

## MATERIALS AND METHODS FOR ENCAPSULATING THERAPEUTICS IN LIPID NANOPARTICLES

### BACKGROUND

#### *Technical Field*

5           The present disclosure generally relates to a novel method for preparing lipid nanoparticles (LNPs) that encapsulate nucleic acid molecules (*e.g.*, oligonucleotides or polynucleotides). The lipid nanoparticles can be prepared using lipid components, such as ionizable lipids, neutral lipids, cholesterol, and polymer conjugated lipids. The lipid nanoparticles that encapsulate nucleic acid molecules facilitate intracellular delivery both *in vitro*  
10   and *in vivo*.

#### *Description of the Related Art*

          There are many challenges associated with storage and eventual delivery of nucleic acids to affect a desired response in a biological system. Nucleic acid-based therapeutics have enormous potential but there remains a need for more effective preparation, storage, and delivery  
15   of nucleic acid-based therapeutics to realize this potential. It has been observed that nucleic acid containing lipid nanoparticles can degrade during the manufacturing process and during storage over time. Although methods exist for formulating nucleic acids with lipid nanoparticles, they involve complicated and/or cumbersome techniques (*e.g.*, sample heating, complex fluid mixing equipment and solutions, buffer exchange by dialysis or TFF, and various analytical  
20   requirements to ensure formulations conform to specification) that require extended exposure of the nucleic acid to adverse conditions and are not readily available to typical treatment environments.

          Accordingly, there exists a need for methods of preparing nucleic acids encompassed in lipid nanoparticles using simplified techniques and conditions that are amenable to "bedside"  
25   preparation of formulations or are less aggressive in the treatment of the nucleic acid payload during manufacturing. The present disclosure provides these and related advantages.

### BRIEF SUMMARY

          In brief, the present disclosure provides methods for preparing lipid nanoparticle formulations that encompass nucleic acid therapeutics (*e.g.*, mRNA) under mild conditions. The

lipid nanoparticles described herein include ionizable lipids, neutral lipids, charged lipids, steroids (including for example, all sterols) and/or their analogs, and/or polymer conjugated lipids. In some instances, the lipid nanoparticles are used to formulate and deliver nucleic acids such as antisense and/or messenger and/or guide RNA. Methods for using lipid nanoparticles following formulation for treatment of various diseases or conditions, such as those caused by infectious entities and/or insufficiency of a protein and/or have a genetic basis, are also provided.

Pharmaceutical compositions (*e.g.*, a vaccine or gene editing product) comprising formulated lipid nanoparticles encompassing nucleic acid molecules are also provided. Such compositions are useful for delivery of the nucleic acid molecules and treatment of various diseases and disorders.

In other embodiments, the present disclosure provides a method for administering a composition comprising a nucleic acid encompassed in a lipid nanoparticle prepared according to methods described herein to a patient in need thereof, the method comprising delivering the composition to the patient. Such methods are useful for inducing expression of a protein in a subject, for example for expressing an antigen for purposes of vaccination, or a gene editing protein for correction of genetic disorders.

These and other aspects of the disclosure will be apparent upon reference to the following detailed description.

## BRIEF DESCRIPTION OF THE DRAWINGS

In the figures, identical reference numbers identify similar elements. The sizes and relative positions of elements in the figures are not necessarily drawn to scale and some of these elements are enlarged and positioned to improve figure legibility. Further, the shapes of the elements as drawn are not intended to convey any information regarding the actual shape of the elements and have been solely selected for ease of recognition in the figures.

FIG. 1 is a depiction of IgG expression as tested using the procedures described in Biological Example 2. Shown from left to right are samples 6a-1, 6b-1, 6a-2, 6b-2, 6a-3, 6b-3, 6a-4, and 6b-4. In the graph, # indicates  $p < 0.01$  for sample 6b-3 vs. sample 6a-3 (one tailed test) and \* indicates  $p < 0.05$ . Samples were tested using a dose of 0.3 mg/kg.

FIG. 2 shows IgG expression as tested using the procedures described in Biological Example 2. Shown from left to right are samples 6a-2 and 6b-2. In the graph,  $p < 0.01$  vs sample 6a-2. Samples were tested using a dose of 1 mg/kg.

FIG. 3 shows a graphical representation of samples tested for lipid adduct formation after storage at -80°C. Samples were prepared and tested as noted in Table 6a and 6b; from left to right in the graph shows samples 6a-1, 6b-1, 6a-2, 6b-2, 6a-3, 6b-3, 6a-4, and 6b-4.

FIG. 4 is a graphical representation of samples tested for lipid adduct formation after storage post dosing (*i.e.*, samples were stored for 2 weeks at 4°C). Samples were prepared and tested as noted in Table 6a and 6b; from left to right in the graph shows samples 6a-1, 6b-1, 6a-2, 6b-2, 6a-3, 6b-3, 6a-4, and 6b-4.

FIG. 5 is a graph showing percentage encapsulation for samples prepared according to Table 6a. The samples shown include (from left to right) sample 6a-1 analyzed by UPLC, sample 6a-1 analyzed by RG, sample 6a-2 analyzed by UPLC, sample 6a-2 analyzed by RG, sample 6a-3 analyzed by UPLC, sample 6a-3 analyzed by RG, sample 6a-4 analyzed by UPLC, and sample 6a-4 analyzed by RG.

FIG. 6 shows a graphical representation of percentages of encapsulation for samples prepared according to Table 6b. The samples shown include (from left to right) sample 6b-1 analyzed by UPLC, sample 6b-1 analyzed by RG, sample 6b-2 analyzed by UPLC, sample 6b-2 analyzed by RG, sample 6b-3 analyzed by UPLC, sample 6b-3 analyzed by RG, sample 6b-4 analyzed by UPLC, and sample 6b-4 analyzed by RG.

FIG. 7 represents data for HAI units measured for a 0.2 µg dose of samples prepared according to Tables 9a and 9b. The graph shows data for (from left to right) samples 9a-1, 9b-1, 9a-2, 9b-2, 9a-3 and 9b-3.

FIG. 8 shows data for HAI units measured for a 0.5 µg dose of samples prepared according to Tables 9a and 9b. The graph shows data for (from left to right) samples 9a-1, 9b-1, 9a-2, 9b-2, 9a-3 and 9b-3.

FIG. 9 illustrates percentage encapsulation compared against loading concentrations at a 10-minute time point. For each concentration (*i.e.*, RNA concentrations of 0.075, 0.15, 0.5, and 1 mg/mL), a percentage encapsulation is shown for (from left to right) samples 10-1, 10-2, and 10-4. Samples 10-1 and 10-2 were not tested concentrations of 0.075

FIG. 10 shows Z-average size (nm) for samples prepared according to Table 10 (sample 10-1 indicated with a square; sample 10-2 indicated with a diamond; sample 10-4 indicated with a circle). Samples were detected at a 10-minute time point.

FIG. 11 shows the physical characteristics of LNPs loaded in 25 mM acetate buffer at pH 5.5 across different RNA concentrations.

FIG. 12 is a graph showing physical characteristics of LNPs loaded in 25 mM acetate buffer at pH 5.9 across different RNA concentrations.

FIG. 13 shows encapsulation efficiency for LNPs loaded using 25 mM acetate buffers at pH 5.5 and pH 5.9 across different RNA concentrations.

5        FIG. 14 shows the effect of acetate concentrations for PFL LNPs at pH 5.9; concentration units for the pH 5.9 acetate concentration are [mM].

FIG. 15 is a graph showing encapsulation of RNA for LNPs prepared with different mol % of PEG lipids over time.

FIG. 16 is a plot of encapsulation efficiency as a function of incubation time for different sized LNPs prepared using PFL methods using ionizable lipid B-45.

FIG. 17 shows changes in particle size (Z-average) after 90-minute incubation time. In the figure, bars show the change in size for LNPs loaded after formation (*i.e.*, the PFL method) and the change in size is shown against the scale on the left; the lines / points show LNP size after loading with RNA against the scale on the right.

15        FIG. 18 shows a comparison of encapsulation efficiency related to particle size over a course of incubation time.

FIG. 19 shows the change in particle size (Z-average) after 90-minute incubation time. In the figure, bars show the change in size for LNPs loaded after formation (*i.e.*, the PFL method) and the change in size is shown against the scale on the left; the lines / points show LNPs after loading with RNA against the scale on the right.

FIG. 20 shows the particle size (Z-average) and PDI for LNPs formulated using buffers at different pH values and ionizable lipid A-15. In the figure, bars show the Z-average size for LNPs across a range of pH values against the scale on the left; the lines / points show LNPs PDI values against the scale on the right.

25        FIG. 21 shows a comparison of encapsulation efficiency across pH values for LNPs prepared using ionizable lipid A-15.

FIG. 22 shows particle size (Z-average) and PDI for LNPs formulated using buffers at different pH values and ionizable lipid B-3. In the figure, bars show the change in size for LNPs across a range of pH values against the scale on the left; the lines / points show LNPs PDI values against the scale on the right.

FIG. 23 shows a comparison of encapsulation efficiency across pH values for LNPs prepared using ionizable lipid B-3.



FIG. 24 shows particle size (Z-average) and PDI for LNPs formulated using buffers at different pH values and ionizable lipid D-1. In the figure, bars show the change in size for LNPs across a range of pH values against the scale on the left; the lines / points show LNPs PDI values against the scale on the right.

5        FIG. 25 shows a comparison of encapsulation efficiency across pH values for LNPs prepared using ionizable lipid D-1.

FIG. 26 shows particle size (Z-average) and PDI for LNPs formulated using buffers at different pH values and ionizable lipid C-18. In the figure, bars show the change in size for LNPs across a range of pH values against the scale on the left; the lines / points show LNPs PDI values  
10 against the scale on the right.

FIG. 27 shows a comparison of encapsulation efficiency across pH values for LNPs prepared using ionizable lipid C-18.

FIG. 28 is a plot of encapsulation efficiency values shown as a function of time as measured by UPLC for a LNP prepared with 1.8 mol% of PEG Lipid 1 and ionizable lipid A-15.  
15 This LNP encapsulated RNA at 97% almost immediately.

FIG. 29 is a plot of encapsulation efficiency values shown as a function of time as measured by UPLC for a LNP prepared with 5 mol% of PEG Lipid 1 and ionizable lipid A-15. Encapsulation improved from 20% to 67% over the course of 10 hours.

FIG. 30 shows serum IgG expression for doses at 0.5 mg/kg. Samples were prepared  
20 according to the description of Formulation Example 18. From left to right, FIG. 30 shows results for samples A, B, C, D, and E, respectively. In FIG. 30, \* represents  $p < 0.05$  vs. T-mix control (sample A) and \*\* represents  $p < 0.01$  between samples D and E.

FIG. 31 shows measurements for size (upper set of lines) and PDI (lower set of lines) for empty LNP samples measured across different time-points after storage at 2-8°C.

25        FIG. 32 shows measurements for size (upper set of lines) and PDI (lower set of lines) for empty LNP samples measured across different time-points after storage at -80°C.

FIG. 33 shows measurements for size (upper set of lines) and PDI (lower set of lines) for samples measured across different time-points after storage at 2-8°C after RNA loading post storage.

30        FIG. 34 shows measurements for size (upper set of lines) and PDI (lower set of lines) for samples measured across different time-points after storage at -80°C after RNA loading post storage.

FIG. 35 shows RNA loading efficiency of stored empty LNPs in terms of encapsulation efficiency. Each time-point (*i.e.*, 1 month, 3 months, and 6 months) shows results for (from left to right) 0.5 mg/mL at 2-8°C, 1.0 mg/mL at 2-8°C, 2.0 mg/mL at 2-8°C, 3.1 mg/mL at 2-8°C, 0.1 mg/mL at -80°C, 0.5 mg/mL at -80°C, 1.0 mg/mL at -80°C, and 2.0 mg/mL at -80°C.

5        FIG. 36A shows the z-average size for samples prepared using T-mixing techniques as detailed in Formulation Example 19.

FIG. 36B shows the PDI for samples prepared using T-mixing techniques as detailed in Formulation Example 19.

10       FIG. 36C shows the total lipid content (mg/mL) for samples prepared using T-mixing techniques as detailed in Formulation Example 19.

FIG. 37A shows the z-average size for samples prepared using PFL methods as detailed in Formulation Example 19.

FIG. 37B shows the PDI for samples prepared using PFL methods as detailed in Formulation Example 19.

15       FIG. 37C shows the total lipid content (mg/mL) for samples prepared using PFL methods as detailed in Formulation Example 19.

FIG. 38A shows the RNA content for samples prepared using T-mixing techniques as detailed in Formulation Example 19.

20       FIG. 38B shows encapsulation efficiency for samples prepared using T-mixing techniques as detailed in Formulation Example 19.

FIG. 38C shows the RNA integrity for samples prepared using T-mixing techniques as detailed in Formulation Example 19.

FIG. 39A shows the RNA content for samples prepared using PFL methods as detailed in Formulation Example 19.

25       FIG. 39B shows encapsulation efficiency for samples prepared using PFL methods as detailed in Formulation Example 19.

FIG. 39C shows the LNP integrity for samples prepared using PFL methods as detailed in Formulation Example 19.

30       FIG. 40 shows LNP z-average size (shown as bars against the left scale) and PDI (shown as points / lines against the right scale) as a function of the N:P ratio.

FIG. 41 shows encapsulation efficiency for LNPs as a function of N:P ratio (from an N:P ratio of 9 to 3).

FIG. 42A shows the RNA encapsulation efficiency of differently sized empty LNP prepared with ionizable lipid B-3 after 10 minutes of incubation with RNA at ambient temperature.

FIG. 42B shows changes in particle size (Z-average) after 10-minute incubation time. In the figure, bars show the change in size for ionizable lipid B-3 LNPs loaded after formation (*i.e.*, the PFL method) and the change in size is shown against the scale on the left; the lines / points show LNP size after loading with RNA against the scale on the right.

FIG. 43A shows LNP z-average size (shown as bars against the left scale) and PDI (shown as points / lines against the right scale) for ionizable lipid B-3 LNP which have been loaded with either siRNA, saRNA, or an mRNA/gRNA mixture using the PFL method.

FIG. 43B shows LNP z-average size (shown as bars against the left scale) and PDI (shown as points / lines against the right scale) for ionizable lipid B-3 LNP which have been loaded with either siRNA, saRNA, or an mRNA/gRNA mixture using the PFL method.

FIG. 43C shows the encapsulation efficiency of LNP loaded with either siRNA, saRNA or an mRNA/gRNA mixture using the PFL method for LNPs prepared with either ionizable lipid B-3 or C-18 (as indicated).

FIG. 44A shows LNP z-average empty LNPs formulated in the presence of an aqueous buffer containing increasing concentrations of sodium chloride.

FIG. 44B shows the z-average size for LNPs formulated using the PFL method in the presence of an aqueous buffer containing increasing concentrations of sodium chloride.

FIG. 44C shows encapsulation efficiency of the LNPs formulated using the PFL method in the presence of increasing concentrations of sodium chloride.

## DETAILED DESCRIPTION

In the following description, certain specific details are set forth to provide a thorough understanding of various embodiments of the disclosure. However, one skilled in the art will understand that the embodiments of the disclosure may be practiced without these details.

The present disclosure is based, in part, upon the discovery that nucleic acid molecules were able to be loaded into lipid nanoparticles in an aqueous solution in the absence of any aiding modality (*e.g.*, a disruptive agent such as ethanol, heat, *etc.*). Applicant unexpectedly discovered that an aqueous solution comprising "empty" lipid nanoparticles could be mixed with nucleic acid molecules (*e.g.*, in solution or dry) and the nucleic acid molecules are then encapsulated into the lipid nanoparticles to yield particles with surprisingly desirable

characteristics (*e.g.*, particle size, polydispersity index (PDI), encapsulation efficiency, loading (RNA / lipid [wt/ $\mu$ m], *etc.*). It was unexpected that a solution of "empty" LNPs at neutral pH (*e.g.*, in phosphate buffered saline or "PBS") could result in desirable encapsulation efficiency and LNP products (*i.e.*, LNPs that encapsulated a nucleic acid-based payload) when the pH of  
5 the solution was shifted to a slightly lower pH than the pKa of the ionizable lipids that made up the LNPs, in the absence of any other aiding modality.

The methods and materials disclosed herein provide advantages when used to prepare lipid nanoparticles (LNPs) for the *in vivo* delivery of an active or therapeutic agent such as a nucleic acid into a cell of a mammal. Some embodiments of the present disclosure provide  
10 methods of preparing nucleic acid-lipid nanoparticle compositions via a *facile* method that can be carried out under mild conditions with minimal equipment.

In some embodiments, the present disclosure provides methods of preparing solutions that enable the formation of improved LNPs for the *ex vivo*, *in vitro*, and *in vivo* delivery of mRNA and/or other polynucleotides. In some embodiments, these improved LNPs are useful for  
15 expression of protein encoded by RNA and/or DNA. In other embodiments, these improved LNPs are useful for upregulation of endogenous protein expression by delivering miRNA inhibitors targeting one specific miRNA or a group of miRNA regulating one target mRNA or several mRNA. In other embodiments, these LNPs are useful for down-regulating (*e.g.*, silencing) the protein levels and/or mRNA levels of target genes. In some other embodiments,  
20 the LNPs are also useful for delivery of mRNA and plasmids for expression of transgenes. In yet other embodiments, the LNPs are useful for inducing a pharmacological effect resulting from expression of a protein, *e.g.*, increased production of red blood cells through the delivery of a suitable erythropoietin mRNA, or protection against infection through delivery of mRNA encoding for a suitable antibody.

25 In some embodiments, the LNPs are useful for gene editing, epigenomic editing, cancer vaccine, Cart-T, gene insertion, Prime editing, or combinations thereof.

The LNPs and compositions comprising the LNPs of the present disclosure may be used for a variety of purposes, including the delivery of encapsulated or associated (*e.g.*, complexed) therapeutic agents such as nucleic acids to cells, both *ex vivo*, *in vitro*, and *in vivo*. Accordingly,  
30 embodiments of the present disclosure provide methods of treating or preventing diseases or disorders in a subject in need thereof by contacting the subject (*e.g.*, via injection) with a LNP that encapsulates or is associated with a suitable therapeutic agent or nucleic acid.

As described herein, embodiments of the LNPs of the present disclosure are particularly useful for the delivery of nucleic acids, including, *e.g.*, mRNA, antisense oligonucleotide, plasmid DNA, microRNA (miRNA), miRNA inhibitors (antagomirs/antimirs), messenger-RNA-interfering complementary RNA (micRNA), DNA, multivalent RNA, dicer substrate RNA, siRNA, circular RNA, self-amplifying RNA, aptamers, closed ended DNA, complementary DNA (cDNA), *etc.* Therefore, the LNPs and compositions comprising the LNPs of the present disclosure may be used to induce expression of a desired protein *ex vivo*, *in vitro*, and *in vivo* by contacting cells with a LNP described herein, wherein the LNP encapsulates or is associated with a nucleic acid that is expressed to produce the desired protein (*e.g.*, a messenger RNA or plasmid encoding the desired protein). Alternatively, the LNPs and compositions comprising the LNPs of the present disclosure may be used to decrease the expression of target genes and proteins both *in vitro* and *in vivo* by contacting cells with a LNP described herein, wherein the LNP encapsulates or is associated with a nucleic acid that reduces target gene expression (*e.g.*, an antisense oligonucleotide or small interfering RNA (siRNA)). The LNPs and compositions comprising the LNPs of the present disclosure may also be used for co-delivery of different nucleic acids (*e.g.*, mRNA and plasmid DNA) separately or in combination, such as may be useful to provide an effect requiring colocalization of different nucleic acids (*e.g.*, mRNA encoding for a suitable gene modifying enzyme and DNA segment(s) for incorporation into the host genome).

Nucleic acids for use with this disclosure may be prepared according to any available technique. For mRNA, the primary methodology of preparation is, but not limited to, enzymatic synthesis (also termed *in vitro* transcription) which currently represents the most efficient method to produce long sequence-specific mRNA. *In vitro* transcription describes a process of template-directed synthesis of RNA molecules from an engineered DNA template comprised of an upstream bacteriophage promoter sequence (*e.g.*, including but not limited to that from the T7, T3, and SP6 coliphage) linked to a downstream sequence encoding the gene of interest. Template DNA can be prepared for *in vitro* transcription from a number of sources with appropriate techniques which are well known in the art including, but not limited to, plasmid DNA and polymerase chain reaction amplification (see Linpinsel, J.L and Conn, G.L., General protocols for preparation of plasmid DNA template and Bowman, J.C., Azizi, B., Lenz, T.K., Ray, P., and Williams, L.D. in RNA *in vitro* transcription and RNA purification by denaturing PAGE in Recombinant and *in vitro* RNA syntheses Methods v. 941 Conn G.L. (ed), New York, N.Y. Humana Press, 2012).

Transcription of the RNA occurs *in vitro* using the linearized DNA template in the presence of the corresponding RNA polymerase and adenosine, guanosine, uridine, and cytidine ribonucleoside triphosphates (rNTPs) under conditions that support polymerase activity while minimizing potential degradation of the resultant mRNA transcripts. *In vitro* transcription can be performed using a variety of commercially available kits including, but not limited to RiboMax Large Scale RNA Production System (Promega), MegaScript Transcription kits (Life Technologies) as well as with commercially available reagents including RNA polymerases and rNTPs. The methodology for *in vitro* transcription of mRNA is well known in the art. (see, e.g., Losick, R., 1972, *In vitro* transcription, Ann Rev Biochem v.41 409-46; Kamakaka, R. T. and Kraus, W. L. 2001. *In Vitro* Transcription. Current Protocols in Cell Biology. 2:11.6:11.6.1–11.6.17; Beckert, B. And Masquida, B., (2010) Synthesis of RNA by *In Vitro* Transcription in RNA in Methods in Molecular Biology v. 703 (Neilson, H. Ed), New York, N.Y. Humana Press, 2010; Brunelle, J.L. and Green, R., 2013, Chapter Five – *In vitro* transcription from plasmid or PCR-amplified DNA, Methods in Enzymology v. 530, 101-114; all of which are incorporated herein by reference).

The desired *in vitro* transcribed mRNA is then purified from the undesired components of the transcription or associated reactions (including unincorporated rNTPs, protein enzyme, salts, short RNA oligos *etc.*). Techniques for the isolation of the mRNA transcripts are well known in the art. Well known procedures include phenol/chloroform extraction or precipitation with either alcohol (ethanol, isopropanol) in the presence of monovalent cations or lithium chloride. Additional, non-limiting examples of purification procedures which can be used include size exclusion chromatography (Lukavsky, P.J. and Puglisi, J.D., 2004, Large-scale preparation and purification of polyacrylamide-free RNA oligonucleotides, RNA v.10, 889-893), silica-based affinity chromatography and polyacrylamide gel electrophoresis (Bowman, J.C., Azizi, B., Lenz, T.K., Ray, P., and Williams, L.D. in RNA *in vitro* transcription and RNA purification by denaturing PAGE in Recombinant and *in vitro* RNA syntheses Methods v. 941 Conn G.L. (ed), New York, N.Y. Humana Press, 2012 ). Purification can be performed using a variety of commercially available kits including, but not limited to SV Total Isolation System (Promega) and *In Vitro* Transcription Cleanup and Concentration Kit (Norgen Biotek).

Furthermore, while reverse transcription can yield large quantities of mRNA, the products can contain several aberrant RNA impurities associated with undesired polymerase activity which may need to be removed from the full-length mRNA preparation. These include short RNAs that result from abortive transcription initiation as well as double-stranded RNA

(dsRNA) generated by RNA-dependent RNA polymerase activity, RNA-primed transcription from RNA templates and self-complementary 3' extension. It has been demonstrated that these contaminants with dsRNA structures can lead to undesired immunostimulatory activity through interaction with various innate immune sensors in eukaryotic cells that function to recognize  
5 specific nucleic acid structures and induce potent immune responses. This in turn, can dramatically reduce mRNA translation since protein synthesis is reduced during the innate cellular immune response. Therefore, additional techniques to remove these dsRNA contaminants have been developed and are known in the art including but not limited to scaleable HPLC purification (see, *e.g.*, Kariko, K., Muramatsu, H., Ludwig, J. And Weissman, D., 2011, Generating the optimal mRNA for therapy: HPLC purification eliminates immune  
10 activation and improves translation of nucleoside-modified, protein-encoding mRNA, Nucl Acid Res, v. 39 e142; Weissman, D., Pardi, N., Muramatsu, H., and Kariko, K., HPLC Purification of *in vitro* transcribed long RNA in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology v.969 (Rabinovich, P.H. Ed), 2013). HPLC purified mRNA has  
15 been reported to be translated at much greater levels, particularly in primary cells and *in vivo*.

A significant variety of modifications have been described in the art which are used to alter specific properties of *in vitro* transcribed mRNA and improve its utility. These include but are not limited to modifications to the 5' and 3'-termini of the mRNA. Endogenous eukaryotic mRNA typically contains a cap structure on the 5'-end of a mature molecule which plays an  
20 important role in mediating binding of the mRNA Cap Binding Protein (CBP), which is in turn responsible for enhancing mRNA stability in the cell and efficiency of mRNA translation. Therefore, highest levels of protein expression are achieved with capped mRNA transcripts. The 5'-cap contains a 5'-5'-triphosphate linkage between the 5'-most nucleotide and guanine nucleotide. The conjugated guanine nucleotide is methylated at the N7 position. Additional  
25 modifications include methylation of the ultimate and penultimate most 5'-nucleotides on the 2'-hydroxyl group.

Multiple distinct cap structures can be used to generate the 5'-cap of *in vitro* transcribed synthetic mRNA. 5'-capping of synthetic mRNA can be performed co-transcriptionally with chemical cap analogs (*i.e.*, capping during *in vitro* transcription). For example, the Anti-Reverse  
30 Cap Analog (ARCA) cap contains a 5'-5'-triphosphate guanine-guanine linkage where one guanine contains an N7 methyl group as well as a 3'-O-methyl group. However, up to 20% of transcripts remain uncapped during this co-transcriptional process and the synthetic cap analog is not identical to the 5'-cap structure of an authentic cellular mRNA, potentially reducing

translatability and cellular stability. Alternatively, synthetic mRNA molecules may also be enzymatically capped post-transcriptionally. These may generate a more authentic 5'-cap structure that more closely mimics, either structurally or functionally, the endogenous 5'-cap which have enhanced binding of cap binding proteins, increased half-life, reduced susceptibility to 5' endonucleases and/or reduced 5' de-capping. Numerous synthetic 5'-cap analogs have been developed and are known in the art to enhance mRNA stability and translatability (see, *e.g.*, Grudzien-Nogalska, E., Kowalska, J., Su, W., Kuhn, A.N., Slepencov, S.V., Darynkiewicz, E., Sahin, U., Jemielity, J., and Rhoads, R.E., Synthetic mRNAs with superior translation and stability properties in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology v.969 (Rabinovich, P.H. Ed), 2013).

On the 3'-terminus, a long chain of adenine nucleotides (poly-A tail) is normally added to mRNA molecules during RNA processing. Immediately after transcription, the 3' end of the transcript is cleaved to free a 3' hydroxyl to which poly-A polymerase adds a chain of adenine nucleotides to the RNA in a process called polyadenylation. The poly-A tail has been extensively shown to enhance both translational efficiency and stability of mRNA (see Bernstein, P. and Ross, J., 1989, Poly (A), poly (A) binding protein and the regulation of mRNA stability, Trends Bio Sci v. 14 373-377; Guhaniyogi, J. And Brewer, G., 2001, Regulation of mRNA stability in mammalian cells, Gene, v. 265, 11-23; Dreyfus, M. And Regnier, P., 2002, The poly (A) tail of mRNAs: Bodyguard in eukaryotes, scavenger in bacteria, Cell, v.111, 611-613).

Poly (A) tailing of *in vitro* transcribed mRNA can be achieved using various approaches including, but not limited to, cloning of a poly (T) tract into the DNA template or by post-transcriptional addition using Poly (A) polymerase. The first case allows *in vitro* transcription of mRNA with poly (A) tails of defined length, depending on the size of the poly (T) tract, but requires additional manipulation of the template. The latter case involves the enzymatic addition of a poly (A) tail to *in vitro* transcribed mRNA using poly (A) polymerase which catalyzes the incorporation of adenine residues onto the 3'-termini of RNA, requiring no additional manipulation of the DNA template, but results in mRNA with poly(A) tails of heterogeneous length. 5'-capping and 3'-poly (A) tailing can be performed using a variety of commercially available kits including, but not limited to Poly (A) Polymerase Tailing kit (EpiCenter), mMMESSAGE mMACHINE T7 Ultra kit and Poly (A) Tailing kit (Life Technologies) as well as with commercially available reagents, various ARCA caps, Poly (A) polymerase, *etc.*

In addition to 5' cap and 3' poly adenylation, other modifications of the *in vitro* transcripts have been reported to provide benefits as related to efficiency of translation and stability. It is



well known in the art that pathogenic DNA and RNA can be recognized by a variety of sensors within eukaryotes and trigger potent innate immune responses. The ability to discriminate between pathogenic and self-DNA and RNA has been shown to be based, at least in part, on structure and nucleoside modifications since most nucleic acids from natural sources contain modified nucleosides. In contrast, *in vitro* synthesized RNA lacks these modifications, thus rendering it immunostimulatory which in turn can inhibit effective mRNA translation as outlined above. The introduction of modified nucleosides into *in vitro* transcribed mRNA can be used to prevent recognition and activation of RNA sensors, thus mitigating this undesired immunostimulatory activity and enhancing translation capacity (see, *e.g.*, Kariko, K. And Weissman, D. 2007, Naturally occurring nucleoside modifications suppress the immunostimulatory activity of RNA: implication for therapeutic RNA development, Curr Opin Drug Discov Devel, v.10 523-532; Pardi, N., Muramatsu, H., Weissman, D., Kariko, K., *In vitro* transcription of long RNA containing modified nucleosides in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology v.969 (Rabinovich, P.H. Ed), 2013); Kariko, K., Muramatsu, H., Welsh, F.A., Ludwig, J., Kato, H., Akira, S., Weissman, D., 2008, Incorporation of Pseudouridine Into mRNA Yields Superior Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability, Mol Ther v.16, 1833-1840. The modified nucleosides and nucleotides used in the synthesis of modified RNAs can be prepared monitored and utilized using general methods and procedures known in the art. A large variety of nucleoside modifications are available that may be incorporated alone or in combination with other modified nucleosides to some extent into the *in vitro* transcribed mRNA (see, *e.g.*, US Publication No. 2012/0251618). *In vitro* synthesis of nucleoside-modified mRNA has been reported to have reduced ability to activate immune sensors with a concomitant enhanced translational capacity.

Other components of mRNA which can be modified to provide benefit in terms of translatability and stability include the 5' and 3' untranslated regions (UTR). Optimization of the UTRs (favorable 5' and 3' UTRs can be obtained from cellular or viral RNAs), either both or independently, have been shown to increase mRNA stability and translational efficiency of *in vitro* transcribed mRNA (see, *e.g.*, Pardi, N., Muramatsu, H., Weissman, D., Kariko, K., *In vitro* transcription of long RNA containing modified nucleosides in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology v.969 (Rabinovich, P.H. Ed), 2013).

In addition to mRNA, other nucleic acid payloads may be used for this disclosure. For oligonucleotides, methods of preparation include but are not limited to chemical synthesis and enzymatic, chemical cleavage of a longer precursor, *in vitro* transcription as described above, *etc.* Methods of synthesizing DNA and RNA nucleotides are widely used and well known in the art (see, *e.g.*, Gait, M. J. (ed.) Oligonucleotide synthesis: a practical approach, Oxford [Oxfordshire], Washington, D.C.: IRL Press, 1984; and Herdewijn, P. (ed.) Oligonucleotide synthesis: methods and applications, Methods in Molecular Biology, v. 288 (Clifton, N.J.) Totowa, N.J.: Humana Press, 2005; both of which are incorporated herein by reference).

For plasmid DNA, preparation for use with this disclosure commonly utilizes but is not limited to expansion and isolation of the plasmid DNA *in vitro* in a liquid culture of bacteria containing the plasmid of interest. The presence of a gene in the plasmid of interest that encodes resistance to a particular antibiotic (penicillin, kanamycin, *etc.*) allows those bacteria containing the plasmid of interest to selectively grow in antibiotic-containing cultures. Methods of isolating plasmid DNA are widely used and well known in the art (see, *e.g.*, Heilig, J., Elbing, K. L. and Brent, R (2001) Large-Scale Preparation of Plasmid DNA. Current Protocols in Molecular Biology. 41:II:1.7:1.7.1–1.7.16; Rozkov, A., Larsson, B., Gillström, S., Björnstedt, R. and Schmidt, S. R. (2008), Large-scale production of endotoxin-free plasmids for transient expression in mammalian cell culture. Biotechnol. Bioeng., 99: 557–566; and US Patent No. 6,197,553). Plasmid isolation can be performed using a variety of commercially available kits including, but not limited to Plasmid Plus (Qiagen), GenJET plasmid MaxiPrep (Thermo) and PureYield MaxiPrep (Promega) kits as well as with commercially available reagents.

Various exemplary embodiments of the lipid nanoparticles and compositions comprising the same, and their use to deliver active or therapeutic agents such as nucleic acids to modulate gene and protein expression, are described in further detail below.

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

Unless the context requires otherwise, throughout the present specification and claims, the word "comprise" and variations thereof, such as, "comprises" and "comprising" are to be construed in an open and inclusive sense, that is, as "including, but not limited to".

Reference throughout this specification to "one embodiment" or "an embodiment" means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present disclosure. Thus, the appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this

specification are not necessarily all referring to the same embodiment. Furthermore, the features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

Unless defined otherwise, all technical and scientific terms used herein have the same  
5 meaning as is commonly understood by one of skill in the art to which this disclosure belongs. As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise.

The phrase "induce expression of a desired protein" refers to the ability of a nucleic acid to increase expression of the desired protein. To examine the extent of protein expression, a test  
10 sample (*e.g.*, a sample of cells in culture expressing the desired protein) or a test mammal (*e.g.*, a mammal such as a human or an animal model such as a rodent (*e.g.*, mouse) or a non-human primate (*e.g.*, monkey) model) is contacted with a nucleic acid (*e.g.*, nucleic acid in combination with a lipid of the present disclosure). Expression of the desired protein in the test sample or test animal is compared to expression of the desired protein in a control sample (*e.g.*, a sample of  
15 cells in culture expressing the desired protein) or a control mammal (*e.g.*, a mammal such as a human or an animal model such as a rodent (*e.g.*, mouse) or non-human primate (*e.g.*, monkey) model) that is not contacted with or administered the nucleic acid. When the desired protein is present in a control sample or a control mammal, the expression of a desired protein in a control sample or a control mammal may be assigned a value of 1.0. In some embodiments, inducing  
20 expression of a desired protein is achieved when the ratio of desired protein expression in the test sample or the test mammal to the level of desired protein expression in the control sample or the control mammal is greater than 1, for example, about 1.1, 1.5, 2.0, 5.0, or 10.0. When a desired protein is not present in a control sample or a control mammal, inducing expression of a desired protein is achieved when any measurable level of the desired protein in the test sample or the test  
25 mammal is detected. One of ordinary skill in the art will understand appropriate assays to determine the level of protein expression in a sample, for example dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, and phenotypic assays, or assays based on reporter proteins that can produce fluorescence or luminescence under appropriate conditions.

30 An "effective amount" or "therapeutically effective amount" of an active agent or therapeutic agent such as a therapeutic nucleic acid is an amount sufficient to produce the desired effect, *e.g.*, an increase or inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of the nucleic acid. An increase in expression of

a target sequence is achieved when any measurable level is detected in the case of an expression product that is not present in the absence of the nucleic acid. In the case where the expression product is present at some level prior to contact with the nucleic acid, an increase in expression is achieved when the fold increase in value obtained with a nucleic acid such as mRNA relative to control is about 1.05, 1.1, 1.2, 1.3, 1.4, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 75, 100, 250, 500, 750, 1000, 5000, 10000 or greater. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with a nucleic acid such as antisense oligonucleotide relative to the control is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%.

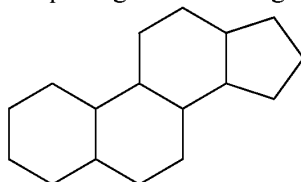
Suitable assays for measuring expression of a target gene or target sequence include, *e.g.*, examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, fluorescence or luminescence of suitable reporter proteins, as well as phenotypic assays known to those of skill in the art.

The term "nucleic acid" as used herein refers to a polymer containing at least two deoxyribonucleotides and/or ribonucleotides in either single- or double-stranded form and includes DNA, RNA, and hybrids thereof. DNA may be in the form of antisense molecules, plasmid DNA, cDNA, PCR products, or vectors. RNA may be in the form of small hairpin RNA (shRNA), messenger RNA (mRNA), antisense RNA, miRNA, micRNA, multivalent RNA, dicer substrate RNA or viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), alleles, orthologs, single nucleotide polymorphisms, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res., 19:5081 (1991); Ohtsuka et al., J. Biol. Chem., 260:2605-2608

(1985); Rossolini et al., Mol. Cell. Probes, 8:91-98 (1994)). "Nucleotides" contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. "Bases" include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural  
5 analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

The term "lipid" refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are generally characterized by being poorly soluble in water, but  
10 soluble in many organic solvents.

A "steroid" is a compound comprising the following carbon skeleton:



Non-limiting examples of steroids include cholesterol, and the like.

An "ionizable lipid" refers to a lipid capable of being charged. In some embodiments, the  
15 ionizable lipid is a cationic lipid. As used herein, a "cationic lipid" refers to a lipid capable of being positively charged. Exemplary cationic lipids include one or more amine group(s) which can or does bear the positive charge. In some embodiments, the cationic lipids are ionizable such that they can exist in a positively charged or neutral form depending on pH. The ionization of the cationic lipid affects the surface charge of the lipid nanoparticle under different pH conditions.  
20 This charge state can influence plasma protein absorption, blood clearance and tissue distribution (Semple, S.C., et al., Adv. Drug Deliv Rev 32:3-17 (1998)) as well as the ability to form endosomolytic non-bilayer structures (Hafez, I.M., et al., Gene Ther 8:1188-1196 (2001)) critical to the intracellular delivery of nucleic acids.

The term "lipid nanoparticle" or "LNP" refers to particles having at least one dimension  
25 on the order of nanometers (*e.g.*, 1-1,000 nm) which comprise components selected from ionizable lipids (*e.g.*, cationic lipids), charged lipids, neutral lipids, steroids, and/or polymer conjugated lipids. In some embodiments, LNPs are included in a formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, mRNA) to a target site of interest (*e.g.*, cell, tissue, organ, tumor, and the like). In some embodiments, the LNPs of the  
30 disclosure comprise a nucleic acid. In some embodiments, the active agent or therapeutic agent, such as a nucleic acid, may be encapsulated in the lipid portion of the lipid nanoparticle or an

aqueous space enveloped by some or all the lipid portion of the lipid nanoparticle, thereby protecting it from enzymatic degradation or other undesirable effects induced by the mechanisms of the host organism or cells, *e.g.*, an adverse immune response.

In various embodiments, the LNPs have a mean diameter of from about 30 nm to about 5 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 10 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm, and are substantially non-toxic. In certain embodiments, nucleic acids, when present in the LNPs, are resistant in aqueous solution to degradation with a nuclease. Lipid nanoparticles comprising nucleic acids and their method of preparation are disclosed in, *e.g.*, U.S. Patent Publication Nos. 2004/0142025, 2007/0042031 and PCT Pub. Nos. WO 2013/016058 and WO 15 2013/086373, the full disclosures of which are herein incorporated by reference in their entirety for all purposes.

As used herein, "encapsulated" refers to a lipid nanoparticle that provides an active agent or therapeutic agent, such as a nucleic acid (*e.g.*, mRNA), with full encapsulation, partial encapsulation, or both. In an embodiment, the nucleic acid molecules (*e.g.*, mRNA) are fully 20 encapsulated in the lipid nanoparticle. In some embodiments, greater than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of the nucleic acid molecules of a sample are fully or partially encapsulated by a lipid nanoparticle.

The term "polymer conjugated lipid" refers to a molecule comprising both a lipid portion and a polymer portion. An example of a polymer conjugated lipid is a pegylated lipid. The term 25 "pegylated lipid" refers to a molecule comprising both a lipid portion and a polyethylene glycol portion. Pegylated lipids are known in the art and include 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG) and the like.

In some embodiments, the polymer conjugated lipid is functionalized to facilitate surface modification of the LNPs. In some embodiments, the polymer conjugated lipid is modified 30 before formation of the LNP. In other embodiments, the polymer conjugated lipid is modified after formation of the LNP. In some embodiments, the modification is the addition of a targeting group (*e.g.*, an antibody). In some embodiments, the modification is the addition of a moiety that decreases clearance of the LNP.

The term "neutral lipid" refers to any of several lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, but are not limited to, phosphatidylcholines such as 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), phosphatidylethanolamines such as 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), sphingomyelins (SM), ceramides, steroids such as sterols and their derivatives. Neutral lipids may be synthetic or naturally derived.

The term "charged lipid" refers to any of a number of lipid species that exist in either a positively charged or negatively charged form independent of the pH within a useful physiological range, *e.g.*, pH ~3 to pH ~9. Charged lipids may be synthetic or naturally derived. Examples of charged lipids include phosphatidylserines, phosphatidic acids, phosphatidylglycerols, phosphatidylinositols, sterol hemisuccinates, dialkyl trimethylammonium-propanes, (*e.g.*, DOTAP, DOTMA), dialkyl dimethylaminopropanes, ethyl phosphocholines, dimethylaminoethane carbamoyl sterols (*e.g.*, DC-Chol).

As used herein, the term "aqueous solution" refers to a composition comprising water. In some embodiments, the aqueous solution consists essentially of water, salts, acids, and bases (*e.g.*, a buffer). In some embodiments, the aqueous solution is phosphate buffered saline. In some embodiments, the aqueous solution is an acetate buffered solution.

"Alkyl" refers to a straight or branched hydrocarbon chain radical consisting solely of carbon and hydrogen atoms that is saturated (*i.e.*, contains no double and/or triple bonds), having from one to thirty-six carbon atoms (C<sub>1</sub>-C<sub>36</sub> alkyl), from one to twenty-four carbon atoms (C<sub>1</sub>-C<sub>24</sub> alkyl), one to sixteen carbon atoms (C<sub>1</sub>-C<sub>16</sub> alkyl), one to twelve carbon atoms (C<sub>1</sub>-C<sub>12</sub> alkyl), six to twenty-four carbon atoms (C<sub>6</sub>-C<sub>24</sub> alkyl), one to eight carbon atoms (C<sub>1</sub>-C<sub>8</sub> alkyl) or one to six carbon atoms (C<sub>1</sub>-C<sub>6</sub> alkyl) and which is attached to the rest of the molecule by a single bond, *e.g.*, methyl, ethyl, n-propyl, 1-methylethyl (iso propyl), n-butyl, n-pentyl, 1,1-dimethylethyl (t-butyl), 3-methylhexyl, 2-methylhexyl, and the like. Unless stated otherwise specifically in the specification, an alkyl group is optionally substituted.

"Alkenyl" refers to a straight or branched hydrocarbon chain radical consisting solely of carbon and hydrogen atoms that contains at least one carbon-carbon double, having from two to twenty-four carbon atoms (C<sub>2</sub>-C<sub>24</sub> alkenyl), two to twelve carbon atoms (C<sub>2</sub>-C<sub>12</sub> alkenyl), six to twenty-four carbon atoms (C<sub>6</sub>-C<sub>24</sub> alkenyl), two to sixteen carbon atoms (C<sub>2</sub>-C<sub>16</sub> alkenyl), four to twelve carbon atoms (C<sub>4</sub>-C<sub>12</sub> alkenyl), two to eight carbon atoms (C<sub>2</sub>-C<sub>8</sub> alkenyl), or two to six

carbon atoms (C<sub>2</sub>-C<sub>6</sub> alkenyl) and which is attached to the rest of the molecule by a single bond, *e.g.*, ethenyl, n-propenyl, 1-methylethenyl, n-butenyl, n-pentenyl, 1,1-dimethylethenyl, 3-methylhexenyl, 2-methylhexenyl, and the like. Unless stated otherwise specifically in the specification, an alkenyl group is optionally substituted.

5 "Alkynyl" refers to a straight or branched hydrocarbon chain radical consisting solely of carbon and hydrogen atoms that contains at least one carbon-carbon triple bond, having from two to twenty-four carbon atoms (C<sub>2</sub>-C<sub>24</sub> alkynyl), two to twelve carbon atoms (C<sub>2</sub>-C<sub>12</sub> alkynyl), two to eight carbon atoms (C<sub>2</sub>-C<sub>8</sub> alkynyl), or two to six carbon atoms (C<sub>2</sub>-C<sub>6</sub> alkynyl) and which is attached to the rest of the molecule by a single bond, *e.g.*, ethynyl, n-propynyl, 1-methylethynyl, 10 n-butynyl, n-pentynyl, 1,1-dimethylethynyl, 3-methylhexynyl, 2-methylhexynyl, and the like. Unless stated otherwise specifically in the specification, an alkynyl group is optionally substituted.

"Alkylene" or "alkylene chain" refers to a straight or branched divalent saturated hydrocarbon chain linking the rest of the molecule to a radical group, consisting solely of carbon 15 and hydrogen. In some embodiments, an alkylene chain has from one to twenty-four carbon atoms (C<sub>1</sub>-C<sub>24</sub> alkylene), one to fifteen carbon atoms (C<sub>1</sub>-C<sub>15</sub> alkylene), one to twelve carbon atoms (C<sub>1</sub>-C<sub>12</sub> alkylene), one to eight carbon atoms (C<sub>1</sub>-C<sub>8</sub> alkylene), one to six carbon atoms (C<sub>1</sub>-C<sub>6</sub> alkylene), four to six carbon atoms (C<sub>4</sub>-C<sub>6</sub> alkylene), two to four carbon atoms (C<sub>2</sub>-C<sub>4</sub> alkylene), one to two carbon atoms (C<sub>1</sub>-C<sub>2</sub> alkylene), *e.g.*, methylene, ethylene, propylene, 20 *n*-butylene, and the like. The alkylene chain is attached to the rest of the molecule through a single bond and to the radical group through a single bond. The points of attachment of the alkylene chain to the rest of the molecule and to the radical group can be through one carbon or any two carbons within the chain. Unless stated otherwise specifically in the specification, an alkylene chain is optionally substituted.

25 "Halo" refers to a halogen substituent (*i.e.*, F, Cl, Br, or I).

The term "substituted" used herein means any of the above groups (*e.g.*, alkyl, alkenyl, and/or alkynyl) wherein at least one hydrogen atom is replaced by a bond to a non-hydrogen atoms such as, but not limited to: a halogen atom, such as F, Cl, Br, and I, cyano, -OH, or -NH<sub>2</sub>. "Optional" or "optionally" (*e.g.*, optionally substituted) means that the subsequently 30 described event or circumstances may or may not occur, and that the description includes instances where said event or circumstance occurs and instances in which it does not. For example, "optionally substituted alkyl" means that the alkyl radical may or may not be substituted and that the description includes both substituted alkyl radicals and alkyl radicals



having no substitution. In some embodiments, "optionally substituted" means a particular radical is substituted with one or more substituents selected from halo (*e.g.*, F, Cl, Br, and I).

This disclosure is also meant to encompass all pharmaceutically acceptable compounds (*e.g.*, ionizable lipids, charged lipids, neutral lipids, polymer conjugated lipids, steroids, *etc.*)

5 being isotopically labelled by having one or more atoms replaced by an atom having a different atomic mass or mass number. Examples of isotopes that can be incorporated into the disclosed compounds include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorous, fluorine, chlorine, and iodine, such as  $^2\text{H}$ ,  $^3\text{H}$ ,  $^{11}\text{C}$ ,  $^{13}\text{C}$ ,  $^{14}\text{C}$ ,  $^{13}\text{N}$ ,  $^{15}\text{N}$ ,  $^{15}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ,  $^{31}\text{P}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{18}\text{F}$ ,  $^{36}\text{Cl}$ ,  $^{123}\text{I}$ , and  $^{125}\text{I}$ , respectively. These radiolabeled compounds could be useful to help determine or  
10 measure the effectiveness of the compounds, by characterizing, for example, the site or mode of action, or binding affinity to pharmacologically important site of action. Certain isotopically labelled compounds, for example, those incorporating a radioactive isotope, are useful in drug and/or substrate tissue distribution studies. The radioactive isotopes tritium, *i.e.*,  $^3\text{H}$ , and carbon-14, *i.e.*,  $^{14}\text{C}$ , are particularly useful for this purpose in view of their ease of incorporation and  
15 ready means of detection.

Substitution with heavier isotopes such as deuterium, *i.e.*,  $^2\text{H}$ , may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or reduced dosage requirements, and hence may be preferred in some circumstances.

Substitution with positron emitting isotopes, such as  $^{11}\text{C}$ ,  $^{18}\text{F}$ ,  $^{15}\text{O}$  and  $^{13}\text{N}$ , can be useful  
20 in Positron Emission Topography (PET) studies for examining substrate receptor occupancy. Isotopically labeled compounds can generally be prepared by conventional techniques known to those skilled in the art or by processes analogous to those described in the Preparations and Examples as set out below using an appropriate isotopically labeled reagent in place of the non-labeled reagent previously employed.

25 This disclosure is also meant to encompass the *in vivo* metabolic products of the disclosed compounds. Such products may result from, for example, the oxidation, reduction, hydrolysis, amidation, esterification, and the like of the administered compound, primarily due to enzymatic processes. Accordingly, the disclosure includes compounds produced by a process comprising administering a compound of this disclosure to a mammal for a period sufficient to  
30 yield a metabolic product thereof. Such products are typically identified by administering a radiolabeled compound of the disclosure in a detectable dose to an animal, such as rat, mouse, guinea pig, monkey, or to human, allowing sufficient time for metabolism to occur, and isolating its conversion products from the urine, blood, or other biological samples.

A "pharmaceutical composition" refers to a formulation of a compound of the disclosure and a medium generally accepted in the art for the delivery of the biologically active compound to mammals, *e.g.*, humans. Such a medium includes all pharmaceutically acceptable carriers, diluents, or excipients therefor.

5 "Effective amount" or "therapeutically effective amount" refers to that amount of a compound of the disclosure which, when administered to a mammal, preferably a human, is sufficient to effect treatment in the mammal, preferably a human. The amount of a lipid nanoparticle of the disclosure which constitutes a "therapeutically effective amount" will vary depending on the compound, the condition and its severity, the manner of administration, and the  
10 age of the mammal to be treated, but can be determined routinely by one of ordinary skill in the art having regard to his own knowledge and to this disclosure.

"Treating" or "treatment" as used herein covers the treatment of the disease or condition of interest in a mammal, preferably a human, having the disease or condition of interest, and includes:

- 15 (i) preventing the disease or condition from occurring in a mammal, in particular, when such mammal is predisposed to the condition but has not yet been diagnosed as having it;
- (ii) inhibiting the disease or condition, *i.e.*, arresting its development;
- (iii) relieving the disease or condition, *i.e.*, causing regression of the disease or condition; or
- 20 (iv) relieving the symptoms resulting from the disease or condition, *i.e.*, relieving pain without addressing the underlying disease or condition. As used herein, the terms "disease" and "condition" may be used interchangeably or may be different in that the particular malady or condition may not have a known causative agent (so that etiology has not yet been worked out) and it is therefore not yet recognized as a disease but only as an undesirable condition or  
25 syndrome, wherein a more or less specific set of symptoms have been identified by clinicians.

The compounds included as components of the lipid nanoparticle or nucleic acid, or their pharmaceutically acceptable salts may contain one or more asymmetric centers and may thus give rise to enantiomers, diastereomers, and other stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (*R*)- or (*S*)- or as (*D*)- or (*L*)- for amino acids. The present  
30 disclosure is meant to include all such possible isomers, as well as their racemic and optically pure forms. Optically active (+) and (-), (*R*)- and (*S*)-, or (*D*)- and (*L*)- isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques, for example, chromatography and fractional crystallization. Conventional techniques for the

preparation/isolation of individual enantiomers include chiral synthesis from a suitable optically pure precursor or resolution of the racemate (or the racemate of a salt or derivative) using, for example, chiral high-pressure liquid chromatography (HPLC). When the compounds described herein contain olefinic double bonds or other centers of geometric asymmetry, and unless  
5 specified otherwise, it is intended that the compounds include both E and Z geometric isomers.

A "stereoisomer" refers to a compound made up of the same atoms bonded by the same bonds but having different three-dimensional structures, which are not interchangeable. The present disclosure contemplates various stereoisomers and mixtures thereof and includes "enantiomers", which refers to two stereoisomers whose molecules are non-superimposable  
10 mirror images of one another.

### ***Methods of Preparation***

As used herein, the "conventional method" for preparing lipid nanoparticles (LNPs) refers to a process that typically includes the following steps:

- (i) mixing RNA (dissolved in aqueous buffer) and LNP components (dissolved in  
15 ethanol) via a T-mixer;
- (ii) removing ethanol and exchanging buffer systems via dialysis or tangential flow filtration (TFF);
- (iii) concentrating the formed RNA containing LNP to about 1 mg/mL via TFF or centrifugation filtration; and
- 20 (iv) diluting with buffer (*e.g.*, PBS and sucrose) and freezing at -80°C.

Step (i) is performed over the course of about 2-5 hours. Step (ii) is performed over about 1-2 days. Step (iii) is performed over about 2-3 hours. Step (iv) is performed over about 0.5 hours.

In an aspect, the disclosure provides novel methods of preparing nucleic acids  
25 encompassed in lipid nanoparticles. The lipid nanoparticles may include components such as ionizable lipids (*e.g.*, cationic lipids), neutral lipids, charged lipids, steroids, and/or polymer conjugated lipids to form lipid nanoparticles. Without wishing to be bound by theory, it is thought that these lipid nanoparticles shield oligonucleotides from degradation in the serum and provide for effective delivery of oligonucleotides to cells *in vitro* and *in vivo*.

30 One embodiment provide a method for encapsulating a payload comprising a nucleic acid within lipid nanoparticles (LNPs), comprising:

i) providing an aqueous first solution comprising a plurality of LNPs comprising a plurality of ionizable lipids, wherein the first solution is substantially free of any destabilizing agents and the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0;

- 5 ii) mixing the first solution with dry payload or payload in a second aqueous solution, wherein the second aqueous solution is substantially free of any destabilizing agents, thereby encapsulating at least a majority of the payload in the LNPs in a resultant solution.

Another embodiment provides a method for encapsulating a payload comprising a nucleic acid within lipid nanoparticles (LNPs), comprising:

- 10 i) providing a dry first composition comprising a plurality of LNPs comprising a plurality of ionizable lipids;
- ii) mixing the first composition with a payload in a second aqueous solution, wherein the second aqueous solution is substantially free of any destabilizing agents, thereby encapsulating at least a majority of the payload in the LNPs in a first solution.

15 One embodiment provides a method for encapsulating a payload comprising a nucleic acid within lipid nanoparticles (LNPs), comprising:

- i) providing a dry first composition comprising a plurality of LNPs comprising a plurality of ionizable lipids;
- ii) mixing the first composition with second composition comprising dry payload;
- 20 iii) mixing the first composition and the second composition together with an aqueous first solution, wherein the first solution is substantially free of any destabilizing agents and the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0, thereby encapsulating at least a majority of the payload in the LNPs.

It was unexpectedly discovered that RNA containing LNP can be prepared using methods  
25 according to present embodiments over a much shorter period than previously known methods.

In some embodiments, a first process of mixing of LNP components (*e.g.*, cationic lipids, neutral lipids, pegylated lipids, and/or cholesterol) is performed followed by second process of dialysis or tangential flow filtration thereby forming a LNP solution comprising lipid nanoparticles (LNP) free of any destabilizing agents (*e.g.*, ethanol).

30 In certain embodiments, the first process is carried out in acetate (aqueous) buffer at a pH ranging from 5.5 to 6.5, from 5.6 to 6.6, from 5.7 to 6.7, from 5.8 to 6.8, from 5.9 to 6.9, from 6.0 to 7.0, from 5.4 to 6.4, from 5.3 to 6.3, from 5.2 to 6.2, from 5.1 to 6.1, or from 5.0 to 6.0. In some embodiments, the first process is carried out in a solution that comprises ethanol.

In some embodiments, the second process is carried out in acetate (aqueous) buffer at a pH ranging from 5.5 to 6.5, from 5.6 to 6.6, from 5.7 to 6.7, from 5.8 to 6.8, from 5.9 to 6.9, from 6.0 to 7.0, from 5.4 to 6.4, from 5.3 to 6.3, from 5.2 to 6.2, from 5.1 to 6.1, or from 5.0 to 6.0. In some embodiments,

5 In some embodiments, the time taken for the first and second processes (together) is less than 48 hours as measured from the mixing the LNP components until a next step in a process is taken (*e.g.*, a third process). In some embodiments, the time taken for the first and second processes (together) is less than 36 hours, less than 24 hours, less than 12 hours, or less than 8 hours. In some embodiments, the first and second processes (together) take more than more than  
10 12 hours, more than 18 hours, more than 24 hours, more than 36 hours, or more than 48 hours.

In certain embodiments, a third process comprises mixing the LNP solution with a (aqueous) solution comprising a payload (*e.g.*, mRNA) thereby encapsulating at least a majority of the payload in the LNPs in a payload-LNP solution.

In some embodiments, the time taken for the third process is less than 4 hours as  
15 measured from the mixing until a next step in a process is taken (*e.g.*, a fourth process). In some embodiments, the time taken for the third process is less than 3 hours, less than 2 hours, less than 1.5 hours, less than 1 hour, less than 30 minutes, less than 15 minutes, or less than 5 minutes. In some embodiments, the third process takes more than 5 minutes, more than 15 minutes, more than 30 minutes, more than 1 hour, more than 1.5 hours, more than 2 hours, or more than 3  
20 hours.

In certain embodiments, the third process is performed at ambient temperature. In some embodiments, the third process is performed at a temperature ranging from 15°C to 30°C, from 20°C to 30°C, from 25°C to 30°C, from 15°C to 25°C, or from 15°C to 20°C.

In some embodiments, a fourth process comprises diluting the payload-LNP solution  
25 with an aqueous buffer (*e.g.*, 2× phosphate buffered saline (PBS)). In certain embodiments, the fourth process further comprises mixing after diluting.

In some embodiments, the time taken for the fourth process is less than 2 hours as measured from the diluting until a next step in a process is taken (*e.g.*, a fifth process). In some embodiments, the time taken for the fourth process is less than 1.5 hours, less than 1 hour, or less  
30 than 30 minutes. In some embodiments, the time taken for the fourth process is more than 30 minutes, more than 1 hour, more than 1.5 hours, or more than 2 hours.

In some embodiments, a fifth process comprises adding a solution comprising sucrose (*e.g.*, an aqueous solution) to the solution produces by a fourth process. In certain embodiments,

the fifth process further comprises mixing after diluting. In some embodiments, the fifth process comprises filtration after diluting. In some embodiments, the aqueous buffer of the fifth process is added in a 1:1 ratio to the payload-LNP solution (*e.g.*, vol : vol or wt : wt). In some embodiments, the solution comprising sucrose further comprises PBS.

5 In some embodiments, the time taken for the fifth process is less than 30 minutes as measured from the adding until the solution produced by the fifth process is placed in a freezer (*e.g.*, a freezer set to -80°C). In some embodiments, the time taken for the fifth process is less than 20 minutes, less than 15 minutes, less than 10 minutes, or less than 5 minutes. In some  
10 more than 5 minutes, more than 10 minutes, more than 15 minutes, more than 20 minutes, or more than 25 minutes.

In some embodiments, RNA (*e.g.*, mRNA) is mixed with LNPs that do not yet encompass a payload. In some embodiments, RNA is mixed at a concentration ranging from 0.05 to 2.0 mg/mL. In some embodiments, RNA is mixed at a concentration ranging from 0.05 to 1.75, from 0.05 to 1.5, from 0.05 to 1.25, from 0.05 to 1.15, from 0.05 to 1.0, from 0.05 to 0.75,  
15 from 0.05 to 0.65, from 0.05 to 0.5, from 0.05 to 0.35, from 0.05 to 0.25, from 0.05 to 0.15, from 0.05 to 0.10, from 0.05 to 0.085, from 0.05 to 0.075 mg/mL. In certain embodiments, RNA is mixed at a concentration ranging from 0.065 to 2.0, from 0.075 to 2.0, from 0.085 to 2.0, from 1.0 to 2.0, from 1.15 to 2.0, from 1.25 to 2.0, from 1.35 to 2.0, from 1.5 to 2.0, from 1.65 to 2.0, from 1.75 to 2.0, from 1.85 to 2.0, or from 1.95 to 2.0 mg/mL. In some more specific  
20 embodiments, RNA is mixed at a maximum concentration of 1.05 mg/mL or 0.065 mg/mL. In some embodiments, the RNA is mixed in an acetate buffer at a pH ranging from 5.25 to 5.75 (*e.g.*, 5.4, 5.5, 5.6, *etc.*). In some embodiments, the RNA is mixed in a phosphate buffer at a pH ranging from 5.6 to 6.0 (*e.g.*, 5.7, 5.8, 5.9, *etc.*).

In some embodiments, the LNPs in the first solution or the first composition do not  
25 encapsulate payload. In certain embodiments, pH of the first solution is less than 7.0. In some embodiments, the pH of the first solution is less than 6.8. In certain embodiments, the pH of the first solution is less than 6.5. In some embodiments, the pH of the first solution is less than 6.4, less than 6.3, less than 6.2, less than 6.1, less than 6.0, less than 5.9, less than 5.8, or less than 5.7. In certain embodiments, the pH of the first solution is less than 5.0. In some embodiments,  
30 the pH of the first solution is less than 5.7. In certain embodiments, the pH of the first solution ranges from 4.5 to 7.4, from 5.5 to 6.5, or from 5.7 to 6.2.

In some embodiments, the difference between the pH of the first solution and the pKa of the ionizable lipids ranges from 0.1 to 1.5, from 0.1 to 1.0, from 0.1 to 0.5, from 0.2 to 0.5, from

0.5 to 0.5, from 0.3 to 0.6, from 0.2 to 0.6, or from 0.4 to 0.8. In certain embodiments, the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0. In some embodiments, the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 0.7. In certain embodiments, the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 0.5, less than 0.3 or less than 0.1. In some embodiments, the difference between the pH of the first solution and the pKa of the ionizable lipids is at least 0.1, at least 0.2, at least 0.3, at least 0.4, at least 0.5, at least 0.6, at least 0.7, at least 0.8, at least 0.9, at least 1.0, at least 1.2, or at least 1.5. In certain embodiments, the difference between the pH of the first solution and pKa of the ionizable lipids ranges from 0.3 to 1.0, from 0.2 to 0.9, from 0.1 to 1.1, from 0.1 to 0.8, or from 0.2 to 0.7.

In some embodiments, an encapsulation efficiency is at least 70%. In certain embodiments, an encapsulation efficiency is at least 80%. In some embodiments, an encapsulation efficiency is at least 85%. In certain embodiments, an encapsulation efficiency is at least 88%. In some embodiments, an encapsulation efficiency is at least 90%. In some embodiments, an encapsulation efficiency is at least 95%.

In certain embodiments, the pH of the resultant solution ranges from 6.0 to 7.8. In some embodiments, the pH of the resultant solution ranges from 6.5 to 7.8. In certain embodiments, the pH of the resultant solution ranges from 7.0 to 7.5.

In some embodiments, the particle size of the LNPs encapsulating the payload in the first or resultant solution ranges from 20 to 120 nm, or from 30 to 90 nm, from 40 to 120 nm, from 50 to 120 nm, or from 30 to 100 nm. In certain embodiments, the particle size of the LNPs encapsulating the payload in the first or resultant solution ranges from 60 to 100 nm. In some embodiments, the particle size of the LNPs encapsulating the payload in the first or resultant solution ranges from 70 to 90 nm. In certain embodiments, the particle size of the LNPs encapsulating the payload in the first or resultant solution is less than 90 nm. In some embodiments, the particle size of the LNPs encapsulating the payload in the first or resultant solution is less than 80 nm. In certain embodiments, the particle size of the LNPs encapsulating the payload in the first or resultant solution is less than 70 nm, less than 60 nm, or less than 50 nm.

In some embodiments, the destabilizing agent in the first or second solution is an organic solvent. In certain embodiments, the destabilizing agent in the first or second solution is methanol, ethanol, isopropyl alcohol, tetrahydrofuran, dimethylformamide, dimethylsulfoxide, acetonitrile, sodium dodecyl sulfate, or combinations thereof.

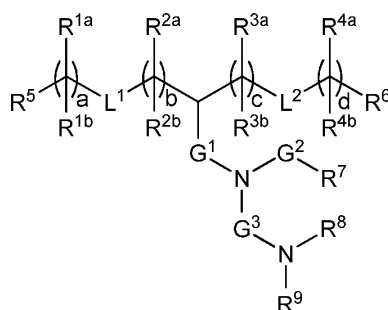
In some embodiments, the temperature of the first solution is less than 27°C, less than 25°C, or less than 22°C immediately before mixing. In certain embodiments, temperature of the second solution is less than 27°C, less than 25°C, or less than 22°C during mixing. In some embodiments, the temperature of the resultant solution is less than 27°C, less than 25°C, or less than 22°C immediately after mixing.

In certain embodiments, the plurality of LNPs in the first solution or the resultant solution comprise 20 to 90 mol% or 30 to 90 mol% of the ionizable lipids. In some embodiments, the plurality of LNPs in the first solution or the resultant solution comprise 40 to 55 mol% of the ionizable lipids. In some embodiments, the plurality of LNPs in the first solution or the resultant solution comprise 46 to 49 mol% of the ionizable lipids. In some embodiments, the plurality of LNPs in the first solution or the resultant solution comprise one or more component selected from neutral lipids, steroids, and polymer conjugated lipids.

In some embodiments, the plurality of LNPs in the first solution or the resultant solution comprise a neutral lipid at a concentration ranging from about 5 to about 15 mol% of the lipid nanoparticle. In certain embodiments, the plurality of LNPs in the first solution or the resultant solution comprise a steroid at a concentration ranging from about 30 to about 50 mol% of the lipid nanoparticle.

In some embodiments, the plurality of LNPs in the first solution or the resultant solution comprise a pegylated lipid at a concentration ranging from 0.1 to 10 mol%, from 0.1 to 5 mol%, from 0.1 to 3 mol%, from 0.1 to 2 mol%, from 0.1 to 1 mol%, 0.5 to 10 mol%, from 0.5 to 5 mol%, from 0.5 to 3 mol%, from 0.5 to 2 mol%, from 0.5 to 1 mol%, 1.0 to 10 mol%, from 1.0 to 5 mol%, from 1.0 to 3 mol%, from 1.0 to 2 mol%, 1.5 to 10 mol%, from 1.5 to 5 mol%, from 1.5 to 3 mol%, from 1.5 to 2 mol%, 2.0 to 10 mol%, from 2.0 to 5 mol%, from 2.0 to 3 mol%, 2.5 to 10 mol%, from 2.5 to 5 mol%, or from 2.5 to 3 mol% of the lipid nanoparticle.

In certain embodiments, the ionizable lipids have the following structure:



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

$L^1$  and  $L^2$  are each independently  $-O(C=O)-$ ,  $-(C=O)O-$ ,  $-C(=O)-$ ,  $-O-$ ,  $-S(O)_x-$ ,



-S-S-, -C(=O)S-, -SC(=O)-, -NR<sup>a</sup>C(=O)-, -C(=O)NR<sup>a</sup>-, -NR<sup>a</sup>C(=O)NR<sup>a</sup>-, -OC(=O)NR<sup>a</sup>-,  
-NR<sup>a</sup>C(=O)O- or a direct bond;

G<sup>1</sup> is C<sub>1</sub>-C<sub>2</sub> alkylene, -(C=O)-, -O(C=O)-, -SC(=O)-, -NR<sup>a</sup>C(=O)- or a direct bond;

G<sup>2</sup> is -C(=O)-, -(C=O)O-, -C(=O)S-, -C(=O)NR<sup>a</sup>- or a direct bond;

5 G<sup>3</sup> is C<sub>1</sub>-C<sub>6</sub> alkylene;

R<sup>a</sup> is H or C<sub>1</sub>-C<sub>12</sub> alkyl;

R<sup>1a</sup> and R<sup>1b</sup> are, at each occurrence, independently either: (a) H or C<sub>1</sub>-C<sub>12</sub> alkyl; or (b) R<sup>1a</sup>  
is H or C<sub>1</sub>-C<sub>12</sub> alkyl, and R<sup>1b</sup> together with the carbon atom to which it is bound is taken together  
with an adjacent R<sup>1b</sup> and the carbon atom to which it is bound to form a carbon-carbon double  
10 bond;

R<sup>2a</sup> and R<sup>2b</sup> are, at each occurrence, independently either: (a) H or C<sub>1</sub>-C<sub>12</sub> alkyl; or (b) R<sup>2a</sup>  
is H or C<sub>1</sub>-C<sub>12</sub> alkyl, and R<sup>2b</sup> together with the carbon atom to which it is bound is taken together  
with an adjacent R<sup>2b</sup> and the carbon atom to which it is bound to form a carbon-carbon double  
bond;

15 R<sup>3a</sup> and R<sup>3b</sup> are, at each occurrence, independently either (a): H or C<sub>1</sub>-C<sub>12</sub> alkyl; or (b) R<sup>3a</sup>  
is H or C<sub>1</sub>-C<sub>12</sub> alkyl, and R<sup>3b</sup> together with the carbon atom to which it is bound is taken together  
with an adjacent R<sup>3b</sup> and the carbon atom to which it is bound to form a carbon-carbon double  
bond;

R<sup>4a</sup> and R<sup>4b</sup> are, at each occurrence, independently either: (a) H or C<sub>1</sub>-C<sub>12</sub> alkyl; or (b) R<sup>4a</sup>  
20 is H or C<sub>1</sub>-C<sub>12</sub> alkyl, and R<sup>4b</sup> together with the carbon atom to which it is bound is taken together  
with an adjacent R<sup>4b</sup> and the carbon atom to which it is bound to form a carbon-carbon double  
bond;

R<sup>5</sup> and R<sup>6</sup> are each independently H or methyl;

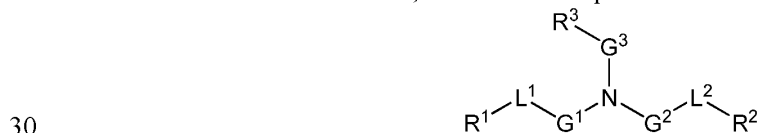
R<sup>7</sup> is C<sub>4</sub>-C<sub>20</sub> alkyl;

25 R<sup>8</sup> and R<sup>9</sup> are each independently C<sub>1</sub>-C<sub>12</sub> alkyl; or R<sup>8</sup> and R<sup>9</sup>, together with the nitrogen  
atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring;

a, b, c and d are each independently an integer from 1 to 24; and

x is 0, 1 or 2.

In certain embodiments, the ionizable lipids have the following structure:



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

one of L<sup>1</sup> or L<sup>2</sup> is -O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -S(O)<sub>x</sub>-, -S-S-, -C(=O)S-,

-SC(=O)-, -NR<sup>a</sup>C(=O)-, -C(=O)NR<sup>a</sup>-, NR<sup>a</sup>C(=O)NR<sup>a</sup>-, -OC(=O)NR<sup>a</sup>- or -NR<sup>a</sup>C(=O)O-, and the other of L<sup>1</sup> or L<sup>2</sup> is -O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -S(O)<sub>x</sub>-, -S-S-, -C(=O)S-, SC(=O)-, -NR<sup>a</sup>C(=O)-, -C(=O)NR<sup>a</sup>-, ,NR<sup>a</sup>C(=O)NR<sup>a</sup>-, -OC(=O)NR<sup>a</sup>- or -NR<sup>a</sup>C(=O)O- or a direct bond;

G<sup>1</sup> and G<sup>2</sup> are each independently unsubstituted C<sub>1</sub>-C<sub>12</sub> alkylene or C<sub>1</sub>-C<sub>12</sub> alkenylene;

5 G<sup>3</sup> is C<sub>1</sub>-C<sub>24</sub> alkylene, C<sub>1</sub>-C<sub>24</sub> alkenylene, C<sub>3</sub>-C<sub>8</sub> cycloalkylene, C<sub>3</sub>-C<sub>8</sub> cycloalkenylene;

R<sup>a</sup> is H or C<sub>1</sub>-C<sub>12</sub> alkyl;

R<sup>1</sup> and R<sup>2</sup> are each independently C<sub>6</sub>-C<sub>24</sub> alkyl or C<sub>6</sub>-C<sub>24</sub> alkenyl;

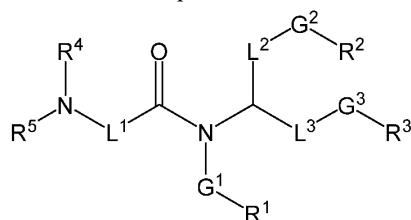
R<sup>3</sup> is H, OR<sup>5</sup>, CN, -C(=O)OR<sup>4</sup>, -OC(=O)R<sup>4</sup> or -NR<sup>5</sup>C(=O)R<sup>4</sup>;

R<sup>4</sup> is C<sub>1</sub>-C<sub>12</sub> alkyl;

10 R<sup>5</sup> is H or C<sub>1</sub>-C<sub>6</sub> alkyl; and

x is 0, 1 or 2.

In some embodiments, the ionizable lipids have the following structure:



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

15 R<sup>1</sup> is optionally substituted C<sub>1</sub>-C<sub>24</sub> alkyl or optionally substituted C<sub>2</sub>-C<sub>24</sub> alkenyl;

R<sup>2</sup> and R<sup>3</sup> are each independently optionally substituted C<sub>1</sub>-C<sub>36</sub> alkyl;

R<sup>4</sup> and R<sup>5</sup> are each independently optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, or R<sup>4</sup> and R<sup>5</sup> join, along with the N to which they are attached, to form a heterocyclyl or heteroaryl;

L<sup>1</sup>, L<sup>2</sup>, and L<sup>3</sup> are each independently optionally substituted C<sub>1</sub>-C<sub>18</sub> alkylene;

20 G<sup>1</sup> is a direct bond, -(CH<sub>2</sub>)<sub>n</sub>O(C=O)-, -(CH<sub>2</sub>)<sub>n</sub>(C=O)O-, or -(C=O)-;

G<sup>2</sup> and G<sup>3</sup> are each independently -(C=O)O- or -O(C=O)-; and

n is an integer greater than 0.

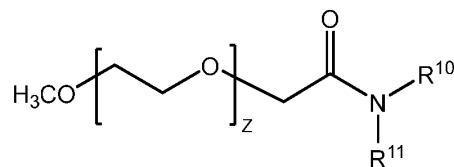
In some embodiments, the ionizable lipids have one of the structures in Table A or B. In certain embodiments, the ionizable lipids have one of the structures in Table C.

25 In certain embodiments, the plurality of LNPs in the first solution or the resultant solution comprise one or more neutral lipids selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE and SM.

In some embodiments, the neutral lipid is DSPC. In some embodiments, the steroid is cholesterol. In certain embodiments, the polymer conjugated lipid is a pegylated lipid. In some

embodiments, the pegylated lipid is PEG-DAG, PEG-PE, PEG-S-DAG, PEG-cer or a PEG dialkylalkoxypropylcarbamate.

In certain embodiments, the pegylated lipid has the following:



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

$\text{R}^{10}$  and  $\text{R}^{11}$  are each independently a straight or branched alkyl, alkenyl, or alkynyl containing from 10 to 30 carbon atoms, wherein each alkyl, alkenyl, or alkynyl is optionally substituted with at least one fluoro; and

$z$  is an integer ranging from 30 to 60.

10 In some embodiments,  $\text{R}^{10}$  and  $\text{R}^{11}$  are each independently straight alkyl chain containing from 12 to 16 carbon atoms. In certain embodiments, the  $z$  ranges from 45 to 50.

In some embodiments, the payload is DNA, siRNA, mini circle RNA, PNA, aptamer, guide RNA, PE guide RNA, saRNA, circular RNA, antisense RNA, messenger RNA, Cas9 mRNA, ribonucleoprotein, or a combination thereof. In certain embodiments, the payload is

15 mRNA.

In some embodiments, the method further comprises administering the payload encapsulated in the LNPs to a patient in need thereof.

One embodiment provides a method for administering a payload encapsulated in a lipid nanoparticle (LNP) to a patient in need thereof, the method comprising administering the lipid  
20 nanoparticle prepared according to any one of the embodiments of this disclosure.

In some embodiments, the administering is performed within 72 hours of the mixing. In certain embodiments, the administering is performed within 24 hours of the mixing. In some embodiments, the administering is performed within 12 hours of the mixing. In certain embodiments, the administering is performed within 4 hours of the mixing.

25 In some embodiments, the amount of payload is less than 1.5 mg per kg of the patient. In certain embodiments, the amount of payload is less than 1.0 mg per kg of the patient. In some embodiments, the amount of payload is less than 0.5 mg per kg of the patient.

In some embodiments, the amount of payload is less than 150 micrograms. In certain embodiments, the amount of payload is less than 100 micrograms or less than 75 micrograms. In  
30 some embodiments, the amount of payload ranges from 1 to 30 micrograms.

One embodiment provides a pharmaceutical composition comprising the LNP produced by the method of this disclosure and a pharmaceutically acceptable diluent or excipient.

Another embodiment provides a kit comprising:

- i) an aqueous first solution comprising a plurality of LNPs comprising a plurality of ionizable lipids, wherein the first solution is substantially free of any destabilizing agents and the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0; and
- ii) dry payload comprising a nucleic acid or payload comprising a nucleic acid in a second aqueous solution that is substantially free of any destabilizing agents.

Another embodiment provides a kit comprising an aqueous first solution comprising a plurality of LNPs comprising a plurality of ionizable lipids, wherein the first solution is substantially free of any destabilizing agents and the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0.

One embodiment provides a kit comprising a dry first composition comprising

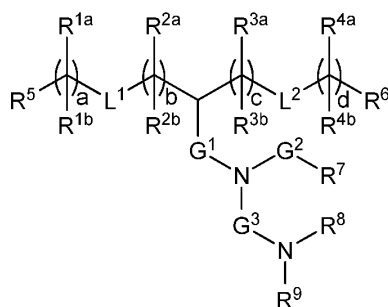
- i) a dry plurality of LNPs comprising a plurality of ionizable lipids; and
- ii) a first solution that is substantially free of any destabilizing agents and the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0.

In some embodiments, the kit further comprises dry payload comprising a nucleic acid or payload comprising a nucleic acid in a second aqueous solution that is substantially free of any destabilizing agents. In some embodiments, the kit further comprises instructions for mixing the first solution with the dry LNPs, the dry payload, the second solution, or combinations thereof.

In some embodiments, a majority of the payload is encapsulated by the LNPs when the first solution is mixed with the dry payload, the second solution, or combinations thereof.

In some embodiments, the kit includes one or more unit doses of a payload comprising a nucleic acid. In some embodiments, the unit dose is less than 1.5 mg per kg of the patient. In some embodiments, the unit dose is less than 1.0 mg per kg of the patient. In certain embodiments, the unit dose is less than 0.5 mg per kg of the patient. In some embodiments, the unit dose is less than 150 micrograms. In certain embodiments, the unit dose is less than 100 micrograms. In certain embodiments, the unit dose is less than 75 micrograms. In some embodiments, the unit dose ranges from 1 to 30 micrograms.

In one embodiment, the ionizable lipid has the following structure:



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

$L^1$  and  $L^2$  are each independently  $-O(C=O)-$ ,  $-(C=O)O-$ ,  $-C(=O)-$ ,  $-O-$ ,  $-S(O)_x-$ ,  $-S-S-$ ,  $-C(=O)S-$ ,  $-SC(=O)-$ ,  $-NR^aC(=O)-$ ,  $-C(=O)NR^a-$ ,  $-NR^aC(=O)NR^a-$ ,

5  $-OC(=O)NR^a-$ ,  $-NR^aC(=O)O-$  or a direct bond;

$G^1$  is  $C_1-C_2$  alkylene,  $-(C=O)-$ ,  $-O(C=O)-$ ,  $-SC(=O)-$ ,  $-NR^aC(=O)-$  or a direct bond;

$G^2$  is  $-C(=O)-$ ,  $-(C=O)O-$ ,  $-C(=O)S-$ ,  $-C(=O)NR^a-$  or a direct bond;

$G^3$  is  $C_1-C_6$  alkylene;

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

10  $R^{1a}$  and  $R^{1b}$  are, at each occurrence, independently either: (a) H or  $C_1-C_{12}$  alkyl; or (b)  $R^{1a}$  is H or  $C_1-C_{12}$  alkyl, and  $R^{1b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{1b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond;

$R^{2a}$  and  $R^{2b}$  are, at each occurrence, independently either: (a) H or  $C_1-C_{12}$  alkyl; or (b)  $R^{2a}$  is H or  $C_1-C_{12}$  alkyl, and  $R^{2b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{2b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond;

$R^{3a}$  and  $R^{3b}$  are, at each occurrence, independently either: (a) H or  $C_1-C_{12}$  alkyl; or (b)  $R^{3a}$  is H or  $C_1-C_{12}$  alkyl, and  $R^{3b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{3b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond;

$R^{4a}$  and  $R^{4b}$  are, at each occurrence, independently either: (a) H or  $C_1-C_{12}$  alkyl; or (b)  $R^{4a}$  is H or  $C_1-C_{12}$  alkyl, and  $R^{4b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{4b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond;

$R^5$  and  $R^6$  are each independently H or methyl;

$R^7$  is  $C_4-C_{20}$  alkyl;

$R^8$  and  $R^9$  are each independently  $C_1$ - $C_{12}$  alkyl; or  $R^8$  and  $R^9$ , together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring;

a, b, c and d are each independently an integer from 1 to 24; and

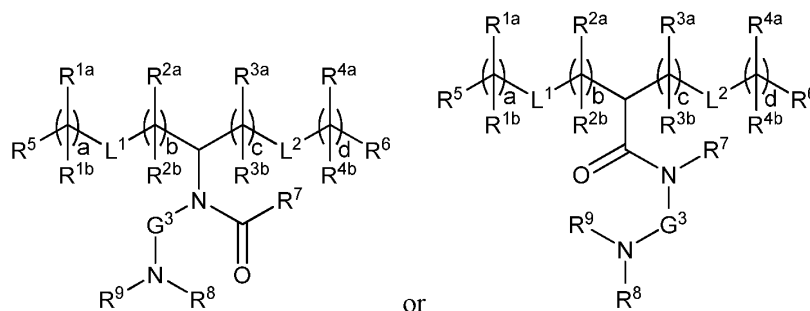
x is 0, 1 or 2.

5 In some embodiments,  $L^1$  and  $L^2$  are each independently  $-O(C=O)-$ ,  $-(C=O)O-$  or a direct bond. In other embodiments,  $G^1$  and  $G^2$  are each independently  $-(C=O)-$  or a direct bond. In some different embodiments,  $L^1$  and  $L^2$  are each independently  $-O(C=O)-$ ,  $-(C=O)O-$  or a direct bond; and  $G^1$  and  $G^2$  are each independently  $-(C=O)-$  or a direct bond.

10 In some different embodiments,  $L^1$  and  $L^2$  are each independently  $-C(=O)-$ ,  $-O-$ ,  $-S(O)_x-$ ,  $-S-S-$ ,  $-C(=O)S-$ ,  $-SC(=O)-$ ,  $-NR^a-$ ,  $-NR^aC(=O)-$ ,  $-C(=O)NR^a-$ ,  $-NR^aC(=O)NR^a$ ,  $-OC(=O)NR^a-$ ,  $-NR^aC(=O)O-$ ,  $-NR^aS(O)_xNR^a-$ ,  $-NR^aS(O)_x-$  or  $-S(O)_xNR^a-$ .

In other of the foregoing embodiments, the ionizable lipid has one of the following

15 structures:



In any of the foregoing embodiments, one of  $L^1$  or  $L^2$  is  $-O(C=O)-$ . For example, in some embodiments each of  $L^1$  and  $L^2$  are  $-O(C=O)-$ .

In some different embodiments of any of the foregoing, one of  $L^1$  or  $L^2$  is  $-(C=O)O-$ . For example, in some embodiments each of  $L^1$  and  $L^2$  is  $-(C=O)O-$ .

In different embodiments, one of  $L^1$  or  $L^2$  is a direct bond. As used herein, a "direct bond" means the group (*e.g.*,  $L^1$  or  $L^2$ ) is absent. For example, in some embodiments each of  $L^1$  and  $L^2$  is a direct bond.

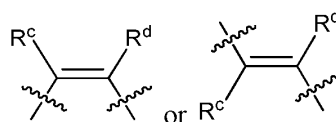
In other different embodiments of the foregoing, for at least one occurrence of  $R^{1a}$  and  $R^{1b}$ ,  $R^{1a}$  is H or  $C_1$ - $C_{12}$  alkyl, and  $R^{1b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{1b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond.

In still other different embodiments, for at least one occurrence of  $R^{4a}$  and  $R^{4b}$ ,  $R^{4a}$  is H or  $C_1$ - $C_{12}$  alkyl, and  $R^{4b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{4b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond.

In more embodiments, for at least one occurrence of  $R^{2a}$  and  $R^{2b}$ ,  $R^{2a}$  is H or  $C_1$ - $C_{12}$  alkyl, and  $R^{2b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{2b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond.

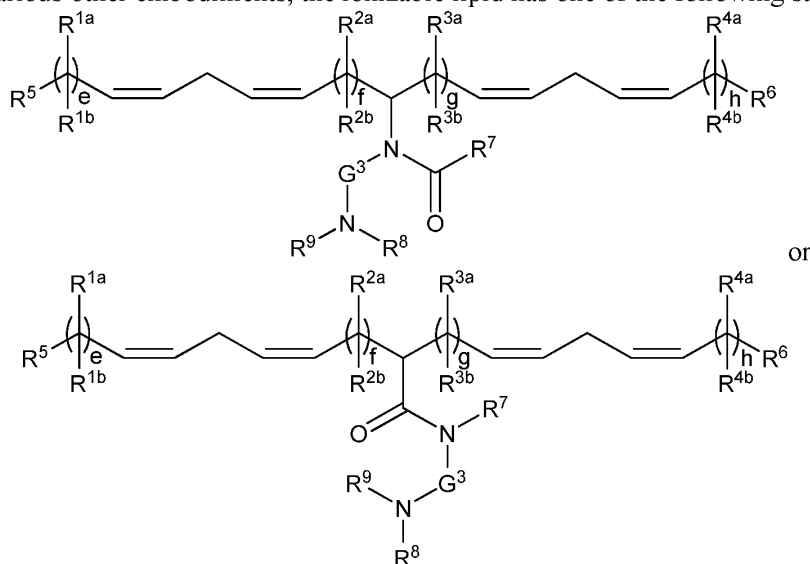
In other different embodiments of any of the foregoing, for at least one occurrence of  $R^{3a}$  and  $R^{3b}$ ,  $R^{3a}$  is H or  $C_1$ - $C_{12}$  alkyl, and  $R^{3b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{3b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond.

It is understood that "carbon-carbon" double bond refers to one of the following structures:



wherein  $R^c$  and  $R^d$  are, at each occurrence, independently H or a substituent. For example, in some embodiments  $R^c$  and  $R^d$  are, at each occurrence, independently H,  $C_1$ - $C_{12}$  alkyl or cycloalkyl, for example H or  $C_1$ - $C_{12}$  alkyl.

In various other embodiments, the ionizable lipid has one of the following structures:



wherein e, f, g and h are each independently an integer from 1 to 12.

In various embodiments, e, f, g and h are each independently an integer from 4 to 10.

In certain embodiments of the foregoing, a, b, c, and d are each independently an integer from 2 to 12 or an integer from 4 to 12. In other embodiments, a, b, c, and d are each independently an integer from 8 to 12 or 5 to 9. In some certain embodiments, a is 0. In some embodiments, a is 1. In other embodiments, a is 2. In more embodiments, a is 3. In yet other  
5 embodiments, a is 4. In some embodiments, a is 5. In other embodiments, a is 6. In more embodiments, a is 7. In yet other embodiments, a is 8. In some embodiments, a is 9. In other embodiments, a is 10. In more embodiments, a is 11. In yet other embodiments, a is 12. In some embodiments, a is 13. In other embodiments, a is 14. In more embodiments, a is 15. In yet other embodiments, a is 16.

10 In some embodiments, b is 1. In other embodiments, b is 2. In more embodiments, b is 3. In yet other embodiments, b is 4. In some embodiments, b is 5. In other embodiments, b is 6. In more embodiments, b is 7. In yet other embodiments, b is 8. In some embodiments, b is 9. In other embodiments, b is 10. In more embodiments, b is 11. In yet other embodiments, b is 12. In some embodiments, b is 13. In other embodiments, b is 14. In more embodiments, b is 15. In yet  
15 other embodiments, b is 16.

In some embodiments, c is 1. In other embodiments, c is 2. In more embodiments, c is 3. In yet other embodiments, c is 4. In some embodiments, c is 5. In other embodiments, c is 6. In more embodiments, c is 7. In yet other embodiments, c is 8. In some embodiments, c is 9. In other embodiments, c is 10. In more embodiments, c is 11. In yet other embodiments, c is 12. In  
20 some embodiments, c is 13. In other embodiments, c is 14. In more embodiments, c is 15. In yet other embodiments, c is 16.

In some certain embodiments, d is 0. In some embodiments, d is 1. In other embodiments, d is 2. In more embodiments, d is 3. In yet other embodiments, d is 4. In some embodiments, d is 5. In other embodiments, d is 6. In more embodiments, d is 7. In yet other  
25 embodiments, d is 8. In some embodiments, d is 9. In other embodiments, d is 10. In more embodiments, d is 11. In yet other embodiments, d is 12. In some embodiments, d is 13. In other embodiments, d is 14. In more embodiments, d is 15. In yet other embodiments, d is 16.

In some embodiments, e is 1. In other embodiments, e is 2. In more embodiments, e is 3. In yet other embodiments, e is 4. In some embodiments, e is 5. In other embodiments, e is 6. In  
30 more embodiments, e is 7. In yet other embodiments, e is 8. In some embodiments, e is 9. In other embodiments, e is 10. In more embodiments, e is 11. In yet other embodiments, e is 12.

In some embodiments, f is 1. In other embodiments, f is 2. In more embodiments, f is 3. In yet other embodiments, f is 4. In some embodiments, f is 5. In other embodiments, f is 6. In



more embodiments, f is 7. In yet other embodiments, f is 8. In some embodiments, f is 9. In other embodiments, f is 10. In more embodiments, f is 11. In yet other embodiments, f is 12.

In some embodiments, g is 1. In other embodiments, g is 2. In more embodiments, g is 3. In yet other embodiments, g is 4. In some embodiments, g is 5. In other embodiments, g is 6. In  
5 more embodiments, g is 7. In yet other embodiments, g is 8. In some embodiments, g is 9. In other embodiments, g is 10. In more embodiments, g is 11. In yet other embodiments, g is 12.

In some embodiments, h is 1. In other embodiments, e is 2. In more embodiments, h is 3. In yet other embodiments, h is 4. In some embodiments, e is 5. In other embodiments, h is 6. In more embodiments, h is 7. In yet other embodiments, h is 8. In some embodiments, h is 9. In  
10 other embodiments, h is 10. In more embodiments, h is 11. In yet other embodiments, h is 12.

In some other various embodiments, a and d are the same. In some other embodiments, b and c are the same. In some other specific embodiments a and d are the same and b and c are the same.

The sum of a and b and the sum of c and d are factors which may be varied to obtain a  
15 lipid having the desired properties. In one embodiment, a and b are chosen such that their sum is an integer ranging from 14 to 24. In other embodiments, c and d are chosen such that their sum is an integer ranging from 14 to 24. In further embodiment, the sum of a and b and the sum of c and d are the same. For example, in some embodiments the sum of a and b and the sum of c and d are both the same integer which may range from 14 to 24. In still more embodiments, a, b, c and d  
20 are selected such that the sum of a and b and the sum of c and d is 12 or greater.

The substituents at  $R^{1a}$ ,  $R^{2a}$ ,  $R^{3a}$ , and  $R^{4a}$  are not particularly limited. In some embodiments, at least one of  $R^{1a}$ ,  $R^{2a}$ ,  $R^{3a}$ , and  $R^{4a}$  is H. In certain embodiments  $R^{1a}$ ,  $R^{2a}$ ,  $R^{3a}$ , and  $R^{4a}$  are H at each occurrence. In certain other embodiments at least one of  $R^{1a}$ ,  $R^{2a}$ ,  $R^{3a}$ , and  $R^{4a}$  is  $C_1$ - $C_{12}$  alkyl. In certain other embodiments at least one of  $R^{1a}$ ,  $R^{2a}$ ,  $R^{3a}$ , and  $R^{4a}$  is  $C_1$ - $C_8$   
25 alkyl. In certain other embodiments at least one of  $R^{1a}$ ,  $R^{2a}$ ,  $R^{3a}$ , and  $R^{4a}$  is  $C_1$ - $C_6$  alkyl. In some of the foregoing embodiments, the  $C_1$ - $C_8$  alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In certain embodiments of the foregoing,  $R^{1a}$ ,  $R^{1b}$ ,  $R^{4a}$  and  $R^{4b}$  are  $C_1$ - $C_{12}$  alkyl at each occurrence.

30 In further embodiments of the foregoing, at least one of  $R^{1b}$ ,  $R^{2b}$ ,  $R^{3b}$  and  $R^{4b}$  is H or  $R^{1b}$ ,  $R^{2b}$ ,  $R^{3b}$  and  $R^{4b}$  are H at each occurrence.

In certain embodiments of the foregoing,  $R^{1b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{1b}$  and the carbon atom to which it is bound to form a

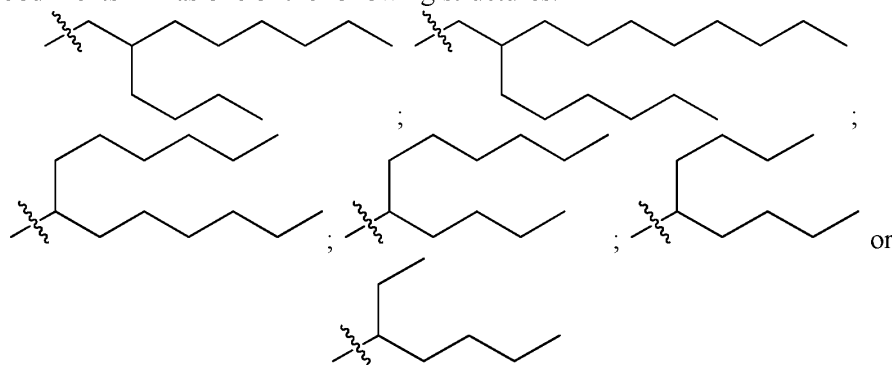
carbon-carbon double bond. In other embodiments of the foregoing  $R^{4b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{4b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond.

The substituents at  $R^5$  and  $R^6$  are not particularly limited in the foregoing embodiments.

- 5 In certain embodiments one of  $R^5$  or  $R^6$  is methyl. In other embodiments each of  $R^5$  or  $R^6$  is methyl.

- The substituents at  $R^7$  are not particularly limited in the foregoing embodiments. In certain embodiments  $R^7$  is  $C_6$ - $C_{16}$  alkyl. In some other embodiments,  $R^7$  is  $C_6$ - $C_9$  alkyl. In some of these embodiments,  $R^7$  is substituted with  $-(C=O)OR^b$ ,  $-O(C=O)R^b$ ,  $-C(=O)R^b$ ,  $-OR^b$ ,  $-S(O)_xR^b$ ,  $-S-SR^b$ ,  $-C(=O)SR^b$ ,  $-SC(=O)R^b$ ,  $-NR^aR^b$ ,  $-NR^aC(=O)R^b$ ,  $-C(=O)NR^aR^b$ ,  $-NR^aC(=O)NR^aR^b$ ,  $-OC(=O)NR^aR^b$ ,  $-NR^aC(=O)OR^b$ ,  $-NR^aS(O)_xNR^aR^b$ ,  $-NR^aS(O)_xR^b$  or  $-S(O)_xNR^aR^b$ , wherein:  $R^a$  is H or  $C_1$ - $C_{12}$  alkyl;  $R^b$  is  $C_1$ - $C_{15}$  alkyl; and x is 0, 1 or 2. For example, in some embodiments  $R^7$  is substituted with  $-(C=O)OR^b$  or  $-O(C=O)R^b$ .

- 15 In various of the foregoing embodiments,  $R^b$  is branched  $C_1$ - $C_{15}$  alkyl. For example, in some embodiments  $R^b$  has one of the following structures:



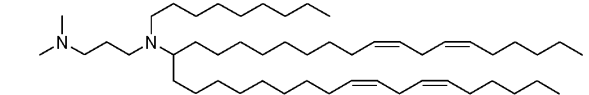
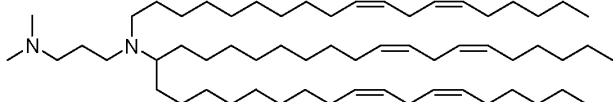
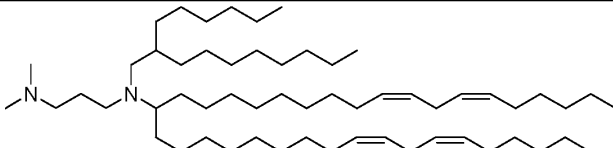
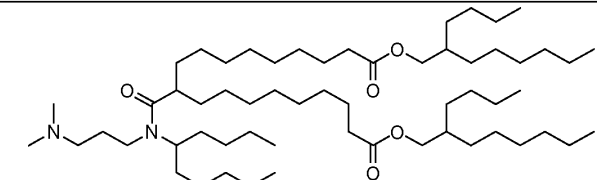
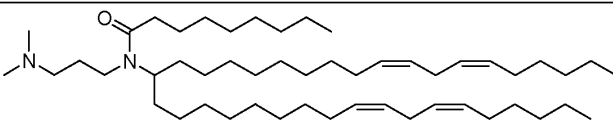
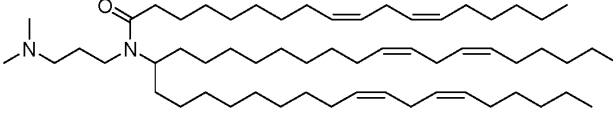
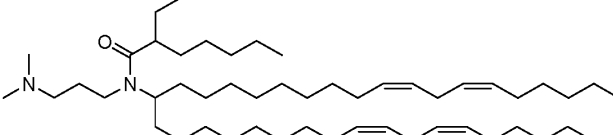
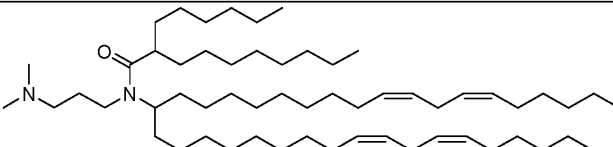
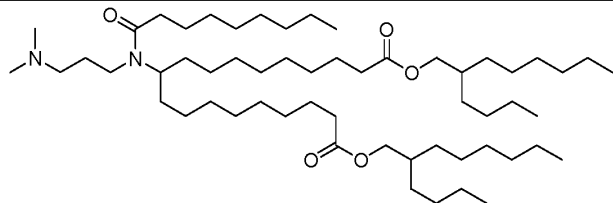
- 20 In certain other of the foregoing embodiments, one of  $R^8$  or  $R^9$  is methyl. In other embodiments, both  $R^8$  and  $R^9$  are methyl.

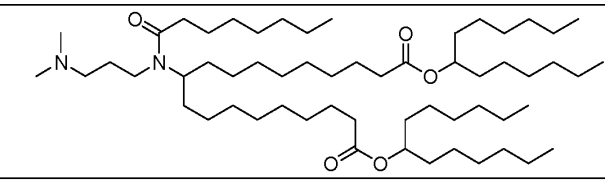
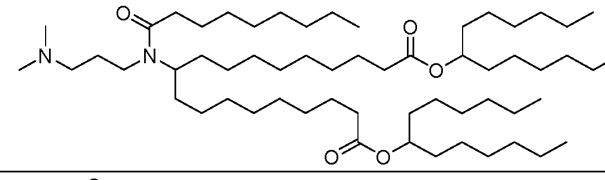
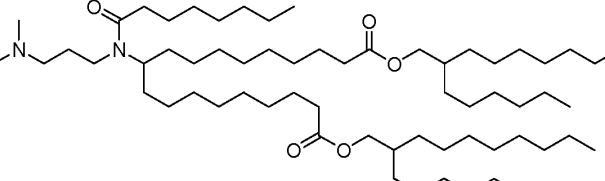
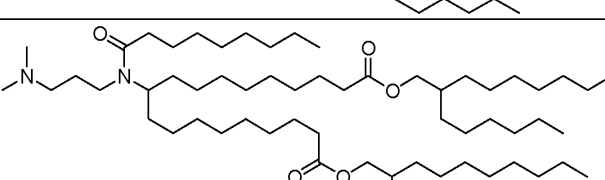
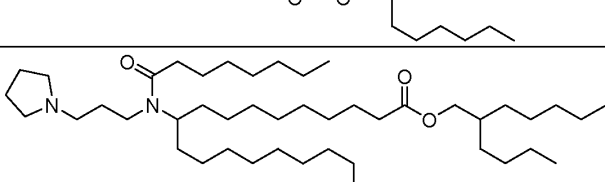
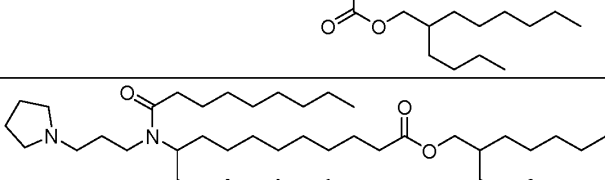
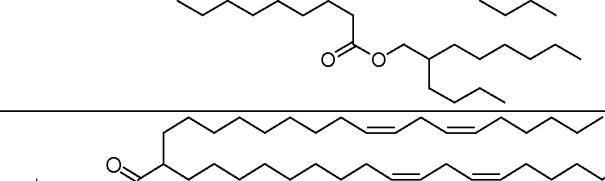
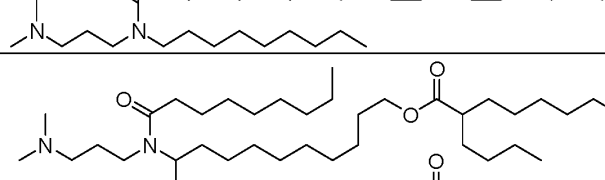
- In some different embodiments,  $R^8$  and  $R^9$ , together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring. In some embodiments of the foregoing,  $R^8$  and  $R^9$ , together with the nitrogen atom to which they are attached, form a 5-membered heterocyclic ring, for example a pyrrolidinyl ring. In some different embodiments of the foregoing,  $R^8$  and  $R^9$ , together with the nitrogen atom to which they are attached, form a 6-membered heterocyclic ring, for example a piperazinyl ring.

In still other embodiments of the foregoing ionizable lipids,  $G^3$  is  $C_2$ - $C_4$  alkylene, for example  $C_3$  alkylene.

In various different embodiments, the ionizable lipid has one of the structures set forth in Table A below.

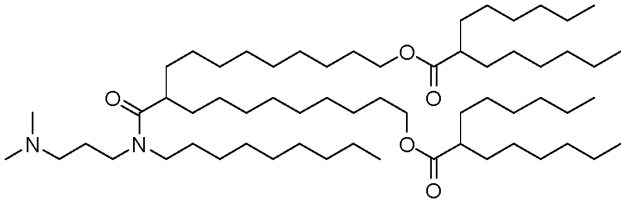
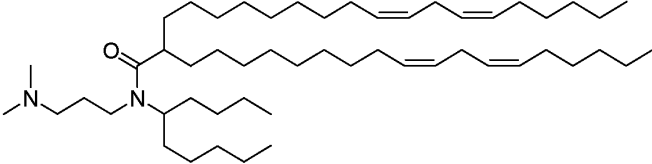
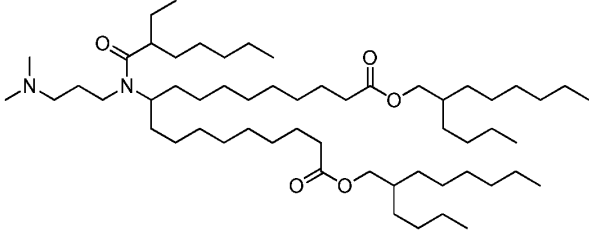
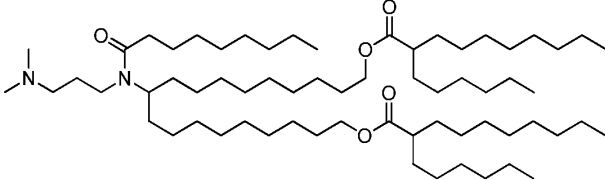
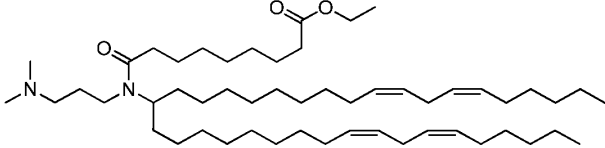
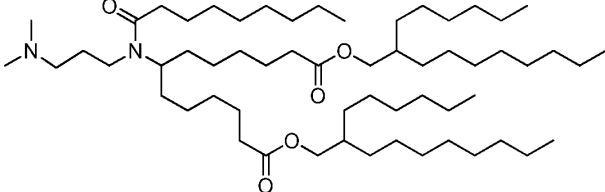
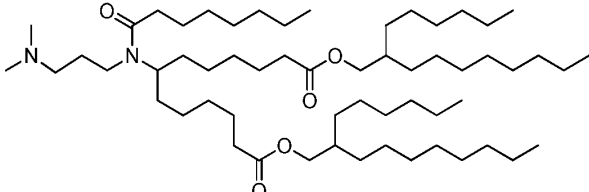
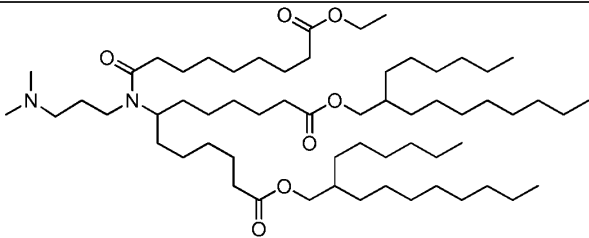
Table A. Representative ionizable lipids

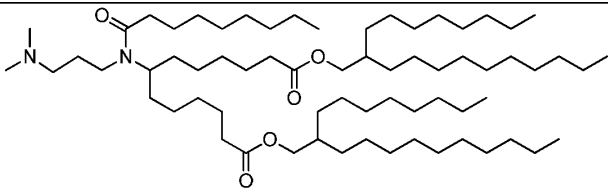
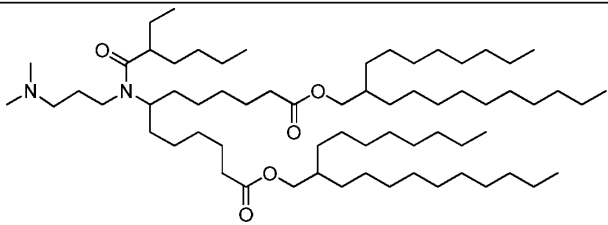
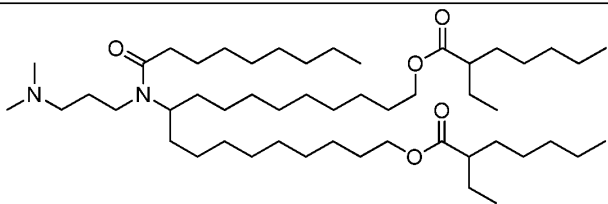
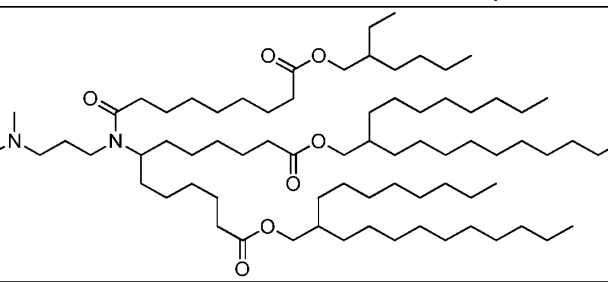
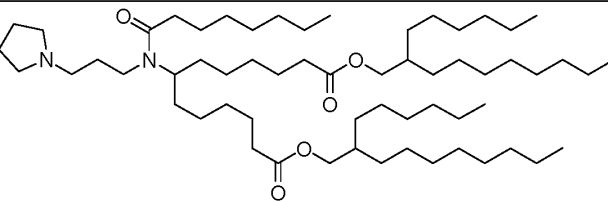
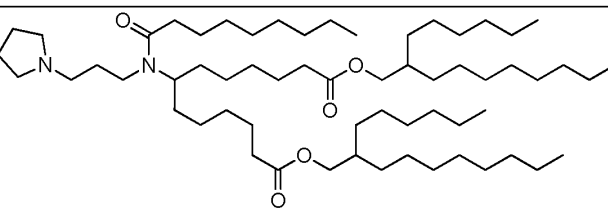
| No. | Structure                                                                            |
|-----|--------------------------------------------------------------------------------------|
| A-1 |    |
| A-2 |    |
| A-3 |    |
| A-4 |   |
| A-5 |  |
| A-6 |  |
| A-7 |  |
| A-8 |  |
| A-9 |  |

| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| A-10 |    |
| A-11 |    |
| A-12 |    |
| A-13 |    |
| A-14 |   |
| A-15 |  |
| A-16 |  |
| A-17 |  |

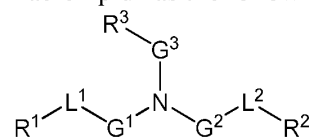
| No.  | Structure |
|------|-----------|
| A-18 |           |
| A-19 |           |
| A-20 |           |
| A-21 |           |
| A-22 |           |
| A-23 |           |
| A-24 |           |

| No.  | Structure |
|------|-----------|
| A-25 |           |
| A-26 |           |
| A-27 |           |
| A-28 |           |
| A-29 |           |
| A-30 |           |
| A-31 |           |
| A-32 |           |

| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| A-33 |    |
| A-34 |    |
| A-35 |    |
| A-36 |   |
| A-37 |  |
| A-38 |  |
| A-39 |  |
| A-40 |  |

| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| A-41 |    |
| A-42 |    |
| A-43 |    |
| A-44 |   |
| A-45 |  |
| A-46 |  |

In one embodiment, the ionizable lipid has the following structure:



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

one of  $\text{L}^1$  or  $\text{L}^2$  is  $-\text{O}(\text{C}=\text{O})-$ ,  $-(\text{C}=\text{O})\text{O}-$ ,  $-\text{C}(=\text{O})-$ ,  $-\text{O}-$ ,  $-\text{S}(\text{O})_x-$ ,  $-\text{S}-\text{S}-$ ,  
 5  $-\text{C}(=\text{O})\text{S}-$ ,  $\text{SC}(=\text{O})-$ ,  $-\text{NR}^a\text{C}(=\text{O})-$ ,  $-\text{C}(=\text{O})\text{NR}^a-$ ,  $\text{NR}^a\text{C}(=\text{O})\text{NR}^a-$ ,  $-\text{OC}(=\text{O})\text{NR}^a-$  or



-NR<sup>a</sup>C(=O)O-, and the other of L<sup>1</sup> or L<sup>2</sup> is -O(C=O)-, -(C=O)O-, -C(=O)-, -O-, -S(O)<sub>x</sub>-,  
 -S-S-, -C(=O)S-, SC(=O)-, -NR<sup>a</sup>C(=O)-, -C(=O)NR<sup>a</sup>-, ,NR<sup>a</sup>C(=O)NR<sup>a</sup>-, -OC(=O)NR<sup>a</sup>- or  
 -NR<sup>a</sup>C(=O)O- or a direct bond;

G<sup>1</sup> and G<sup>2</sup> are each independently unsubstituted C<sub>1</sub>-C<sub>12</sub> alkylene or C<sub>1</sub>-C<sub>12</sub> alkenylene;

5 G<sup>3</sup> is C<sub>1</sub>-C<sub>24</sub> alkylene, C<sub>1</sub>-C<sub>24</sub> alkenylene, C<sub>3</sub>-C<sub>8</sub> cycloalkylene, C<sub>3</sub>-C<sub>8</sub> cycloalkenylene;

R<sup>a</sup> is H or C<sub>1</sub>-C<sub>12</sub> alkyl;

R<sup>1</sup> and R<sup>2</sup> are each independently C<sub>6</sub>-C<sub>24</sub> alkyl or C<sub>6</sub>-C<sub>24</sub> alkenyl;

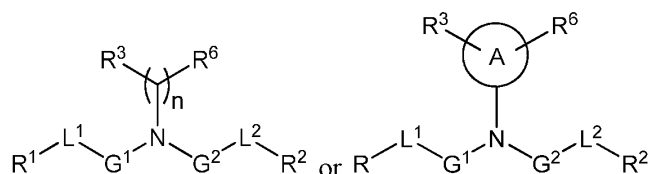
R<sup>3</sup> is H, OR<sup>5</sup>, CN, -C(=O)OR<sup>4</sup>, -OC(=O)R<sup>4</sup> or -NR<sup>5</sup>C(=O)R<sup>4</sup>;

R<sup>4</sup> is C<sub>1</sub>-C<sub>12</sub> alkyl;

10 R<sup>5</sup> is H or C<sub>1</sub>-C<sub>6</sub> alkyl; and

x is 0, 1 or 2.

In some of the foregoing embodiments, the ionizable lipid has one of the following structures:



15 wherein:

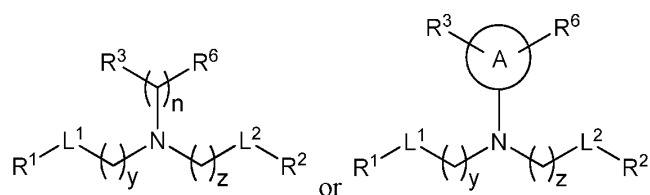
A is a 3 to 8-membered cycloalkyl or cycloalkylene ring;

R<sup>6</sup> is, at each occurrence, independently H, OH or C<sub>1</sub>-C<sub>24</sub> alkyl; and

n is an integer ranging from 1 to 15.

In other embodiments of the foregoing, the ionizable lipid has one of the following

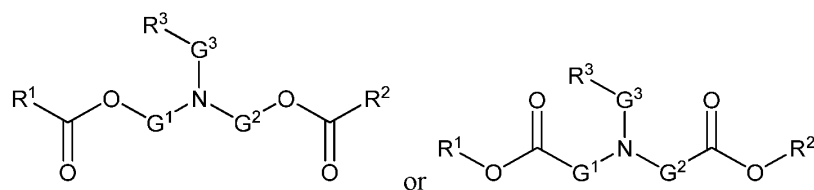
20 structures:



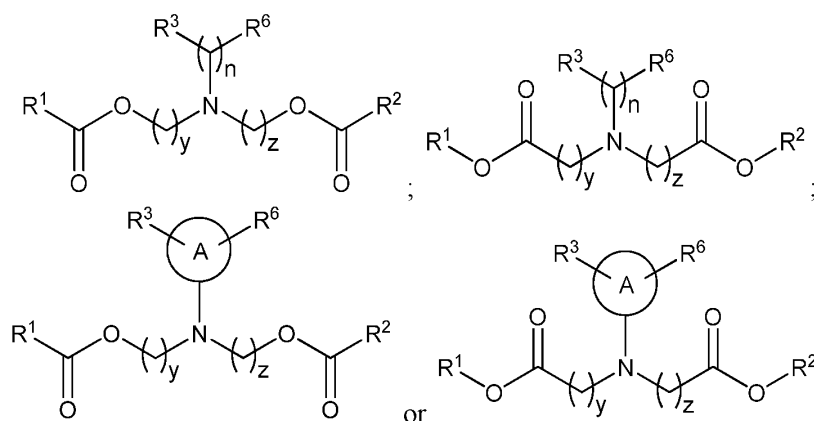
wherein y and z are each independently integers ranging from 1 to 12.

In any of the foregoing embodiments, one of L<sup>1</sup> or L<sup>2</sup> is -O(C=O)-. For example, in some  
 embodiments each of L<sup>1</sup> and L<sup>2</sup> are -O(C=O)-. In some different embodiments of any of the  
 25 foregoing, L<sup>1</sup> and L<sup>2</sup> are each independently -(C=O)O- or -O(C=O)-. For example, in some  
 embodiments each of L<sup>1</sup> and L<sup>2</sup> is -(C=O)O-.

In some different embodiments of the foregoing, the ionizable lipid has one of the  
 following structures:



In some of the foregoing embodiments, the ionizable lipid has one of the following structures:



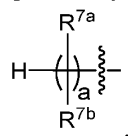
In some of the foregoing embodiments,  $n$  is an integer ranging from 2 to 12, for example from 2 to 8 or from 2 to 4. For example, in some embodiments,  $n$  is 3, 4, 5 or 6. In some embodiments,  $n$  is 3. In some embodiments,  $n$  is 4. In some embodiments,  $n$  is 5. In some embodiments,  $n$  is 6.

In some other of the foregoing embodiments,  $y$  and  $z$  are each independently an integer ranging from 2 to 10. For example, in some embodiments,  $y$  and  $z$  are each independently an integer ranging from 4 to 9 or from 4 to 6.

In some of the foregoing embodiments,  $\text{R}^6$  is H. In other of the foregoing embodiments,  $\text{R}^6$  is  $\text{C}_1\text{-C}_{24}$  alkyl. In other embodiments,  $\text{R}^6$  is OH.

In some embodiments,  $\text{G}^3$  is unsubstituted. In other embodiments,  $\text{G}^3$  is substituted. In various different embodiments,  $\text{G}^3$  is linear  $\text{C}_1\text{-C}_{24}$  alkylene or linear  $\text{C}_1\text{-C}_{24}$  alkenylene.

In some other foregoing embodiments,  $\text{R}^1$  or  $\text{R}^2$ , or both, is  $\text{C}_6\text{-C}_{24}$  alkenyl. For example, in some embodiments,  $\text{R}^1$  and  $\text{R}^2$  each, independently have the following structure:



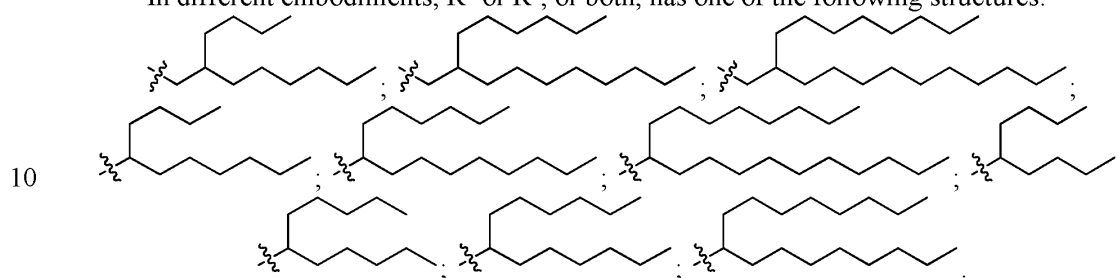
wherein:

$\text{R}^{7a}$  and  $\text{R}^{7b}$  are, at each occurrence, independently H or  $\text{C}_1\text{-C}_{12}$  alkyl; and  $a$  is an integer from 2 to 12,

wherein  $R^{7a}$ ,  $R^{7b}$  and  $a$  are each selected such that  $R^1$  and  $R^2$  each independently comprise from 6 to 20 carbon atoms. For example, in some embodiments  $a$  is an integer ranging from 5 to 9 or from 8 to 12.

In some of the foregoing embodiments, at least one occurrence of  $R^{7a}$  is H. For example, in some embodiments,  $R^{7a}$  is H at each occurrence. In other different embodiments of the foregoing, at least one occurrence of  $R^{7b}$  is  $C_1$ - $C_8$  alkyl. For example, in some embodiments,  $C_1$ - $C_8$  alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In different embodiments,  $R^1$  or  $R^2$ , or both, has one of the following structures:

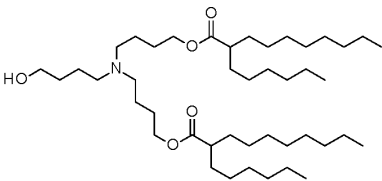
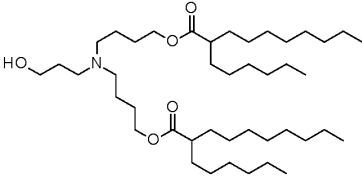
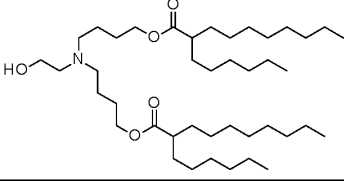
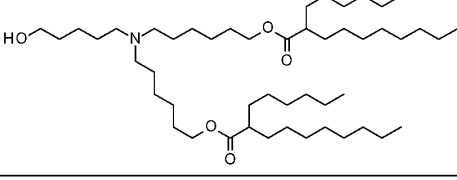
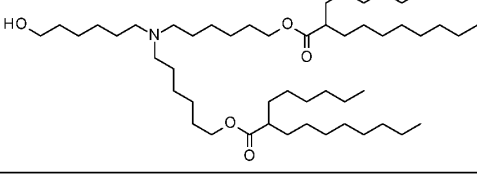
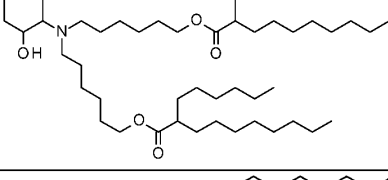
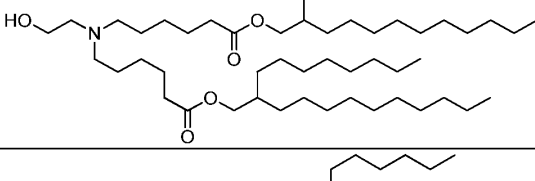
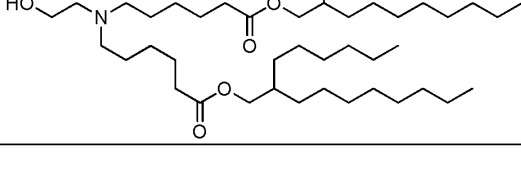


In some of the foregoing embodiments,  $R^3$  is OH, CN,  $-C(=O)OR^4$ ,  $-OC(=O)R^4$  or  $-NHC(=O)R^4$ . In some embodiments,  $R^4$  is methyl or ethyl.

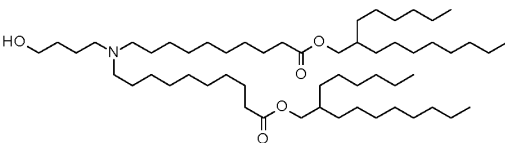
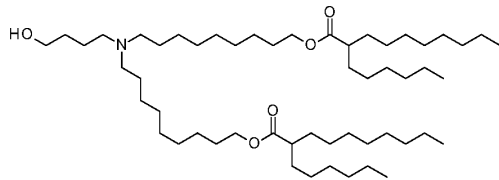
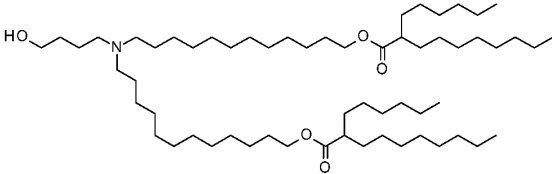
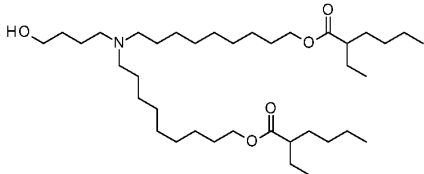
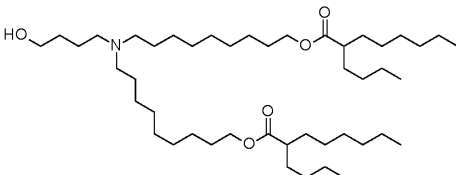
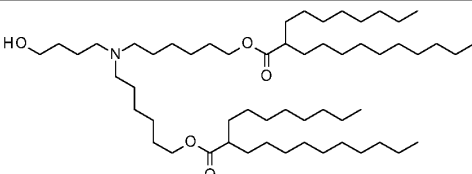
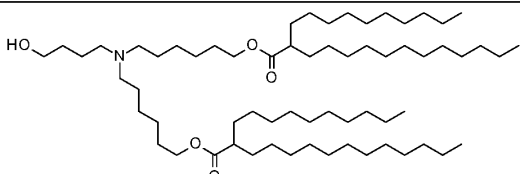
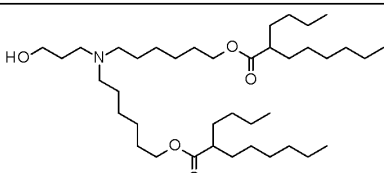
In various different embodiments, the ionizable lipid has one of the structures set forth in Table C below.

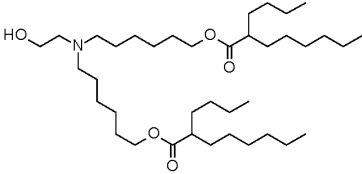
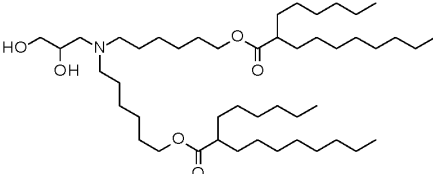
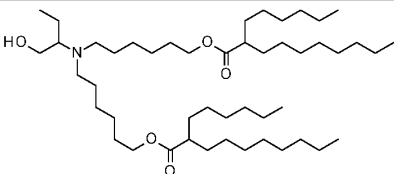
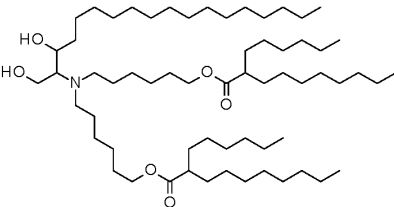
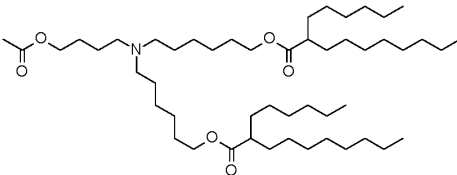
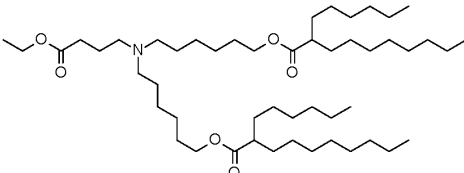
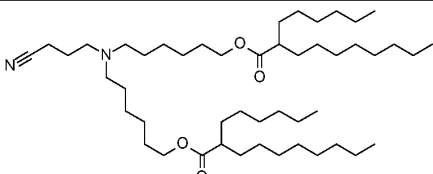
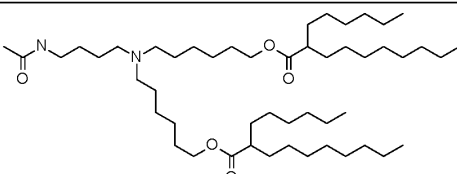
**Table B. Representative ionizable lipids**

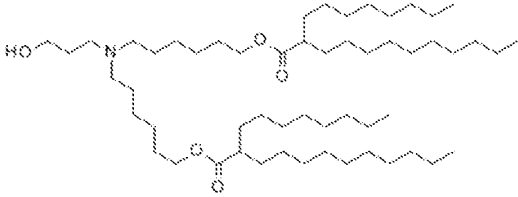
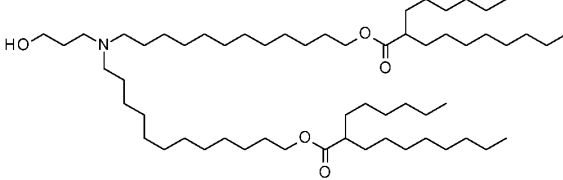
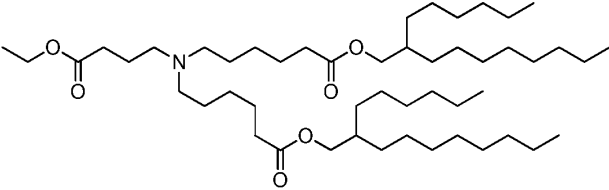
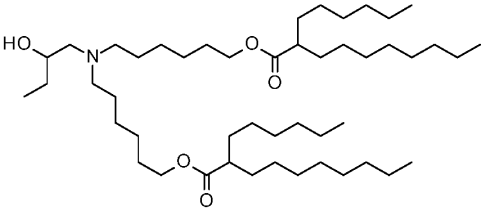
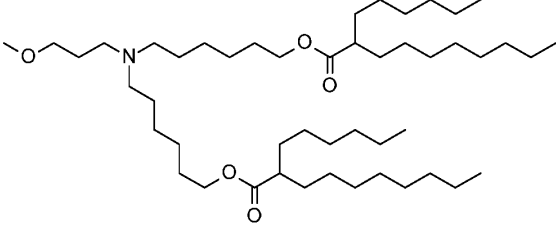
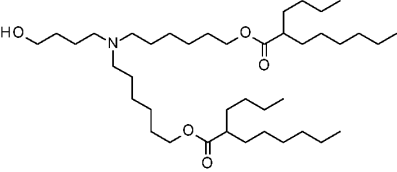
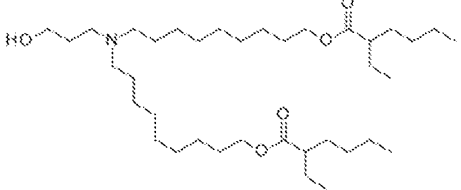
| No. | Structure |
|-----|-----------|
| B-1 |           |
| B-2 |           |
| B-3 |           |

| No.  | Structure                                                                                                                                                 |
|------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|
| B-4  |  <chem>CCCCCCCCC(=O)OCCN(CCCOC(=O)CCCCCCCC)CCOC(=O)CCCCCCCC</chem>      |
| B-5  |  <chem>CCCCCCCCC(=O)OCCN(CCCOC(=O)CCCCCCCC)CCOC(=O)CCCCCCCC</chem>      |
| B-6  |  <chem>CCCCCCCCC(=O)OCCN(CCCOC(=O)CCCCCCCC)CCOC(=O)CCCCCCCC</chem>      |
| B-7  |  <chem>CCCCCCCCC(=O)OCCN(CCCCOC(=O)CCCCCCCC)CCCOC(=O)CCCCCCCC</chem>   |
| B-8  |  <chem>CCCCCCCCC(=O)OCCN(CCCCOC(=O)CCCCCCCC)CCCCOC(=O)CCCCCCCC</chem> |
| B-9  |  <chem>CCCCCCCCC(=O)OCCN(C1CCCCC1C(O)CC)CCOC(=O)CCCCCCCC</chem>       |
| B-10 |  <chem>CCCCCCCCC(=O)OCCN(CCCOC(=O)CCCCCCCC)CCOC(=O)CCCCCCCC</chem>    |
| B-11 |  <chem>CCCCCCCCC(=O)OCCN(CCCOC(=O)CCCCCCCC)CCOC(=O)CCCCCCCC</chem>    |

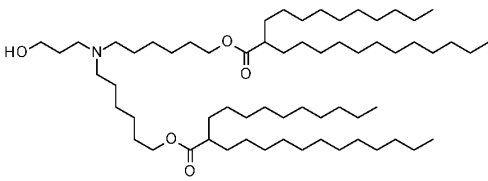
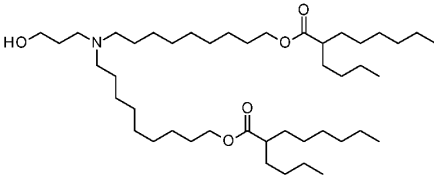
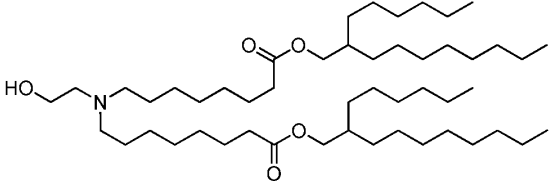
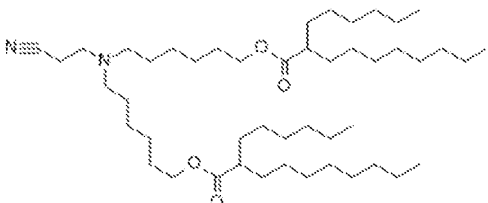
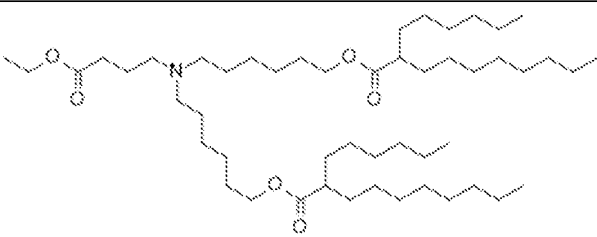
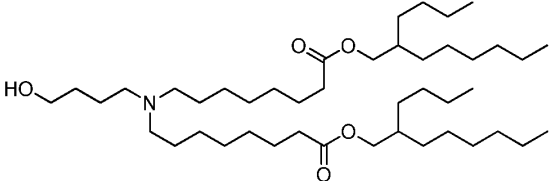
| No.  | Structure |
|------|-----------|
| B-12 |           |
| B-13 |           |
| B-14 |           |
| B-15 |           |
| B-16 |           |
| B-17 |           |
| B-18 |           |
| B-19 |           |
| B-20 |           |

| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| B-21 |    |
| B-22 |    |
| B-23 |    |
| B-24 |    |
| B-25 |  |
| B-26 |  |
| B-27 |  |
| B-28 |  |

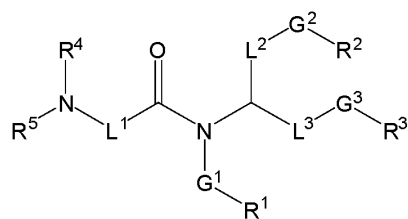
| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| B-29 |    |
| B-30 |    |
| B-31 |    |
| B-32 |   |
| B-33 |  |
| B-34 |  |
| B-35 |  |
| B-36 |  |

| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| B-37 |    |
| B-38 |    |
| B-39 |    |
| B-40 |   |
| B-41 |  |
| B-42 |  |
| B-43 |  |



| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| B-44 |    |
| B-45 |    |
| B-46 |    |
| B-47 |   |
| B-48 |  |
| B-49 |  |

In one embodiment, the ionizable lipid has the following structure:



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

$\text{R}^1$  is optionally substituted  $\text{C}_1$ - $\text{C}_{24}$  alkyl or optionally substituted  $\text{C}_2$ - $\text{C}_{24}$  alkenyl;

$R^2$  and  $R^3$  are each independently optionally substituted  $C_1$ - $C_{36}$  alkyl;

$R^4$  and  $R^5$  are each independently optionally substituted  $C_1$ - $C_6$  alkyl, or  $R^4$  and  $R^5$  join, along with the N to which they are attached, to form a heterocyclyl or heteroaryl;

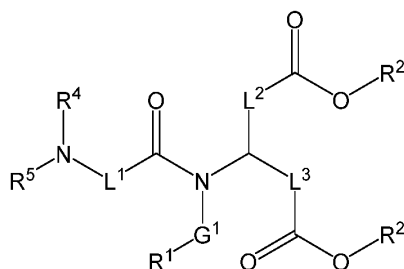
$L^1$ ,  $L^2$ , and  $L^3$  are each independently optionally substituted  $C_1$ - $C_{18}$  alkylene;

5  $G^1$  is a direct bond,  $-(CH_2)_nO(C=O)-$ ,  $-(CH_2)_n(C=O)O-$ , or  $-(C=O)-$ ;

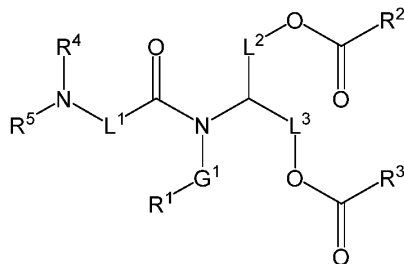
$G^2$  and  $G^3$  are each independently  $-(C=O)O-$  or  $-O(C=O)-$ ; and

$n$  is an integer greater than 0.

In some embodiments, the ionizable lipid has the following structure:



10 In some embodiments, the ionizable lipid has the following structure:



In some embodiments,  $R^1$  is optionally substituted  $C_6$ - $C_{18}$  alkyl or  $C_{14}$ - $C_{18}$  alkenyl. In certain embodiments,  $R^1$  is  $C_8$  alkyl,  $C_9$  alkyl,  $C_{10}$  alkyl,  $C_{12}$  alkyl,  $C_{14}$  alkyl, or  $C_{16}$  alkyl. In some more specific embodiments,  $R^1$  is  $C_{16}$  alkenyl. In certain more specific embodiments,  $R^1$  is unbranched. In some embodiments,  $R^1$  is branched. In certain embodiments,  $R^1$  is unsubstituted.

In some embodiments,  $G^1$  is a direct bond,  $-(CH_2)_nO(C=O)-$ , or  $-(CH_2)_n(C=O)O-$ . In certain embodiments,  $G^1$  is a direct bond. In some more specific embodiments,  $G^1$  is  $-(CH_2)_n(C=O)O-$  and  $n$  is greater than 1. In some embodiments,  $n$  is 1-20. In some embodiments  $n$  is 1-10. In some embodiments  $n$  is 5-11. In some embodiments,  $n$  is 6-10. In certain more specific embodiments,  $n$  is 5, 6, 7, 8, 9, or 10. In some embodiments,  $n$  is 5. In some embodiments,  $n$  is 6. In some embodiments,  $n$  is 7. In certain embodiments,  $n$  is 8. In some embodiments,  $n$  is 9. In some embodiments,  $n$  is 10.

In some embodiments,  $L^1$  is  $C_1$ - $C_6$  alkylene. In certain embodiments,  $L^1$  is  $C_2$  alkylene,  $C_3$  alkylene, or  $C_4$  alkylene. In some more specific embodiments,  $L^1$  is unbranched. In certain more specific embodiments,  $L^1$  is unsubstituted.

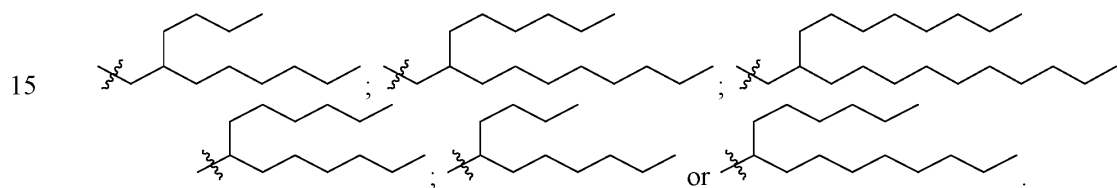
In some embodiments,  $R^2$  is  $C_8$ - $C_{24}$  alkyl. In some embodiments,  $R^3$  is  $C_8$ - $C_{24}$  alkyl. In some more specific embodiments,  $R^2$  and  $R^3$  are both  $C_8$ - $C_{24}$  alkyl. In some embodiments,  $R^2$  and  $R^3$  are each independently  $C_{11}$  alkyl,  $C_{12}$  alkyl,  $C_{13}$  alkyl,  $C_{14}$  alkyl,  $C_{15}$  alkyl,  $C_{16}$  alkyl,  $C_{18}$  alkyl, or  $C_{20}$  alkyl. In certain embodiments,  $R^2$  is branched. In more specific embodiments,  $R^3$  is branched. In some more specific embodiments,  $R^2$  and  $R^3$  each independently have one of the following structures:



wherein:

$R^6$  and  $R^7$  are each independently  $C_2$ - $C_{12}$  alkyl.

In some embodiments,  $R^2$  and  $R^3$  each independently have one of the following structures:



In some embodiments,  $L^2$  and  $L^3$  are each independently  $C_4$ - $C_{10}$  alkylene. In certain embodiments,  $L^2$  and  $L^3$  are both  $C_5$  alkylene. In some more specific embodiments,  $L^2$  and  $L^3$  are both  $C_6$  alkylene. In certain embodiments,  $L^2$  and  $L^3$  are both  $C_8$  alkylene. In some more specific embodiments,  $L^2$  and  $L^3$  are both  $C_9$  alkylene. In some embodiments,  $L^2$  is unbranched. In some embodiments,  $L^3$  is unbranched. In more specific embodiments,  $L^2$  is unsubstituted. In some embodiments,  $L^2$  is unsubstituted.

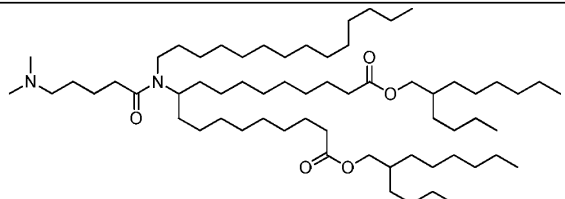
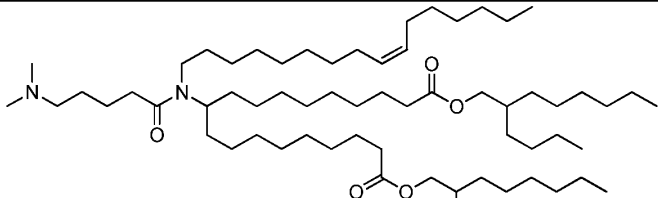
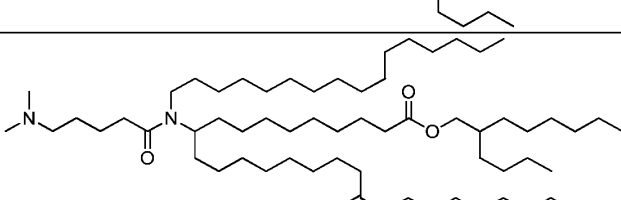
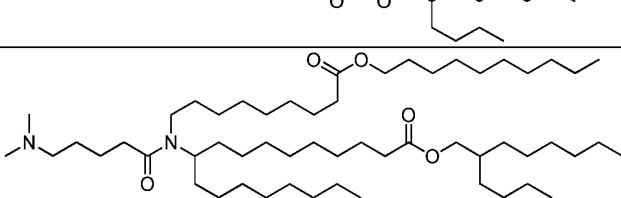
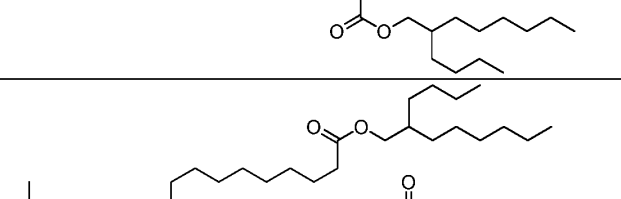
In some embodiments,  $R^4$  and  $R^5$  are each independently  $C_1$ - $C_6$  alkyl. In more specific embodiments,  $R^4$  and  $R^5$  are both methyl. In certain embodiments,  $R^4$  and  $R^5$  are both ethyl. In certain embodiments,  $R^4$  is methyl and  $R^5$  is n-butyl. In some embodiments,  $R^4$  and  $R^5$  are both n-butyl. In different embodiments,  $R^4$  is methyl and  $R^5$  is n-hexyl.

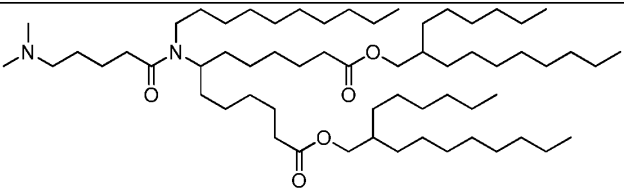
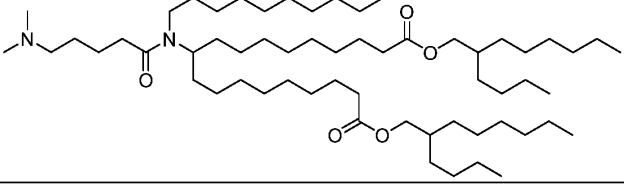
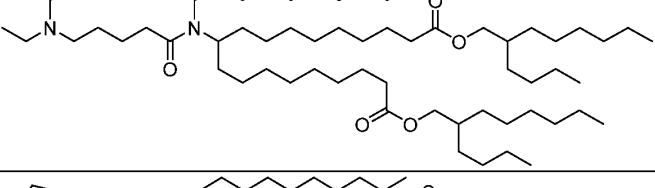
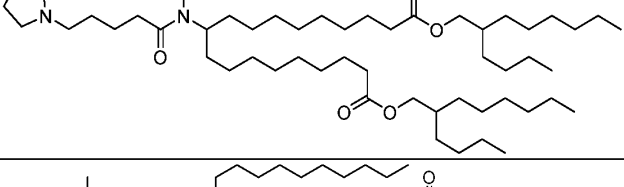
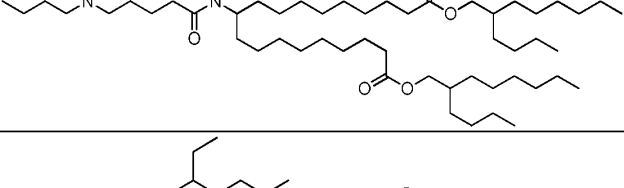
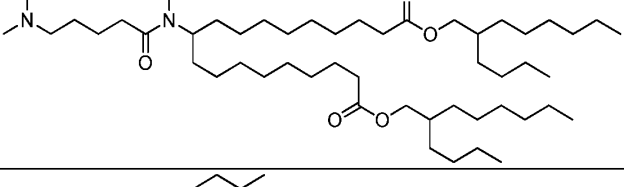
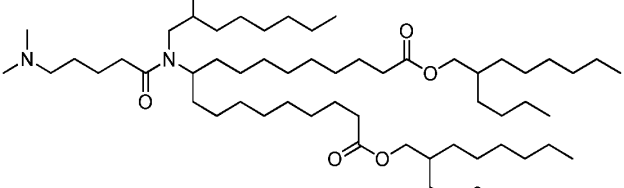
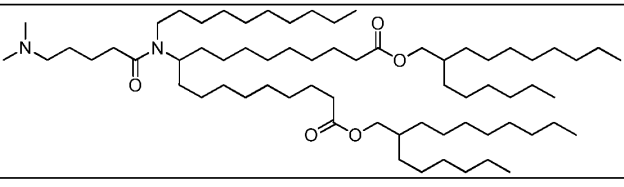
In some embodiments,  $R^4$  and  $R^5$  join, along with the N to which they are attached, to form a heterocyclyl. In certain embodiments, the heterocyclyl is a 5-membered heterocyclyl. In some embodiments, the heterocyclyl has the following structure:

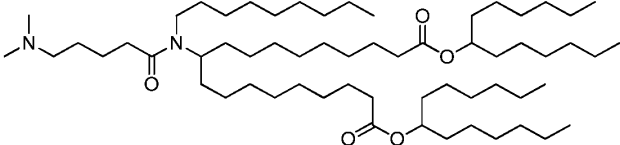
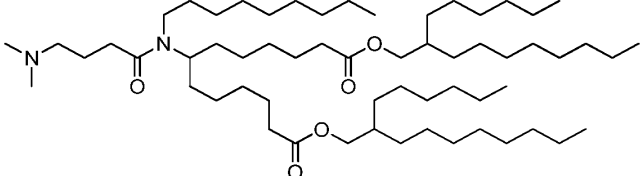
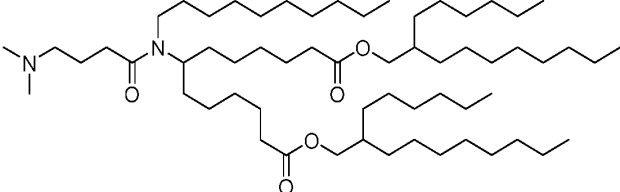
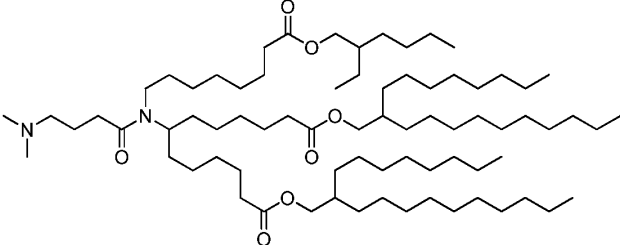
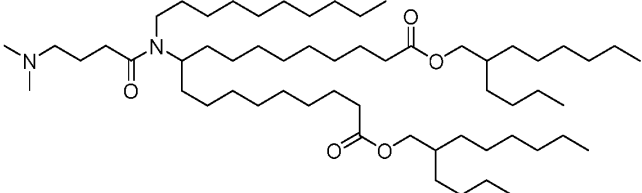
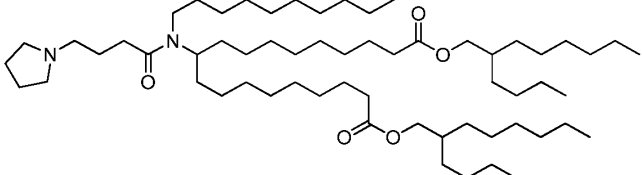
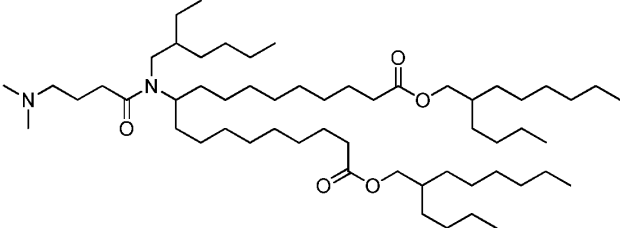
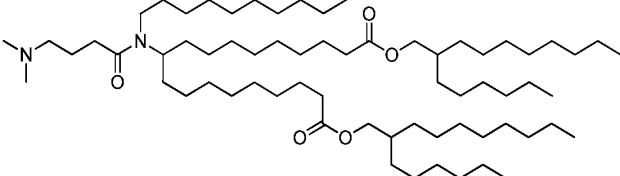


In various different embodiments, the ionizable lipid has one of the structures set forth in Table C below.

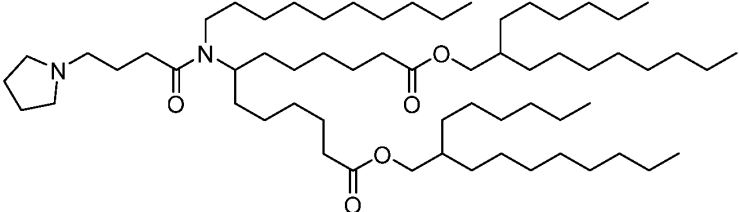
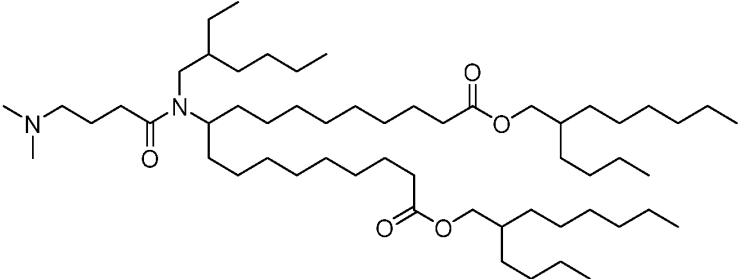
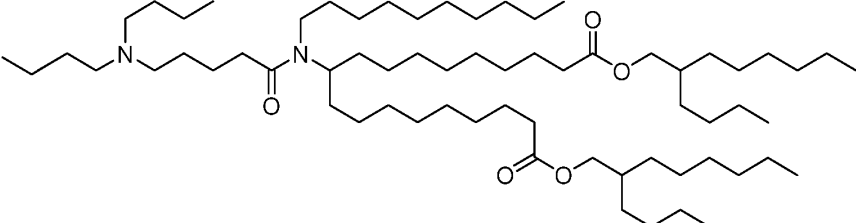
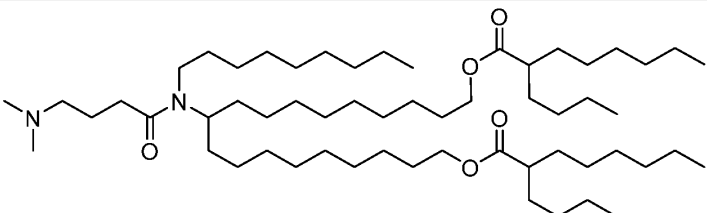
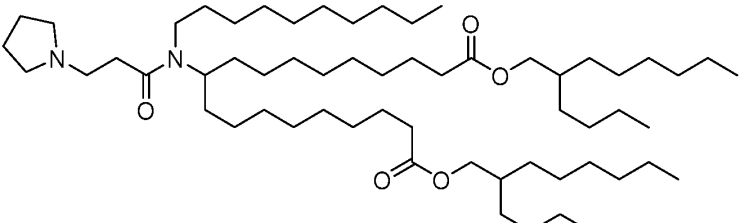
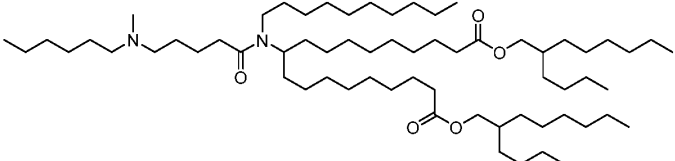
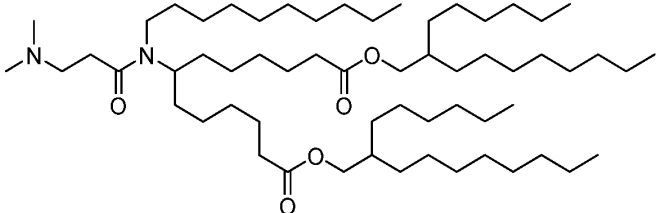
### Table C. Representative ionizable lipids

| No. | Structure                                                                            |
|-----|--------------------------------------------------------------------------------------|
| C-1 |    |
| C-2 |    |
| C-3 |   |
| C-4 |  |
| C-5 |  |

| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| C-6  |    |
| C-7  |    |
| C-8  |    |
| C-9  |   |
| C-10 |  |
| C-11 |  |
| C-12 |  |
| C-13 |  |

| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| C-14 |    |
| C-15 |    |
| C-16 |    |
| C-17 |   |
| C-18 |  |
| C-19 |  |
| C-20 |  |
| C-21 |  |

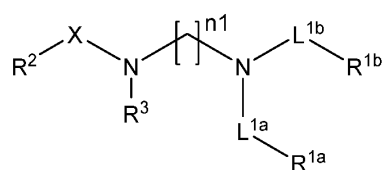
| No.  | Structure |
|------|-----------|
| C-22 |           |
| C-23 |           |
| C-24 |           |
| C-25 |           |
| C-26 |           |
| C-27 |           |
| C-28 |           |

| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| C-29 |    |
| C-30 |    |
| C-31 |   |
| C-32 |  |
| C-33 |  |
| C-34 |  |
| C-35 |  |



| No.  | Structure |
|------|-----------|
| C-36 |           |
| C-37 |           |
| C-38 |           |
| C-39 |           |
| C-40 |           |

One embodiment provides an ionizable lipid having the following structure:



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

$\text{L}^{1a}$  and  $\text{L}^{1b}$  are each independently optionally substituted  $\text{C}_3\text{-C}_{12}$  alkyl;

5  $\text{R}^{1a}$  is  $\text{-C(=O)OR}^{4a}$  or  $\text{-O(C=O)R}^{4a}$ ,

$\text{R}^{1b}$  is  $\text{-C(=O)OR}^{4b}$  or  $\text{-O(C=O)R}^{4b}$ ;

$\text{R}^2$  is  $\text{-NR}^6\text{(C=O)R}^5$ ,  $\text{-(C=O)N(R}^6\text{)R}^5$  or  $\text{-(C=O)OR}^7$ ;

$\text{R}^3$  and  $\text{R}^6$  are each independently hydrogen or optionally substituted  $\text{C}_1\text{-C}_{12}$  alkyl;

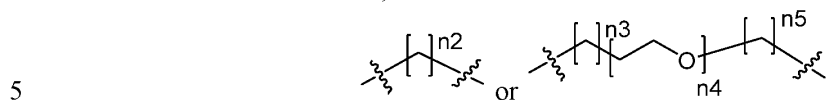
$\text{R}^{4a}$ ,  $\text{R}^{4b}$ , and  $\text{R}^5$  are each independently optionally substituted alkyl;

$R^7$  is optionally substituted  $C_1$ - $C_6$  alkyl or optionally substituted arylalkyl;

$n_1$  is 2, 3, 4, 5, or 6; and

X is  $C_2$ - $C_6$  alkylene or  $C_4$ - $C_{20}$  alkyleneoxide.

In some embodiments, wherein X is:



wherein:

$n_2$  is 2, 3, 4, 5, or 6;

$n_3$  is 0, 1, 2, 3, or 4;

$n_4$  is 2, 3, or 4; and

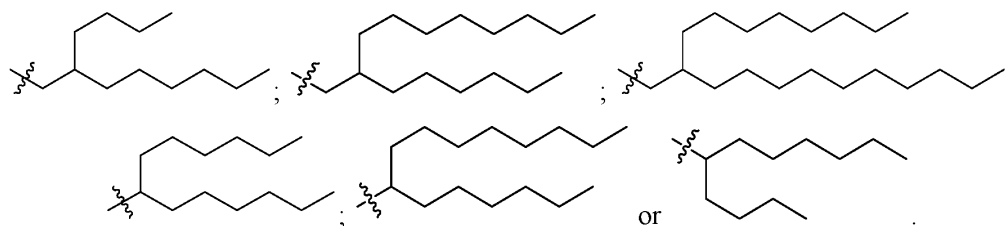
10  $n_5$  is 2, 3, 4, or 5.

In some embodiments,  $L^{1a}$  is  $C_5$ - $C_9$  alkyl. In certain embodiments,  $L^{1b}$  is  $C_5$ - $C_9$  alkyl. In some embodiments,  $L^{1a}$  is  $C_5$ -,  $C_6$ -,  $C_7$ -, or  $C_9$ -alkyl. In certain embodiments,  $L^{1b}$  is  $C_5$ -,  $C_6$ -,  $C_7$ -, or  $C_9$ -alkyl. In some embodiments,  $L^{1a}$  is  $C_5$ -alkyl. In certain embodiments,  $L^{1a}$  is  $C_6$ -alkyl. In some embodiments,  $L^{1a}$  is  $C_7$ -alkyl. In certain embodiments,  $L^{1a}$  is  $C_9$ -alkyl. In some  
15 embodiments,  $L^{1b}$  is  $C_5$ -alkyl. In certain embodiments,  $L^{1b}$  is  $C_6$ -alkyl. In some embodiments,  $L^{1b}$  is  $C_7$ -alkyl. In certain embodiments,  $L^{1b}$  is  $C_9$ -alkyl. In some embodiments,  $L^{1a}$  is unsubstituted. In certain embodiments,  $L^{1b}$  is unsubstituted. In some embodiments,  $L^{1a}$  is unbranched. In certain embodiments,  $L^{1b}$  is unbranched.

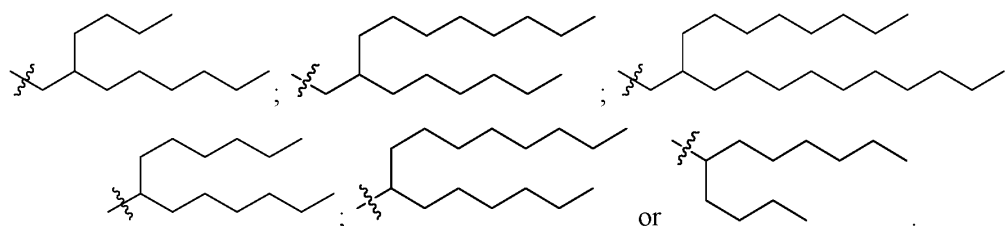
In some embodiments, one of  $R^{1a}$  is  $-O(C=O)R^{4a}$ . In certain embodiments, one of  $R^{1a}$  is  $-(C=O)OR^{4a}$ . In some embodiments,  $R^{1b}$  is  $-O(C=O)R^{4b}$ . In certain embodiments, one of  $R^{1b}$  is  $-(C=O)OR^{4b}$ .  
20

In some embodiments,  $R^{4a}$  is  $C_8$ - $C_{24}$ -alkyl. In certain embodiments,  $R^{4a}$  is  $C_{10}$ - $C_{18}$ -alkyl. In certain embodiments,  $R^{4a}$  is  $C_{11}$ - $C_{16}$ -alkyl. In some embodiments,  $R^{4a}$  is  $C_{11}$ -alkyl. In certain embodiments,  $R^{4a}$  is  $C_{15}$ -alkyl. In some embodiments,  $R^{4a}$  is  $C_{16}$ -alkyl. In certain embodiments,  $R^{4b}$  is  $C_8$ - $C_{24}$ -alkyl. In some embodiments,  $R^{4b}$  is  $C_{10}$ - $C_{18}$ -alkyl. In certain embodiments,  $R^{4b}$  is  $C_{11}$ - $C_{16}$ -alkyl. In some embodiments,  $R^{4b}$  is  $C_{11}$ -alkyl. In certain embodiments,  $R^{4b}$  is  $C_{15}$ -alkyl. In some embodiments,  $R^{4b}$  is  $C_{16}$ -alkyl.  
25

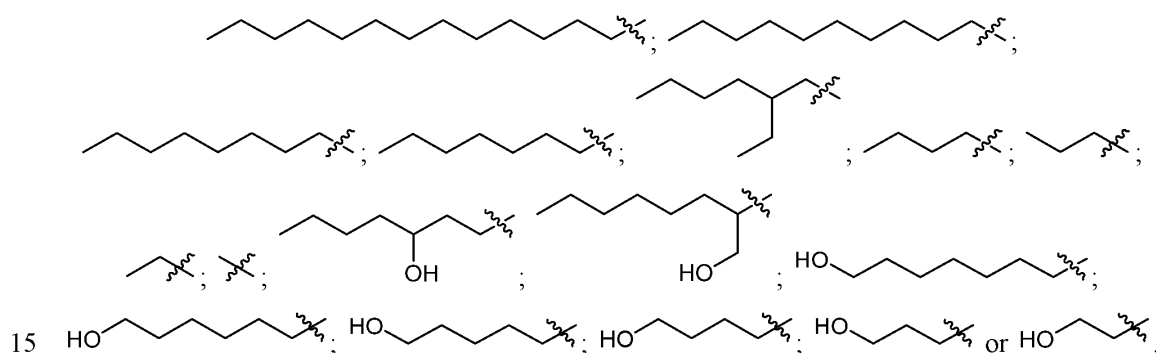
In certain embodiments,  $R^{4a}$  is branched. In some embodiments,  $R^{4b}$  is branched. In certain embodiments,  $R^{4a}$  is unsubstituted. In some embodiments,  $R^{4b}$  is unsubstituted. In certain  
30 embodiments,  $R^{4a}$  has one of the following structures:



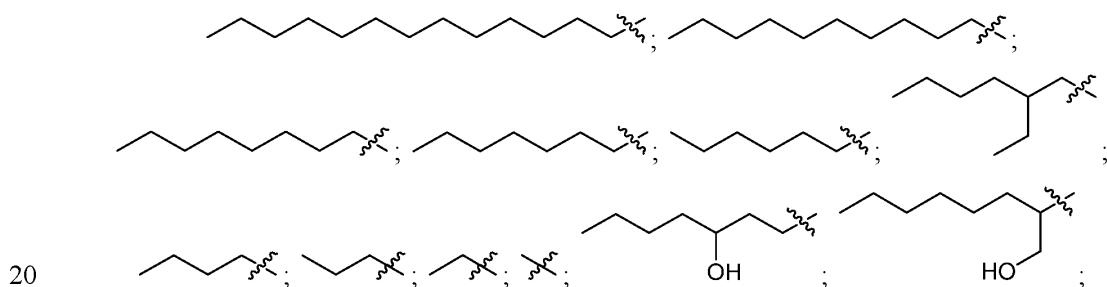
In some embodiments,  $R^{4b}$  has one of the following structures:

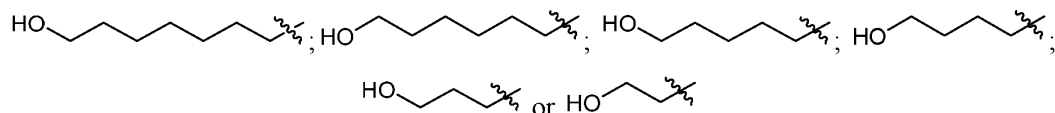


In some embodiments,  $R^2$  is  $-NR^6(C=O)R^5$ . In certain embodiments,  $R^2$  is  $-(C=O)N(R^6)R^5$ . In some embodiments,  $R^5$  is  $C_2$ - $C_{16}$ -alkyl. In certain embodiments,  $R^5$  is  $C_4$ - $C_{13}$ -alkyl. In some embodiments,  $R^5$  is  $C_4$ -,  $C_7$ -,  $C_8$ -,  $C_{10}$ -, or  $C_{13}$ -alkyl. In certain embodiments,  $R^5$  is unsubstituted. In some embodiments,  $R^5$  is substituted with hydroxyl. In some embodiments,  $R^5$  is branched. In certain embodiments,  $R^5$  is unbranched. In some embodiments,  $R^5$  has one of the following structures:



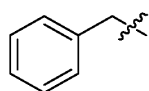
In certain embodiments,  $R^5$  is unbranched. In some embodiments,  $R^5$  has one of the following structures:



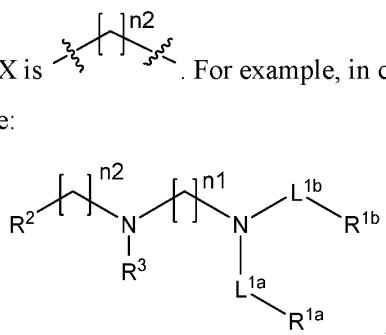


In some embodiments, R<sup>7</sup> is C<sub>1</sub>-C<sub>3</sub> alkyl or C<sub>7</sub>-C<sub>16</sub> arylalkyl. In certain embodiments, R<sup>7</sup> is C<sub>7</sub>-C<sub>16</sub> arylalkyl. In some embodiments, R<sup>7</sup> is C<sub>1</sub>-C<sub>3</sub> alkyl. In some embodiments, R<sup>7</sup> is unsubstituted.

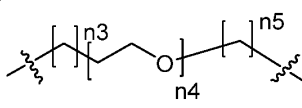
15



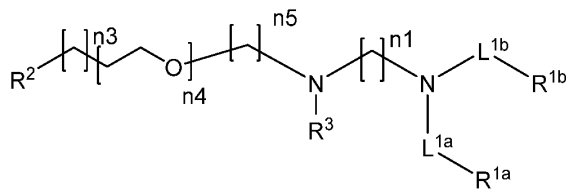
25 lipid has the following structure:



or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof. For example, in some of these embodiments  $n_2$  is 3, 4, or 5.

In other embodiments X is . In some such embodiments, the

ionizable lipid has the following structure:



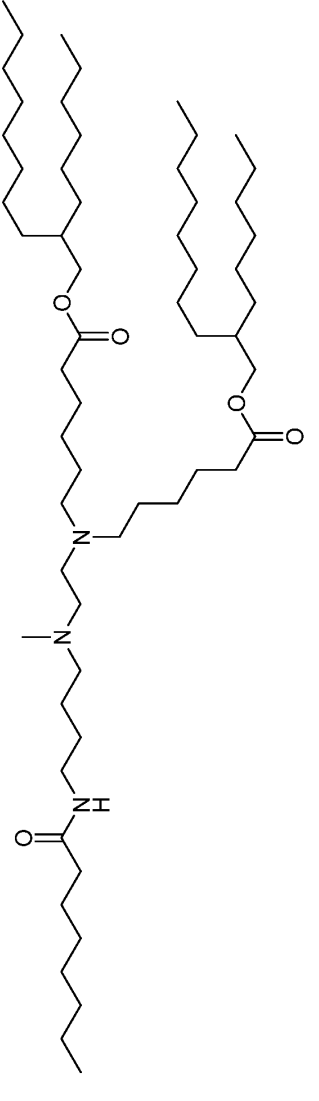
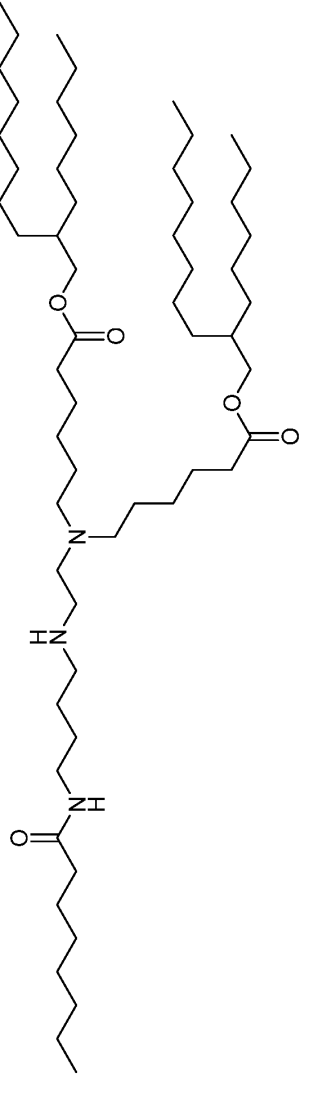
5

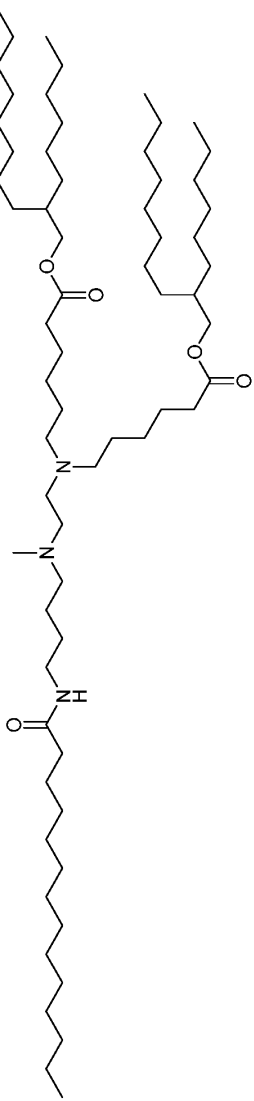
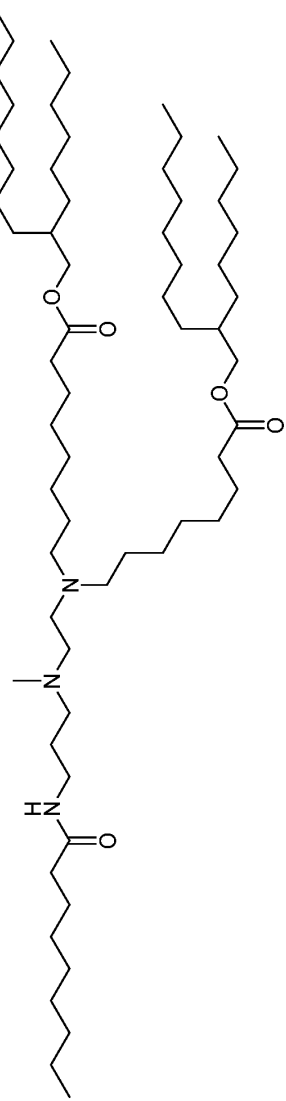
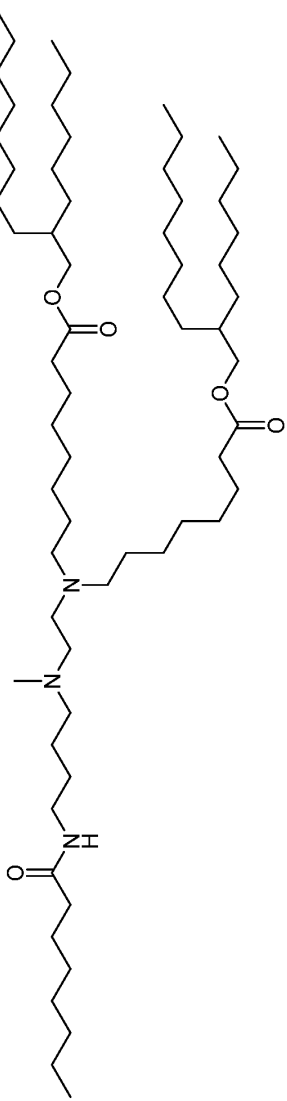
or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof. In different of these embodiments,  $n_3$  is 0 or 1. In other embodiments,  $n_4$  is 2 or 3. In some other different embodiments,  $n_5$  is 3.

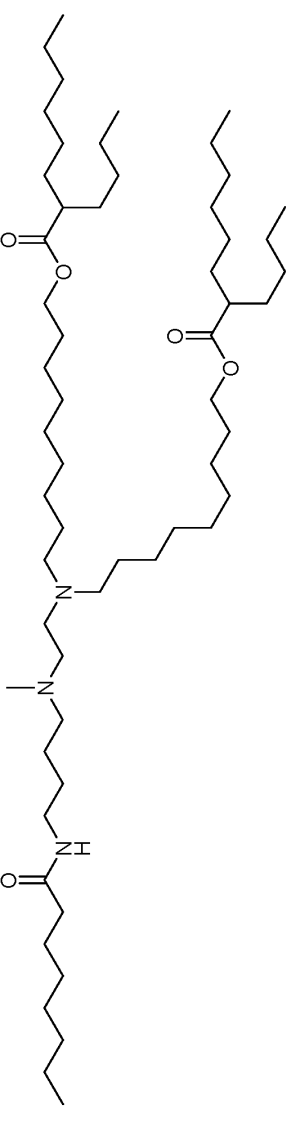
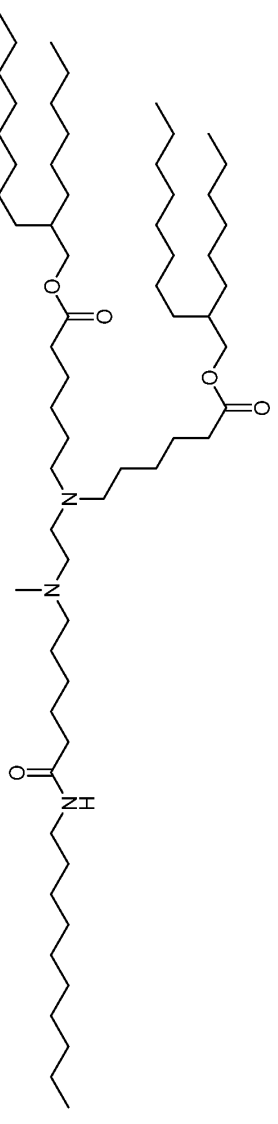
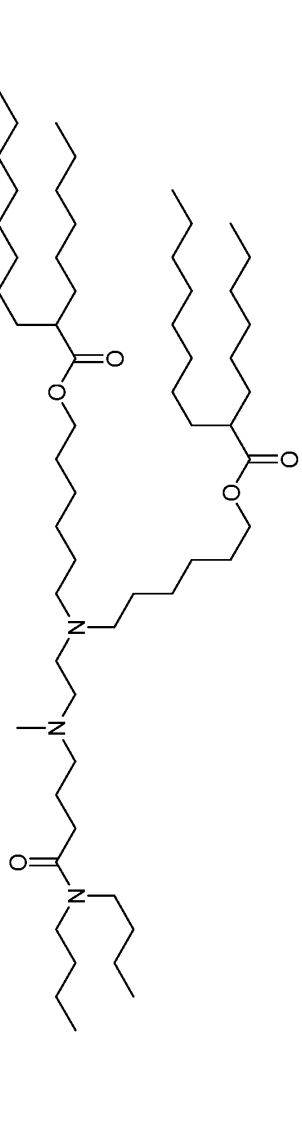
In some embodiments,  $n_1$  is 3, 4, or 5. In certain embodiments,  $n_1$  is 2.

10 In some embodiments, the ionizable lipid has one of the structures set forth in Table D below or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof.

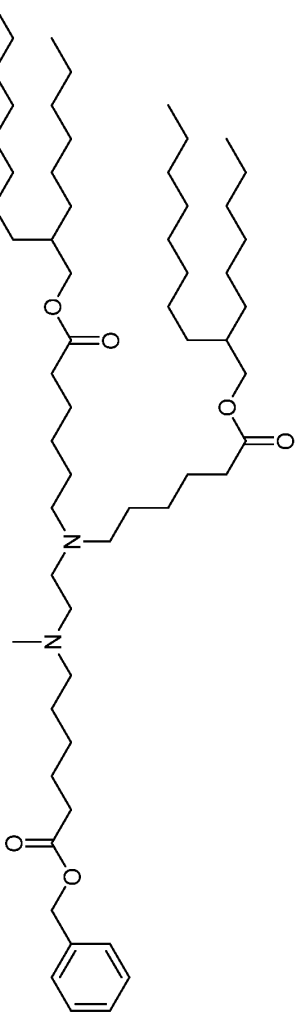
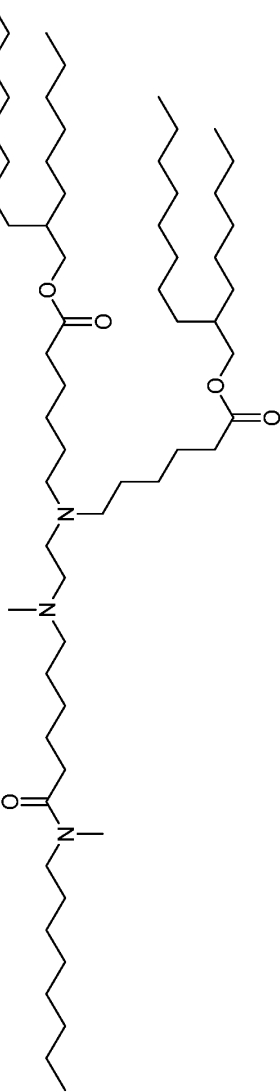
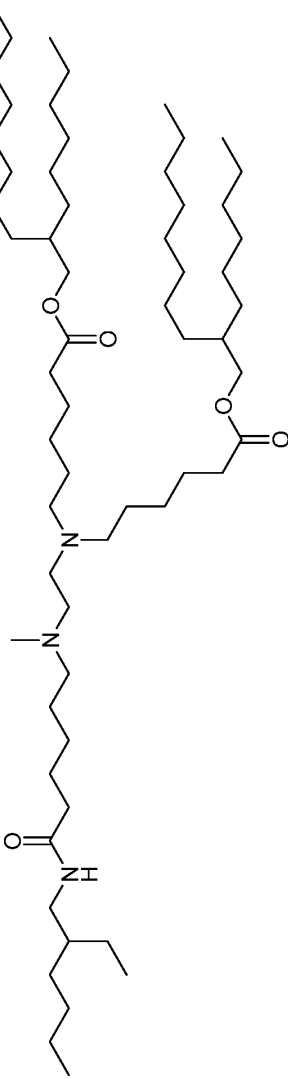
Table D. Representative ionizable lipids

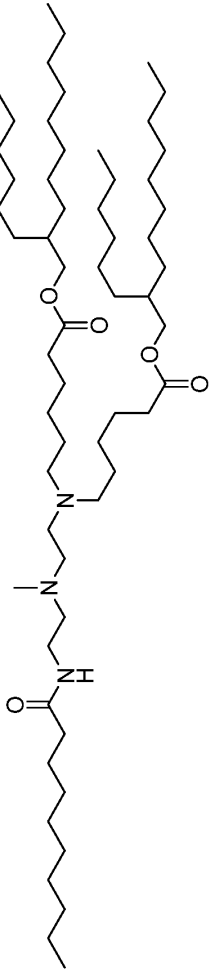
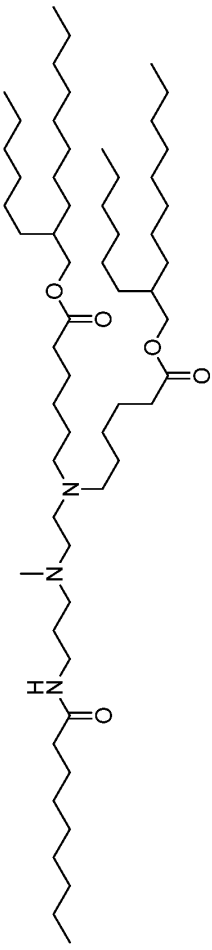
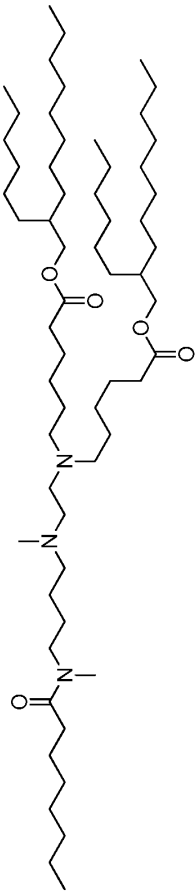
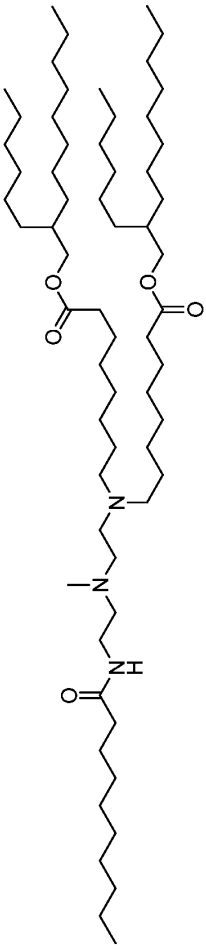
| No. | Structure                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
|-----|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| D-1 |  <p>The chemical structure of lipid D-1 is a symmetrical molecule. It features a central nitrogen atom (N) bonded to two identical side chains. Each side chain consists of a 10-carbon alkyl chain (represented by a zigzag line) attached to a carbonyl group (C=O). This carbonyl group is further attached to an oxygen atom (O), which is then connected to a 10-carbon alkyl chain (represented by a zigzag line). The central nitrogen atom is also bonded to two hydrogen atoms (H).</p>  |
| D-2 |  <p>The chemical structure of lipid D-2 is a symmetrical molecule. It features a central nitrogen atom (N) bonded to two identical side chains. Each side chain consists of a 10-carbon alkyl chain (represented by a zigzag line) attached to a carbonyl group (C=O). This carbonyl group is further attached to an oxygen atom (O), which is then connected to a 10-carbon alkyl chain (represented by a zigzag line). The central nitrogen atom is also bonded to two hydrogen atoms (H).</p> |

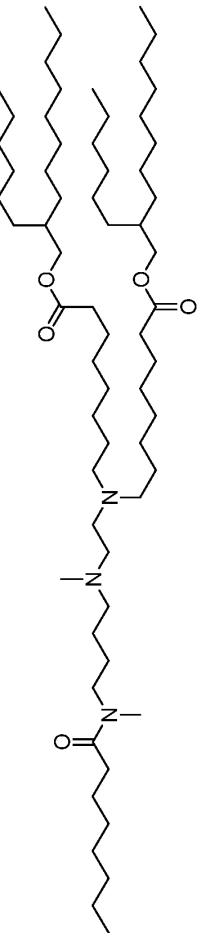
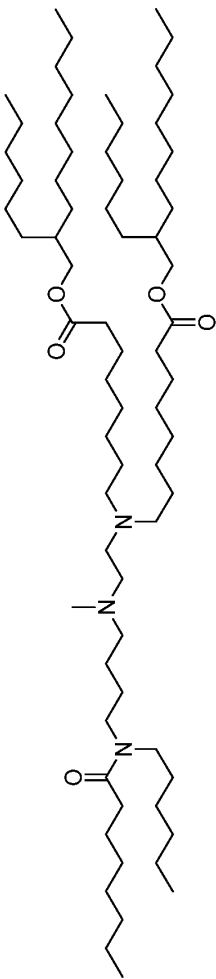
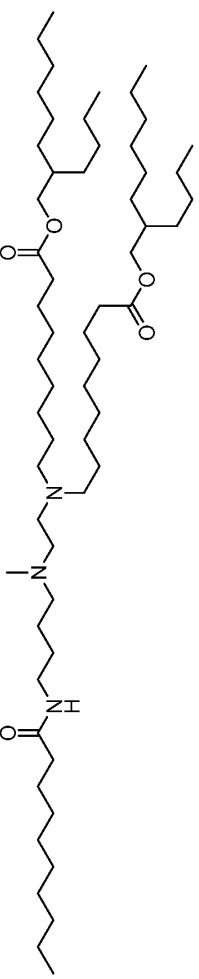
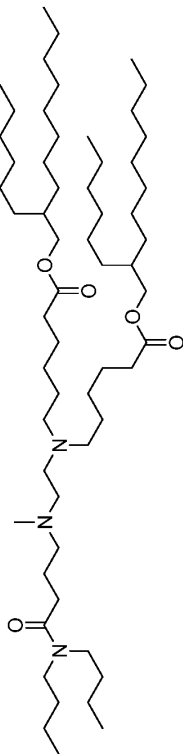
| Structure |                                                                                     |
|-----------|-------------------------------------------------------------------------------------|
| No.       |                                                                                     |
| D-3       |   |
| D-4       |   |
| D-5       |  |

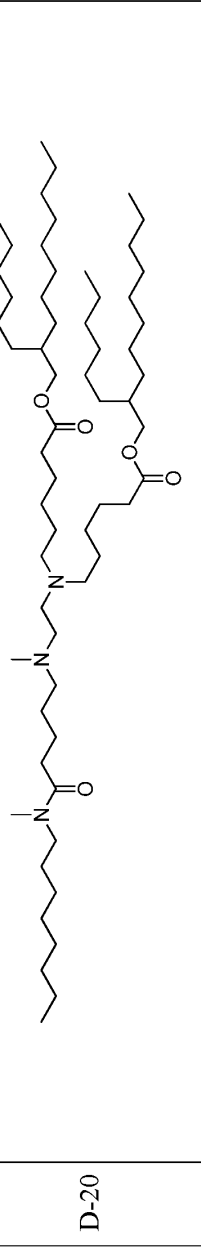
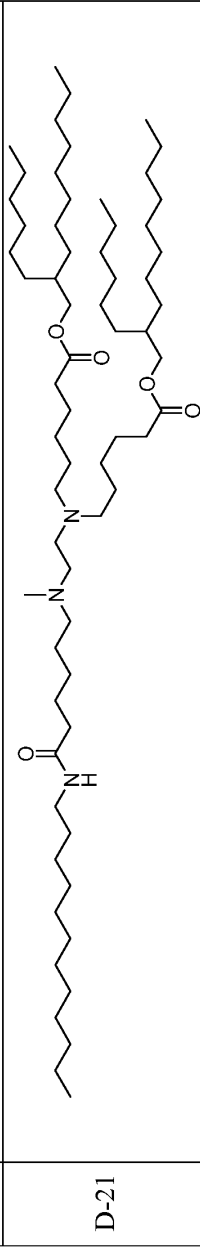
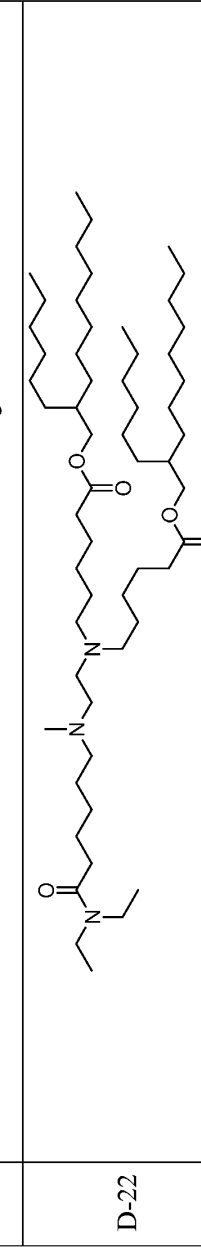
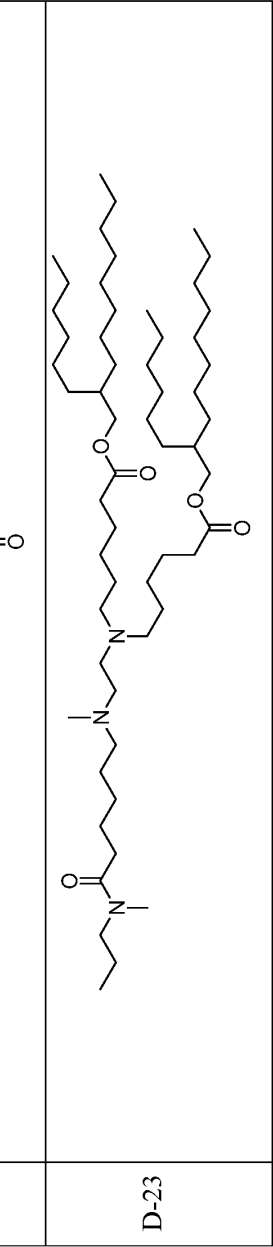
| No. | Structure                                                                           |
|-----|-------------------------------------------------------------------------------------|
| D-6 |   |
| D-7 |   |
| D-8 |  |

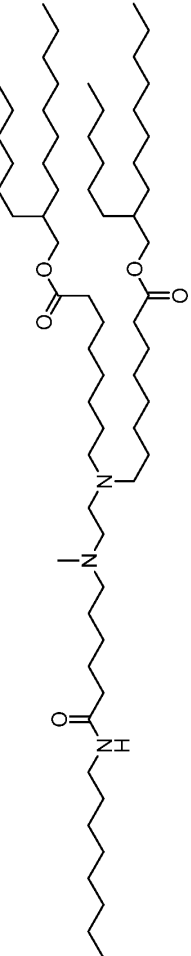
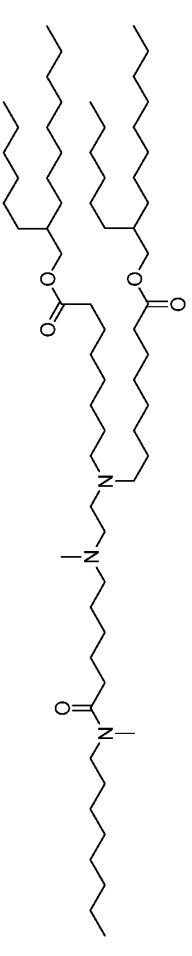
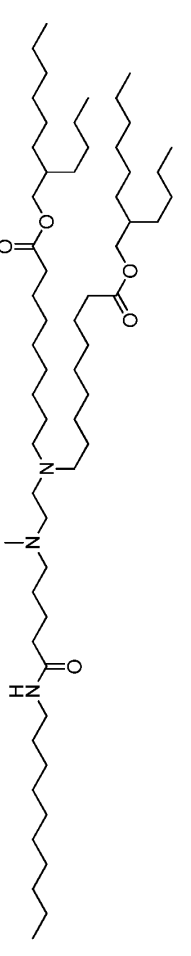
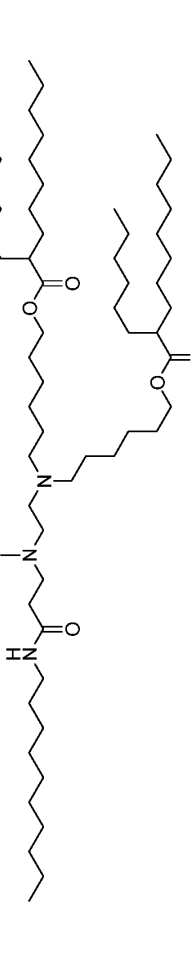


| Structure |                                                                                     |
|-----------|-------------------------------------------------------------------------------------|
| No.       |                                                                                     |
| D-9       |   |
| D-10      |   |
| D-11      |  |

| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| D-12 |    |
| D-13 |    |
| D-14 |    |
| D-15 |  |

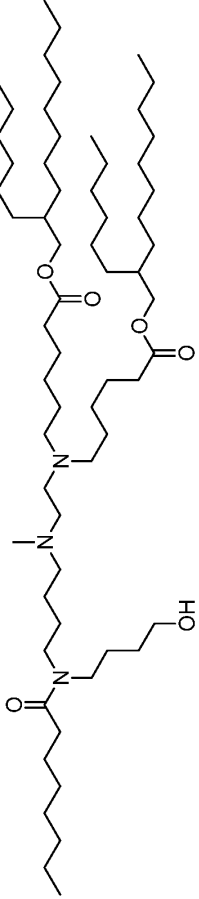
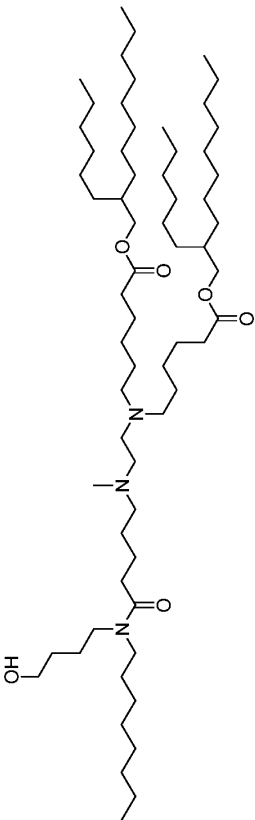
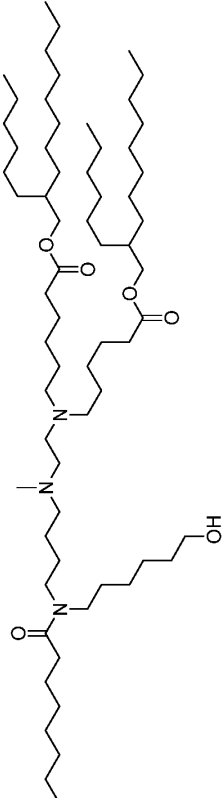
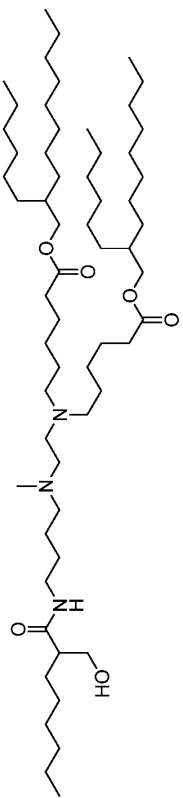
| No.  | Structure                                                                            |
|------|--------------------------------------------------------------------------------------|
| D-16 |    |
| D-17 |    |
| D-18 |    |
| D-19 |  |

| No.  | Structure                                                                           |
|------|-------------------------------------------------------------------------------------|
| D-20 |   |
| D-21 |   |
| D-22 |   |
| D-23 |  |

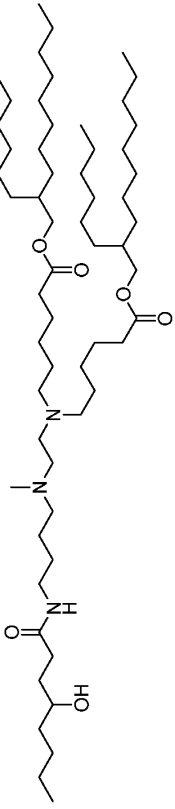
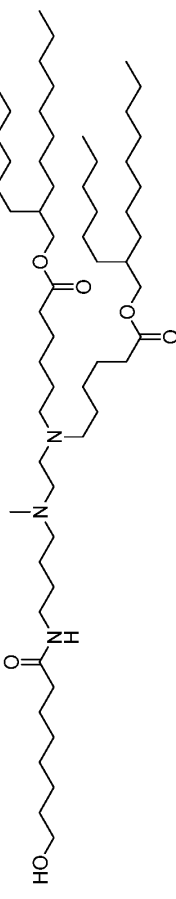
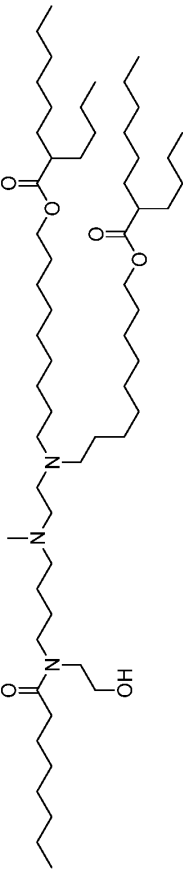
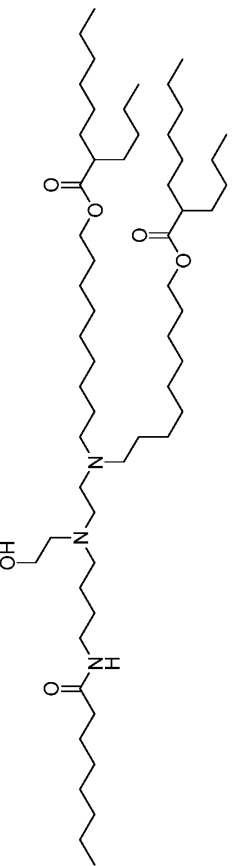
| No.  | Structure                                                                           |
|------|-------------------------------------------------------------------------------------|
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| D-25 |   |
| D-26 |   |
| D-27 |  |

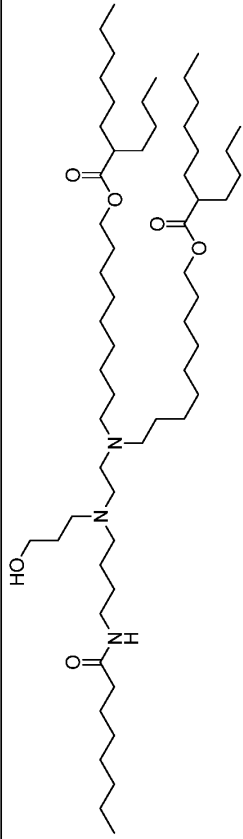
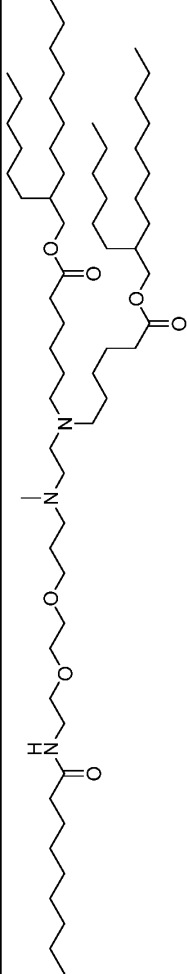
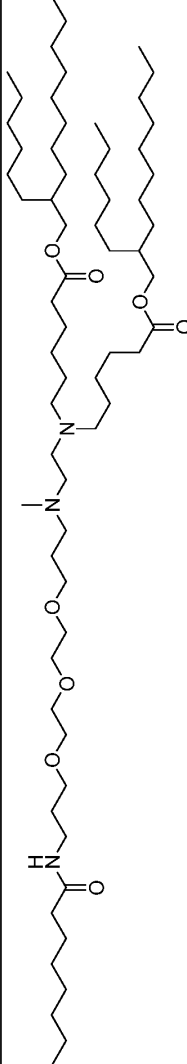
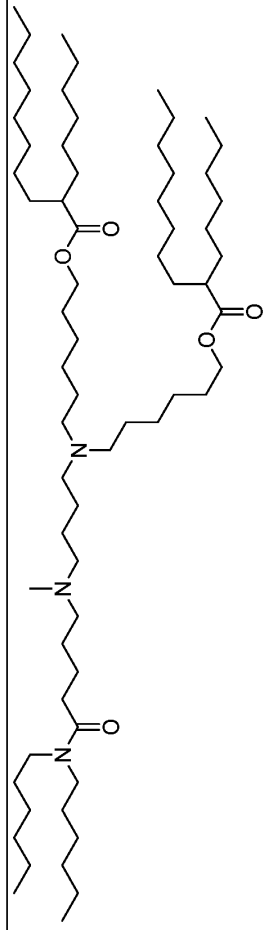
| No.  | Structure |
|------|-----------|
| D-28 |           |
| D-29 |           |
| D-30 |           |
| D-31 |           |

| No.  | Structure |
|------|-----------|
| D-32 |           |
| D-33 |           |
| D-34 |           |
| D-35 |           |

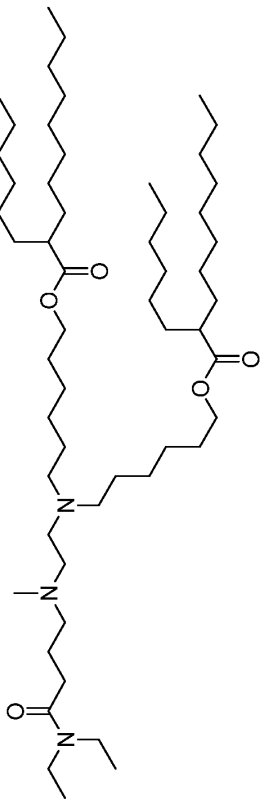
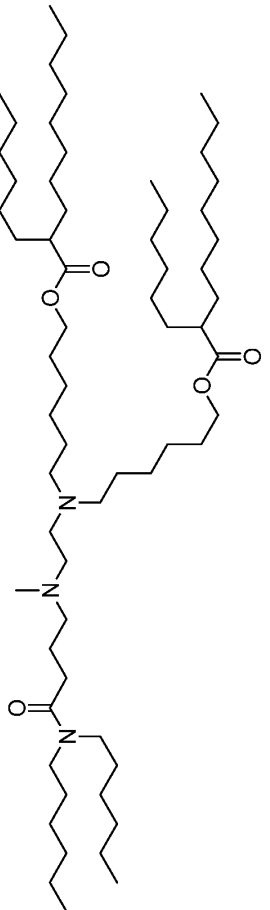
| No.  | Structure                                                                            |
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| D-37 |    |
| D-38 |   |
| D-39 |  |



| No.  | Structure                                                                           |
|------|-------------------------------------------------------------------------------------|
| D-40 |   |
| D-41 |   |
| D-42 |   |
| D-43 |  |

| No.  | Structure                                                                           |
|------|-------------------------------------------------------------------------------------|
| D-44 |   |
| D-45 |   |
| D-46 |   |
| D-47 |  |

| No.  | Structure |
|------|-----------|
| D-48 |           |
| D-49 |           |
| D-50 |           |
| D-51 |           |

| No.  | Structure                                                                                                                                                                                                                                                                                                                                                                                                                                                               |
|------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| D-52 |  <p>Chemical structure of D-52: A complex molecule featuring a central tertiary amine (N) connected to a dimethylammonium salt (N<sup>+</sup>Me<sub>2</sub>) and a long alkyl chain. The long alkyl chain is terminated by a carboxylate group (COO<sup>-</sup>) and a long alkyl chain. The molecule also contains a long alkyl chain and a carboxylate group (COO<sup>-</sup>).</p> |
| D-53 |  <p>Chemical structure of D-53: A complex molecule featuring a central tertiary amine (N) connected to a dimethylammonium salt (N<sup>+</sup>Me<sub>2</sub>) and a long alkyl chain. The long alkyl chain is terminated by a carboxylate group (COO<sup>-</sup>) and a long alkyl chain. The molecule also contains a long alkyl chain and a carboxylate group (COO<sup>-</sup>).</p> |

LNPs prepared according to the present disclosure may be used for delivery of therapeutic agents, such as nucleic acids. The components of the LNPs are present in an amount that is effective to form an LNP, encapsulate a therapeutic agent (*e.g.*, a nucleic acid) under mild conditions, and deliver a therapeutic agent. In some embodiments, the delivery facilitates treating a particular disease or condition of interest. Appropriate concentrations and dosages can be readily determined by one skilled in the art.

An embodiment provides a composition comprising a lipid nanoparticle a therapeutic agent and optionally additional lipid excipients. In some embodiments, the composition further comprises one or more component selected from ionizable lipids, neutral lipids, steroids, and polymer conjugated lipids.

In some embodiments, the therapeutic agent comprises a nucleic acid. In certain embodiments, the nucleic acid is selected from antisense and messenger RNA. In some embodiments, the nucleic acid is mRNA. In some embodiments, the composition is a vaccine (*e.g.*, a COVID-19 vaccine).

Exemplary ionizable lipids (*e.g.*, cationic lipids) and their synthesis can be found in the following publications:

US Patent Nos. US 9,738,593; US 10,221,127; US 10,166,298; US 11,357,856; US 11,712,481; US 11,453,639;

US Patent Publication Nos: US 2018/0185516; US 2022/0106257;

PCT Publication Nos. WO 2017/117528; WO 2016/176330; WO 2018/191719; WO 2018/200943; WO 2019/036000; WO 2019/036028; WO 2019/036030; WO 2019/036008; WO 2019/089828; WO 2020/061426; WO 2020/081938; WO 2021/030701; WO 2023/114944; WO 2023/114939; WO 2023/114943, the disclosures of which are hereby incorporated by reference.

Lipids (*e.g.*, ionizable lipids) and their method of preparation are disclosed in, *e.g.*, U.S.

Patent Nos. 8,569,256, 5,965,542 and U.S. Patent Publication Nos. US 2016/0199485, US 2016/0009637, US 2015/0273068, US 2015/0265708, US 2015/0203446, US 2015/0005363, US 2014/0308304, US 2014/0200257, US 2013/086373, US 2013/0338210, US 2013/0323269, US 2013/0245107, US 2013/0195920, US 2013/0123338, US 2013/0022649, US 2013/0017223, US 2012/0295832, US 2012/0183581, US 2012/0172411, US 2012/0027803, US 2012/0058188, US 2011/0311583, US 2011/0311582, US 2011/0262527, US 2011/0216622, US 2011/0117125, US 2011/0091525, US 2011/0076335, US 2011/0060032, US 2010/0130588, US 2007/0042031, US 2006/0240093, US 2006/0083780, US 2006/0008910, US 2005/0175682, US 2005/017054, US 2005/0118253, US 2005/0064595, US 2004/0142025, US 2007/0042031, US 1999/009076 and PCT Pub. Nos. WO 99/39741, WO 2017/004143, WO 2017/075531, WO 2015/199952, WO

2014/008334, WO 2013/086373, WO 2013/086322, WO 2013/016058, WO 2013/086373, WO 2011/141705, and WO 2001/07548, the full disclosures of which are herein incorporated by reference in their entirety for all purposes.

Other exemplary lipids and their manufacture are described in the art, for example in the following publications:

US Patent Application Publication No. US 2012/0276209,  
 Semple et al., 2010, Nat Biotechnol., 28(2):172-176;  
 Akinc et al., 2010, Mol Ther., 18(7): 1357-1364;  
 Basha et al., 2011, Mol Ther, 19(12): 2186-2200;  
 Leung et al., 2012, J Phys Chem C Nanomater Interfaces, 116(34): 18440-18450;  
 Lee et al., 2012, Int J Cancer., 131(5): E781-90;  
 Belliveau et al., 2012, Mol Ther nucleic Acids, 1: e37;  
 Jayaraman et al., 2012, Angew Chem Int Ed Engl., 51(34): 8529-8533;  
 Mui et al., 2013, Mol Ther Nucleic Acids. 2, e139; Maier et al., 2013, Mol Ther., 21(8):  
 1570-1578; and

Tam et al., 2013, Nanomedicine, 9(5): 665-74,  
 each of which are incorporated by reference in their entirety. Lipids and their manufacture can be found, for example, in U.S. Pub. No. 2015/0376115 and 2016/0376224, both of which are incorporated herein by reference.

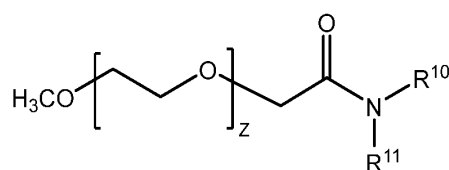
In certain embodiments, the lipid nanoparticle comprises one or more neutral lipids selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE and SM. In some embodiments, the neutral lipid is DSPC. In some embodiments, the molar ratio of ionizable lipid to the neutral lipid ranges from about 2:1 to about 8:1. In certain embodiments, the steroid is cholesterol. In some embodiments, the molar ratio of ionizable lipid to cholesterol ranges from about 2:1 to about 1:1. In certain embodiments, the molar ratio of ionizable lipid to cholesterol ranges from about 5:1 to about 1:1 or from about 2:1 to about 1:1.

In certain embodiments, the polymer conjugated lipid is a pegylated lipid. In various embodiments, the polymer conjugated lipid is a pegylated lipid. For example, some embodiments include a pegylated diacylglycerol (PEG-DAG) such as  
 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG), a pegylated phosphatidylethanolamine (PEG-PE), a PEG succinate diacylglycerol (PEG-S-DAG) such as 4-O-(2',3'-di(tetradecanoyloxy)propyl)-1-O-(ω-methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG), a pegylated ceramide (PEG-cer), or a PEG dialkoxypopylcarbamate such as ω-

methoxy(polyethoxy)ethyl-N-(2,3-di(tetradecanoxy)propyl)carbamate or 2,3-di(tetradecanoxy)propyl-N-(ω-methoxy(polyethoxy)ethyl)carbamate.

In some embodiments, the molar ratio of ionizable lipid to the pegylated lipid ranges from about 100:1 to about 10:1 or from about 100:1 to about 25:1. In some embodiments, the molar ratio of ionizable lipid to pegylated lipid ranges from about 100:1 to about 20:1 or from about 100:1 to about 10:1. In some embodiments, the pegylated lipid is PEG-DMG. In some embodiments, the pegylated lipid is PEG-DAG, PEG-PE, PEG-S-DAG, PEG-cer or a PEG dialkylalkoxypropylcarbamate.

In some embodiments, the lipid nanoparticle comprises at least one pegylated lipid having a structure:



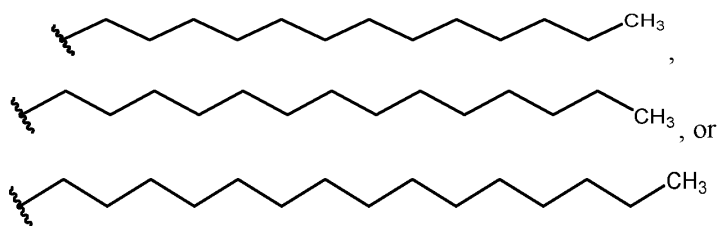
or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof, wherein:

$\text{R}^{10}$  and  $\text{R}^{11}$  are each independently a straight or branched alkyl, alkenyl, or alkynyl containing from 10 to 30 carbon atoms, wherein each alkyl, alkenyl, or alkynyl is optionally substituted with at least one fluoro; and

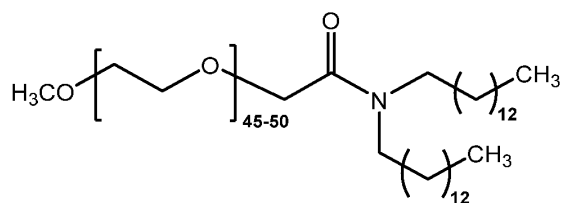
$z$  is an integer ranging from 30 to 60.

In some embodiments,  $\text{R}^{10}$  and  $\text{R}^{11}$  are each independently straight, alkyl chains containing from 12 to 16 carbon atoms, wherein each alkyl is optionally substituted with at least one fluoro. In certain embodiments,  $\text{R}^{10}$  and  $\text{R}^{11}$  are each independently straight alkyl chains containing from 12 to 16 carbon atoms.

In some embodiments,  $\text{R}^4$  and  $\text{R}^5$  are each independently:



In some embodiments, wherein  $z$  is an integer ranging from 45 to 50. In some embodiments, wherein  $z$  is an integer ranging from 42 to 48. In some embodiments, the at least one pegylated lipid has the following structure ("PEG Lipid 1"):



"PEG Lipid 1"

or a pharmaceutically acceptable salt or stereoisomer thereof.

In some embodiments, the lipid nanoparticle comprises a plurality of pegylated lipids. In some embodiments, the plurality of pegylated lipids has an average value of  $z$  ranging from 40 to 55. In some embodiments, the plurality of pegylated lipids has an average value of  $z$  ranging from 40 to 50, or 42 to 48. In some embodiments, the plurality of pegylated lipids has an average value of  $z$  ranging from 30 to 55, 30 to 50, 30 to 45, 30 to 40, or 30 to 35. In some embodiments, the plurality of lipids has an average value of  $z$  ranging from 35 to 55, 40 to 55, 42 to 55, 45 to 55, or 48 to 55.

Synthesis of pegylated lipids can be found in US Patent No. 9,738,593, the disclosure of which is hereby incorporated by reference.

As used herein, "mol percent," "mole percent," or "mol%" refers to a component's molar percentage relative to the total number of mols of all components of a lipid nanoparticle excluding a therapeutic agent (*e.g.*, total mols of ionizable lipid(s), neutral lipid(s), steroid(s), and/or polymer conjugated lipid(s)).

In some embodiments, the ionizable lipid is present at a concentration ranging from about 20 to about 70 mol% of the lipid nanoparticle. In some embodiments, the ionizable lipid is present at a concentration ranging from about 35 to about 70 mol%, from about 40 to about 60 mol%, from about 45 to about 50 mol%, from about 45 to about 49 mol%, from about 40 to about 55 mol%, or from about 46 to about 48 mol% of the lipid nanoparticle.

In some embodiments, the neutral lipid is present at a concentration ranging from about 5 to about 15 mol% of the lipid nanoparticle. In some embodiments, the neutral lipid is present at a concentration ranging from about 7 to about 12 mol%, from about 6 to about 11 mol%, or from about 8 to about 13 mol% of the lipid nanoparticle.

In some embodiments, the steroid is present at a concentration ranging from about 30 to about 60 mol% of the lipid nanoparticle. In some embodiments, the steroid is present at a concentration ranging from about 40 to about 50 mol%, from about 41 to about 49 mol%, or from about 46 to about 44 mol%.



In some embodiments, the concentration of the pegylated lipid ranges from about 3.5 to about 5.5 mol% of the lipid nanoparticle. In some embodiments, the concentration of the pegylated lipid ranges from about 1.0 to about 3.0 mol% of the lipid nanoparticle. In some embodiments, the concentration of the pegylated lipid ranges from about 1.0 to about 2.5 mol% of the lipid nanoparticle.

Administration of the lipid nanoparticles of the disclosure can be carried out via any of the accepted modes of administration of agents for serving similar utilities. The pharmaceutical compositions of the disclosure may be formulated into preparations in solid, semi-solid, liquid, or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suspensions, suppositories, injections, inhalants, gels, microspheres, and aerosols. Typical routes of administering such pharmaceutical compositions include, without limitation, oral, topical, transdermal, inhalation, parenteral, sublingual, buccal, rectal, vaginal, and intranasal. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intradermal, intrasternal injection or infusion techniques. Pharmaceutical compositions of the disclosure are formulated to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that will be administered to a subject or patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of a compound of the disclosure in aerosol form may hold a plurality of dosage units. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington: The Science and Practice of Pharmacy*, 20th Edition (Philadelphia College of Pharmacy and Science, 2000). The composition to be administered will, in any event, contain a therapeutically effective amount of a therapeutic agent (*e.g.*, a nucleic acid) of the disclosure, or a pharmaceutically acceptable salt thereof, for treatment of a disease or condition of interest in accordance with the teachings of this disclosure.

In some embodiments, the administration is a local administration (*e.g.*, direct injection into an eye or brain).

Some embodiments provide a pharmaceutical composition comprising the lipid nanoparticles (*e.g.*, dry or in an aqueous solution) as defined herein. Certain embodiments provide a pharmaceutical composition comprising the lipid nanoparticles (*e.g.*, dry or in an aqueous solution) and the payload (*e.g.*, dry or in an aqueous solution) as defined herein. Some other embodiments, provide a pharmaceutical composition comprising the payload (*e.g.*, dry or in an aqueous solution) as defined herein. The composition may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the

active ingredient (*e.g.*, mRNA). The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage. The administration may be via a syringe injection, or an intravenous drip and the unit dose may be contained with a suitable container (*e.g.*, a bottle, vial, ampoule, bag, *etc.*). The unit dose may be administered over a short period (*e.g.*, under 5 seconds or under 30 seconds) or over a longer period (*e.g.*, over the course of hours or days). In some embodiments, unit dose comprises a payload that is mRNA and is administered as a single injection. In some embodiments, the unit dose is less than 100 micrograms. In certain embodiments, the unit dose is less than 100 mg per kg (of the patient). In some embodiments, the unit dose is contained in a syringe.

In certain embodiments, a pharmaceutical composition of the disclosure is in the form of an aqueous solution. In some embodiments, the aqueous solution comprises one or more carrier(s), which may be liquid or dissolve in liquid. In some embodiments, the carrier(s) facilitate aerosol delivery, which is useful in, for example, inhalatory administration. In some embodiments, the administration is via inhalation and the composition is aerosolized. In some embodiments, the composition is administered to treat surfaces with mucosa.

In some embodiments, the administration targets extrahepatic tissue by prolonged circulation. In some embodiments, the administration targets extrahepatic tissue and the LNP includes a targeting moiety (*e.g.*, an antibody).

In some embodiments, the pharmaceutical composition is in the form of a liquid (*e.g.*, an aqueous solution), for example, an elixir, syrup, solution, emulsion, or suspension. The liquid may be for delivery by intravenous injection or intramuscular injection, as two examples. In a composition intended to be administered by injection, one or more of surfactant(s), preservative(s), wetting agent(s), dispersing agent(s), suspending agent(s), buffer(s), stabilizer(s), isotonic agent(s), or combinations thereof may be included.

The liquid pharmaceutical compositions of the disclosure, whether they be solutions, suspensions, or a similar form, may include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose; agents to act as cryoprotectants such as sucrose or trehalose. The

parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

5 In some embodiments, a liquid pharmaceutical composition of the disclosure intended for parenteral administration should contain an amount of a therapeutic agent of the disclosure such that a suitable dosage will be obtained.

The pharmaceutical composition of the disclosure may be intended for topical administration, in which case the carrier may suitably comprise a solution, emulsion, ointment or gel base. The base, for example, may comprise one or more of the following: petrolatum, lanolin, 10 polyethylene glycols, bee wax, mineral oil, diluents such as water and alcohol, and emulsifiers and stabilizers. Thickening agents may be present in a pharmaceutical composition for topical administration. If intended for transdermal administration, the composition may include a transdermal patch or iontophoresis device.

The pharmaceutical composition of the disclosure may be intended for rectal 15 administration, in the form, for example, of a suppository, which will melt in the rectum and release the drug. The composition for rectal administration may contain an oleaginous base as a suitable nonirritating excipient. Such bases include, without limitation, lanolin, cocoa butter, and polyethylene glycol.

The pharmaceutical composition of the disclosure may also include an agent that binds to 20 the surface of the lipid nanoparticle of the disclosure and thereby assists in the delivery of the therapeutic agent. Suitable agents that may act in this capacity include a monoclonal or polyclonal antibody, or a protein.

The pharmaceutical composition of the disclosure may consist of dosage units that can be administered as an aerosol. The term aerosol is used to denote a variety of systems ranging from 25 those of colloidal nature to systems consisting of pressurized packages. Delivery may be by a liquefied or compressed gas or by a suitable pump system that dispenses the active ingredients. Aerosols of compounds of the disclosure may be delivered in single phase, bi-phasic, or tri-phasic systems to deliver the active ingredient(s). Delivery of the aerosol includes the necessary container, activators, valves, sub-containers, and the like, which together may form a 30 kit. One skilled in the art, without undue experimentation may determine preferred aerosols.

The pharmaceutical compositions of the disclosure may be prepared by methodology well known in the pharmaceutical art. For example, a pharmaceutical composition intended to be administered by injection can be prepared by combining the lipid nanoparticles of the disclosure with sterile, distilled water or other carrier to form a solution. A surfactant may be added to

facilitate the formation of a homogeneous solution or suspension. Surfactants are compounds that non-covalently interact with the compound of the disclosure to facilitate dissolution or homogeneous suspension of the compound in the aqueous delivery system.

The compositions of the disclosure, or their pharmaceutically acceptable salts, are administered in a therapeutically effective amount, which will vary depending upon a variety of factors including the activity of the specific therapeutic agent employed; the metabolic stability and length of action of the therapeutic agent; the age, body weight, general health, sex, and diet of the patient; the mode and time of administration; the rate of excretion; the drug combination; the severity of the particular disorder or condition; and the subject undergoing therapy.

Compositions of the disclosure may also be administered simultaneously with, prior to, or after administration of one or more other therapeutic agents. Such combination therapy includes administration of a single pharmaceutical dosage formulation of a composition of the disclosure and one or more additional active agents, as well as administration of the composition of the disclosure and each active agent in its own separate pharmaceutical dosage formulation. For example, a composition of the disclosure and the other active agent can be administered to the patient together in a single dosage composition (*e.g.*, an injection), or each agent administered in separate dosage formulations. Where separate dosage formulations are used, the compounds of the disclosure and one or more additional active agents can be administered at essentially the same time, *i.e.*, concurrently, or at separately staggered times, *i.e.*, sequentially; combination therapy is understood to include all these regimens.

Preparation methods for the above compounds and compositions are described herein below and/or known in the art.

### ***Preparation of Lipid Nanoparticle Components***

It will be appreciated by those skilled in the art that in the process described or incorporated herein the functional groups of intermediate compounds may need to be protected by suitable protecting groups. Such functional groups include hydroxy, amino, mercapto and carboxylic acid. Suitable protecting groups for hydroxy include trialkylsilyl or diarylalkylsilyl (for example, *t*-butyldimethylsilyl, *t*-butyldiphenylsilyl or trimethylsilyl), tetrahydropyranyl, benzyl, and the like. Suitable protecting groups for amino, amidino and guanidino include *t*-butoxycarbonyl, benzyloxycarbonyl, and the like. Suitable protecting groups for mercapto include -C(O)-R'' (where R'' is alkyl, aryl or arylalkyl), *p*-methoxybenzyl, trityl and the like. Suitable protecting groups for carboxylic acid include alkyl, aryl, or arylalkyl esters. Protecting groups may be added or removed in accordance with standard techniques, which are known to

one skilled in the art and as described herein. The use of protecting groups is described in detail in Green, T.W. and P.G.M. Wutz, *Protective Groups in Organic Synthesis* (1999), 3<sup>rd</sup> Ed., Wiley. As one of skill in the art would appreciate, the protecting group may also be a polymer resin such as a Wang resin, Rink resin or a 2-chlorotrityl-chloride resin.

5           Furthermore, all compounds of this disclosure which exist in free base or acid form can be converted to their pharmaceutically acceptable salts by treatment with the appropriate inorganic or organic base or acid by methods known to one skilled in the art. Salts of lipid components of the disclosure can be converted to their free base or acid form by standard techniques.

10           It is understood that one skilled in the art may be able to make the ionizable lipids disclosed or incorporated herein using referenced methods or by combining referenced methods with those known to one skilled in the art. In general, starting components for making ionizable lipids may be obtained from sources such as Sigma Aldrich, Lancaster Synthesis, Inc., Maybridge, Matrix Scientific, TCI, and Fluorochem USA, *etc.* or synthesized according to  
15           sources known to those skilled in the art (see, for example, *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, 5<sup>th</sup> edition (Wiley, December 2000)) or prepared as described in this disclosure.

## FORMULATION EXAMPLE 1

### LIPID NANOPARTICLE FORMATION

20           The following parameters and procedures were used to test loading of lipid nanoparticles with nucleic acids following formation of lipid nanoparticles (*i.e.*, instead of encapsulating the nucleic acid during or concomitant with the lipid nanoparticle formation process – so called "post formulation loading," "post formation loading," or "PFL"). More specifically, for the PFL method the LNPs were formed, lipid solubilizing agents necessary to the formation step (*e.g.*,  
25           ethanol) were removed, and the resultant LNPs were then combined with a payload (*e.g.*, mRNA) under specific conditions thereby encapsulating the payload. For these experiments, different methods and steps for loading, and different post-loading neutralization methods were used.

            LNP samples for control samples were prepared using standard methods with specific variations as described herein. These general methods are as described elsewhere (*e.g.*, US Patent  
30           No. 11,453,639) with variations specified herein.

## A) Preparation of Empty LNP

Individual stock solutions of each of distearoyl phosphatidylcholine (DSPC), cholesterol and PEG Lipid 1 were prepared by weighing out each lipid powder and dissolving in ethanol to achieve a concentration of approximately 10 mg/mL lipid. Using these stock solutions, a mixture  
5 containing the lipids in a molar ratio of 47.5:10:40.7:1.8 for ionizable lipid C-18/ DSPC/ cholesterol/ PEG Lipid 1 was prepared by adding appropriate amounts of the stock solutions to 27.6 mg of ionizable lipid C-18 neat oil. The final lipid mixture was diluted with ethanol to achieve a final total lipid concentration of 11.83 mM.

## B) Preparation of mRNA payload

10 mRNA encoding hAAT was thawed at room temperature and the RNA concentration was verified by measuring its absorbance at 260 nm assuming a conversion factor of 40 µg/OD<sub>260</sub>. This yielded a measured concentration of 1.08 mg/mL. The RNA was then diluted in 10 mM Phosphate buffer, pH 7.4 to a final concentration of 0.100 mg/mL.

## C) Preparation of LNP with RNA stock solution at neutral pH

15 LNP were prepared by mixing the lipid solution with the aqueous solution of RNA diluted in 10 mM phosphate buffer, pH 7.4 as generally described elsewhere (*e.g.*, US Patent No. 11,453,639). Briefly, the solutions were combined at a 3:1 ratio of aqueous to organic (*i.e.*, RNA solution to lipid solution) using a T-mixing system at flow rates 30 mL/min and of 10 mL/min for the aqueous and organic solutions, respectively. As shown in Table 1 below, when the LNP  
20 are formed as described with the RNA stock at pH 7.4, the RNA is not encapsulated during the mixing process and the resulting LNP suspension is comprised of empty LNP with RNA in solution external to the LNP. This mixture of empty LNP and external RNA was separated into 2 aliquots, and both were dialyzed in 1.5 L of 10 mM phosphate, pH 7.4 for 1.5 hours. The first aliquot (Aliquot 1 in Table 1 below) was transferred to 6 L of Dulbecco's Phosphate Buffered  
25 Saline pH 7.4 (DPBS) and dialyzed until the samples were collected the next day. The second aliquot (Aliquot 2 in the Table 1 below) was transferred to 2 L of 25 mM Acetate buffer, pH 5.9 and dialyzed for 11.5 hours, after which the sample was transferred to 1 L of DPBS and allowed to dialyze for 6 hours. After the dialysis procedure was completed for Aliquot 2, both LNP  
30 formulations were removed from dialysis and filtered (0.2 µm), then analyzed to determine particle size (by dynamic light scattering) and RNA encapsulation efficiency (by Ribogreen). The results are shown in Table 1, below.

**Table 1: The effect of intermediate dialysis in Acetate pH 5.9 on post formation loading**

| Sample Description                                                                                               | Z-Avg (nm) | PDI   | Encapsulation Efficiency (%) |
|------------------------------------------------------------------------------------------------------------------|------------|-------|------------------------------|
| Aliquot 1: LNP prepared with DPBS pH 7.4 and dialyzed with PBS pH 7.4                                            | 62         | 0.059 | 0                            |
| Aliquot 2: LNP prepared with DPBS pH 7.4 and dialyzed with Acetate pH 5.9 before final dialysis with DPBS pH 7.4 | 117        | 0.096 | 95                           |

#### Summary

This data demonstrates that a combination of lipids with RNA at neutral pH does not result in encapsulation during the mixing process, but exposure of the resulting mixture to a lower pH condition results in encapsulation after the LNP has initially formed. Moreover, RNA encapsulation in this way does not require any additional aids such as a solvent, surfactant or elevated temperature.

### FORMULATION EXAMPLE 2

#### A) Preparation of Empty LNP

Individual stock solutions of each of distearoyl phosphatidylcholine (DSPC), cholesterol and PEG Lipid 1 were prepared by weighing out each lipid powder and dissolving in ethanol to achieve a concentration of approximately 10 mg/mL lipid. Using these stock solutions, a mixture containing the lipids in a molar ratio of 47.5:10:40.7:1.8 for ionizable lipid C-18/DSPC/cholesterol/PEG Lipid 1 was prepared by adding appropriate amounts of the stock solutions to 30.3 mg of ionizable lipid C-18 neat oil. The final lipid mixture was diluted with ethanol to achieve a final total lipid concentration of 11.83 mM.

#### B) Preparation of mRNA payload

mRNA encoding firefly luciferase was thawed at room temperature and the RNA concentration was verified by measuring its absorbance at 260 nm assuming a conversion factor of 40 µg/OD<sub>260</sub>. This yielded a measured concentration of 1.04 mg/mL. The RNA then was diluted in 10 mM Phosphate buffer, pH 7.4 to a final concentration of 0.100 mg/mL.

#### C) Preparation of LNP

LNP were prepared by mixing the lipid solution with the aqueous solution of RNA diluted in 10 mM phosphate buffer, pH 7.4 as generally described elsewhere (*e.g.*, US Patent No. 11,453,639). Briefly, the solutions were combined at a 3:1 ratio of aqueous to organic (*i.e.*, RNA solution to lipid solution) using a T-mixing system at flow rates 30 mL/min and of 10 mL/min for the aqueous and organic solutions, respectively. As demonstrated in Formulation Example 1

and confirmed in the first row of Table 2 below, when the LNP are formed as described with the RNA stock at pH 7.4, the RNA is not encapsulated during the mixing process and the resulting LNP suspension is comprised of empty LNP with RNA in solution external to the LNP. This mixture of empty LNP and external RNA was separated into 5 aliquots and dialyzed using 12-14 kDa regenerated cellulose dialysis tubing in 1.4 L of 10 mM phosphate buffered saline pH 7.4 for 2 hours. After 2 hours of dialysis in 10 mM phosphate pH 7.4 one of the dialysis bags was transferred into 5L of DPBS and the remaining 4 dialysis bags were transferred to 2L of 25 mM acetate buffer pH 5.9. At each of the time points shown in the Table 2, one of the LNP samples dialyzing in 25 mM acetate buffer was removed, a 200  $\mu$ L aliquot drawn for in-process analysis of particle size, encapsulation efficiency and pH, and the remaining LNP was transferred to DPBS pH 7.4 to dialyze overnight. After ~16 hours (*i.e.*, dialysis overnight), all the LNP formulations were collected from dialysis and filtered (0.2  $\mu$ m), then analyzed to determine particle size (by dynamic light scattering) and RNA encapsulation efficiency (by Ribogreen). The results for both in process analyses at the intermediate stage after dialysis in acetate pH 5.9, and the corresponding final products after dialysis in DPBS are shown in Table 2.

**Table 2: The effect of intermediate dialysis time in Acetate pH 5.9**

|                                       | After intermediate dialysis in Acetate pH 5.9 |       |                              |           | After final dialysis in PBS pH 7.4 |       |                              |
|---------------------------------------|-----------------------------------------------|-------|------------------------------|-----------|------------------------------------|-------|------------------------------|
| Time of Acetate pH 5.9 Dialysis (min) | Z-Avg (nm)                                    | PDI   | Encapsulation Efficiency (%) | Sample pH | Z-Avg (nm)                         | PDI   | Encapsulation Efficiency (%) |
| 0                                     | n/a                                           | n/a   | n/a                          | n/a       | 55                                 | 0.029 | 3                            |
| 30                                    | 61                                            | 0.054 | 30                           | 7.00      | 61                                 | 0.042 | 24                           |
| 60                                    | 62                                            | 0.052 | 51                           | 6.78      | 69                                 | 0.036 | 53                           |
| 120                                   | 70                                            | 0.048 | 93                           | 6.16      | 71                                 | 0.002 | 93                           |
| 180                                   | 70                                            | 0.060 | 95                           | 6.00      | 71                                 | 0.021 | 93                           |

#### Summary

This data demonstrates that encapsulation of nucleic acid can be achieved after the LNP has already formed by exposing the mixture of empty LNP and external RNA to an acidic pH. The encapsulation of RNA occurs without any additional aids (*e.g.*, such as surfactants or solvents or elevated temperature, or requiring encapsulation to occur concomitant with the initial LNP formation.

The data show that the pH of the sample is lowered with time of dialysis in acetate pH 6, which then correlates with significantly increased encapsulation and concomitant minor increases in size, but no effect on polydispersity which remains homogeneously low throughout.



**FORMULATION EXAMPLE 3****LIPID NANOPARTICLE FORMATION****A) Preparation of Empty LNP**

A lipid mixture was prepared from appropriate additions of individual lipid stock solutions (10 mg/mL) to the ionizable lipid. The final lipid mixture contained the constituent lipids (ionizable lipid C-18, DSPC, cholesterol, PEG Lipid 1) in a 47.5:10:40.7:1.8 molar ratio at a total lipid concentration of 23.65 mM. To prepare the empty LNP an aqueous solution of 25 mM acetate buffer pH 5.9 (*i.e.*, without RNA) was combined with the lipid solution at a 3:1 ratio of aqueous to organic (*i.e.*, aqueous buffer solution to organic lipid solution) using a T-mixing system at flow rates 30 mL/min and of 10 ml/min for the aqueous and organic solutions, respectively. The LNP suspension was collected and allowed to sit at room temperature for approximately 15 min then ethanol was removed by overnight dialysis of the empty LNP in 25 mM acetate buffer (pH 5.9) at a volume 200-times that of the LNP suspension. Following dialysis, the empty LNP were filtered (0.2  $\mu$ m) and analyzed to determine physical attributes. Empty LNP samples were stored at 2-8°C for two days and then analyzed for particle size and lipid concentration. Total lipid concentration was calculated by first determining the cholesterol concentration using a cholesterol E enzymatic assay and then using the theoretical molar ratio of cholesterol relative to all the lipid components to calculate the total lipid concentration. The total lipid concentration was used to calculate the amount of lipid needed to prepare RNA-loaded LNP at an aminolipid nitrogen to nucleic acid phosphate (N:P) ratio of 6.

**B) Loading of LNP with mRNA payload**

mRNA encoding firefly luciferase was thawed at room temperature and a concentration of 1.09 mg/mL was found by measuring absorbance at 260 nm assuming a conversion factor of 40  $\mu$ g/OD<sub>260nm</sub>. The empty LNP solution was diluted in 25 mM acetate buffer, pH 5.9 to achieve a concentration of 3.17 mM total lipid. RNA (0.686 mL, 1.09 mg/mL) was added to the LNP solution by pipetting followed by gently inverting the tube several times. This produced an RNA-LNP mixture with a total lipid concentration of 2.96 mM and an RNA concentration of 0.075 mg/mL. The RNA-LNP mixture was allowed to incubate at room temperature and aliquots (1 mL) were taken at 10, 30, 50, 70 and 90 minutes. At each time point the RNA-LNP aliquot was added to 19 mL of 1 $\times$  DPBS. The PBS-diluted RNA-LNP was mixed gently by inversion, then the sample was analyzed for particle size and RNA encapsulation (Table 3). In addition to this dilution timecourse, further aliquots of RNA-LNP (1 mL) were transferred to dialysis bags and dialyzed in DPBS (at 200-times the volume) at the 10, 30, 70, 90 min timepoints. The remaining empty LNP were also dialyzed against PBS from time 0. The dialysis process was

stirred overnight at room temperature, after which the empty LNP formulations were filtered (0.2  $\mu\text{m}$ ) and then analyzed to determine particle size and RNA encapsulation.

**Table 3: Physical attributes of LNP following 20-fold direct dilution of the PFL RNA-LNP with DPBS.**

|                                         | Post Direct Dilution with PBS pH 7.4 |       |                            | Post dialysis overnight in PBS pH 7.4 |       |                            |
|-----------------------------------------|--------------------------------------|-------|----------------------------|---------------------------------------|-------|----------------------------|
| Incubation Time in Acetate pH 5.9 at RT | Z-Avg (nm)                           | PDI   | Encapsulation Efficiency % | Z-Avg (nm)                            | PDI   | Encapsulation Efficiency % |
| N/A; Empty LNP                          | 51                                   | 0.188 | N/A                        | 50                                    | 0.063 | N/A                        |
| 10                                      | 72                                   | 0.126 | 96.4                       | 63                                    | 0.050 | 99.2                       |
| 30                                      | 71                                   | 0.087 | 97.3                       | 64                                    | 0.034 | 98.8                       |
| 50                                      | 69                                   | 0.110 | 98.4                       | -                                     | -     | -                          |
| 70                                      | 69                                   | 0.145 | 98.3                       | 64                                    | 0.035 | 98.9                       |
| 90                                      | 69                                   | 0.103 | 99.5                       | 64                                    | 0.054 | 99.0                       |

#### 5 Summary

This data demonstrates that encapsulation of nucleic acid can be achieved after the LNP has already formed by introducing the RNA to a suspension of LNP held at a mildly acidic pH of 5.9 and then bringing the mixture back to a neutral pH suitable for *in vivo* administration. The encapsulation of RNA occurs without any additional aids such as surfactants or solvents or temperature, or requiring encapsulation to occur when the LNP are forming.

Preparation of RNA-LNP by direct addition of RNA to a suspension of empty LNP followed by direct dilution in DPBS to neutralize the pH, yields RNA-LNP of a reasonable size and polydispersity with high RNA encapsulation. The data indicates the encapsulation process is effectively complete (*e.g.*, greater than 95% encapsulation) within 10 minutes although marginal improvement is observed for longer incubation times prior to direct dilution. There is a trend to smaller particle size and higher encapsulation efficiency with additional incubation time prior to direct dilution.

The corresponding samples that are neutralized via dialysis show no trends with pH 5.9 incubation time, again indicating the encapsulation process is complete within the shortest incubation time investigated here (10 minutes). The results generally show smaller sizes, lower polydispersity than corresponding direct dilution samples, and effectively maximum encapsulation efficiency for all incubation times investigated here.

**FORMULATION EXAMPLE 4****LIPID NANOPARTICLE FORMATION**

The following example shows results for the PFL method using different sample neutralization methods post-loading for a range of formulations based on different ionizable cationic lipids.

**A) Preparation of Empty LNP**

Lipid mixtures were prepared from appropriate additions of individual lipid stock solutions (10 mg/mL) to a separate weighing of neat ionizable lipid for each of three ionizable cationic lipids: ionizable lipid A-15, ionizable lipid B-3, and ionizable lipid C-18. The final lipid mixture contained the constituent lipids (ionizable lipid, DSPC, cholesterol, PEG Lipid 1) in a 47.5:10:40.7:1.8 molar ratio at a total lipid concentration of 23.65 mM. To prepare the empty LNP, an aqueous solution of 25 mM acetate buffer pH 5.9 (*i.e.*, without RNA) was combined with the lipid solution at a 3:1 ratio of aqueous to organic (*i.e.*, aqueous buffer solution to organic lipid solution) using a T-mixing system at flow rates of 30 mL/min and of 10 mL/min for the aqueous and organic solutions, respectively. The LNP suspension was collected and allowed to sit at room temperature for approximately 15 min then ethanol was removed by overnight dialysis of the empty LNP in 25 mM acetate buffer (pH 5.9) at a volume 200-times that of the LNP suspension. Following dialysis, the empty LNP were filtered (0.2 µm) and analyzed for particle size and lipid concentration. Total lipid concentration was calculated by first determining the cholesterol concentration using a cholesterol E enzymatic assay and then using the theoretical molar ratio of cholesterol relative to all the lipid components to calculate the total lipid concentration. The total lipid concentration was used to calculate the amount of lipid needed to prepare RNA-loaded LNP at an aminolipid nitrogen to nucleic acid phosphate (N:P) ratio of 6.

**B) Loading of LNP with mRNA payload**

A 1:1 molar ratio mixture of 2 mRNA encoding the heavy chain and light chain of an anti-flu IgG antibody was thawed out at room temperature and diluted in nuclease-free water to a working concentration of approximately 1 mg/mL. The RNA concentration was verified by measuring its absorbance at 260 nm assuming a conversion factor of 40 µg/OD<sub>260</sub>. This yielded a measured concentration of 1.12 mg/mL RNA. The empty LNP solution was added to a polypropylene conical tube and was diluted in 25 mM acetate buffer, pH 5.9 to achieve a concentration of 3.17 mM total lipid. 0.806 mL of the 1.12 mg/mL RNA stock was then added to the LNP solution by pipetting followed by gently inverting the tube several times. This produced an RNA-LNP mixture with a total lipid concentration of 2.96 mM and an RNA concentration of 0.075 mg/mL. The RNA-LNP mixture was incubated at room temperature for 90 min.

## C) Processing and analysis of the RNA-LNP (neutralization and concentration)

After the incubation period in acetate at pH 5.9 the RNA-LNP mixture was split and either diluted or dialyzed in neutral pH buffer solutions to return the sample to a neutral pH. In this experiment three different methods were investigated for this neutralization step:

5            5× Dilution with 1× DPBS: 1 volume of LNP mixture was added to 4 volumes of DPBS in a polypropylene conical tube. The sample was then concentrated to ~0.6 mg/mL RNA by ultracentrifugation and stored at 2-8 °C overnight.

              2× Dilution with 2× DPBS: 1 volume of LNP mixture was added to an equal volume of 2× concentrated DPBS. The sample was then concentrated to ~1 mg/mL RNA by  
10 ultracentrifugation and stored at 2-8 °C overnight.

              Dialysis in DPBS: the LNP mixture was loaded into a dialysis bag and dialyzed against a 1× DPBS at 200-times the volume of the RNA-LNP mixture in acetate buffer overnight. After dialysis, the sample was then concentrated to ~1 mg/mL RNA by ultracentrifugation.

## D) Analysis of all LNP

15            RNA-LNP samples were analyzed for particle size, RNA encapsulation efficiency as well as lipid and RNA content using UPLC methods.

## E) Test of Freeze/Thaw Stability

              After processing as described above, all samples were diluted with DPBS and spiked with an amount of 1.2 M sucrose cryoprotectant appropriate to achieve 300 mM sucrose and 0.8  
20 mg/mL RNA in preparation for frozen storage at -80°C. However, samples processed by 5× dilution in DPBS were not sufficiently concentrated by ultracentrifugation to reach this final target of 0.8 mg/mL RNA, and in these instances the samples received the minimum required dilution with cryoprotectant to achieve 300 mM sucrose in the final solution, with total RNA concentrations for those samples indicated in tables below. After this dilution the samples were  
25 passed through a filter (0.2 µm) and aliquoted as required. RNA-LNP formulations were then analyzed for particle size, RNA encapsulation efficiency as well as lipid and RNA concentration following a single free/thaw cycle.

**Table 4a: Data generated using Formulations based on ionizable lipid A-15 – Size, polydispersity, encapsulation, and drug-to-lipid ratio.**

| Sample | Description                                                         | Z-Avg (nm) | PDI   | Encapsulation % | Total mRNA/Lipid (mg/µmol) |
|--------|---------------------------------------------------------------------|------------|-------|-----------------|----------------------------|
| 4a-1   | Empty ionizable lipid A-15 LNP stock - post acetate pH 5.9 dialysis | 49         | 0.101 | N/A             | N/A                        |

| Sample | Description                                                                       | Z-Avg (nm) | PDI   | Encapsulation % | Total mRNA/Lipid (mg/μmol) |
|--------|-----------------------------------------------------------------------------------|------------|-------|-----------------|----------------------------|
| 4a-2   | Empty LNP control - post DPBS dialysis and concentration (final)                  | 56         | 0.093 | N/A             | N/A                        |
| 4a-3   | Empty LNP control – Final sample post freeze/thaw                                 | 62         | 0.066 | N/A             | N/A                        |
| 4a-4   | Add mRNA to 4a-1, incubate 90 minutes, then 5-fold dilution in 1× DPBS            | 80         | 0.093 | -               | -                          |
| 4a-5   | Post concentration (final)                                                        | 77         | 0.046 | -               | -                          |
| 4a-6   | Post storage overnight at 2-8 °C                                                  | 78         | 0.032 | 97              | 0.028                      |
| 4a-7   | Final sample at 0.38 mg/mL post freeze/thaw                                       | 78         | 0.031 | 98              | 0.026                      |
| 4a-8   | Add mRNA to A, incubate 90 minutes then 2-fold dilution in 2× DPBS                | 78         | 0.060 | -               | -                          |
| 4a-9   | Post concentration (final)                                                        | 80         | 0.035 | -               | -                          |
| 4a-10  | Post storage overnight at 2-8 °C                                                  | 76         | 0.044 | 97              | 0.029                      |
| 4a-11  | Final sample at 0.8 mg/mL post freeze/thaw                                        | 77         | 0.071 | 98              | 0.025                      |
| 4a-12  | Add mRNA to 4a-1, incubate 90min, post dialysis in DPBS and concentration (final) | 75         | 0.018 | 99              | 0.029                      |
| 4a-13  | Final sample at 0.8 mg/mL post freeze/thaw                                        | 75         | 0.033 | 98              | 0.027                      |

**Table 4b: Data generated using Formulations Based on ionizable lipid B-3 – Size, polydispersity, encapsulation, and drug-to-lipid ratio.**

| Sample | Description                                                            | Z-Avg (nm) | PDI   | Encapsulation % | Total mRNA/Lipid (mg/μmol) |
|--------|------------------------------------------------------------------------|------------|-------|-----------------|----------------------------|
| 4b-1   | Empty ionizable lipid B-3 LNP stock - post acetate pH 5.9 dialysis     | 52         | 0.085 | N/A             | N/A                        |
| 4b-2   | Empty LNP control - post DPBS dialysis and concentration (final)       | 56         | 0.073 | N/A             | N/A                        |
| 4b-3   | Empty LNP control – Final sample post freeze/thaw                      | 66         | 0.113 | N/A             | N/A                        |
| 4b-4   | Add mRNA to 4b-1, incubate 90 minutes, then 5-fold dilution in 1× DPBS | 76         | 0.064 | -               | -                          |
| 4b-5   | Post concentration (final)                                             | 73         | 0.026 | -               | -                          |
| 4b-6   | Post storage overnight at 2-8 °C                                       | 73         | 0.017 | 88%             | 0.028                      |
| 4b-7   | Final sample at 0.38 mg/mL post freeze/thaw                            | 74         | 0.022 | 87%             | 0.025                      |

| Sample | Description                                                                       | Z-Avg (nm) | PDI   | Encapsulation % | Total mRNA/Lipid (mg/μmol) |
|--------|-----------------------------------------------------------------------------------|------------|-------|-----------------|----------------------------|
| 4b-8   | Add mRNA to 4b-1, incubate 90 minutes then 2-fold dilution in 2× DPBS             | 74         | 0.051 | -               | -                          |
| 4b-9   | Post concentration (final)                                                        | 73         | 0.047 | -               | -                          |
| 4b-10  | Post storage overnight at 2-8 °C                                                  | 74         | 0.019 | 88%             | 0.027                      |
| 4b-11  | Final sample at 0.8 mg/mL post freeze/thaw                                        | 73         | 0.065 | 90%             | 0.025                      |
| 4b-12  | Add mRNA to 4b-1, incubate 90min, post dialysis in DPBS and concentration (final) | 73         | 0.002 | 92%             | 0.028                      |
| 4b-13  | Final sample at 0.8 mg/mL post freeze/thaw                                        | 72         | 0.029 | 90%             | 0.025                      |

**Table 4c: Data generated using Formulations based on ionizable lipid C-18 – Size, encapsulation, and drug-to-lipid ratio.**

| Sample | Description                                                                       | Z-Avg (nm) | PDI   | Encapsulation % | Total mRNA/Lipid (mg/μmol) |
|--------|-----------------------------------------------------------------------------------|------------|-------|-----------------|----------------------------|
| 4c-1   | Empty ionizable lipid C-18 LNP stock – post acetate pH 5.9 dialysis               | 48         | 0.053 | N/A             | N/A                        |
| 4c-2   | Empty LNP control – post DPBS dialysis and concentration (final)                  | 55         | 0.048 | N/A             | N/A                        |
| 4c-3   | Empty LNP control – Final sample post freeze/thaw                                 | 66         | 0.027 | N/A             | N/A                        |
| 4c-4   | Add mRNA to 4c-1, incubate 90 minutes, then 5-fold dilution in 1× DPBS            | 74         | 0.097 | -               | -                          |
| 4c-5   | Post concentration (final)                                                        | 76         | 0.045 | -               | -                          |
| 4c-6   | Post storage overnight at 2-8 °C                                                  | 73         | 0.080 | 99%             | 0.028                      |
| 4c-7   | Final sample at 0.38 mg/mL post freeze/thaw                                       | 75         | 0.067 | 99%             | 0.025                      |
| 4c-8   | Add mRNA to 4c-1, incubate 90 minutes then 2-fold dilution in 2× DPBS             | 74         | 0.079 | -               | -                          |
| 4c-9   | Post concentration (final)                                                        | 75         | 0.051 | -               | -                          |
| 4c-10  | Post storage overnight at 2-8 °C                                                  | 73         | 0.068 | 99%             | 0.028                      |
| 4c-11  | Final sample at 0.8 mg/mL post freeze/thaw                                        | 76         | 0.086 | 99%             | 0.025                      |
| 4c-12  | Add mRNA to 4c-1, incubate 90min, post dialysis in DPBS and concentration (final) | 70         | 0.051 | 100%            | 0.028                      |

| Sample | Description                                | Z-Avg (nm) | PDI   | Encapsulation % | Total mRNA/Lipid (mg/ $\mu$ mol) |
|--------|--------------------------------------------|------------|-------|-----------------|----------------------------------|
| 4c-13  | Final sample at 0.8 mg/mL post freeze/thaw | 78         | 0.017 | 99%             | 0.025                            |

#### Summary

Tables 4a-c demonstrate that Size/PDI, RNA encapsulation and drug to lipid ratios were all generally within desirable parameters across formulations based on ionizable lipids from three chemically distinct chemical classes, regardless of the variations in post loading sample neutralization strategies employed here. In all cases, the empty LNP stocks displayed a smaller mean size that grew by ~15-25 nm in the final state. All samples were stable with respect to freeze/thaw *i.e.*, minor if any growth in size or PDI after freeze/thaw and no impact on encapsulation efficiency.

It is noted that formulations based on ionizable lipid B-3 demonstrated lower encapsulation efficiency at ~90% than the ~97% or greater observed for other formulations in this study. The apparent pKa of ionizable lipid B-3 is 6.09 compared to 6.35 for ionizable lipid A-15 and 6.45 for ionizable lipid C-18.

### FORMULATION EXAMPLE 5

#### LIPID NANOPARTICLE FORMATION

The following example demonstrates alternative PFL conditions for improved encapsulation with formulation based on ionizable lipid B-3 and demonstrates the PFL method for formulations based on a related ionizable lipid B-45 using the original conditions.

##### A) Preparation of Empty LNP

Lipid mixtures were prepared from appropriate additions of individual lipid stock solutions (10 mg/mL) to a separate weighing of neat ionizable lipid for each of two ionizable cationic lipids: ionizable lipid B-3 and ionizable lipid B-45. The final lipid mixture contained the constituent lipids (ionizable lipid, DSPC, cholesterol, PEG Lipid 1) in a 47.5:10:40.7:1.8 molar ratio at a total lipid concentration of 23.65 mM. This experiment used acetate buffer at 2 different pHs; a lower pH acetate buffer (pH 5.5) was used for ionizable lipid B-3 LNP, whereas the same pH 5.9 buffer as used in earlier examples was used for ionizable lipid B-45. To prepare the empty LNP, an aqueous solution of 25 mM acetate buffer at the pH described above for the corresponding ionizable lipid (*i.e.*, without RNA) was combined with the lipid solution at a 3:1 ratio of aqueous to organic (*i.e.*, aqueous buffer solution to organic lipid solution) using a T-mixing system at flow rates 30 mL/min and of 10 ml/min for the aqueous and organic solutions,

respectively. Suspensions were allowed to rest at room temperature for approximately 15 minutes after mixing was completed. Following this step, ethanol was removed by overnight dialysis of the empty LNP in 25 mM acetate buffer (for ionizable lipid B-3 pH 5.5; for ionizable lipid B-45 pH 5.9) at a volume 200× greater than that of the LNP preparation (using 12-14 kDa regenerated cellulose dialysis tubing). After dialysis, the empty LNP formulations were filtered using a 0.2 µm syringe filter and analyzed to determine particle size (by dynamic light scattering) and lipid concentration. Total lipid concentration was calculated by first determining the cholesterol concentration using a cholesterol E enzymatic assay and then using the theoretical molar ratio of cholesterol relative to all the lipid components to calculate the total lipid concentration. The total lipid concentration was used to calculate the amount of lipid needed to prepare RNA-loaded LNP at an aminolipid nitrogen to nucleic acid phosphate (N:P) ratio of 6.

#### B) Loading of LNP with mRNA payload

A 1:1 molar ratio mixture of 2 mRNA encoding the heavy chain and light chain of an anti-flu IgG antibody was thawed out at room temperature and diluted in nuclease-free water to a working concentration of approximately 1 mg/mL. The RNA concentration was verified by measuring its absorbance at 260 nm assuming a conversion factor of 40 µg/OD260. This yielded a measured concentration of 1.12 mg/mL RNA. Each of the empty LNP solutions were added to respective tubes and were diluted with either pH 5.5 or pH 5.9 acetate buffer (25mM) for ionizable lipid B-3 or ionizable lipid B-45, respectively, to achieve a concentration of 3.17 mM total lipid in both cases. RNA stock (0.806 mL, 1.12 mg/mL) was then added to each of the LNP solutions by pipetting and gently inverting the tube several times following RNA addition. This produced an RNA-LNP solution with a total lipid concentration of 2.96 mM and an RNA concentration of 0.075 mg/mL. The RNA-LNP mixture was then incubated at room temperature for 90 min.

#### 25 C) Processing and analysis of the RNA-LNP (neutralization and concentration)

After the incubation period in acetate buffer as described above, the RNA-LNP mixture was split and either diluted or dialyzed in neutral pH buffer solutions to return the sample to a neutral pH. In this experiment two different methods were investigated for this neutralization step:

30 2× PBS dialysis dilution: After the incubation period the mixture was added to an equal volume of 2× DPBS in a polypropylene conical tube. The DPBS diluted RNA-LNP was analyzed for particle size, RNA encapsulation efficiency as well as lipid and RNA concentration.



Dialysis in DPBS: Following incubation the LNP mixture was loaded into a dialysis bag and dialyzed against a 1× DPBS solution (pH 7.4) at 200× the volume of the LNP mixture in acetate buffer.

#### D) Analysis of all LNP

- 5 All final LNP samples were analyzed for particle size, RNA encapsulation efficiency as well as lipid and RNA content as previously described.

#### E) Test of Freeze/Thaw stability

- After processing as described above, all samples were diluted with DPBS and spiked with an amount of 1.2 M sucrose cryoprotectant appropriate to achieve 300 mM sucrose and ~0.75 mg/mL RNA in preparation for frozen storage at -80°C. After this dilution the samples were passed through a filter (0.2 µm) and aliquoted as required. RNA-LNP formulations were then analyzed for particle size, RNA encapsulation efficiency as well as lipid and RNA concentration following a single free/thaw cycle.

15 **Table 5a: Data generated using Cationic Lipid B-3 (pH 5.5) – Size, encapsulation, and drug-to-lipid ratio.**

| Sample | Description                                                            | Z-Avg (nm) | PDI   | Encapsulation % | Total mRNA/Lipid (mg/µmol) |
|--------|------------------------------------------------------------------------|------------|-------|-----------------|----------------------------|
| 5a-1   | Empty ionizable lipid B-3 LNP stock - post acetate pH 5.5 dialysis     | 57         | 0.125 | N/A             | N/A                        |
| 5a-2   | Empty LNP control - post DPBS dialysis and concentration (final)       | 57         | 0.111 | N/A             | N/A                        |
| 5a-3   | Empty LNP control -- Final sample post freeze/thaw                     | 69         | 0.097 | N/A             | N/A                        |
| 5a-4   | Add mRNA to 5a-1, incubate 90 minutes, then 2-fold dilution in 2× DPBS | 81         | 0.140 | -               | -                          |
| 5a-5   | Post concentration (final)                                             | 78         | 0.078 | -               | -                          |
| 5a-6   | Post storage overnight at 2-8 °C                                       | 77         | 0.076 | 99              | 0.023                      |
| 5a-7   | Final sample post freeze/thaw                                          | 82         | 0.118 | 97              | 0.028                      |
| 5a-8   | Add mRNA to 5a-1, incubate 90 minutes, then dialysis in DPBS overnight | 75         | 0.077 | -               | -                          |
| 5a-9   | Post concentration (final)                                             | 76         | 0.077 | 98              | 0.023                      |
| 5a-10  | Final sample post freeze/thaw                                          | 80         | 0.092 | 97              | 0.026                      |

**Table 5b: Data generated using ionizable lipid B-45 (pH 5.9) – Size, encapsulation, and drug-to-lipid ratio.**

| Sample | Description                                                                    | Z-Avg (nm) | PDI   | Encapsulation % | Total mRNA/Lipid (mg/ $\mu$ mol) |
|--------|--------------------------------------------------------------------------------|------------|-------|-----------------|----------------------------------|
| 5b-1   | Empty ionizable lipid B-45 LNP stock - post acetate pH 5.5 dialysis            | 75         | 0.103 | N/A             | N/A                              |
| 5b-2   | Empty LNP control - post DPBS dialysis and concentration (final)               | 76         | 0.132 | N/A             | N/A                              |
| 5b-3   | Empty LNP control – Final sample post freeze/thaw                              | 76         | 0.077 | N/A             | N/A                              |
| 5b-4   | Add mRNA to 5b-1, incubate 90 minutes, then 2-fold dilution in 2 $\times$ DPBS | 90         | 0.092 | -               | -                                |
| 5b-5   | Post concentration (final)                                                     | 89         | 0.005 | -               | -                                |
| 5b-6   | Post storage overnight at 2-8 $^{\circ}$ C                                     | 88         | 0.077 | 97              | 0.024                            |
| 5b-7   | Final sample post freeze/thaw                                                  | 90         | 0.080 | 96              | 0.026                            |
| 5b-8   | Add mRNA to 5b-1, incubate 90 minutes, then dialysis in DPBS overnight         | 88         | 0.021 | -               | -                                |
| 5b-9   | Post concentration (final)                                                     | 87         | 0.078 | 97              | 0.024                            |
| 5b-10  | Final sample post freeze/thaw                                                  | 90         | 0.054 | 96              | 0.028                            |

#### Summary

The data here demonstrate that the adjustment of pH for the acetate incubation step for the ionizable lipid B-3 based formulation results in high encapsulation consistent with other formulations incubated at pH 5.9 such as shown in earlier examples and for the formulation in this example based on ionizable lipid B-45 which has a related chemical structure but a higher apparent pKa of 6.26 (vs. 6.09 for ionizable lipid B-3). This indicates that maximum encapsulation by the PFL method is dependent on a minimum difference between the apparent pKa of the ionizable lipid and the PFL incubation buffer pH.

### FORMULATION EXAMPLE 6

#### HEAD-TO-HEAD IN VIVO COMPARISON 1

This study was commissioned to compare properties of LNP prepared using conventional methods or according to embodiments of the methods described herein (*e.g.*, Formulation Example 1). Each formulation was prepared using a ratio of 47.5:10:40.7:1.8 for ionizable lipid / DSPC / cholesterol / PEG Lipid 1

4 formulations were prepared according to conventional methods (*e.g.*, T-mixing, TFF, concentration, *etc.*) according to the parameters described below:

**Table 6a: Parameters for formulations prepared according to conventional methods**

| Sample | Ionizable lipid | Formulation pH |
|--------|-----------------|----------------|
| 6a-1   | A-15            | 6              |
| 6a-2   | B-3             | 4              |
| 6a-3   | B-45            | 5.9            |
| 6a-4   | C-18            | 6              |

Test samples were also prepared by mixing LNP components via a T-mixer in pH 5.9 acetate buffer to yield empty LNPs, dialyzed with pH 5.9 acetate buffer to remove ethanol, mixed with mRNA at room temperature to yield encapsulated mRNA, diluted with 2× PBS (1:1 ratio) and concentrated via ultrafiltration (Amicon), and diluted for freezing with PBS / sucrose mixture. 4 formulations were prepared as noted below.

**Table 6b: Parameters for formulations prepared according to methods of the present**

disclosure

| Sample | Ionizable Lipid | Formulation pH |
|--------|-----------------|----------------|
| 6b-1   | A-15            | 5.9            |
| 6b-2   | B-3             | 5.9            |
| 6b-3   | B-45            | 5.9            |
| 6b-4   | C-18            | 5.9            |

Samples were tested according to the procedure described in Biological Example 2 below using intravenous dosing. Additionally, all LNPs were submitted for a full analysis including RNA integrity, lipid-adduct formation, cryo-electron microscopy, encapsulation detection via UPLC, *etc.* Samples prepared according to the parameters detailed in Table 6a and 6b are described below.

**Table 6c: Physical characteristics for samples 6a-1 through 6a-4 and 6b-1 through 6b-4**

| Sample                        | 6a-1  | 6b-1  | 6a-2  | 6b-2  | 6a-3  | 6b-3  | 6a-4  | 6b-4  |
|-------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| Size (nm) <sup>†</sup>        | 71    | 81    | 73    | 74    | 64    | 87    | 75    | 77    |
| PDI                           | 0.006 | 0.042 | 0.023 | 0.064 | 0.065 | 0.046 | 0.099 | 0.016 |
| Encapsulation (%)             | 97    | 99    | 90    | 97    | 96    | 96    | 98    | 99    |
| mRNA : lipid (wt : $\mu$ mol) | 0.026 | 0.026 | 0.025 | 0.026 | 0.025 | 0.024 | 0.023 | 0.025 |

<sup>†</sup> Intensity mean particle diameter

As illustrated in FIG. 1 and FIG. 2, both types of formulations showed comparable activity at low doses for sample 6a-1 vs. sample 6b-1 and for sample 6a-2 vs. sample 6b-2. Sample 6b-3 was more active than sample 6a-3.

## FORMULATION EXAMPLE 7

5

### LIPID ADDUCT ASSESSMENT

Samples were tested for the formation of lipid adducts after storage at -80°C. It was discovered that lipid adducts are initially worse for samples prepared using conventional methods (*e.g.*, samples as shown in Table 6a) compared to methods prepared according to embodiments described herein (*e.g.*, samples as shown in Table 6b). Results for lipid adduct testing are shown in FIG. 3 (freshly ran from -80°C) and FIG. 4 (same samples post-dose and storage for 2 weeks at 4°C).

## FORMULATION EXAMPLE 8

### ENCAPSULATION ASSESSMENT

This procedure was used to check that RNA is encapsulated and confirm that RNA is not just adsorbed to the outer surface of the LNP. RiboGreen analysis was performed after dosing (*i.e.*, after about 1 week of storage at 4°C) and a UPLC assay was performed 6 days later with samples being stored at 4°C in the interim.

RNA encapsulation was similar for both the conventional methods as well as samples prepared according to methods described herein (*e.g.*, Formulation Example 1) according to both the RG analysis and the UPLC assay. Samples tested are shown in Table 6a and 6b. One notable exception is sample 6a-2 appears to show particularly low encapsulation when analyzed by UPLC.

## FORMULATION EXAMPLE 9

### IN VIVO COMPARISON FOR INTRAMUSCULAR DELIVERY

This procedure was used to compare properties of LNPs prepared using conventional methods or methods according to embodiments described herein (*e.g.*, Formulation Example 1) when these formulations are used for intramuscular delivery.

3 formulations were prepared according to conventional methods (*e.g.*, T-mixing, TFF, concentration, *etc.*) according to the parameters described below:

**Table 9a: Parameters for formulations prepared according to conventional methods**

| Sample | Ionizable lipid | Formulation pH |
|--------|-----------------|----------------|
| 9a-1   | B-3             | 4              |
| 9a-2   | A-39            | 5.9            |
| 6a-3   | D-1             | 5.9            |

Test samples were also prepared by mixing LNP components via a T-mixer in pH 5.9 acetate buffer to yield empty LNPs, dialyzed with pH 5.9 acetate buffer to remove ethanol, mixed with mRNA at room temperature to yield encapsulated mRNA, diluted with 2× PBS (1:1 ratio) and concentrated via ultrafiltration (Amicon), and diluted for freezing with PBS / sucrose mixture. 3 formulations were prepared as noted below.

**Table 9b: Parameters for formulations prepared according to methods of the present disclosure**

| Sample | Ionizable Lipid | Formulation pH |
|--------|-----------------|----------------|
| 9b-1   | B-3             | 5.9            |
| 9b-2   | A-39            | 5.9            |
| 9b-3   | D-1             | 5.9            |

All samples were prepared using PR8HA mRNA and activity was studied in mice using a prime / boost HAI immunogenicity assay when dosed intramuscularly (IM). All LNP preparations were fully analyzed (*e.g.*, RNA integrity, lipid adduct testing, *etc.*).

Analysis of the prepared samples showed the following data:

**Table 9c: Physical characteristics for samples 9a-1 through 9a-3 and 9b-1 through 9b-3**

| Sample                        | 9a-1  | 9b-1  | 9a-2  | 9b-2  | 9a-3  | 9b-3  |
|-------------------------------|-------|-------|-------|-------|-------|-------|
| Size (nm) <sup>†</sup>        | 84    | 81    | 85    | 95    | 80    | 96    |
| PDI                           | 0.044 | 0.043 | 0.017 | 0.091 | 0.057 | 0.101 |
| Encapsulation (%)             | 81    | 94    | 96    | 97    | 95    | 96    |
| mRNA : lipid (wt : $\mu$ mol) | 0.018 | 0.022 | 0.020 | 0.021 | 0.019 | 0.021 |
| Lipid adducts (%)             | 3.1   | 0.9   | 2.2   | 0.5   | 10.3  | 1.6   |
| RNA integrity (%) (RNA = 78)  | 66    | 72    | 72    | 70    | 72    | 65    |

<sup>†</sup> Intensity mean particle diameter

In sum, LNP samples prepared according to Tables 9a and 9b showed similar immunogenicity independent of preparation method with the notable exception of sample 9b-3 at a dose of 0.5  $\mu$ g, which had a lower titre. At low dose (0.2  $\mu$ g), samples 9a-1 and 9b-1 were less

active than samples 9a-2 and 9b-2 (1.9×) or samples 9a-3 and 9b-3 (3.2×). At higher dose (0.5 µg), immunogenicity was similar for all ionizable lipids.

Size, PDI, encapsulation, and lipid adduct formation generally looks good for samples described in Table 9b. Sizes for these samples are slightly larger than samples prepared according to Table 9a. *In vivo* activity was similar for both preparations made according to Table 9a and Table 9b across the range of lipids. Lipid adduct data shows RNA integrity and indicates surprisingly reduced levels for samples prepared according to embodiments of methods described herein (*e.g.*, Formulation Example 1; see, *e.g.*, Table 9c, FIG. 3). UPLC encapsulation assay confirms good encapsulation in all formulations prepared according to embodiments of methods described herein (*e.g.*, Formulation Example 1). Cryo-electron microscope data appears similar for all methods of preparation.

## FORMULATION EXAMPLE 10

### STUDY OF ENCAPSULATION AS A FUNCTION OF RNA MIXING CONCENTRATION

Concentration of RNA at mixing was studied to determine encapsulation efficiency of LNPs prepared according to embodiments of methods described herein (*e.g.*, Formulation Example 1). Namely, test samples were prepared by mixing LNP components via a T-mixer in pH 5.9 acetate or pH 5.5 acetate buffer to yield empty LNPs, dialyzed with pH 5.9 acetate or pH 5.5 acetate buffer to remove ethanol, mixed with mRNA at room temperature to yield encapsulated mRNA, diluted with 2× PBS (1:1 ratio) and concentrated via ultrafiltration (Amicon), and diluted for freezing with PBS / sucrose mixture. 3 formulations were prepared as noted below.

**Table 10: Parameters for formulations prepared according to methods of the present disclosure**

| Sample | Ionizable Lipid | Formulation pH |
|--------|-----------------|----------------|
| 10-1   | A-15            | 5.9            |
| 10-2   | B-3             | 5.5            |
| 10-4   | C-18            | 5.9            |

Samples were then mixed with mRNA and encapsulation was determined for a range of concentrations (*i.e.*, for RNA concentrations of 0.15, 0.5, and 1 mg/mL for Lipids A-15 and B-3 and RNA concentrations 0.075, 0.15, 0.5, and 1 mg/mL for Lipid C-18). Results for encapsulation and LNP size vs. loading concentrations are shown in FIGs. 9 and 10, respectively. In sum, empty LNP can be mixed with RNA across the range studied, yielding similar particle sizes of RNA loaded LNP.

## FORMULATION EXAMPLE 11

## STUDY OF BUFFER SPECIES

Several buffers were tested to determine which buffer systems were desirable for preparing LNPs according to the present disclosure. Namely, the following buffer systems were tested:

- citrate buffer: 5, 50 mM pH 6.0 / 5.9
- acetate buffer: 25 mM pH 5.9, pH 5.5
- phosphate buffer: 5, 10, 20, and 50 mM pH 5.8, pH 5.5

Generally, acetate and phosphate buffer systems worked well. Citrate buffers showed encapsulation below 90% and were more challenging to neutralize with phosphate buffered saline (PBS) than the other buffers tested.

**Table 11a: Physical results for citrate buffer systems using a LNP formulated with A-15 and 2.5 % pegylated lipid (pre-dilution with PBS)**

| Buffer               | Encapsulation at 20 minutes (%) | Encapsulation at 60 minutes (%) | Size post incubation with RNA (nm) | PDI  |
|----------------------|---------------------------------|---------------------------------|------------------------------------|------|
| 50 mM citrate (pH 4) | 89                              | 93                              | 127 (87%)<br>63 (13%)              | 0.10 |
| 50 mM citrate (pH 6) | 29                              | 21                              | 121                                | 0.11 |

**Table 11b: Physical results for citrate buffer systems using a LNP formulated with A-15 and 2.5 % pegylated lipid (post-dilution with PBS)**

| Buffer               | Encapsulation (%) | pH   | Size post dilution with PBS (nm) | PDI  |
|----------------------|-------------------|------|----------------------------------|------|
| 50 mM citrate (pH 4) | 55                | 5.43 | 122 (90%)<br>56 (10%)            | 0.11 |
| 50 mM citrate (pH 6) | 21                | 6.15 | 114.8                            | 0.11 |

Two different pHs (5.5 and 5.9) were tested for acetate buffer systems. These buffer systems were tested across different RNA loading concentrations and tested for size (FIGs. 11 and 12) and encapsulation efficiency (FIG. 13). The acetate buffer system at pH 5.9 generally led to smaller particle size and lower polydispersity than the pH 5.5 buffer.

**FORMULATION EXAMPLE 12****STUDY OF BUFFER CONCENTRATION (ACETATE)**

Several buffer concentrations were tested according to embodiments of the disclosure for particle size, PDI, and encapsulation efficiency. RNA-LNP were prepared by adding RNA, buffer, then empty LNPs. RNA loading concentration was 1.0 mg/mL. Formulations were prepared using compound A-15 as the cationic lipid with other components and concentrations as described in Formulation Example 1. Empty LNPs were formulated then stored at 4 °C two weeks prior to mixing. Results are shown in FIG. 14.

**FORMULATION EXAMPLE 13****RNA LOADING AND PEG CONTENT**

PEGylated lipid content of empty LNP was explored to determine whether PEG content influenced loading RNA. LNPs were prepared using ionizable lipid C-18 with a range of different PEG lipid concentrations. Concentrations and physical characteristics are presented below as determined by RG and DLS.

**Table 13a: PEG concentrations and data related to the same**

| PEG concentration (mol %) | Z-Average (nm) | PDI   | Encapsulation (%) |
|---------------------------|----------------|-------|-------------------|
| 5                         | 71             | 0.134 | 50.8              |
| 2.5                       | 73             | 0.087 | 98.2              |
| 1.8                       | 78             | 0.092 | 98.6              |
| 0.5                       | 88             | 0.074 | 98.4              |

LNP loading was determined after 10 minutes of mixing for the data in Table 15a above. Additionally. The 5 mol % sample was tested over a time course as noted by the data below.

**Table 13b: Time course for 5 mol% PEG**

| Time       | Z-Average (nm) | PDI   | Encapsulation (%) |
|------------|----------------|-------|-------------------|
| 10 minutes | 78             | 0.159 | 57.7              |
| 30 minutes | 81             | 0.182 | 68.6              |
| 4 hours    | 88             | 0.197 | 89.2              |
| 8 hours    | 88             | 0.207 | 94.9              |
| 24 hours   | 89             | 0.216 | 96.6              |

As evidenced by the data above, LNP with higher percentages of PEG in their composition take longer to load RNA but do reach a high level of encapsulation over time.



This was investigated further by testing a range of PEG content from 1.8 to 8% PEG with the same ionizable lipid (C-18). Data is shown in FIG. 15, demonstrating the RNA loading of empty LNP for 1.8, 4.0, 6.0, 8.0% PEG content over a period of 24 h. Of the samples studied, RNA loading of over 90% can be achieved after 24 h incubation for all LNP except for the 8% PEG version which reached ~70%. This demonstrates the effect of higher PEG% on the loading of RNA in the PFL process.

#### FORMULATION EXAMPLE 14

##### RNA LOADING OF EMPTY LNP AT DIFFERENT PH

Empty LNP (A-15, B3, D-1, C-18) were studied for loading at different buffer pH by assessing the resulting RNA loaded LNP size and encapsulation efficiency. FIG. 20, 22, 24, and 26 show the particle size (Z-average) and PDI for RNA loaded LNP formulated using buffers at different pH values using ionizable lipids A-15, B-3, D-1, C-18, respectively. In the figures, bars show the Z-average size for LNPs across a range of pH values against the scale on the left; the lines / points show LNPs PDI values against the scale on the right.

FIG. 21, 23, 25, and 27 show a comparison of encapsulation efficiency across pH values for LNPs prepared using the same range of ionizable lipids (A-15, B-3, D-1, C-18, respectively).

#### FORMULATION EXAMPLE 15

##### UPLC ENCAPSULATION

UPLC can be used to assess the degree of encapsulation during the PFL loading process as has been demonstrated in data presented as FIG. 28 and 29. FIG. 28 shows encapsulation efficiency measured by UPLC as a function of time for a LNP prepared with 1.8 mol% of PEG Lipid 1 and ionizable lipid A-15. This LNP encapsulated RNA at 97% almost immediately.

FIG. 29 is a plot of encapsulation efficiency values shown as a function of time as measured by UPLC for a LNP prepared with 5 mol% of PEG Lipid 1 and ionizable lipid A-15. Encapsulation improved from 20% to 67% over the course of 10 hours.

#### FORMULATION EXAMPLE 16

##### RNASE TREATMENT

The goal of this study to determine the amount of free RNA and if free RNA is accessible to RNase degradation by AGE. RNases include:

RNase T1: cleaves after G, inhibited by metal ions, optimal at 37°C

Benzonase: cleaves at all positions, requires  $Mg^{2+}$ , optimal at 37°C

RNase A: cleaves at pyrimidines C and U, very stable, optimal at 37°C

RNase I: cleaves at all positions, requires NaCl, optimal at 37°C

S1 Nuclease: cleaves at all positions but only single stranded, optimal at 37°C

5 RNase T1 was found to leave a visible band in the middle of the gel if any unencapsulated RNA was present. This was demonstrated by studying a 6% PEG LNP (see, *e.g.*, Formulation Example 13) before it had fully loaded (~1 h incubation). This band gradually decreased in intensity over time as more of the RNA loaded inside the LNP and so was protected from digestion by the RNase.

10 In another experiment, a 1.8% PEG empty LNP was loaded and tested after incubation for just 10 min. No band in the gel corresponding to unloaded RNA was observed. This is further support that the RNA is encapsulated within the LNP and not just adhered to the outside.

## FORMULATION EXAMPLE 17

### LOW PEG LIPID CONTENT

15 A study was performed to determine the effect of PEG lipid concentration on LNP formulations. Empty LNPs were prepared using different concentrations of PEG Lipid 1 and an ionizable lipid. Physical characteristics were tested of the empty LNPs as well as after a 10-minute incubation with fLuc RNA (RNA loaded LNPs). Results are shown in Tables 17a-d for ionizable lipid C-18; Tables 17e-g for ionizable lipid B-3; and Tables 17h-j for ionizable lipid D-  
20 1.

**Table 17a: Z-average and poly dispersity index values are shown for empty LNPs prepared using different concentrations of PEG Lipid 1 and ionizable lipid C-18.**

| PEG Lipid 1<br>Concentration (mol%) | Z-Average<br>(nm) | PDI   |
|-------------------------------------|-------------------|-------|
| 1.8                                 | 56                | 0.107 |
| 1                                   | 66                | 0.079 |
| 0.75                                | 71                | 0.066 |
| 0.5                                 | 78                | 0.072 |

**Table 17b: Z-average and poly dispersity index values are shown for RNA loaded LNPs after 10-minute incubation with RNA, prepared using different concentrations of PEG Lipid 1 and ionizable lipid C-18.**

| PEG Lipid 1 Concentration (mol%) | Z-Average (nm) | PDI   | Encapsulation (%) |
|----------------------------------|----------------|-------|-------------------|
| 1.8                              | 66             | 0.038 | 99                |
| 1                                | 76             | 0.018 | 99                |
| 0.75                             | 82             | 0.052 | 99                |
| 0.5                              | 87             | 0.071 | 99                |

**Table 17c: Z-average, PDI and encapsulation efficiency values are shown for RNA loaded LNPs after storage of the precursor empty LNPs for 4 weeks at 2-8°C and a 10-minute incubation prepared using different concentrations of PEG Lipid 1 and ionizable lipid C-18.**

| PEG Lipid 1 Concentration (mol%) | Z-Average (nm) | PDI   | Encapsulation (%) |
|----------------------------------|----------------|-------|-------------------|
| 1.8                              | 67             | 0.039 | 99                |
| 1.0                              | 76             | 0.011 | 99                |
| 0.75                             | 81             | 0.028 | 99                |
| 0.5                              | 87             | 0.026 | 99                |

**Table 17d: Z-average, PDI and encapsulation efficiency values are shown for RNA loaded LNPs after storage of the precursor empty LNPs for 8 weeks at 2-8°C and a 10-minute incubation with RNA prepared using different concentrations of PEG Lipid 1 and ionizable lipid C-18.**

| PEG Lipid 1 Concentration (mol%) | Z-Average (nm) | PDI   | Encapsulation (%) |
|----------------------------------|----------------|-------|-------------------|
| 1.8                              | 69             | 0.072 | 98                |
| 1.0                              | 78             | 0.005 | 99                |
| 0.75                             | 83             | 0.051 | 99                |
| 0.5                              | 91             | 0.082 | 99                |

**Table 17e: Z-average and poly dispersity index values are shown for empty LNPs prepared using different concentrations of PEG Lipid 1 and ionizable lipid B-3.**

| PEG Lipid 1 Concentration (mol%) | Z-Average (nm) | PDI   |
|----------------------------------|----------------|-------|
| 1.0                              | 80             | 0.243 |
| 0.5                              | 87             | 0.130 |

**Table 17f: Z-average and poly dispersity index values are shown for RNA loaded LNPs after 10-minute incubation with RNA prepared using different concentrations of PEG Lipid 1 and ionizable lipid B-3.**

| PEG Lipid 1 Concentration (mol%) | Z-Average (nm) | PDI   | Encapsulation (%) |
|----------------------------------|----------------|-------|-------------------|
| 1.0                              | 90             | 0.094 | 99                |
| 0.5                              | 106            | 0.155 | 99                |

**Table 17g: Z-average and poly dispersity index values are shown for RNA loaded LNPs after storage of the precursor empty LNPs for 4 weeks at 2-8°C and a 10-minute incubation with RNA prepared using different concentrations of PEG Lipid 1 and ionizable lipid B-3.**

| PEG Lipid 1 Concentration (mol%) | Z-Average (nm) | PDI   | Encapsulation (%) |
|----------------------------------|----------------|-------|-------------------|
| 1.0                              | 92             | 0.082 | 97                |
| 0.5                              | 102            | 0.118 | 97                |

**Table 17h: Z-average and poly dispersity index values are shown for empty LNPs prepared using different concentrations of PEG Lipid 1 and ionizable lipid D-1.**

| PEG Lipid 1 Concentration (mol%) | Z-Average (nm) | PDI   |
|----------------------------------|----------------|-------|
| 1.0                              | 69             | 0.119 |
| 0.5                              | 82             | 0.120 |

**Table 17i: Z-average and poly dispersity index values are shown for RNA loaded LNPs after 10-minute incubation with RNA prepared using different concentrations of PEG Lipid 1 and ionizable lipid D-1.**

| PEG Lipid 1 Concentration (mol%) | Z-Average (nm) | PDI   | Encapsulation (%) |
|----------------------------------|----------------|-------|-------------------|
| 1.0                              | 79             | 0.136 | 98                |
| 0.5                              | 94             | 0.059 | 99                |

**Table 17j: Z-average and poly dispersity index values are shown for RNA loaded LNPs after storage of the precursor empty LNPs for 4 weeks at 2-8°C and a 10-minute incubation with RNA prepared using different concentrations of PEG Lipid 1 and ionizable lipid D-1.**

| PEG Lipid 1 Concentration (mol%) | Z-Average (nm) | PDI   | Encapsulation (%) |
|----------------------------------|----------------|-------|-------------------|
| 1.0                              | 87             | 0.112 | 97                |
| 0.5                              | 102            | 0.068 | 99                |

Empty LNPs can be prepared using low concentrations of PEG lipid. The samples prepared can be stored at 4°C for at least 4 weeks with minimal aggregation. Empty LNPs with low PEG lipid concentration can be rapidly loaded with RNA. The physical attributes of RNA loaded LNPs are essentially unchanged after storage of the empty LNP at 2-8°C for a month or more. These studies indicate that this technique of loading LNPs in a point-of-care situation would enable the use of LNPs with lower PEG lipid composition than with conventional LNP systems (*e.g.*, RNA is loaded into an empty LNP by a medical professional just prior to administration).

## FORMULATION EXAMPLE 18

### POINT OF CARE STUDY

This study was employed to assess the efficacy of formulations prepared using PFL methods in a "point-of-care" situation (*e.g.*, where an RNA and empty LNP are mixed immediately or shortly before injection / administration). Results are displayed graphically in FIG. 30.

LNPs were prepared with ionizable lipid C-18 and the following sample parameters were used.

**Table 18a: Sample parameters for "point-of-care" samples prepared and tested.**

| Sample         | Description                                                                                    | mRNA addition   |
|----------------|------------------------------------------------------------------------------------------------|-----------------|
| A              | T-mixture prepared as control sample                                                           | Prior to onsite |
| B              | PFL LNP prepared in advance from same empty stock as samples C-E and frozen                    | Prior to onsite |
| C <sup>+</sup> | mRNA-LNP in acidic buffer (pH 5.9), diluted with saline to match dilution with samples D and E | Onsite          |
| D <sup>+</sup> | mRNA-LNP diluted with PBS to dosing volume (pH 7.4)                                            | Onsite          |
| E <sup>+</sup> | mRNA-LNP diluted with PBS prepared by separate operator (pH 7.4)                               | Onsite          |

<sup>+</sup> Empty LNPs were stored at 4°C – RNA-LNP was prepared just prior to administration

Samples (0.5 mg/kg) were then used to dose mice (n = 5 mice per formulation) and serum IgG concentrations were detected. The results are presented in FIG. 30 and in the table below.

5 **Table 18b: Results for samples prepared according to the descriptions noted in Table 21a.**

| Sample | Serum IgG concentration (µg/mL) | vs. sample A (fold change) | vs. sample B (fold change) | %CV |
|--------|---------------------------------|----------------------------|----------------------------|-----|
| A      | 76.0                            | 1.0                        | 0.7                        | 17  |
| B      | 106.6                           | 1.4                        | 1.0                        | 19  |
| C      | 78.8                            | 1.0                        | 0.7                        | 17  |
| D      | 112.8                           | 1.5                        | 1.1                        | 13  |
| E      | 81.6                            | 1.1                        | 0.8                        | 8   |

Overall, this data successfully demonstrated comparability for point-of-care LNP preparation compared with a traditional LNP preparation (*e.g.*, sample A).

## FORMULATION EXAMPLE 19

### STABILITY STUDY – EMPTY LNP

10 Empty LNP stability and subsequent loading was tested to determine efficacy of such samples. Empty LNPs were stored at 2-8°C and -80°C and samples were tested at time points of 48 hours, 7 days, 1 month, 3 months, 6 months, and onward. Data was collected for LNP size and PDI. As an initial study, ionizable lipid A-15 was used. Results are illustrated in FIGs. 31-32 for empty LNP and FIGs. 33-35 for RNA loaded LNP after storage as empty LNP. As shown in FIG.  
15 35, all encapsulation efficiencies were greater than 95% at all time-points, even after 6 months of storage. In all, samples loaded with RNA had good physical attributes following storage of the empty LNP. This is true for samples stored at 2-8°C as well as samples stored at -80°C.

In summary, empty LNPs store well at both 2-8°C and -80°C. Following storage, all empty LNPs were successfully loaded with RNA to generate loaded LNPs with good particle  
20 characteristics.

## FORMULATION EXAMPLE 20

### STABILITY STUDY – LOADED LNP

Empty LNPs were loaded with fLuc RNA at 0.15 mg/mL via the PFL method described herein as well as traditional T-mixing methods and stored at -80°C at an RNA concentration of 1  
25 mg/mL. The cationic lipids used are as described below.

**Table 20: Physical characteristics of samples prepared using PFL methods compared to samples prepared using conventional methods (*i.e.*, T-mixing) at timepoint 0**

| Cationic Lipid | PFL Samples    |       |                   | T-mixed Samples |       |                   |
|----------------|----------------|-------|-------------------|-----------------|-------|-------------------|
|                | Z-Average (nm) | PDI   | Encapsulation (%) | Z-Average (nm)  | PDI   | Encapsulation (%) |
| A-15           | 79             | 0.028 | 97                | 79              | 0.03  | 89                |
| B-3            | 85             | 0.095 | 96                | 69              | 0.08  | 84                |
| B-45           | 86             | 0.066 | 95                | 61              | 0.003 | 84                |
| D-1            | 96             | 0.114 | 97                | 76              | 0.028 | 96                |
| C-18           | 80             | 0.064 | 98                | 75              | 0.029 | 97                |

Samples were tested every 2 months for various physical properties (*e.g.*, size, PDI, RNA content, encapsulation efficiency, RNA integrity).

- 5 Results for the T-mixed samples are shown in FIGs. 36A-C (z-average, PDI, and total lipids, respectively) and FIGs. 38A-C (RNA content, encapsulation efficiency, integrity, respectively). Data for samples prepared using the PFL method is shown in FIGs. 37A-C (z-average, PDI, and total lipids, respectively) and FIGs. 39A-C (RNA content, encapsulation efficiency, integrity, respectively). In all, sample stability for PFL RNA-LNP samples are
- 10 comparable to that for RNA-LNP samples prepared using T-mixing techniques over the course of 6-months.

## FORMULATION EXAMPLE 21

### EMPTY LNP PARTICLE SIZE AND SUBSEQUENT LOADING EFFICIENCY

- Empty LNP of identical composition prepared with one ionizable lipid of A-15, B-3 or B-45 were each formulated to possess distinctive particle sizes. These empty LNP were loaded with
- 15 fLuc RNA at 0.075 mg/mL via the PFL method described herein. FIG. 16, 18, and 42A shows the encapsulation efficiency of the RNA loaded particles for each ionizable lipid (B-45, A-15, B-3, respectively). FIG. 17, 19, and 42B shows the Z-average size of the loaded particles as well as the magnitude of the size change between the loaded and empty LNP for each ionizable lipid (B-45, A-15, B-3, respectively).
- 20

## FORMULATION EXAMPLE 22

### NUCLEIC ACID VARIANTS

- To assess whether the PFL method can be utilized as means of successfully encapsulating a range of different nucleic acids, empty LNPs were formulated using either ionizable lipid B-3
- 25 or ionizable lipid C-18 and were subsequently loaded with the following nucleic acids:

siRNA (Invitrogen silencer negative control),

Cas9 mRNA/gRNA mixture at a 1:1 ratio (mg/mg): Cas9 gRNA; or

saRNA: encoding for the Covid-19 pre-fusion spike protein

These nucleic acids were loaded into empty LNP to achieve a final nucleic acid

5 concentration of 0.075 mg/mL and incubated at room temperature for 10 minutes prior to neutralization with 2× DPBS. Particle size and PDI of the RNA loaded LNPs are shown in FIG. 43A and FIG. 43B for ionizable lipid B-3 and ionizable lipid C-18, respectively. Encapsulation efficiency for both lipid compositions is shown in FIG. 43C. All nucleic acids encapsulated with >90% efficiency regardless of the lipid species used in the formulation. Particle size was  
10 somewhat dependent on the type of nucleic acid used, with the saRNA and siRNA loaded LNPs being larger than those formulated with the Cas9 mRNA/gRNA mix. LNP loaded with siRNA showed low polydispersity (around 0.01) regardless of lipid species, whilst LNP loaded with saRNA tended to be more polydisperse (0.098 for ionizable lipid B-3 and 0.11 for ionizable lipid C-18).

15

### FORMULATION EXAMPLE 23

#### EFFECT OF NaCl CONCENTRATION

A study was conducted to determine the effect of sodium chloride concentration on RNA-LNP formulated via the PFL method. Empty LNPs were prepared using either ionizable lipid B-3 or ionizable lipid C-18 in 25 mM acetate pH 5.5 or pH 5.9 respectively. Each of these acetate  
20 buffers contained the following amounts of sodium chloride: 0 mM, 50 mM, 137 mM, 500 mM. The particle size (Z-average) of the empty LNP formulations is shown in FIG. 44A. Particles showed a slight decrease in size when formulated in the presence of 50 mM NaCl, however, particle size increased as NaCl concentration was raised further. Empty LNP were then loaded with fLuc RNA at 0.075 mg/mL using the PFL method as described herein. FIG. 44B and FIG.  
25 44C show the particle size (z-average) and encapsulation efficiency of the loaded LNP respectively. Loaded particle size was almost identical between LNP formulated with 50 mM NaCl or in formulation prepared in the absence of NaCl. However, a noticeable size increase was observed when formulated at 137 mM NaCl. Formulation at 500 mM NaCl yielded particles with sizes identical to those of the empty LNP. Encapsulation efficiency dropped to zero for both  
30 ionizable lipids tested when formulated with 500 mM NaCl. Encapsulation generally remained high for the other concentrations that were tested, with only a slight drop observed for the



ionizable lipid C-18 LNP formulated at 137 mM NaCl. In summary, all LNPs tested can be formulated at NaCl concentrations of up to 137 mM.

#### FORMULATION EXAMPLE 24

##### EFFECT OF N:P ON PFL LOADING

5           To investigate the effect of N:P ratio on the PFL method a series of different N:P ratios were used from 9 to 3 (9, 7.5, 6, 4.5, 3), using ionizable lipid C-18. FIG. 40 shows LNP z-average size and PDI as a function of the N:P ratio and FIG. 41 shows encapsulation efficiency for the same LNP as a function of N:P ratio.

          There is a clear inverse trend for increasing RNA loaded LNP size for decreasing N:P.  
10       Encapsulation does not appear to be affected by N:P in this range, showing near quantitative loading across the range studied.

#### FORMULATION EXAMPLE 25

##### SUMMARY

          LNPs made using the PFL method have comparable behavior to methods prepared using  
15       conventional (*e.g.*, T-mixing) techniques. A PFL method works over a broad range of missing concentrations (*i.e.*, empty LNP to RNA) with high success rates. In some embodiments, pH is a critical component for successful loading and can be dependent on the cationic lipid pKa. In some embodiments, the concentration of PEG (*e.g.*, the PEGylated lipid) in the LNP shell affects the speed of the RNA loading.

20

#### BIOLOGICAL EXAMPLE 1

##### EFFICACY ASSESSMENT

          The following protocol is used to determine efficacy of nucleic acid molecules encapsulated in lipid nanoparticle formulations according to the present disclosure using an *in*  
25       *vivo* luciferase mRNA expression model in rodents.

          Lipid nanoparticles encompassing nucleic acids are prepared according to the example described above. Studies are performed in 6–8-week-old female C57BL/6 mice (Charles River) 8–10-week-old CD-1 (Harlan) mice (Charles River) according to guidelines established by an institutional animal care committee (ACC) and the Canadian Council on Animal Care (CCAC).  
30       Varying doses of mRNA-lipid nanoparticle are systemically administered by tail vein injection

and animals euthanized at a specific time point (*e.g.*, 4 hours) post-administration. Liver and spleen are collected in pre-weighed tubes, weights determined, immediately snap frozen in liquid nitrogen, and stored at -80 °C until processing for analysis.

For liver, approximately 50 mg is dissected for analyses in 2 mL FastPrep tubes (MP Biomedicals, Solon OH). ¼" ceramic sphere (MP Biomedicals) is added to each tube and 500 µL of Glo Lysis Buffer – GLB (Promega, Madison WI) equilibrated to room temperature is added to liver tissue. Liver tissues are homogenized with the FastPrep24 instrument (MP Biomedicals) at  $2 \times 6.0$  m/s for 15 seconds. Homogenate is incubated at room temperature for 5 minutes prior to a 1:4 dilution in GLB and assessed using SteadyGlo Luciferase assay system (Promega). Specifically, 50 µL of diluted tissue homogenate is reacted with 50 µL of SteadyGlo substrate, shaken for 10 seconds followed by 5-minute incubation and then quantitated using a CentroXS<sup>3</sup> LB 960 luminometer (Berthold Technologies, Germany). The amount of protein assayed is determined by using the BCA protein assay kit (Pierce, Rockford, IL). Relative luminescence units (RLU) are then normalized to total µg protein assayed. To convert RLU to ng luciferase a standard curve is generated with QuantiLum Recombinant Luciferase (Promega).

The FLuc mRNA (L-6107) from Trilink Biotechnologies expresses a luciferase protein, originally isolated from the firefly, *photinus pyralis*. FLuc is commonly used in mammalian cell culture to measure both gene expression and cell viability. It emits bioluminescence in the presence of the substrate, luciferin. This capped and polyadenylated mRNA is fully substituted with 5-methylcytidine and pseudouridine.

Activity is determined by measuring luciferase expression in the liver 4 hours following administration via tail vein injection. The activity is compared at a dose of 1.0, 0.3, or 0.1 mg mRNA/kg and expressed as ng luciferase/g liver measured 4 hours after administration.

## BIOLOGICAL EXAMPLE 2

### IMMUNOGLOBULIN G (IgG) mRNA IN VIVO EVALUATION USING LIPID NANOPARTICLE COMPOSITIONS

Studies are performed in 6–8-week-old CD-1/ICR mice (Envigo) according to guidelines established by an institutional animal care committee (ACC) and the Canadian Council on Animal Care (CCAC). Varying doses of mRNA-lipid nanoparticle are systemically administered by tail vein injection and animals euthanized at a specific time point (*e.g.*, 24 hours) post-administration. The whole blood is collected, and the serum subsequently separated by

centrifuging the tubes of the whole blood at  $2000 \times g$  for 10 minutes at  $4^{\circ}\text{C}$  and stored at  $-80^{\circ}\text{C}$  until use for analysis.

For immunoglobulin G (IgG) ELISA (Life Diagnostics Human IgG ELISA kit), the serum samples are diluted at 100 to 15000 folds with  $1\times$  diluent solution. 100  $\mu\text{L}$  of diluted  
5 serum is dispensed into anti-human IgG coated 96-well plate in duplicate alongside human IgG standards and incubated in a plate shaker at 150 rpm at  $25^{\circ}\text{C}$  for 45 minutes. The wells are washed 5 times with  $1\times$  wash solution using a plate washer (400  $\mu\text{L}/\text{well}$ ). 100  $\mu\text{L}$  of HRP conjugate is added into each well and incubated in a plate shaker at the same condition above. The wells are washed 5 times again with  $1\times$  wash solution using a plate washer (400  $\mu\text{L}/\text{well}$ ).  
10 100  $\mu\text{L}$  of TMB reagent is added into each well and incubated in a plate shaker at the same condition above. The reaction is stopped by adding 100  $\mu\text{L}$  of Stop solution to each well. The absorbance is read at 450 nm ( $A_{450}$ ) with a microplate reader. The amount of human IgG in mouse serum is determined by plotting  $A_{450}$  values for the assay standard against human IgG concentration.

15

### BIOLOGICAL EXAMPLE 3

#### DETERMINATION OF $\text{pK}_a$ OF FORMULATED LIPIDS

The  $\text{pK}_a$  of each lipid is determined in lipid nanoparticles using an assay based on fluorescence of 2-(p-toluidino)-6-naphthalene sulfonic acid (TNS). Lipid nanoparticles comprising a ionizable lipid / DSPC / cholesterol / PEG Lipid 1 (50/10/38.5/1.5 or 47.5:10:40.7:1.8 mol%) in  
20 PBS at a concentration of 0.4 mM total lipid are prepared using an in-line process according to known methods. TNS is prepared as a 100  $\mu\text{M}$  stock solution in distilled water. Vesicles are diluted to 24  $\mu\text{M}$  lipid in 2 mL of buffered solutions containing 10 mM HEPES, 10 mM MES, 10 mM ammonium acetate, and 130 mM NaCl, where the pH ranged from 2.5 to 11. An aliquot of the TNS solution is added to give a final concentration of 1  $\mu\text{M}$  and following vortex mixing  
25 fluorescence intensity is measured at room temperature in a SLM Aminco Series 2 Luminescence Spectrophotometer using excitation and emission wavelengths of 321 nm and 445 nm. A sigmoidal best fit analysis is applied to the fluorescence data and the  $\text{pK}_a$  is measured as the pH giving rise to half-maximal fluorescence intensity.

30

### BIOLOGICAL EXAMPLE 4

#### IMMUNOGENICITY AFTER INTRAMUSCULAR (I.M.) ADMINISTRATION OF LNPs

BALB/c mice were injected intramuscularly into quadriceps with 30 µL LNP-mRNA encoding Influenza A/Puerto Rico/8/1934 Hemagglutinin (HA). formulation at 0.2 or 0.5 µg mRNA dose on Day 0 and Day 14. Blood samples were collected on Day -1 13 and terminal bleed collected at Day 28 and processed to serum. Immunogenicity of LNP formulations was determined by Hemagglutination Inhibition (HAI) Assay.

*Hemagglutination Inhibition (HAI) Assay*

The samples were diluted 1:4 with Receptor Destroying Enzyme II (RDE) to inactivate non-specific inhibitors of hemagglutination present in sera. Samples were incubated for 18 hours at 37°C followed by further incubation for 30 minutes at 56°C to inactivate the enzyme. RDE-treated samples were diluted 1:10 in 0.85% NaCl. A 1% suspension of turkey red blood cells (TRBCs) was prepared in PBS. Non-specific agglutinins were detected in samples by incubating RDE-treated sera (25 µL), 25 µL PBS and 50 µL 1% TRBC suspension for 30 min at room temperature. If RBCs settle completely in serum-containing wells then serum sample is acceptable for use in the HAI assay.

Verified RDE-treated sera samples were serially diluted in duplicate two-fold in PBS for a total of 12 dilutions ranging from 20 – 40960. Inactivated IFV-A/PR/8/34 (Charles River Laboratories, #10100782) was prepared to a concentration equating to 4 hemagglutinin (HA) units and incubated with sera in a 1:1 ratio for 30 minutes at room temperature. The serum/antigen mixture was incubated with the 1% TRBC suspension in a 1:1 ratio for 30 minutes at room temperature. TRBCs only and antigen only controls were included.

Plates were then analyzed and scored. Each well was examined for the presence of hemagglutination. Inhibition of hemagglutination indicates the presence of HA-specific antibodies. For each serum sample, the highest dilution that completely inhibited hemagglutination was recorded as the HA titer.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification, including U.S. Provisional Patent Application No. 63/560,490, filed on March 1, 2024, are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications, and publications to provide yet further embodiments.

These and other changes can be made to the embodiments considering the above-detailed description. In general, in the following claims, the terms used should not be construed to limit

the claims to the specific embodiments disclosed in the specification and the claims but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

## CLAIMS

1. A method for encapsulating a payload comprising a nucleic acid within lipid nanoparticles (LNPs), comprising:
  - i) providing an aqueous first solution comprising a plurality of LNPs comprising a plurality of ionizable lipids, wherein the first solution is substantially free of any destabilizing agents and the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0; and
  - ii) mixing the first solution with dry payload or payload in a second aqueous solution, wherein the second aqueous solution is substantially free of any destabilizing agents, thereby encapsulating at least a majority of the payload in the LNPs in a resultant solution.
2. A method for encapsulating a payload comprising a nucleic acid within lipid nanoparticles (LNPs), comprising:
  - i) providing a dry first composition comprising a plurality of LNPs comprising a plurality of ionizable lipids; and
  - ii) mixing the first composition with a payload in a second aqueous solution, wherein the second aqueous solution is substantially free of any destabilizing agents, thereby encapsulating at least a majority of the payload in the LNPs in a first solution.
3. A method for encapsulating a payload comprising a nucleic acid within lipid nanoparticles (LNPs), comprising:
  - i) providing a dry first composition comprising a plurality of LNPs comprising a plurality of ionizable lipids;
  - ii) mixing the first composition with a second composition comprising dry payload; and
  - iii) mixing the first composition and the second composition together with an aqueous first solution, wherein the first solution is substantially free of any destabilizing agents and the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0, thereby encapsulating at least a majority of the payload in the LNPs.

4. The method of any one of claims 1-3, wherein the LNPs in the first solution or the first composition do not encapsulate payload.
5. The method of any one of claims 1-4, wherein the pH of the first solution is less than 7.0.
6. The method of any one of claims 1-4, wherein the pH of the first solution is less than 6.8.
7. The method of any one of claims 1-4, wherein the pH of the first solution is less than 6.5.
8. The method of any one of claims 1-4, wherein the pH of the first solution is less than 6.4, less than 6.3, less than 6.2, less than 6.1, less than 6.0, less than 5.9, less than 5.8, or less than 5.7.
9. The method of any one of claims 1-4, wherein the pH of the first solution is less than 5.0.
10. The method of any one of claims 1-4, wherein the pH of the first solution is less than 5.7.
11. The method of any one of claims 1-4, wherein the pH of the first solution ranges from 4.5 to 7.4, from 5.5 to 6.5, or from 5.7 to 6.2.
12. The method of any one of claims 1-11, wherein the difference between the pH of the first solution and the pKa of the ionizable lipids ranges from 0.1 to 1.5, from 0.1 to 1.0, from 0.1 to 0.5, from 0.2 to 0.5, from 0.5 to 0.5, from 0.3 to 0.6, from 0.2 to 0.6, or from 0.4 to 0.8.
13. The method of any one of claims 1-11, wherein the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0.

14. The method of any one of claims 1-11, wherein the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 0.7.

15. The method of any one of claims 1-11, wherein the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 0.5, less than 0.3 or less than 0.1.

16. The method of any one of claims 1-11, wherein the difference between the pH of the first solution and the pKa of the ionizable lipids is at least 0.1, at least 0.2, at least 0.3, at least 0.4, at least 0.5, at least 0.6, at least 0.7, at least 0.8, at least 0.9, at least 1.0, at least 1.2, or at least 1.5.

17. The method of any one of claims 1-11, wherein the difference between the pH of the first solution and pKa of the ionizable lipids ranges from 0.3 to 1.0, from 0.2 to 0.9, from 0.1 to 1.1, from 0.1 to 0.8, or from 0.2 to 0.7.

18. The method of any one of claims 1-17, wherein an encapsulation efficiency is at least 70%.

19. The method of any one of claims 1-17, wherein an encapsulation efficiency is at least 80%.

20. The method of any one of claims 1-17, wherein an encapsulation efficiency is at least 85%.

21. The method of any one of claims 1-17, wherein an encapsulation efficiency is at least 88%.

22. The method of any one of claims 1-17, wherein an encapsulation efficiency is at least 90%.

23. The method of any one of claims 1-17, wherein an encapsulation efficiency is at least 95%.



24. The method of claim 1, wherein the pH of the resultant solution ranges from 6.0 to 7.8.
25. The method of claim 1, wherein the pH of the resultant solution ranges from 6.5 to 7.8.
26. The method of claim 1, wherein the pH of the resultant solution ranges from 7.0 to 7.5.
27. The method of any one of claims 1-26, wherein the particle size of the LNPs encapsulating the payload in the first or resultant solution ranges from 20 to 200 nm, from 20 to 120 nm, from 30 to 90 nm, from 40 to 120 nm, from 50 to 120 nm, or from 30 to 100 nm.
28. The method of any one of claims 1-26, wherein the particle size of the LNPs encapsulating the payload in the first or resultant solution ranges from 60 to 100 nm.
29. The method of any one of claims 1-26, wherein the particle size of the LNPs encapsulating the payload in the first or resultant solution ranges from 70 to 90 nm.
30. The method of any one of claims 1-26, wherein the particle size of the LNPs encapsulating the payload in the first or resultant solution is less than 90 nm.
31. The method of any one of claims 1-26, wherein the particle size of the LNPs encapsulating the payload in the first or resultant solution is less than 80 nm.
32. The method of any one of claims 1-26, wherein the particle size of the LNPs encapsulating the payload in the first or resultant solution is less than 70 nm, less than 60 nm, or less than 50 nm.
33. The method of any one of claims 1-32, wherein the destabilizing agent in the first or second solution is an organic solvent.

34. The method of any one of claims 1-33, wherein the destabilizing agent in the first or second solution is methanol, ethanol, isopropyl alcohol, tetrahydrofuran, dimethylformamide, dimethylsulfoxide, acetonitrile, sodium dodecyl sulfate, or combinations thereof.

35. The method of any one of claims 1-34, wherein the temperature of the first solution is less than 27°C, less than 25°C, or less than 22°C immediately before mixing.

36. The method of any one of claims 1-35, wherein the temperature of the second solution is less than 27°C, less than 25°C, or less than 22°C during mixing.

37. The method of claim 1, wherein the temperature of the resultant solution is less than 27°C, less than 25°C, or less than 22°C immediately after mixing.

38. The method of any one of claims 1-37, wherein the plurality of LNPs in the first solution or the resultant solution comprise 20 to 90 mol% or 30 to 90 mol% of the ionizable lipids.

39. The method of any one of claims 1-37, wherein the plurality of LNPs in the first solution or the resultant solution comprise 40 to 55 mol% of the ionizable lipids.

40. The method of any one of claims 1-37, wherein the plurality of LNPs in the first solution or the resultant solution comprise 46 to 49 mol% of the ionizable lipids.

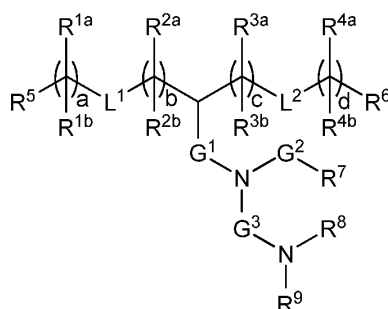
41. The method of any one of claims 1-40, wherein the plurality of LNPs in the first solution or the resultant solution comprise one or more component selected from neutral lipids, steroids, and polymer conjugated lipids.

42. The method of any one of claim 1-41, wherein the plurality of LNPs in the first solution or the resultant solution comprise a neutral lipid at a concentration ranging from about 5 to about 15 mol% of the lipid nanoparticle.

43. The method of any one of claims 1-42, wherein the plurality of LNPs in the first solution or the resultant solution comprise a steroid at a concentration ranging from about 30 to about 50 mol% of the lipid nanoparticle.

44. The method of any one of claims 1-43, wherein the plurality of LNPs in the first solution or the resultant solution comprise a pegylated lipid at a concentration ranging from 0.1 to 10 mol%, from 0.1 to 5 mol%, from 0.1 to 3 mol%, from 0.1 to 2 mol%, from 0.1 to 1 mol%, 0.5 to 10 mol%, from 0.5 to 5 mol%, from 0.5 to 3 mol%, from 0.5 to 2 mol%, from 0.5 to 1 mol%, 1.0 to 10 mol%, from 1.0 to 5 mol%, from 1.0 to 3 mol%, from 1.0 to 2 mol%, 1.5 to 10 mol%, from 1.5 to 5 mol%, from 1.5 to 3 mol%, from 1.5 to 2 mol%, 2.0 to 10 mol%, from 2.0 to 5 mol%, from 2.0 to 3 mol%, 2.5 to 10 mol%, from 2.5 to 5 mol%, or from 2.5 to 3 mol% of the lipid nanoparticle.

45. The method of any one of claims 1-44, wherein the ionizable lipids have the following structure:



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

$L^1$  and  $L^2$  are each independently  $-O(C=O)-$ ,  $-(C=O)O-$ ,  $-C(=O)-$ ,  $-O-$ ,  $-S(O)_x-$ ,  $-S-S-$ ,  $-C(=O)S-$ ,  $-SC(=O)-$ ,  $-NR^aC(=O)-$ ,  $-C(=O)NR^a-$ ,  $-NR^aC(=O)NR^a-$ ,  $-OC(=O)NR^a-$ ,  $-NR^aC(=O)O-$  or a direct bond;

$G^1$  is  $C_1-C_2$  alkylene,  $-(C=O)-$ ,  $-O(C=O)-$ ,  $-SC(=O)-$ ,  $-NR^aC(=O)-$  or a direct bond;

$G^2$  is  $-C(=O)-$ ,  $-(C=O)O-$ ,  $-C(=O)S-$ ,  $-C(=O)NR^a-$  or a direct bond;

$G^3$  is  $C_1-C_6$  alkylene;

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

$R^{1a}$  and  $R^{1b}$  are, at each occurrence, independently either: (a) H or  $C_1-C_{12}$  alkyl; or (b)  $R^{1a}$  is H or  $C_1-C_{12}$  alkyl, and  $R^{1b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{1b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond;

$R^{2a}$  and  $R^{2b}$  are, at each occurrence, independently either: (a) H or  $C_1$ - $C_{12}$  alkyl; or (b)  $R^{2a}$  is H or  $C_1$ - $C_{12}$  alkyl, and  $R^{2b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{2b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond;

$R^{3a}$  and  $R^{3b}$  are, at each occurrence, independently either (a): H or  $C_1$ - $C_{12}$  alkyl; or (b)  $R^{3a}$  is H or  $C_1$ - $C_{12}$  alkyl, and  $R^{3b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{3b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond;

$R^{4a}$  and  $R^{4b}$  are, at each occurrence, independently either: (a) H or  $C_1$ - $C_{12}$  alkyl; or (b)  $R^{4a}$  is H or  $C_1$ - $C_{12}$  alkyl, and  $R^{4b}$  together with the carbon atom to which it is bound is taken together with an adjacent  $R^{4b}$  and the carbon atom to which it is bound to form a carbon-carbon double bond;

$R^5$  and  $R^6$  are each independently H or methyl;

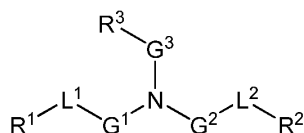
$R^7$  is  $C_4$ - $C_{20}$  alkyl;

$R^8$  and  $R^9$  are each independently  $C_1$ - $C_{12}$  alkyl; or  $R^8$  and  $R^9$ , together with the nitrogen atom to which they are attached, form a 5, 6 or 7-membered heterocyclic ring;

a, b, c and d are each independently an integer from 1 to 24; and

x is 0, 1 or 2.

46. The method of any one of claims 1-44, wherein the ionizable lipids have the following structure:



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

one of  $L^1$  or  $L^2$  is  $-O(C=O)-$ ,  $-(C=O)O-$ ,  $-C(=O)-$ ,  $-O-$ ,  $-S(O)_x-$ ,  $-S-S-$ ,  $-C(=O)S-$ ,  $-SC(=O)-$ ,  $-NR^aC(=O)-$ ,  $-C(=O)NR^a-$ ,  $NR^aC(=O)NR^a-$ ,  $-OC(=O)NR^a-$  or  $-NR^aC(=O)O-$ , and the other of  $L^1$  or  $L^2$  is  $-O(C=O)-$ ,  $-(C=O)O-$ ,  $-C(=O)-$ ,  $-O-$ ,  $-S(O)_x-$ ,  $-S-S-$ ,  $-C(=O)S-$ ,  $SC(=O)-$ ,  $-NR^aC(=O)-$ ,  $-C(=O)NR^a-$ ,  $NR^aC(=O)NR^a-$ ,  $-OC(=O)NR^a-$  or  $-NR^aC(=O)O-$  or a direct bond;

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

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

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

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

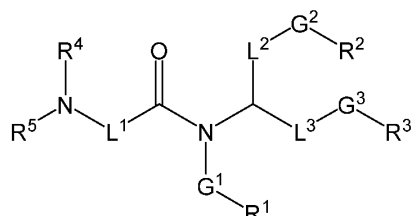
$R^3$  is H,  $OR^5$ , CN,  $-C(=O)OR^4$ ,  $-OC(=O)R^4$  or  $-NR^5C(=O)R^4$ ;

R<sup>4</sup> is C<sub>1</sub>-C<sub>12</sub> alkyl;

R<sup>5</sup> is H or C<sub>1</sub>-C<sub>6</sub> alkyl; and

x is 0, 1 or 2.

47. The method of any one of claims 1-44, wherein the ionizable lipids have the following structure:



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

R<sup>1</sup> is optionally substituted C<sub>1</sub>-C<sub>24</sub> alkyl or optionally substituted C<sub>2</sub>-C<sub>24</sub> alkenyl;

R<sup>2</sup> and R<sup>3</sup> are each independently optionally substituted C<sub>1</sub>-C<sub>36</sub> alkyl;

R<sup>4</sup> and R<sup>5</sup> are each independently optionally substituted C<sub>1</sub>-C<sub>6</sub> alkyl, or R<sup>4</sup> and R<sup>5</sup> join,

along with the N to which they are attached, to form a heterocyclcyl or heteroaryl;

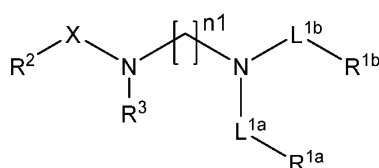
L<sup>1</sup>, L<sup>2</sup>, and L<sup>3</sup> are each independently optionally substituted C<sub>1</sub>-C<sub>18</sub> alkylene;

G<sup>1</sup> is a direct bond, -(CH<sub>2</sub>)<sub>n</sub>O(C=O)-, -(CH<sub>2</sub>)<sub>n</sub>(C=O)O-, or -(C=O)-;

G<sup>2</sup> and G<sup>3</sup> are each independently -(C=O)O- or -O(C=O)-; and

n is an integer greater than 0.

48. The method of any one of claims 1-44, wherein the ionizable lipids have the following structure:



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

L<sup>1a</sup> and L<sup>1b</sup> are each independently optionally substituted C<sub>3</sub>-C<sub>12</sub> alkyl;

R<sup>1a</sup> is -C(=O)OR<sup>4a</sup> or -O(C=O)R<sup>4a</sup>;

R<sup>1b</sup> is -C(=O)OR<sup>4b</sup> or -O(C=O)R<sup>4b</sup>;

R<sup>2</sup> is -NR<sup>6</sup>(C=O)R<sup>5</sup>, -(C=O)N(R<sup>6</sup>)R<sup>5</sup> or -(C=O)OR<sup>7</sup>;

R<sup>3</sup> and R<sup>6</sup> are each independently hydrogen or optionally substituted C<sub>1</sub>-C<sub>12</sub> alkyl;

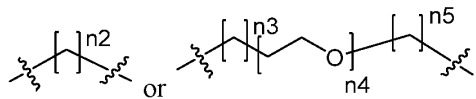
R<sup>4a</sup>, R<sup>4b</sup>, and R<sup>5</sup> are each independently optionally substituted alkyl;

$R^7$  is optionally substituted  $C_1$ - $C_6$  alkyl or optionally substituted arylalkyl;

$n_1$  is 2, 3, 4, 5, or 6; and

X is  $C_2$ - $C_6$  alkylene or  $C_4$ - $C_{20}$  alkyleneoxide.

In some embodiments, wherein X is:



wherein:

$n_2$  is 2, 3, 4, 5, or 6;

$n_3$  is 0, 1, 2, 3, or 4;

$n_4$  is 2, 3, or 4; and

$n_5$  is 2, 3, 4, or 5.

49. The method of any one of claims 1-44, wherein the ionizable lipids have one of the structures in Table A or B.

50. The method of any one of claims 1-44, wherein the ionizable lipids have one of the structures in Table C.

51. The method of any one of claims 1-44, wherein the ionizable lipids have one of the structures in Table D.

52. The method of any one of claims 1-51, wherein the plurality of LNPs in the first solution or the resultant solution comprise one or more neutral lipids selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE and SM.

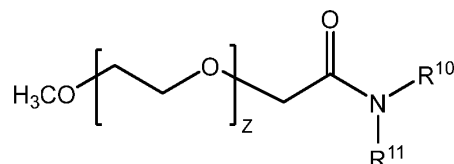
53. The method of claim 52, wherein the neutral lipid is DSPC.

54. The method of any one of claims 41-53, wherein the steroid is cholesterol.

55. The method of any one of claims 41-54, wherein the polymer conjugated lipid is a pegylated lipid.

56. The method of claim 55, wherein the pegylated lipid is PEG-DAG, PEG-PE, PEG-S-DAG, PEG-cer or a PEG dialkyoxypropylcarbamate.

57. The method of claim 55, wherein the pegylated lipid has the following:



or a pharmaceutically acceptable salt or stereoisomer thereof, wherein:

$\text{R}^{10}$  and  $\text{R}^{11}$  are each independently a straight or branched alkyl, alkenyl, or alkynyl containing from 10 to 30 carbon atoms, wherein each alkyl, alkenyl, or alkynyl is optionally substituted with at least one fluoro; and

$z$  is an integer ranging from 30 to 60.

58. The method of claim 57, wherein  $\text{R}^{10}$  and  $\text{R}^{11}$  are each independently straight alkyl chain containing from 12 to 16 carbon atoms.

59. The method of any one of claims 57-58, wherein the  $z$  ranges from 45 to 50.

60. The method of any one of claims 1-59, wherein the payload is DNA, siRNA, mini circle RNA, PNA, aptamer, guide RNA, PE guide RNA, saRNA, circular RNA, antisense RNA, messenger RNA, Cas9 mRNA, ribonucleoprotein, or a combination thereof.

61. The method of any one of claims 1-59, wherein the payload is mRNA.

62. The method of any one of claims 1-61, wherein the method further comprises administering the payload encapsulated in the LNPs to a patient in need thereof.

63. A method for administering a payload encapsulated in a lipid nanoparticle (LNP) to a patient in need thereof, the method comprising administering the lipid nanoparticle prepared according to any one of claims 1-61 to the patient.

64. The method of any one of claims 62-63, wherein the administering is performed within 72 hours of the mixing.

65. The method of any one of claims 62-63, wherein the administering is performed within 24 hours of the mixing.

66. The method of any one of claims 62-63, wherein the administering is performed within 12 hours of the mixing.

67. The method of any one of claims 62-63, wherein the administering is performed within 4 hours of the mixing.

68. The method of any one of claims 62-67, wherein the amount of payload is less than 1.5 mg per kg of the patient.

69. The method of any one of claims 62-67, wherein the amount of payload is less than 1.0 mg per kg of the patient.

70. The method of any one of claims 62-67, wherein the amount of payload is less than 0.5 mg per kg of the patient.

71. The method of any one of claims 1-70, wherein the amount of payload is less than 150 micrograms.

72. The method of any one of claims 1-70, wherein the amount of payload is less than 100 micrograms or less than 75 micrograms.

73. The method of any one of claims 1-70, wherein the amount of payload ranges from 1 to 30 micrograms.

74. A pharmaceutical composition comprising the LNP produced by the method of any one of claims 1-61 and a pharmaceutically acceptable diluent or excipient.

75. A kit comprising  
i) an aqueous first solution comprising a plurality of LNPs comprising a plurality of ionizable lipids, wherein the first solution is substantially free of any destabilizing agents and the



difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0; and

ii) dry payload comprising a nucleic acid or payload comprising a nucleic acid in a second aqueous solution that is substantially free of any destabilizing agents.

76. A kit comprising an aqueous first solution comprising a plurality of LNPs comprising a plurality of ionizable lipids, wherein the first solution is substantially free of any destabilizing agents and the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0.

77. A kit comprising a dry first composition comprising

i) a dry plurality of LNPs comprising a plurality of ionizable lipids; and  
ii) a first solution that is substantially free of any destabilizing agents and the difference between the pH of the first solution and the pKa of the ionizable lipids is less than 1.0.

78. The kit of claim 77, wherein the kit further comprises dry payload comprising a nucleic acid or payload comprising a nucleic acid in a second aqueous solution that is substantially free of any destabilizing agents.

79. The kit of any one of claims 75-78, wherein the kit further comprises instructions for mixing the first solution with the dry LNPs, the dry payload, the second solution, or combinations thereof.

80. The kit of any one of claims 75-79, wherein a majority of the payload is encapsulated by the LNPs when the first solution is mixed with the dry payload, the second solution, or combinations thereof.

81. The kit of any one of claims 75-80, wherein the kit includes one or more unit doses of a payload comprising a nucleic acid.

82. The kit of claim 81, wherein the unit dose is less than 1.5 mg per kg of the patient.

83. The kit of claim 81, wherein the unit dose is less than 1.0 mg per kg of the patient.

- 84. The kit of claim 81, wherein the unit dose is less than 0.5 mg per kg of the patient.
- 85. The kit of claim 81, wherein the unit dose is less than 150 micrograms.
- 86. The kit of claim 81, wherein the unit dose is less than 100 micrograms.
- 87. The kit of claim 81, wherein the unit dose is less than 75 micrograms.
- 88. The kit of claim 81, wherein the unit dose ranges from 1 to 30 micrograms.