

LYOPHILIZED FORMULATIONS AND LIQUID FORMULATIONS OF LIPID NANOPARTICLES

TECHNICAL FIELD

5 The present invention relates to novel formulations of lipid nanoparticles. In particular, the present invention relates to lyophilized formulations and liquid formulations of lipid nanoparticles.

BACKGROUND

10 Lipid nanoparticles (LNPs) are the most clinically advanced non-viral gene delivery system. Lipid nanoparticles safely and effectively deliver nucleic acids, overcoming a major barrier preventing the development and use of genetic medicines. Lipid nanoparticles have successfully entered the clinic for the delivery of mRNA. Lipid nanoparticles encapsulating mRNA have been widely used as COVID-19 vaccines during pandemics. However, mRNA is
15 sensitive to hydrolysis and has limited thermostability. Most of the time, ultracold storage is required for long-term storage of mRNA-LNP vaccines to slow down mRNA degradation. However, ultracold freezers are not feasible for some rural areas.

 Lyophilization is considered to be useful to extend mRNA-LNP shelf-life by removing water from the formulation. The dry formulation could be stored at 2-8 °C for couple years.

20 Therefore, it is desired to develop lyophilized formulations of mRNA-LNP.

 However, as a fragile system, mRNA-LNP could be hugely impacted during the physical stress of sublimation. In most of the case, particle size increases significantly, and a certain amount of mRNA leaks out during lyophilization. This potentially would impact the in vivo activity of mRNA vaccines.

25 Thus, there is a need for lyophilized formulations of mRNA-LNP with relatively higher encapsulation efficiency and suitable particle sizes.

SUMMARY OF THE INVENTION

 The inventors have now discovered that specific cryoprotectant combinations can protect
30 mRNA-LNP from lyophilization stress. The present invention is based on such an unexpected discovery.

 In a first aspect, the present invention provides a lyophilized formulation of lipid nanoparticles (also referred to as "lyophilized LNP formulation" herein) comprising, relative to the total weight of the lyophilized formulation,

- 35 (A) 0.2-20 wt.% of lipid nanoparticles comprising a cationic lipid; and
 (B) a cryoprotectant combination comprising
 (i) 75-99 wt.% of sucrose; and
 (ii) 0.1-10 wt.% of a non-polar amino acid.

 The present invention also provides a liquid formulation of lipid nanoparticles (also
40 referred to as "liquid LNP formulation" herein) which can be used to prepare the lyophilized LNP formulation disclosed herein.

 In a second aspect, the present invention provides a liquid formulation of lipid nanoparticles comprising, by weight relative to the total volume of the liquid formulation:

- (A) 0.001-0.2% w/v of lipid nanoparticles comprising a cationic lipid; and

(B) a cryoprotectant combination comprising

(i) 0.001-40% w/v of sucrose, and

(ii) 0.01-10% w/v of a non-polar amino acid.

5 The inventors have now found that specific cryoprotectant combinations comprising sucrose and a non-polar amino acid can protect mRNA-LNP from lyophilization stress so that the particle size of mRNA-LNP will not change significantly, for example the change of particle size of mRNA-LNP before and after lyophilization is less than 10 nm.

10 The inventors have also found that with specific cryoprotectant combinations comprising sucrose and a non-polar amino acid, lyophilized formulations of mRNA-LNP obtained have suitable particle sizes, for example, 80-100 nm, and relatively higher encapsulation efficiency, for example, not lower than 85%, so that in vivo bioactivity of the mRNA can be greatly maintained.

15 The inventors have further found that with specific cryoprotectant combinations comprising sucrose, a non-polar amino acid and salts or Tris, lyophilized formulations of mRNA-LNP obtained have high purity of RNA and reduced levels of lipid adducts during the storage.

Other characteristics and advantages of the invention will emerge more clearly on reading the description and the examples that follow.

20 **BRIEF DESCRIPTION OF THE DRAWINGS**

The present invention will be described and explained in detail in conjunction with the drawings hereinafter.

Fig. 1 shows pre-lyo and post-lyo sizes as well as polydispersity index of LNPs of CE.10-17.

25 Fig. 2 shows encapsulation efficiencies of LNPs of CE.10-17.

Fig. 3 shows pre-lyo and post-lyo sizes as well as polydispersity index of LNPs of IE.16-20.

Fig. 4 shows encapsulation efficiencies of LNPs of IE. 12 and 16-19.

30 Fig. 5 shows pre-lyo and post-lyo sizes as well as polydispersity index of LNPs of IE.12 and 20-21.

Fig. 6 shows encapsulation efficiencies of LNPs of IE. 12 and 20-21.

Fig. 7 shows *in-vivo* hEPO expression levels (μg/ml) measured from the tested group treated with mRNA LNPs before and after lyophilization in inventive example 22.

35 Fig. 8 shows *in-vivo* hEPO expression levels (μg/ml) measured from the tested group treated with mRNA LNPs before and after lyophilization in inventive examples 23-26.

Fig. 9 shows pre-lyo and post-lyo sizes of LNPs of IE.27-31.

Fig. 10 shows encapsulation efficiencies of LNPs of IE.27-31.

Fig. 11 shows RNA purity (%) of LNPs of CE.19 and IE.32 stored at 25 °C for a certain period of time.

40 Fig. 12 shows lipid adduct percentage of LNPs of CE.19 and IE.32 stored at 25 °C for a certain period of time.

Fig. 13 shows lipid adduct percentage of LNPs of IE.33-36 and CE.20 stored at 37 °C for a certain period of time.

DETAILED DESCRIPTION OF THE INVENTION

Unless described otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those skilled in the field the present invention belongs to.

5 When the definition of a term in the present description conflicts with the meaning as commonly understood by those skilled in the field the present invention belongs to, the definition described herein shall apply.

As used herein, the term “comprising” is to be interpreted as encompassing all specifically mentioned features as well as optional, additional, unspecified ones.

10 As used herein, the use of the term “comprising” also discloses the embodiment wherein no features other than the specifically mentioned features are present (i.e. “consisting of”).

Unless otherwise specified, all numerical values expressing amount of ingredients and the like which are used in the description and claims are to be understood as being modified by the term “about”. Accordingly, unless indicated to the contrary, the numerical values and
15 parameters described herein are approximate values which are capable of being changed according to the desired purpose as required.

As used herein and unless otherwise specified, the term “lipid” refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are generally characterized by being poorly soluble in water, but soluble in many nonpolar organic solvents.
20 While lipids generally have poor solubility in water, there are certain categories of lipids (e.g., lipids modified by polar groups, e.g., DMG-PEG2000) that have limited aqueous solubility and can dissolve in water under certain conditions. Known types of lipids include biological molecules such as fatty acids, waxes, sterols, fat-soluble vitamins, monoglycerides, diglycerides, triglycerides, and phospholipids. Lipids can be divided into at least three classes:
25 (1) “simple lipids”, which include fats and oils as well as waxes; (2) “compound lipids”, which include phospholipids and glycolipids (e.g., DMPE-PEG2000); and (3) “derived lipids”, such as steroids. Further, as used herein, lipids also encompass lipidoid compounds. The term “lipidoid compound”, also simply “lipidoid”, refers to a lipid-like compound (e.g. an amphiphilic compound with lipid-like physical properties).

30 The term “lipid nanoparticle” or “LNP” refers to a particle having at least one dimension on the order of nanometers (nm) (e.g., 1 to 1,000 nm), which contains one or more types of lipid molecules. The LNP provided herein can further contain at least one non-lipid payload molecule (e.g., one or more nucleic acid molecules). In some embodiments, the LNP comprises a non-lipid payload molecule either partially or completely encapsulated inside a lipid shell.
35 Particularly, in some embodiments, wherein the payload is a negatively charged molecule (e.g., mRNA encoding a viral protein), and the lipid components of the LNP comprise at least one cationic lipid. Without being bound by the theory, it is contemplated that the cationic lipids can interact with the negatively charged payload molecules and facilitate incorporation and/or encapsulation of the payload into the LNP during LNP formation. Other lipids that can form
40 part of a LNP as provided herein include but are not limited to neutral lipids and charged lipids, such as steroids, polymer conjugated lipids, and various zwitterionic lipids.

The term “cationic lipid” refers to a lipid that is either positively charged at any pH value or hydrogen ion activity of its environment, or capable of being positively charged in response to the pH value or hydrogen ion activity of its environment (e.g., the environment of its intended

use). Thus, the term “cationic” encompasses both “permanently cationic” and “cationisable”. In certain embodiments, the positive charge in a cationic lipid results from the presence of a quaternary nitrogen atom. In certain embodiments, the cationic lipid comprises a zwitterionic lipid that assumes a positive charge in the environment of its intended use (e.g., at physiological pH). In preferred embodiments, the cationic lipid is ionizable cationic lipid. The term “ionizable cationic lipid” refers to an ionizable lipid that is positively charged at acidic pH to condense RNAs into a composition, such as LNPs, but is neutral at physiological pH to minimize toxicity.

The term “polymer conjugated lipid” refers to a molecule comprising both a lipid portion and a polymer portion. An example of a polymer conjugated lipid is a pegylated lipid (PEG-lipid), in which the polymer portion comprises a polyethylene glycol.

The term “neutral lipid” encompasses any lipid molecules existing in uncharged forms or neutral zwitterionic forms at a selected pH value or within a selected pH range. In some embodiments, the selected useful pH value or range corresponds to the pH condition in an environment of the intended uses of the lipids, such as the physiological pH. As non-limiting examples, neutral lipids that can be used in connection with the present disclosure include, but are not limited to, phosphatidylcholines such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), phosphatidylethanolamines such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 2-((2,3-bis(oleoyloxy)propyl)dimethylammonio)ethyl hydrogen phosphate (DOCP), sphingomyelins (SM), ceramides, steroids such as sterols and their derivatives. Neutral lipids as provided herein may be synthetic or derived (isolated or modified) from a natural source or compound.

The term “charged lipid” encompasses any lipid molecules that exist in either positively charged or negatively charged forms at a selected pH or within a selected pH range. In some embodiments, the selected pH value or range corresponds to the pH condition in an environment of the intended uses of the lipids, such as the physiological pH. As non-limiting examples, charged lipids that can be used in connection with the present disclosure include, but are not limited to, phosphatidylserines, phosphatidic acids, phosphatidylglycerols, phosphatidylinositols, sterol hemisuccinates, dialkyl trimethylammonium-propanes, (e.g., DOTAP, DOTMA), dialkyl dimethylaminopropanes, ethyl phosphocholines, dimethylaminoethane carbamoyl sterols (e.g., DC-Chol), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine sodium salt (DOPS-Na), 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) sodium salt (DOPG-Na), and 1,2-dioleoyl-sn-glycero-3-phosphate sodium salt (DOPA-Na). Charged lipids as provided herein may be synthetic or derived (isolated or modified) from a natural source or compound.

As used herein, and unless otherwise specified, the term “pharmaceutically acceptable carrier, diluent or excipient” includes without limitation any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, or emulsifier which has been approved by the United States Food and Drug Administration as being acceptable for use in humans or domestic animals.

The term “composition” is intended to encompass a product containing the specified ingredients (e.g., a lipid compound provided herein, and/or a mRNA molecule provided herein) in, optionally, the specified amounts.

The term “polynucleotide” or “nucleic acid”, as used interchangeably herein, refers to polymers of nucleotides of any length and includes, e.g., DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. Nucleic acid can be in either single- or double-stranded forms. As used herein and unless otherwise specified, “nucleic acid” also includes nucleic acid mimics such as locked nucleic acids (LNAs), peptide nucleic acids (PNAs), and morpholinos. “Oligonucleotide”, as used herein, refers to short synthetic polynucleotides that are generally, but not necessarily, fewer than about 200 nucleotides in length. The terms “oligonucleotide” and “polynucleotide” are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides. Unless specified otherwise, the left-hand end of any single-stranded polynucleotide sequence disclosed herein is the 5' end; the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA transcript that are 5' to the 5' end of the RNA transcript are referred to as “upstream sequences”; sequence regions on the DNA strand having the same sequence as the RNA transcript that are 3' to the 3' end of the RNA transcript are referred to as “downstream sequences”.

An “isolated nucleic acid” is a nucleic acid, for example, an RNA, DNA, or a mixed nucleic acids, which is substantially separated from other genome DNA sequences as well as proteins or complexes such as ribosomes and polymerases, which naturally accompany a native sequence. An “isolated” nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. Moreover, an “isolated” nucleic acid molecule, such as an mRNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. In a specific embodiment, one or more nucleic acid molecules encoding an antigen as described herein are isolated or purified. The term embraces nucleic acid sequences that have been removed from their naturally occurring environment, and includes recombinant or cloned DNA or RNA isolates and chemically synthesized analogues or analogues biologically synthesized by heterologous systems. A substantially pure molecule may include isolated forms of the molecule.

The term “encoding nucleic acid” or grammatical equivalents thereof as it is used in reference to nucleic acid molecule encompasses (a) a nucleic acid molecule in its native state or when manipulated by methods well known to those skilled in the art that can be transcribed to produce mRNA which is then translated into a peptide and/or polypeptide, and (b) the mRNA molecule itself. The antisense strand is the complement of such a nucleic acid molecule, and the encoding sequence can be deduced therefrom. The term “coding region” refers to a portion in an encoding nucleic acid sequence that is translated into a peptide or polypeptide. The term “untranslated region” or “UTR” refers to the portion of an encoding nucleic acid that is not

translated into a peptide or polypeptide. Depending on the orientation of a UTR with respect to the coding region of a nucleic acid molecule, a UTR is referred to as the 5'-UTR if located to the 5'-end of a coding region, and a UTR is referred to as the 3'-UTR if located to the 3'-end of a coding region.

5 The term "mRNA" as used herein refers to a message RNA molecule comprising one or more open reading frame (ORF) that can be translated by a cell or an organism provided with the mRNA to produce one or more peptide or protein product. The region containing the one or more ORFs is referred to as the coding region of the mRNA molecule. In certain embodiments, the mRNA molecule further comprises one or more untranslated regions (UTRs).

10 The term "functional nucleotide analog" as used herein refers to a modified version of a canonical nucleotide A, G, C, U or T that (a) retains the base-pairing properties of the corresponding canonical nucleotide, and (b) contains at least one chemical modification to (i) the nucleobase, (ii) the sugar group, (iii) the phosphate group, or (iv) any combinations of (i) to (iii), of the corresponding natural nucleotide. As used herein, base pairing encompasses not
15 only the canonical Watson-Crick adenine-thymine, adenine-uracil, or guanine-cytosine base pairs, but also base pairs formed between canonical nucleotides and functional nucleotide analogs or between a pair of functional nucleotide analogs, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a modified nucleobase and a canonical nucleobase or between two complementary modified
20 nucleobase structures. For example, a functional analog of guanosine (G) retains the ability to base-pair with cytosine (C) or a functional analog of cytosine. One example of such non-canonical base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine, or uracil. As described herein, a functional nucleotide analog can be either naturally occurring or non-naturally occurring. Accordingly, a nucleic acid molecule containing a
25 functional nucleotide analog can have at least one modified nucleobase, sugar group and/or internucleoside linkage. Exemplary chemical modifications to the nucleobases, sugar groups, or internucleoside linkages of a nucleic acid molecule are provided herein.

 All publications, patent applications, accession numbers, and other references cited in this specification are herein incorporated by reference in their entirety as if each individual
30 publication or patent application were specifically and individually indicated to be incorporated by reference. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided can be different from the actual publication dates
35 which need to be independently confirmed.

Lyophilized LNP formulations

 In the first aspect, the present invention provides a lyophilized LNP formulation comprising relative to the total weight of the lyophilized formulation,

- 40 (A) 0.2-20 wt.% of lipid nanoparticles comprising a cationic lipid; and
 (B) a cryoprotectant combination comprising
 (i) 75-99 wt.% of sucrose; and
 (ii) 0.1-10 wt.% of a non-polar amino acid.

Lipid nanoparticles

In some embodiments, the lyophilized LNP formulation comprises 0.2-20 wt.%, for example, 0.3-18 wt.%, 0.4-16 wt.%, 0.5-12 wt.%, 0.6-10 wt.%, or 0.2-1 wt.%, 0.2-0.8 wt.%, 0.25-0.7 wt.%, of the lipid nanoparticles, relative to the total weight of the lyophilized LNP formulation.

The particle size of the LNP in the lyophilized LNP formulation is from about 80 nm to about 100 nm, preferably from about 80 nm to about 95 nm, for example, from about 82 nm to about 95 nm.

As used herein, the particle size means a mean size, which can be determined by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern UK) using a 173° backscatter detection mode.

The lipid nanoparticle comprises a cationic lipid.

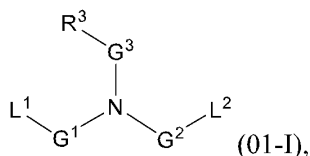
The lipid nanoparticle may further comprise one or more of a structural lipid, a phospholipid, and a polymer conjugated lipid.

As used herein, unless other specified, the mol percent of a lipid is calculated based on the total mole number of all lipids present in the nanoparticle.

The cationic lipid includes at least one of the following Series 01-06 of compounds (and sub-formulas thereof).

Series 01 of compounds

In some embodiments, the cationic lipid of the present invention comprises at least one of those disclosed in International Application Publication No. WO2021204175, the entire teachings of which are incorporated herein by reference. Specifically, the cationic lipid comprises a compound represented by Formula (01-I):



or a pharmaceutically acceptable salt, prodrug or stereoisomer thereof, wherein:

G¹ and G² are each independently a bond, C₂-C₁₂ alkylene, or C₂-C₁₂ alkenylene, wherein one or more -CH₂- in the alkylene or alkenylene is optionally replaced by -O-;

L¹ is -OC(=O)R¹, -C(=O)OR¹, -OC(=O)OR¹, -C(=O)R¹, -OR¹, -S(O)_xR¹, -S-SR¹, -C(=O)SR¹, -SC(=O)R¹, -NR^aC(=O)R¹, -C(=O)NR^bR^c, -NR^aC(=O)NR^bR^c, -OC(=O)NR^bR^c, -NR^aC(=O)OR¹, -SC(=S)R¹, -C(=S)SR¹, -C(=S)R¹, -CH(OH)R¹, -P(=O)(OR^b)(OR^c), -(C₆-C₁₀ arylene)-R¹, -(6- to 10-membered heteroarylene)-R¹, or R¹;

L² is -OC(=O)R², -C(=O)OR², -OC(=O)OR², -C(=O)R², -OR², -S(O)_xR², -S-SR², -C(=O)SR², -SC(=O)R², -NR^dC(=O)R², -C(=O)NR^eR^f, -NR^dC(=O)NR^eR^f, -OC(=O)NR^eR^f, -NR^dC(=O)OR², -SC(=S)R², -C(=S)SR², -C(=S)R², -CH(OH)R², -P(=O)(OR^e)(OR^f), -(C₆-C₁₀ arylene)-R², -(6- to 10-membered heteroarylene)-R², or R²;

R¹ and R² are each independently C₆-C₃₂ alkyl or C₆-C₃₂ alkenyl;

R^a, R^b, R^d, and R^e are each independently H, C₁-C₂₄ alkyl, or C₂-C₂₄ alkenyl;

R^c and R^f are each independently C₁-C₃₂ alkyl or C₂-C₃₂ alkenyl;

G³ is C₂-C₂₄ alkylene, C₂-C₂₄ alkenylene, C₃-C₈ cycloalkylene, or C₃-C₈ cycloalkenylene;

R^3 is $-N(R^4)R^5$;

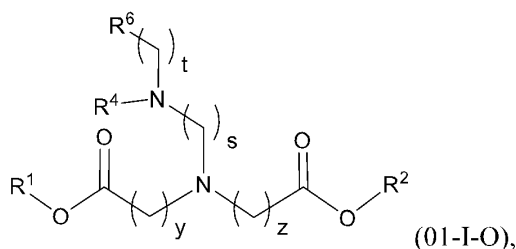
R^4 is C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, 4- to 8-membered heterocyclyl, or C_6 - C_{10} aryl; or R^4 , G^3 or part of G^3 , together with the nitrogen to which they are attached form a cyclic moiety;

- 5 R^5 is C_1 - C_{12} alkyl or C_3 - C_8 cycloalkyl; or R^4 , R^5 , together with the nitrogen to which they are attached form a cyclic moiety;

x is 0, 1 or 2; and

wherein each alkyl, alkenyl, cycloalkyl, cycloalkenyl, heterocyclyl, aryl, alkylene, alkenylene, cycloalkylene, cycloalkenylene, arylene, heteroarylene, and cyclic moiety is
10 independently optionally substituted.

In one embodiment, the cationic lipid comprises a compound of Formula (01-I-O):



wherein y and z are each independently an integer from 2 to 12,

s is an integer from 2 to 24,

- 15 t is an integer from 1 to 12, and

R^1 and R^2 are each independently C_6 - C_{32} alkyl or C_6 - C_{32} alkenyl;

R^4 is C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, 4- to 8-membered heterocyclyl, or C_6 - C_{10} aryl;

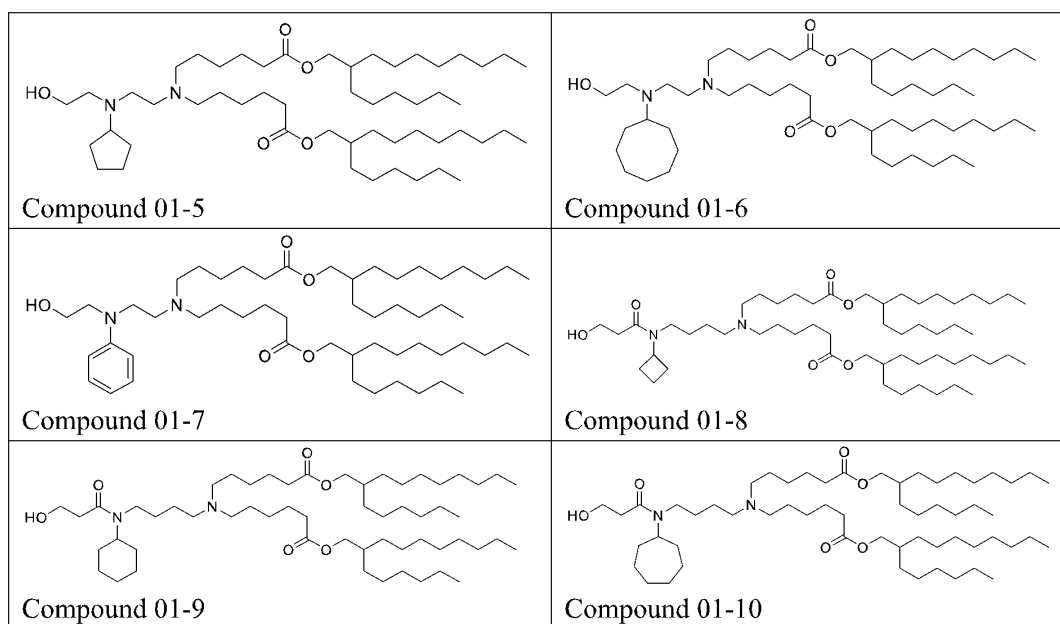
R^6 is hydrogen or hydroxyl,

or a pharmaceutically acceptable salt, prodrug or stereoisomer thereof.

- 20 In one embodiment, the cationic lipid comprises a compound in Table 01-1, or a pharmaceutically acceptable salt, prodrug or stereoisomer thereof.

Table 01-1

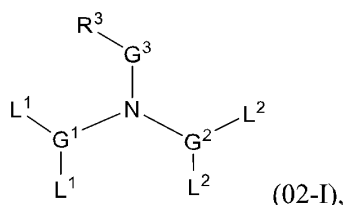
Compound 01-1	Compound 01-2
Compound 01-3	Compound 01-4



Series 02 of compounds

In one embodiment, the cationic lipid contained in the compositions, nanoparticle compositions, or nanoparticles provided herein is a cationic lipid described in International Patent Publication No. WO2023138611A1, the entirety of which is incorporated herein by reference.

In some embodiments, the cationic lipid of the present invention comprises a compound of Formula (02-I):



or a pharmaceutically acceptable salt, prodrug or stereoisomer thereof, wherein:

G^1 and G^2 are each independently C_2 - C_{12} alkylene or C_2 - C_{12} alkenylene, wherein one or more $-CH_2-$ in G^1 and G^2 is optionally replaced by $-O-$, $-C(=O)O-$, or $-OC(=O)-$;

each L^1 is independently $-OC(=O)R^1$, $-C(=O)OR^1$, $-OC(=O)OR^1$, $-C(=O)R^1$, $-OR^1$, $-S(O)_xR^1$, $-S-SR^1$, $-C(=O)SR^1$, $-SC(=O)R^1$, $-NR^aC(=O)R^1$, $-C(=O)NR^bR^c$, $-NR^aC(=O)NR^bR^c$, $-OC(=O)NR^bR^c$, $-NR^aC(=O)OR^1$, $-SC(=S)R^1$, $-C(=S)SR^1$, $-C(=S)R^1$, $-CH(OH)R^1$, $-P(=O)(OR^b)(OR^c)$, $-NR^aP(=O)(OR^b)(OR^c)$;

each L^2 is independently $-OC(=O)R^2$, $-C(=O)OR^2$, $-OC(=O)OR^2$, $-C(=O)R^2$, $-OR^2$, $-S(O)_xR^2$, $-S-SR^2$, $-C(=O)SR^2$, $-SC(=O)R^2$, $-NR^dC(=O)R^2$, $-C(=O)NR^eR^f$, $-NR^dC(=O)NR^eR^f$, $-OC(=O)NR^eR^f$, $-NR^dC(=O)OR^2$, $-SC(=S)R^2$, $-C(=S)SR^2$, $-C(=S)R^2$, $-CH(OH)R^2$, $-P(=O)(OR^e)(OR^f)$, $-NR^dP(=O)(OR^e)(OR^f)$;

R^1 and R^2 are each independently C_6 - C_{24} alkyl or C_6 - C_{24} alkenyl;

R^a , R^b , R^d , and R^e are each independently H, C_1 - C_{24} alkyl, or C_2 - C_{24} alkenyl;

R^c and R^f are each independently C_1 - C_{24} alkyl or C_2 - C_{24} alkenyl;

G^3 is C_2 - C_{24} alkylene, C_2 - C_{24} alkenylene, C_3 - C_8 cycloalkylene, or C_3 - C_8 cycloalkenylene;

R^3 is $-N(R^4)R^5$ or $-OR^6$;

R^4 is C_1 - C_{12} alkyl, C_2 - C_{12} alkenyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, C_6 - C_{10} aryl, or
5 4- to 8-membered heterocycloalkyl;

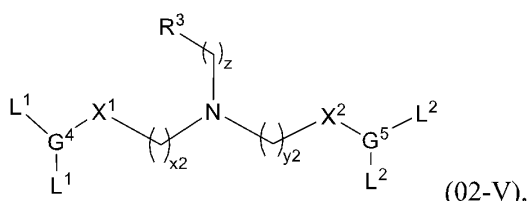
R^5 is hydrogen, C_1 - C_{12} alkyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, C_6 - C_{10} aryl, or 4- to 8-membered heterocycloalkyl;

R^6 is hydrogen, C_1 - C_{12} alkyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, or C_6 - C_{10} aryl;

x is 0, 1, or 2; and

10 wherein each alkyl, alkenyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, aryl, alkylene, alkenylene, cycloalkylene, and cycloalkenylene is independently optionally substituted.

In one embodiment, the cationic lipid comprises a compound of Formula (02-V):



wherein X^1 and X^2 are each independently a bond, $-O-$, $-C(=O)O-$, or $-OC(=O)-$;

15 z is an integer from 2 to 12;

each L^1 is independently $-OC(=O)R^1$, $-C(=O)OR^1$, $-OC(=O)OR^1$, $-C(=O)R^1$, $-OR^1$, $-S(O)_xR^1$, $-S-SR^1$, $-C(=O)SR^1$, $-SC(=O)R^1$, $-NR^aC(=O)R^1$, $-C(=O)NR^bR^c$, $-NR^aC(=O)NR^bR^c$, $-OC(=O)NR^bR^c$, $-NR^aC(=O)OR^1$, $-SC(=S)R^1$, $-C(=S)SR^1$, $-C(=S)R^1$, $-CH(OH)R^1$, $-P(=O)(OR^b)(OR^c)$, $-NR^aP(=O)(OR^b)(OR^c)$;

20 each L^2 is independently $-OC(=O)R^2$, $-C(=O)OR^2$, $-OC(=O)OR^2$, $-C(=O)R^2$, $-OR^2$, $-S(O)_xR^2$, $-S-SR^2$, $-C(=O)SR^2$, $-SC(=O)R^2$, $-NR^dC(=O)R^2$, $-C(=O)NR^eR^f$, $-NR^dC(=O)NR^eR^f$, $-OC(=O)NR^eR^f$, $-NR^dC(=O)OR^2$, $-SC(=S)R^2$, $-C(=S)SR^2$, $-C(=S)R^2$, $-CH(OH)R^2$, $-P(=O)(OR^e)(OR^f)$, $-NR^dP(=O)(OR^e)(OR^f)$;

R^3 is $-N(R^4)R^5$ or $-OR^6$;

25 R^4 is C_1 - C_{12} alkyl, C_2 - C_{12} alkenyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, C_6 - C_{10} aryl, or 4- to 8-membered heterocycloalkyl;

R^5 is hydrogen, C_1 - C_{12} alkyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, C_6 - C_{10} aryl, or 4- to 8-membered heterocycloalkyl;

R^6 is hydrogen, C_1 - C_{12} alkyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, or C_6 - C_{10} aryl;

30 x_2 is an integer from 2 to 5 when X^1 is $-O-$, $-C(=O)O-$, or $-OC(=O)-$, and x_2 is an integer from 2 to 6 when X^1 is a bond;

y_2 is an integer from 2 to 5 when X^2 is $-O-$, $-C(=O)O-$, or $-OC(=O)-$, and y_2 is an integer from 2 to 6 when X^2 is a bond;

G^4 and G^5 are each independently C_2 - C_6 alkylene;

35 or a pharmaceutically acceptable salt, prodrug or stereoisomer thereof.

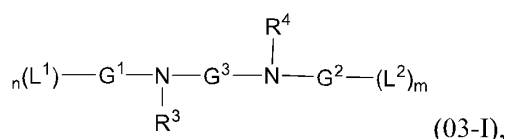
In one embodiment, the cationic lipid comprises a compound in Table 02-1, or a pharmaceutically acceptable salt, prodrug or stereoisomer thereof.

Table 02-1

Compound 02-1	Compound 02-2
Compound 02-3	Compound 02-4
Compound 02-5	Compound 02-6
Compound 02-7	Compound 02-8
Compound 02-9	Compound 02-10

Series 03 of compounds

- 5 In some embodiments, the cationic lipids of the present invention comprises at least one of those disclosed in International Application Publication No. WO2022152109A2, the entire teachings of which are incorporated herein by reference. Specifically, the cationic lipid comprises a compound represented by Formula (03-I):



or a pharmaceutically acceptable salt, prodrug or stereoisomer thereof, wherein:

G^1 and G^2 are each independently a bond, $\text{C}_2\text{-C}_{12}$ alkylene, or $\text{C}_2\text{-C}_{12}$ alkenylene, wherein one or more $-\text{CH}_2-$ in G^1 and G^2 is optionally replaced by $-\text{O}-$;

5 each L^1 is independently $-\text{OC}(=\text{O})\text{R}^1$, $-\text{C}(=\text{O})\text{OR}^1$, $-\text{OC}(=\text{O})\text{OR}^1$, $-\text{C}(=\text{O})\text{R}^1$, $-\text{OR}^1$, $-\text{S}(\text{O})_x\text{R}^1$, $-\text{S}-\text{SR}^1$, $-\text{C}(=\text{O})\text{SR}^1$, $-\text{SC}(=\text{O})\text{R}^1$, $-\text{NR}^a\text{C}(=\text{O})\text{R}^1$, $-\text{C}(=\text{O})\text{NR}^b\text{R}^c$, $-\text{NR}^a\text{C}(=\text{O})\text{NR}^b\text{R}^c$, $-\text{OC}(=\text{O})\text{NR}^b\text{R}^c$, $-\text{NR}^a\text{C}(=\text{O})\text{OR}^1$, $-\text{SC}(=\text{S})\text{R}^1$, $-\text{C}(=\text{S})\text{SR}^1$, $-\text{C}(=\text{S})\text{R}^1$, $-\text{CH}(\text{OH})\text{R}^1$, $-\text{P}(=\text{O})(\text{OR}^b)(\text{OR}^c)$, $-\text{NR}^a\text{P}(=\text{O})(\text{OR}^b)(\text{OR}^c)$, $-(\text{C}_6\text{-C}_{10} \text{ arylene})\text{-R}^1$, $-(6\text{- to } 10\text{-membered heteroarylene})\text{-R}^1$, $-(4\text{- to } 8\text{-membered heterocyclylene})\text{-R}^1$, or R^1 ;

10 each L^2 is independently $-\text{OC}(=\text{O})\text{R}^2$, $-\text{C}(=\text{O})\text{OR}^2$, $-\text{OC}(=\text{O})\text{OR}^2$, $-\text{C}(=\text{O})\text{R}^2$, $-\text{OR}^2$, $-\text{S}(\text{O})_x\text{R}^2$, $-\text{S}-\text{SR}^2$, $-\text{C}(=\text{O})\text{SR}^2$, $-\text{SC}(=\text{O})\text{R}^2$, $-\text{NR}^d\text{C}(=\text{O})\text{R}^2$, $-\text{C}(=\text{O})\text{NR}^e\text{R}^f$, $-\text{NR}^d\text{C}(=\text{O})\text{NR}^e\text{R}^f$, $-\text{OC}(=\text{O})\text{NR}^e\text{R}^f$, $-\text{NR}^d\text{C}(=\text{O})\text{OR}^2$, $-\text{SC}(=\text{S})\text{R}^2$, $-\text{C}(=\text{S})\text{SR}^2$, $-\text{C}(=\text{S})\text{R}^2$, $-\text{CH}(\text{OH})\text{R}^2$, $-\text{P}(=\text{O})(\text{OR}^e)(\text{OR}^f)$, $-\text{NR}^d\text{P}(=\text{O})(\text{OR}^e)(\text{OR}^f)$, $-(\text{C}_6\text{-C}_{10} \text{ arylene})\text{-R}^2$, $-(6\text{- to } 10\text{-membered heteroarylene})\text{-R}^2$, $-(4\text{- to } 8\text{-membered heterocyclylene})\text{-R}^2$, or R^2 ;

15 R^1 and R^2 are each independently $\text{C}_6\text{-C}_{24}$ alkyl or $\text{C}_6\text{-C}_{24}$ alkenyl;

R^a , R^b , R^d , and R^e are each independently H, $\text{C}_1\text{-C}_{24}$ alkyl, or $\text{C}_2\text{-C}_{24}$ alkenyl;

R^c and R^f are each independently $\text{C}_1\text{-C}_{24}$ alkyl or $\text{C}_2\text{-C}_{24}$ alkenyl;

G^3 is $\text{C}_2\text{-C}_{12}$ alkylene or $\text{C}_2\text{-C}_{12}$ alkenylene, wherein part or all of alkylene or alkenylene is optionally replaced by $\text{C}_3\text{-C}_8$ cycloalkylene, $\text{C}_3\text{-C}_8$ cycloalkenylene, $\text{C}_3\text{-C}_8$ cycloalkynylene, 4- to 8-membered heterocyclylene, $\text{C}_6\text{-C}_{10}$ arylene, or 5- to 10-membered heteroarylene;

20 R^3 is hydrogen, $\text{C}_1\text{-C}_{12}$ alkyl, $\text{C}_2\text{-C}_{12}$ alkenyl, $\text{C}_2\text{-C}_{12}$ alkynyl, $\text{C}_3\text{-C}_8$ cycloalkyl, $\text{C}_3\text{-C}_8$ cycloalkenyl, $\text{C}_3\text{-C}_8$ cycloalkynyl, 4- to 8-membered heterocyclyl, $\text{C}_6\text{-C}_{10}$ aryl, or 5- to 10-membered heteroaryl; or R^3 , G^1 or part of G^1 , together with the nitrogen to which they are attached form a cyclic moiety; or R^3 , G^3 or part of G^3 , together with the nitrogen to which they are attached form a cyclic moiety;

25 R^4 is $\text{C}_1\text{-C}_{12}$ alkyl or $\text{C}_3\text{-C}_8$ cycloalkyl;

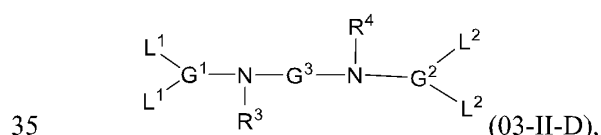
x is 0, 1, or 2;

n is 1 or 2;

m is 1 or 2; and

30 wherein each alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aryl, heteroaryl, alkylene, alkenylene, cycloalkylene, cycloalkenylene, cycloalkynylene, heterocyclylene, arylene, heteroarylene, and cyclic moiety is independently optionally substituted.

In one embodiment, the cationic lipid comprises a compound of Formula (03-II-D):

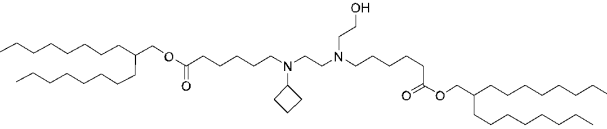
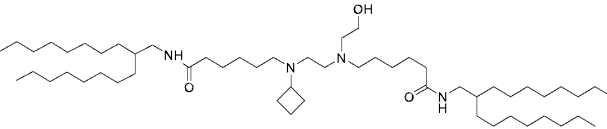
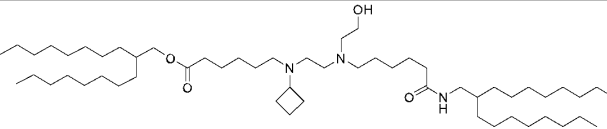
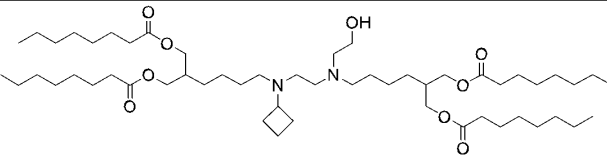
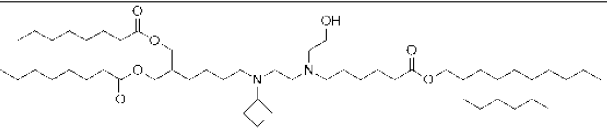
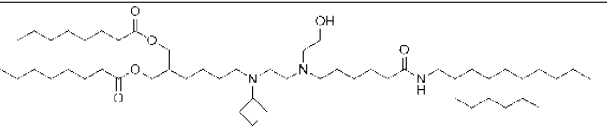
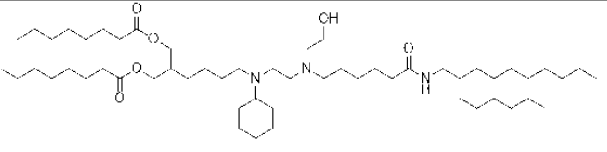
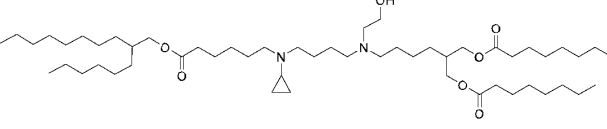


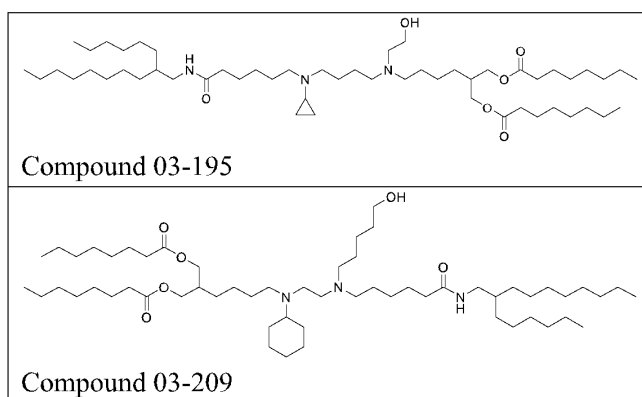
or a pharmaceutically acceptable salt, prodrug or stereoisomer thereof,

wherein G^1 , G^2 , G^3 , L^1 , L^2 , R^3 , and R^4 are as defined above.

In one embodiment, the cationic lipid comprises a compound in Table 03-1, or a pharmaceutically acceptable salt, prodrug or stereoisomer thereof.

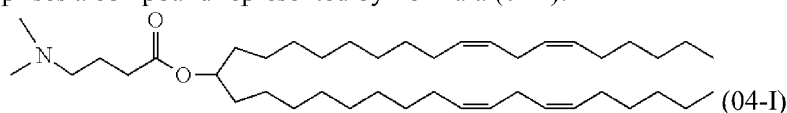
Table 03-1.


Compound 03-10

Compound 03-11

Compound 03-15

Compound 03-71

Compound 03-135

Compound 03-136

Compound 03-140

Compound 03-190



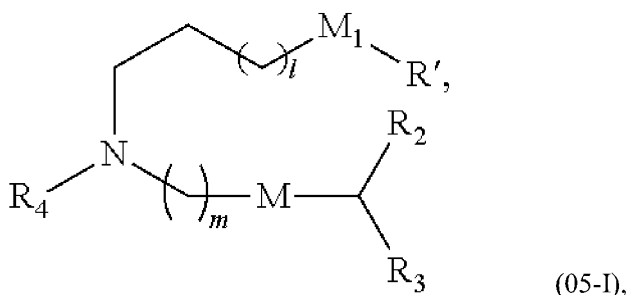
Series 04 of compounds

In some embodiments, the cationic lipid of the present invention comprises at least one of those disclosed in International Application Publication No. WO2010144740, the entire teachings of which are incorporated herein by reference. For example, the cationic lipid comprises a compound represented by Formula (04-I):



Series 05 of compounds

In some embodiments, the cationic lipid of the present invention comprises at least one of those disclosed in U.S. Patent Nos. US10442756B2, US9868691B2, and US9868692B2, the entire teachings of which are incorporated herein by reference. For example, the cationic lipid comprises a compound represented by Formula (05-I):



or a salt or isomer thereof, wherein:

l is selected from 1, 2, 3, 4, and 5;

m is selected from 5, 6, 7, 8, and 9;

M₁ is a bond or M';

R₄ is unsubstituted C₁₋₃ alkyl, or -(CH₂)_nQ, in which Q is OH, -NHC(S)N(R)₂, -NHC(O)N(R)₂, -N(R)C(O)R, -N(R)S(O)₂R, -N(R)R₈, -NHC(=NR₉)N(R)₂, -NHC(=CHR₉)N(R)₂, -OC(O)N(R)₂, -N(R)C(O)OR, -N(OR)C(O)R, -N(OR)S(O)₂R, -N(OR)C(O)OR, -N(OR)C(O)N(R)₂, -N(OR)C(S)N(R)₂, -N(OR)C(=NR₉)N(R)₂, -N(OR)C(=CHR₉)N(R)₂, or heteroaryl, and n is selected from 1, 2, 3, 4, or 5;

M and M' are independently selected from -C(O)O-, -OC(O)-, -C(O)N(R')-, -P(O)(OR')O-,

-S-S-, an aryl group, and a heteroaryl group; and R₂ and R₃ are both C₁₋₁₄ alkyl, or C₂₋₁₄ alkenyl, R₈ is selected from the group consisting of C₃₋₆ carbocycle and heterocycle;

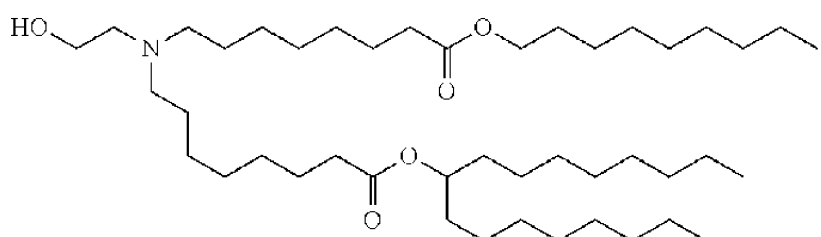
R₉ is selected from the group consisting of H, CN, NO₂, C₁₋₆ alkyl, -OR, -S(O)₂R, -S(O)₂N(R)₂, C₂₋₆ alkenyl, C₃₋₆ carbocycle and heterocycle;

5 each R is independently selected from the group consisting of C₁₋₃ alkyl, C₂₋₃ alkenyl, and H; and R' is a linear alkyl.

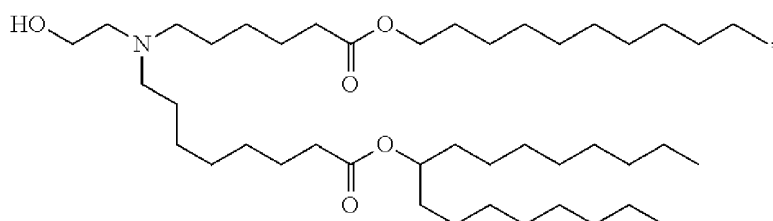
In some embodiments, R' is a linear C₁₋₁₈ alkyl, for example, C₁ alkyl, C₂ alkyl, C₃ alkyl, C₄ alkyl, C₅ alkyl, C₆ alkyl, C₇ alkyl, C₈ alkyl, C₉ alkyl, C₁₀ alkyl, C₁₁ alkyl, C₁₂ alkyl, C₁₃ alkyl, C₁₄ alkyl, C₁₅ alkyl, C₁₆ alkyl, C₁₇ alkyl and C₁₈ alkyl, preferably C₉ alkyl and C₁₁ alkyl.

10 In certain embodiments, the cationic lipid comprises at least one of compounds of Formula (A), (B):

(Formula A)



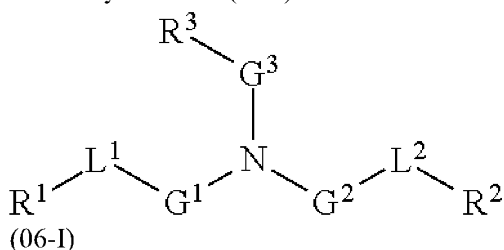
(Formula B)



15 or a salt or isomer thereof.

Series 06 of compounds

In some embodiments, the cationic lipid of the present invention comprises at least one of those disclosed in U.S. Patent No. US10166298B2, the entire teachings of which are incorporated herein by reference. For example, the cationic lipid comprises a compound represented by Formula (06-I):



25 or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein: one of L¹ or L² is -O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -S(O)_x-, -S-S-, -C(=O)S-, SC(=O)-, -NR^aC(=O)-, -C(=O)NR^a-, NR^aC(=O)NR^a-, -OC(=O)NR^a- or -NR^aC(=O)O-, and the other of

L^1 or L^2 is $-\text{O}(\text{C}=\text{O})-$, $-(\text{C}=\text{O})\text{O}-$, $-\text{C}(\text{=O})-$, $-\text{O}-$, $-\text{S}(\text{O})_{\text{x}}-$, $-\text{S}-\text{S}-$, $-\text{C}(\text{=O})\text{S}-$, $\text{SC}(\text{=O})-$, $-\text{NR}^{\text{a}}\text{C}(\text{=O})-$, $-\text{C}(\text{=O})\text{NR}^{\text{a}}-$, $\text{NR}^{\text{a}}\text{C}(\text{=O})\text{NR}^{\text{a}}-$, $-\text{OC}(\text{=O})\text{NR}^{\text{a}}-$ or $-\text{NR}^{\text{a}}\text{C}(\text{=O})\text{O}-$ or a direct bond;

G^1 and G^2 are each independently unsubstituted C_1 - C_{12} alkylene or C_1 - C_{12} alkenylene;

G^3 is C_1 - C_{24} alkylene, C_1 - C_{24} alkenylene, C_3 - C_8 cycloalkylene, C_3 - C_8 cycloalkenylene;

5 R^{a} is H or C_1 - C_{12} alkyl;

R^1 and R^2 are each independently C_6 - C_{24} alkyl or C_6 - C_{24} alkenyl;

R^3 is H, OR^5 , CN, $-\text{C}(\text{=O})\text{OR}^4$, $-\text{OC}(\text{=O})\text{R}^4$ or $-\text{NR}^5\text{C}(\text{=O})\text{R}^4$;

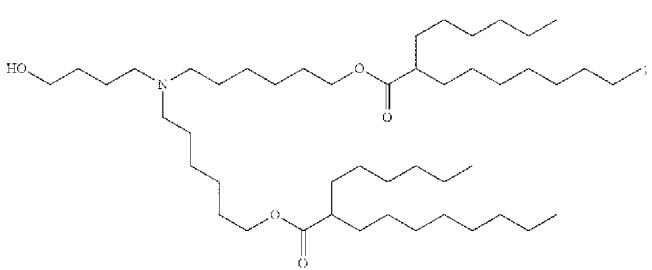
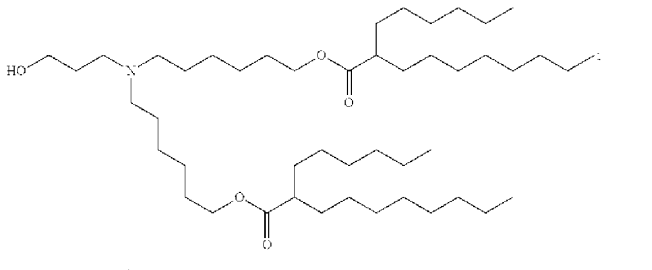
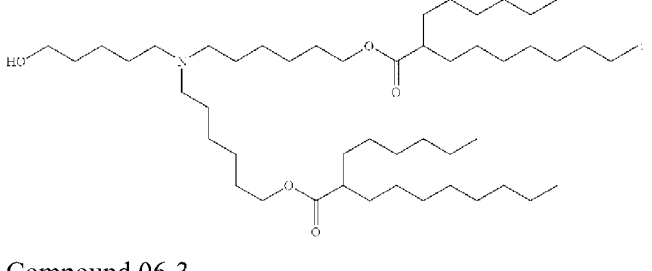
R^4 is C_1 - C_{12} alkyl;

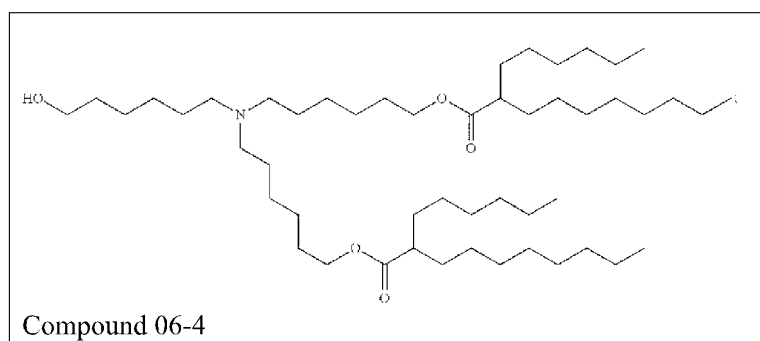
R^5 is H or C_1 - C_6 alkyl; and

10 x is 0, 1 or 2.

In one embodiment, the cationic lipid is a compound in Table 06-1, or a pharmaceutically acceptable salt, prodrug or stereoisomer thereof.

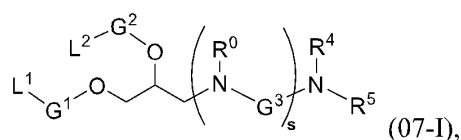
Table 06-1

 Compound 06-1
 Compound 06-2
 Compound 06-3



Series 07 of compounds

In some embodiments, the cationic lipid of the present invention comprises at least one of those disclosed in International Patent Application No. PCT/CN2022/094227, the entirety of which is incorporated herein by reference. For example, the cationic lipid comprises a compound represented by Formula (07-I):



or a pharmaceutically acceptable salt, prodrug, or stereoisomer thereof, wherein:

G^1 and G^2 are each independently a bond, C_2 - C_{12} alkylene, or C_2 - C_{12} alkenylene;

L^1 is $-OC(=O)R^1$, $-C(=O)OR^1$, $-OC(=O)OR^1$, $-C(=O)R^1$, $-OR^1$, $-S(O)_xR^1$, $-S-SR^1$, $-C(=O)SR^1$, $-SC(=O)R^1$, $-NR^aC(=O)R^1$, $-C(=O)NR^bR^c$, $-NR^aC(=O)NR^bR^c$, $-OC(=O)NR^bR^c$, $-NR^aC(=O)OR^1$, $-SC(=S)R^1$, $-C(=S)SR^1$, $-C(=S)R^1$, $-CH(OH)R^1$, $-P(=O)(OR^b)(OR^c)$, $-(C_6-C_{10} \text{ arylene})-R^1$, $-(6\text{- to } 10\text{-membered heteroarylene})-R^1$, or R^1 ;

L^2 is $-OC(=O)R^2$, $-C(=O)OR^2$, $-OC(=O)OR^2$, $-C(=O)R^2$, $-OR^2$, $-S(O)_xR^2$, $-S-SR^2$, $-C(=O)SR^2$, $-SC(=O)R^2$, $-NR^dC(=O)R^2$, $-C(=O)NR^eR^f$, $-NR^dC(=O)NR^eR^f$, $-OC(=O)NR^eR^f$, $-NR^dC(=O)OR^2$, $-SC(=S)R^2$, $-C(=S)SR^2$, $-C(=S)R^2$, $-CH(OH)R^2$, $-P(=O)(OR^e)(OR^f)$, $-(C_6-C_{10} \text{ arylene})-R^2$, $-(6\text{- to } 10\text{-membered heteroarylene})-R^2$, or R^2 ;

R^1 and R^2 are each independently C_5 - C_{32} alkyl or C_5 - C_{32} alkenyl;

R^a , R^b , R^d , and R^e are each independently H, C_1 - C_{24} alkyl, or C_2 - C_{24} alkenyl;

R^c and R^f are each independently C_1 - C_{32} alkyl or C_2 - C_{32} alkenyl;

R^0 is C_1 - C_{12} alkyl, C_2 - C_{12} alkenyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, C_6 - C_{10} aryl, or 4- to 8-membered heterocycloalkyl;

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

R^4 is C_1 - C_{12} alkyl, C_2 - C_{12} alkenyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, C_6 - C_{10} aryl, or 4- to 8-membered heterocycloalkyl;

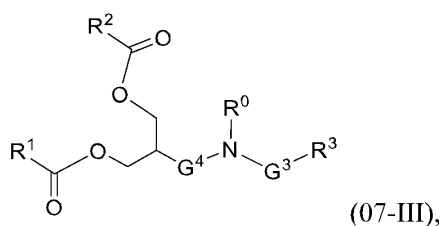
R^5 is C_1 - C_{12} alkyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, C_6 - C_{10} aryl, or 4- to 8-membered heterocycloalkyl;

x is 0, 1, or 2;

s is 0 or 1; and

wherein each alkyl, alkenyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, aryl, alkylene, alkenylene, arylene, and heteroarylene, is independently optionally substituted.

In some embodiments, the cationic lipid is a compound of Formula (07-III):



or a pharmaceutically acceptable salt, prodrug, or stereoisomer thereof, wherein:

R^1 and R^2 are each independently C_5 - C_{32} alkyl or C_5 - C_{32} alkenyl;

R^0 is C_1 - C_{12} alkyl, C_2 - C_{12} alkenyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, C_6 - C_{10} aryl, or
 5 4- to 8-membered heterocycloalkyl;

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

G^4 is C_2 - C_{12} alkylene or C_2 - C_{12} alkenylene;

R^3 is $-N(R^4)R^5$ or $-OR^6$;

R^4 is C_1 - C_{12} alkyl, C_2 - C_{12} alkenyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, C_6 - C_{10} aryl, or
 10 4- to 8-membered heterocycloalkyl;

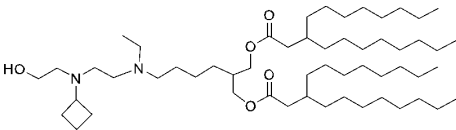
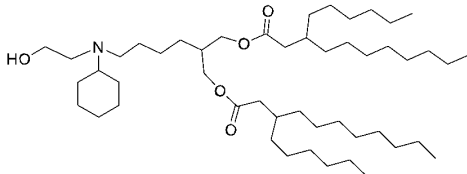
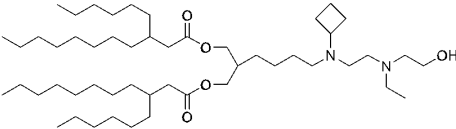
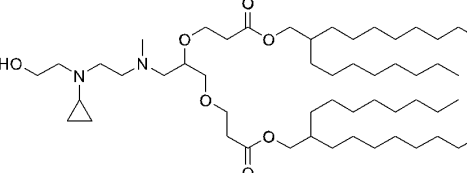
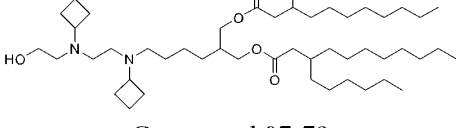
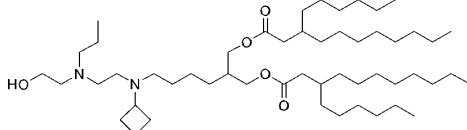
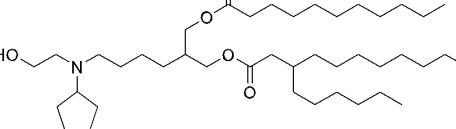
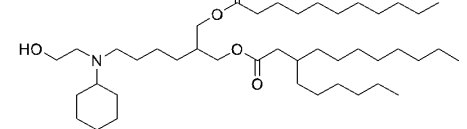
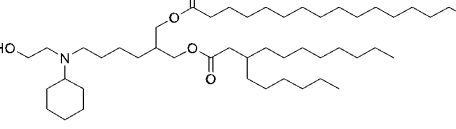
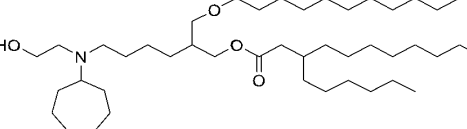
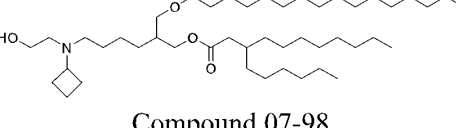
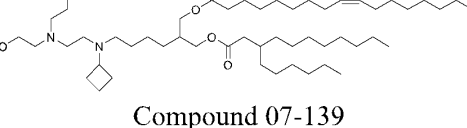
R^5 is C_1 - C_{12} alkyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, C_6 - C_{10} aryl, or 4- to 8-membered heterocycloalkyl; or R^4 and R^5 , together with the nitrogen to which they are attached form a cyclic moiety;

R^6 is hydrogen, C_1 - C_{12} alkyl, C_3 - C_8 cycloalkyl, C_3 - C_8 cycloalkenyl, or C_6 - C_{10} aryl; and
 15 wherein each alkyl, alkenyl, cycloalkyl, cycloalkenyl, heterocycloalkyl, aryl, alkylene, alkenylene, and cyclic moiety is independently optionally substituted.

In some embodiments, the cationic lipid is a compound in Table 4, or a pharmaceutically acceptable salt, prodrug, or stereoisomer thereof.

Table 07-1

Compound 07-1	Compound 07-2
Compound 07-7	Compound 07-8
Compound 07-65	Compound 07-66

 Compound 07-67	 Compound 07-68
 Compound 07-69	 Compound 07-70
 Compound 07-79	 Compound 07-80
 Compound 07-84	 Compound 07-85
 Compound 07-86	 Compound 07-92
 Compound 07-98	 Compound 07-139

• Structural Lipids

Without being bound by the theory, it is contemplated that structural lipids can stabilize the amphiphilic structure of a nanoparticle, such as but not limited to the lipid bilayer structure of a nanoparticle. Exemplary structural lipids that can be used in connection with the present disclosure include but are not limited to steroid, such as cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, tomatine, ursolic acid, alpha-tocopherol, and combinations thereof. In certain embodiments, the structural lipid is cholesterol. In some embodiments, the structural lipid is selected from cholesterol, a corticosteroid (such as prednisolone, dexamethasone, prednisone, and hydrocortisone), and a combination thereof.

In one embodiment, the lipid nanoparticles used in the present invention comprise a steroid or steroid analogue. In one embodiment, the lipid nanoparticles comprise cholesterol. In one embodiment, the lipid nanoparticles comprise a steroid, which is present in a concentration ranging from 13 to 55 mole percent, from 20 to 50 mole percent, from 30 to 50 mole percent, from 32 to 50 mole percent, from 39 to 49 mole percent, from 40 to 46 mole percent, from 40 to 44 mole percent, from 40 to 42 mole percent, from 42 to 44 mole percent, or from 44 to 46 mole percent. In one embodiment, the lipid nanoparticles comprise a steroid, which is present in a concentration of 40, 41, 42, 43, 44, 45, or 46 mole percent.

In one embodiment, the lipid nanoparticles comprise a steroid, and the molar ratio of cationic lipid to the steroid ranges from 0.6 to 2.75, or from 1.0 to 1.5. In one embodiment, the lipid nanoparticles comprise a steroid such as cholesterol, and the molar ratio of cationic lipid to cholesterol ranges from about 1.0 to 1.5. In one embodiment, the lipid nanoparticles comprise a steroid, and the steroid is present in a concentration ranging from 20 to 50 mol percent of the steroid.

• Phospholipids

Without being bound by the theory, it is contemplated that phospholipids may assemble into one or more lipid bilayers structures. Exemplary phospholipids that can form part of the lipid nanoparticles useful in the present invention include but are not limited to 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-3-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), and sphingomyelin. In certain embodiments, the LNPs include DSPC. In certain embodiments, the LNPs include DOPE. In some embodiments, the LNPs include both DSPC and DOPE.

Additional exemplary phospholipids include, for example, dipalmitoylphosphatidylglycerol (DPPG), palmitoyl-oleoyl-phosphatidylethanolamine (POPE) and dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1-stearoyl-2-oleoylphosphatidylethanolamine (SOPE), and 1,2-di-laidoyl-sn-glycero-3-phosphoethanolamine (transDOPE). In one embodiment, the lipid nanoparticles comprise 1,2-distearoyl-sn-glycero-3-phosphocholine

(DSPC). In one embodiment, the lipid nanoparticles comprise a phospholipid selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE, and SM.

In one embodiment, the lipid nanoparticles comprise a phospholipid selected from phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS),
 5 phosphatidic acid (PA), and phosphatidylglycerol (PG).

Additionally phospholipids that can form part of the present LNPs also include those described in WO2017/112865, the entire content of which is hereby incorporated by reference in its entirety.

In one embodiment, the lipid nanoparticles comprise a phospholipid, and the phospholipid
 10 is present in a concentration ranging from 2 to 50 mol percent, from 5 to 40 mol percent, from 5 to 15 mol percent, from 5 to 10 mol percent, from 7 to 13 mol percent, or from 9 to 11 mol percent. In one embodiment, the lipid nanoparticles comprise a phospholipid, and the phospholipid is present in a concentration of about 9.5, 10 or 10.5 mol percent.

In one embodiment, the lipid nanoparticles comprise a phospholipid, and the molar ratio
 15 of the cationic lipid to the phospholipid ranges from about 0.5 to about 11, or about 1.3 to about 6. In one embodiment, the lipid nanoparticles comprise a phospholipid, and the molar ratio of the cationic lipid to the phospholipid ranges from about 4 to about 7, from about 4.5 to about 6, or from about 4.5 to 5.5.

20 • Polymer conjugated lipids

In some embodiments, the lipid component of the LNPs useful in the present invention can include one or more polymer conjugated lipids, such as PEGylated lipids (PEG lipids). Without being bound by the theory, it is contemplated that a polymer conjugated lipid component in LNPs can improve of colloidal stability and/or reduce protein absorption of the
 25 nanoparticles. Exemplary polymer conjugated lipids that can be used in connection with the present disclosure include but are not limited to PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, PEG-modified dialkylglycerols, and combinations thereof. For example, a PEG lipid may be PEG-c-DOMG, PEG-DMG, PEG-DLPE, PEG-DMPE, PEG-DPPC, PEG-DSPE, Ceramide-PEG2000, or Chol-PEG2000.

In one embodiment, the lipid nanoparticles comprise a PEGylated lipid. For example, in some embodiments, the lipid nanoparticles comprise a polymer conjugated lipid selected from PEGylated diacylglycerol (PEG-DAG) such as 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG), a PEGylated phosphatidylethanolamine (PEG-PE), a PEG
 35 succinate diacylglycerol (PEG-S-DAG) such as 4-O-(2',3'-di(tetradecanoyloxy)propyl)-1-O-(ω -methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG), a PEGylated ceramide (PEG-cer), a PEG dialkoxypentylcarbamate such as ω -methoxy(polyethoxy)ethyl-N-(2,3-di(tetradecanoyloxy)propyl)carbamate and 2,3-di(tetradecanoyloxy)propyl-N-(ω -methoxy(polyethoxy)ethyl)carbamate. In one embodiment, the lipid nanoparticles comprise
 40 DMG-PEG. In one embodiment, the lipid nanoparticles comprise PEG-PE.

In one embodiment, the lipid nanoparticles comprise a polymer conjugated lipid, which is present in a concentration ranging from 1.0 to 2.5 mole percent. In one embodiment, the lipid nanoparticles comprise a polymer conjugated lipid, which is present in a concentration of about 1.7 mole percent. In one embodiment, the lipid nanoparticles comprise a polymer conjugated

lipid, which is present in a concentration of about 1.5 mole percent. In one embodiment, the lipid nanoparticles comprise a polymer conjugated lipid, and the molar ratio of cationic lipid to the polymer conjugated lipid ranges from about 20 to about 100. In one embodiment, the lipid nanoparticles comprise a polymer conjugated lipid, and the molar ratio of cationic lipid to the polymer conjugated lipid ranges from about 25 to about 50. In one embodiment, the lipid nanoparticles comprise a polymer conjugated lipid, and the molar ratio of cationic lipid to the polymer conjugated lipid ranges from about 25 to about 40.

In one embodiment, the lipid nanoparticle comprises, relative to the total mole number of all lipids present in the nanoparticle:

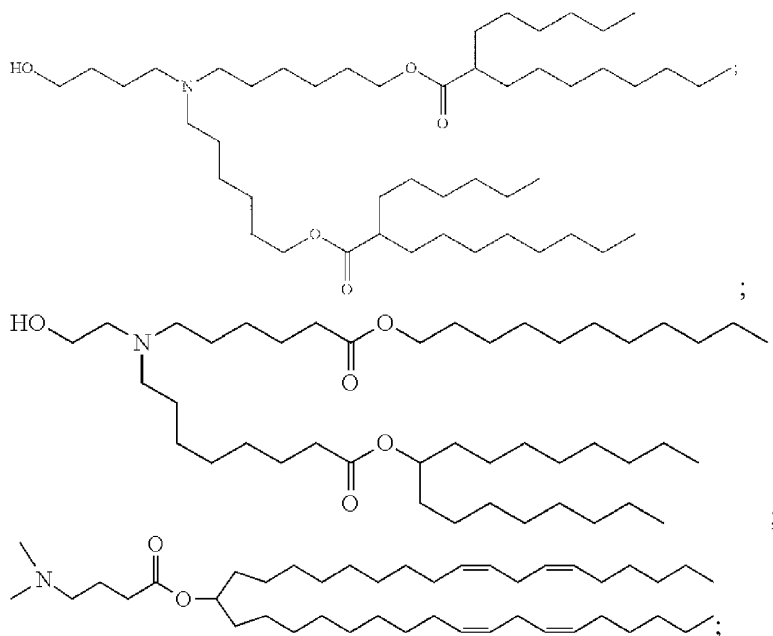
- (a) from about 30 mol % to about 55 mol % of a cationic lipid;
- (b) from about 20 mol % to about 50 mol % of a structural lipid;
- (c) from about 5 mol % to about 40 mol % of a phospholipid; and
- (d) from about 0.5 mol % to about 5 mol % of a polymer conjugated lipid.

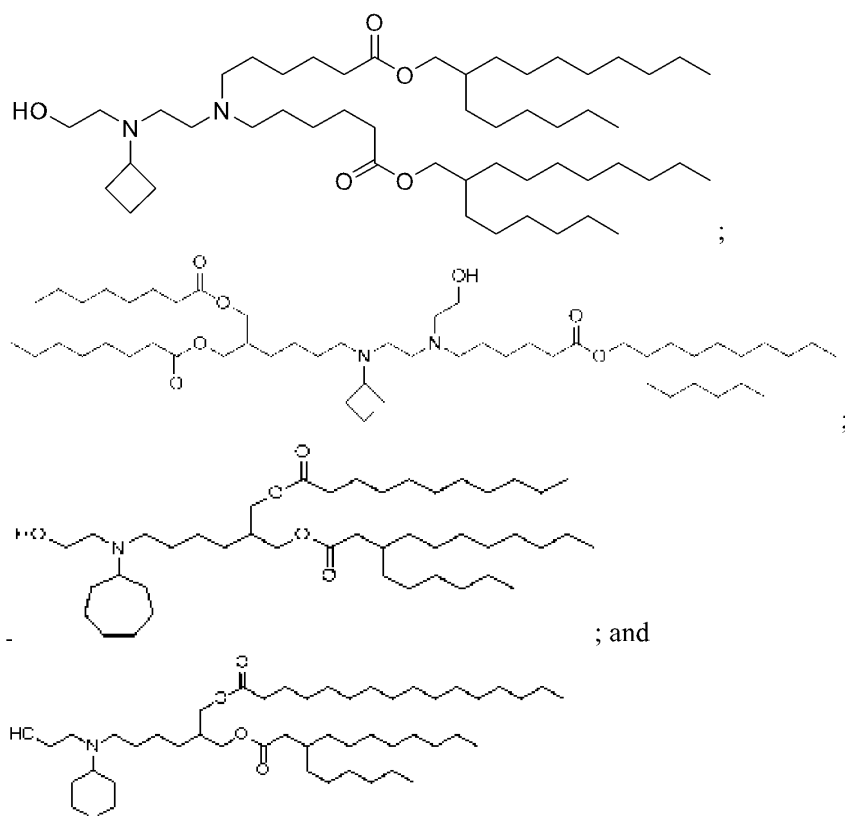
In one embodiment, the lipid nanoparticle comprises, relative to the total mole number of all lipids present in the nanoparticle:

- (a) from about 30 mol % to about 55 mol % of a cationic lipid;
- (b) from about 20 mol % to about 50 mol % of a steroid;
- (c) from about 5 mol % to about 40 mol % of a phospholipid; and
- (d) from about 0.5 mol % to about 3 mol % of a polymer conjugated lipid.

In one embodiment, the lipid nanoparticle comprises a cationic lipid, DSPC, cholesterol, and PEG-lipid.

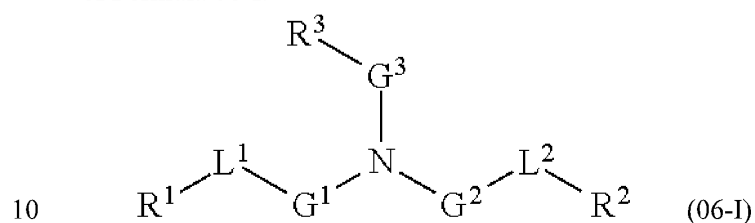
In one embodiment, the lipid nanoparticle comprises 30- 55 mol% of a cationic lipid, 5 - 40 mol% of DSPC, 20-50 mol% of cholesterol, and 0.5 -3 mol% of PEG-lipid, relative to the total mole number of all lipids in the nanoparticle, preferably the cationic lipid is selected from the following compounds:





5

In some embodiments, the lyophilized LNP formulation comprises 0.1-10 wt.%, preferably 0.1-1 wt.%, more preferably 0.2-0.7 wt.%, for example, 0.35 wt.%, relative to the total weight of the lyophilized LNP formulation, of a cationic lipid selected from compounds of Formula 06-I:



10

wherein

L^1 and L^2 is $-O(C=O)-$;

G^1 and G^2 are each independently unsubstituted C_4-C_8 alkylene;

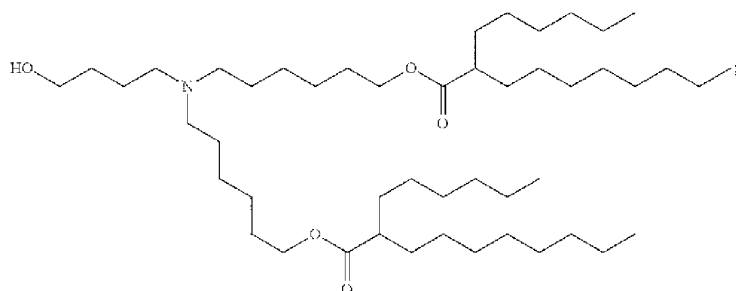
G^3 is C_3-C_8 alkylene;

15

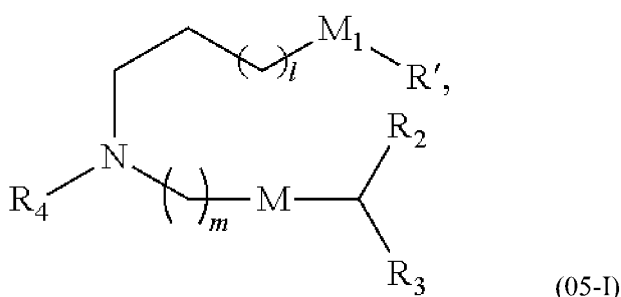
R^1 and R^2 are each independently $C_{12}-C_{22}$ alkyl;

R^3 is H or OH,

preferably the cationic lipid is compound of the following formula:



In some embodiments, the lyophilized LNP formulation comprises 0.1-10 wt.%, preferably 0.1-1 wt.%, more preferably 0.2-0.7 wt.%, for example, 0.32 wt.% or 0.33 wt.%, relative to the total weight of the lyophilized LNP formulation, of a cationic lipid selected from compounds of Formula 05-I:



wherein

l is selected from 1, 2, 3, 4, and 5;

m is selected from 5, 6, 7, 8, and 9;

M_1 is $-C(O)O-$;

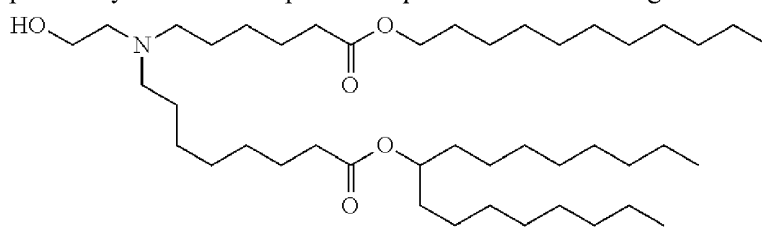
R_4 is $-(CH_2)_nOH$, and n is selected from 1, 2, 3, 4, or 5;

M is $-OC(O)-$;

R_2 and R_3 are both C_{6-10} alkyl; and

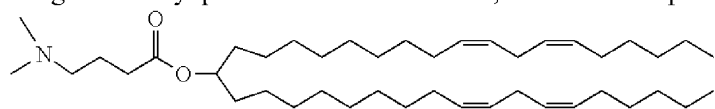
R' is a linear alkyl;

preferably the cationic lipid is compound of the following formula B:



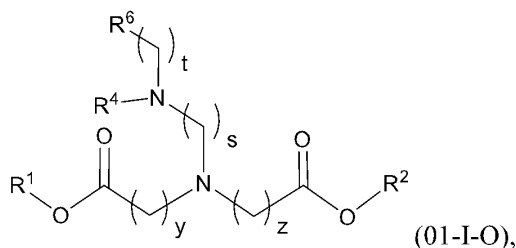
(Formula B).

In some embodiments, the lyophilized LNP formulation comprises 0.1-10 wt.%, preferably 0.1-1 wt.%, more preferably 0.2-0.7 wt.%, for example, 0.29 wt.%, relative to the total weight of the lyophilized LNP formulation, of a cationic lipid of the following formula:



In some embodiments, the lyophilized LNP formulation comprises 0.1-10 wt.%, preferably 0.1-1 wt.%, more preferably 0.2-0.7 wt.%, for example, 0.25 wt.%, 0.26 wt.%, 0.38

wt.% or 0.39 wt.%, relative to the total weight of the lyophilized LNP formulation, of a cationic lipid selected from compounds of Formula (01-I-O):



wherein y and z are each independently an integer from 4 to 6,

s is an integer from 2 to 4,

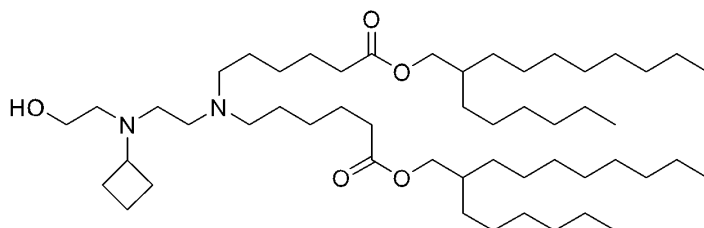
t is an integer from 1 to 3, and

R¹ and R² are each independently C₁₂-C₂₂ alkyl;

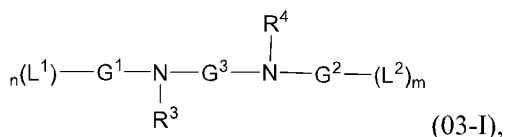
R⁴ is C₃-C₈ cycloalkyl;

R⁶ is hydrogen or hydroxyl,

preferably the cationic lipid is compound of the following formula:



In some embodiments, the lyophilized LNP formulation comprises 0.1-10 wt.%, preferably 0.1-1 wt.%, more preferably 0.2-0.7 wt.%, for example, 0.40 wt.%, relative to the total weight of the lyophilized LNP formulation, of a cationic lipid selected from compounds of Formula (03-I):



wherein

G¹ and G² are each independently C₃-C₈ alkylene;

each L¹ is independently -OC(=O)R¹;

each L² is independently -C(=O)OR²;

R¹ is independently C₆-C₁₀ alkyl;

R² is independently C₁₂-C₂₂ alkyl;

G³ is C₂-C₁₂ alkylene;

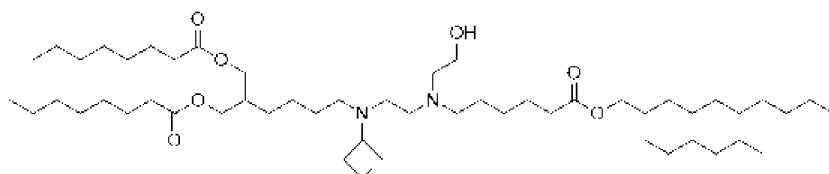
R³ is C₃-C₈ cycloalkyl;

R⁴ is C₁-C₄ hydroxylalkyl;

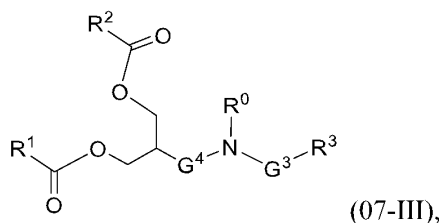
n is 2;

m is 1,

preferably the cationic lipid is compound of the following formula:



In some embodiments, the lyophilized LNP formulation comprises 0.1-10 wt.%, preferably 0.1-1 wt.%, more preferably 0.2-0.7 wt.%, for example, 0.33 wt.% or 0.35 wt.%, relative to the total weight of the lyophilized LNP formulation, of a cationic lipid selected from compounds of Formula (07-III):



wherein

R^1 and R^2 are each independently C_6 - C_{22} alkyl;

R^0 is C_3 - C_8 cycloalkyl;

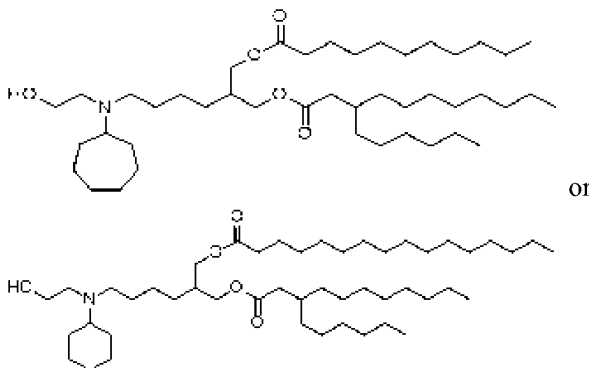
G^3 is C_2 - C_6 alkylene;

G^4 is C_2 - C_6 alkylene;

R^3 is $-OR^6$;

R^6 is hydrogen;

preferably the cationic lipid is compound of the following formula:



Cryoprotectant combination

The cryoprotectant combination in the lyophilized LNP formulation comprises sucrose and a non-polar amino acid.

In some embodiments, the lyophilized LNP formulation comprises 78-99 wt.%, for example, 80-98.5 wt.%, 82-98 wt.%, 84-97.5 wt.%, 85-97.5 wt.%, 87-97.5 wt.%, 89-97.5 wt.%, 91-97.5 wt.%, or 93-97.5 wt.% of sucrose, relative to the total weight of the lyophilized LNP formulation.

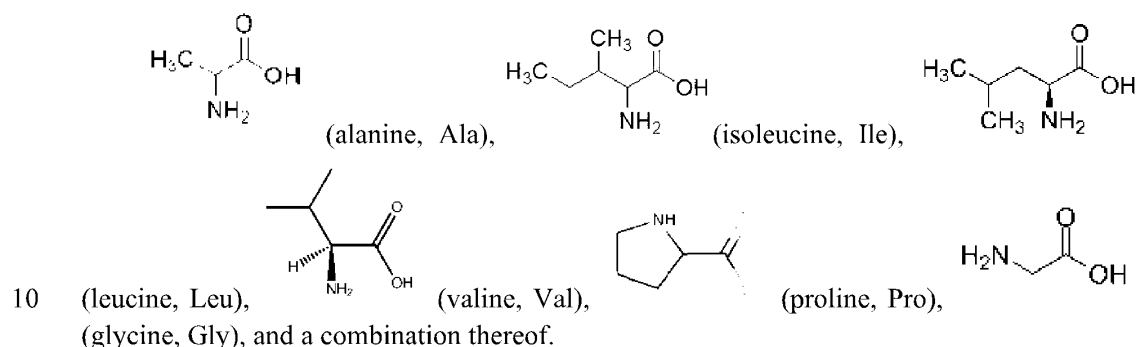
In some embodiments, the lyophilized LNP formulation comprises 93.39 wt.%, 93.85 wt.%, 94.20 wt.%, 94.22 wt.%, 94.25 wt.%, 94.27 wt.%, 94.3 wt.%, 94.36 wt.%, 94.41 wt.%, 94.44 wt.%, 94.55 wt.%, 94.62 wt.%, 94.64 wt.%, 94.89 wt.%, 95.14 wt.%, 95.40 wt.%, 95.96

wt.%, 96.48 wt.%, 96.58 wt.%, 96.78 wt.%, 97.04 wt.%, 97.08 wt.%, 97.31 wt.%, 97.58 wt.%, 97.68 wt.%, 97.84 wt.%, 98.16 wt.% or 98.73 wt.% of sucrose, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 0.2-9 wt.%, for example, 0.4-9 wt.%, 0.6-9 wt.%, 0.8-8 wt.%, 1-7 wt.%, 1.5-6 wt.%, 1.8-5 wt.%, or 2-4.8 wt.% of the non-polar amino acid, relative to the total weight of the lyophilized LNP formulation.

Non-polar amino acid

The non-polar amino acid used in the present invention is selected from



In some embodiments, the lyophilized LNP formulation comprises 0.4-6 wt.%, preferably 1-4 wt.% or 1-3 wt.% of alanine, relative to the total weight of the lyophilized LNP formulation. In some embodiments, the lyophilized LNP formulation comprises 1.70 wt.%, 1.74 wt.% or 3.42 wt.% of alanine, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 0.6-9 wt.%, preferably 1-5 wt.% of isoleucine, relative to the total weight of the lyophilized LNP formulation. In some embodiments, the lyophilized LNP formulation comprises 2.48 wt.% or 2.48 wt.% of isoleucine relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 0.6-9 wt.%, preferably 1-5 wt.% of leucine, relative to the total weight of the lyophilized LNP formulation. In some embodiments, the lyophilized LNP formulation comprises 2.48 wt.%, 2.54 wt.%, 4.95 wt.% or 4.96 wt.% of leucine, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 0.2-9 wt.%, for example, 0.5-8 wt.% or 1.5-6 wt.% of valine, relative to the total weight of the lyophilized LNP formulation. In some embodiments, the lyophilized LNP formulation comprises 0.38 wt.%, 0.58 wt.%, 0.76 wt.%, 1.15 wt.%, 1.51 wt.%, 2.22 wt.%, 2.24 wt.%, 2.27 wt.%, 2.97 wt.%, 4.38 wt.%, 4.40 wt.%, 4.42 wt.%, 4.43 wt.% or 4.45 wt.% of valine, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 0.5-8 wt.%, preferably 1-5 wt.% of proline, relative to the total weight of the lyophilized LNP formulation. In some embodiments, the lyophilized LNP formulation comprises 2.24 wt.% of proline, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 0.3-5 wt.%, preferably 0.8-3 wt.% of glycine, relative to the total weight of the lyophilized LNP formulation. In some embodiments, the lyophilized LNP formulation comprises 1.47 wt.% or 2.90 wt.% of glycine, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 1.5-4 wt.% of valine and 1-3 wt.% of alanine, relative to the total weight of the lyophilized LNP formulation. In some embodiments, the lyophilized LNP formulation comprises 2.24 wt.% of valine and 1.70 wt.% of alanine, relative to the total weight of the lyophilized LNP formulation.

5 In some embodiments, the lyophilized LNP formulation comprises 1.5-4 wt.% of valine and 1.5-4 wt.% of leucine, relative to the total weight of the lyophilized LNP formulation. In some embodiments, the lyophilized LNP formulation comprises 2.22 wt.% of valine and 2.48 wt.% of leucine, relative to the total weight of the lyophilized LNP formulation.

10 In some embodiments, the lyophilized LNP formulation comprises 1.5-4 wt.% of valine and 1.5-4 wt.% of isoleucine, relative to the total weight of the lyophilized LNP formulation. In some embodiments, the lyophilized LNP formulation comprises 2.22 wt.% of valine and 2.48 wt.% of isoleucine, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 95-98.5 wt.% of sucrose and 1-4 wt.% of alanine, relative to the total weight of the lyophilized LNP formulation.

15 In some embodiments, the lyophilized LNP formulation comprises 95-98.5 wt.% of sucrose and 1-3 wt.% of alanine, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 93-98 wt.% of sucrose and 1.5-4 wt.% of isoleucine, relative to the total weight of the lyophilized LNP formulation.

20 In some embodiments, the lyophilized LNP formulation comprises 93-98 wt.% of sucrose and 1.5-4 wt.% of leucine, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 93-99 wt.% of sucrose and 0.3-6 wt.% of valine, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 93-99 wt.% of sucrose and 0.4-6 wt.% of valine, relative to the total weight of the lyophilized LNP formulation.

25 In some embodiments, the lyophilized LNP formulation comprises 96-98 wt.% of sucrose and 1.5-3 wt.% of proline, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 97-98.5 wt.% of sucrose and 1-2 wt.% of glycine, relative to the total weight of the lyophilized LNP formulation.

30 In some embodiments, the lyophilized LNP formulation comprises 94-96 wt.% of sucrose, 1.5-3 wt.% of valine and 1-2.5 wt.% of alanine, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 93-95 wt.% of sucrose, 1.5-3 wt.% of valine and 1.5-3 wt.% of leucine, relative to the total weight of the lyophilized LNP formulation.

35 In some embodiments, the lyophilized LNP formulation comprises 93-95 wt.% of sucrose, 1.5-3 wt.% of valine and 1.5-3 wt.% of isoleucine, relative to the total weight of the lyophilized LNP formulation.

Salts or Tris

40 The lyophilized LNP formulation may further comprise a salt. In some embodiments, the lyophilized LNP formulation comprises 0.01-3 wt.%, for example, 0.1-3 wt.%, 0.15-2 wt.%, 0.2-1.5 wt.%, or 0.2-1.3 wt.% of a salt, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 0.1-3 wt.%, for example, 0.15-2 wt.%, 0.2-1.5 wt.%, or 0.2-1.3 wt.% of a salt, relative to the total weight of the lyophilized LNP formulation.

Representative salt useful in the present invention include, but are not limited to tris-HCl, KCl, NaCl, K₂HPO₄, KH₂PO₄, sodium citrate, sodium acetate, and a combination thereof.

In some embodiments, the salt is selected from tris-HCl, KCl, NaCl, sodium citrate, sodium acetate, and a combination thereof.

In some embodiments, the salt is selected from tris-HCl, KCl, NaCl, K₂HPO₄, KH₂PO₄, sodium citrate, sodium acetate, and a combination thereof.

In some embodiments, the lyophilized LNP formulation comprises 0.1-3 wt.%, for example, 0.28 wt.%, 0.55 wt.%, 0.74 wt.%, 0.75 wt.%, 0.76 wt.% or 1.10 wt.% of NaCl, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 0.1-3 wt.%, for example, 0.35 wt.% or 0.70 wt.% of KCl, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the salt is selected from KCl, NaCl, and a combination thereof.

In some embodiments, the salt is selected from KCl, NaCl, K₂HPO₄, KH₂PO₄, and a combination thereof.

In some embodiments, the lyophilized LNP formulation comprises 0.03 wt.% of K₂HPO₄, 0.66 wt.% of KH₂PO₄ and 0.74 wt.% of NaCl, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 0.03 wt.% of K₂HPO₄, 0.67 wt.% of KH₂PO₄ and 0.75 wt.% of NaCl, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the lyophilized LNP formulation comprises 0.03 wt.% of K₂HPO₄, 0.68 wt.% of KH₂PO₄ and 0.76 wt.% of NaCl, relative to the total weight of the lyophilized LNP formulation.

The lyophilized LNP formulation may further comprise Tris.

As used herein, "Tris" means the compound of tri(hydroxymethyl)aminomethane.

In some embodiments, the lyophilized LNP formulation comprises 0.1-3 wt.%, for example, 0.9-2 wt.%, 1.2-1.8 wt.%, or 1.4-1.7 wt.% of Tris, relative to the total weight of the lyophilized LNP formulation. In some embodiments, the lyophilized LNP formulation comprises 1.58 wt.% of Tris, relative to the total weight of the lyophilized LNP formulation.

In some embodiments, the non-polar amino acids are combined with salts or Tris as a combo system.

In an embodiment, the lyophilized LNP formulation comprises 97.58 wt.% of sucrose and 1.74 wt.% of alanine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 96.78 wt.% of sucrose and 2.54 wt.% of isoleucine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 96.78 wt.% of sucrose and 2.54 wt.% of leucine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 97.04 wt.% of sucrose and 2.27 wt.% of valine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 97.08 wt.% of sucrose and 2.24 wt.% of proline, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 97.84 wt.% of sucrose and 1.47 wt.% of glycine, by weight relative to the total weight of the lyophilized formulation.

5 In an embodiment, the lyophilized LNP formulation comprises 98.73 wt.% of sucrose and 0.58 wt.% of valine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 98.16 wt.% of sucrose and 1.15 wt.% of valine, by weight relative to the total weight of the lyophilized formulation.

10 In an embodiment, the lyophilized LNP formulation comprises 94.89 wt.% of sucrose and 4.45 wt.% of valine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 94.55 wt.% of sucrose, 4.43 wt.% of valine and 0.35 wt.% of KCl, by weight relative to the total weight of the lyophilized formulation.

15 In an embodiment, the lyophilized LNP formulation comprises 94.22 wt.% of sucrose, 4.42 wt.% of valine and 0.70 wt.% of KCl, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 94.62 wt.% of sucrose, 4.43 wt.% of valine and 0.28 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation.

20 In an embodiment, the lyophilized LNP formulation comprises 94.36 wt.% of sucrose, 4.42 wt.% of valine and 0.55 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation.

25 In an embodiment, the lyophilized LNP formulation comprises 93.85 wt.% of sucrose, 4.40 wt.% of valine and 1.10 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 94.25 wt.% of sucrose, 4.42 wt.% of valine and 0.70 wt.% of KCl, by weight relative to the total weight of the lyophilized formulation.

30 In an embodiment, the lyophilized LNP formulation comprises 94.27 wt.% of sucrose, 4.42 wt.% of valine and 0.70 wt.% of KCl, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 94.3 wt.% of sucrose, 4.42 wt.% of valine and 0.70 wt.% of KCl, by weight relative to the total weight of the lyophilized formulation.

35 In an embodiment, the lyophilized LNP formulation comprises 94.20 wt.% of sucrose, 4.4 wt.% of valine and 0.70 wt.% of KCl, by weight relative to the total weight of the lyophilized formulation.

40 In an embodiment, the lyophilized LNP formulation comprises 95.40 wt.% of sucrose, 2.24 wt.% of valine and 1.70 wt.% of alanine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 94.64 wt.% of sucrose, 2.22 wt.% of valine and 2.48 wt.% of leucine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 94.64 wt.% of sucrose, 2.22 wt.% of valine and 2.48 wt.% of isoleucine, by weight relative to the total weight of the lyophilized formulation.

5 In an embodiment, the lyophilized LNP formulation comprises 95.96 wt.% of sucrose and 3.42 wt.% of alanine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 96.48 wt.% of sucrose and 2.897 wt.% of glycine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 94.41 wt.% of sucrose and 4.95 wt.% of leucine, by weight relative to the total weight of the lyophilized formulation.

10 In an embodiment, the lyophilized LNP formulation comprises 96.48 wt.% of sucrose and 2.90 wt.% of glycine, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 94.4 wt.% of sucrose and 4.96 wt.% of leucine, by weight relative to the total weight of the lyophilized formulation.

15 In an embodiment, the lyophilized LNP formulation comprises 93.39 wt.% of sucrose, 4.38 wt.% of valine and 1.58 wt.% of Tris, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 95.14 wt.% of sucrose, 2.97 wt.% of valine, 0.03 wt.% of K_2HPO_4 , 0.66 wt.% of KH_2PO_4 and 0.74 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation.

20 In an embodiment, the lyophilized LNP formulation comprises 96.58 wt.% of sucrose, 1.51 wt.% of valine, 0.03 wt.% of K_2HPO_4 , 0.67 wt.% of KH_2PO_4 and 0.75 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation.

25 In an embodiment, the lyophilized LNP formulation comprises 97.31 wt.% of sucrose, 0.76 wt.% of valine, 0.03 wt.% of K_2HPO_4 , 0.68 wt.% of KH_2PO_4 and 0.76 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation.

In an embodiment, the lyophilized LNP formulation comprises 97.68 wt.% of sucrose, 0.38 wt.% of valine, 0.03 wt.% of K_2HPO_4 , 0.68 wt.% of KH_2PO_4 and 0.76 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation.

30 Therapeutical and/or prophylactic agents

The lyophilized LNP formulation of the present invention may further comprise a therapeutic and/or prophylactic agent in the lipid nanoparticles.

35 For example, the weight ratio of the lipid to the therapeutic and/or prophylactic agent in the lyophilized LNP formulation is from about 10: 1 to about 60: 1, or about 2: 1 to about 30: 1, e.g., or 20:1 to about 30:1.

The lyophilized formulation may be stored and diluted before or during administration. For example, the formulation for administration has about 0.01 mg/mL to about 2 mg/mL (e.g., about 0.01 mg/mL, about 0.025 mg/mL, about 0.05 mg/mL, about 0.075 mg/mL, about 0.1 mg/mL, about 0.3 mg/mL, about 0.5 mg/mL, about 1 mg/mL, about 1.5 mg/mL, about 2 mg/mL, 40 about 0.025-1 mg/mL or about 0.05-1 mg/mL, or about 0.5-1 mg/mL) of a therapeutic and/or prophylactic agent.

In specific embodiments, the therapeutic agent comprises a vaccine composition (e.g., a genetic vaccine) as described herein. In some embodiments, the therapeutic agent comprises a compound capable of eliciting immunity against one or more target conditions or disease. In

some embodiments, the target condition is related to or caused by infection by a pathogen, such as a coronavirus (e.g. 2019-nCoV), influenza, measles, human papillomavirus (HPV), rabies, meningitis, whooping cough, tetanus, plague, hepatitis, and tuberculosis. In some embodiments, the therapeutic agent comprises a nucleic acid sequence (e.g., mRNA) encoding a pathogenic protein characteristic for the pathogen, or an antigenic fragment or epitope thereof. The vaccine, upon administration to a vaccinated subject, allows for expression of the encoded pathogenic protein (or the antigenic fragment or epitope thereof), thereby eliciting immunity in the subject against the pathogen.

In some embodiments, the target condition is related to or caused by neoplastic growth of cells, such as a cancer. In some embodiments, the therapeutic agent comprises a nucleic acid sequence (e.g., mRNA) encoding a tumor associated antigen (TAA) characteristic for the cancer, or an antigenic fragment or epitope thereof. The vaccine, upon administration to a vaccinated subject, allows for expression of the encoded TAA (or the antigenic fragment or epitope thereof), thereby eliciting immunity in the subject against the neoplastic cells expressing the TAA.

The therapeutic and/or prophylactic agent may be e.g., a nucleic acid such as a ribonucleic acid (RNA), for example an mRNA.

For example, the mRNA has at least 30 nucleotides in length (e.g., at least 300 nucleotides in length).

In some embodiments, the mRNA is selected from rabies mRNA, respiratory syncytial virus mRNA, human erythropoietin mRNA, varicella zoster virus mRNA, all optionally modified with 1-N-Methyl-Pseudouridine, and a combination thereof.

In some embodiments, the mRNA is selected from rabies mRNA, respiratory syncytial virus mRNA with 1-N-Methyl-Pseudouridine modification, rabies mRNA with 1-N-Methyl-Pseudouridine modification, human erythropoietin mRNA, varicella zoster virus mRNA with 1-N-Methyl Pseudouridine modification, and a combination thereof.

In some embodiments, the mRNA is selected from rabies mRNA, respiratory syncytial virus mRNA with 1-N-Methyl-Pseudouridine modification, rabies mRNA with 1-N-Methyl-Pseudouridine modification, human erythropoietin mRNA, and a combination thereof.

The lyophilized LNP formulation comprising a therapeutic and/or prophylactic agent of the present invention has relatively higher encapsulation efficiency, for example, at least 85%, so that the *in vivo* bioactivity of the therapeutic and/or prophylactic agent can be greatly maintained.

As used herein, “encapsulation efficiency” means the weight percent of therapeutic and/or prophylactic agents encapsulated by lipids, relative to the total weight of therapeutic and/or prophylactic agents.

In some embodiments, the encapsulation efficiency of the lyophilized LNP formulation is at least 87%, at least 90%, at least 92%, or at least 94%.

Preparation of lyophilized LNP formulations

The lyophilized LNP formulation can be prepared by a known process in the art.

Taking a lyophilized LNP formulation comprising an mRNA as an example, a lipid is solubilized in a solvent such as ethanol. The mRNA is diluted in 10 to 50 mM citrate buffer, pH = 3-5. The LNPs are prepared at a total lipid to mRNA weight ratio of approximately 10:1

to 30:1 by mixing the lipid solution with the aqueous mRNA solution at a volume ratio of 1:3 using a microfluidic apparatus, total flow rate ranging from 9-30 mL/min. Solvent such as ethanol is then removed and replaced by PBS using dialysis. After dialysis, LNP is buffer exchanged to cryoprotectant buffers optionally with a salt or Tris to obtain a liquid LNP formulation and then filtered.

Lyophilization can be performed in a glass chamber of a Pilot Freeze Dryer (Christ Epsilon 2-10D LSCplus Lyophilizer). LNPs are frozen at -40 – -60 °C for 3 hours, followed by a primary dry cycle at -25 – -35 °C /20 – 100 mTorr for 48 – 60 hours. Next, during a secondary dry cycle, LNPs are warmed to 5- 25 °C/20 -100 mTorr and held for 12-24 hours. Vials were capped with stoppers at vacuum, and additionally capped with aluminum caps and transferred to 2-8 °C for long-term storage.

Liquid LNP formulations

In the second aspect, the present invention provides a liquid formulation of lipid nanoparticles (also called as “liquid LNP formulation”) comprising, by weight relative to the total volume of the liquid formulation:

(A) 0.001-0.2% w/v of lipid nanoparticles comprising a cationic lipid; and

(B) a cryoprotectant combination comprising

(i) 0.001-40% w/v of sucrose, and

(ii) 0.01-10% w/v of a non-polar amino acid.

The liquid LNP formulation can be used to prepare the lyophilized LNP formulation in the first aspect of the present invention.

The particle size of the LNP in the liquid LNP formulation is from about 50 nm to about 140 nm, preferably from about 60 nm to about 120 nm.

The definition for cationic lipid and the non-polar amino acid are the same as defined above regarding the lyophilized LNP formulation.

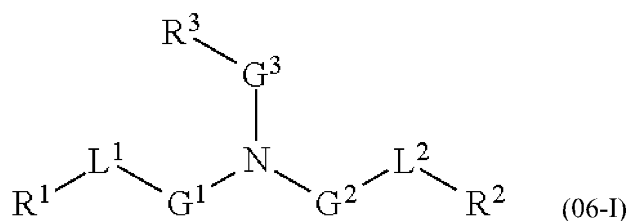
In some embodiments, the liquid LNP formulation comprises 0.001-0.2% w/v, for example, 0.001-0.1% w/v, 0.1-0.2 % w/v, 0.1-0.15 % w/v, 0.001-0.05% w/v, 0.001-0.03% w/v, 0.001-0.01% w/v, 0.01-0.1% w/v, or 0.05-0.1% w/v of the lipid nanoparticle, by weight relative to the total volume of the liquid LNP formulation.

The lipid nanoparticle may further comprise one or more of a structural lipid, a phospholipid, and a polymer conjugated lipid.

The definitions for the structural lipid, the phospholipid, and the polymer conjugated lipid for the same as those regarding the lyophilized LNP formulation.

If any of structural lipids, phospholipids, and polymer conjugated lipids presents, the mole ratios thereof to the cationic lipid is the same as those regarding the lyophilized LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.01-1 % w/v, preferably 0.01-0.1 % w/v, more preferably 0.02-0.07% w/v, for example, 0.038% w/v, by weight relative to the total volume of the liquid LNP formulation, of a cationic lipid selected from compounds of Formula 06-I :



wherein

L^1 and L^2 is $-O(C=O)-$;

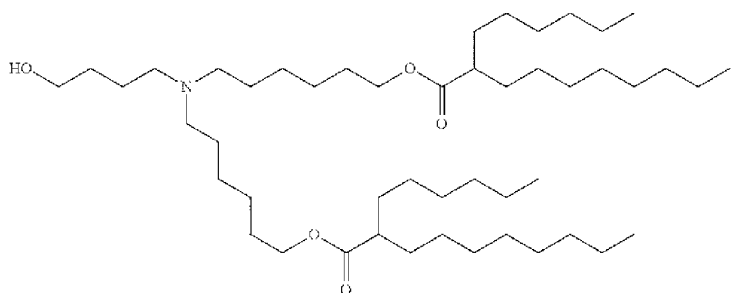
G^1 and G^2 are each independently unsubstituted C_4 - C_8 alkylene;

5 G^3 is C_3 - C_8 alkylene;

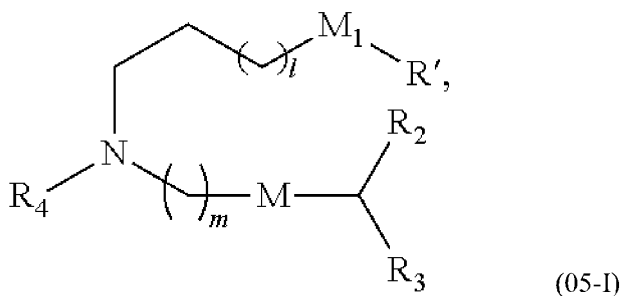
R^1 and R^2 are each independently C_{12} - C_{22} alkyl;

R^3 is H or OH,

preferably the cationic lipid is compound of the following formula:



10 In some embodiments, the liquid LNP formulation comprises 0.01-1 % w/v, preferably 0.01-0.1 % w/v, more preferably 0.02-0.07% w/v, for example, 0.034% w/v or 0.035% w/v, by weight relative to the total volume of the liquid LNP formulation, of a cationic lipid selected from compounds of Formula 05-I:



15 wherein

l is selected from 1, 2, 3, 4, and 5;

m is selected from 5, 6, 7, 8, and 9;

M_1 is $-C(O)O-$;

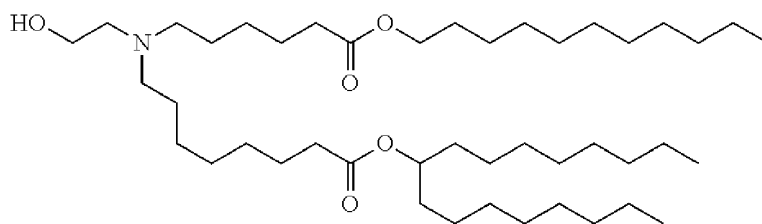
R_4 is $-(CH_2)_nOH$, and n is selected from 1, 2, 3, 4, or 5;

20 M is $-OC(O)-$;

R_2 and R_3 are both C_{6-10} alkyl; and

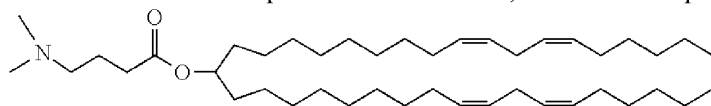
R' is a linear alkyl;

preferably the cationic lipid is compound of the following formula B:



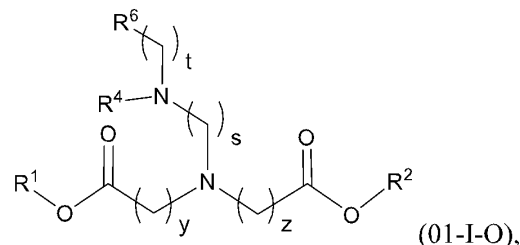
(Formula B).

In some embodiments, the liquid LNP formulation comprises 0.01-1% w/v, preferably 0.01-0.1 % w/v, more preferably 0.02-0.07% w/v, for example, 0.032% w/v, by weight relative to the total volume of the liquid LNP formulation, of a cationic lipid of the following formula:



5

In some embodiments, the liquid LNP formulation comprises 0.01-1% w/v, preferably 0.01-0.1 % w/v, more preferably 0.02-0.07% w/v, for example, 0.04% w/v or 0.041% w/v, by weight relative to the total volume of the liquid LNP formulation, of a cationic lipid selected from compounds of Formula (01-I-O):



10

wherein y and z are each independently an integer from 4 to 6,

s is an integer from 2 to 4,

t is an integer from 1 to 3, and

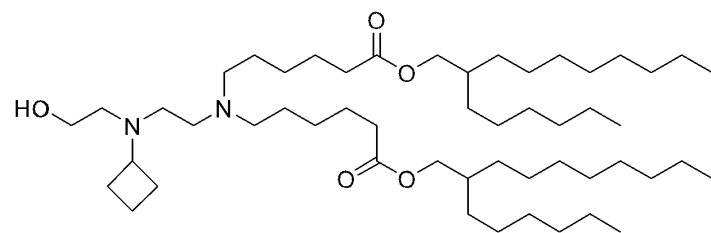
R¹ and R² are each independently C₁₂-C₂₂ alkyl;

15

R⁴ is C₃-C₈ cycloalkyl;

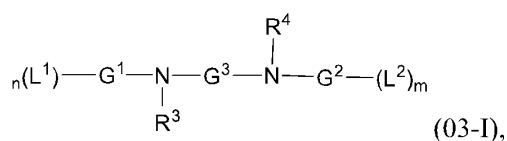
R⁶ is hydrogen or hydroxyl,

preferably the cationic lipid is compound of the following formula:



In some embodiments, the liquid LNP formulation comprises 0.01-1% w/v, preferably 0.01-0.1 % w/v, more preferably 0.02-0.07% w/v, for example, 0.043% w/v, by weight relative to the total volume of the liquid LNP formulation, of a cationic lipid selected from compounds of Formula (03-I):

20



wherein

G^1 and G^2 are each independently C_3 - C_8 alkylene;

each L^1 is independently $-OC(=O)R^1$;

5 each L^2 is independently $-C(=O)OR^2$;

R^1 is independently C_6 - C_{10} alkyl;

R^2 is independently C_{12} - C_{22} alkyl;

G^3 is C_2 - C_{12} alkylene;

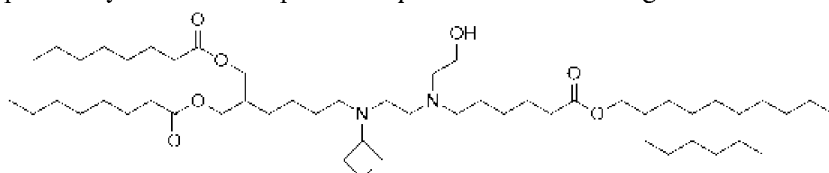
R^3 is C_3 - C_8 cycloalkyl;

10 R^4 is C_1 - C_4 hydroxylalkyl;

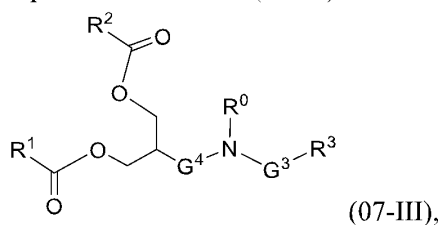
n is 2;

m is 1,

preferably the cationic lipid is compound of the following formula:



15 In some embodiments, the liquid LNP formulation comprises 0.01-1% w/v, preferably 0.01-0.1 % w/v, more preferably 0.02-0.07% w/v, for example, 0.034% w/v or 0.037 w/v, by weight relative to the total volume of the liquid LNP formulation, of a cationic lipid selected from compounds of Formula (07-III):



20 wherein

R^1 and R^2 are each independently C_6 - C_{22} alkyl;

R^0 is C_3 - C_8 cycloalkyl;

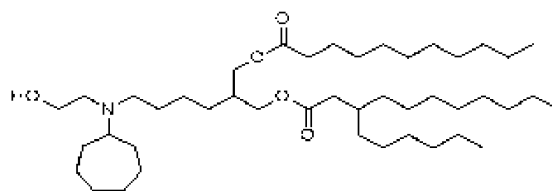
G^3 is C_2 - C_6 alkylene;

G^4 is C_2 - C_6 alkylene;

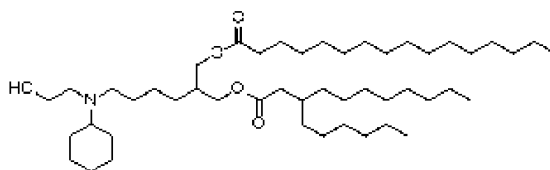
25 R^3 is $-OR^6$;

R^6 is hydrogen;

preferably the cationic lipid is compound of the following formula:



or



In some embodiments, the liquid LNP formulation comprises 0.01-35% w/v, for example, 0.1-30% w/v, 1-30% w/v, 2-25% w/v, 3-20% w/v, 4-15% w/v, 5-14% w/v, 6-13% w/v, 7-12% w/v, 8-11% w/v, 9-10% w/v, or 9.3-10 % w/v of sucrose, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 10% w/v of sucrose, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.04-1% w/v, for example, 0.06-0.9% w/v, 0.08-0.8% w/v, 0.1-0.7% w/v, 0.15-0.6% w/v, 0.18-0.5% w/v, or 0.2-0.5% w/v of a non-polar amino acid, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.04-0.6 % w/v, preferably 0.1-0.3% w/v of alanine, by weight relative to the total volume of the liquid LNP formulation. In some embodiments, the liquid LNP formulation comprises 0.18 % w/v, 0.23 % w/v or 0.36 % w/v of alanine, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.06-0.9 % w/v, preferably 0.1-0.5% w/v of isoleucine, by weight relative to the total volume of the liquid LNP formulation. In some embodiments, the liquid LNP formulation comprises 0.26 % w/v of isoleucine, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.06-0.9 % w/v, preferably 0.1-0.5% w/v of leucine, by weight relative to the total volume of the liquid LNP formulation. In some embodiments, the liquid LNP formulation comprises 0.26 % w/v or 0.52 % w/v of leucine, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.05-0.8 % w/v, preferably 0.15-0.6% w/v of valine, by weight relative to the total volume of the liquid LNP formulation. In some embodiments, the liquid LNP formulation comprises 0.06 % w/v, 0.12 % w/v, 0.23 % or 0.47 % w/v of valine, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.05-0.8 % w/v, preferably 0.1-0.5% w/v of proline, by weight relative to the total volume of the liquid LNP formulation. In some embodiments, the liquid LNP formulation comprises 0.23 % w/v of proline, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.03-0.5 % w/v, preferably 0.08-0.3% w/v of glycine, by weight relative to the total volume of the liquid LNP formulation. In some embodiments, the liquid LNP formulation comprises 0.15 % w/v or 0.30 % w/v of glycine, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.15-0.4 % w/v of valine and 0.1-0.3 % w/v of alanine, by weight relative to the total volume of the liquid LNP formulation. In some embodiments, the liquid LNP formulation comprises 0.23 % w/v of valine and 0.18 % w/v of alanine, by weight relative to the total volume of the liquid LNP formulation.

- In some embodiments, the liquid LNP formulation comprises 0.15-0.4 % w/v of valine and 0.15-0.4 % w/v of leucine, by weight relative to the total volume of the liquid LNP formulation. In some embodiments, the liquid LNP formulation comprises 0.23 % w/v of valine and 0.26 % w/v of leucine, by weight relative to the total volume of the liquid LNP formulation.
- 5 In some embodiments, the liquid LNP formulation comprises 0.15-0.4 % w/v of valine and 0.15-0.4 % w/v of isoleucine, by weight relative to the total volume of the liquid LNP formulation. In some embodiments, the liquid LNP formulation comprises 0.23 % w/v of valine and 0.26 % w/v of isoleucine, by weight relative to the total volume of the liquid LNP formulation.
- 10 In some embodiments, the liquid LNP formulation comprises 9.5-10 % w/v of sucrose and 0.1-0.4 % w/v of alanine, by weight relative to the total volume of the liquid LNP formulation.
- In some embodiments, the liquid LNP formulation comprises 9.5-10 % w/v of sucrose and 0.1-0.3 % w/v of alanine, by weight relative to the total volume of the liquid LNP formulation.
- In some embodiments, the liquid LNP formulation comprises 9.3-10 % w/v of sucrose and
- 15 0.15-0.4 % w/v of isoleucine, by weight relative to the total volume of the liquid LNP formulation.
- In some embodiments, the liquid LNP formulation comprises 9.3-10 % w/v of sucrose and 0.15-0.4 % w/v of leucine, relative to the total weight of the liquid LNP formulation.
- In some embodiments, the liquid LNP formulation comprises 9.3-10 % w/v of sucrose and
- 20 0.03-0.6 wt.% of valine, by weight relative to the total volume of the liquid LNP formulation.
- In some embodiments, the liquid LNP formulation comprises 9.3-10 % w/v of sucrose and 0.04-0.6 % w/v of valine, by weight relative to the total volume of the liquid LNP formulation.
- In some embodiments, the liquid LNP formulation comprises 9.6-10 % w/v of sucrose and 0.15-0.3 % w/v of proline, by weight relative to the total volume of the liquid LNP formulation.
- 25 In some embodiments, the liquid LNP formulation comprises 9.7-10 % w/v of glycine and 0.1-0.2 % w/v of glycine, by weight relative to the total volume of the liquid LNP formulation.
- In some embodiments, the liquid LNP formulation comprises 9.4-10 % w/v of sucrose, 0.15-0.3 % w/v of valine and 0.1-0.25 % w/v of alanine, by weight relative to the total volume of the liquid LNP formulation.
- 30 In some embodiments, the liquid LNP formulation comprises 9.3-10 % w/v of sucrose, 0.15-0.3 % w/v of valine and 0.15-0.3 % w/v of leucine, by weight relative to the total volume of the liquid LNP formulation.
- In some embodiments, the liquid LNP formulation comprises 9.3-10 % w/v of sucrose, 0.15-0.3 % w/v of valine and 0.15-0.3 % w/v of isoleucine, by weight relative to the total
- 35 volume of the liquid LNP formulation.
- The liquid LNP formulation may further comprise a salt.
- The salt is the same as defined above regarding the lyophilized LNP formulation.
- In some embodiments, the liquid LNP formulation comprises 0.001-0.3% w/v, for example, 0.01-0.3% w/v, 0.015-0.2 % w/v, 0.02-0.15 % w/v, or 0.02-0.13 % w/v of a salt, by
- 40 weight relative to the total volume of the liquid LNP formulation.
- In some embodiments, the liquid LNP formulation comprises 0.01-0.3% w/v, for example, 0.015-0.2 % w/v, 0.02-0.15 % w/v, or 0.02-0.13 % w/v of a salt, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.01-0.3% w/v, for example, 0.03 % w/v, 0.06 % w/v, 0.117 % w/v, or 0.12 % w/v of NaCl, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.01-0.3% w/v, for example, 0.04 % w/v or 0.07 % w/v of KCl, by weight relative to the total volume of the liquid LNP formulation.

In some embodiments, the liquid LNP formulation comprises 0.005% w/v of K_2HPO_4 , 0.10 % w/v of KH_2PO_4 and 0.117 % w/v of NaCl, by weight relative to the total volume of the liquid LNP formulation.

The liquid LNP formulation may further comprise Tris.

In some embodiments, the liquid LNP formulation comprises 0.01-0.3% w/v, for example, 0.015-0.25 % w/v, 0.12-0.22 % w/v, or 0.15-0.20 % w/v of Tris, by weight relative to the total volume of the liquid LNP formulation. In some embodiments, the liquid LNP formulation comprises 0.17% w/v of Tris, by weight relative to the total volume of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.18% w/v of alanine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.26% w/v of isoleucine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.26% w/v of leucine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.23% w/v of valine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.23% w/v of proline, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.15% w/v of glycine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.06% w/v of valine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.12% w/v of valine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.23% w/v of valine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.47% w/v of valine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.47% w/v of valine and 0.04% w/v of KCl, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.47% w/v of valine and 0.07% w/v of KCl, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.47% w/v of valine and 0.03% w/v of NaCl, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.47% w/v of valine and 0.06% w/v of NaCl, by weight relative to the total weight of the liquid LNP formulation.

5 In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.47% w/v of valine and 0.12% w/v of NaCl, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.23% w/v of valine, by weight relative to the total weight of the liquid LNP formulation.

10 In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.23% w/v of valine and 0.18% w/v of alanine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.23% w/v of valine and 0.26% w/v of leucine, by weight relative to the total weight of the liquid LNP formulation.

15 In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.23% w/v of valine and 0.26% w/v of isoleucine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.36% w/v of alanine, by weight relative to the total weight of the liquid LNP formulation.

20 In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.30% w/v of glycine, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose and 0.52% w/v of leucine, by weight relative to the total weight of the liquid LNP formulation.

25 In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.47% w/v of valine and 0.17% w/v of Tris, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.47% w/v of valine, 0.005% w/v of K_2HPO_4 , 0.10% w/v of KH_2PO_4 and 0.117% w/v of NaCl, by weight relative to the total weight of the liquid LNP formulation.

30 In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.23% w/v of valine, 0.005% w/v of K_2HPO_4 , 0.10% w/v of KH_2PO_4 and 0.117% w/v of NaCl, by weight relative to the total weight of the liquid LNP formulation.

35 In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.12% w/v of valine, 0.005% w/v of K_2HPO_4 , 0.10% w/v of KH_2PO_4 and 0.117% w/v of NaCl, by weight relative to the total weight of the liquid LNP formulation.

In an embodiment, the liquid LNP formulation comprises 10% w/v of sucrose, 0.06% w/v of valine, 0.005% w/v of K_2HPO_4 , 0.10% w/v of KH_2PO_4 and 0.117% w/v of NaCl, by weight relative to the total weight of the liquid LNP formulation.

40 The liquid LNP formulation of the present invention may further comprise a therapeutic and/or prophylactic agent in the lipid nanoparticles.

The therapeutic and/or prophylactic agent is the same as defined above regarding the lyophilized LNP formulation.

For example, the weight ratio of the lipid to the therapeutic and/or prophylactic agent in the lyophilized LNP formulation is from about 10: 1 to about 60: 1, or about 2: 1 to about 30: 1, e.g., or 20:1 to about 30:1.

In some embodiments, the liquid formulation disclosed herein comprises about 0.025 mg/mL to about 4 mg/mL (e.g., about 0.025 mg/mL, about 0.04 mg/mL, about 0.05 mg/mL, about 0.075 mg/mL, about 0.1 mg/mL, about 0.2 mg/mL, about 0.25 mg/mL, about 0.4 mg/mL, about 0.5 mg/mL, about 0.75 mg/mL, about 1 mg/mL, about 1.5 mg/mL, about 2 mg/mL, about 3 mg/mL, about 4 mg/mL, about 0.025-0.4 mg/mL or about 0.05-0.2 mg/mL, about 0.05-0.1 mg/mL about 0.25-2 mg/mL or about 0.5-2 mg/mL, or about 0.5-1 mg/mL) of a therapeutic and/or prophylactic (e.g., an mRNA), e.g., prior to lyophilization.

The liquid LNP formulation comprising a therapeutic and/or prophylactic agent of the present invention has an encapsulation efficiency of at least 75%.

In some embodiments, the encapsulation efficiency of the liquid LNP formulation is at least 77%, at least 79%, at least 81%, or at least 83%.

The examples that follow are given as non-limiting illustrations of the present invention.

EXPERIMENTAL

Inventive Examples (IE) 1-6 and Comparative Examples (CE) 1-17

Lyophilized nanoparticles encapsulating rabies mRNA (SEQ ID NO: 2) according to inventive examples 1-6 and comparative examples 1-17 were prepared as follows.

Specified amount of the lipid components (about 30 to 55 mol percent of compound C1, which is compound 01-1 in Table 01-1, as cationic lipid; from about 5 to 40 mol percent of DSPC; between about 20 to 50 mol percent of cholesterol; and a DMG-PEG) was solubilized in ethanol. In examples below, except other specified, the lipid components and their molar ratios are the same.

The Rabies mRNA was diluted in 10 to 50 mM citrate buffer, pH = 3-5. In examples below, except other specified, the mRNA is the same.

The LNPs were prepared at a total lipid to mRNA weight ratio of approximately 10:1 to 30:1 by mixing the ethanolic lipid solution with the aqueous mRNA solution at a volume ratio of 1:3 using a microfluidic apparatus, total flow rate ranging from 9-30 mL/min. In all examples, except other specified, the concentration of mRNA in the liquid LNP formulation is about 0.003% w/v, by weight relative to the total volume of the liquid LNP formulation. Ethanol was then removed and replaced by PBS using dialysis. After dialysis, LNP was buffer exchanged to cryoprotectant buffers (see Tables 1-3) to obtain a liquid LNP formulation and then filtered through a 0.2 µm sterile filter. LNP was loaded into type I glass vials.

Lyophilization was performed in a glass chamber of a Pilot Freeze Dryer (Christ Epsilon 2-10D LSCplus Lyophilizer). LNPs were frozen at -40 – -60 °C for 3 hours, followed by a primary dry cycle at -25 – -35 °C/20 – 100 mTorr for 48 – 60 hours. Next, during a secondary dry cycle, LNPs were warmed to 5- 25 °C/20 -100 mTorr and held for 12-24 hours. Vials were capped with stoppers at vacuum, and additionally capped with aluminum caps and transferred to 2-8 °C for long-term storage. In all examples, except other specified, the concentration of mRNA in the lyophilized LNP formulation is about 0.03 wt.%, relative to the total weight of the lyophilized LNP formulation.

Particle size, polydispersity index (PDI), and encapsulation efficiency of LNPs were characterized as follows.

Lipid nanoparticle size were determined by dynamic light scattering using a Malvern Zetasizer Nano ZS (Malvern UK) using a 173° backscatter detection mode. To measure the size and PDI of lipid nanoparticle, formulations were diluted 20-fold in PBS and transferred 1 mL in measurement cuvette.

The encapsulation efficiency (EE%) of lipid nanoparticles was determined using a Quant-it Ribogreen RNA quantification assay kit (Thermo Fisher Scientific, UK) according to the manufacturer's instructions. In order to determine free RNA and total RNA fluorescence intensity, LNP formulations were diluted to 0.5 µg/mL in Tris-EDTA and 0.1% Triton respectively.

Ribogreen reagent were diluted 200-fold with Tris-EDTA buffer and mixed at the same volume as diluted LNP formulation. Fluorescence intensity was measured at room temperature in a Molecular Devices Spectramax iD3 spectrometer using excitation and emission wavelengths of 488 nm and 525 nm. Encapsulation efficiency was calculated based on the ratio of encapsulated to total RNA fluorescence intensity.

The results obtained were summarized in Tables 1 and 2 and Figs. 1 and 2.

Table 1

Ex.	Lyophilization	Salts	Cryoprotectants	Size (nm)	ΔS (nm)	PDI	EE (%)
CE.1	before(%w/v)	0.24 Tris HCl	10 Sucrose	81.59	8.62	-	-
	after (%w/w)	2.35 Tris HCl	96.97 Sucrose	90.21		0.104	80.0
CE.2	before(%w/v)	0.24 HEPES	10 Sucrose	64.87	33.93	-	-
	after (%w/w)	2.31 HEPES	97.01 Sucrose	98.80		0.185	83.0
CE.3	before(%w/v)	0.20 MES	10 Sucrose	64.83	28.46	-	-
	after (%w/w)	2.07 MES	97.24 Sucrose	93.29		0.193	84.3
CE.4	before(%w/v)	0.24 Tris HCl	10 Sucrose 0.1 PEG200	79.79	9.24	-	-
	after (%w/w)	2.33 Tris HCl	96.04 Sucrose 0.96 PEG200	89.03		0.115	79.9
CE.5	before(%w/v)	0.24 Tris HCl	10 Sucrose 0.1 PEG400	81.02	8.63	-	-
	after (%w/w)	2.33 Tris HCl	96.04 Sucrose 0.96 PEG400	89.65		0.119	81.4
CE.6	before(%w/v)	0.24 Tris HCl	10 Sucrose 0.1 PEG600	80.30	6.43	-	-
	after (%w/w)	2.33 Tris HCl	96.04 Sucrose 0.96 PEG600	86.73		0.080	81.1

ΔS= Size change after lyophilization.

In comparative examples (CE) 1-6, the concentration of cationic lipid in the liquid LNP formulation is about 0.04% w/v, by weight relative to the total volume of the lipid LNP formulation, and the concentration of cationic lipid in the lyophilized LNP formulation is about 0.38-0.39 wt.%, relative to the total volume of the lyophilized LNP formulation.

It can be seen from Table 1 that conventional salts and cryoprotectants (e.g., Tris/sucrose, HEPES/sucrose, MES/sucrose) yield different size change and encapsulation efficiency lower than 85%.

Table 2

Ex.	Lyophilization	Cryoprotectants	Size (nm)	ΔS (nm)	PDI	EE (%)
IE.1	before(%w/v)	0.18 Alanine + 10 Sucrose	79.47	4.48	-	-
	after (%w/w)	1.74 Alanine + 97.58 Sucrose	83.95		0.096	92.4
IE.2	before(%w/v)	0.26 Isoleucine + 10 Sucrose	80.49	6.23	-	-
	after (%w/w)	2.54 Isoleucine + 96.78 Sucrose	86.72		0.158	92.6
IE.3	before(%w/v)	0.26 Leucine + 10 Sucrose	80.41	8.06	-	-
	after (%w/w)	2.54 Leucine + 96.78 Sucrose	88.47		0.191	91.9
IE.4	before(%w/v)	0.23 Valine + 10 Sucrose	79.57	5.67	-	-
	after (%w/w)	2.27 Valine + 97.04 Sucrose	85.24		0.143	90.3
IE.5	before(%w/v)	0.23 Proline + 10 Sucrose	80.3	6.23	-	-
	after (%w/w)	2.24 Proline + 97.08 Sucrose	86.53		0.119	92.3
IE.6	before(%w/v)	0.15 Glycine + 10 Sucrose	80.05	9.99	-	-
	after (%w/w)	1.47 Glycine + 97.84 Sucrose	90.04		0.151	92.8
CE.7	before(%w/v)	0.35 Arginine + 10 Sucrose	73.16	27.84	-	-
	after (%w/w)	3.34 Arginine + 95.98 Sucrose	101		0.290	73.5
CE.8	before(%w/v)	0.29 Lysine + 10 Sucrose	75.2	34.4	-	-
	after (%w/w)	2.84 Lysine + 96.5 Sucrose	109.6		0.392	70.3
CE.9	before(%w/v)	0.31 Histidine + 10 Sucrose	75.74	21.09	-	-
	after (%w/w)	2.99 Histidine + 96.33 Sucrose	96.83		0.128	83.9

5 In inventive examples (IE) 1-6 and comparative examples (CE) 7-9, the concentration of cationic lipid in the liquid LNP formulation is about 0.04% w/v, by weight relative to the total volume of the lipid LNP formulation, and the concentration of cationic lipid in the lyophilized LNP formulation is about 0.38-0.39 wt.%, relative to the total volume of the lyophilized LNP formulation.

10 It can be seen from Table 2 that non-polar amino acids (e.g., Val, Ala, Leu, Ile, Pro and Gly) together with sucrose can minimize particle size change ($\Delta \text{Size} \leq 10$ nm) and maintain high mRNA encapsulation (>90%) post-lyophilization. By contrast, alkaline amino acids (e.g., Arg, Lys, His) and sucrose leads to significantly size increases and low EE%, which indicates not all amino acids can be good cryoprotectants for mRNA-LNP.

Table 3

Ex.	Lyophilization	Cationic lipids	Cryoprotectants
CE.10	before(%w/v)	0.041 Compound C1	0.23 Valine + 10 Trehalose
	after (%w/w)	0.39 Compound C1	2.27 Valine + 97.04 Trehalose
CE.11	before(%w/v)	0.041 Compound C1	0.23 Valine + 10 Maltose
	after (%w/w)	0.39 Compound C1	2.27 Valine + 97.04 Maltose
CE.12	before(%w/v)	0.041 Compound C1	0.23 Valine + 10 Lactose
	after (%w/w)	0.39 Compound C1	2.27 Valine + 97.04 Lactose

CE.13	before(%w/v)	0.041 Compound C1	0.23 Valine + 10 Mannitol
	after (%w/w)	0.39 Compound C1	2.27 Valine + 97.04 Mannitol
CE.14	before(%w/v)	0.041 Compound C1	10 Sucrose
	after (%w/w)	0.40 Compound C1	99.30 Sucrose
CE.15	before(%w/v)	0.041 Compound C1	10 Trehalose
	after (%w/w)	0.40 Compound C1	99.30 Trehalose
CE.16	before(%w/v)	0.041 Compound C1	10 Maltose
	after (%w/w)	0.40 Compound C1	99.30 Maltose
CE.17	before(%w/v)	0.041 Compound C1	10 Lactose
	after (%w/w)	0.40 Compound C1	99.30 Lactose

Compound C1 is compound 01-1 in Table 01-1.

Pre-lyo and post-lyo sizes as well as polydispersity index of LNPs of CE.10-17 obtained with cryoprotectants in Table 3 are shown in Fig. 1.

Encapsulation efficiencies of LNPs of CE.10-17 obtained with cryoprotectants in Table 3 are shown in Fig. 2.

It can be seen from Figures 1 and 2 that other sugars or polyols other than sucrose (e.g., trehalose, maltose, lactose, or mannitol) cannot protect mRNA-LNP from lyophilization stress.

Inventive Examples (IE) 7-10 and Comparative Example (CE) 18

Lyophilized nanoparticles encapsulating hEPO mRNA (SEQ ID NO: 1) according to inventive examples 7-10 and comparative example 18 were prepared with cryoprotectants in Table 4 according to the process described above.

Particle size and encapsulation efficiency of LNPs before and after lyophilization were characterized. The results were summarized in Table 4.

Table 4

Ex.	Lyophilization	Cationic lipids	Cryoprotectants	Size (nm)	ΔS (nm)	EE (%)	ΔEE (%)
IE.7	before(%w/v)	0.041 Compound C1	0.06 Valine + 10 Sucrose	80	4.33	97.3	-3.3
	after (%w/w)	0.39 Compound C1	0.58 Valine + 98.73Sucrose	84.33		94.0	
IE.8	before(%w/v)	0.041 Compound C1	0.12 Valine + 10 Sucrose	81.17	4.96	97.9	-4.0
	after (%w/w)	0.39 Compound C1	1.15 Valine + 98.16 Sucrose	86.13		93.9	
IE.9	before(%w/v)	0.041 Compound C1	0.23 Valine + 10 Sucrose	78.09	7.27	96.4	-3.9
	after (%w/w)	0.39 Compound C1	2.27 Valine + 97.04 Sucrose	85.36		92.5	
IE.10	before(%w/v)	0.041 Compound C1	0.47 Valine + 10 Sucrose	78.51	6.94	96.6	-4.3
	after (%w/w)	0.38 Compound C1	4.45 Valine + 94.89Sucrose	85.45		92.3	

CE.18	before(%w/v)	0.041 Compound C1	0.82 Valine + 10 Sucrose	77.26	16.33	96.3	-4.9
	after (%w/w)	0.37 Compound C1	7.53 Valine + 91.83 Sucrose	93.59		91.4	

It can be seen from Table 4 that when the molar concentration of valine is below 0.8 % w/v, especially 0.06-0.5 % w/v, by weight relative to the total volume of the liquid LNP formulation, mRNA-LNP size and EE% can be well maintained.

5

Inventive Examples (IE) 11-15

Lyophilized nanoparticles encapsulating rabies mRNA according to inventive examples 11-15 were prepared with salts and cryoprotectants in Table 5 according to the process described above.

10 Particle sizes, and encapsulation efficiency of LNPs before and after lyophilization were characterized. The results were summarized in Table 5.

Table 5

Ex.	Lyophilization	Salts	Cryoprotectants	Size (nm)	ΔS (nm)	EE (%)	ΔEE (%)
IE.10	before(%w/v)	-	0.47 Valine + 10 Sucrose	78.51	6.94	96.6	-4.3
	after (%w/w)	-	4.45 Valine + 94.89 Sucrose	85.45		92.3	
IE.11	before(%w/v)	0.04 KCl	0.47 Valine + 10 Sucrose	80.22	5.34	95.7	-7.3
	after (%w/w)	0.35 KCl	4.43 Valine + 94.55 Sucrose	85.56		88.4	
IE.12	before(%w/v)	0.07 KCl	0.47 Valine + 10 Sucrose	82.26	3.54	96.5	-9.1
	after (%w/w)	0.70 KCl	4.42 Valine + 94.22 Sucrose	85.8		87.4	
IE.13	before(%w/v)	0.03 NaCl	0.47 Valine + 10 Sucrose	82.88	6.01	96.1	-7.9
	after (%w/w)	0.28 NaCl	4.43 Valine + 94.62 Sucrose	88.89		88.2	
IE.14	before(%w/v)	0.06 NaCl	0.47 Valine + 10 Sucrose	81.17	7.19	97.0	-7.6
	after (%w/w)	0.55 NaCl	4.42 Valine + 94.36 Sucrose	88.36		89.4	
IE.15	before(%w/v)	0.12 NaCl	0.47 Valine + 10 Sucrose	81.28	8.60	97.3	-8.4
	after (%w/w)	1.10 NaCl	4.40 Valine + 93.85 Sucrose	89.88		88.9	

In inventive examples (IE) 10-15, the concentration of cationic lipid in the liquid LNP formulation is 0.041% w/v, by weight relative to the total volume of the lipid LNP formulation, and the concentration of cationic lipid in the lyophilized LNP formulation is 0.38 wt.%, relative to the total volume of the lyophilized LNP formulation.

- 5 It can be seen from Table 4 that addition of salts (e.g., KCl or NaCl) in the valine system doesn't impact particle size and EE%. On top of that, salts can provide certain amount of ionic strength and better stability for in-process samples.

Inventive Examples (IE) 16-19

- 10 Lyophilized nanoparticles encapsulating rabies mRNA according to inventive examples 16-19 were prepared with cationic lipids instead of compound C1 and cryoprotectants in Table 6 according to the process described above.

Table 6

Ex.	Lyophilization	Cationic lipids	Salts	Cryoprotectants
IE.16	before(%w/v)	0.038 ALC0315	0.07 KCl	0.47 Valine + 10 Sucrose
	after (%w/w)	0.35 ALC0315	0.70 KCl	4.42 Valine + 94.25 Sucrose
IE.17	before(%w/v)	0.035 SM102	0.07 KCl	0.47 Valine + 10 Sucrose
	after (%w/w)	0.32 SM102	0.70 KCl	4.42 Valine + 94.27 Sucrose
IE.18	before(%w/v)	0.032 MC3	0.07 KCl	0.47 Valine + 10 Sucrose
	after (%w/w)	0.29 MC3	0.70 KCl	4.42 Valine + 94.3 Sucrose
IE.12	before(%w/v)	0.041 Compound C1	0.07 KCl	0.47 Valine + 10 Sucrose
	after (%w/w)	0.38 Compound C1	0.70 KCl	4.42 Valine + 94.22 Sucrose
IE.19	before(%w/v)	0.043 Compound C2	0.07 KCl	0.47 Valine + 10 Sucrose
	after (%w/w)	0.40 Compound C2	0.70 KCl	4.4 Valine + 94.20 Sucrose

ALC0315 is compound 06-1 in Table 06-1.

- 15 SM102 is compound of formula B in Series 05 of compounds.

MC3 is compound 04-I in Series 04 of compounds.

Compound C1 is compound 01-1 in Table 01-1.

Compound C2 is compound 03-135 in Table 03-1.

- 20 Particle sizes, polydispersity index, and encapsulation efficiency of LNPs before and after lyophilization were characterized.

Particle sizes and polydispersity index of LNPs before and after lyophilization obtained with lipids and cryoprotectants in Table 6 were shown in Fig. 3.

Encapsulation efficiencies of LNPs before and after lyophilization obtained with lipids and cryoprotectants in Table 6 were shown in Fig. 4.

- 25 It can be seen from Figs.3 and 4 that LNP with different ionizable lipids can be well protected.

Inventive Examples (IE) 20-21

- 30 Lyophilized nanoparticles encapsulating different mRNA according to inventive examples 20-21 were prepared with lipids, mRNA and cryoprotectants in Table 7 according to the process described above.

Table 7

Ex.	Lyophilization	Salts	Cryoprotectants	mRNA
IE.20	before(%w/v)	0.07 KCl	0.47 Valine + 10 Sucrose	0.003 RSV N1-Methyl mRNA
	after (%w/w)	0.70 KCl	4.42 Valine + 94.22 Sucrose	0.03 RSV N1-Methyl mRNA
IE.12	before(%w/v)	0.07 KCl	0.47 Valine + 10 Sucrose	0.003 Rabies mRNA
	after (%w/w)	0.70 KCl	4.42 Valine + 94.22 Sucrose	0.03 Rabies mRNA
IE.21	before(%w/v)	0.07 KCl	0.47 Valine + 10 Sucrose	0.003 Rabies N1-Methyl mRNA
	after (%w/w)	0.70 KCl	4.42 Valine + 94.22 Sucrose	0.03 Rabies N1-Methyl mRNA

Rabies N1-Methyl mRNA (SEQ ID NO: 3): rabies mRNA with 1-N-Methyl-Pseudouridine modification.

RSV N1-Methyl mRNA (SEQ ID NO: 4): respiratory syncytial virus mRNA with 1-N-Mehtyl-Pseudouridine modification.

In inventive examples (IE) 20-21, the concentration of cationic lipid in the liquid LNP formulation is 0.041% w/v, by weight relative to the total volume of the lipid LNP formulation, and the concentration of cationic lipid in the lyophilized LNP formulation is 0.38 wt.%, relative to the total volume of the lyophilized LNP formulation.

Particle sizes, polydispersity index, and encapsulation efficiency of LNPs before and after lyophilization were characterized.

Particle sizes and polydispersity index of LNPs obtained with lipids, mRNA and cryoprotectants in Table 7 before and after lyophilization were shown in Fig. 5.

Encapsulation efficiencies of LNPs obtained with lipids, mRNA and cryoprotectants in Table 7 before and after lyophilization were shown in Fig. 6.

It can be seen from Figs.5 and 6 that non-polar amino acids and sucrose as cryoprotectants have wide applicability in different mRNA-LNPs.

Inventive Example (IE) 22

Lyophilized nanoparticles encapsulating human erythropoietin (hEPO) mRNA according to inventive example 22 was prepared with a lipid and cryoprotectants in Table 8 according to the process described above.

Table 8

Ex.	Lyophilization	Salts	Cryoprotectants	mRNA
IE.22	before(%w/v)	0.07 KCl	0.47 Valine + 10 Sucrose	0.003 hEPO mRNA
	after (%w/w)	0.70 KCl	4.42 Valine + 94.22 Sucrose	0.03 hEPO mRNA

In inventive example (IE) 22, the concentration of cationic lipid in the liquid LNP formulation is 0.041% w/v, by weight relative to the total volume of the lipid LNP formulation,

and the concentration of cationic lipid in the lyophilized LNP formulation is 0.38 wt.%, relative to the total volume of the lyophilized LNP formulation.

LNPs pre- and post-lyophilization were systemically administered to 6-8 week-old female ICR mice (Xipuer-Bikai, Shanghai) at 0.4 mg/kg dose by tail vein injection. Mice were euthanized by CO₂ overdoses at 6 hours post administration, and blood samples were taken for hEPO measurement. Specifically, serum was separated from total blood by centrifugation at 5000g for 10 minutes at 4 °C, snap-frozen and stored at -80 °C for analysis.

The serum hEPO level was measured using an ELSA assay with a commercial kit (DEP00, R&D systems) according to manufacturer's instructions. The hEPO expression levels (μg/ml) measured from the tested group treated with mRNA LNPs before and after lyophilization were plotted in FIG.7.

Statistically, it can be seen from Fig. 7 that no significant difference was observed between protein expression level before and after LNP lyophilization, indicating that the cryoprotectant combination has no impact on the *in-vivo* activity of mRNA-LNP.

Inventive Examples (IE) 23-26

Lyophilized nanoparticles encapsulating human erythropoietin (hEPO) mRNA according to inventive examples 23-26 was prepared with lipids and cryoprotectants in Table 8 according to the process described above.

Particle sizes and encapsulation efficiency of LNPs before and after lyophilization were characterized. The results were summarized in Table 9.

Table 9

Ex.	Lyophilization	Cryoprotectants	Size (nm)	ΔS (nm)	EE (%)	ΔEE (%)
IE.23	before(%w/v)	0.23 Valine + 10 Sucrose	83.36	6.04	94.3	-3
	after (%w/w)	2.27 Valine + 97.04 Sucrose	89.40		91.3	
IE.24	before(%w/v)	0.23 Valine + 0.18 Alanine +10 Sucrose	79.75	6.25	96.5	-4.1
	after (%w/w)	2.24 Valine+ 1.70 Alanine + 95.40Sucrose	86.00		92.4	
IE.25	before(%w/v)	0.23 Valine +0.26 Leucine +10 Sucrose	78.48	6.74	96.4	-3.8
	after (%w/w)	2.22 Valine + 2.48 Leucine + 94.64 Sucrose	85.22		92.6	
IE.26	before(%w/v)	0.23 Valine + 0.26 Isoleucine + 10Sucrose	78.95	5.85	96.1	-3.8
	after (%w/w)	2.22 Valine +2.48 Isoleucine + 94.64 Sucrose	84.81		92.3	

In inventive examples (IE) 23-26, the concentration of cationic lipid in the liquid LNP formulation is about 0.04% w/v, by weight relative to the total volume of the lipid LNP formulation, and the concentration of cationic lipid in the lyophilized LNP formulation is about 0.38-0.39 wt.%, relative to the total volume of the lyophilized LNP formulation.

It can be seen from Table 9 that valine and other non-polar amino acids together with sucrose can protect mRNA-LNP from lyophilization stress. LNP characterizations maintain post-lyophilization.

LNPs pre- and post-lyophilization were systemically administered to 6-8 week old female ICR mice (Xipuer-Bikai, Shanghai) at 0.4 mg/kg dose by tail vein injection. Mice were euthanized by CO₂ overdoses at 6 hours post administration, and blood samples were taken for hEPO measurement as mentioned above for invention example 22. The hEPO expression levels (μg/ml) measured from the tested group treated with mRNA LNPs before and after lyophilization were plotted in FIG.8.

It can be seen from FIG.8 that the hEPO expression level (μg/ml) is not negatively impacted for all cryoprotectants.

Inventive Examples (IE) 27-31

Lyophilized nanoparticles encapsulating rabies mRNA according to inventive examples 27 - 31 were prepared with ionizable lipids instead of compound C1 and cryoprotectants in Table 10 according to the process described above.

Table 10

Ex.	Lyophilization	Ionizable lipids	Cryoprotectants
IE.27	before(%w/v)	0.034 Compound C3	0.36 Alanine + 10 Sucrose
	after (%w/w)	0.33 Compound C3	3.42 Alanine + 95.96 sucrose
IE.28	before(%w/v)	0.034 Compound C3	0.30 Glycine + 10 sucrose
	after (%w/w)	0.33 Compound C3	2.897 Glycine + 96.48 sucrose
IE.29	before(%w/v)	0.037 Compound C4	0.52 Leucine + 10 sucrose
	after (%w/w)	0.35 Compound C4	4.95 Leucine + 94.41 Sucrose
IE.30	before(%w/v)	0.034 SM102	0.30 Glycine + 10 sucrose
	after (%w/w)	0.33 SM102	2.90 Glycine + 96.48 Sucrose
IE.31	before(%w/v)	0.034 SM102	0.52 Leucine + 10 sucrose
	after (%w/w)	0.32 SM102	4.96 Leucine + 94.44 Sucrose

Compound C3 is Compound 07-92 in Table 07-1.

Compound C4 is Compound 07-86 in Table 07-1.

SM102 is compound of formula B in Series 05 of compounds.

Particle sizes and encapsulation efficiency of LNPs before and after lyophilization were characterized.

Particle sizes of LNPs before and after lyophilization obtained with lipids and cryoprotectants in Table 10 were shown in Fig. 9.

Encapsulation efficiencies of LNPs before and after lyophilization obtained with lipids and cryoprotectants in Table 10 were shown in Fig. 10.

It can be seen from Figs. 9 and 10 that LNP with different ionizable lipids can be well protected.

Invention Example (IE) 32-36 and Comparative Example (CE) 19-20

Lyophilized nanoparticles encapsulating mRNA according to inventive examples 32-36 and comparative examples 19-20 were prepared with cryoprotectants in Table 11 according to the process described above.

5

Table 11

Ex.	Lyophilization	Cationic lipids	Cryoprotectants	mRNA
IE.32	before(%w/v)	0.04 Compound C1	0.17 Tris + 0.47 Valine + 10 Sucrose	VZV N1-Methyl mRNA
	after (%w/w)	0.38 Compound C1	1.58 Tris + 4.38 Valine + 93.39 Sucrose	VZV N1-Methyl mRNA
CE.19	before(%w/v)	0.04 Compound C1	0.17 Tris + 10 Sucrose	VZV N1-Methyl mRNA
	after (%w/w)	0.39 Compound C1	1.66 Tris + 97.66 Sucrose	VZV N1-Methyl mRNA
IE.33	before(%w/v)	0.04 Compound C1	0.005 K ₂ HPO ₄ + 0.10 KH ₂ PO ₄ + 0.117 NaCl + 0.47 Valine + 10 Sucrose	Rabies N1-Methyl mRNA
	after (%w/w)	0.25 Compound C1	0.03 K ₂ HPO ₄ + 0.66 KH ₂ PO ₄ + 0.74 NaCl + 2.97 Valine + 95.14 Sucrose	Rabies N1-Methyl mRNA
IE.34	before(%w/v)	0.04 Compound C1	0.005 K ₂ HPO ₄ + 0.10 KH ₂ PO ₄ + 0.117 NaCl + 0.23 Valine + 10 Sucrose	Rabies N1-Methyl mRNA
	after (%w/w)	0.26 Compound C1	0.03 K ₂ HPO ₄ + 0.67 KH ₂ PO ₄ + 0.75 NaCl + 1.51 Valine + 96.58 Sucrose	Rabies N1-Methyl mRNA
IE.35	before(%w/v)	0.04 Compound C1	0.005 K ₂ HPO ₄ + 0.10 KH ₂ PO ₄ + 0.117 NaCl + 0.12 Valine + 10 Sucrose	Rabies N1-Methyl mRNA
	after (%w/w)	0.26 Compound C1	0.03 K ₂ HPO ₄ + 0.68 KH ₂ PO ₄ + 0.76 NaCl + 0.76 Valine + 97.31 Sucrose	Rabies N1-Methyl mRNA
IE.36	before(%w/v)	0.04 Compound C1	0.005 K ₂ HPO ₄ + 0.10 KH ₂ PO ₄ + 0.117 NaCl + 0.06 Valine + 10 Sucrose	Rabies N1-Methyl mRNA

	after (%w/w)	0.26 Compound C1	0.03 K ₂ HPO ₄ + 0.68 KH ₂ PO ₄ + 0.76 NaCl + 0.38 Valine + 97.68 Sucrose	Rabies N1-Methyl mRNA
CE.20	before(%w/v)	0.04 Compound C1	0.005 K ₂ HPO ₄ + 0.10 KH ₂ PO ₄ + 0.117 NaCl + 10 Sucrose	Rabies N1-Methyl mRNA
	after (%w/w)	0.26 Compound C1	0.04 K ₂ HPO ₄ + 0.69 KH ₂ PO ₄ + 0.77 NaCl + 98.06 Sucrose	Rabies N1-Methyl mRNA

Compound C1 is compound 01-1 in Table 01-1.

VZV N1-Methyl mRNA (SEQ ID NO: 5): varicella zoster virus mRNA with 1-N-Methyl-Pseudouridine modification.

Rabies N1-Methyl mRNA: rabies mRNA with 1-N-Methyl-Pseudouridine modification.

5 The purity of RNA and the levels of lipid adducts of LNPs after lyophilization, following further storage at a certain temperature for a certain period of time, were characterized.

For invention example 32 and comparative example 19, lyophilized LNPs were stored at 25 °C for up to 4 weeks, and samples were collected at Week 0, 1, 2 and 4 to measure lipid adducts and RNA purity. For invention examples 33 to 36 and comparative example 20, 10 lyophilized LNPs were stored at 37 °C for up to 7 days, and samples were collected at Day 0, 3 and 7 to measure lipid adducts.

To measure RNA purity and the levels of lipid adduct, mRNA was extracted from the mRNA-LNP formulation by isopropanol precipitation. The mRNA-LNP was diluted 10-fold with ammonium acetate in isopropanol, vortexed briefly, and centrifuged for 15min. The pellet 15 was washed and dried in vacuum, and resuspended in 100 µL RNase-free water at room temperature.

To measure lipid adducts, extracted mRNA samples were separated on a DNAPac Reverse Phase column with 4-µm particles and dimensions of 2.1 × 100 mm (Thermo Fisher Scientific). Mobile phase A consisted of 50 mM dibutylammonium acetate and 10 mM triethylammonium acetate, while mobile phase B consisted of 50% acetonitrile, 50 mM dibutylammonium acetate, 20 and 100 mM triethylammonium acetate. Separation was accomplished by step-gradient with an initial 1.5-minute hold at 25% B, a 1-minute gradient from 25–45% B, a 12.5-minute gradient from 45–100% B, and a 0.5- minute gradient and hold at 100% B. Approximately 2 µg of mRNA was loaded on the column. mRNA was detected by UV at 260 nm. Lipid adducts are 25 quantified as the relative percent of late peak area over the total chromatographic peak area.

To measure RNA purity, extracted mRNA samples were tested on a Fragment Analyzer (Agilent Technologies), which was an automated capillary electrophoresis system equipped with an LED light source and charged-coupled device detector. The RNA Analysis Kit (Agilent Technologies DNF-489-0500) was required to perform the experiment. Extracted mRNA 30 samples were denatured at 70 °C for 2min and cooled on ice immediately, keeping the denatured samples on ice prior to the analysis. Denatured RNA samples were electrokinetically injected at 5 kV for 4 s, and electrophoresis was performed for 45 min at 8 kV. An RNA ladder was similarly analyzed as a calibrator for nucleotide size. Data were analyzed using PROSize 2.0 software (Agilent Technologies).

The purities of RNA of LNPs after lyophilization obtained with lipids and cryoprotectants from invention example 32 and comparative example 19, following further storage at 25 °C for 0 to 4 weeks, were shown in Fig. 11.

It can be seen from Fig. 11 that non-polar amino acids together with Tris and sucrose could
5 slow down RNA degradation during storage at 25 °C compared to using only Tris/sucrose.

The levels of lipid adducts of LNPs after lyophilization obtained with lipids and cryoprotectants from invention example 32 and comparative example 19, following further storage at 25°C for 0 to 4 weeks, were shown in Fig. 12.

It can be seen from Fig. 12 that non-polar amino acids together with Tris and sucrose could
10 minimize lipid adducts formation during storage at 25 °C compared to using only Tris/sucrose.

The levels of lipid adducts of LNPs after lyophilization obtained with lipids and cryoprotectants from invention examples 33 to 36 and comparative example 20, following further storage at 37°C for 0 to 7 days, were shown in Fig. 13.

It can be seen from Fig. 13 that addition of valine in $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4/\text{NaCl}/\text{Sucrose}$ buffer helps to reduce lipid adduct formation during storage at 37 °C. The levels of lipid adducts are dependent on valine concentration. When the molar concentration of valine is 0.06-0.5 % w/v, by weight relative to the total volume of the liquid LNP formulation, the levels of lipid adducts can remain low. The higher valine concentration, the lower the level of lipid adducts.

A number of embodiments and examples of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, the descriptions in the Experimental section and examples are intended to illustrate but not limit the scope of invention described in the claims.

25

SEQUENCES

In the following sequences, “Ψ” denotes “m1Ψ (1-methyl-pseudouridine)” unless
30 otherwise indicated. The abbreviation is for the clarity of the sequences.

SEQ ID NO: 1 (hEPO mRNA)

GGGGAUCCAAGCUUAGCUUGCUUGUUCUUUUUGCAGAAGCUCAGAAUAAA
CGCUCAACUUUGGCGCCGCCACCAUGGGUGUGCAUGAGUGUCCCGCUUGGCUU
35 UGGCUGUUAUUAUCAUUACUUCCUUAACCUCUUGGCCUGCCUGUCUUAGGCGC
CCCUCUCGUUUAAUCUGUGAUAGCCGUGUGUUAGAGAGAUACUUGCUUGAGG
CUAAAGAGGCCGAGAAUUAUACUACCGGUUGUGCUGAACAUUGCUCGCUGAAC
GAGAACAUAUACCGUGCCCGACACCAAAGUUAACUUCUAUGCUUGGAAACGUAU
GGAGGUGGGUCAGCAGGCCGUUGAGGUAUGGCAGGGUUUGGCCCUUCUUUCGG
40 AGGCCGUGUACGUGGUCAGGCUCUCUUGGUUAACUCUUCUCAGCCUUGGGAG
CCUUUACAGUUAUUAUGUUAUAAAGCCGUUAGCGGCUUGAGAAGUUUGACCAC
UUUGCUUCGGGCUCUGGGUGCUCAAAAAGAGGCUAUUUCACCCCUGAUGCUG
CUAGCGCUGCUCCACUUAAGAACCAUCACUGCCGACACUUUUCGUAAAUUGUUU
CGUGUUUAUAGCAAUUUCUUACGUGGCAAACUGAAAUUAUACACUGGCGAGGC

UUGCAGAACUGGUGACAGAUGAUAAAGAAUUCUAGGCUGGAGCCUCGGUGGCCA
UGCUCUUGCCCCUUGGGCCUCCCCCAGCCCCUCCUCCCCUCCUGCACCCGU
ACCCCCGUGGUCUUUGAAUAAAGUCUGAGUGGGCGGCACUAGUAAAAAAAAA
AAA
5 AA
AA

SEQ ID NO: 2 (Rabies mRNA)

GGGGAUCCAAGCUUAGCUUGCUUGUUCUUUUUGCAGAAGCUCAGAAUAAA
10 CGCUCAACUUUGGCGCCGCCACCAUGGUGCCUCAGGCCUGUUGUUUGUGCCA
CUGCUGGGCUUUAGCCUGUGUUUUGGCAAAUCCCCAUCUACACCAUCCCCGA
UGAGCUGGGCCCUUGGAGCCCUAUCGACAUCACCAUCUCAGCUGCCCCAACA
ACCUGGUGGUGGAAGAUGAGGGCUGCACCAAUUCUGAGCGAGUUCAGCUACAUG
GAACUGAAGGUGGGCUAUUUCAGCGCCAUCAAGGUGAACGGCUUCACCUGUAC
15 AGGCGUCGUGACCGAGGCUGAGACAUACACCAACUUCGUGGGAUACGUGACAA
CCACCUUCAAGAGAAAGCACUUCAGGCCAACACCUGACGCCUGCAGAGCCGCC
UACAACUGGAAGAUGGCCGGCGAUCCACGGUACGAGGAAAGCCUGCACAACCC
UUAUCCUGAUUACCACUGGCUGAGAACAGUGCGGACAACAAAGGAAAGCCUGA
UUAUUAUCAGCCCCAGCGUGACCGACCUGGACCCUACGACAAGUCUCUGCAC
20 AGCAGAGUGUUUCCUGGAGGGAAGUGCUCGCGCAUACCGUGUCCUCUACCUA
CUGCAGCACCAUACGACUACACCAUCUGGAUGCCUGAGAACCCAGACCCA
GAACCCCUUGCGACAUCUUCACCAACAGCCGCGGAAAACGCGCUUCUAAGGGC
AACAAGACAUGCGGCUUCGUCGACGAGAGAGGCCUGUACAAGAGCCUAAAAGG
CGCUUGUAGACUGAAGCUGUGCGGCGUGUUAGGCCUGAGACUGAUGGACGGCA
25 CGUGGGUCGCCAUGCAGACCAGCGACGAGACAAAGUGGUGCCCUCCUGAUCAG
CUGGUGAACCUUGCAUGAUUUCCGGAGCGACGAGAUCGAGCACCUUGGUGGUGGA
AGAACUUGUUAAGAAGCGGGAAGAGUGCCUGGACGCCCUUGGAAAGCAUCAUGA
CCACCAAGUCCGUGUCCUUCAGACGGCUGAGCCACCUGCGGAAACUGGUUCCU
GGCUUCGGCAAGGCCUUAUACCAUCUUAACAAGACACUGAUGGAAGCCGACGC
30 UCACUACAAGAGCGUUAAGAACCUGGAACGAGAUUAUCCCUUCUAAGGGCUGUC
UGAAAGUGGGCGGCAGAUGCCACCCCCACGUGAAUGGCGUGUUCUUAACGGC
AUCAUCCUGGGACCUGAUGGCCACGUGCUGAUCCCCGAGAUGCAAAGCAGCCU
GCUGCAGCAACACAUGGAACUGCUGAAGUCCUCUGUGAUCCACUGAUGCACC
CUCUGGCCGAUCCUAGCACCGUGUUAAGGAAGGCGACGAGGCCGAGGACUUC
35 GUGGAGGUGCACCUUGCCUGACGUGUACAAGCAGAUUCUCUGGCGUGGACCUGGG
ACUGCCCCAAUUGGGGAAAAUACGUGCUGAUGACCGCCGGAGCCAUGAUCGGCC
UGGUGCUGAUCUUUAGCCUGAUGACAUGGUGUCGGAGAGCCAACCGGCCUGAG
UCCAAGCAGAGAAGCUUCGGCGGCACCGGCAGAAACGUGUCUGUGACCAGCCA
GUCUGGCAAGGUGAUCCCCAGCUGGGAGUCUUAACAAUUCUGGCGGACAGACAA
40 GACUGUGAUAAAGAAUUCUAGGCUGGAGCCUCGGUGGCCAUGCUUCUUGCCCCU
UGGGCCUCCCCCAGCCCCUCCUCCCCUCCUGCACCCGUACCCCGUGGUCUU
UGAAUAAAGUCUGAGUGGGCGGCACUAGUAAAAAAAAAAAAAAAAAAAAAAAAA
AAA
AAA

SEQ ID NO: 3 (Rabies N1-Methyl mRNA)

GGGGAΨCCAAGCΨΨAGCΨΨGCTΨΨGΨΨCΨΨΨΨΨGCAGAAGCΨCAGAAΨAA
ACGCΨCAACΨΨΨGGCGCCGCCACCAΨGGΨGCTΨCAGGCCCCΨGΨΨGΨΨΨGΨGCT
5 ACΨGCTΨGGGCΨΨΨAGCCΨGΨGΨΨΨΨGGCAAAΨΨCCCAAΨCΨACACCAΨCCCCG
AΨGAGCΨGGGCCCCΨΨGGAGCCCCΨAΨCGACAΨCCACCAΨCΨCAGCΨGCCCCAAC
AACCΨGGΨGGΨGGAAGAΨGAGGGCΨGCACCAAΨCΨGAGCGAGΨΨCAGCΨACA
ΨGGAACΨGAAGGΨGGGCΨAΨAΨCAGCGCCAΨCAAGGΨGAACGGCΨΨCACCΨG
ΨACAGGCGΨCGΨGACCGAGGCΨGAGACAΨACACCAACΨΨCGΨGGGAΨACGΨGA
10 CAACCACCΨΨCAAGAGAAAGCACΨΨCAGGCCAACACCΨGACGCCΨGCAGAGCC
GCCΨACAACΨΨGGAAGAΨGGCCGGCGAΨCCACGGΨACGAGGAAAGCCΨGCACAA
CCCΨΨAΨCCΨGAΨΨACCACΨGGCΨGAGAACAGΨGCGGACAACAAAGGAAAGCC
ΨGAΨΨAΨΨAΨCAGCCCCAGCGΨGACCGACCΨGGACCCΨΨACGACAAGΨCΨCΨG
CACAGCAGAGΨGΨΨΨCCΨGGAGGGAAGΨGCΨCCGGCAΨCACCΨGΨCCΨCΨAC
15 CΨACΨGCAGCACCAAΨCACGACΨACACCAΨCΨGGAΨGCCΨGAGAACCCAGAC
CCAGAACCCCΨΨGCGACAΨCΨΨCACCAACAGCCGCGGAAAACGCGCΨΨCΨAAG
GGCAACAAGACAΨGCGGCΨΨCGΨCGACGAGAGAGGCCΨGΨACAAGAGCCΨAAA
AGGCGCΨΨGΨAGACΨGAAGCΨGΨGCGGCGΨGΨΨAGGCCΨGAGACΨGAΨGGAC
GGCACGΨGGGΨCGCCAΨGCAGACCAGCGACGAGACAAAGΨGGΨGCCΨΨCCΨGA
20 ΨCAGCΨGGΨGAACCΨGCAΨGAΨΨΨCCGGAGCGACGAGAΨCGAGCACCCΨGGΨGG
ΨGGAAGAACΨΨGΨΨAAGAAGCGGGAAGAGΨGCCΨGGACGCCΨΨGGAAGCAΨ
CAΨGACCACCAAGΨCCGΨGΨCCΨΨCAGACGGCΨGAGCCACCΨΨGCGGAAACΨGG
ΨΨCCΨGGCΨΨCGGCAAGGCCΨAΨACCAΨCΨΨCAACAAGACACΨGAΨGGAAGCC
GACGCΨCACΨACAAGAGCGΨΨAGAACCΨGGAACGAGAΨΨAΨCCCΨΨCΨAAGGG
25 CΨGΨCΨGAAAGΨGGGCGGCAGAΨGCCACCCCCACGΨGAAΨGGCGΨGΨΨCΨΨCA
ACGGCAΨCAΨCCΨΨGGGACCΨGAΨGGCCACGΨGCΨGAΨCCCCGAGAΨGCAAAGC
AGCCΨGCΨGCAGCAACACAΨGGAACΨGCΨGAAGΨCCΨCΨGΨGAΨCCCACΨGAΨ
GCACCCΨCΨGGCCGAΨCCΨAGCACCGΨGΨΨCAAGGAAGGCGACGAGGCCGAGG
ACΨΨCGΨGGAGGΨGCACCΨGCCΨGACGΨGΨACAAGCAGAΨCΨCΨGGCGΨGGAC
30 CΨGGGACΨGCCCCAAΨΨGGGGA AAAAΨACGΨGCΨGAΨGACCGCCGGAGCCAΨGAΨ
CGGCCΨGGΨGCΨGAΨCΨΨΨAGCCΨGAΨGACAΨGGΨGΨCGGAGAGCCAACCGGC
CΨGAGΨCCAAGCAGAGAAGCΨΨCGGCGGCACCGGCAGAAACGΨGΨCΨGΨGACC
AGCCAGΨCΨGGCAAGGΨGAΨCCCCAGCΨGGGAGΨCΨΨACAAAΨCΨGGCGGACA
GACAAGACΨGΨGAΨAAGAAΨΨCΨAGGCΨGGAGCCΨCGGΨGGCCAΨGCΨΨCΨΨ
35 GCCCCΨΨGGGCCΨCCCCCCAGCCCCΨCCΨCCCCΨCCΨGCACCCGΨACCCCCGΨ
GGΨCΨΨΨGAΨAAAGΨCΨGAGΨGGGCGGCACΨAGΨAAAAAAAAAAAAAAAAAAAA
AA
AA

40 SEQ ID NO: 4 (RSV N1-Methyl mRNA)

GGGGAΨCCAAGCΨΨAGCΨΨGCTΨΨGΨΨCΨΨΨΨΨGCAGAAGCΨCAGAAΨAA
ACGCΨCAACΨΨΨGGCGCCGCCACCAΨGGAGCΨGCΨGAΨCCΨGAAGGCΨAAΨGC
CAΨΨACCACCAΨΨCΨGACCGCCGΨGACAΨΨCΨGCΨΨCGGAGCGGGCAGAAC
ΨCACCAGGAGΨΨCΨACCAGAGCACCΨGΨΨCΨGCCGΨCAGCAAGGGGΨACCΨG

ΨCCGCCCCΨGCGGACAGGAYGGYAYACCΨCAGΨGAYCACAAYΨGAGCΨGΨCCAA
 ΨAYΨAAGGAGAAΨAAGΨGCAAΨGGGACΨGACGCCAAGGΨGAAGCΨGAYCAAG
 CAGGAGCΨGGACAAGΨACAAGAACGCCGΨGACAGAGCΨGCAGCΨGCΨCAΨGCA
 GAGCACCCCCGCCACCGGGAGΨGGCAGCGCCAΨCΨGCAGCGGCGΨGGCCGΨCΨ
 5 GCAAGGΨGCΨGCACCCΨCGAGGGCGAGGΨGAAΨAAGAΨCAAGAGΨGCΨCΨGCΨ
 GAGCACΨAACAAGGCΨGΨCGΨGΨCΨCΨGΨCCAACGGCGΨGΨCΨGΨGCΨGACCCΨ
 ΨCAAGGΨCCΨGGACCΨGAAGAAΨΨACAΨΨGACAAGCAGCΨCCΨGCCCAΨCCΨG
 AACAAGCAGAGCΨGΨΨCΨAΨCΨCCAAYAYΨΨGAGACAGΨΨAΨCGAGΨΨΨCAGC
 AGAAGAACAACAGGCΨGCΨΨGAGAYCACACGGGAGΨΨCAGCGΨGAACGCΨGG
 10 GGΨGACCACΨCCCGΨGΨCCACΨΨACAΨGCΨGACCAACΨCAGAGCΨGCΨGΨCCC
 ΨGAYCAACGACAYGCCΨAYΨACAAACGACCAGAAGAAGΨΨGAYΨGAGCAACAAC
 GΨGCAGAYCGΨGAGACAGCAGΨCΨΨACAGCAΨCAΨGΨGCAΨCAΨCAAGGAGG
 AGGΨGCΨGGCGΨACGΨGGΨGCAGCΨGCCCCΨGΨAYGGGGΨCAΨCGACACCCΨΨ
 ΨGΨΨGGAAGCΨGCACACΨΨCΨCCCCΨGΨGCACGACCAAYACGAAGGAGGGCAG
 15 CAACAΨCΨGΨCΨGACCCGGACAGAYCGGGGCΨGGΨAYΨGCGACAAΨGCCGGCΨ
 CCGΨGΨCCΨΨCΨΨCCCCAGGCΨGAGACΨΨGCAAGGΨGCAGAGΨAACCGGGΨG
 ΨΨCΨGCGACACCAΨGAACΨCCCCGGACΨCΨGCCΨCCGAGGΨGAACCΨGΨGCAA
 CGΨGGACAΨΨΨΨCAACCCCAAGΨACGAΨΨGCAAGAYCAΨGACΨAGCAAGACCG
 ACGΨGΨCΨΨCΨAGCGΨGAYΨACCAGCCΨGGGGGCGAYΨGΨGΨCGΨGCΨACGG
 20 GAAGACGAAGΨGΨACAGCΨΨCCAACAAGAACAGGGGCAΨCAΨCAAGACCΨΨCΨ
 CΨAAΨGGGΨGCGACΨACGΨCAGCAAΨAAGGGCGΨCGACACCGΨCAGCGΨGGGG
 AACACCΨΨGΨACΨGCGΨGAACAAGCAGGAGGGCAAGAGCCΨGΨACGΨGAAGG
 GGGAGCCCCAYΨAYCAACΨΨCΨACGACCCCCΨGGΨGΨΨCCCCAGCGACGAGΨΨC
 GACGCCΨCΨAYCAGCCAGGΨGAACGAGAAGAYCAAΨCAGΨCCCΨGGCCΨΨCAΨ
 25 ΨAGGAAGΨCΨGAYΨGAGCΨGCΨGCACAACGΨGAACGCCGGGAAGAGCACΨACGA
 AYAYCAΨGAYΨCACGACGAYCAΨCAΨCGΨGAYCAΨΨGΨGAYΨCCΨGCΨCAGCCΨG
 AYCGCCGΨGGGGCΨGCΨCCΨGΨACΨGCAAGGCCAGGAGCACCCCCGΨGACCCΨ
 GΨCCAAGGAYCAGCΨGAGCGGGAΨCAACAAΨAYCGCΨΨΨCAGCAACΨGAYAAAG
 AAΨΨCΨAGGCΨGGAGCCΨCGΨGGCCAΨGCΨΨCΨΨGCCΨΨGGGCCΨCCCCC
 30 CAGCCCCΨCCΨCCCCΨCCΨGCACCCGΨACCCCCGΨGGΨCΨΨΨGAAΨAAAGΨC
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 AA
 AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

35 SEQ ID NO: 5 (VZV N1-Methyl mRNA)

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5 GCACAΨCΨGCCΨGAAGCAΨACCACCΨGCΨΨCCAGGACGΨGGΨGGΨGGACGΨGG
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AA
30 AA

CLAIMS

1. A lyophilized formulation of lipid nanoparticles comprising, relative to the total weight of the lyophilized formulation,

- (A) 0.2-20 wt.% of lipid nanoparticles comprising a cationic lipid; and
- (B) a cryoprotectant combination comprising
 - (i) 75-99 wt.% of sucrose; and
 - (ii) 0.1-10 wt.% of a non-polar amino acid.

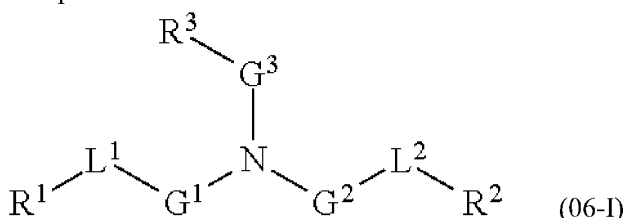
2. The lyophilized formulation of claim 1, wherein the particle size of the lipid nanoparticles is from 80 nm to 100 nm, preferably from 80 nm to 95 nm, or from 82 nm to 95 nm.

3. The lyophilized formulation of claim 1, wherein the lipid nanoparticle comprises, relative to the total mole number of all lipids present in the nanoparticle:

- (a) from about 30 mol % to about 55 mol % of a cationic lipid;
 - (b) from about 20 mol % to about 50 mol % of a structural lipid;
 - (c) from about 5 mol % to about 40 mol % of a phospholipid; and
 - (d) from about 0.5 mol % to about 5 mol % of a polymer conjugated lipid;
- preferably

30-55 mol% of a cationic lipid, 5-40 mol% of DSPC, 20-50 mol% of cholesterol, and 0.5-3 mol% of PEG-lipid, relative to the total mole number of all lipids in the nanoparticle.

4. The lyophilized formulation of any one of claims 1 to 3, wherein the lyophilized formulation comprises 0.1-10 wt.%, preferably 0.1-1 wt.%, more preferably 0.2-0.7 wt.%, relative to the total weight of the lyophilized LNP formulation, of a cationic lipid selected from compounds of Formula 06-I:



wherein

L¹ and L² is -O(C=O)-;

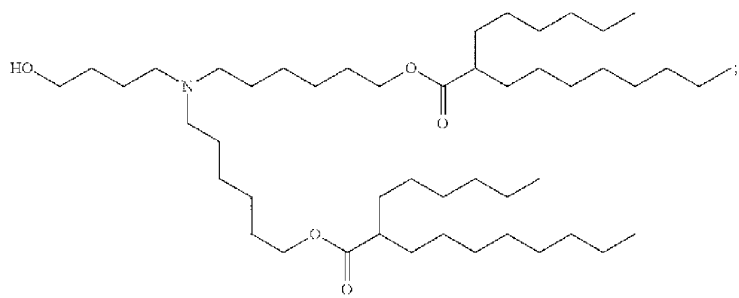
G¹ and G² are each independently unsubstituted C₄-C₈ alkylene;

G³ is C₃-C₈ alkylene;

R¹ and R² are each independently C₁₂-C₂₂ alkyl;

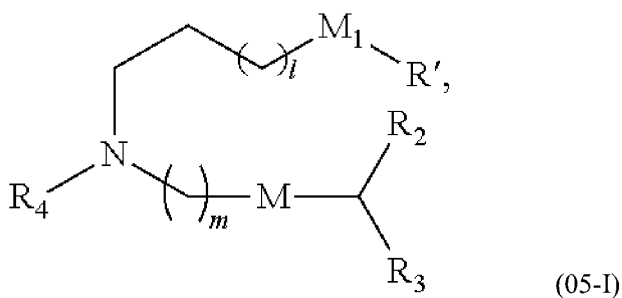
R³ is H or OH,

preferably the cationic lipid is compound of the following formula:



and/or

compounds of Formula 05-I:



wherein

l is selected from 1, 2, 3, 4, and 5;

m is selected from 5, 6, 7, 8, and 9;

M₁ is -C(O)O-;

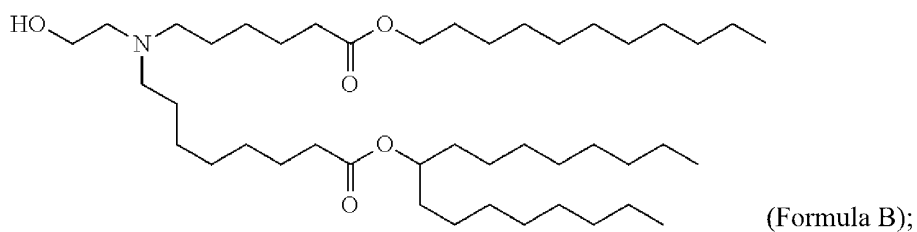
R₄ is -(CH₂)_nOH, and n is selected from 1, 2, 3, 4, or 5;

M is -OC(O)-;

R₂ and R₃ are both C₆₋₁₀ alkyl; and

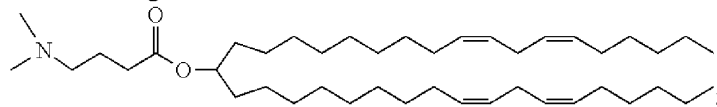
R' is a linear alkyl;

preferably the cationic lipid is compound of the following formula B:



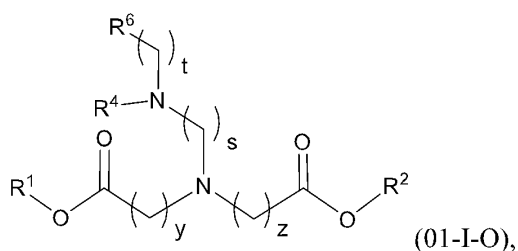
and/or

the following formula:



and/or

compounds of Formula (01-I-O):



wherein y and z are each independently an integer from 4 to 6,

s is an integer from 2 to 4,

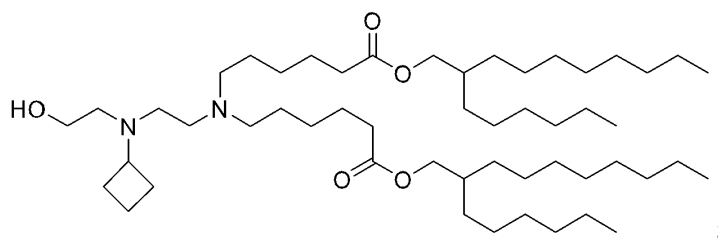
t is an integer from 1 to 3, and

R¹ and R² are each independently C₁₂-C₂₂ alkyl;

R⁴ is C₃-C₈ cycloalkyl;

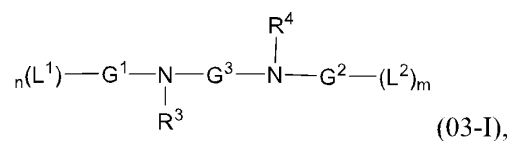
R⁶ is hydrogen or hydroxyl,

preferably the cationic lipid is compound of the following formula:



and/or

compounds of Formula (03-I):



wherein

G¹ and G² are each independently C₃-C₈ alkylene;

each L¹ is independently -OC(=O)R¹;

each L² is independently -C(=O)OR²;

R¹ is independently C₆-C₁₀ alkyl;

R² is independently C₁₂-C₂₂ alkyl;

G³ is C₂-C₁₂ alkylene;

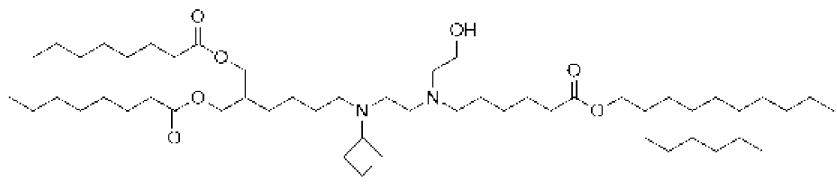
R³ is C₃-C₈ cycloalkyl;

R⁴ is C₁-C₄ hydroxylalkyl;

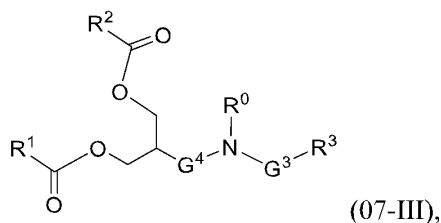
n is 2;

m is 1,

preferably the cationic lipid is compound of the following formula:



and/or
compounds of Formula (07-III):



wherein

R^1 and R^2 are each independently C_6 - C_{22} alkyl;

R^0 is C_3 - C_8 cycloalkyl;

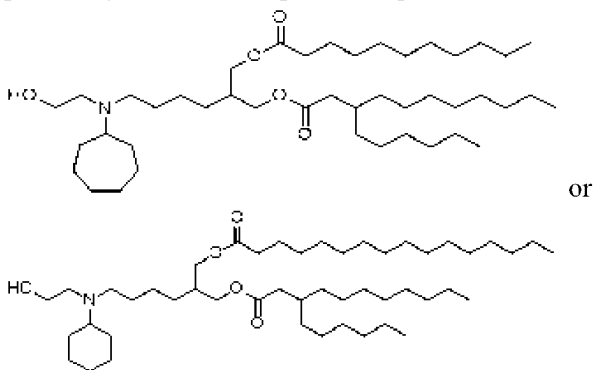
G^3 is C_2 - C_6 alkylene;

G^4 is C_2 - C_6 alkylene;

R^3 is $-OR^6$;

R^6 is hydrogen;

preferably the cationic lipid is compound of the following formula:



5. The lyophilized formulation of any one of claims 1 to 4, wherein the lyophilized formulation comprises 78-99 wt.%, for example, 80-98.5 wt.%, 82-98 wt.%, 84-97.5 wt.%, 85-97.5 wt.%, 87-97.5 wt.%, 89-97.5 wt.%, 91-97.5 wt.%, or 93-97.5 wt.% of sucrose, relative to the total weight of the lyophilized formulation.

6. The lyophilized formulation of any one of claims 1 to 5, wherein the lyophilized formulation comprises 0.2-9 wt.%, for example, 0.4-9 wt.%, 0.6-9 wt.%, 0.8-8 wt.%, 1-7 wt.%, 1.5-6 wt.%, 1.8-5 wt.%, or 2-4.8 wt.% of the non-polar amino acid, relative to the total weight of the lyophilized formulation.

7. The lyophilized formulation of any one of claims 1 to 6, wherein the non-polar amino acid is selected from alanine, isoleucine, leucine, valine, proline, glycine, and a combination thereof.

8. The lyophilized formulation of any one of claims 1 to 6, wherein the lyophilized formulation comprises

0.4-6 wt.%, for example, 1-4 wt.% or 1-3 wt.% of alanine, relative to the total weight of the lyophilized formulation;
and/or
0.6-9 wt.%, preferably 1-5 wt.% of isoleucine, relative to the total weight of the lyophilized formulation;
and/or
0.6-9 wt.%, preferably 1-5 wt.% of leucine, relative to the total weight of the lyophilized LNP formulation;
and/or
0.2-9 wt.%, for example, 0.5-8 wt. or 1.5-6 wt.% of valine, relative to the total weight of the lyophilized formulation;
and/or
0.5-8 wt.%, preferably 1-5 wt.% of proline, relative to the total weight of the lyophilized formulation;
and/or
0.3-5 wt.%, preferably 0.8-3 wt.% of glycine, relative to the total weight of the lyophilized formulation;
and/or
1.5-4 wt.% of valine and 1-3 wt.% of alanine, relative to the total weight of the lyophilized formulation;
and/or
1.5-4 wt.% of valine and 1.5-4 wt.% of leucine, relative to the total weight of the lyophilized formulation;
and/or
1.5-4 wt.% of valine and 1.5-4 wt.% of isoleucine, relative to the total weight of the lyophilized formulation.

9. The lyophilized formulation of any one of claims 1 to 8, wherein the lyophilized formulation comprises 0.01-3 wt.%, 0.1-3 wt.%, 0.15-2 wt.%, 0.2-1.5 wt.%, or 0.2-1.3 wt.% of a salt or Tris, by weight relative to the total weight of the lyophilized formulation.

10. The lyophilized formulation of claim 9, wherein the salt is selected from tris-HCl, KCl, NaCl, K₂HPO₄, KH₂PO₄, sodium citrate, sodium acetate, and a combination thereof.

11. The lyophilized formulation of any one of claims 1 to 10, wherein the lyophilized formulation comprises

80-98.5 wt.% of sucrose, 0.2-9 wt.% of valine and 0.01-3 wt.% of a salt or Tris, by weight relative to the total weight of the lyophilized formulation;

for example,

93.39 wt.% of sucrose, 4.38 wt.% of valine and 1.58 wt.% of Tris, by weight relative to the total weight of the lyophilized formulation; or

95.14 wt.% of sucrose, 2.97 wt.% of valine, 0.03 wt.% of K₂HPO₄, 0.66 wt.% of KH₂PO₄ and 0.74 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation; or

96.58 wt.% of sucrose, 1.51 wt.% of valine, 0.03 wt.% of K_2HPO_4 , 0.67 wt.% of KH_2PO_4 and 0.75 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation; or

97.31 wt.% of sucrose, 0.76 wt.% of valine, 0.03 wt.% of K_2HPO_4 , 0.68 wt.% of KH_2PO_4 and 0.76 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation; or

97.68 wt.% of sucrose, 0.38 wt.% of valine, 0.03 wt.% of K_2HPO_4 , 0.68 wt.% of KH_2PO_4 and 0.76 wt.% of NaCl, by weight relative to the total weight of the lyophilized formulation.

12. The lyophilized formulation of any one of claims 1 to 11, wherein the lyophilized formulation further comprises a therapeutic and/or prophylactic agent in the lipid nanoparticles.

13. The lyophilized formulation of claim 12, wherein the weight ratio of the lipid to the therapeutic and/or prophylactic agent is from 10: 1 to 60: 1, or 2: 1 to 30: 1, or 20:1 to 30:1.

14. The lyophilized formulation of claim 12 or 13, wherein the therapeutic and/or prophylactic agent is a nucleic acid,

for example,

a ribonucleic acid,

preferably

a messenger RNA,

for example,

rabies mRNA, respiratory syncytial virus mRNA, human erythropoietin mRNA, varicella zoster virus mRNA, all optionally modified with 1-N-Methyl-Pseudouridine, and a combination thereof.

15. The lyophilized formulation of any one of claims 1 to 14, wherein the encapsulation efficiency of the lyophilized formulation is at least 85%, at least 87%, at least 90%, at least 92%, or at least 94%.

16. A liquid formulation of lipid nanoparticles comprising, by weight relative to the total volume of the liquid formulation:

(A) 0.001-0.2% w/v of lipid nanoparticles comprising a cationic lipid; and

(B) a cryoprotectant combination comprising

(i) 0.001-40% w/v of sucrose, and

(ii) 0.01-10% w/v of a non-polar amino acid.

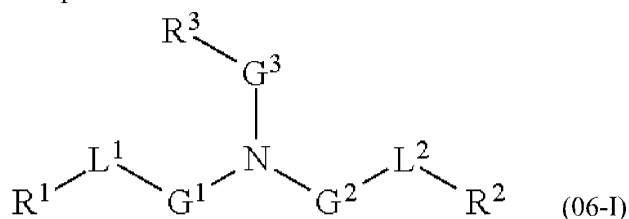
17. The liquid formulation of claim 16, wherein the particle size of the lipid nanoparticles is about 50 nm to about 140 nm, preferably from about 60 nm to about 120 nm.

18. The liquid formulation of claim 16 or 17, wherein the liquid formulation comprises 0.001-0.2% w/v, 0.001-0.1% w/v, 0.1-0.2 % w/v, 0.1-0.15 % w/v, 0.001-0.05% w/v, 0.001-0.03% w/v, 0.001-0.01% w/v, 0.01-0.1% w/v, or 0.05-0.1% w/v of the lipid nanoparticle, by weight relative to the total volume of the LNP formulation.

19. The liquid formulation of any one of claims 16 to 18, wherein the lipid nanoparticle comprises 30-55 mol % of a cationic lipid, 20-50 mol % of a structural lipid; 5-40 mol % of a phospholipid; and from 0.5-5 mol % of a polymer conjugated lipid, preferably 30- 55 mol% of a cationic lipid, 5-40 mol% of DSPC, 20-50 mol% of cholesterol, and 0.5 -3 mol% of PEG-lipid, relative to the total mole number of all lipids in the nanoparticle.

20. The liquid formulation of any one of claims 16 to 19, wherein the liquid formulation comprises 0.01-1 % w/v, preferably 0.01-0.1 % w/v, more preferably 0.02-0.07% w/v, by weight relative to the total volume of the liquid LNP formulation, of a cationic lipid selected from

compounds of Formula 06-I :



wherein

L¹ and L² is -O(C=O)-;

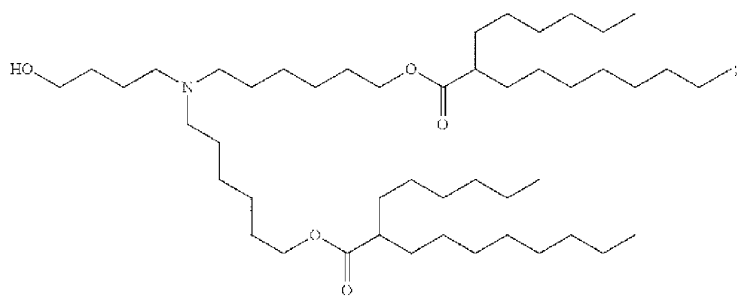
G¹ and G² are each independently unsubstituted C₄-C₈ alkylene;

G³ is C₃-C₈ alkylene;

R¹ and R² are each independently C₁₂-C₂₂ alkyl;

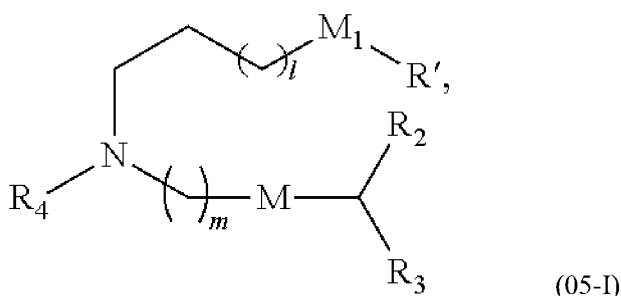
R³ is H or OH,

preferably the cationic lipid is compound of the following formula:



and/or

compounds of Formula 05-I:



wherein

l is selected from 1, 2, 3, 4, and 5;

m is selected from 5, 6, 7, 8, and 9;

M₁ is -C(O)O-;

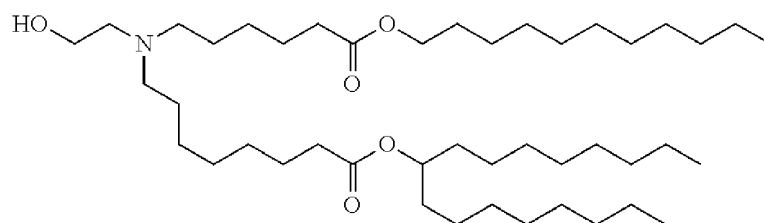
R₄ is -(CH₂)_nOH, and n is selected from 1, 2, 3, 4, or 5;

M is -OC(O)-;

R₂ and R₃ are both C₆₋₁₀ alkyl; and

R' is a linear alkyl;

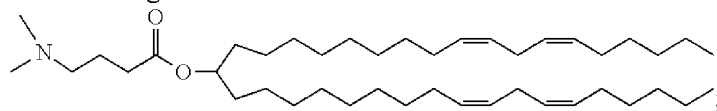
preferably the cationic lipid is compound of the following formula B:



(Formula B);

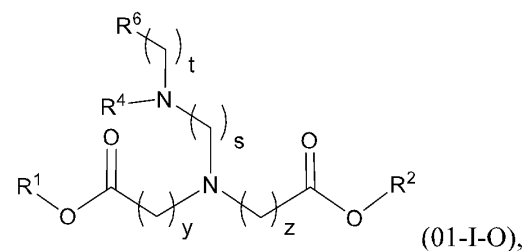
and/or

the following formula:



and/or

compounds of Formula (01-I-O):



wherein y and z are each independently an integer from 4 to 6,

s is an integer from 2 to 4,

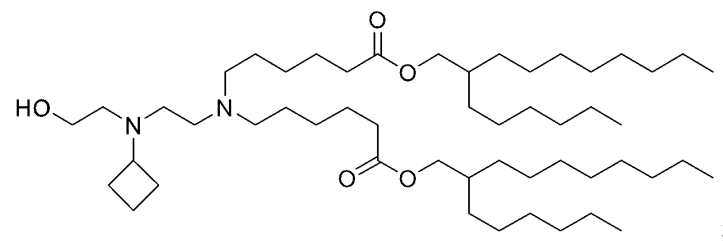
t is an integer from 1 to 3, and

R¹ and R² are each independently C₁₂-C₂₂ alkyl;

R⁴ is C₃-C₈ cycloalkyl;

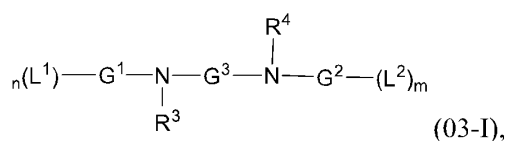
R⁶ is hydrogen or hydroxyl,

preferably the cationic lipid is compound of the following formula:



and/or

compounds of Formula (03-I):



wherein

G^1 and G^2 are each independently C_3 - C_8 alkylene;

each L^1 is independently $-OC(=O)R^1$;

each L^2 is independently $-C(=O)OR^2$;

R^1 is independently C_6 - C_{10} alkyl;

R^2 is independently C_{12} - C_{22} alkyl;

G^3 is C_2 - C_{12} alkylene;

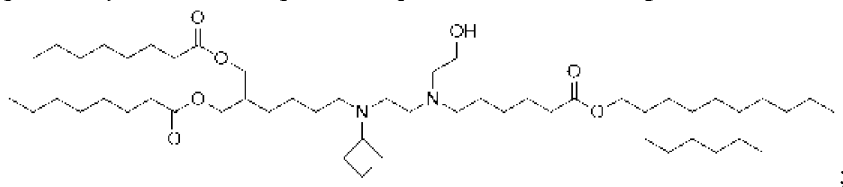
R^3 is C_3 - C_8 cycloalkyl;

R^4 is C_1 - C_4 hydroxylalkyl;

n is 2;

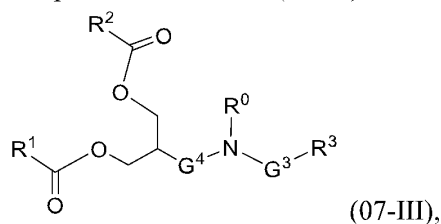
m is 1,

preferably the cationic lipid is compound of the following formula:



and/or

compounds of Formula (07-III):



wherein

R^1 and R^2 are each independently C_6 - C_{22} alkyl;

R^0 is C_3 - C_8 cycloalkyl;

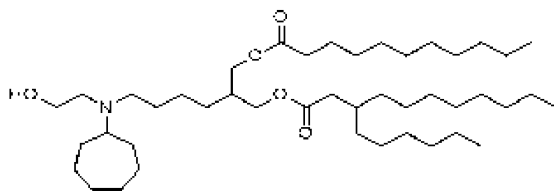
G^3 is C_2 - C_6 alkylene;

G^4 is C_2 - C_6 alkylene;

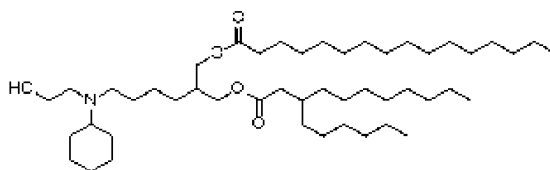
R^3 is $-OR^6$;

R^6 is hydrogen;

preferably the cationic lipid is compound of the following formula:



or



21. The liquid formulation of any one of claims 16 to 20, wherein the liquid formulation comprises 0.01-35% w/v, for example, 0.1-30% w/v, 1-30% w/v, 2-25% w/v, 3-20% w/v, 4-15% w/v, 5-14% w/v, 6-13% w/v, 7-12% w/v, 8-11% w/v, 9-10% w/v, or 9.3-10 % w/v of sucrose, by weight relative to the total volume of the liquid LNP formulation.

22. The liquid formulation of any one of claims 16 to 21, wherein the liquid formulation comprises 0.04-1% w/v, for example, 0.06-0.9% w/v, 0.08-0.8% w/v, 0.1-0.7% w/v, 0.15-0.6% w/v, 0.18-0.5% w/v, or 0.2-0.5% w/v of a non-polar amino acid, by weight relative to the total volume of the liquid LNP formulation.

23. The liquid formulation of any one of claims 16 to 22, wherein the non-polar amino acid is selected from alanine, isoleucine, leucine, valine, proline, glycine, and a combination thereof.

24. The liquid formulation of any one of claims 16 to 22, wherein the liquid formulation comprises

0.04-0.6 % w/v, for example, 0.1-0.4% w/v or 0.1-0.3% w/v of alanine, by weight relative to the total volume of the liquid formulation;

and/or

0.06-0.9 % w/v, preferably 0.1-0.5% w/v of isoleucine, by weight relative to the total volume of the liquid formulation;

and/or

0.06-0.9 % w/v, for example, 0.1-0.6% w/v or 0.1-0.5% w/v of leucine, by weight relative to the total volume of the liquid formulation;

and/or

0.05-0.8 % w/v, preferably 0.15-0.6% w/v of valine, by weight relative to the total volume of the liquid formulation;

and/or

0.05-0.8 % w/v, preferably 0.1-0.5% w/v of proline, by weight relative to the total volume of the liquid formulation;

and/or

0.03-0.5 % w/v, preferably 0.08-0.3% w/v of glycine, by weight relative to the total volume of the liquid formulation;

and/or

0.15-0.4 % w/v of valine and 0.1-0.3 % w/v of alanine, by weight relative to the total volume of the liquid formulation;

and/or

0.15-0.4 % w/v of valine and 0.15-0.4 % w/v of leucine, by weight relative to the total volume of the liquid formulation;

and/or

0.15-0.4 % w/v of valine and 0.15-0.4 % w/v of isoleucine, by weight relative to the total volume of the liquid formulation.

25. The liquid formulation of any one of claims 16 to 24, wherein the liquid formulation comprises 0.001-0.3%, 0.01-0.3% w/v, 0.015-0.2 % w/v, 0.02-0.15 % w/v, or 0.02-0.13 % w/v of a salt or Tris, by weight relative to the total weight of the liquid formulation.

26. The liquid formulation of claim 25, wherein the salt is selected from tris-HCl, KCl, NaCl, K₂HPO₄, KH₂PO₄, sodium citrate, sodium acetate, and a combination thereof.

27. The liquid formulation of any one of claims 16 to 26, wherein the liquid formulation comprises

4-15% w/v of sucrose, 0.05-0.8 % w/v of valine and 0.001-0.3% w/v of a salt or Tris, by weight relative to the total weight of the liquid formulation;

for example,

10% w/v of sucrose, 0.47% w/v of valine and 0.17% w/v of Tris, by weight relative to the total weight of the liquid formulation; or

10% w/v of sucrose, 0.47% w/v of valine, 0.005% w/v of K₂HPO₄, 0.10% w/v of KH₂PO₄ and 0.117% w/v of NaCl, by weight relative to the total weight of the liquid formulation; or

10% w/v of sucrose, 0.23% w/v of valine, 0.005% w/v of K₂HPO₄, 0.10% w/v of KH₂PO₄ and 0.117% w/v of NaCl, by weight relative to the total weight of the liquid formulation; or

10% w/v of sucrose, 0.12% w/v of valine, 0.005% w/v of K₂HPO₄, 0.10% w/v of KH₂PO₄ and 0.117% w/v of NaCl, by weight relative to the total weight of the liquid formulation; or

10% w/v of sucrose, 0.06% w/v of valine, 0.005% w/v of K₂HPO₄, 0.10% w/v of KH₂PO₄ and 0.117% w/v of NaCl, by weight relative to the total weight of the liquid formulation.

28. The liquid formulation of any one of claims 16 to 27, wherein the lipid formulation further comprises a therapeutic and/or prophylactic agent in the lipid nanoparticles.

29. The liquid formulation of claim 28, wherein the weight ratio of the lipid to the therapeutic and/or prophylactic agent is from 10: 1 to 60: 1, or 2: 1 to 30: 1, or 20:1 to 30:1.

30. The liquid formulation of claim 28 or 29, wherein the therapeutic and/or prophylactic agent is a nucleic acid,

for example,

a ribonucleic acid,

preferably

a messenger RNA,

for example,

rabies mRNA, respiratory syncytial virus mRNA, human erythropoietin mRNA, varicella zoster virus mRNA, all optionally modified with 1-N-Methyl-Pseudouridine, and a combination thereof.

31. The liquid formulation of any one of claims 16-30, wherein the encapsulation efficiency of the liquid formulation is at least 75%, at least 77%, at least 79%, at least 81%, or at least 83%.