

POLYVINYLPYRROLIDONE STABILIZING AGENTS, COMPOSITIONS, USES, AND MANUFACTURING METHODS THEREOF

CROSS REFERENCE TO RELATED APPLICATION

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 63/537,974, filed September 12, 2023, which is incorporated by reference in its entirety herein.

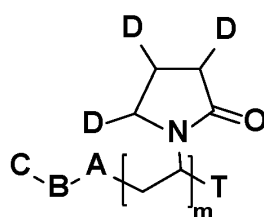
BACKGROUND

[0002] Lipid nanoparticle (LNP) formulations and nucleic acid-containing lipid nanoparticle (NALNP) formulations are used for a variety of applications, particularly medical applications such as oligonucleotide-based therapeutics, *e.g.*, vaccines, immunogenic cell incorporation, and gene therapy. LNP and NALNP formulations can also be used for antibiotics and vitamins, among other uses.

[0003] However, there is a need for improved LNP and NALNP formulations. The present invention provides for ameliorating at least some of the disadvantages of the prior art. These and other advantages of the present invention will be apparent from the description as set forth below.

BRIEF SUMMARY

[0004] In one aspect, the invention provides a stabilizing agent having the structure of formula (I):

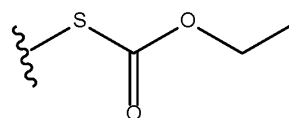


(I)

wherein A is S, NH, O, C₁₋₈ substituted or unsubstituted alkyl or heteroalkyl group, a hydrophilic group, a glyceryl group, a heterocyclic group, a phospholipid group, a ceramide group, an ionizable group, a triazole group, or a combination thereof; B is CH₂, triazole group or a C₁₋₁₈ amino oxobutanoate group or a C₁₋₁₈ substituted or unsubstituted alkyl or heteroalkyl group; C is a C₁₋₅₀ substituted or unsubstituted heteroalkyl group, C₁₋₅₀ unsaturated heteroalkyl group, C₁₋₅₀

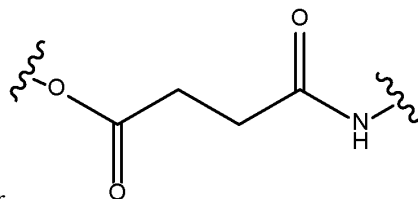
heterocyclyl group, C₁₋₅₀ charged heteroalkyl group, or a combination thereof; T is hydrogen, a C₁₋₁₈ carbonothioate group, C₁₋₁₈ substituted or unsubstituted alkyl or heteroalkyl group, a C₁₋₁₈ ionizable group, a C₁₋₁₈ targeting group, a hydrophilic group, an amphiphilic group, or a combination thereof; D is H, C₁₋₅ substituted or unsubstituted alkyl or heteroalkyl group, or hydroxyl group and m is an integer from 15 to 500.

[0005] In some embodiments, A is sulfur or a triazole group; B is a CH₂, triazole group, or a C₃-C₆ amino oxobutanoate group; T is hydrogen, a C₁-C₅ carbonothioate group, a C₁-C₅ ionizable group, a C₁-C₅ targeting group, a hydrophilic group, an amphiphilic group, or a combination thereof; and m is an integer from 15 to 400.



[0006] In some embodiments, T is hydrogen, methyl, , a peptide, an antibody, a sugar, an oligosaccharide, an aminoglycoside, a sterol, phenyl boronic acid, or a combination thereof.

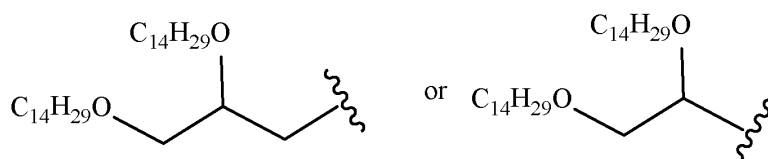
[0007] In some embodiments, A is sulfur or a triazole group.



[0008] In some embodiments, B is CH₂ or

[0009] In some embodiments, C is a C₁₋₃₅ substituted or unsubstituted heteroalkyl group, C₁₋₃₅ unsaturated heteroalkyl group, C₁₋₃₅ heterocyclcyl group, C₁₋₃₅ charged heteroalkyl group, or a combination thereof.

[0010] In some embodiments, C is



[0011] In some embodiments, the stabilizing agent is SAF29, SAF29A, SAF29B, SAF29C, SAF34, SAF102, SAF102A, SAF102B, SAF103, SAF104, SAF123, SAF124, SAF125, SAF126, SAF127, SAF133, SAF134, SAF135, SAF136, SAF137, SAF138, SAF139, SAF141, SAF149, SAF150, SAF154, SAF167, SAF178, SAF190, SAF191, or a combination thereof.

[0012] In some embodiments, the stabilizing agent of formula (I) is used for stabilizing a lipid nanoparticle or liposome.

[0013] In one aspect, the invention provides a lipid nanoparticle composition comprising: (a) an ionizable lipid; (b) two or more lipids; and (c) the stabilizing agent of formula (I).

[0014] In some embodiments, the two or more lipids include a structural lipid, a sterol, or a combination thereof.

[0015] In some embodiments, the lipid nanoparticle composition consists essentially of: (a) an ionizable lipid; (b) two lipids; and (c) the stabilizing agent.

[0016] In some embodiments, the lipid nanoparticle composition is substantially free of PEG or PEG-R, where R is any atom or molecule covalently attached to PEG.

[0017] In some embodiments, the structural lipid is neutrally charged, positively charged, or negatively charged.

[0018] In some embodiments, the ionizable lipid is DODMA, DLin-MC3-DMA, DLin-KC2-DMA, BOCHD-C3-DMA, C12-200, PNI 516, PNI 127, PNI 550, PNI 580, PNI 659, PNI 728, PNI 762, or a combination thereof.

[0019] In some embodiments, the structural lipid includes diacylphosphatidylcholines, diacylphosphatidylethanolamines, diacylphosphatidylglycerols, ceramides, sphingomyelins, dihydrosphingomyelins, cephalins, cerebrosides, or a combination thereof.

[0020] In some embodiments, the structural lipid comprises distearoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, dioleoyl-phosphatidylethanolamine, palmitoyloleoylphosphatidylcholine, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine, palmitoyloleoyl-phosphatidylethanolamine, dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate, dipalmitoyl phosphatidyl ethanolamine, dimyristoylphosphoethanolamine, distearoyl-phosphatidylethanolamine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-methyl, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N,N-dimethyl, 1,2-dielaidoyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-oleoyl-phosphatidylethanol amine, 1,2-dielaidoyl-sn-glycero-3-phosphoethanolamine, distearoylphosphatidylcholine, dioleoylphosphatidylglycerol, dipalmitoylphosphatidylglycerol, palmitoyloleyolphosphatidylglycerol, cardiolipin, phosphatidylinositol, diacylphosphatidylserine, diacylphosphatidic acid, monosialoganglioside GM1, or a combination thereof.

- [0021] In some embodiments, the sterol includes cholesterol, beta-sitosterol, 20-alpha-hydroxysterol, phytosterol, or a combination thereof.
- [0022] In some embodiments, the stabilizing agent has a molecular weight of about 500 Da to about 50,000 Da.
- [0023] In some embodiments, the lipid nanoparticle composition comprises about 20 to about 70 mol% ionizable lipid, about 1 to about 25 mol% structural lipid, about 28 to about 50 mol% sterol, and about 0.1 to about 10 mol% stabilizing agent.
- [0024] In one aspect, the invention provides a lipid nanoparticle including the lipid nanoparticle composition and a nucleic acid.
- [0025] In some embodiments, the nucleic acid is encapsulated by the lipid nanoparticle composition.
- [0026] In some embodiments, the nucleic acid includes an antisense oligonucleotide, a siRNA, a miRNA, a self-amplifying RNA (samRNA or saRNA), a self-replicating DNA, an LNA, a DNA, a replicon, an mRNA, a guide RNA, a transposon, a single gene, a vector, a plasmid, a viral particle, an AAV, a complex of RNA and RNA-binding protein, or a combination thereof.
- [0027] In some embodiments, the nucleic acid is an antigen encoded mRNA for prophylactic or therapeutic vaccine, a nucleic acid for gene therapy, or a nucleic acid for immunogenic cell incorporation, wherein the immunogenic cell is a T cell.
- [0028] In some embodiments, the lipid nanoparticle diameter is about 15 nm to about 500 nm.
- [0029] In some embodiments, the lipid nanoparticle has a polydispersity index of about 0.01 to about 0.40.
- [0030] In some embodiments, the lipid nanoparticle has an encapsulation efficiency of about 50% to about 100%.
- [0031] In one aspect, the invention provides a pharmaceutical composition including the lipid nanoparticle composition and a pharmaceutically acceptable carrier.
- [0032] In another aspect, the invention provides a method for preparing the lipid nanoparticle, the method comprising: (a) forming the lipid nanoparticle composition by combining an ionizable lipid, a structural lipid, a sterol, and stabilizing agent; (b) preparing the

lipid nanoparticle by combining the lipid nanoparticle composition and the nucleic acid using a microfluidic mixer; and (c) purifying the lipid nanoparticle.

[0033] In some embodiments, the lipid nanoparticle composition and the nucleic acid are combined using a flow ratio of about 1:1 to about 10:1 by volume (aqueous phase: organic phase) at a N/P ratio of about 2 to about 20, and a total flow rate of about 2 to about 2000 mL/min.

[0034] In some embodiments, the aqueous phase comprises a low pH buffer. In some embodiments, the aqueous phase includes a citrate or acetate buffer.

[0035] In some embodiments, the organic phase comprises 1,4-dioxane, tetrahydrofuran, acetone, acetonitrile, dimethyl sulfoxide, dimethylformamide, acids, alcohols, or a combination thereof.

[0036] In some embodiments, the organic phase is an alcohol and the alcohol includes aqueous or anhydrous alcohol, wherein the alcohol is a primary, secondary, or tertiary alcohol having from 1 to 12 branched or unbranched carbons.

[0037] In another aspect, the invention provides use of the lipid nanoparticle or the pharmaceutical composition for preventing, treating, or ameliorating conditions or diseases including administering the lipid nanoparticle as a vaccine or as a treatment to prevent or reduce the severity of a contagion, administering the lipid nanoparticle as a gene therapeutic, or administering the lipid nanoparticle to an immunogenic cell for the treatment of cancer or an infection.

[0038] Further and alternative aspects and features of the disclosed principles will be appreciated from the following detailed description. As will be appreciated, the compositions and methods disclosed herein are capable of being carried out and used in other and different embodiments, and capable of being modified in various respects. Accordingly, it is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and do not restrict the scope of the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0039] **FIG. 1A** is a bar graph that demonstrates the level of Luciferase expressed by HEK293 cells 24 h after being treated with either untreated Fluc mRNA LNPs or Fluc mRNA LNPs containing PEG-DMG, SAF29, or SAF34 as the stabilizing agent, as described herein. The

lipid composition used included PNI 516-V02 (lipid concentration: 25 mM), which was 47.5 mol% ionizable lipid, 12.5 mol % structural lipid, 38.5 mol % sterol, and 1.5 mol % stabilizing agent.

[0040] FIG. 1B is a bar graph that demonstrates the levels of Luciferase expressed by T cells 24 h after being treated with either untreated Fluc mRNA LNPs or Fluc mRNA LNPs containing PEG-DMG, SAF29, or SAF34 as the stabilizing agent, as described herein. The lipid composition used included PNI 516-V02 (lipid concentration: 25 mM), which was 47.5 mol % ionizable lipid, 12.5 mol % structural lipid, 38.5 mol % sterol, and 1.5 mol % stabilizing agent.

[0041] FIG. 2A is a schematic representation that shows the hEPO expression study in mice. In this study, four mice per group were injected with EPO-expressing LNPs containing either PBS, SAF29, SAF34, SAF103, or SAF104 as the stabilizing agent, as described herein. hEPO expression levels were measured 24 h post administration.

[0042] FIG. 2B includes dot plots that demonstrates hEPO expression levels (24 h post administration) in C57BL/6 mice sera following IV administration of EPO-expressing LNPs containing either PBS, SAF29, SAF34, SAF103, or SAF104 as the stabilizing agent, as described herein. The mice were administered with 0.25 mg/kg and 200 µL/20 g mouse dose of recombinant human EPO-encoded mRNA-LNPs using PNI 516 in V02 composition (N/P-8). The lipid composition used included PNI 516-V02 (lipid concentration: 25 mM), which was 47.5 mol % ionizable lipid, 12.5 mol % structural lipid, 38.5 mol % sterol, and 1.5 mol % stabilizing agent.

[0043] FIG. 3A is a schematic representation that shows the SARS-CoV-2 vaccine study in mice. In this study, mice were injected with SARS-CoV-2 expressing LNPs containing either PBS, SAF29, or SAF34 as the stabilizing agent, as described herein, and SARS-CoV-2 antigen specific IgG levels were measured 21 and 42 days after IV administration.

[0044] FIG. 3B is a dot plot that demonstrates the SARS-CoV-2 spike protein specific IgG level in sera of mice treated with A5 saRNA lipid nanoparticles (1 µg/50 µl/20 g mouse/injection) with different stabilizing agents (PBS, SAF29, or SAF34), 14 days post boost-injection (42 days post prime-injection). The lipid composition used included PNI 516-V02 (lipid concentration: 25 mM), which was 47.5 mol % ionizable lipid, 12.5 mol % structural lipid, 38.5 mol % sterol, and 1.5 mol % stabilizing agent.

[0045] FIG. 4 is a bar graph that demonstrates the knock out (KO) efficiency in T cells following 24 h treatment (10,000 cells/100 μ L, dose: 4 μ g/million cells) with T cell receptor (TCR) single guide RNA (sgRNA)/Cas9 mRNA LNPs with polyvinylpyrrolidone-based stabilizers. The compositions used were 40 mol % PNI 762, 20 mol % DSPC, 37.5 mol % cholesterol, 2.5 mol % stabilizer. SAF190 and SAF191 outperformed PEG-DMG stabilizers in the *ex vivo* experiments.

[0046] FIG. 5 are line graphs that demonstrate the *in vitro* potency of various polyvinylpyrrolidone -based LNPs (lipid composition: 40 mol % PNI 762, 20 mol % DSPC, 37.5 mol % cholesterol, 2.5 mol % stabilizer), with the payload of enhanced green fluorescent protein (eGFP) mRNA. The cell lines tested included BHK. The primary cells used for the *ex vivo* studies were human T cells. SAF190 and SAF191 outperformed PEG-DMG in both the *in vitro* and *ex vivo* experiments.

[0047] FIGS. 6A-6B are bar graphs that demonstrate the *ex vivo* activity of various LNPs. (FIG. 6A) Transfection efficiency (TE) % and (FIG. 6B) mean fluorescence intensity (MFI) for various polyvinylpyrrolidone -based LNPs (lipid composition: 40 mol % PNI 762, 20 mol % DSPC, 37.5 mol % cholesterol, 2.5 mol % stabilizer), with the payload of enhanced green fluorescent protein (eGFP) mRNA 24 h post treatment analyzed by flow cytometry. The cell line tested included BHK, U937, HeLa and Jurkat. The primary cells used for *ex vivo* studies were human T cells. SAF190 and SAF191 outperformed PEG-DMG in both the *in vitro* and *ex vivo* experiments.

[0048] FIGS. 7A-7B are dot plots that demonstrate SARS-CoV-2 spike protein specific IgG expression in C57BL/6 mice on day 21 and day 42 following IM administration of 1 μ g/mouse dose of SARS-CoV-2 spike protein encoded saRNA-containing LNPs formed based on V62 compositions (40 mol % PNI 516, 12.5 mol % DSPC, 46 mol % cholesterol, 1.5 mol % stabilizer) (N/P-8). The stabilizers that equally performed/outperformed PEG-DMG in the V62 compositions *in vivo* included SAF124, SAF 125, SAF 127, SAF 136, SAF 137, SAF 138, SAF 139, SAF 149, SAF 150, SAF 154, and SAF 178.

[0049] FIG. 8 is a dot plot that demonstrates hEPO expression levels (6 h post administration) in C57BL/6 mice following subcutaneous administration of 0.25 mg/kg dose of recombinant human EPO-encoded mRNA-LNPs formed using the composition of 40 mol % PNI 516, 12.5 mol % DSPC, 46 mol % cholesterol, 1.5 mol % stabilizer (N/P-8).

[0050] FIG. 9 is a bar graph that demonstrates firefly luciferase (Fluc) protein expression levels (4 h post administration) in Hsd:ICR female mice following IV administration of 0.1 mg/kg dose of Fluc-encoded mRNA-LNPs formed using the composition of 40 mol % PNI 516, 12.5 mol % DSPC, 46 mol % cholesterol, 1.5 mol % stabilizer (N/P-8).

[0051] FIG. 10 is a dot plot that demonstrates hEPO expression levels (6 h and 24 h post administration) in C57BL/6 mice following IV administration of 0.25 mg/kg dose of recombinant human EPO-encoded mRNA-LNPs using 40 mol % PNI 516, 12.5 mol % DSPC, (47.5-x) mol % cholesterol, x mol % stabilizer; N/P-8). Data showed that increasing the SAF191 mol% resulted in higher *in vivo* efficacy.

DETAILED DESCRIPTION

[0052] I. Introduction

[0053] The disclosure provides stabilizing agents and lipid nanoparticle (LNP) compositions including the stabilizing agents, as well as methods for preparing the stabilizing agents and lipid nanoparticles. These lipid nanoparticle (LNP) compositions may be configured to encapsulate nucleic acids. The lipid nanoparticle compositions for encapsulating nucleic acids may comprise an ionizable lipid, two or more lipids, and a polyvinylpyrrolidone stabilizing agent as discussed herein. Advantageously, in contrast with LNP compositions including polyethylene glycol (PEG) or PEGylated lipid (polyethylene glycol (PEG)-lipid conjugate) as stabilizing agents, LNP and NALNP formulations with polyvinylpyrrolidone stabilizing agent in accordance with the invention are free of PEG and free of PEGylated lipids. The inventors have found that the inventive stabilizing agents allow for LNPs that have higher compatibility and safety and lower immunogenicity when compared to PEG or PEGylated lipid based LNP products. In some cases, the PEGylated lipid is PEG-R, where R is any atom or molecule. In some cases, R is DMG, DSG, DSPE, DOPE, or DPPE. In some cases, the PEGylated lipid includes DMG-PEG, DSG-PEG, DSPE-PEG, DOPE-PEG, or DPPE-PEG.

[0054] The resulting encapsulated LNP formulations may be used in a variety of applications, particularly medical applications such as oligonucleotide-based therapeutics, *e.g.*, vaccines, immunogenic cell incorporation, and gene therapy. The compositions surprisingly and unexpectedly provide benefits including more potent protein expression when compared to traditional PEG based products.

[0055] To facilitate an understanding of the present disclosure, a number of terms and phrases are defined below.

[0056] **II. Definitions**

[0057] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods described herein belong. Any reference to standard methods refers to the most recent available version of the method at the time of filing of this disclosure unless otherwise indicated.

[0058] For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

[0059] All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

[0060] The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful and is not intended to exclude other embodiments from the scope of the invention.

[0061] The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Such terms will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements.

[0062] The singular form "a", "an" and "the" include plural referents unless the context clearly dictates otherwise. These articles refer to one or to more than one (i.e., to at least one). The use of the term "at least one" followed by a list of one or more items (for example, "at least one of A and B") is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly contradicted by context. As used herein, the term "or" is generally employed in its usual sense including "and/or" unless the content clearly dictates otherwise. The term "and/or" means any one or more of the items in the list joined by "and/or". As an example, "x and/or y" means any element of the three-element set {(x), (y), (x, y)}. In other words, "x and/or y" means "one or both of x and y". As another example, "x, y, and/or z" means any element of the seven-

element set $\{(x), (y), (z), (x, y), (x, z), (y, z), (x, y, z)\}$. In other words, "x, y and/or z" means "one or more of x, y and z".

[0063] Where ranges are given, endpoints include all numbers subsumed within that range (*e.g.*, 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.80, 4, 5, etc.). Furthermore, unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the disclosure, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. Herein, "up to" a number (for example, up to 50) includes the number (for example, 50). The term "in the range" or "within a range" (and similar statements) includes the endpoints of the stated range.

[0064] Reference throughout this specification to "one aspect (or embodiment)," "an aspect (or embodiment)," "certain aspects (or embodiments)," or "some aspects (or embodiments)," etc., means that a particular feature, configuration, composition, or characteristic described in connection with the aspect is included in at least one aspect of the disclosure. Thus, the appearances of such phrases in various places throughout this specification are not necessarily referring to the same embodiment of the disclosure. Furthermore, the particular features, configurations, compositions, or characteristics may be combined in any suitable manner in one or more aspects.

[0065] Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." As used herein in connection with a measured quantity, the term "about" refers to that variation in the measured quantity as would be expected by the skilled artisan making the measurement and exercising a level of care commensurate with the objective of the measurement and the precision of the measuring equipment used. The term "about" as used in connection with a numerical value throughout the specification and the claims denotes an interval of accuracy, familiar and acceptable to a person skilled in the art. In general, such interval of accuracy is $\pm 10\%$. Thus, "about" can be understood to be within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1 %, 0.5%, 0.1 %, 0.05%, 0.01 %, or 0.001 % greater or less than the stated value. Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by

the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[0066] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

[0067] The term "exemplary" means serving as a non-limiting example, instance, or illustration. As utilized herein, the terms "e.g.," and "for example" set off lists of one or more non-limiting aspects, examples, instances, or illustrations.

[0068] As used herein, the term "substantially" refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. Biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term "substantially" is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena. For example, "substantially" may refer to being within at least about 20%, alternatively at least about 10%, alternatively at least about 5% of a characteristic or property of interest.

[0069] The term "administering" as used herein refers to the physical introduction of an agent to a subject, such as a lipid nanoparticle disclosed herein, using any of the various methods and delivery systems known to those skilled in the art. Exemplary routes of administration for the formulations disclosed herein include intravenous, intramuscular, subcutaneous, intraperitoneal, spinal, or other parenteral routes of administration, for example by injection or infusion. The phrase "parenteral administration" means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intralymphatic, intralesional, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion, as well as in vivo electroporation. In some embodiments, the formulation is administered via a non-parenteral route, *e.g.*, orally. Other non-parenteral routes include a topical, epidermal or mucosal route of administration, for example, intranasally, vaginally,

rectally, sublingually or topically. Administering can also be performed, for example, once, a plurality of times, and/or over one or more extended periods.

[0070] The term "cancer" refers to a broad group of various diseases characterized by the uncontrolled growth of abnormal cells in the body. Unregulated cell division and growth results in the formation of malignant tumors that invade neighboring tissues and may also metastasize to distant parts of the body through the lymphatic system or bloodstream. A "cancer" or "cancer tissue" can include a tumor.

[0071] The term "in vitro" refers to events occurring in an artificial environment, *e.g.*, in a test tube, reaction vessel, cell culture, etc., rather than within a multi-cellular organism. The term "in vitro cell" refers to any cell which is cultured *ex vivo*. In particular, an in vitro cell can include a T cell. The term "*in vivo*" refers to events that occur within a multi-cellular organism, such as a human or a non-human animal.

[0072] The term "nucleic acid" refers to any polymeric chain of nucleotides. A nucleic acid may be DNA, RNA, or a combination thereof. In some embodiments, a nucleic acid comprises one or more natural nucleic acid residues. In some embodiments, a nucleic acid comprises of one or more nucleic acid analogs. In some embodiments, nucleic acids are prepared by one or more of isolation from a natural source, enzymatic synthesis by polymerization based on a complementary template (*in vivo* or *in vitro*), reproduction in a recombinant cell or system, and chemical synthesis. In some embodiments, a nucleic acid is at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 or more residues long (*e.g.*, 20 to 100, 20 to 500, 20 to 1000, 20 to 2000, or 20 to 5000 or more residues). In some embodiments, a nucleic acid is partly or wholly single stranded; in some embodiments, a nucleic acid is partly or wholly double stranded. In some embodiments a nucleic acid has a nucleotide sequence comprising at least one element that encodes, or is the complement of a sequence that encodes, a polypeptide.

[0073] The term "pharmaceutically acceptable" refers to a molecule or composition that, when administered to a recipient, is not deleterious to the recipient thereof, or that any deleterious effect is outweighed by a benefit to the recipient thereof. With respect to a carrier, diluent, or excipient used to formulate a composition as disclosed herein, a pharmaceutically acceptable carrier, diluent, or excipient must be compatible with the other ingredients of the

composition and not deleterious to the recipient thereof, or any deleterious effect must be outweighed by a benefit to the recipient. The term "pharmaceutically acceptable carrier" means a pharmaceutically- acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent encapsulating material, involved in carrying or transporting an agent from one portion of the body to another (*e.g.*, from one organ to another). Each carrier present in a pharmaceutical composition must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the patient, or any deleterious effect must be outweighed by a benefit to the recipient. Some examples of materials which may serve as pharmaceutically acceptable carriers comprise: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; pH buffered solutions; polyesters, polycarbonates and/or polyanhydrides; and other non-toxic compatible substances employed in pharmaceutical formulations.

[0074] "Treatment" or "treating" of a subject refers to any type of intervention or process performed on, or the administration of an active agent to, the subject with the objective of reversing, alleviating, ameliorating, inhibiting, slowing down or preventing the onset, progression, development, severity or recurrence of a symptom, complication or condition, or biochemical indicia associated with a disease. In one embodiment, "treatment" or "treating" includes a partial remission. In another embodiment, "treatment" or "treating" includes a complete remission. In some embodiments, treatment may be of a subject who does not exhibit signs of the relevant disease, disorder and/or condition and/or of a subject who exhibits only early signs of the disease, disorder, and/or condition. In some embodiments, such treatment may be of a subject who exhibits one or more established signs of the relevant disease, disorder and/or condition. In some embodiments, treatment may be of a subject who has been diagnosed as suffering from the relevant disease, disorder, and/or condition. In some embodiments, treatment may be of a subject known to have one or more susceptibility factors that are

statistically correlated with increased risk of development of the relevant disease, disorder, and/or condition.

[0075] A "disease", as used herein, is a state of health of a subject wherein the subject cannot maintain homeostasis, and wherein if the disease is not ameliorated, the subject's health continues to deteriorate. In contrast, a "disorder" is a state of health in which the subject is able to maintain homeostasis, but in which the subject's state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the subject's state of health. A disease or disorder is "alleviated" if the severity of a sign or symptom of the disease or disorder, the frequency with which such a sign or symptom is experienced by a subject, or both, is reduced.

[0076] As used herein, the terms "subject", "individual", and "patient" are interchangeable, and relate to vertebrates, preferably mammals. For example, mammals in the context of the disclosure are humans, non-human primates, domesticated animals such as dogs, cats, sheep, cattle, goats, pigs, horses, etc., laboratory animals such as mice, rats, rabbits, guinea pigs, etc., as well as animals in captivity such as animals in zoos. The term "animal" as used herein includes humans. The term "subject" may also include a patient, i.e., an animal, having a disease. In exemplary aspects, a subject, individual, or patient refers to a human (*e.g.*, a man, a woman, or a child).

[0077] As used herein, the term "preventing a disease" in a subject means, for example, to stop the development of one or more clinical symptoms of a disease or disorder in a subject before they occur or are detectable. Preferably, the disease or disorder does not develop at all, i.e., no symptoms of the disease or disorder are detectable. In some aspects, it can also mean delaying or slowing of the development of one or more symptoms of the disease or disorder. Alternatively, or in addition, it can mean decreasing the severity of one or more subsequently developed symptoms.

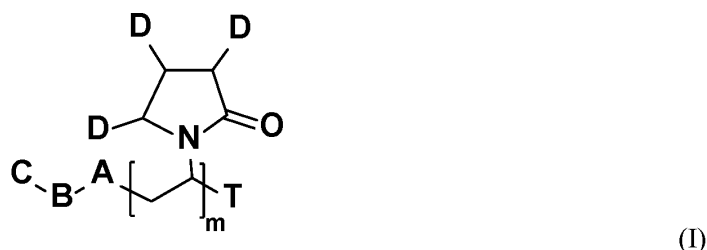
[0078] The invention is defined in the claims. However, below is a non-exhaustive listing of non-limiting exemplary aspects. Any one or more of the features of these aspects may be combined with any one or more features of another example, embodiment, or aspect described herein.

[0079] III. Stabilizing Agents

[0080] Lipid nanoparticles including liposomes, cubosomes, hexosomes, solid lipid nanoparticles, and nanostructured lipid carriers can require steric stabilizers to maintain colloidal stability in an aqueous medium and improve pharmacokinetics and biodistribution profiles. One indication of colloidal stability is the polydispersion index (PDI), which needs to be sufficiently low such that aggregation of the nanoparticles does not occur. A high PDI would indicate a decrease in the stability and viability of the nanoparticles over time.

[0081] Currently, polyethylene glycol (PEG)-lipid conjugates are the most commonly employed stabilizers in these systems. However, this PEGylated class of stabilizers can elicit an undesirable immunogenic response and impede cell interactions with nanoparticles containing a PEG layer. Surprisingly, the new class of stabilizers having a polyvinylpyrrolidone group that the inventors developed have favorable profiles (*e.g.*, size, polydispersion index values, organ selectivity, and encapsulation efficiency) as described herein without eliciting the same immunogenic response and without impeding cell interactions compared to PEGylated stabilizers.

[0082] Methods for generating these polyvinylpyrrolidone stabilizers are described herein. The polyvinylpyrrolidone stabilizers have the general structure of formula (I):



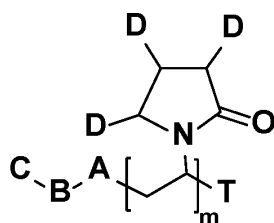
wherein **A** is S, NH, O, C₁₋₈ substituted or unsubstituted alkyl or heteroalkyl group, a hydrophilic group, a glyceryl group, a heterocyclic group, a phospholipid group, a ceramide group, an ionizable group, a triazole group, or a combination thereof; **B** is CH₂, a triazole group or a C₁₋₁₈ amino oxobutanoate group or a C₁₋₁₈ substituted or unsubstituted alkyl or heteroalkyl group; **C** is a C₁₋₅₀ substituted or unsubstituted heteroalkyl group, C₁₋₅₀ unsaturated heteroalkyl group, C₁₋₅₀ heterocyclic group, C₁₋₅₀ charged heteroalkyl group, or a combination thereof; **T** is hydrogen, a C₁₋₁₈ carbonothioate group, C₁₋₁₈ substituted or unsubstituted alkyl or heteroalkyl group, a C₁₋₁₈ ionizable group, a C₁₋₁₈ targeting group, a hydrophilic group, an amphiphilic group, or a combination thereof; **D** is H, C₁₋₅ substituted or unsubstituted alkyl or heteroalkyl group, or hydroxyl group and *m* is an integer from 15 to 500.

[0083] In some embodiments, C is a C₁₋₄₅ substituted or unsubstituted heteroalkyl group, C₁₋₄₅ unsaturated heteroalkyl group, C₁₋₄₅ heterocyclyl group, C₁₋₄₅ charged heteroalkyl group, or a combination thereof. In some embodiments, C is a C₁₋₄₀ substituted or unsubstituted heteroalkyl group, C₁₋₄₀ unsaturated heteroalkyl group, C₁₋₄₀ heterocyclyl group, C₁₋₄₀ charged heteroalkyl group, or a combination thereof. In some embodiments, C is a C₁₋₃₅ substituted or unsubstituted heteroalkyl group, C₁₋₃₅ unsaturated heteroalkyl group, C₁₋₃₅ heterocyclyl group, C₁₋₃₅ charged heteroalkyl group, or a combination thereof. In some embodiments, C is a C₁₋₃₀ substituted or unsubstituted heteroalkyl group, C₁₋₃₀ unsaturated heteroalkyl group, C₁₋₃₀ heterocyclyl group, C₁₋₃₀ charged heteroalkyl group, or a combination thereof. In some embodiments, C is a C₁₋₂₅ substituted or unsubstituted heteroalkyl group, C₁₋₂₅ unsaturated heteroalkyl group, C₁₋₂₅ heterocyclyl group, C₁₋₂₅ charged heteroalkyl group, or a combination thereof. In some embodiments, C is a C₁₋₂₀ substituted or unsubstituted heteroalkyl group, C₁₋₂₀ unsaturated heteroalkyl group, C₁₋₂₀ heterocyclyl group, C₁₋₂₀ charged heteroalkyl group, or a combination thereof. In some embodiments, C is a C₁₋₁₅ substituted or unsubstituted heteroalkyl group, C₁₋₁₅ unsaturated heteroalkyl group, C₁₋₁₅ heterocyclyl group, C₁₋₁₅ charged heteroalkyl group, or a combination thereof. In some embodiments, C is a C₁₋₁₀ substituted or unsubstituted heteroalkyl group, C₁₋₁₀ unsaturated heteroalkyl group, C₁₋₁₀ heterocyclyl group, C₁₋₁₀ charged heteroalkyl group, or a combination thereof.

[0084] In some embodiments, A is sulfur or a triazole group; B is CH₂, a triazole group or a C₃₋₆ amino oxobutanoate group; C is a C₃₀₋₃₁ substituted or unsubstituted heteroalkyl group, C₃₀₋₃₁ unsaturated heteroalkyl group, C₃₀₋₃₁ heterocyclyl group, C₃₀₋₃₁ charged heteroalkyl group, or a combination thereof; T is hydrogen, a C₁₋₅ carbonothioate group, a C₁₋₅ ionizable group, a C₁₋₅ targeting group, a hydrophilic group, an amphiphilic group, or a combination thereof; and m is an integer from 15 to 400.

[0085] IV. Lipid Nanoparticle Compositions

[0086] In an aspect of the invention, the lipid nanoparticle composition comprises (a) an ionizable lipid; (b) two or more lipids; and (c) a stabilizing agent of formula (I):

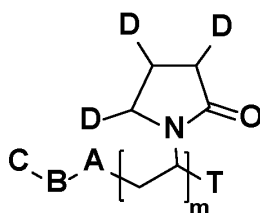


(I)

wherein A is S, NH, O, C₁₋₈ substituted or unsubstituted alkyl or heteroalkyl group, a hydrophilic group, a glyceryl group, a heterocyclic group, a phospholipid group, a ceramide group, an ionizable group, a triazole group, or a combination thereof; B is CH₂, a triazole group or a C₁₋₁₈ amino oxobutanoate group or a C₁₋₁₈ substituted or unsubstituted alkyl or heteroalkyl group; C is a C₁₋₅₀ substituted or unsubstituted heteroalkyl group, C₁₋₅₀ unsaturated heteroalkyl group, C₁₋₅₀ heterocyclic group, C₁₋₅₀ charged heteroalkyl group, or a combination thereof; T is hydrogen, a C₁₋₁₈ carbonothioate group, C₁₋₁₈ substituted or unsubstituted alkyl or heteroalkyl group, a C₁₋₁₈ ionizable group, a C₁₋₁₈ targeting group, a hydrophilic group, an amphiphilic group, or a combination thereof; D is H, C₁₋₅ substituted or unsubstituted alkyl or heteroalkyl group, or hydroxyl group and m is an integer from 15 to 500.

[0087] In some embodiments, A is sulfur or a triazole group; B is CH₂ or a C₃₋₆ amino oxobutanoate group; C is a C₃₀₋₃₁ substituted or unsubstituted heteroalkyl group, C₃₀₋₃₁ unsaturated heteroalkyl group, C₃₀₋₃₁ heterocyclyl group, C₃₀₋₃₁ charged heteroalkyl group, or a combination thereof; T is hydrogen, a C₁₋₅ carbonothioate group, a C₁₋₅ ionizable group, a C₁₋₅ targeting group, a hydrophilic group, an amphiphilic group, or a combination thereof; D is H, hydroxyl group or ethyl group and m is an integer from 15 to 400.

[0088] In an aspect of the invention, the lipid nanoparticle composition consists essentially of (a) an ionizable lipid; (b) two or more lipids; and (c) a stabilizing agent of formula (I):



(I).

[0089] "Consisting essentially of" is meant to include any elements listed after the phrase, and may include other additional elements limited to those that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the

phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they materially affect the activity or action of the listed elements.

[0090] As used herein, the term "targeting ligand" or "targeting group" can be in the form of a moiety capable of specifically binding to a molecule on the surface of a target cell, such as a cell within a target tissue of interest. In certain embodiments, the targeting ligand is a peptide, antibody, sugar, oligosaccharide, aminoglycoside, sterol, phenyl boronic acid, or a combination thereof.

[0091] In some embodiments of the invention, the hydrophilic group can be in the form of a molecule with a water soluble portion that can either carry a formal charge, ionic, or can be neutral, non-ionic.

[0092] In some embodiments of the invention, the ionizable group can be in the form of a molecule which, either by its intrinsic chemical nature, or as a function of the medium and/or of the pH of the medium in which it is present, may be in ionic form.

[0093] In some embodiments of the invention, the amphiphilic group can be in the form of a molecule that possesses both hydrophilic and hydrophobic properties.

[0094] As used herein, the term "alkyl group" or "alkyl" can be in the form of a branched, straight-chained (linear), or cyclic hydrocarbon group, having the specified number of carbon atoms, usually from 1 to about 18 or from 1 to about 50 carbon atoms. Exemplary alkyls include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, pentyl, iso-amyl, hexyl, octyl cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cyclooctyl, and the like. In some embodiments, the alkyl may be substituted. Substituted alkyls are alkyls in which at least one hydrogen atom of the alkyl has been substituted with at least a non-hydrogen group, such as a hydrocarbyl group, a heteroatom, or a heteroatom containing group, such as halogen (such as Br, Cl, F or I) or at least one functional group such as -NR^{*}₂, -OR^{*}, -SeR^{*}, -TeR^{*}, -PR^{*}₂, -AsR^{*}₂, -SbR^{*}₂, -SR^{*}, -BR^{*}₂, -SiR^{*}, -SiR^{*}₃, -GeR^{*}, -GeR^{*}₃, -SnR^{*}, -SnR^{*}₃, -PbR^{*}₃, and the like, where each R^{*} is independently a hydrocarbyl or halocarbyl radical, and two or more R^{*} may join together to form a substituted or unsubstituted saturated, partially unsaturated or aromatic cyclic or polycyclic ring structure, or where at least one heteroatom has been inserted within a hydrocarbyl ring.

[0095] The term "branched alkyl" means that the alkyl group contains a tertiary or quaternary carbon (a tertiary carbon is a carbon atom bound to three other carbon atoms. A quaternary carbon is a carbon atom bound to four other carbon atoms). For example, 3,5,5-trimethylhexylphenyl is an alkyl group (hexyl) having three methyl branches (hence, one tertiary and one quaternary carbon) and thus is a branched alkyl bound to a phenyl group. Unless otherwise indicated a branched alkyl includes all isomers thereof.

[0096] As used herein, the term "heteroalkyl group" or "heteroalkyl" can be in the form of an alkyl group described herein further in which one or more of the carbon atoms (and any associated hydrogen atoms) are each independently replaced with the same or different heteroatom or heteroatomic group.

[0097] For example, heteroalkyl may include 1, 2, 3, 4, 5, or 6 heteroatomic groups, *e.g.*, 1 heteroatomic group. Heteroatoms include, but are not limited to, N, P, O, S, etc, and combinations thereof. Heteroatomic groups include, but are not limited to, -NR-, -O-, -S-, -PH-, -P(O)₂-, -S(O)-, -S(O)₂-, and the like, where R is H, alkyl, aryl, cycloalkyl, heteroalkyl, heteroaryl or cycloheteroalkyl. The term "heteroalkyl" includes heterocycloalkyl (a cyclic heteroalkyl group), alkyl-heterocycloalkyl (a linear or branched aliphatic group attached to a cyclic heteroalkyl group), and the like. Heteroalkyl groups include, but are not limited to, -OCH₃, -CH₂OCH₃, -SCH₃, -CH₂SCH₃, -NRCH₃, -CH₂NRCH₃, and the like, where R is hydrogen, alkyl, aryl, arylalkyl, heteroalkyl, or heteroaryl, each of which may be optionally substituted. A heteroalkyl group comprises from 1 to about 10 carbon and hetero atoms, *e.g.*, from 1 to 6 carbon and hetero atoms.

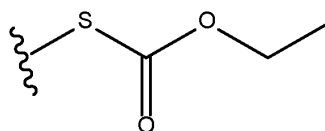
[0098] As used herein, the term "cycloalkyl group" or "cycloalkyl" is a subset of "alkyl" and may be in the form of a saturated partially saturated cyclic group of from 3 to about 10 carbon atoms and no ring heteroatoms and having a single ring or multiple rings including fused, bridged, and spiro ring systems, having the specified number of carbon atoms, usually from 1 to about 18 or from 1 to about 35 carbon atoms. For multiple ring systems having aromatic and non-aromatic rings that have no ring heteroatoms, the term "cycloalkyl" applies when the point of attachment is at a non- aromatic carbon atom (*e.g.*, 5, 6, 7, 8-tetrahydronaphthalene-5-yl). The term "cycloalkyl" includes cycloalkenyl groups. Examples of cycloalkyl groups include, for instance, adamantyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclooctyl, and cyclohexenyl.

[0099] As used herein, the term “heterocyclyl group” can be in the form of a cycloalkyl group, described herein, containing from 1 to about 6 heteroatoms chosen from N, O, S, or combinations thereof with remaining ring atoms being about 3 carbon to about 8 carbon atoms. Examples of cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, or cyclohexyl. A heterocyclic ring is a ring having a heteroatom in the ring structure as opposed to a heteroatom substituted ring where a hydrogen on a ring atom is replaced with a heteroatom. For example, tetrahydrofuran is a heterocyclic ring and 4-N,N-dimethylamino-phenyl is a heteroatom substituted ring.

[00100] Substituted heterocyclic means a heterocyclic group where at least one hydrogen atom of the heterocyclic radical has been substituted with at least a non-hydrogen group, such as a hydrocarbyl group, a heteroatom, or a heteroatom containing group, such as halogen (such as Br, Cl, F or I) or at least one functional group such as -NR*₂, -OR*, -SeR*, -TeR*, -PR*₂, -ASR*₂, -SbR*₂, -SR*, -BR*₂, -SiR*, -SiR*₃, -GeR*, -GeR*₃, -SnR*, -SnR*₃, -PbR*₃, and the like, where each R* is independently a hydrocarbyl or halocarbyl radical.

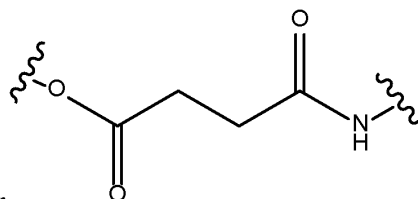
[00101] As used herein, the term “ceramide” can be in the form of a family of lipid molecules composed of a sphingosine and a fatty acid. Generally, the long-chain sphingoid base is linked to a fatty acid via an amide bond.

[00102] In some embodiments, T is hydrogen, methyl, a peptide, an antibody, a sugar, an



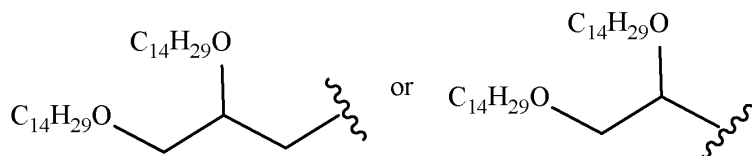
oligosaccharide, an aminoglycoside, a sterol, , phenyl boronic acid, or a combination thereof.

[00103] In some embodiments, A is sulfur or a triazole group.



[00104] In some embodiments, B is CH₂ or

[00105] In some embodiments, C is



[00106] In some embodiments, the lipid nanoparticle composition comprises two or more lipids comprising of a structural lipid, a sterol, or a combination thereof.

[00107] Any suitable ionizable lipid can be present in the lipid nanoparticle composition and lipid nanoparticle. An ionizable lipid is a lipid that is cationic or becomes ionizable (protonated) as the pH is lowered below the pKa of the ionizable group of the lipid, but is more neutral at higher pH values. At pH values below the pKa, the lipid is able to associate with negatively charged nucleic acids (*e.g.*, oligonucleotides). Ionizable lipid includes lipids that assume a positive charge on pH decrease from physiological pH, or lipids that carry a net positive charge at a selective pH.

[00108] In some embodiments, the lipid nanoparticle composition or lipid nanoparticle comprise one or more ionizable lipids, *e.g.*, two or more ionizable lipids, three or more ionizable lipids, or four or more ionizable lipids. In some embodiments, the ionizable lipid is DODMA (1,2-dioleoyloxy-3-dimethylaminopropane), DLin-MC3-DMA (O-(Z,Z,Z,Z-heptatriaconta-6,9,26,29-tetraen-19-yl)-4-(N,N-dimethylamino)), DLin-KC2-DMA (2-dilinoleyl-4-dimethylaminoethyl- [1,3]-dioxolane), BOCHD-C3-DMA (4-(dimethylamino)-,9-(2-octylcyclopropyl)-1-[8-(2 octylcyclopropyl) octyl]nonyl ester), C12-200 (1,1'-[[2-[4-[2-[[2-bis(2-hydroxydodecyl)amino]ethyl](2-hydroxydodecyl)amino]ethyl]-1-piperazinyl]ethyl]imino]bis-2-dodecanol), PNI 516 (Z)-3-(2-((1,17-bis(2-octylcyclopropyl)heptadecan-9-yl)oxy)-2-oxoethyl)-2-(pent-2-en-1-yl)cyclopentyl 4-(dimethylamino)butanoate, PNI 127 (2R,3S,4R)-2-(((1,4-dimethylpiperidine-4-carbonyl)oxy)methyl)tetrahydrofuran-3,4-diyl (9E,9'E,12E,12'E)-bis(octadeca-9,12-dienoate), PNI 550 3-(2-((1,17-bis(2-octylcyclopropyl)heptadecan-9-yl)oxy)-2-oxoethyl)cyclopentyl 4-(dimethylamino)butanoate, PNI 580 (2S,3R,4R)-2-(((4-(dimethylamino)butanoyl)oxy)methyl)tetrahydrofuran-3,4-diyl bis(2-hexyldecanoate), PNI 659 ((2R,3R,4S)-3,4-bis((2-hexyldecyl)oxy)tetrahydrofuran-2-yl)methyl 4-(dimethylamino)butanoate, PNI 728 ((2R,3R,4S)-3,4-bis((2-hexyldecyl)oxy)tetrahydrofuran-2-yl)methyl 2-(dimethylamino)ethyl)carbamate, PNI 762 ((2R,3R,4S)-3,4-bis((2-hexyldecyl)oxy)tetrahydrofuran-2-yl)methyl (2-

(diethylamino)ethyl)carbamate, or a combination thereof. In some embodiments, the ionizable lipid is PNI 516, PNI 127, PNI 550, PNI 580, PNI 659, PNI 728, PNI 762, or a combination thereof.

[00109] The ionizable lipid can be present in the lipid nanoparticle composition or lipid nanoparticle in any suitable amount or concentration. In some embodiments, the ionizable lipid is present at a concentration of about 20 to about 70 mol%, *e.g.*, about 20 mol%, about 25 mol%, about 30 mol%, about 35 mol%, about 40 mol%, about 45 mol%, about 50 mol%, about 55 mol%, about 60 mol%, about 65 mol%, or about 70 mol%, or a concentration within a range defined by any two of the foregoing values. In some cases, the ionizable lipid is present at a concentration of more than about 10 mol%, more than about 12 mol%, more than about 14 mol%, more than about 16 mol%, more than about 18 mol%, more than about 20 mol%, more than about 22 mol%, more than about 24 mol%, more than about 26 mol%, more than about 28 mol%, or more than about 30 mol%. In some cases, the ionizable lipid is present at a concentration of less than about 80 mol%, less than about 78 mol%, less than about 76 mol%, less than about 74 mol%, less than about 72 mol%, less than about 70 mol%, less than about 68 mol%, less than about 66 mol%, less than about 64 mol%, less than about 62 mol%, less than about 60 mol%, less than about 58 mol%, less than about 56 mol%, less than about 54 mol%, less than about 52 mol%, or less than about 50 mol%.

[00110] In some embodiments, the lipid nanoparticle composition comprises a structural lipid. Any suitable structural lipid can be present in the lipid nanoparticle composition and lipid nanoparticle. A structural lipid, or phospholipid, supports the formation of particles during manufacture. In various embodiments, the structural lipid includes one or more neutrally charged, positively charged, or negatively charged molecules. In some embodiments, the structural lipid has a net negative charge. In some embodiments, the structural lipid has a net neutral charge. In some embodiments, the structural lipid has a net positive charge.

[00111] In some embodiments, the lipid nanoparticle composition or lipid nanoparticle comprise one or more structural lipids, *e.g.*, two or more structural lipids, three or more structural lipids, or four or more structural lipids. In some embodiments, the structural lipid comprises diacylphosphatidylcholines, diacylphosphatidylethanolamines, diacylphosphatidylglycerols, ceramides, sphingomyelins, dihydrosphingomyelins, cephalins, cerebroside, or a combination thereof. In some embodiments, the structural lipid is distearoylphosphatidylcholine,

dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, dioleoyl-phosphatidylethanolamine, palmitoyl-oleoylphosphatidylcholine, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine, palmitoyl-oleoyl-phosphatidylethanolamine, dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate, dipalmitoyl phosphatidyl ethanolamine, dimyristoylphosphoethanolamine, distearoyl-phosphatidylethanolamine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-methyl, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N,N-dimethyl, 1,2-di-oleoyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-oleoyl-phosphatidylethanolamine, 1,2-di-oleoyl-sn-glycero-3-phosphoethanolamine, distearoylphosphatidylcholine, or a combination thereof.

[00112] In some embodiments, the structural lipid comprises any suitable lipid that is negatively charged (anionic) at physiological pH. In certain embodiments, the structural lipid comprises dioleoylphosphatidylglycerol, dipalmitoylphosphatidylglycerol, palmitoyl-oleoylphosphatidylglycerol, cardiolipin, phosphatidylinositol, diacylphosphatidylserine, diacylphosphatidic acid, monosialoganglioside GM1, or a combination thereof. In some embodiments, the structural lipid is distearoylphosphatidylcholine.

[00113] The structural lipid can be present in the lipid nanoparticle composition in any suitable amount. In some embodiments the structural lipid is present in the lipid nanoparticle composition at a concentration of about 1 to about 25 mol%, *e.g.*, about 1 mol%, about 2 mol %, about 3 mol %, about 4 mol%, about 5 mol%, about 6 mol%, about 7 mol%, about 8 mol%, about 9 mol%, about 10 mol%, about 11 mol%, about 12 mol%, about 13 mol%, about 14 mol%, about 15 mol%, about 16 mol%, about 17 mol%, about 18 mol%, about 19 mol%, about 20 mol%, about 21 mol%, about 22 mol%, about 23 mol%, about 24 mol%, or about 25 mol%, or a concentration within a range defined by any two of the aforementioned values. In some cases, the structural lipid is present in the lipid nanoparticle composition at a concentration of less than about 1%. In some cases, the structural lipid is present in the lipid nanoparticle composition at a concentration of more than about 20 mol %, more than about 21 mol %, more than about 22 mol %, more than about 23 mol %, more than about 24 mol %, more than about 25 mol %, more than about 26 mol %, more than about 27 mol %, more than about 28 mol %, more than about 29 mol %, more than about 30 mol %, more than about 31 mol %, more than about 32 mol %, more than about 33 mol %, more than about 34 mol %, or more than about 35 mol %.

[00114] In some embodiments, the lipid nanoparticle composition comprises a sterol. Any suitable sterol can be present in the lipid nanoparticle composition. In some embodiments, the lipid nanoparticle composition comprises one or more sterols *e.g.*, two or more sterols, three or more sterols, or four or more sterols. In some embodiments, the sterol is cholesterol, beta-sitosterol, 20-alpha-hydroxysterol, phytosterol, or a combination thereof. In some embodiments the sterol is cholesterol.

[00115] The sterol can be present in the lipid nanoparticle composition in any suitable amount. In some embodiments the sterol is present in the lipid nanoparticle composition a concentration of about 28 to about 50 mol%, *e.g.*, about 28 mol%, about 29 mol%, about 30 mol%, about 31 mol%, about 32 mol%, about 33 mol%, about 34 mol%, about 35 mol%, about 36 mol%, about 37 mol%, about 38 mol%, about 39 mol%, about 40 mol%, about 41 mol%, about 42 mol%, about 43 mol%, about 44 mol%, about 45 mol%, about 46 mol%, about 47 mol%, about 48 mol%, about 49 mol%, or about 50 mol%, or a concentration within a range defined by any two of the aforementioned values. In some cases, the sterol is present at a concentration of more than about 18 mol%, more than about 20 mol%, more than about 22 mol%, more than about 24 mol%, more than about 26 mol%, more than about 28 mol%, or more than about 30 mol%. In some cases, the sterol is present at a concentration of less than about 60 mol%, less than about 58 mol%, less than about 56 mol%, less than about 54 mol%, less than about 52 mol%, less than about 50 mol%, less than about 48 mol%, less than about 46 mol%, less than about 44 mol%, less than about 42 mol%, less than about 40 mol%, less than about 38 mol%, less than about 36 mol%, less than about 34 mol%, less than about 32 mol%, or less than about 30 mol%.

[00116] In some embodiments, the lipid nanoparticle composition comprises one or more stabilizing agents, *e.g.*, two or more stabilizing agents, three or more stabilizing agents, or four or more stabilizing agents.

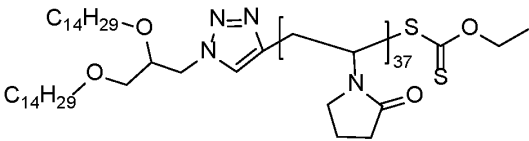
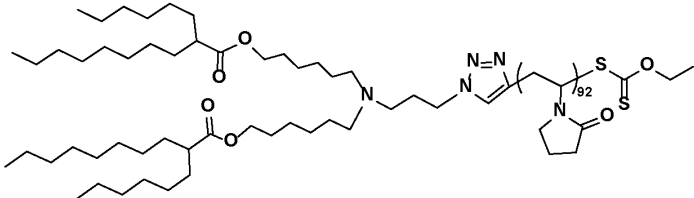
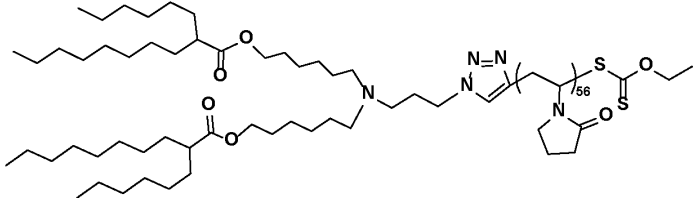
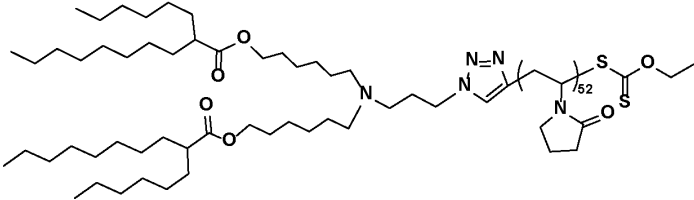
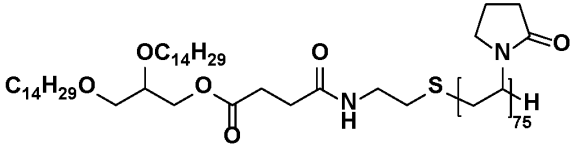
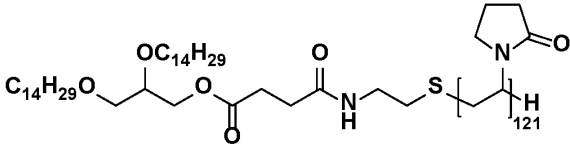
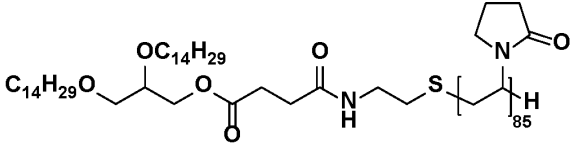
[00117] Any suitable stabilizing agent can be used in the lipid nanoparticle composition. In some embodiments the stabilizing agent is SAF29, SAF29A, SAF29B, SAF29C, SAF30, SAF34, SAF102, SAF102A, SAF102B, SAF103, SAF 104, SAF123, SAF124, SAF125, SAF126, SAF127, SAF133, SAF134, SAF135, SAF136, SAF137, SAF138, SAF139, SAF141, SAF149, SAF150, SAF154, SAF167, SAF178, SAF190, SAF191, or a combination thereof.

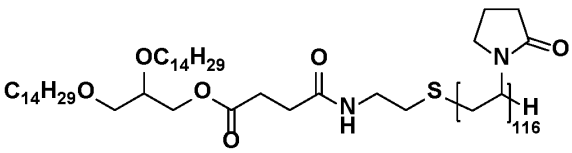
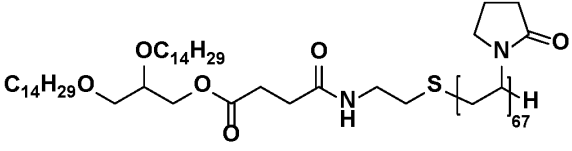
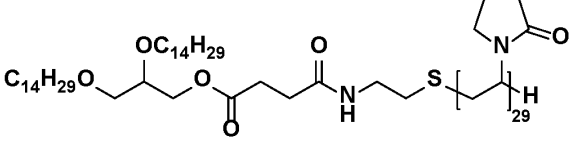
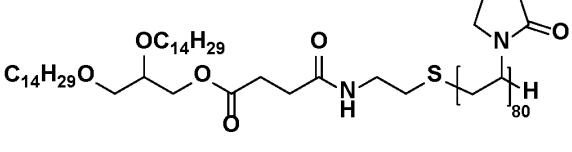
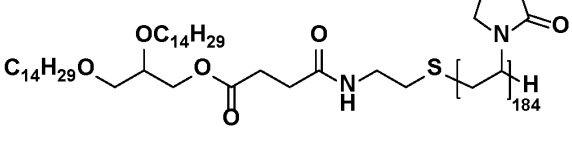
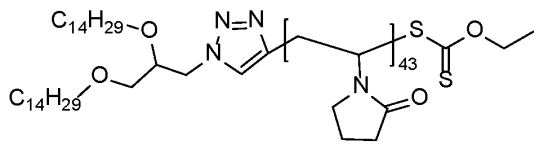
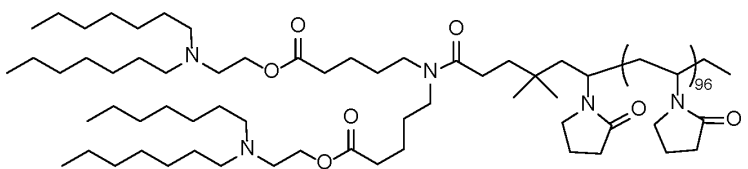
Table 1 provides the structures of selected stabilizing agents.

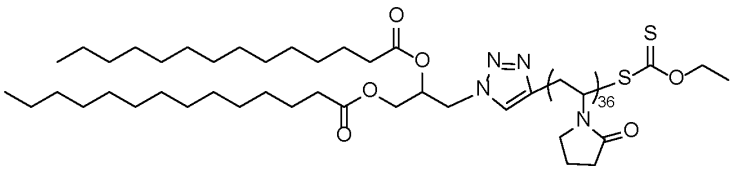
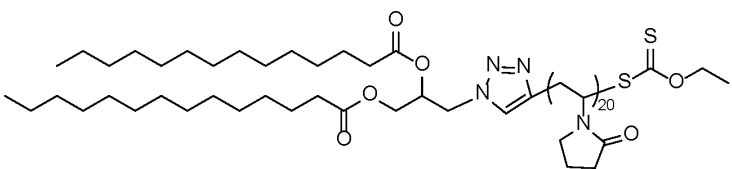
Table 1

SAF #	Structure	MWt (Da)
SAF29		9204
SAF29A		8408
SAF29B		8120
SAF29C		8498
SAF30		42282
SAF34		10425
SAF102		4873
SAF102A		6224

SAF102B		5614
SAF103		5761
SAF104		2875
SAF123		6705
SAF124		9521
SAF125		4860
SAF126		5546

SAF127		4374
SAF133		11162
SAF 134		7161
SAF135		6717
SAF136		9119
SAF137		14231
SAF138		10230

SAF139		13675
SAF141		8229
SAF149		4000
SAF150		9670
SAF154		21233
SAF167		5546
SAF178		11603

SAF190		4695
SAF191		2920

[00118] The stabilizing agent can have any suitable molecular weight. In some embodiments, the stabilizing agent has a molecular weight of about 500 to about 50000 Da, *e.g.*, about 500 Da, about 600 Da, about 700 Da, about 800 Da, about 900 Da, about 1000 Da, about 2000 Da, about 4000 Da, about 6000 Da, about 8000 Da, about 10000 Da, about 12000 Da, about 14000 Da, about 16000 Da, about 18000 Da, about 20000 Da, about 22000 Da, about 24000 Da, about 26000 Da, about 28000 Da, about 30000 Da, about 32000 Da, about 34000 Da, about 36000 Da, about 38000 Da, about 40000 Da, about 42000 Da, about 44000 Da, about 46000 Da, about 48000 Da, or about 50000 Da, or a molecular weight defined by the range of any two of the foregoing values. In some cases, the stabilizing agent has a molecular weight of less than about 1000 Da. In some cases, the stabilizing agent has a molecular weight of more than about 1000 Da, about 2000 Da, about 4000 Da, about 6000 Da, about 8000 Da, or about 10000 Da. In some cases, the stabilizing agent has a molecular weight of less than about 60000 Da, about 58000 Da, about 56000 Da, about 54000 Da, about 52000 Da, about 50000 Da, about 48000 Da, about 46000 Da, about 44000 Da, about 42000 Da, or about 40000 Da.

[00119] The stabilizing agent can be present in any suitable concentration. In some cases, the stabilizing agent has a concentration from about 0.1 mol% to about 10 mol%, *e.g.*, about 0.1 mol%, about 0.2 mol%, about 0.3 mol%, about 0.4 mol%, about 0.5 mol%, about 0.6 mol%, about 0.7 mol%, about 0.8 mol%, about 0.9 mol%, about 1 mol%, about 1.2 mol%, about 1.4 mol%, about 1.6 mol%, about 1.8 mol%, about 2 mol%, about 2.5 mol%, about 3 mol%, about 3.5 mol%, about 4 mol%, about 4.5 mol%, about 5 mol%, about 5.5 mol%, about 6 mol%, about 6.5 mol%, about 7 mol%, about 7.5 mol%, about 8 mol%, about 8.5 mol%, about 9 mol%, about 9.5 mol%, about 10 mol%, or a concentration defined by a range of any two of

the foregoing values. In some cases, the stabilizing agent has a concentration of more than about 0.02 mol%, more than about 0.04 mol%, more than about 0.06 mol%, more than about 0.08 mol%, more than about 0.1 mol%, more than about 0.2 mol%, more than about 0.3 mol%, more than about 0.4 mol%, more than about 0.5 mol%, more than about 0.6 mol%, more than about 0.7 mol%, more than about 0.8 mol%, more than about 0.9 mol%, or more than about 1.0 mol%. In some cases, the stabilizing agent has a concentration of less than about 20 mol%, less than about 18 mol%, less than about 16 mol%, less than about 14 mol%, less than about 12 mol%, less than about 10 mol%, less than about 9 mol%, less than about 8 mol%, less than about 7 mol%, less than about 6 mol%, less than about 5 mol%, less than about 4 mol%, or less than about 3 mol%.

[00120] In some embodiments, the lipid nanoparticle composition comprises about 20 to about 70 mol% ionizable lipid, about 1 to about 25 mol% structural lipid, about 28 to about 50 mol% sterol, and about 0.1 to about 5 mol% stabilizing agent. In certain embodiments, the lipid nanoparticle composition comprises about 47.5 mol% ionizable lipid, about 12.5 mol% structural lipid, about 38.5 mol% sterol, and about 1.5 mol% stabilizing agent. In some embodiments, the lipid nanoparticle composition comprises about 40 mol% ionizable lipid, about 12.5 mol% structural lipid, about 46 mol% sterol, and about 1.5 mol% stabilizing agent.

[00121] In some embodiments, the lipid nanoparticle composition is used in the formation of a lipid nanoparticle in embodiments of the methods described herein. In some embodiments, the diameter of the lipid nanoparticle is about 15 nm to about 500 nm, *e.g.*, about 15 nm, about 25 nm, about 50 nm, about 75 nm, about 100 nm, about 125 nm, about 150 nm, about 175 nm, about 200 nm, about 225 nm, about 250 nm, about 275 nm, about 300 nm, about 325 nm, about 350 nm, about 375 nm, about 400 nm, about 425 nm, about 450 nm, about 475 nm, or about 500 nm, or a diameter defined by a range of any two of the foregoing values. Such diameters can be useful for improving the tissue targeting and biodistribution of the lipid nanoparticles. In some cases, the diameter of the lipid nanoparticle is more than about 10 nm, more than about 15 nm, more than about 20 nm, more than about 25 nm, more than about 30 nm, more than about 35 nm, more than about 40 nm, or more than about 45 nm. In some cases, the diameter of the lipid nanoparticle is less than about 700 nm, less than about 675 nm, less than about 650 nm, less than about 625 nm, less than about 600 nm, less than about 575 nm, less than about 550 nm, less than about 525 nm, less than about 500 nm, less than about 475 nm, less than about 450 nm, less than

about 425 nm, less than about 400 nm, less than about 375 nm, less than about 350 nm, less than about 325 nm, or less than about 300 nm.

[00122] Embodiments of the lipid nanoparticle described herein can have any suitable polydispersity index. In some embodiments, the lipid nanoparticle has a polydispersity index of from about 0.01 to about 0.40, *e.g.*, about 0.01, about 0.02, about 0.03, about 0.04, about 0.05, about 0.06, about 0.07, about 0.08, about 0.09, about 0.10, about 0.11, about 0.12, about 0.13, about 0.14, about 0.15, about 0.16, about 0.17, about 0.18, about 0.19, about 0.20, about 0.21, about 0.22, about 0.23, about 0.24, about 0.25, about 0.26, about 0.27, about 0.28, about 0.29, about 0.30, about 0.31, about 0.32, about 0.33, about 0.34, about 0.35, about 0.36, about 0.37, about 0.38, about 0.39, or about 0.40, or polydispersity index defined by a range of any two of the foregoing values.

[00123] Embodiments of the lipid nanoparticle described herein can have any suitable encapsulation efficiency. Encapsulation efficiency refers to the percentage of nucleic acid that is successfully entrapped into the lipid nanoparticle. In some embodiments, the lipid nanoparticle has an encapsulation efficiency from about 50% to about 100%, *e.g.*, about 50%, about 52%, about 54%, about 56%, about 58%, about 60%, about 62%, about 64%, about 66%, about 68%, about 70%, about 72%, about 74%, about 76%, about 78%, about 80%, about 82%, about 84%, about 86%, about 88%, about 90%, about 92%, about 94%, about 96%, about 98%, or about 100%, or an encapsulation efficiency defined by a range of any two of the foregoing values.

[00124] The invention also provides a method for preparing embodiments of the lipid nanoparticle as described herein comprising (a) forming the lipid nanoparticle composition by combining prescribed amounts of ionizable lipid, structural lipid, sterol and stabilizing agent; (b) preparing the lipid nanoparticle by combining the lipid nanoparticle composition and the nucleic acid using a microfluidic mixer; and (c) purifying the lipid nanoparticle.

[00125] Any suitable method of mixing can be used to combine the prescribed amounts of ionizable lipid, structural lipid, sterol, stabilizing agent, and nucleic acid. Any suitable method can be used to combine the prescribed amounts of ionizable lipid, structural lipid, sterol, and stabilizing agent. In some embodiments, the ionizable lipid, structural lipid, sterol, and stabilizing agent are combined by mixing. In some embodiments, the mixing is done using a microfluidic mixer. In some embodiments, the prescribed amounts of ionizable lipid, structural lipid, sterol, and stabilizing agent are as described herein. In some embodiments, the ionizable

lipid, structural lipid, sterol, stabilizing agent, and nucleic acid are combined by standard T-tube mixing techniques, turbulent mixing, titration mixing, agitation promoting ordered self-assembly, or passive mixing of all the elements with self-assembly of elements into nanoparticles. A variety of methods have been developed to formulate lipid nanoparticles containing genetic drugs.

[00126] In some embodiments, microfluidic mixing devices, which can involve mixing two or more types of fluids together uniformly in a microfluidic chip, such as the NanoAssemblr® Spark™, NanoAssemblr® Ignite™, and NanoAssemblr® Blaze™, NanoAssemblr® GMP system, and NanoAssemblr® commercial formulation system are used. In some embodiments, the lipid nanoparticles formed by using a microfluidic mixing device has an encapsulation efficiency from about 90 to about 100%.

[00127] Any suitable method can be used to combine the lipid nanoparticle composition and the nucleic acid. In some embodiments, the lipid nanoparticle composition and the nucleic acid are combined by mixing. In some embodiments, the mixing is done using a microfluidic mixer. In some embodiments, the microfluidic mixer comprises a first and second stream of reagents, which feed into the microfluidic mixer, and lipid nanoparticles are collected from the outlet.

[00128] In some embodiments, the first stream includes a payload in a first solvent. In some embodiments, the payload may include a nucleic acid. In some cases, the payload may include a therapeutic agent. The combination of the payload in a first solvent can be described as the aqueous phase. Any suitable first solvent can be used. Suitable first solvents include solvents in which the payload is soluble and that are miscible with the second solvent. In some embodiments, the first solvent comprises aqueous buffers. In some embodiments, the aqueous buffer is a low pH buffer. In some embodiments, the low pH buffer is a citrate or acetate buffer.

[00129] In some embodiments, the second stream includes embodiments of the lipid nanoparticle composition as described herein in a second solvent. The combination of the lipid nanoparticle composition and the second solvent can be described as the organic phase. Any suitable second solvent can be used. Suitable second solvents include solvents in which the ionizable lipids according to embodiments of the invention are soluble, and that are miscible with the first solvent. In some embodiments, the second solvent comprises one or more solvents, two or more solvents, three or more solvents, or four or more solvents. In some embodiments, the second solvent comprises 1,4-dioxane, tetrahydrofuran, acetone, acetonitrile, dimethyl

sulfoxide, dimethylformamide, acids, alcohols, or a combination thereof. In some embodiments, the second solvent comprises aqueous or anhydrous alcohols. In some cases, the alcohol is a primary, secondary, or tertiary alcohol having from 1 to 12 branched or unbranched carbons (*e.g.*, methanol, ethanol, n-propanol, isopropanol, n-butanol, 2-methyl 1-propanol, 2-butanol, 2-methylpropan-2-ol).

[00130] In some embodiments, a suitable device for mixing includes one or more microchannels (*i.e.*, a channel having its greatest dimension less than 1 millimeter). In some embodiments, the microchannel has a diameter from about 20 to about 300 μm . In some embodiments, at least one region of the microchannel has a principal flow direction and one or more surfaces having at least one groove or protrusion defined therein, the groove or protrusion having an orientation that forms an angle with the principal direction (*e.g.*, a staggered herringbone mixer) or a bifurcating toroidal flow mixer. To achieve maximal mixing rates, it is advantageous to avoid undue fluidic resistance prior to the mixing region. In some embodiments, a device has non-microfluidic channels having dimensions greater than 1000 μm , to deliver the fluids to a single mixing channel.

[00131] Any suitable flow ratio can be used to combine the lipid nanoparticle composition and the nucleic acid. In some embodiments, the lipid nanoparticle composition and the nucleic acid are combined using a flow ratio of about 1:1 (or 1) to about 10:1 (or 10) (aqueous phase: organic phase) by volume, *e.g.*, about 1, about 2, about 3, about 4, about 5, about 6, or about 7, about 8, about 9, about 10, or a flow ratio defined by a range of any two of the aforementioned values. In some cases, the flow ratio is more than about 0.5. In some cases, the flow ratio is less than about 20, less than about 18, less than about 16, less than about 14, less than about 12, less than about 10, or less than about 8. Any suitable N/P ratio can be used to combine the lipid nanoparticle composition and the nucleic acid. The N/P ratio is the ratio of positively-chargeable polymer amine (N = nitrogen) groups to negatively-charged nucleic acid phosphate (P) groups. In some embodiments, the lipid nanoparticle composition and the nucleic acid are combined at a N/P ratio from about 2 to about 20, *e.g.*, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, or about 20, or at an N/P ratio defined by a range of any two of the aforementioned values. In some case, the N/P ratio is more than about 1, more than about 2, more than about 3, more than about 4, more than about 5, more than about 6, more than about 7,

more than about 8, or more than about 9. In some case, the N/P ratio is less than about 40, less than about 38, less than about 36, less than about 34, less than about 32, less than about 30, less than about 28, less than about 26, less than about 24, less than about 22, less than about 20, less than about 18, less than about 16, less than about 14, less than about 12, or less than about 10. Any suitable total flow rate can be used to combine the lipid nanoparticle composition and the nucleic acid. In some embodiments, the lipid nanoparticle composition and the nucleic acid are combined with a total flow rate of the organic phase and aqueous phase from about 2 to about 2000 mL/min, *e.g.*, about 2 mL/min, about 4 mL/min, about 6 mL/min, about 8 mL/min, about 10 mL/min, about 20 mL/min, about 40 mL/min, about 60 mL/min, about 80 mL/min, or about 100 mL/min, about 120 mL/min, about 140 mL/min, about 160 mL/min, about 180 mL/min, about 200 mL/min, about 220 mL/min, about 240 mL/min, about 260 mL/min, about 280 mL/min, about 300 mL/min, about 350 mL/min, about 400 mL/min, about 450 mL/min, or about 500 mL/min, about 550 mL/min, about 600 mL/min, about 650 mL/min, about 700 mL/min, about 750 mL/min, about 800 mL/min, about 850 mL/min, or about 900 mL/min, about 950 mL/min, about 1000 mL/min, about 1100 mL/min, about 1200 mL/min, about 1300 mL/min, about 1400 mL/min, about 1500 mL/min, about 1600 mL/min, about 1700 mL/min, about 1800 mL/min, about 1900 mL/min, about 2000 mL/min, or a total flow rate defined by a range of any two of the foregoing values. In some cases, the total flow rate is more than about 1 mL/min, 2 mL/min, 4 mL/min, 6 mL/min, 8 mL/min, 10 mL/min, 20 mL/min, or 40 mL/min. In some case, the total flow rate is less than about 3000 mL/min, less than about 2800 mL/min, less than about 2600 mL/min, less than about 2400 mL/min, less than about 2200 mL/min, less than about 2100 mL/min, less than about 2000 mL/min, less than about 1800 mL/min, less than about 1600 mL/min, less than about 1500 mL/min, less than about 1400 mL/min, less than about 1200 mL/min, less than about 1000 mL/min, or less than about 800 mL/min. In some embodiments, the lipid nanoparticle composition and the nucleic acid are combined using a flow ratio from about 1:1 (or 1) to about 10: 1 (or 10) by volume (aqueous phase: organic phase) at a N/P ratio from about 2 to about 20, and a total flow rate from about 2 to about 2000 mL/min. In some embodiments, the flow rate is 3 (aqueous phase: organic phase) to optimize for a particular payload or molar ratio of lipid components.

[00132] Any suitable method of purifying the lipid nanoparticles can be used. In some embodiments, the purifying is done using a filter or a centrifuge.

[00133] V. Methods of Use

[00134] In some embodiments, a payload is encapsulated by an exemplary lipid nanoparticle composition. Any suitable payload can be present in the lipid nanoparticle composition. The payload may include a nucleic acid. In some cases, the payload may include a therapeutic agent. The nucleic acid may be a substance intended to have a direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease, or to have direct effect in restoring, correcting or modifying physiological functions, or to act as a research reagent. Exemplary nucleic acids include any oligonucleotide or polynucleotide whose delivery into a cell causes a desirable effect. The nucleic acid can be single-stranded DNA or RNA, or double-stranded DNA or RNA, or DNA-RNA hybrids, or combinations thereof. In some embodiments, the lipid nanoparticle comprises one or more nucleic acids, two or more nucleic acids, three or more nucleic acids, or four or more nucleic acids. Including more than one nucleic acid may be beneficial in some embodiments (*e.g.*, gene editing). In some embodiments, the nucleic acid is an antisense oligonucleotide, a siRNA, a miRNA, a self-amplifying RNA (samRNA or saRNA), a self-replicating DNA, an LNA, a DNA, a replicon, an mRNA, a guide RNA, a transposon, a single gene, a vector, a plasmid, a viral particle, an AAV, a complex of RNA and RNA-binding protein(s), or a combination thereof. In some embodiments, the nucleic acid is an antigen encoded mRNA.

[00135] In some embodiments, the lipid nanoparticle composition is a therapeutic composition, such as an mRNA-based therapeutic composition. The therapeutic composition may optionally include one or more therapeutically acceptable carriers, diluents, or excipients such as salts, buffering agents, preservatives, antiadherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes, emollients, emulsifiers, fillers, film formers or coatings, flavors, fragrances, glidants, lubricants, sorbents, suspending or dispersing agents, sweeteners, waters of hydration, and/or other therapeutic agents. As used herein, the term “excipient” means any pharmaceutically acceptable additive, carrier, diluent, adjuvant, or other ingredient, other than the active pharmaceutical ingredient (API) (and typically in addition to components of the delivery vehicle compositions), suitably selected with respect to the intended form of administration, and consistent with conventional pharmaceutical practices. The disclosed compounds can be administered to a subject or patient in a therapeutically effective amount. The complexes can be administered alone or as part of a pharmaceutically acceptable composition or

formulation. In addition, the compositions can be administered all at once, as for example, by a bolus injection, multiple times, or delivered substantially uniformly over a period of time. It is also noted that the dose of the compound can be varied over time.

[00136] In some embodiments, the lipid nanoparticle composition may encapsulate an antigen encoded mRNA and be used as a vaccine. In some embodiments, the antigen encoded mRNA is for a prophylactic or therapeutic vaccine. A vaccine may be referred to as a substance used to stimulate the production of antibodies and provide immunity against one or several diseases, prepared from the causative agent of a disease, its products, or a synthetic substitute. The vaccine may further comprise one or more immunologic adjuvants. As used herein, the term "immunologic adjuvant" refers to a compound or a mixture of compounds that acts to accelerate, prolong, enhance or modify immune responses when used in conjugation with an immunogen (*e.g.*, neoantigens). Adjuvant may be non-immunogenic when administered to a host alone, but that augments the host's immune response to another antigen when administered conjointly with that antigen. Specifically, the terms "adjuvant" and "immunologic adjuvant" are used interchangeably in the present disclosure. Adjuvant-mediated enhancement and/or extension of the duration of the immune response can be assessed by any method known in the art including without limitation one or more of the following: (i) an increase in the number of antibodies produced in response to immunization with the adjuvant/antigen combination versus those produced in response to immunization with the antigen alone; (ii) an increase in the number of T cells recognizing the antigen or the adjuvant; and (iii) an increase in the level of one or more cytokines. Adjuvants may be aluminum based adjuvants including but not limiting to aluminum hydroxide and aluminum phosphate; saponins such as steroid saponins and triterpenoid saponins; bacterial flagellin and some cytokines such as GM-CSF. Adjuvants selection may depend on antigens, vaccines, and routes of administrations.

[00137] In some aspects, adjuvants improve the adaptive immune response to a vaccine antigen by modulating innate immunity or facilitating transport and presentation. Adjuvants act directly or indirectly on antigen presenting cells (APCs) including dendritic cells (DCs). Adjuvants may be ligands for toll-like receptors (TLRs) and can directly affect DCs to alter the strength, potency, speed, duration, bias, breadth, and scope of adaptive immunity. In other instances, adjuvants may signal via proinflammatory pathways and promote immune cell infiltration, antigen presentation, and effector cell maturation. This class of adjuvants includes

mineral salts, oil emulsions, nanoparticles, and polyelectrolytes and comprises colloids and molecular assemblies exhibiting complex, heterogeneous structures. In one example, the composition further comprises pidotimod as an adjuvant. In another example, the composition further comprises CpG as an adjuvant.

[00138] In some cases, the lipid nanoparticle composition is used in gene therapy. Gene therapy is a medical technique that produces a therapeutic effect through the manipulation of gene expression or through altering the biological properties of cells. In some cases, a gene encoding a therapeutic protein for incorporation into the host's DNA or a mRNA encoding the therapeutic protein is administered to treat a disease, where the disease is the result of a missing protein and/or missing activity of the protein. In some cases, a new gene or mRNA is supplied, which may enhance a cell's function without modifying the genes that cause the disease. In other cases, an antisense oligonucleotide (ASO) or small interfering RNA (siRNA) is used as a therapeutic to silence the activity of a variant protein causing a disease.

[00139] Gene therapy can be performed on a somatic cell level or a germline cell level. Gene therapy can be performed *ex vivo* or *in vivo*. Gene therapy can be employed by various gene editing techniques (*e.g.*, CRISPR, homologous recombination, zinc finger nucleases, TALEN).

[00140] In some cases, the nucleic acid is for incorporation into an immunogenic cell. In some cases, the immunogenic cell includes a T cell. In some embodiments, the immunogenic cell can be engineered to express a receptor to a specific antigen or neoantigen, engineered to enhance the immunogenic response or the immunogenic cell, and engineered to decrease proteins associated with an adverse response such as neurotoxicity (*e.g.*, reduction of cytokines to ameliorate the effects of cytokine release syndrome). In some embodiments, the lipid nanoparticle is in an anhydrous form. In some embodiments, the lipid nanoparticle is in an anhydrous form consisting of a lyophilized cake. In some embodiments, the lipid nanoparticle is in a reconstituted form. In a reconstituted form, a lyophilized lipid nanoparticle may have a pharmaceutically acceptable carrier added to the lyophilized lipid nanoparticle.

[00141] In some embodiments, the lipid nanoparticle and the pharmaceutically acceptable carrier are a pharmaceutical composition. Examples of pharmaceutically acceptable carrier are provided herein.

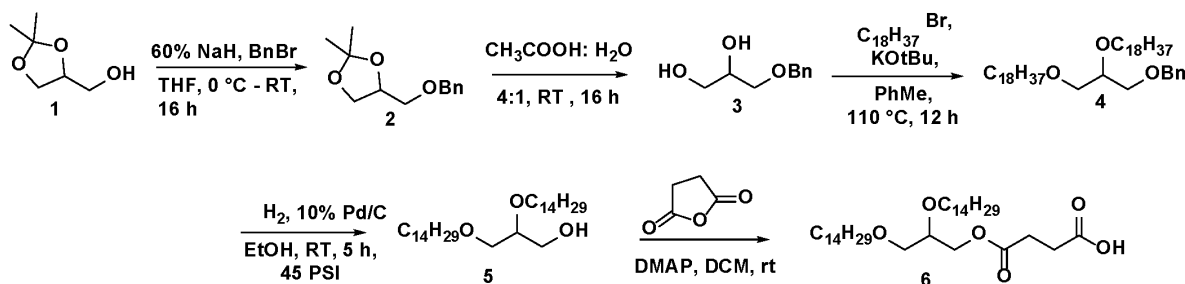
ABBREVIATIONS

- [00142] A5 saRNA SARS Cov2: Self-amplified RNA encoding viral replicase genes in addition to the SARS Cov2 antigen gene(s)
- [00143] C: Degrees Celsius
- [00144] Cas9 mRNA: Cas9 messenger RNA from Trilink Biotechnologies (San Diego, CA; L-7606-1000 CleanCap Cas9 mRNA, 1mg)
- [00145] Chol: cholesterol
- [00146] EE: Encapsulation efficiency
- [00147] eGFP-mRNA: Enhanced green fluorescent protein mRNA derived from Aequorea Victoria
- [00148] FLuc-mRNA: Firefly luciferase protein mRNA
- [00149] h: Hour(s)
- [00150] hEPO: Human erythropoietin
- [00151] hEPO-mRNA: Human erythropoietin protein mRNA
- [00152] HPLC: High performance liquid chromatography
- [00153] iL: Ionizable Lipid
- [00154] IM: intramuscular administration
- [00155] IV: intravenous administration
- [00156] KO: knock out
- [00157] LNP: lipid nanoparticles
- [00158] MFI: Median fluorescence intensity
- [00159] min: Minute(s)
- [00160] mRNA: messenger RNA
- [00161] N/P: nitrogen to phosphorous ratio
- [00162] NALNP: nucleic acid-containing lipid nanoparticles
- [00163] NAT: Nucleic acid therapeutic
- [00164] PBS: Phosphate buffered saline
- [00165] PCSK9 gRNA: PCSK9 gene guide RNA
- [00166] PDI: polydispersity index
- [00167] PNI 127: (2R,3S,4R)-2-(((1,4-dimethylpiperidine-4-carbonyl)oxy)methyl)tetrahydrofuran-3,4-diyl (9E,9'E,12E,12'E)-bis(octadeca-9,12-dienoate)

- [00168] PNI 516: (Z)-3-(2-((1,17-bis(2-octylcyclopropyl) heptadecan-9-yl) oxy)-2-oxoethyl)-2-(pent-2-en-1-yl)cyclopentyl 4-(dimethylamino)butanoate
- [00169] PNI 550: 3-(2-((1,17-bis(2-octylcyclopropyl)heptadecan-9-yl)oxy)-2-oxoethyl)cyclopentyl 4-(dimethylamino)butanoate
- [00170] PNI 580: (2S,3R,4R)-2-(((4-(dimethylamino)butanoyl) oxy)methyl)tetrahydrofuran-3,4-diyl bis(2-hexyldecanoate)
- [00171] PNI 659: ((2R,3R,4S)-3,4-bis((2-hexyldecyl)oxy)tetrahydrofuran-2-yl)methyl 4-(dimethylamino)butanoate
- [00172] PNI 728: ((2R,3R,4S)-3,4-bis((2-hexyldecyl)oxy)tetrahydrofuran-2-yl)methyl 2-(dimethylamino)ethyl)carbamate
- [00173] PNI 762: ((2R,3R,4S)-3,4-bis((2-hexyldecyl)oxy)tetrahydrofuran-2-yl)methyl (2-(diethylamino)ethyl)carbamate
- [00174] RT: room temperature
- [00175] SARS-CoV-2: severe acute respiratory syndrome coronavirus 2
- [00176] sgRNA = single guide RNA
- [00177] TCR sgRNA: T cell receptor single guide RNA
- [00178] TTR sgRNA: Transthyretin single guide RNA from Integrated DNA Technologies (Coralville, IA) according to sequence (CCCAUACUCCUACAGCACCA)
- [00179] V02: lipid nanoparticle composition of ionizable lipid:structural lipid:sterol:stabilizing agent (47.5:12.5:38.5:1.5 mol%)
- [00180] V62: lipid nanoparticle composition of ionizable lipid:structural lipid:sterol:stabilizing agent (40:12.5:46:1.5 mol%)
- [00181] wt: Weight
- [00182] The following examples further illustrate the invention but, should not be construed in any way as limiting its scope.

EXAMPLE 1

- [00183] This example demonstrates an illustrative synthetic pathway to access lipid alcohol 6, which is needed for the synthesis of the stabilizing agents.



Scheme 1. Synthesis of compound 6

[00184] Step 1: Synthesis of compound 2

[00185] A 2000-mL two neck round bottom flask was charged with (2,2-dimethyl-1,3-dioxolan-4-yl) methanol (50.0 g, 0.378 mol) in THF (1000 mL) under argon atmosphere, 60% NaH (20.0 g, 0.833 mol) was added at 0 °C, and stirred for 30 min. (Bromomethyl)benzene (77.72 g, 0.454 mol) was added drop wise at the same temperature, and then stirred the reaction mixture at RT for 16 h and completion of the reaction was monitored by TLC. The reaction mixture was diluted with aqueous NH₄Cl (500 mL) and extracted with EtOAc (2 x 600 mL), washed with brine solution (80 mL) and the organic layer was dried over Na₂SO₄ and evaporated on the rotary evaporator to afford the compound 3 (90 g) as a crude product. The formation of the desired product (yellow liquid) was confirmed by ¹H NMR. ¹H NMR (CDCl₃) 400 MHz: δ ppm: 1.36 (s, 3H), 1.42 (s, 3H), 3.45-3.57(m, 2H), 3.72-3.76 (m, 1H), 4.04-4.07 (m, 1H), 4.30 (m, 1H, J = 6 Hz), 4.57 (d, 2H, J = 6.4 Hz), 7.29-7.35 (m, 5H).

[00186] Step 2: Synthesis of compound 3

[00187] To a 250-mL two neck round bottom flask, 4-((benzyloxy)methyl)-2,2-dimethyl-1,3-dioxolane (45.0 g, 202.44 mmol), water (62 mL), and acetic acid (250 mL) was added and stirred at RT for 16 h. The reaction progress was monitored by TLC. Then, the reaction mixture was concentrated on the rotary evaporator to afford the crude compound. The crude compound was purified by CombiFlash using 2.0% MeOH in DCM as eluent to obtain compound 4 (30.0 g, yield- 81.32%) as a brown liquid. The desired product was analyzed and confirmed by ¹H NMR. ¹H NMR (CDCl₃) 400 MHz: δ ppm: 3.56- 3.65 (m, 3H), 3.69-3.72 (dd, 1H, J1 = 11.4 Hz, J2 = 3.6Hz), 3.88-3.91 (m, 1H), 4.55 (br s, 2H), 7.30-7.36 (m, 5H).

[00188] Step 3: Synthesis of compound 4

[00189] A 1000-mL two neck round bottom flask was charged with 3-(benzyloxy) propane-1,2-diol (30.0 g, 164.83 mmol), dissolved in 450 mL of toluene under argon atmosphere at RT.

To this, potassium *t*-butoxide (74.0 g, 659 mmol) and bromo tetradecane (137.0 g, 494.0 mmol) were added and then the reaction mixture was kept at 110 °C and stirred for 12 h. The progress of the reaction was monitored by TLC. After completion, the reaction mixture was cooled to RT, concentrated, and toluene removed under reduced vacuum. To this, 100 mL of water and 100 mL of ethyl acetate were added, and the organic layer was separated. The aqueous layer was again washed with ethyl acetate (2x 250 mL). The organic layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The crude compound was purified by flash column chromatography by eluting with 14% EtOAc in hexane to get compound 5 (60.0 g) (yield, 63.8%) as an off-white solid. ¹H NMR (CDCl₃) 400 MHz: δ ppm: 0.88 (t, 6H, J = 6.8 Hz) 1.18-1.31 (m, 45H), 1.51-1.58(m, 4H), 3.42 (t, 2H, J = 6.4 Hz), 3.50-3.59 (m, 6H), 4.55 (br s, 2H), 7.28-7.34 (m, 5H).

[00190] Step 4: Synthesis of compound 5

[00191] A 500-mL parr vessel was charged with 2, 3-bis (tetradecyloxy) propan-1-ol (20 g, 34.782) in 250 mL of ethanol, 4.5 g of 10% Pd/C and the parr shaker was hydrogenated at 50 psi of hydrogen gas for 5 h. The progress of the reaction was monitored by TLC. The reaction mixture was filtered through the celite bed washed with 200 mL of ethanol. The filtrate was concentrated under reduced pressure to get compound 6 (15.0 g) (yield 91%) as a brown liquid. The obtained desired product was confirmed by ¹H NMR. ¹H NMR (CDCl₃) 400 MHz: δ ppm: 0.88 (t, 6H, J = 6.8 Hz) 1.18-1.31 (m, 46H), 1.51-1.60 (m, 4H), 3.42 (m, 1H), 3.43-3.59 (m, 6H), 3.61-3.63 (m, 3H).

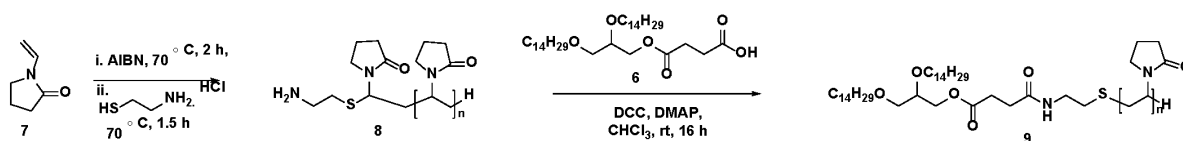
[00192] Step 5: Synthesis of compound 6

[00193] A 250-mL two neck RBF was charged with 2,3-bis(tetradecyloxy)propan-1-ol (5.0 g, 10.31 mmol) in DCM (100 ml), dihydrofuran-2,5-dione (2.064 g, 20.62 mmol) and 4-dimethylaminopyridine (2.52 g, 20.62 mmol) at RT and then the reaction mixture was stirred at RT for 16 h. After completion of the reaction, the reaction mixture was quenched with water, extracted with DCM (2x 100 mL) and washed with brine solution (20 mL). The DCM layer was dried over Na₂SO₄ and evaporated on the rotavapor to afford the crude product. Crude product was purified by combi flash by using 5-7% EtOAc in hexane as eluents to afford desired compound 6 as an off-white solid, which was confirmed by ¹H NMR. ¹H NMR (CDCl₃) 400 MHz: δ ppm: 0.880 (t, 6H, J = 7.2 Hz), 1.15-1.38 (m, 45 H), 1.48-1.59 (m, 4H), 2.60-2.72 (m,

4H), 3.38-3.50 (m, 4H), 3.54 (t, 2H, $J = 6.4$ Hz), 3.58-3.65 (m, 1H), 4.13 (dd, 1H, $J_1 = 11.6$ Hz, $J_2 = 4.0$ Hz), 4.26 (dd, 1H, $J_1 = 11.6$ Hz, $J_2 = 4.0$ Hz).

EXAMPLE 2

[00194] This example demonstrates an illustrative synthetic pathway to access stabilizing agents SAF 29 and 34

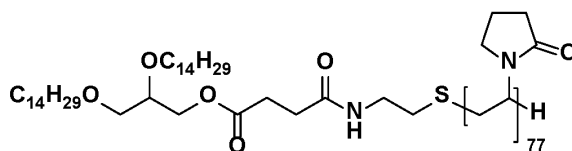


Scheme 2. General synthetic procedures of SAF 29, and 34

[00195] Synthesis of Compound 8

[00196] A 50 mL two neck RBF was charged with vinylpyrrolidone (1.7 mL, 15.2 mmol) under nitrogen atmosphere in dry 1,4-dioxane (20 mL) and then recrystallized azobisisobutyronitrile (AIBN; 67 mg, 0.027 mol to monomer) was added and stirred for 2 h at 70 °C. Next, 2-aminoethanethiol hydrochloride (cysteamine hydrochloride) (60 mg, 0.035 mol to monomer) was added to the system as chain transferring agent and the reaction mixture was stirred for another 1.5 h at 70 °C. Solvent was then evaporated under reduced pressure and crude polymer was dissolved in minimum volume of DCM and then precipitated three times in cold diethyl ether to get 1.2 g (yield, 70.5%) of compound 8 as a brown solid. ^1H NMR shows a trace of monomer impurity. ^1H NMR (CDCl_3) 400 MHz: δ ppm: 4.0- 3.60 (br S, 1H, $-\text{CH}-\text{NCO}$, polymer backbone), 3.0-3.40 (2H, $-\text{CH}_2-\text{NCO}-$), 2.17- 2.50 (2H, $-\text{NCOCH}_2-$), 1.32-2.17 ($-\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CO}-$, $-\text{CH}_2-\text{CH}-\text{NCO}-$ (polymer backbone)). GPC analysis showed a monomodal peak in the chromatogram (retention time at 7.80 min). This polymer was used for the next step without any further purification.

SAF 29

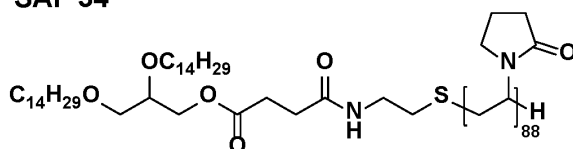


[00197]

[00198] 4-(2,3-bis(tetradecyloxy)propoxy)-4-oxobutanoic acid (284 mg, 0.48 mmol) was dissolved in chloroform (6 mL) and its carboxyl group was activated by DCC (80 mg, 0.38 mmol) for 1 h at room temperature. Amine terminated-PVP (650 mg, 0.32 mmol) was dissolved

in chloroform (4 mL) and added to the above lipid reaction mixture and stirred at RT for 16 h. The solvent was evaporated under reduced pressure and the obtained residue was dissolved in DCM to precipitate out the urea by-product. The by-product urea was removed by filtration. The obtained DCM solution was concentrated and precipitated in cold diethyl ether to get a brown solid as a precipitate. DMAP catalyst was removed by dissolving polymer solution in DCM and washing with diluted HCl. The organic layer was dried on sodium sulfate and concentrated using a rotary evaporator to get a brown solid. The desired product was formed (brown solid) and confirmed by ^1H NMR and GPC. Yield: 260 mg (31.3%). ^1H NMR (400 MHz, MeOD) δ 0.80 (t, 6H), 1.2 (m, 50H), 1.4 (br, 37H), 1.7 (br, 99H), 2 (br, 131H), 3.2 (br, 127H), 3.5 (br, 36H), 3.8 (br, 70H). The molecular weight determined from ^1H NMR using end group analysis was 9204 Da. The retention time determined by GPC (Shodex KD-802 300 by 8.0 mm, Mobile phase: 0.1 mM LiBr in DMF, 60 °C, concentration of the sample: 10 mg/mL) was 7.8 min.

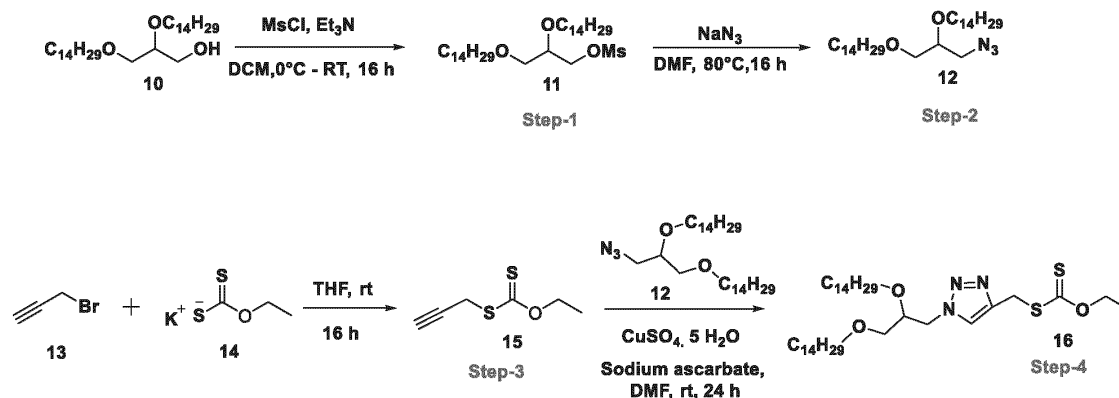
SAF 34



[00199] 4-(2,3-bis(tetradecyloxy)propoxy)-4-oxobutanoic acid (420 mg, 0.71 mmol) was dissolved in chloroform (6 mL) and its carboxyl group was activated by DCC (148 mg, 0.71 mmol) for 1 h at room temperature. Amine terminated-PVP (600 mg, 0.60 mmol) was dissolved in chloroform (4 mL). Then, it was added to the above lipid reaction mixture and stirred at RT for 16 h. The solvent was evaporated under reduced pressure and the by-product urea was removed using filtration. The obtained DCM solution was concentrated and precipitated 2-3 times in cold diethyl ether to get a brown solid as a precipitate. DMAP catalyst was removed by dissolving polymer solution in DCM and washing with diluted HCl and concentrated to get a brown solid. The desired product was formed (brown solid) and confirmed by ^1H NMR and GPC. Yield: 150 mg (15.7%). ^1H NMR (400 MHz, MeOD) δ 0.80 (t, 6H), 1.1 (br, 10H), 1.3 (br, 51H), 1.4 (br, 42H), 1.7 (br, 116H), 2 (br, 146H), 2.4 (br, 150H), 3.1 (br, 158H), 3.5 (br, 32H), 3.9 (br, 72H), 5.3 (m, 10H), 6.7 (br, 2H), 8.2 (br, 1H). The molecular weight determined from ^1H NMR using end group analysis was 10425 Da. The retention time determined by GPC (Shodex™ KD-802 300 by 8.0 mm, Mobile phase: 0.1 mM LiBr in DMF, 60 °C, concentration of the sample: 10 mg/mL) was 7.8 min.

EXAMPLE 3

[00200] This example demonstrates an illustrative synthetic pathway synthesis of the thiol lipid initiator 7, which is needed for the synthesis of SAF102, 103 and 104.



[00201] Step-1: Synthesis of compound 11

[00202] A 250 mL two neck round bottom flask charged with 2,3-bis(tetradecyloxy)propan-1-ol (5.0 g, 10.31 mmol) in DCM (100.0 mL), triethylamine (5.8 mL, 41.24 mmol) was added followed by mesyl chloride (1.2 mL, 15.46 mmol) were added at 0 °C, the reaction mixture was then stirred at RT for 16 h. After the completion of the reaction, reaction mixture was cooled to 0 °C, quenched with aqueous NaHCO₃ solution and extracted with DCM (2x 100 mL) The organic layer was washed with water and brine solution and then evaporated on the rotary evaporator to afford the crude compound (5.7 g, crude), which was confirmed by ¹H NMR and used in the next step without further purification. ¹H NMR (CDCl₃) 400 MHz: δ ppm: 0.88 (t, 6H, J=7.2 Hz), 1.18 (s, 2H), 1.20-1.38 (m, 53 H), 1.48-1.58 (m, 3 H), 3.04 (s, 3H), 3.38-3.45 (m, 2H), 3.46-3.52 (m, 2H), 3.56 (t, 2H, J = 6.4 Hz), 3.62-3.70 (m, 1H), 4.25(dd, 1H, J1 = 11.2 HZ, J2= 6.0 Hz), 4.39 (dd, 1H, J1 = 10.8 HZ, J2= 3.6 Hz)

[00203] Step-2: Synthesis of compound 12

[00204] A 250 mL two neck round bottom flask was charged with 2,3-bis(tetradecyloxy)propyl methanesulfonate (5.7 g, 10.12 mmol) in DMF (100.0 mL), sodium azide (0.724 g, 11.13 mmol) was added at RT, and then the reaction mixture was stirred at 80 °C for 16 h. The completion of the reaction was monitored by TLC. The reaction mixture was cooled to 0 °C, quenched with ice cold water and extracted with EtOAc (2x 200 mL) The organic layer was washed with water and brine solution and then evaporated on the rotary evaporator to

afford the crude compound. This crude mixture was purified using Combi-Flash column chromatography using 5% EtOAc in hexanes as eluents to obtain 4.0 g (yield 77 %) of desired compound 3 as a light brown color liquid. ¹H NMR (CDCl₃) 400 MHz: δ ppm: 0.87 (t, 6H, J = 7.2 Hz), 1.18 (s, 2H), 1.20-1.38 (m, 48 H), 1.50-1.60 (m, 6H), 3.28-3.36 (m, 2H), 3.42 (t, 3H, J = 6.8 Hz), 3.46-3.62 (m, 4H).

[00205] Step-3. Synthesis of compound 15

[00206] A 50 mL two neck RB flask, charged with potassium ethyl xanthogenate (1.00 g, 6.23 mmol) and dissolved in 10 mL dry THF. The solution was cooled to 0 °C using ice bath and propargyl bromide (1.01 g, 8.54 mmol) was added drop by drop and then the reaction mixture solution was stirred at room temperature overnight. Reaction completion was monitored by TLC. After 16 h completion of reaction, the crude reaction mixture was analyzed by using ¹H NMR. The crude reaction mixture was filtered to remove the KBr salt and the solvent evaporated under reduced pressure to get a dark red color solution. This crude mixture was purified using Combi-Flash column chromatography using hexane as eluent to obtain 900 mg (yield 90%) of desired compound 6 as a light-yellow color liquid. ¹H NMR (CDCl₃) 400 MHz: δ ppm: 1.43 (t, 3H, J = 7.6 Hz), 2.23 (t, 1H, J = 2.4 Hz), 3.87 (d, 2H, J = 2.8 Hz), 4.67 (q, 4H, J = 6.8 Hz).

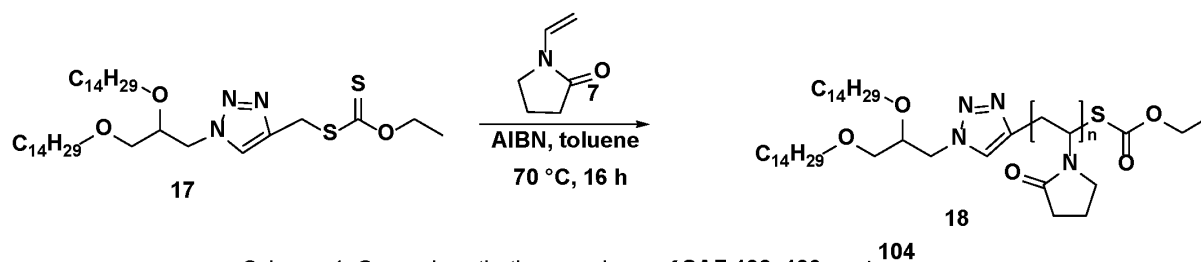
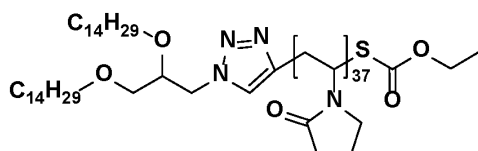
[00207] Step-4: Synthesis of compound 16

[00208] A 25 mL Schlenk tube, charged with O-ethyl-S-(prop-2-yn-1-yl) carbonodithioate (200 mg, 1.25 mmol), 1-(3-azido-2-(tetradecyloxy)propoxy)tetradecane (609 mg, 1.19 mmol) and sodium ascorbate (61.8 mg, 0.311 mmol) was dissolved in 2 ml of DMF. Then, CuSO₄·5H₂O (19 mg, 0.062 mmol) was added under an argon atmosphere. The reaction mixture turned a brown color and was stirred for 24 h. The reaction was kept under continuous argon positive pressure. After 24 h, the reaction mixture solvent was evaporated under reduced pressure to get crude reaction mixture. Crude reaction mixture was dissolved in dichloromethane and washed with brine solution and then the organic layer was evaporated under reduced pressure. The crude compound was then purified by combi flash column chromatography. The compound was eluted using hexane followed by 20 % EtOAc in hexanes as eluent to afford the desired compound 7 (350 mg, yield 41.8%) as a colorless liquid. ¹H NMR (CDCl₃) 400 MHz, δ ppm: 0.880 (t, 6H, J = 7.2 Hz), 1.15-1.38 (m, 45 H), 1.39-1.50 (m, 5H), 3.25 (q, 1H, J = 13.2 Hz), 3.39-3.54 (m, 5H), 4.32-4.35 (dd, 1H, J₁ = 14.0 Hz, J₂ = 4.0 Hz), 4.48-4.60 (dd, 1H, J = 14 Hz), 4.623-4.67(q, 2H, J = 14.4 Hz). HPLC (ELSD): 99 %.

EXAMPLE 4

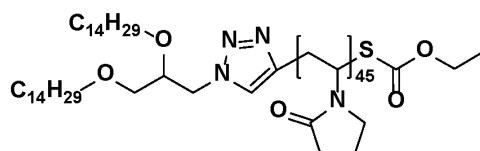
[00209] This example demonstrates an illustrative synthetic pathway for the synthesis of SAF102, SAF103 and SAF104.

[00210] Synthesis of SAF102, SAF103 and SAF104:

**SAF 102**

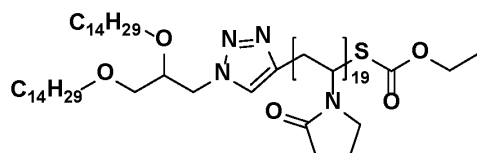
[00211] A 30 mL Schlenk tube, charged with S-((1-(2,3-bis(tetradecyloxy)propyl)-1H-1,2,3-triazol-4-yl)methyl) O-ethyl carbonodithioate (480 mg, 0.716 mmol) RAFT initiator, 1-vinyl-2-pyrrolidinone (1227 mg, 11.04 mmol) and recrystallized AIBN (82 mg, 0.499 mmol) were dissolved in 3 mL of dry toluene. The solution was then degassed by purging with argon for 30 min and then heated under argon atmosphere with a preheated oil bath at 70 °C for 21 h. The reaction was stopped after 21 h. The crude reaction mixture solvent was evaporated under reduced pressure and dissolved in minimal DCM solvent and precipitated in cold ether. The precipitation was repeated three times to remove the monomer impurity. The desired product was formed and confirmed by ¹H NMR. Yield: 900 mg. (46.15 %). ¹H NMR (400 MHz, MeOD) δ 0.80 (t, 6H), 1.3 (m, 73H), 1.7 (br, 54H), 2.3 (br, 175H), 3.2 (br, 82H), 3.9 (br, 38H), 4.3 (br, 2H), 4.6 (br, 2H). The molecular weight determined from ¹H NMR using end group analysis was 4873 Da. The retention time determined by GPC (Shodex™ KD-802 300 by 8.0 mm, Mobile phase: 0.1 mM LiBr in DMF, 60 °C, concentration of the sample: 10 mg/mL) was 9.3 min.

SAF 103



[00212] A 30 mL Schlenk tube, charged with S-((1-(2,3-bis(tetradecyloxy)propyl)-1H-1,2,3-triazol-4-yl)methyl) O-ethyl carbonodithioate (120 mg, 0.179 mmol) RAFT initiator, 1-vinyl-2-pyrrolidinone (409 mg, 3.68 mmol), and recrystallized AIBN (20.6 mg, 0.125 mmol) in 4 mL of dry toluene. The solution was degassed by purging with argon for 30 min then heated under argon atmosphere with a preheated oil bath at 70 °C for 16 h. The reaction was stopped after 12 h. Crude ¹H NMR spectrum showed still unreacted monomer present in the reaction mixture after 12 h. The solvent was evaporated under reduced pressure and dissolved in a minimal amount of DCM solvent and precipitated in cold ether. The precipitation was repeated three times to remove the monomer impurity. The desired product was formed and confirmed by ¹H NMR. Yield: 170 mg. (34.9 %). ¹H NMR (400 MHz, MeOD) δ 0.80 (t, 6H), 1.5 (m, 154H), 2.3 (br, 256H), 3.3 (br, 106H), 3.8 (br, 46H), 4.3 (br, 1H), 4.6 (br, 2H). The molecular weight as determined from ¹H NMR using end group analysis was 5761 Da. The retention time determined by GPC (Shodex™ KD-802 300 by 8.0 mm, Mobile phase: 0.1 mM LiBr in DMF, 60 °C, concentration of the sample: 10 mg/mL) was 9.1 min.

SAF 104



[00213] A 30 mL Schlenk tube, charged with S-((1-(2,3-bis(tetradecyloxy)propyl)-1H-1,2,3-triazol-4-yl)methyl) O-ethyl carbonodithioate (400 mg, 0.716 mmol) RAFT initiator, 1-vinyl-2-pyrrolidinone (682 mg, 6.13 mmol), and recrystallized AIBN (69 mg, 0.419 mmol) in 3 mL of dry toluene. The solution was degassed by purging with argon for 30 min then heated under argon atmosphere with a preheated oil bath at 70 °C for 21 h. The reaction was stopped after 21 h. The crude reaction mixture solvent was evaporated under reduced pressure and dissolved in a minimal amount of DCM solvent and precipitated in cold ether. This was repeated three times to remove the monomer impurity. The desired product was formed and confirmed by ¹H NMR.

Yield: 280 mg. (28.34 %). ^1H NMR (400 MHz, MeOD) δ 0.80 (t, 6H), 1.3 (m, 56H), 1.9 (br, 88H), 2.4 (br, 44H), 3.3 (br, 48H), 4 (br, 20H), 4.6 (br, 2H). The molecular weight as determined from ^1H NMR using end group analysis was 2875 Da. The retention time determined by GPC (Shodex KD-802 300 by 8.0 mm, Mobile phase: 0.1 mM LiBr in DMF, 60 °C, concentration of the sample: 10 mg/mL) was 9.689 min.

[00214] The rest of the stabilizing agents with different monomer units varying $n = 3$ to 1000 can be synthesized by altering the ratios of monomer units and initiator.

EXAMPLE 5

[00215] This example demonstrates an illustrative manner of preparing the lipid nanoparticles (LNP).

[00216] Components of the lipid nanoparticle composition including an ionizable lipid, structural lipid, sterol and stabilizing agent were mixed together in different molar ratios. Stabilizing agent means any agent including PEG-DMG or a polyvinylpyrrolidone stabilizing agent to stabilize the LNP formation. Lipid nanoparticle compositions were prepared in ethanol by combining prescribed amounts of lipids from individual lipid stocks in ethanol. LNPs were then prepared by running the lipid nanoparticle composition and nucleic acid through a NanoAssemblr® Ignite™ microfluidic mixer.

[00217] Lipid nanoparticles (LNPs) formulations were produced by mixing the lipid nanoparticle composition (PNI 516-V02; lipid concentration: 25 mM) with the nucleic acid (*e.g.*, mRNA and saRNA) solution (100 mM acetate buffer; pH 4) via a microfluidic mixer from the NanoAssemblr® platform at an aqueous:organic solution ratio of 3:1 vol. The formulations were diluted with 25x PBS followed by storing at 4 °C for 30 min. To purify LNPs, formulations were centrifuged utilizing UF Amicon tubes at 2200 x g for 45 min. EPO mRNA LNPs were stored at 4 °C until use. A5 saRNA LNPs were frozen at -80 °C until use.

[00218] The mixing of nucleic acids and lipid nanoparticle compositions occurred as follows. Ionizable lipids, structural lipids, sterol, and a stabilizing agent were mixed at a molar ratio of either V02 or V62 in 100% ethanol. The aqueous phase was prepared by diluting nucleic acids such as mRNA/saRNA/pDNA solutions in 100 mM sodium acetate buffer (pH 4). The solutions were combined using the NanoAssemblr® Ignite™ with an Ignite™ NxGen™ (DVBM) cartridge (Precision Nanosystems) at a flow ratio of 3:1 (aqueous phase: organic phase) at N/P

ratio of 8 or 10, unless otherwise noted, with a total flow rate of 12 mL/min. The resulting LNPs were diluted 25-40 times in 1x PBS (pH 7.4) and the mixture was subjected to downstream processing. Downstream processing included ethanol removal through dialysis in PBS (pH 7), or using Amicon™ centrifugal filters (Millipore, USA) at 2500 RPM, or using tangential flow filtration systems. Particles were concentrated to required target dose. This example demonstrated the preparation of the lipid nanoparticles (LNP) for testing.

EXAMPLE 6

[00219] This example demonstrates illustrative methods used to determine the size, polydispersity index (PDI), and encapsulation efficiency (EE) of the lipid nanoparticles (LNP).

[00220] Size and PDI of the LNPs was measured by Dynamic Light Scattering (DLS) using a ZetaSizer™ Nano ZS™ (Malvern Instruments). He/Ne laser of 633 nm wavelength was used as the light source. Data were measured from the scattered intensity data conducted in backscattering detection mode (measurement angle = 173°). 0.5 to 2 µL of the sample was placed in a cuvette and diluted with PBS (0.3 mL). Measurements were an average of 10 runs of two cycles each per sample. Z -Average size was reported as the particle size and is defined as the harmonic intensity averaged particle diameter. EE of the LNPs was measured by Quanti-iT™ RiboGreen® RNA reagent.

[00221] Size, PDI and EE of lipid nanoparticles with polyvinylpyrrolidone stabilizing agents and different payloads were measured and shown in Table 2. The lipid nanoparticle composition used for EPO mRNA and A5 saRNA studies include PNI 516-V02 (lipid concentration: 25 mM). The lipid nanoparticle composition used for the TTR sgRNA/Cas9 mRNA study include PNI 516-V62 (lipid concentration: 25 mM). The N/P ratio used for EPO mRNA and A5 saRNA formulations was 8. The N/P ratio used for TTR sgRNA/Cas9 mRNA formulation was 6. The sgRNA:Cas9 mRNA was 1:1 by wt. The LNP size was measured by dynamic light scattering (DLS) using a ZetaSizer™ Nano ZS™ (Malvern Instruments, UK). The EE measurements were determined by a modified Ribogreen™ assay (Quanti-iT RiboGreen™ RNA assay kit, Thermo Fisher).

[00222] **Table 2.** Physiochemical properties and encapsulation efficiency (EE%) of LNPs formulated by varying different PVP stabilizing agents in V02 using PNI 516 and PNI 580 for various nucleic acid payloads.

Table 2

#Stabilizing agent	Composition	Nucleic acid	Size (nm)	PDI	EE (%)
SAF29	PNI 516-V02	EPO mRNA	110.5	0.04	97.5
		A5 saRNA	132.1	0.02	94.5
	PNI 580-V62	TTR gRNA-Cas9 mRNA	164.8	0.04	93.8
		PCSK9 gRNA-Cas9 mRNA	128.4	0.05	87.9
SAF34	PNI 516-V02	EPO mRNA	85.3	0.04	97.5
		A5 saRNA	114.3	0.04	93.8
	PNI 580-V62	TTR gRNA-Cas9 mRNA	123.1	0.03	92.1
SAF102	PNI 516-V02	EPO mRNA	86.1	0.08	98.6
	PNI 516-V62	A5 saRNA	87.7	0.07	99.2
SAF103	PNI 516-V02	EPO mRNA	78.8	0.06	99.4
	PNI 516-V62	A5 saRNA	74.7	0.07	99.4
SAF104	PNI 516-V02	EPO mRNA	92.0	0.06	98.6
	PNI 516-V62	A5 saRNA	91.6	0.05	99.1
SAF29	PNI 516-V01	FLuc mRNA	144.8	0.03	98.2
	PNI 516-CT10	FLuc mRNA	100.6	0.04	98.7
	PNI 516-V02	FLuc mRNA	134.2	0.03	97.0
SAF34	PNI 516-V01	FLuc mRNA	146.2	0.05	98.8
	PNI 516-CT10	FLuc mRNA	61.5	0.10	96.2
	PNI 516-V02	FLuc mRNA	109.6	0.05	100.3
SAF150	PNI 659 - V135	GFP mRNA	79	0.166	96
	PNI 728 - V135	GFP mRNA	87	0.182	96
	PNI 762 - V135	GFP mRNA	129	0.060	98
	PNI 659 - V142	GFP mRNA	102	0.226	94
	PNI 728 - V142	GFP mRNA	84	0.314	95
	PNI 762 - V142	GFP mRNA	114	0.149	93
	PNI 659 - V144	GFP mRNA	91	0.129	99
	PNI 728 - V144	GFP mRNA	112	0.209	96
	PNI 762 - V144	GFP mRNA	131	0.220	96
SAF167	PNI 659 - V142	GFP mRNA	94	0.232	96
	PNI 728 - V142	GFP mRNA	106	0.234	93
	PNI 762 - V142	GFP mRNA	101	0.250	96
	PNI 659 - V143	GFP mRNA	123	0.314	97

	PNI 728 - V143	GFP mRNA	113	0.307	97
	PNI 762 - V143	GFP mRNA	179	0.243	96
	PNI 659 - V148	GFP mRNA	106	0.165	96
	PNI 728 - V148	GFP mRNA	115	0.200	96
	PNI 762 - V148	GFP mRNA	117	0.137	95

Table 3. Lipid Nanoparticle Compositions

Composition	Ratios
CT10	40 mol% iL/ 20 mol% DSPC/ 37.5 mol% Chol/ 2.5 mol% Stabilizing agent
V01	47.5 mol% iL/ 12.5 mol% DOPE/ 37.5 mol% Chol/1.5 mol% Stabilizing agent
V02	47.5 mol% iL/12.5 mol% DSPC/38.5 mol% Chol/1.5 mol% Stabilizing agent
V62	40 mol% iL/12.5 mol% DSPC/46 mol% Chol/1.5 mol% Stabilizing agent
V135	17.37 mol% iL / 41.11 mol % DSPC /41.11 mol % Chol /0.41 mol % Stabilizing agent
V138	24.32 mol% iL / 36.49 mol % DSPC /36.49 mol % Chol /2.7 mol % Stabilizing agent
V139	24.46 mol% iL / 57.88 mol % DSPC /15.51 mol % Chol /2.14 mol % Stabilizing agent
V140	24.46 mol% iL / 15.51 mol % DSPC /57.88 mol % Chol /2.14 mol % Stabilizing agent
V141	24.7 mol% iL/ 36.7 mol% DSPC/ 36.7 mol% Chol/ 1.4 mol% Stabilizing agent
V142	24.85 mol% iL / 58.81 mol % DSPC /15.76 mol % Chol /0.58 mol % Stabilizing agent
V143	24.85 mol% iL / 15.76 mol % DSPC /58.81 mol % Chol /0.58 mol % Stabilizing agent
V146	39.13 mol% iL / 58.7 mol % DSPC /0 mol % Chol /2.17 mol % Stabilizing agent
V148	43.64 mol% iL / 27.67 mol % DSPC /27.67 mol % Chol /1.02 mol % Stabilizing agent

EXAMPLE 7

[00223] This example demonstrates an illustrative protocol for Luciferase quantification.

[00224] HEK 293 cells were seeded on 96-well plate at 12,000 cells/well (100 μ L/well) for 24 h. The cells were treated with Fluc mRNA LNPs (dose: 25 ng/well) for 24 h. Afterwards, cell viability was quantified using ONE-Glo+Tox assay according to the manufacturer protocol (Fischer Sci, Catalogue no. PRE7110). SAF 29 and 34 showed higher cell viability and potent Luciferase protein expression *in vitro* (both HEK 293 and T-cells) which is significantly higher than for PEG-DMG containing LNPs (**FIGS. 1A-1B**). This example demonstrated the Luciferase quantification protocol.

EXAMPLE 8

[00225] This example demonstrates an illustrative procedure used for the erythropoietin (EPO) expression evaluation of EPO-expressing LNPs *in vivo*.

[00226] LNPs were intravenously injected into mice (6-week old female C57BL/6 mice) at a single dose of 0.25 mg/kg. The sera samples were collected 6 h post-injection via the tail nick method. For serum preparation, after collection of the whole blood, the blood was allowed to clot by leaving the collection tube at room temperature for 15-30 minutes. The clot was removed by centrifuging the tubes at 1000-2000 x g for 10 min at 4 °C. The clear golden-yellow color supernatant was carefully removed and transferred to a sterile screw-capped clear polypropylene tube on ice. The serum was then stored at -80 °C until further use. The terminal blood collection was performed 24 h post injection. The Erythropoietin (EPO) protein level in sera samples was determined using the Ella kit (ProteinSimple™, Catalog # SPCKB-PS-000487). The LNPs containing SAF 29, 34, 103, and 104 showed potent EPO expression in mice (**FIGS. 2A-2B**) post 24 h IV administration. These PVP based stabilizing agents showed more potent hEPO expression in mice compared to traditional LNPs with PEG-DMG stabilizing agents. SAF 29, 34, 103, and 104 containing LNPs showed more potent EPO expression in mice compared to PEG-DMG *in vivo*.

EXAMPLE 9

[00227] This example demonstrates an illustrative procedure used for the SARS-CoV-2 expression evaluation of SARS-CoV-2 expressing A5 PNI saRNA-LNPs *in vivo*.

[00228] LNPs were intramuscularly injected into groups of four mice (N =4, 6-week old male BALB/c mice) at a prime dose of 0.05 mg/kg (1 µg/50 µL/20-g mouse) on Day 0 and a booster dose of 0.05 mg/kg (1 µg/50 µL/20-g mouse) on Day 28 (7 days after the first sera collection). The sera samples were collected on Day-21 and Day-42 post-prime injection. For serum preparation, after collection of the whole blood, the blood was allowed to clot by leaving the collection tube at room temperature for 15-30 minutes. The clot was removed by centrifuging the tubes at 1000-2000 x g for 10 min at 4 °C. The clear golden-yellow color supernatant was carefully removed and transferred to a sterile screw-capped clear polypropylene tube on ice. The serum is then stored at -80 °C until further use. The SARS-CoV-2 antigen specific IgG level in sera was determined using enzyme-linked immunoassay (ELISA) assay. SAF 29 and 34 containing LNPs induced the potent expression of SARS-CoV-2 spike protein specific IgGs titers in mice (**FIGS. 3A-3B**) along with booster response. Further, these SAF containing LNPs showed improved immune response in mice compared to PEG-DMG containing LNPs

(PEGylated LNPs). SAF 29 and 34 containing LNPs induced the expression of SARS-CoV-2 spike protein specific IgGs and booster response was observed. This example demonstrated the procedure used for the SARS-CoV-2 expression evaluation of SARS-CoV-2 expressing A5 PNI saRNA-LNPs *in vivo*.

EXAMPLE 10

[00229] This example illustrates the physio-chemical properties of selected compositions. Size and PDI of the LNPs were measured by Dynamic Light Scattering (DLS) using a ZetaSizer™ Nano ZS™ (Malvern Instruments). He/Ne laser of 633 nm wavelength was used as the light source. Data were measured from the scattered intensity data conducted in backscattering detection mode (measurement angle = 173°). Measurements were an average of 10 runs of two cycles each per sample. Z - Average size was reported as the particle size and is defined as the harmonic intensity averaged particle diameter. Encapsulation efficiency (EE) of the LNPs was measured by Quant-iT™ RiboGreen® RNA reagent.

[00230] **Table 4.** Attributes of various LNPs formed based on polyvinylpyrrolidone-based stabilizers.

Stabilizer	Payload	MW (Da)	Composition (% is mol%)	Size		PDI		EE (%)	
				Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
PEG-DMG	GFP mRNA	2509	40% PNI 762, 20% DSPC, 37.5% cholesterol, 2.5% stabilizer	57.23	56.84	0.18	0.1473	98.7	99.0
PEG-DMG	TCR sgRNA/Cas 9 mRNA	2509	40% PNI 762, 20% DSPC, 37.5% cholesterol, 2.5% stabilizer	63.1	65.3	0.16	0.11	96.5	98.50
PEG-DMG	Fluc mRNA	2509	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	71.5	68.6	0.10	0.18	100.0	99.6
SAF29	Fluc mRNA	9204	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	116.0	115.1	0.06	0.01	98.9	99.2

SAF103	Fluc mRNA	5640	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	78.0	75.7	0.08	0.09	99.2	99.0
SAF123	A5 saRNA	6705	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	228.4	97.7	0.23	0.19	99.0	99.1
SAF124	A5 saRNA	9521	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	287.0	81.0	0.29	0.19	94.2	93.8
SAF125	A5 saRNA	4860	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	79.1	76.2	0.05	0.08	99.0	99.0
SAF127	A5 saRNA	4374	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	83.3	75.9	0.19	0.24	99.2	99.2
SAF133	A5 saRNA	1116 2	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	87.2	85.2	0.15	0.06	97.2	97.2
SAF134	A5 saRNA	7161	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	75.6	73.3	0.06	0.08	97.7	97.5
SAF135	A5 saRNA	6717	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	74.2	71.7	0.11	0.11	98.1	98.0
SAF136	A5 saRNA	9119	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	134.0	129.9	0.16	0.04	99.4	99.4
SAF137	A5 saRNA	1423 1	40% PNI 516, 12.5% DSPC, 46%	105.8	94.5	0.16	0.29	99.0	99.0

			cholesterol, 1.5% stabilizer						
SAF138	A5 saRNA	1023 0	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	122.0	117.5	0.08	0.05	99.3	99.3
SAF139	A5 saRNA	1367 5	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	110.0	108.3	0.05	0.03	99.1	99.1
SAF149	A5 saRNA	4000	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	123.5	121.4	0.05559	0.03464	99.26 5	100.37
SAF150	A5 saRNA	9670	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	90.66 5	88.75	0.1069	0.02801	98.41	98.195
SAF154	A5 saRNA	2123 3	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	118.1 5	115.85	0.04626 5	0.02506 5	99.18	101.08 5
SAF178	A5 saRNA	1160 3	40% PNI 516, 12.5% DSPC, 46% cholesterol, 1.5% stabilizer	189.7	183.15	0.04354 5	0.02979 7	94.61 5	93.1
SAF190	GFP mRNA	4695	40% PNI 762, 20% DSPC, 37.5% cholesterol, 2.5% stabilizer	69.89	68.41	0.09692	0.05263	98.6	98.7
SAF190	TCR sgRNA/Cas 9 mRNA	4695	40% PNI 762, 20% DSPC, 37.5% cholesterol, 2.5% stabilizer	74.9	76.8	0.10	0.15	97.8	98.20
SAF191	GFP mRNA	2920	40% PNI 762, 20% DSPC, 37.5% cholesterol, 2.5% stabilizer	83.01	80.3	0.07944	0.1089	99.4	99.5

SAF191	TCR sgRNA/Cas 9 mRNA	2920	40% PNI 762, 20% DSPC, 37.5% cholesterol, 2.5% stabilizer	122.3	125.9	0.08	0.10	98.8	97.80
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EXAMPLE 11

[00231] This example illustrates the T cell knock out studies performed *ex vivo*.

Cryopreserved primary human pan T cells were thawed, activated using CD3/CD28/CD2 T cell activator and cultured (96 well-plate, 0.2 mL/well, 0.125 million cell/well) for 3 days in the incubator (37 °C, 95% humidity and 5% CO₂). Afterwards, cells were treated with the TCR sgRNA/cas9 mRNA loaded LNPs (4 µg/well) for 24 h followed by washing for activator removal. The washed cells were kept in the complete media (IL-2 containing T cell expansion media) for 3 days in the incubator followed by staining and flow cytometry analysis. For cell staining, cells were stained with Anti-CD3 (PE conjugate), Anti-TCR α/β (FITC conjugate) and FVS660 dye (APC conjugate) according to the manufacturer's protocols. The number of live cells and TCR α/β + cells were determined using CytExpert software and the T cell receptor (TCR) knockout efficiency (%) of the LNP samples was calculated and shown in **FIG. 4**.

EXAMPLE 12

[00232] This example illustrates the *in vitro* studies of GFP expression. BHK cells were cultured and seeded (96 well-plate, 0.1 mL/well, 12,000 cell/well). Afterwards, cells were treated with the GFP mRNA loaded LNPs for 24 h followed by analysis by fluorescence microscopy. The results are shown in **FIG. 5**.

EXAMPLE 13

[00233] This example illustrates the T cell GFP *ex vivo* expression studies. Cryopreserved primary human pan T cells were thawed, activated using CD3/CD28/CD2 T cell activator and cultured (96 well-plate, 0.2 mL/well, 0.125 million cell/well) for 3 days in the incubator (37 °C, 95% humidity and 5% CO₂). Afterwards, cells were treated with the GFP mRNA loaded LNPs (4 µg/well) for 24 h followed by staining and flow cytometry analysis. For cell staining, cells were stained with FVS660 dye (BD Biosciences) according to the manufacturer's protocols. The data were interpreted using CytExpert software and the transfection efficiency (TE) % and mean fluorescence intensity (MFI) of the LNP samples were calculated and shown in **FIGS. 6A-6B**.

EXAMPLE 14

[00234] This example illustrates the *in vivo* vaccine studies. This study describes the procedure used for the SARS-CoV-2 expression evaluation of SARS-CoV-2 expressing A5 PNI saRNA-LNPs *in vivo*. LNPs were intramuscularly injected into mice (6-week old male BALB/c mice) at a prime dose of 0.05 mg/kg (1 µg/20-g mouse) on Day 0 and a booster dose of 0.05 mg/kg (1 µg/20-g mouse) on Day 28 (7 days after the first sera collection). The sera samples were collected 21 and 42 days post-prime injection. For serum preparation, after collection of the whole blood, the blood was allowed to clot by leaving the collection tube at room temperature for 15-30 minutes. The clot was removed by centrifuging the tubes at 1000-2000 x g for 10 min at 4 °C. The clear golden-yellow color supernatant was carefully removed and transferred to a sterile screw-capped clear polypropylene tube on ice. The serum was then stored at – 80 °C until further use. The SARS-CoV-2 antigen specific IgG level in sera was determined using enzyme-linked immunoassay (ELISA) assay and shown in **FIGS. 7A-7B**.

EXAMPLE 15

[00235] This example illustrates the *in vivo* subcutaneous studies. This study describes the procedure used for the erythropoietin (EPO) expression evaluation of EPO-expressing LNPs *in vivo*. LNPs were subcutaneously injected into mice (6-week old female C57 BL6 mice) at a single dose of 0.25 mg/kg. The sera samples were collected 6 h post-injection via the tail nick method. For serum preparation, after collection of the whole blood, the blood was allowed to clot by leaving the collection tube at room temperature for 15-30 minutes. The clot was removed by centrifuging the tubes at 1000-2000 x g for 10 min at 4 °C. The clear golden-yellow color supernatant was carefully removed and transferred to a sterile screw-capped clear polypropylene tube on ice. The serum was then stored at -80 °C until further use. The terminal blood collection was performed 24 h post injection. The Erythropoietin (EPO) protein level in sera samples was determined using the Ella kit (ProteinSimple, Catalog # SPCKB-PS-000487) and shown in **FIG. 8**.

EXAMPLE 16

[00236] This example illustrates the *in vivo* biodistribution (BD) studies. This study describes the procedure used for the firefly-luciferase (Fluc) expression evaluation of Fluc-expressing LNPs *in vivo*. LNPs were intravenously injected into mice (6-week old female Hsd:IRC mice) at

a single dose of 0.1 mg/kg. Four hours after administration, mice were given an intraperitoneal injection of D-luciferin substrate solution (150 mg/kg, 200 μ L/20g) followed by anesthetizing with isoflurane and imaged at 12 minutes post-administration with the exposure time of 30 s. Post-imaging, blood and organs (liver, lung, spleen, kidney, heart) were collected, rinsed with PBS, and imaged *ex vivo* with exposure times of 1 s, 5 s, 10 s, and 30 s. Liver and blood (500 μ L) were imaged separately. Bioluminescence values were quantified by measuring photon flux (photons/second) using the Living Image® software program for both *in vivo* and *ex vivo* imaging. **FIG. 9** shows that the polyvinylpyrrolidones had higher extrahepatic selectivity than PEG-DMG.

EXAMPLE 17

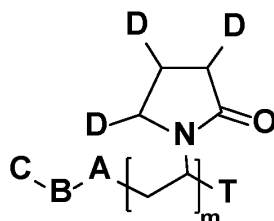
[00237] This example illustrates *in vivo* expression of hEPO. hEPO expression levels were measured (6 h and 24 h post administration) in C57BL/6 mice following IV administration of 0.25 mg/kg dose of recombinant human EPO-encoded mRNA-LNPs using 40 mol% PNI 516, 12.5 mol % DSPC, (47.5-x) mol % cholesterol, x mol % stabilizer; N/P-8). **FIG. 10** showed that increasing the stabilizer mol% of SAF191 resulted in higher *in vivo* efficacy.

[00238] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[00239] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIM(S):

1. A stabilizing agent comprising a structure of formula (I):

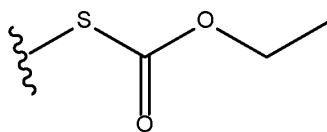


(I)

wherein A is S, NH, O, C₁₋₈ substituted or unsubstituted alkyl or heteroalkyl group, a hydrophilic group, a glyceryl group, a heterocyclic group, a phospholipid group, a ceramide group, an ionizable group, a triazole group, or a combination thereof; B is CH₂, a triazole group or a C₁₋₁₈ amino oxobutanoate group or a C₁₋₁₈ substituted or unsubstituted alkyl or heteroalkyl group; C is a C₁₋₅₀ substituted or unsubstituted heteroalkyl group, C₁₋₅₀ unsaturated heteroalkyl group, C₁₋₅₀ heterocyclic group, C₁₋₅₀ charged heteroalkyl group, or a combination thereof; T is hydrogen, a C₁₋₁₈ carbonothioate group, C₁₋₁₈ substituted or unsubstituted alkyl or heteroalkyl group, a C₁₋₁₈ ionizable group, a C₁₋₁₈ targeting group, a hydrophilic group, an amphiphilic group, or a combination thereof; D is H, C₁₋₅ substituted or unsubstituted alkyl or heteroalkyl group, or hydroxyl group and m is an integer from 15 to 500.

2. The stabilizing agent of claim 1, wherein A is sulfur or a triazole group; B is CH₂, triazole group, or a C₃₋₆ amino oxobutanoate group; T is hydrogen, a C₁₋₅ carbonothioate group, a C₁₋₅ ionizable group, a C₁₋₅ targeting group, a hydrophilic group, an amphiphilic group, or a combination thereof; and m is an integer from 15 to 400.

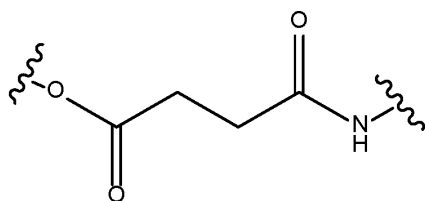
3. The stabilizing agent of claims 1 or 2, wherein T is hydrogen, methyl,



, a peptide, an antibody, a sugar, an oligosaccharide, an aminoglycoside, a sterol, phenyl boronic acid, or a combination thereof.

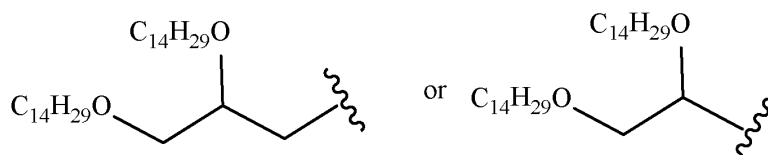
4. The stabilizing agent of any one of claims 1-3, wherein A is sulfur or a triazole group.

5. The stabilizing agent of any one of claims 1-4, wherein B is CH₂ or



6. The stabilizing agent of any one of claims 1-5, wherein C is a C₁₋₃₅ substituted or unsubstituted heteroalkyl group, C₁₋₃₅ unsaturated heteroalkyl group, C₁₋₃₅ heterocyclyl group, C₁₋₃₅ charged heteroalkyl group, or a combination thereof.

7. The stabilizing agent of any one of claims 1-6, wherein C is



8. The stabilizing agent of any one of claims 1-7, wherein the stabilizing agent is SAF29, SAF29A, SAF29B, SAF29C, SAF30, SAF34, SAF102, SAF102A, SAF102B, SAF103, SAF104, SAF123, SAF124, SAF125, SAF126, SAF127, SAF133, SAF134, SAF135, SAF136, SAF137, SAF138, SAF139, SAF141, SAF149, SAF150, SAF154, SAF167, SAF178, SAF190, SAF191, or a combination thereof.

9. The stabilizing agent of any one of claims 1-8, wherein the stabilizing agent is used for stabilizing a lipid nanoparticle or liposome.

10. A lipid nanoparticle composition comprising: (a) an ionizable lipid; (b) two or more lipids; (c) and the stabilizing agent of any one of claims 1-9.

11. The lipid nanoparticle composition of claim 9, wherein the two or more lipids comprises a structural lipid, a sterol, or a combination thereof.

12. The lipid nanoparticle composition of claim 10 or 11 consisting essentially of: (a) an ionizable lipid; (b) two lipids; (c) and the stabilizing agent of any one of claims 1-9.

13. The lipid nanoparticle composition of claim 12, wherein the lipid nanoparticle composition is substantially free of PEG or PEG-R, wherein R is any atom or molecule covalently attached to PEG.

14. The lipid nanoparticle composition of any one of claims 10-13, wherein the structural lipid is neutrally charged, positively charged, or negatively charged.

15. The lipid nanoparticle composition of any one of claims 10-14, wherein the ionizable lipid is DODMA, DLin-MC3-DMA, DLin-KC2-DMA, BOCHD-C3-DMA, C12-200, PNI 516, PNI 127, PNI 550, PNI 580, PNI 659, PNI 728, PNI 762, or a combination thereof.

16. The lipid nanoparticle composition of any one of claims 10-15, wherein the structural lipid comprises diacylphosphatidylcholines, diacylphosphatidylethanolamines, diacylphosphatidylglycerols, ceramides, sphingomyelins, dihydrosphingomyelins, cephalins, cerebroside, or a combination thereof.

17. The lipid nanoparticle composition of any one of claims 10-16, wherein the structural lipid comprises distearoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, dioleoyl-phosphatidylethanolamine, palmitoyloleoylphosphatidylcholine, 1-stearoyl-2-oleoyl-sn-glycero-3-phosphocholine, palmitoyloleoyl-phosphatidylethanolamine, dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate, dipalmitoyl phosphatidyl ethanolamine, dimyristoylphosphoethanolamine, distearoyl-phosphatidylethanolamine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-methyl, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N,N-dimethyl, 1,2-dielaoidyl-sn-glycero-3-phosphoethanolamine, 1-stearoyl-2-oleoyl-phosphatidylethanolamine, 1,2-dielaoidyl-sn-glycero-3-phosphoethanolamine, distearoylphosphatidylcholine, dioleoylphosphatidylglycerol, dipalmitoylphosphatidylglycerol, palmitoyloleoylphosphatidylglycerol, cardiolipin, phosphatidylinositol, diacylphosphatidylserine, diacylphosphatidic acid, monosialoganglioside GM1, or a combination thereof.

18. The lipid nanoparticle composition of any one of claims 10-17, wherein the sterol comprises cholesterol, beta-sitosterol, 20-alpha-hydroxysterol, phytosterol, or a combination thereof.

19. The lipid nanoparticle composition of any one of claims 10-18, wherein the stabilizing agent has a molecular weight of about 500 Da to about 50,000 Da.

20. The lipid nanoparticle composition of any one of claims 10-19, wherein the lipid nanoparticle composition comprises about 20 to about 70 mol% ionizable lipid, about 1 to about 25 mol% structural lipid, about 28 to about 50 mol% sterol, and about 0.1 to about 10 mol% stabilizing agent.

21. A lipid nanoparticle comprising the lipid nanoparticle composition of any one of claims 10-20 and a nucleic acid.

22. The lipid nanoparticle of claim 21, wherein the nucleic acid is encapsulated by the lipid nanoparticle composition.

23. The lipid nanoparticle of claim 21 or 22, wherein the nucleic acid comprises an antisense oligonucleotide, a siRNA, a miRNA, a self-amplifying RNA (samRNA or saRNA), a self-replicating DNA, an LNA, a DNA, a replicon, an mRNA, a guide RNA, a transposon, a single gene, a vector, a plasmid, a viral particle, an AAV, a complex of RNA and RNA-binding protein, or a combination thereof.

24. The lipid nanoparticle of any one of claims 21-23, wherein the nucleic acid is an antigen encoded mRNA for prophylactic or therapeutic vaccine, a nucleic acid for gene therapy, or a nucleic acid for immunogenic cell incorporation, wherein the immunogenic cell is a T cell.

25. The lipid nanoparticle of any one of claims 21-24, wherein the lipid nanoparticle diameter is about 15 nm to about 500 nm.

26. The lipid nanoparticle of any one of claims 21-25, wherein the lipid nanoparticle has a polydispersity index of about 0.01 to about 0.40.

27. The lipid nanoparticle of any one of claims 21-26, wherein the lipid nanoparticle has an encapsulation efficiency of about 50% to about 100%.

28. A pharmaceutical composition comprising the lipid nanoparticle of any one of claims 21-27 and a pharmaceutically acceptable carrier.

29. A method for preparing the lipid nanoparticle of any one of claims 21-27 or the pharmaceutical composition of claim 28, the method comprising:

- (i) forming the lipid nanoparticle composition by combining the ionizable lipid, structural lipid, sterol, and stabilizing agent;
- (ii) preparing the lipid nanoparticle by combining the lipid nanoparticle composition and the nucleic acid using a microfluidic mixer; and
- (iii) purifying the lipid nanoparticle.

30. The method of claim 29, wherein the lipid nanoparticle composition and the nucleic acid are combined using a flow ratio of about 1:1 to about 10:1 by volume (aqueous phase: organic phase) at a N/P ratio of about 2 to about 20, and a total flow rate of about 2 to about 2000 mL/min.

31. The method of claim 30, wherein the aqueous phase comprises a low pH buffer.
32. The method of claims 30 or 31, wherein the aqueous phase comprises a citrate or acetate buffer.
33. The method of any one of claims 30-32, wherein the organic phase comprises 1,4-dioxane, tetrahydrofuran, acetone, acetonitrile, dimethyl sulfoxide, dimethylformamide, acids, alcohols, or a combination thereof.
34. The method of any one of claims 30-33, wherein the organic phase is an alcohol and the alcohol comprises aqueous or anhydrous alcohol, wherein the alcohol is a primary, secondary, or tertiary alcohol having from 1 to 12 branched or unbranched carbons.
35. The use of the lipid nanoparticle of any one of claims 21-27 or the pharmaceutical composition of claim 28 for preventing, treating, or ameliorating conditions or diseases including administering the lipid nanoparticle as a vaccine or as a treatment to prevent or reduce the severity of a contagion, administering the lipid nanoparticle as a gene therapeutic, or administering the lipid nanoparticle to an immunogenic cell for the treatment of cancer or an infection.