, Fanconi's anemia, retinitis pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, mucopolysaccharide storage diseases (*e.g.,* Hurler syndrome (MPS Type I), Scheie syndrome (MPS Type I S), Hurler-Scheie syndrome (MPS Type I H-S), Hunter syndrome (MPS Type II), Sanfilippo Types A, B, C, and D (MPS Types III A, B, C, and D), Morquio Types A and B (MPS IVA and MPS IVB), Maroteaux-Lamy syndrome (MPS Type VI), Sly syndrome (MPS Type VII), hyaluronidase deficiency (MPS Type IX)), Niemann-Pick Disease Types A/B, C1 and C2, Schindler disease, GM2-gangliosidosis Type II (Sandhoff Disease), Tay-Sachs disease, Metachromatic Leukodystrophy, Krabbe disease, Mucolipidosis Type I, II/III and IV, Sialidosis Types I and II, Glycogen Storage

disease Types I and II (Pompe disease), Gaucher disease Types I, II and III, Fabry disease, cystinosis, Batten disease, Aspartylglucosaminuria, Salla disease, Danon disease (LAMP-2 deficiency), Lysosomal Acid Lipase (LAL) deficiency, neuronal ceroid lipofuscinoses (CLN1-8, INCL, and LINCL), sphingolipidoses, galactosialidosis, amyotrophic lateral sclerosis (ALS), Parkinson's disease, Alzheimer's disease, Huntington's disease, spinocerebellar ataxia, spinal muscular atrophy, Friedreich's ataxia, Duchenne muscular dystrophy (DMD), Becker muscular dystrophies (BMD), dystrophic epidermolysis bullosa (DEB), ectonucleotide pyrophosphatase 1 deficiency, generalized arterial calcification of infancy (GACI), Leber Congenital Amaurosis, Stargardt macular dystrophy (ABCA4), ornithine transcarbamylase (OTC) deficiency, Usher syndrome, alpha-1 antitrypsin deficiency, and Cathepsin A deficiency.

~~114. The method of claim 113, wherein the genetic disorder is Leber congenital amaurosis (LCA) 10.~~

~~115. The method of claim 113, wherein the genetic disorder is Stargardt macular dystrophy.~~

~~116. The method of claim 113, wherein the genetic disorder is hemophilia A (Factor VIII deficiency).~~

~~117. The method of claim 113, wherein the genetic disorder is hemophilia B (Factor IX deficiency).~~

~~118. The method of claim 113, wherein the genetic disorder is Wilson's disease.~~

~~119. The method of claim 113, wherein the genetic disorder is Gaucher disease.~~

~~120. The method of claim 113, wherein the genetic disorder is phenylketonuria (PKU).~~

~~121. The method of claim 113, wherein the genetic disorder is hyaluronidase deficiency.~~

~~122. The method of any one of claims 111-121, further comprising administering an immunosuppressant.~~

~~123. The method of claim 122, wherein the immunosuppressant is dexamethasone.~~

124. The method of any one of claims 111-123, wherein the subject exhibits a diminished immune response level against the pharmaceutical composition, as compared to an immune response level observed with an LNP comprising MC3 as a main cationic lipid, wherein the immune response level against the pharmaceutical composition is at least 50% lower than the level observed with the LNP comprising MC3.

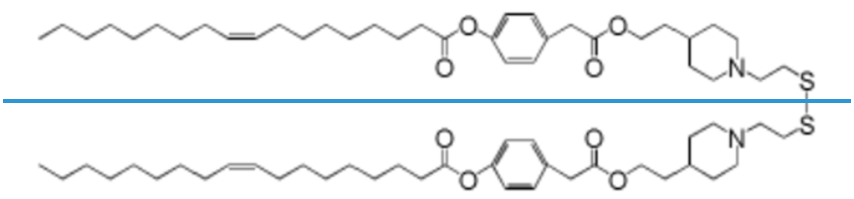
125. The method of claim 124, wherein the immune response is measured by detecting the levels of a pro-inflammatory cytokine or chemokine.

126. The method of claim 125, wherein the pro-inflammatory cytokine or chemokine is selected from the group consisting of IL-6, IFNα, IFNγ, IL-18, TNFα, IP-10, MCP-1, MIP1α, MIP1β, and RANTES.

127. The method of claim 125, wherein at least one of the pro-inflammatory cytokines is under a detectable level in serum of the subject at 6 hours after the administration of the pharmaceutical composition.

128. The method of any one of claims 111-127, wherein the LNP comprising a SS-cleavable lipid and the closed-ended DNA (ceDNA) is not phagocytosed; or exhibits diminished phagocytic levels by at least 50% as compared to phagocytic levels of LNPs comprising MC3 as a main cationic lipid administered at a similar condition.

129. The method of claim 128, wherein the SS-cleavable lipid is a lipid having the structure:



or a pharmaceutically acceptable salt thereof.

130. The method of claim 129, wherein the LNP further comprises cholesterol and a PEGylated lipid.

131. The method of claim 130, wherein the LNP further comprises a noncationic lipid.

~~132. The method of claim 131, wherein the noncationic lipid is selected from the group consisting of dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), and dioleoyl phosphatidylethanolamine (DOPE).~~

~~133. The method of any of claims 130-132, wherein the LNP further comprises N-Acetylgalactosamine (GalNAc).~~

~~134. The method of claim 133, wherein the GalNAc is conjugated to the PEGylated lipid and the PEGylated lipid conjugated to the GalNAc is present in the LNP at a molar percentage of 0.5%.~~

~~135. A method of increasing therapeutic nucleic acid targeting to the liver of a subject in need of treatment, the method comprising administering to the subject an effective amount of the pharmaceutical composition according to any one of claims 1-110, wherein the LNP comprises a rigid therapeutic nucleic acid (rTNA), an ss-cleavable lipid, a sterol, and a polyethylene glycol (PEG) and N-Acetylgalactosamine (GalNAc).~~

~~136. The method of claim 135, wherein the PEG is 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG).~~

~~137. The method of claim 135, wherein the LNP further comprises a non-cationic lipid.~~

~~138. The method of claim 137, wherein the non-cationic lipid is selected from the group consisting of dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), and dioleoyl phosphatidylethanolamine (DOPE).~~

~~139. The method of claim 135, wherein the GalNAc is conjugated to the PEGylated lipid and the PEGylated lipid conjugated to the GalNAc is present in the LNP at a molar percentage of 0.5%.~~

~~140. The method of claim 135, wherein the subject is suffering from a genetic disorder.~~

~~141. The method of claim 140, wherein the genetic disorder is hemophilia A (Factor VIII deficiency).~~

~~142. The method of claim 140, wherein the genetic disorder is hemophilia B (Factor IX deficiency).~~

143. The method of claim 140, wherein the genetic disorder is phenylketonuria (PKU).

144. The method of claim 135, wherein the rigid therapeutic nucleic acid is selected from the group consisting of minigenes, plasmids, minicircles, small interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotides (ASO), ribozymes, ceDNA, ministring, doggybone™, protelomere closed ended DNA, or dumbbell linear DNA, dicer-substrate dsRNA, small hairpin RNA (shRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, DNA viral vectors, viral RNA vector, non-viral vector and any combination thereof.

145. The method of claim 135, wherein the rigid therapeutic nucleic acid is a ceDNA.

146. The method of claim 145, wherein the ceDNA comprises an expression cassette comprising a promoter sequence and a transgene.

147. The method of claim 146, wherein the ceDNA comprises at least one inverted terminal repeat (ITR) flanking either the 5' or the 3' end of said expression cassette.

148. The method of claim 135, wherein the ceDNA is selected from the group consisting of a CELiD, a MIDGE, a ministering DNA, a dumbbell shaped linear duplex closed-ended DNA comprising two hairpin structures of ITRs in the 5' and 3' ends of an expression cassette, or a doggybone™ DNA, wherein the ceDNA is capsid free and linear duplex DNA.

149. A method of mitigating a complement response in a subject in need of treatment with a rigid therapeutic nucleic acid (rTNA), the method comprising administering to the subject an effective amount of the pharmaceutical composition according to any of the previous claims, wherein the LNP comprises the rTNA, a cationic lipid, a sterol, and a PEGylated lipid.

150. The method of claim 149, wherein the subject is suffering from a genetic disorder.

151. The method of claim 150, wherein the genetic disorder is selected from the group consisting of sickle-cell anemia, melanoma, hemophilia A (clotting factor VIII (FVIII) deficiency) and hemophilia B (clotting factor IX (FIX) deficiency), cystic fibrosis (CFTR), familial hypercholesterolemia (LDL receptor defect), hepatoblastoma, Wilson's disease, phenylketonuria (PKU), congenital hepatic porphyria, inherited disorders of hepatic metabolism, Lesch Nyhan syndrome, sickle cell anemia, thalassaemias, xeroderma pigmentosum, Fanconi's anemia, retinitis pigmentosa, ataxia telangiectasia, Bloom's syndrome, retinoblastoma, mucopolysaccharide storage

diseases (*e.g.*, Hurler syndrome (MPS Type I), Scheie syndrome (MPS Type I S), Hurler-Scheie syndrome (MPS Type I H-S), Hunter syndrome (MPS Type II), Sanfilippo Types A, B, C, and D (MPS Types III A, B, C, and D), Morquio Types A and B (MPS IVA and MPS IVB), Maroteaux-Lamy syndrome (MPS Type VI), Sly syndrome (MPS Type VII), hyaluronidase deficiency (MPS Type IX)), Niemann-Pick Disease Types A/B, C1 and C2, Schindler disease, GM2-gangliosidosis Type II (Sandhoff Disease), Tay-Sachs disease, Metachromatic Leukodystrophy, Krabbe disease, Mucolipidosis Type I, II/III and IV, Sialidosis Types I and II, Glycogen Storage disease Types I and II (Pompe disease), Gaucher disease Types I, II and III, Fabry disease, cystinosis, Batten disease, Aspartylglucosaminuria, Salla disease, Danon disease (LAMP-2 deficiency), Lysosomal Acid Lipase (LAL) deficiency, neuronal ceroid lipofuscinoses (CLN1-8, INCL, and LINCL), sphingolipidoses, galactosialidosis, amyotrophic lateral sclerosis (ALS), Parkinson's disease, Alzheimer's disease, Huntington's disease, spinocerebellar ataxia, spinal muscular atrophy, Friedreich's ataxia, Duchenne muscular dystrophy (DMD), Becker muscular dystrophies (BMD), dystrophic epidermolysis bullosa (DEB), ectonucleotide pyrophosphatase-1 deficiency, generalized arterial calcification of infancy (GACI), Leber Congenital Amaurosis, Stargardt macular dystrophy (ABCA4), ornithine transcarbamylase (OTC) deficiency, Usher syndrome, alpha-1 antitrypsin deficiency, and Cathepsin A deficiency.

152. The method of any one of claims 149-151, wherein the rigid therapeutic nucleic acid is selected from the group consisting of minigenes, plasmids, minicircles, small interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotides (ASO), ribozymes, ceDNA, ministring, doggybone™, protelomere closed ended DNA, or dumbbell linear DNA, dicer substrate dsRNA, small hairpin RNA (shRNA), asymmetrical interfering RNA (aiRNA), microRNA (miRNA), mRNA, tRNA, rRNA, DNA viral vectors, viral RNA vector, non-viral vector and any combination thereof.

153. The method of claim 152, wherein the rigid therapeutic nucleic acid is a ceDNA, wherein the ceDNA is selected from the group consisting of a CELiD, a MIDGE, a ministering DNA, a dumbbell shaped linear duplex closed-ended DNA comprising two hairpin structures of ITRs in the 5' and 3' ends of an expression cassette, or a doggybone™ DNA, wherein the ceDNA is capsid free and linear duplex DNA.

154. The method of any one of claims 149-153, wherein the PEGylated lipid is 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG).

155. The method of claim 154, wherein the PEG is present in the LNP at a molar percentage of about 2 to 4%.

~~156. The method of claim 155, wherein the PEG is present in the LNP at a molar percentage of about 3%.~~

~~157. The method of any one of claims 149-156, wherein the LNP further comprises a non-cationic lipid.~~

~~158. The method of claim 157, wherein the non-cationic lipid is selected from the group consisting of dioleoylphosphatidylcholine (DOPC), distearoylphosphatidylcholine (DSPC), and dioleoyl-phosphatidylethanolamine (DOPE).~~