

LIPID NANOPARTICLE FORMULATIONS FOR MRNA DELIVERY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of, and priority to U.S. Provisional Application Serial Number 63/025,355, filed on May 15, 2020, the disclosure of which is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] Messenger RNA (mRNA) therapy is becoming an increasingly important approach for the treatment of a variety of diseases. Messenger RNA therapy involves administration of messenger RNA to a patient in need of therapy for production of a protein encoded by the mRNA within the patient's body. Lipid encapsulated mRNA formulations, such as lipid nanoparticle (LNP) compositions show high degree of cellular uptake and protein expression. Lipid nanoparticle formulations traditionally use ethanol as a solvent for the lipid solution which is then mixed with an mRNA solution.

[0003] However, the use of flammable solvents such as ethanol pose safety risks and increase production costs, particularly in large-scale applications. In addition, low volume LNP formulations that are more suitable for dosing and reduce downstream processing volumes and costs, are also currently difficult to obtain using ethanol as a solvent. Low volume LNP formulations are also desirable as they permit bedside mixing to include other routes of administration, for example, subcutaneous or intramuscular.

SUMMARY OF THE INVENTION

[0004] There is a need for stable, safe, cost-effective ethanol-free LNP formulations that have a high mRNA encapsulation efficiency for efficient delivery in therapeutic use. The present invention provides, among other things, a stable, safe, cost-effective method of encapsulating messenger RNA in lipid nanoparticles without the use of flammable solvents that yields LNPs with high encapsulation efficiency for mRNA delivery in therapeutic applications. In one aspect, the present invention provides a safer and more cost-effective method for large-scale manufacturing

processes. In another aspect, the present invention provides a method for producing LNP formulations in low volumes that not only reduce downstream processing in manufacturing but are also suitable for dosing and bedside mixing facilitating multiple administration routes including subcutaneous and intramuscular. The invention is based on the surprising discovery that mixing an mRNA solution and a lipid solution in the presence of an amphiphilic polymer forms mRNA encapsulated within LNPs (mRNA-LNPs) in a LNP formulation solution. The present invention provides, among other things, a safe, efficient and cost-effective process for preparing a composition comprising mRNA-loaded lipid nanoparticles.

[0005] In one aspect, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles (LNPs) comprising a step of mixing (a) an mRNA solution comprising one or more mRNAs with (b) a lipid solution comprising one or more cationic lipids, one or more non-cationic lipids, and one or more PEG-modified lipids, and wherein the step of mixing the mRNA solution and the lipid solution comprises mixing in the presence of an amphiphilic polymer to form mRNA encapsulated within LNPs (mRNA-LNPs) in a LNP formulation solution. In some embodiments, the lipid solution comprises three lipid components. In some embodiments, the lipid solution comprises four lipid components. In particular embodiments, the four lipid components of the lipid solution are a PEG-modified lipid, a cationic lipid (*e.g.* ML-2 or MC-3), cholesterol, and a helper (*e.g.* non-cationic) lipid (*e.g.* DSPC or DOPE).

[0006] In some embodiments, the amphiphilic polymer comprises pluronics, polyvinyl pyrrolidone, polyvinyl alcohol, polyethylene glycol (PEG), or combinations thereof. Accordingly, in some embodiments, the amphiphilic polymer comprises pluronics. In some embodiments, the amphiphilic polymer comprises polyvinyl pyrrolidone. In some embodiments, the amphiphilic polymer comprises polyethylene glycol. .

[0007] In some embodiments, the PEG is triethylene glycol monomethyl ether (mTEG). In some embodiments, the PEG is methoxy polyethylene glycol (mPEG). In some embodiments, the PEG is tetraethylene glycol monomethyl ether. In some embodiments, the PEG is pentaethylene glycol monomethyl ether. In some embodiments, the PEG is a combination of mTEG, mPEG, tetraethylene glycol monomethyl ether, and/or pentaethylene glycol monomethyl ether.

[0008] In some embodiments, the step of mixing the mRNA solution and the lipid solution yields PEG at a concentration of greater than 25% volume/volume.

[0009] In some embodiments, the step of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 50% volume/volume. In some embodiments, the step

of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 45% volume /volume. In some embodiments, the step of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 40% volume /volume. In some embodiments, the step of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 35% volume /volume. In some embodiments, the step of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 30% volume /volume. In some embodiments, the step of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 25% volume /volume. In some embodiments, the step of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 20% volume /volume. In some embodiments, the step of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 15% volume /volume. In some embodiments, the step of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 10% volume /volume. In some embodiments, the step of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 5% volume /volume. In some embodiments, the step of mixing the mRNA solution and the lipid solution comprises PEG at a concentration of about 1% volume /volume. In particular embodiments, the PEG is mTEG. A particularly suitable final concentration of mTEG in the mRNA-LNP formulation is about 55-65% volume /volume, for example about 50% volume /volume. As shown in the examples, this final concentration of mTEG maintains mRNA solubility and stability and allows reduced processing volumes and ease of manufacture of the formulations on a larger scale.

[0010] In some embodiments, the mRNA solution comprises less than 5 mM of citrate, and wherein the mRNA-LNPs have an encapsulation efficiency of greater than 60%. In some embodiments, the mRNA solution comprises less than 5 mM of citrate, and wherein the mRNA-LNPs have an encapsulation efficiency of greater than 70%. In some embodiments, the mRNA solution comprises less than 5 mM of citrate, and wherein the mRNA-LNPs have an encapsulation efficiency of greater than 80%. In some embodiments, the mRNA solution comprises less than 5 mM of citrate, and wherein the mRNA-LNPs have an encapsulation efficiency of greater than 90%. In some embodiments, the mRNA solution comprises less than 5 mM of citrate, and wherein the mRNA-LNPs have an encapsulation efficiency of greater than 95%. In some embodiments, the mRNA solution comprises less than 5 mM of citrate, and wherein the mRNA-LNPs have an encapsulation efficiency of greater than 99%.

[0011] In some embodiments, the mRNA solution and/or the lipid solution are at about ambient temperature.

[0012] In some embodiments, the ambient temperature is less than about 35 °C. In some embodiments, the ambient temperature is less than about 32 °C. In some embodiments, the ambient temperature is less than about 30 °C. In some embodiments, the ambient temperature is less than about 28 °C. In some embodiments, the ambient temperature is less than about 26 °C. In some embodiments, the ambient temperature is less than about 25 °C. In some embodiments, the ambient temperature is less than about 24 °C. In some embodiments, the ambient temperature is less than about 23 °C. In some embodiments, the ambient temperature is less than about 22 °C. In some embodiments, the ambient temperature is less than about 21 °C. In some embodiments, the ambient temperature is less than about 20 °C. In some embodiments, the ambient temperature is less than about 19 °C. In some embodiments, the ambient temperature is less than about 18 °C. In some embodiments, the ambient temperature is less than about 16 °C.

[0013] In some embodiments, the ambient temperature ranges from about 15-35 °C. In some embodiments, the ambient temperature ranges from about 16-32 °C. In some embodiments, the ambient temperature ranges from about 17-30 °C. In some embodiments, the ambient temperature ranges from about 18-30 °C. In some embodiments, the ambient temperature ranges from about 18-32 °C. In some embodiments, the ambient temperature ranges from about 20-28 °C. In some embodiments, the ambient temperature ranges from about 20-26 °C. In some embodiments, the ambient temperature ranges from about 20-25 °C. In some embodiments, the ambient temperature ranges from about 23-25 °C. In some embodiments, the ambient temperature ranges from about 21-24 °C. In some embodiments, the ambient temperature ranges from about 21-23 °C. In some embodiments, the ambient temperature ranges from about 21-26 °C.

[0014] In some embodiments, the ambient temperature is about 16 °C. In some embodiments, the ambient temperature is about 18 °C. In some embodiments, the ambient temperature is about 20 °C. In some embodiments, the ambient temperature is about 21 °C. In some embodiments, the ambient temperature is about 22 °C. In some embodiments, the ambient temperature is about 23 °C. In some embodiments, the ambient temperature is about 24 °C. In some embodiments, the ambient temperature is about 25 °C. In some embodiments, the ambient temperature is about 26 °C. In some embodiments, the ambient temperature is about 27 °C. In some embodiments, the ambient temperature is about 28 °C. In some embodiments, the ambient temperature is about 30 °C. In some embodiments, the ambient temperature is about 31 °C. In some embodiments, the ambient temperature is about 32 °C. In some embodiments, the ambient temperature is about 35 °C.

[0015] In some embodiments, the one or more non-cationic lipids is selected from distearoylphosphatidylcholine (DSPC). In some embodiments, the one or more non-cationic lipids is dioleoylphosphatidylcholine (DOPC). In some embodiments, the one or more non-cationic lipids is dipalmitoylphosphatidylcholine (DPPC). In some embodiments, the one or more non-cationic lipids is dioleoylphosphatidylglycerol (DOPG). In some embodiments, the one or more non-cationic lipids is dipalmitoylphosphatidylglycerol (DPPG). In some embodiments, the one or more non-cationic lipids is dioleoylphosphatidylethanolamine (DOPE). In some embodiments, the one or more non-cationic lipids is palmitoyloleoylphosphatidylcholine (POPC). In some embodiments, the one or more non-cationic lipids is palmitoyloleoyl-phosphatidylethanolamine (POPE). In some embodiments, the one or more non-cationic lipids is dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-l-carboxylate (DOPE-mal). In some embodiments, the one or more non-cationic lipids is dipalmitoyl phosphatidyl ethanolamine (DPPE). In some embodiments, the one or more non-cationic lipids is dimyristoylphosphoethanolamine (DMPE). In some embodiments, the one or more non-cationic lipids is distearoyl-phosphatidyl-ethanolamine (DSPE). In some embodiments, the one or more non-cationic lipids is phosphatidylserine. In some embodiments, the one or more non-cationic lipids is sphingolipids. In some embodiments, the one or more non-cationic lipids is cerebrosides. In some embodiments, the one or more non-cationic lipids is gangliosides. In some embodiments, the one or more non-cationic lipids is 16-O-monomethyl PE. In some embodiments, the one or more non-cationic lipids is 16-O-dimethyl PE. In some embodiments, the one or more non-cationic lipids is 18-1-trans PE. In some embodiments, the one or more non-cationic lipids is l-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE).

[0016] In some embodiments, the mRNA solution further comprises trehalose. In some embodiments, the mRNA solution comprises 20% trehalose. In some embodiments, the mRNA solution comprises 15% trehalose. In some embodiments, the mRNA solution comprises 10% trehalose. In some embodiments, the mRNA solution comprises 5% trehalose.

[0017] In some embodiments, the process does not require a step of heating the mRNA solution and the lipid solution prior to the mixing step.

[0018] In some embodiments, the mRNA solution comprises greater than about 1 g of mRNA per 12 L of the mRNA solution. In some embodiments, the mRNA solution comprises greater than about 1 g of mRNA per 10 L of the mRNA solution. In some embodiments, the mRNA solution comprises about 1 g of mRNA per 8 L of the mRNA solution. In some embodiments, the

mRNA solution comprises greater than about 1 g of mRNA per 6 L of the mRNA solution. In some embodiments, the mRNA solution comprises about 1 g of mRNA per 4 L of the mRNA solution. In some embodiments, the mRNA solution comprises about 1 g of mRNA per 2 L of the mRNA solution. In some embodiments, the mRNA solution comprises greater than about 1 g of mRNA per 1 L of the mRNA solution.

[0019] In some embodiments, the concentration of mRNA in the mRNA solution is greater than about 0.05 mg/mL. In some embodiments, the concentration of mRNA in the mRNA solution is greater than about 0.1 mg/mL. In some embodiments, the concentration of mRNA in the mRNA solution is greater than about 0.125 mg/mL. In some embodiments, the concentration of mRNA in the mRNA solution is greater than about 0.25 mg/mL. In some embodiments, the concentration of mRNA in the mRNA solution is greater than about 0.5 mg/mL. In some embodiments, the concentration of mRNA in the mRNA solution is greater than about 1.0 mg/mL. In some embodiments, the concentration of mRNA in the mRNA solution is greater than about 1.5 mg/mL. In some embodiments, the concentration of mRNA in the mRNA solution is greater than about 2.0 mg/mL. In some embodiments, the concentration of mRNA in the mRNA solution is between about 0.05 mg/mL and about 0.5 mg/mL. In particular embodiments, the concentration of mRNA in the mRNA solution is between about 0.1 mg/mL to about 0.5 mg/mL, for example about 0.1 mg/mL or about 0.35 mg/mL.

[0020] In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of between 1:1 and 10:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of between 2:1 and 6:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of about 2:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of about 3:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of about 4:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of about 5:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of about 6:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of greater than about 2:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of greater than about 3:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of greater than about 4:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of greater than about 5:1. In some embodiments, the mRNA solution and the lipid solution are mixed at a ratio (v/v) of greater than about 6:1. In some embodiments, the mRNA solution and the lipid solution (*e.g.* about 100%

mTEG-lipid solution) are mixed at a ratio (v/v) of 1-8:1, for example 1-4:1. In particular embodiments, the mRNA solution and the lipid solution (e.g. about 100% mTEG-lipid solution) are mixed at a ratio (v/v) of about 1:1. As shown in the examples, this ratio of mRNA solution to the lipid solution maintains mRNA solubility and stability and allows reduced processing volumes and ease of manufacture of the formulations on a larger scale.

[0021] In some embodiments, the mRNA solution has a pH between 2.5 and 5.5. In some embodiments, the mRNA solution has a pH between 3.0 and 5.0. In some embodiments, the mRNA solution has a pH between 3.5 and 4.5. In some embodiments, the mRNA solution has a pH of about 3.0. In some embodiments, the mRNA solution has a pH of about 3.5. In some embodiments, the mRNA solution has a pH of about 4.0. In some embodiments, the mRNA solution has a pH of about 4.5. In some embodiments, the mRNA solution has a pH of about 5.0. In some embodiments, the mRNA solution has a pH of about 5.5.

[0022] In some embodiments, the step of mixing occurs in a total volume of between about 3 and 10 mL. In some embodiments, the step of mixing occurs in a total volume of between about 1 and 10 mL. In some embodiments, the step of mixing occurs in a total volume of between about 1 and 15 mL. In some embodiments, the step of mixing occurs in a total volume of between about 1 mL. In some embodiments, the step of mixing occurs in a total volume of between about 2 mL. In some embodiments, the step of mixing occurs in a total volume of between about 3 mL. In some embodiments, the step of mixing occurs in a total volume of between about 4 mL. In some embodiments, the step of mixing occurs in a total volume of between about 5 mL. In some embodiments, the step of mixing occurs in a total volume of between about 6 mL. In some embodiments, the step of mixing occurs in a total volume of between about 7 mL. In some embodiments, the step of mixing occurs in a total volume of between about 8 mL. In some embodiments, the step of mixing occurs in a total volume of between about 9 mL. In some embodiments, the step of mixing occurs in a total volume of between about 10 mL. In some embodiments, the step of mixing occurs in a total volume of between about 12 mL. In some embodiments, the step of mixing occurs in a total volume of between about 13 mL. In some embodiments, the step of mixing occurs in a total volume of between about 14 mL. In some embodiments, the step of mixing occurs in a total volume of between about 15 mL.

[0023] In some embodiments, the process does not comprise an alcohol.

[0024] In some embodiments, the process further comprises a step of incubating the mRNA-LNPs. In some embodiments, the process further comprises a step of incubating the

mRNA-LNPs post-mixing. In some embodiments, the mRNA-LNPs are incubated at a temperature of between 21 °C and 65 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of between 25 °C and 60 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of between 30 °C and 55 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of between 35 °C and 50 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 26 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 30 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 31 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 32 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 35 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 36 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 38 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 40 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 42 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 45 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 50 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 55 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 60 °C. In some embodiments, the mRNA-LNPs are incubated at a temperature of about 65 °C.

[0025] In some embodiments, the mRNA-LNPs are incubated for greater than about 20 minutes. In some embodiments, the mRNA-LNPs are incubated for greater than about 30 minutes. In some embodiments, the mRNA-LNPs are incubated for greater than about 40 minutes. In some embodiments, the mRNA-LNPs are incubated for greater than about 50 minutes. In some embodiments, the mRNA-LNPs are incubated for greater than about 60 minutes. In some embodiments, the mRNA-LNPs are incubated for greater than about 70 minutes. In some embodiments, the mRNA-LNPs are incubated for greater than about 80 minutes. In some embodiments, the mRNA-LNPs are incubated for greater than about 90 minutes. In some embodiments, the mRNA-LNPs are incubated for greater than about 100 minutes. In some embodiments, the mRNA-LNPs are incubated for greater than about 120 minutes. In some embodiments, the mRNA-LNPs are incubated for about 30 minutes. In some embodiments, the mRNA-LNPs are incubated for about 40 minutes. In some embodiments, the mRNA-LNPs are incubated for about 50 minutes. In some embodiments, the mRNA-LNPs are incubated for about 60 minutes. In some embodiments, the mRNA-LNPs are incubated for about 70 minutes. In some

embodiments, the mRNA-LNPs are incubated for about 80 minutes. In some embodiments, the mRNA-LNPs are incubated for about 90 minutes. In some embodiments, the mRNA-LNPs are incubated for about 100 minutes. In some embodiments, the mRNA-LNPs are incubated for about 120 minutes. In some embodiments, the mRNA-LNPs are incubated for about 150 minutes. In some embodiments, the mRNA-LNPs are incubated for about 180 minutes.

[0026] In some embodiments, the lipid solution does not comprise an alcohol.

[0027] In some embodiments, the lipid solution further comprises one or more cholesterol-based lipids.

[0028] In some embodiments, the mRNA-LNPs are purified by Tangential Flow Filtration.

[0029] In some embodiments, the mRNA-LNPs have an average size less than 200 nm. In some embodiments, the mRNA-LNPs have an average size less than 150 nm. In some embodiments, the mRNA-LNPs have an average size less than 100 nm. In some embodiments, the mRNA-LNPs have an average size less than 95 nm. In some embodiments, the mRNA-LNPs have an average size less than 90 nm. In some embodiments, the mRNA-LNPs have an average size less than 85 nm. In some embodiments, the mRNA-LNPs have an average size less than 80 nm. In some embodiments, the mRNA-LNPs have an average size less than 75 nm. In some embodiments, the mRNA-LNPs have an average size less than 70 nm. In some embodiments, the mRNA-LNPs have an average size less than 65 nm. In some embodiments, the mRNA-LNPs have an average size less than 60 nm. In some embodiments, the mRNA-LNPs have an average size less than 55 nm. In some embodiments, the mRNA-LNPs have an average size less than 50 nm. In some embodiments, the mRNA-LNPs have an average size less than 45 nm. In some embodiments, the mRNA-LNPs have an average size less than 40 nm. In some embodiments, the mRNA-LNPs have an average size less than 35 nm. In some embodiments, the mRNA-LNPs have an average size ranging from 35 nm to 65 nm. In some embodiments, the mRNA-LNPs have an average size ranging from 40-70 nm. In some embodiments, the mRNA-LNPs have an average size ranging from 40 nm to 60 nm. In some embodiments, the mRNA-LNPs have an average size ranging from 45 nm to 55 nm.

[0030] In some embodiments, the lipid nanoparticles have a PDI of less than about 0.3. In some embodiments, the lipid nanoparticles have a PDI of less than about 0.2. In some embodiments, the lipid nanoparticles have a PDI of less than about 0.18. In some embodiments, the lipid nanoparticles have a PDI of less than about 0.15. In some embodiments, the lipid

nanoparticles have a PDI of less than about 0.12. In some embodiments, the lipid nanoparticles have a PDI of less than about 0.10.

[0031] In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 60%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 65%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 70%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 75%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 80%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 85%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 90%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 95%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 96%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 97%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 98%. In some embodiments, the encapsulation efficiency of the mRNA-LNPs is greater than about 99%.

[0032] In some embodiments, the mRNA-LNPs have a N/P ratio of between 1 to 10. In some embodiments, the mRNA-LNPs have a N/P ratio of between 2 to 6. In some embodiments, the mRNA-LNPs have a N/P ratio of about 4. In some embodiments, the mRNA solution and the lipid solution are mixed at a N/P ratio of between 1 to 10. In some embodiments, the mRNA solution and the lipid solution are mixed at a N/P ratio of between 2 to 6. In some embodiments, the mRNA solution and the lipid solution are mixed at a N/P ratio of about 2. In some embodiments, the mRNA solution and the lipid solution are mixed at a N/P ratio of about 4. In some embodiments, the mRNA solution and the lipid solution are mixed at a N/P ratio of about 6. In particular embodiments, the mRNA solution and lipid solution are mixed at a N/P ratio of about 4. As shown in the examples, such an N/P ratio yielded LNPs of suitable size and encapsulation efficiencies for therapeutic use.

[0033] In some embodiments, 5 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 10 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 15 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 20 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 25 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 30 g or more of

mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 40 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 50 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 75 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 100 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 150 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 200 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 250 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 500 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 750 g or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 1 kg or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 5 kg or more of mRNA is encapsulated in lipid nanoparticles in a single batch. In some embodiments, 10 kg or more of mRNA is encapsulated in lipid nanoparticles in a single batch.

[0034] In some embodiments, the mRNA solution and the lipid solution are mixed by a pulse-less flow pump. In some embodiments, the pump is a gear pump. In some embodiments, the pump is a centrifugal pump.

[0035] In some embodiments, the mRNA solution is mixed at a flow rate ranging from about 150-250 ml/minute, 250-500 ml/minute, 500-1000 ml/minute, 1000-2000 ml/minute, 2000-3000 ml/minute, 3000-4000 ml/minute, 4000-5000 ml/minute, 6000-8000 ml/minute, 8000-10000 ml/minute or 10000-12000 ml/minute.

[0036] In some embodiments, the mRNA solution is mixed at a flow rate of about 100 ml/minute, about 200 ml/minute, about 500 ml/minute, about 800 ml/minute, about 1000 ml/minute, about 1200 ml/minute, about 2000 ml/minute, about 3000 ml/minute, about 4000 ml/minute, about 5000 ml/minute, about 6000 ml/minute, about 8000 ml/minute, about 10000 ml/minute, about 12000 ml/minute, or about 15000 ml/minute.

[0037] In some embodiments, the mRNA solution is mixed at a flow of about 100 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 200 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 400 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 500 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 600 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 800

ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 1000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 1200 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 1400 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 1600 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 1800 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 2000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 2400 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 3000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 4000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 5000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 6000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 7000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 8000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 9000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 10000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 12000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow of about 15000 ml/minute.

[0038] In some embodiments, the lipid solution is mixed at a flow rate ranging from about 25-75 ml/minute, about 75-200 ml/minute, about 200-350 ml/minute, about 350-500 ml/minute, about 500-650 ml/minute, about 650-850 ml/minute, or about 850-1000 ml/minute. In some embodiments, the lipid solution is mixed at a flow rate of about 50 ml/minute, about 100 ml/minute, about 150 ml/minute, about 200 ml/minute, about 250 ml/minute, about 300 ml/minute, about 350 ml/minute, about 400 ml/minute, about 450 ml/minute, about 500 ml/minute, about 550 ml/minute, about 600 ml/minute, about 650 ml/minute, about 700 ml/minute, about 750 ml/minute, about 800 ml/minute, about 850 ml/minute, about 900 ml/minute, about 950 ml/minute, about 1000 ml/minute, about 1200 ml/minute, or about 1500 ml/minute.

[0039] In some embodiments, the flow rate of the mRNA solution is same as the flow rate of the lipid solution. In some embodiments, the flow rate of the mRNA solution is 2 times greater than the flow rate of the lipid solution. In some embodiments, the flow rate of the mRNA solution is 3 times greater than the flow rate of the lipid solution. In some embodiments, the flow rate of the mRNA solution is 4 times greater than the flow rate of the lipid solution. In some

embodiments, the flow rate of the mRNA solution is 4.5 times greater than the flow rate of the lipid solution. In some embodiments, the flow rate of the mRNA solution is 5 times greater than the flow rate of the lipid solution. In some embodiments, the flow rate of the mRNA solution is 5.5 times greater than the flow rate of the lipid solution. In some embodiments, the flow rate of the mRNA solution is 6 times greater than the flow rate of the lipid solution. In some embodiments, the flow rate of the mRNA solution is 8 times greater than the flow rate of the lipid solution. In some embodiments, the flow rate of the mRNA solution is 10 times greater than the flow rate of the lipid solution.

[0040] In some embodiments, a composition comprising mRNA encapsulated in lipid nanoparticles is prepared by the process.

[0041] In some embodiments, the composition comprises 1 g or more of mRNA. In some embodiments, the composition comprises 5 g or more of mRNA. In some embodiments, the composition comprises 10 g or more of mRNA. In some embodiments, the composition comprises 15 g or more of mRNA. In some embodiments, the composition comprises 20 g or more of mRNA. In some embodiments, the composition comprises 25 g or more of mRNA. In some embodiments, the composition comprises 50 g or more of mRNA. In some embodiments, the composition comprises 75 g or more of mRNA. In some embodiments, the composition comprises 100 g or more of mRNA. In some embodiments, the composition comprises 125 g or more of mRNA. In some embodiments, the composition comprises 150 g or more of mRNA. In some embodiments, the composition comprises 250 g or more of mRNA. In some embodiments, the composition comprises 500 g or more of mRNA. In some embodiments, the composition comprises 1 kg or more of mRNA.

[0042] In some embodiments, the mRNA comprises one or more modified nucleotides.

[0043] In some embodiments, the mRNA is unmodified.

[0044] In some embodiments, the mRNA is greater than about 0.5 kb. In some embodiments, the mRNA is greater than about 1 kb. In some embodiments, the mRNA is greater than about 2 kb. In some embodiments, the mRNA is greater than about 3 kb. In some embodiments, the mRNA is greater than about 4 kb. In some embodiments, the mRNA is greater than about 5 kb. In some embodiments, the mRNA is greater than about 6 kb. In some embodiments, the mRNA is greater than about 8 kb. In some embodiments, the mRNA is greater than about 10 kb. In some embodiments, the mRNA is greater than about 20 kb. In some

embodiments, the mRNA is greater than about 30 kb. In some embodiments, the mRNA is greater than about 40 kb. In some embodiments, the mRNA is greater than about 50 kb.

[0045] In some embodiments, the lipid solution comprises four lipid components. In some embodiments, the lipid solution comprises a PEG-modified lipid, a cationic lipid (*e.g.* ML-2 or MC-3), a helper (*e.g.* non-cationic) lipid (*e.g.* DSPC or DOPE), and optionally cholesterol. In some embodiments, the ratio of cationic lipid(s) to non-cationic lipid(s) to cholesterol-based lipid(s) to PEG-modified lipid(s) in the LNPs is 35-55:5-35:20-40:1-15. In particular embodiments, a lipid solution with mTEG as the solvent (*e.g.*, 100% mTEG) and an aqueous solution of mRNA (*e.g.*, a citrate buffer) are mixed at a volumetric ratio of 1:1-4 (for example about 1:1), with a final concentration of mRNA of about 0.05-0.5 mg/mL, and the ratio of cationic lipid(s) to non-cationic lipid(s) to cholesterol-based lipid(s) to PEG-modified lipid(s) in the LNPs is 35-55:25-35:20-40:1-15 (for example about 40:30:25:5), such that the cationic lipid(s) to mRNA N/P ratio is about 2-6 (*e.g.* about 4). As shown in the examples, these preparations are particularly suitable for use in the formulations of the invention as they ensure suitable mRNA-LNP size and encapsulation efficacy. Furthermore, such mRNA-LNP formulations having high lipid and mRNA concentrations are advantageous in reducing processing volumes and thereby increasing ease of processing in manufacturing.

[0046] In some embodiments, the mRNA is purified using low amounts of volatile organic compounds or no volatile organic compounds. In some embodiments, the mRNA is purified in a process free of volatile organic compounds. In some embodiments, the mRNA is purified in a process free of alcohol. In some embodiments, the mRNA is purified using an isopropyl alcohol-free process. In some embodiments, the mRNA is purified using a benzyl alcohol-free process.

[0047] In some embodiments, the mRNA is purified and encapsulated in an LNP in a process free of volatile organic compounds. In some embodiments, the mRNA is purified and encapsulated in an LNP in a process free of alcohol. In some embodiments, the mRNA is encapsulated in an LNP in a process that does not comprise volatile organic compounds. In some embodiments, the mRNA is encapsulated in an LNP in a process that does not comprise alcohol.

[0048] Other features, objects, and advantages of the present invention are apparent in the detailed description, drawings and claims that follow. It should be understood, however, that the detailed description, the drawings, and the claims, while indicating embodiments of the present invention, are given by way of illustration only, not limitation. Various changes and modifications within the scope of the invention will become apparent to those skilled in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

[0049] The following figures are for illustration purposes only and not for limitation.

[0050] **FIG. 1** is a graph that depicts average radiance p/s/cm²/sr from mice that were administered firefly luciferase (FFL) mRNA-LNPs that were encapsulated either in an ethanol-free formulation (i.e., mTEG) or in that were encapsulated in an ethanol-containing formulation. Furthermore, The data also show data obtained from formulations that were made at high volumes (1:4 lipid solution to mRNA solution) or in low volumes (1:1 lipid solution to mRNA solution).

[0051] **FIG. 2** is a graph that depicts the total ornithine transcarbamylase (OTC) in ng/mg of total protein from mice that were administered OTC mRNA-LNPs that were encapsulated in an ethanol-free formulation (i.e. mTEG). The data also show data obtained from formulations that were made at high volumes (1:4 lipid solution to mRNA solution) or in low volumes (1:1 lipid solution to mRNA solution).

DEFINITIONS

[0052] In order for the present invention to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms are set forth throughout the specification. The publications and other reference materials referenced herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference.

[0053] The terms “or more”, “at least”, “more than”, and the like, e.g., “at least one” are understood to include but not be limited to at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149 or 150, 200, 300, 400, 500, 600, 700,

800, 900, 1000, 2000, 3000, 4000, 5000 or more than the stated value. Also included is any greater number or fraction in between.

[0054] Conversely, the term “no more than” includes each value less than the stated value. For example, “no more than 100 nucleotides” includes 100, 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 79, 78, 77, 76, 75, 74, 73, 72, 71, 70, 69, 68, 67, 66, 65, 64, 63, 62, 61, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, and 0 nucleotides. Also included is any lesser number or fraction in between.

[0055] The terms “plurality”, “at least two”, “two or more”, “at least second”, and the like, are understood to include but not limited to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149 or 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000 or more. Also included is any greater number or fraction in between.

[0056] *Amino acid:* As used herein, the term “amino acid,” in its broadest sense, refers to any compound and/or substance that can be incorporated into a polypeptide chain. In some embodiments, an amino acid has the general structure $\text{H}_2\text{N}-\text{C}(\text{H})(\text{R})-\text{COOH}$. In some embodiments, an amino acid is a naturally occurring amino acid. In some embodiments, an amino acid is a synthetic amino acid; in some embodiments, an amino acid is a d-amino acid; in some embodiments, an amino acid is an l-amino acid. “Standard amino acid” refers to any of the twenty standard l-amino acids commonly found in naturally occurring peptides. “Nonstandard amino acid” refers to any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or obtained from a natural source. As used herein, “synthetic amino acid” encompasses chemically modified amino acids, including but not limited to salts, amino acid derivatives (such as amides), and/or substitutions. Amino acids, including carboxy- and/or amino-terminal amino acids in peptides, can be modified by methylation, amidation, acetylation, protecting groups, and/or substitution with other chemical groups that can change the peptide’s

circulating half-life without adversely affecting their activity. Amino acids may participate in a disulfide bond. Amino acids may comprise one or posttranslational modifications, such as association with one or more chemical entities (*e.g.*, methyl groups, acetate groups, acetyl groups, phosphate groups, formyl moieties, isoprenoid groups, sulfate groups, polyethylene glycol moieties, lipid moieties, carbohydrate moieties, biotin moieties, *etc.*). The term “amino acid” is used interchangeably with “amino acid residue,” and may refer to a free amino acid and/or to an amino acid residue of a peptide. It will be apparent from the context in which the term is used whether it refers to a free amino acid or a residue of a peptide.

[0057] *Animal*: As used herein, the term “animal” refers to any member of the animal kingdom. In some embodiments, “animal” refers to humans, at any stage of development. In some embodiments, “animal” refers to non-human animals, at any stage of development. In certain embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, insects, and/or worms. In some embodiments, an animal may be a transgenic animal, genetically-engineered animal, and/or a clone.

[0058] *Approximately or about*: As used herein, the term “approximately” or “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term “approximately” or “about” refers to be within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, 0.01%, or 0.001% of the stated value. Unless otherwise clear from the context, all numerical values provided herein are modified by the term “approximately” or “about”.

[0059] *Batch*: As used herein, the term “batch” refers to a quantity or amount of mRNA purified at one time, *e.g.*, purified according to a single manufacturing order during the same cycle of manufacture. A batch may refer to an amount of mRNA purified in one reaction.

[0060] *Biologically active*: As used herein, the phrase “biologically active” refers to a characteristic of any agent that has activity in a biological system, and particularly in an organism. For instance, an agent that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active.

[0061] *Comprising*: As used herein, the term “comprising,” or variations such as “comprises” or “comprising,” will be understood to imply the inclusion of a stated element,

integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

[0062] *Combining:* As used herein, the term “combining” is interchangeably used with mixing or blending. Combining refers to putting together discrete LNP particles having distinct properties in the same solution, for example, combining an mRNA-LNP and an empty LNP, to obtain an mRNA-LNP composition. In some embodiments, the combining of the two LNPs is performed at a specific ratio of the components being combined. In some embodiments, the resultant composition obtained from the combining has a property distinct from any one or both of its components.

[0063] *Delivery:* As used herein, the term “delivery” encompasses both local and systemic delivery. For example, delivery of mRNA encompasses situations in which an mRNA is delivered to a target tissue and the encoded protein is expressed and retained within the target tissue (also referred to as “local distribution” or “local delivery”), and situations in which an mRNA is delivered to a target tissue and the encoded protein is expressed and secreted into patient’s circulation system (*e.g.*, serum) and systematically distributed and taken up by other tissues (also referred to as “systemic distribution” or “systemic delivery”). In some embodiments, delivery is pulmonary delivery, *e.g.*, comprising nebulization.

[0064] *dsRNA:* As used herein, the term “dsRNA” refers to the production of complementary RNA sequences during an *in vitro* transcription (IVT) reaction. Complimentary RNA sequences can be produced for a variety of reasons including, for example, short abortive transcripts that can hybridize to complimentary sequences in the nascent RNA strand, short abortive transcripts acting as primers for RNA dependent DNA independent RNA transcription, and possible RNA polymerase template reversal.

[0065] *Efficacy:* As used herein, the term “efficacy,” or grammatical equivalents, refers to an improvement of a biologically relevant endpoint, as related to delivery of mRNA that encodes a relevant protein or peptide.

[0066] *Encapsulation:* As used herein, the term “encapsulation,” or its grammatical equivalent, refers to the process of confining a nucleic acid molecule within a nanoparticle.

[0067] *Expression:* As used herein, “expression” of a nucleic acid sequence refers to translation of an mRNA into a polypeptide (*e.g.*, heavy chain or light chain of antibody), assemble multiple polypeptides (*e.g.*, heavy chain or light chain of antibody) into an intact protein (*e.g.*,

antibody) and/or post-translational modification of a polypeptide or fully assembled protein (e.g., antibody). In this application, the terms “expression” and “production,” and grammatical equivalent, are used inter-changeably.

[0068] *Functional:* As used herein, a “functional” biological molecule is a biological molecule in a form in which it exhibits a property and/or activity by which it is characterized.

[0069] *Improve, increase, or reduce:* As used herein, the terms “improve,” “increase” or “reduce,” or grammatical equivalents, indicate values that are relative to a baseline measurement, such as a measurement in the same individual prior to initiation of the treatment described herein, or a measurement in a control subject (or multiple control subject) in the absence of the treatment described herein. A “control subject” is a subject afflicted with the same form of disease as the subject being treated, who is about the same age as the subject being treated.

[0070] *Impurities:* As used herein, the term “impurities” refers to substances inside a confined amount of liquid, gas, or solid, which differ from the chemical composition of the target material or compound. Impurities are also referred to as “contaminants.”

[0071] *In Vitro:* As used herein, the term “*in vitro*” refers to events that occur in an artificial environment, e.g., in a test tube or reaction vessel, in cell culture, etc., rather than within a multi-cellular organism.

[0072] *In Vivo:* As used herein, the term “*in vivo*” refers to events that occur within a multi-cellular organism, such as a human and a non-human animal. In the context of cell-based systems, the term may be used to refer to events that occur within a living cell (as opposed to, for example, *in vitro* systems).

[0073] *Isolated:* As used herein, the term “isolated” refers to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% of the other components with which they were initially associated. In some embodiments, isolated agents are about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a

substance is “pure” if it is substantially free of other components. As used herein, calculation of percent purity of isolated substances and/or entities should not include excipients (*e.g.*, buffer, solvent, water, *etc.*).

[0074] *Liposome*: As used herein, the term “liposome” refers to any lamellar, multilamellar, or solid nanoparticle vesicle. Typically, a liposome as used herein can be formed by mixing one or more lipids or by mixing one or more lipids and polymer(s). In some embodiments, a liposome suitable for the present invention contains a cationic lipid(s) and optionally non-cationic lipid(s), optionally cholesterol-based lipid(s), and/or optionally PEG-modified lipid(s).

[0075] *Local distribution or delivery*: As used herein, the terms “local distribution,” “local delivery,” or grammatical equivalent, refer to tissue specific delivery or distribution. Typically, local distribution or delivery requires a peptide or protein (*e.g.*, enzyme) encoded by mRNAs be translated and expressed intracellularly or with limited secretion that avoids entering the patient’s circulation system.

[0076] *messenger RNA (mRNA)*: As used herein, the term “messenger RNA (mRNA)” refers to a polynucleotide that encodes at least one polypeptide. mRNA as used herein encompasses both modified and unmodified RNA. mRNA may contain one or more coding and non-coding regions. mRNA can be purified from natural sources, produced using recombinant expression systems and optionally purified, chemically synthesized, *etc.* Where appropriate, *e.g.*, in the case of chemically synthesized molecules, mRNA can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, backbone modifications, *etc.* An mRNA sequence is presented in the 5’ to 3’ direction unless otherwise indicated. In some embodiments, an mRNA is or comprises natural nucleosides (*e.g.*, adenosine, guanosine, cytidine, uridine); nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, 2-thiocytidine, pseudouridine, and 5-methylcytidine); chemically modified bases; biologically modified bases (*e.g.*, methylated bases); intercalated bases; modified sugars (*e.g.*, 2’-fluororibose, ribose, 2’-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (*e.g.*, phosphorothioates and 5’-N-phosphoramidite linkages).

[0077] *mRNA integrity*: As used herein, the term “mRNA integrity” generally refers to the quality of mRNA. In some embodiments, mRNA integrity refers to the percentage of mRNA that is not degraded after a purification process. mRNA integrity may be determined using methods well known in the art, for example, by RNA agarose gel electrophoresis (e.g., Ausubel et al., John Wiley & Sons, Inc., 1997, Current Protocols in Molecular Biology).

[0078] *N/P Ratio*: As used herein, the term “N/P ratio” refers to a molar ratio of positively charged molecular units in the cationic lipids in a lipid nanoparticle relative to negatively charged molecular units in the mRNA encapsulated within that lipid nanoparticle. As such, N/P ratio is typically calculated as the ratio of moles of amine groups in cationic lipids in a lipid nanoparticle relative to moles of phosphate groups in mRNA encapsulated within that lipid nanoparticle. For example, a 4-fold molar excess of cationic lipid per mol mRNA is referred to as an “N/P ratio” of about 4.

[0079] *Nucleic acid*: As used herein, the term “nucleic acid,” in its broadest sense, refers to any compound and/or substance that is or can be incorporated into a polynucleotide chain. In some embodiments, a nucleic acid is a compound and/or substance that is or can be incorporated into a polynucleotide chain via a phosphodiester linkage. In some embodiments, “nucleic acid” refers to individual nucleic acid residues (e.g., nucleotides and/or nucleosides). In some embodiments, “nucleic acid” refers to a polynucleotide chain comprising individual nucleic acid residues. In some embodiments, “nucleic acid” encompasses RNA as well as single and/or double-stranded DNA and/or cDNA. Furthermore, the terms “nucleic acid,” “DNA,” “RNA,” and/or similar terms include nucleic acid analogs, *i.e.*, analogs having other than a phosphodiester backbone. For example, the so-called “peptide nucleic acids,” which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention. The term “nucleotide sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and/or encode the same amino acid sequence. Nucleotide sequences that encode proteins and/or RNA may include introns. Nucleic acids can be purified from natural sources, produced using recombinant expression systems and optionally purified, chemically synthesized, *etc.* Where appropriate, *e.g.*, in the case of chemically synthesized molecules, nucleic acids can comprise nucleoside analogs such as analogs having chemically modified bases or sugars, backbone modifications, *etc.* A nucleic acid sequence is presented in the 5' to 3' direction unless otherwise indicated. In some embodiments, a nucleic acid is or comprises natural nucleosides (e.g., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine); nucleoside

analogues (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyladenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine); chemically modified bases; biologically modified bases (e.g., methylated bases); intercalated bases; modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (e.g., phosphorothioates and 5'-N-phosphoramidite linkages). In some embodiments, the present invention is specifically directed to "unmodified nucleic acids," meaning nucleic acids (e.g., polynucleotides and residues, including nucleotides and/or nucleosides) that have not been chemically modified in order to facilitate or achieve delivery.

[0080] *Patient:* As used herein, the term "patient" or "subject" refers to any organism to which a provided composition may be administered, e.g., for experimental, diagnostic, prophylactic, cosmetic, and/or therapeutic purposes. Typical patients include animals (e.g., mammals such as mice, rats, rabbits, non-human primates, and/or humans). In some embodiments, a patient is a human. A human includes pre- and post-natal forms.

[0081] *Pharmaceutically acceptable:* The term "pharmaceutically acceptable" as used herein, refers to substances that, within the scope of sound medical judgment, are suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0082] *Pharmaceutically acceptable salt:* Pharmaceutically acceptable salts are well known in the art. For example, S. M. Berge et al., describes pharmaceutically acceptable salts in detail in *J. Pharmaceutical Sciences* (1977) 66:1-19. Pharmaceutically acceptable salts of the compounds of this invention include those derived from suitable inorganic and organic acids and bases. Examples of pharmaceutically acceptable, nontoxic acid addition salts are salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid or with organic acids such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid or malonic acid or by using other methods used in the art such as ion exchange. Other pharmaceutically acceptable salts include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate,

formate, fumarate, glucoheptonate, glycerophosphate, gluconate, hemisulfate, heptanoate, hexanoate, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like. Salts derived from appropriate bases include alkali metal, alkaline earth metal, ammonium and $N^+(C_{1-4} \text{ alkyl})_4$ salts. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like. Further pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations formed using counter ions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, sulfonate and aryl sulfonate. Further pharmaceutically acceptable salts include salts formed from the quaternization of an amine using an appropriate electrophile, e.g., an alkyl halide, to form a quaternized alkylated amino salt.

[0083] *Precipitation:* As used herein, the term “precipitation” (or any grammatical equivalent thereof) refers to the formation of a solid in a solution. When used in connection with mRNA, the term “precipitation” refers to the formation of insoluble or solid form of mRNA in a liquid.

[0084] *Prematurely aborted RNA sequences:* The terms “prematurely aborted RNA sequences”, “short abortive RNA species”, “shortmers”, and “long abortive RNA species” as used herein, refers to incomplete products of an mRNA synthesis reaction (e.g., an *in vitro* synthesis reaction). For a variety of reasons, RNA polymerases do not always complete transcription of a DNA template; e.g., RNA synthesis terminates prematurely. Possible causes of premature termination of RNA synthesis include quality of the DNA template, polymerase terminator sequences for a particular polymerase present in the template, degraded buffers, temperature, depletion of ribonucleotides, and mRNA secondary structures. Prematurely aborted RNA sequences may be any length that is less than the intended length of the desired transcriptional product. For example, prematurely aborted mRNA sequences may be less than 1000 bases, less than 500 bases, less than 100 bases, less than 50 bases, less than 40 bases, less than 30 bases, less than 20 bases, less than 15 bases, less than 10 bases or fewer.

[0085] *Salt:* As used herein the term “salt” refers to an ionic compound that does or may result from a neutralization reaction between an acid and a base.

[0086] *Systemic distribution or delivery:* As used herein, the terms “systemic distribution,” “systemic delivery,” or grammatical equivalent, refer to a delivery or distribution mechanism or approach that affect the entire body or an entire organism. Typically, systemic distribution or delivery is accomplished via body’s circulation system, *e.g.*, blood stream. Compared to the definition of “local distribution or delivery.”

[0087] *Subject:* As used herein, the term “subject” refers to a human or any non-human animal (*e.g.*, mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate). A human includes pre- and post-natal forms. In many embodiments, a subject is a human being. A subject can be a patient, which refers to a human presenting to a medical provider for diagnosis or treatment of a disease. The term “subject” is used herein interchangeably with “individual” or “patient.” A subject can be afflicted with or is susceptible to a disease or disorder but may or may not display symptoms of the disease or disorder.

[0088] *Substantially:* As used herein, the term “substantially” refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term “substantially” is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0089] *Substantially free:* As used herein, the term “substantially free” refers to a state in which relatively little or no amount of a substance to be removed (*e.g.*, prematurely aborted RNA sequences) are present. For example, “substantially free of prematurely aborted RNA sequences” means the prematurely aborted RNA sequences are present at a level less than approximately 5%, 4%, 3%, 2%, 1.0%, 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1% or less (w/w) of the impurity. Alternatively, “substantially free of prematurely aborted RNA sequences” means the prematurely aborted RNA sequences are present at a level less than about 100 ng, 90 ng, 80 ng, 70 ng, 60 ng, 50 ng, 40 ng, 30 ng, 20 ng, 10 ng, 1 ng, 500 pg, 100 pg, 50 pg, 10 pg, or less.

[0090] *Target tissues:* As used herein, the term “target tissues” refers to any tissue that is affected by a disease to be treated. In some embodiments, target tissues include those tissues that display disease-associated pathology, symptom, or feature.

[0091] *Therapeutically effective amount:* As used herein, the term “therapeutically effective amount” of a therapeutic agent means an amount that is sufficient, when administered to a subject suffering from or susceptible to a disease, disorder, and/or condition, to treat,

diagnose, prevent, and/or delay the onset of the symptom(s) of the disease, disorder, and/or condition. It will be appreciated by those of ordinary skill in the art that a therapeutically effective amount is typically administered via a dosing regimen comprising at least one unit dose.

[0092] *Treating:* As used herein, the term “treat,” “treatment,” or “treating” refers to any method used to partially or completely alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of and/or reduce incidence of one or more symptoms or features of a particular disease, disorder, and/or condition. Treatment may be administered to a subject who does not exhibit signs of a disease and/or exhibits only early signs of the disease for the purpose of decreasing the risk of developing pathology associated with the disease.

[0093] *Yield:* As used herein, the term “yield” refers to the percentage of mRNA recovered after encapsulation as compared to the total mRNA as starting material. In some embodiments, the term “recovery” is used interchangeably with the term “yield”.

[0094] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this application belongs and as commonly used in the art to which this application belongs; such art is incorporated by reference in its entirety. In the case of conflict, the present Specification, including definitions, will control.

DETAILED DESCRIPTION

[0095] The present invention provides, among other things, methods and compositions for formulations comprising mRNA encapsulated in lipid nanoparticles without the use of ethanol or other flammable solvents in the formulation. Accordingly, this disclosure provides methods of making and using stable, safe, cost-effective ethanol-free LNP formulations that have a high mRNA encapsulation efficiency for efficient mRNA delivery for therapeutic use.

[0096] Various aspects of the invention are described in detail in the following sections. The use of sections is not meant to limit the invention. Each section can apply to any aspect of the invention. In this application, the use of “or” means “and/or” unless stated otherwise.

Liposomes Encapsulating mRNA (mRNA-LNP)

[0097] The method of encapsulating mRNA into lipid nanoparticles disclosed herein can be applied to various techniques, which are presently known in the art. Various methods are

described in published U.S. Application No. US 2011/0244026, published U.S. Application No. US 2016/0038432, published U.S. Application No. US 2018/0153822, published U.S. Application No. US 2018/0125989 and U.S. Provisional Application No. 62/877,597, filed July 23, 2019 and can be used to practice the present invention, all of which are incorporated herein by reference. A conventional method of encapsulating mRNA comprises mixing mRNA with a mixture of lipids, without first pre-forming the lipids into lipid nanoparticles, as described in US 2016/0038432, also known as Process A. Alternatively, another process of encapsulating messenger RNA (mRNA) by mixing pre-formed lipid nanoparticles with mRNA, as described in US 2018/0153822, is known as Process B.

[0098] For the delivery of nucleic acids, achieving high encapsulation efficiencies is important to protect the drug substance (e.g., mRNA) and reduce loss of activity *in vivo*. Thus, enhancement of expression of a protein or peptide encoded by the mRNA and its therapeutic effect is highly correlated with mRNA encapsulation efficiency.

[0099] To achieve high encapsulation efficiency using Process A, the process typically includes heating or applying heat to one or more of the solutions in 10 mM citrate buffer to achieve or maintain a temperature greater than ambient temperature. As described in a published U.S. Application No. US 2016/0038432, heating one or more solutions increases mRNA encapsulation efficiency and recovery rate. Furthermore, Process A typically includes 10-100 mM citrate as a buffer in mRNA and/or lipid solutions. Alternatively, high encapsulation rate can be achieved in a process without heating the mRNA and/or the lipid solutions prior to mixing, by using low concentration of citrate (i.e., ≤ 5 mM) in the mRNA solution.

mRNA Solution

[0100] Various methods may be used to prepare an mRNA solution suitable for the present invention. In some embodiments, mRNA may be directly dissolved in a buffer solution described herein. In some embodiments, an mRNA solution may be generated by mixing an mRNA stock solution with a buffer solution prior to mixing with a lipid solution for encapsulation. In some embodiments, an mRNA solution may be generated by mixing an mRNA stock solution with a buffer solution immediately before mixing with a lipid solution for encapsulation. In some embodiments, a suitable mRNA stock solution may contain mRNA in water at a concentration at or greater than about 0.2 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1.0 mg/ml, 1.2 mg/ml, 1.4 mg/ml, 1.5 mg/ml, or 1.6 mg/ml, 2.0 mg/ml, 2.5 mg/ml, 3.0 mg/ml, 3.5 mg/ml, 4.0 mg/ml, 4.5 mg/ml, or 5.0 mg/ml. In some embodiments, a suitable mRNA stock solution contains

the mRNA at a concentration at or greater than about 1 mg/ml, about 10 mg/ml, about 50 mg/ml, or about 100 mg/ml. In some embodiments, the mRNA stock solution contains mRNA in water at a concentration of between about 0.05 mg/mL and about 0.5 mg/mL. In particular embodiments, the mRNA stock solution contains mRNA in water at a concentration of about 0.1 mg/mL to about 0.5 mg/mL, for example about 0.1 mg/mL or about 0.35 mg/mL.

[0101] Typically, a suitable mRNA solution may also contain a buffering agent and/or salt. Generally, buffering agents can include HEPES, ammonium sulfate, sodium bicarbonate, sodium citrate, sodium acetate, potassium phosphate and sodium phosphate. In some embodiments, suitable concentration of the buffering agent may range from about 0.1 mM to 100 mM, 0.5 mM to 90 mM, 1.0 mM to 80 mM, 2 mM to 70 mM, 3 mM to 60 mM, 4 mM to 50 mM, 5 mM to 40 mM, 6 mM to 30 mM, 7 mM to 20 mM, 8 mM to 15 mM, or 9 to 12 mM. In some embodiments, suitable concentrations of the buffering agent may range from 2.0 mM to 4.0 mM.

[0102] In some embodiments, a buffer solution comprises less than about 5 mM of citrate. In some embodiments, a buffer solution comprises less than about 3 mM of citrate. In some embodiments, a buffer solution comprises less than about 1 mM of citrate. In some embodiments, a buffer solution comprises less than about 0.5 mM of citrate. In some embodiments, a buffer solution comprises less than about 0.25 mM of citrate. In some embodiments, a buffer solution comprises less than about 0.1 mM of citrate. In some embodiments, a buffer solution does not comprise citrate.

[0103] Exemplary salts can include sodium chloride, magnesium chloride, and potassium chloride. In some embodiments, suitable concentration of salts in an mRNA solution may range from about 1 mM to 500 mM, 5 mM to 400 mM, 10 mM to 350 mM, 15 mM to 300 mM, 20 mM to 250 mM, 30 mM to 200 mM, 40 mM to 190 mM, 50 mM to 180 mM, 50 mM to 170 mM, 50 mM to 160 mM, 50 mM to 150 mM, or 50 mM to 100 mM. Salt concentration in a suitable mRNA solution is or greater than about 1 mM, 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, or 100 mM.

[0104] In some embodiments, a buffer solution comprises about 300 mM NaCl. In some embodiments, a buffer solution comprises about 200 mM NaCl. In some embodiments, a buffer solution comprises about 175 mM NaCl. In some embodiments, a buffer solution comprises about 150 mM NaCl. In some embodiments, a buffer solution comprises about 100 mM NaCl. In some embodiments, a buffer solution comprises about 75 mM NaCl. In some embodiments, a buffer

solution comprises about 50 mM NaCl. In some embodiments, a buffer solution comprises about 25 mM NaCl.

[0105] In some embodiments, a suitable mRNA solution may have a pH ranging from about 3.5-6.5, 3.5-6.0, 3.5-5.5, 3.5-5.0, 3.5-4.5, 4.0-5.5, 4.0-5.0, 4.0-4.9, 4.0-4.8, 4.0-4.7, 4.0-4.6, or 4.0-4.5. In some embodiments, a suitable mRNA solution may have a pH of or no greater than about 3.5, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5.0, 5.2, 5.4, 5.6, 5.8, 6.0, 6.1, 6.3, and 6.5.

[0106] In some embodiments, a buffer solution has a pH of about 5.0. In some embodiments, a buffer solution has a pH of about 4.8. In some embodiments, a buffer solution has a pH of about 4.7. In some embodiments, a buffer solution has a pH of about 4.6. In some embodiments, a buffer solution has a pH of about 4.5. In some embodiments, a buffer solution has a pH of about 4.4. In some embodiments, a buffer solution has a pH of about 4.3. In some embodiments, a buffer solution has a pH of about 4.2. In some embodiments, a buffer solution has a pH of about 4.1. In some embodiments, a buffer solution has a pH of about 4.0. In some embodiments, a buffer solution has a pH of about 3.9. In some embodiments, a buffer solution has a pH of about 3.8. In some embodiments, a buffer solution has a pH of about 3.7. In some embodiments, a buffer solution has a pH of about 3.6. In some embodiments, a buffer solution has a pH of about 3.5. In some embodiments, a buffer solution has a pH of about 3.4.

[0107] In some embodiments, an mRNA stock solution is mixed with a buffer solution using a pump. Exemplary pumps include but are not limited to pulse-less flow pumps, gear pumps, peristaltic pumps and centrifugal pumps.

[0108] Typically, the buffer solution is mixed at a rate greater than that of the mRNA stock solution. For example, the buffer solution may be mixed at a rate at least 1x, 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x, 15x, or 20x greater than the rate of the mRNA stock solution. In some embodiments, a buffer solution is mixed at a flow rate ranging between about 100-6000 ml/minute (e.g., about 100-300 ml/minute, 300-600 ml/minute, 600-1200 ml/minute, 1200-2400 ml/minute, 2400-3600 ml/minute, 3600-4800 ml/minute, 4800-6000 ml/minute, or 60-420 ml/minute). In some embodiments, a buffer solution is mixed at a flow rate of or greater than about 60 ml/minute, 100 ml/minute, 140 ml/minute, 180 ml/minute, 220 ml/minute, 260 ml/minute, 300 ml/minute, 340 ml/minute, 380 ml/minute, 420 ml/minute, 480 ml/minute, 540 ml/minute, 600 ml/minute, 1200 ml/minute, 2400 ml/minute, 3600 ml/minute, 4800 ml/minute, or 6000 ml/minute.

[0109] In some embodiments, an mRNA stock solution is mixed at a flow rate ranging between about 10-600 ml/minute (e.g., about 5-50 ml/minute, about 10-30 ml/minute, about 30-60 ml/minute, about 60-120 ml/minute, about 120-240 ml/minute, about 240-360 ml/minute, about 360-480 ml/minute, or about 480-600 ml/minute). In some embodiments, an mRNA stock solution is mixed at a flow rate of or greater than about 5 ml/minute, 10 ml/minute, 15 ml/minute, 20 ml/minute, 25 ml/minute, 30 ml/minute, 35 ml/minute, 40 ml/minute, 45 ml/minute, 50 ml/minute, 60 ml/minute, 80 ml/minute, 100 ml/minute, 200 ml/minute, 300 ml/minute, 400 ml/minute, 500 ml/minute, or 600 ml/minute.

[0110] In some embodiments, the mRNA stock solution is mixed at a flow rate ranging between about 10-30 ml/minute, about 30-60 ml/minute, about 60-120 ml/minute, about 120-240 ml/minute, about 240-360 ml/minute, about 360-480 ml/minute, or about 480-600 ml/minute. In some embodiments, the mRNA stock solution is mixed at a flow rate of about 20 ml/minute, about 40 ml/minute, about 60 ml/minute, about 80 ml/minute, about 100 ml/minute, about 200 ml/minute, about 300 ml/minute, about 400 ml/minute, about 500 ml/minute, or about 600 ml/minute.

[0111] In some embodiments, an mRNA solution is at an ambient temperature. In some embodiments, an mRNA solution is at a temperature of about 20-25 °C. In some embodiments, an mRNA solution is at a temperature of about 21-23 °C. In some embodiments, an mRNA solution is not heated prior mixing with a lipid solution. In some embodiments, an mRNA solution is kept at an ambient temperature.

Lipid Solution

[0112] According to the present invention, a lipid solution contains a mixture of lipids suitable to form lipid nanoparticles for encapsulation of mRNA. According to the present invention, in some embodiments, a suitable lipid solution does not contain ethanol, isopropanol, or any other flammable organic solvent.

[0113] A suitable lipid solution may contain a mixture of desired lipids at various concentrations. For example, a suitable lipid solution may contain a mixture of desired lipids at a total concentration of or greater than about 0.1 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml, 3.0 mg/ml, 4.0 mg/ml, 5.0 mg/ml, 6.0 mg/ml, 7.0 mg/ml, 8.0 mg/ml, 9.0 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, 50 mg/ml, or 100 mg/ml. In some embodiments, a suitable lipid solution may contain a mixture of desired lipids at a total concentration ranging from about 0.1-100 mg/ml, 0.5-90 mg/ml, 1.0-80 mg/ml, 1.0-70 mg/ml, 1.0-60 mg/ml, 1.0-50 mg/ml, 1.0-40

mg/ml, 1.0-30 mg/ml, 1.0-20 mg/ml, 1.0-15 mg/ml, 1.0-10 mg/ml, 1.0-9 mg/ml, 1.0-8 mg/ml, 1.0-7 mg/ml, 1.0-6 mg/ml, or 1.0-5 mg/ml. In some embodiments, a suitable lipid solution may contain a mixture of desired lipids at a total concentration up to about 100 mg/ml, 90 mg/ml, 80 mg/ml, 70 mg/ml, 60 mg/ml, 50 mg/ml, 40 mg/ml, 30 mg/ml, 20 mg/ml, or 10 mg/ml.

[0114] Any desired lipids may be mixed at any ratios suitable for encapsulating mRNAs. In some embodiments, a suitable lipid solution contains a mixture of desired lipids including cationic lipids, helper lipids (e.g. non cationic lipids and/or cholesterol lipids), amphiphilic block copolymers (e.g. poloxamers) and/or PEGylated lipids. In some embodiments, a suitable lipid solution contains a mixture of desired lipids including one or more cationic lipids, one or more helper lipids (e.g. non cationic lipids and/or cholesterol lipids) and one or more PEGylated lipids. In some embodiments, the lipid solution comprises three lipid components. In some embodiments, the lipid solution comprises four lipid components. In particular embodiments, the three or four lipid components of the lipid solution are a PEG-modified lipid, a cationic lipid (e.g. ML-2 or MC-3), a helper (e.g. non-cationic) lipid (e.g. DSPC or DOPE), and optionally cholesterol.

[0115] In some embodiments, a lipid solution is at an ambient temperature. In some embodiments, a lipid solution is at a temperature of about 20-25 °C. In some embodiments, a lipid solution is at a temperature of about 21-23 °C. In some embodiments, a lipid solution is not heated prior mixing with a lipid solution. In some embodiments, a lipid solution is kept at an ambient temperature.

[0116] In certain embodiments, provided compositions comprise a liposome wherein the mRNA is associated on both the surface of the liposome and encapsulated within the same liposome. For example, during preparation of the compositions of the present invention, cationic liposomes may associate with the mRNA through electrostatic interactions.

[0117] In some embodiments, the compositions and methods of the invention comprise mRNA encapsulated in a liposome. In some embodiments, the one or more mRNA species may be encapsulated in the same liposome. In some embodiments, the one or more mRNA species may be encapsulated in different liposomes. In some embodiments, the mRNA is encapsulated in one or more liposomes, which differ in their lipid composition, molar ratio of lipid components, size, charge (zeta potential), targeting ligands and/or combinations thereof. In some embodiments, the one or more liposome may have a different composition of sterol-based cationic lipids, neutral lipid, PEG-modified lipid and/or combinations thereof. In some embodiments the one or more

liposomes may have a different molar ratio of cholesterol-based cationic lipid, neutral lipid, and PEG-modified lipid used to create the liposome.

Process of Encapsulation

[0118] As used herein, a process for formation of mRNA-loaded lipid nanoparticles (mRNA-LNPs) is used interchangeably with the term “mRNA encapsulation” or grammatical variants thereof. In some embodiments, mRNA-LNPs are formed by mixing an mRNA solution with a lipid solution, wherein the mRNA solution and/or the lipid solution are kept at ambient temperature prior to mixing.

[0119] In some embodiments, an mRNA solution and a lipid solution are mixed into a solution such that the mRNA becomes encapsulated in the lipid nanoparticle. Such a solution is also referred to as a formulation or encapsulation solution.

[0120] In some embodiments, for example, an LNP formulation without ethanol according to the present invention may be compared to a conventional ethanol LNP formulation or encapsulation solution that includes a solvent such as ethanol. In previous LNP formulations which used ethanol as a solvent, the formulation comprised ethanol at about 10%-40% volume. Other previous LNP formulations used isopropyl alcohol as a solvent at about 10% to about 40% volume. In contrast, in some embodiments, the instant invention provides a method of LNP encapsulation that does not comprise flammable solvents.

[0121] Accordingly, in some embodiments, a suitable formulation or encapsulation solution of the present invention does not include a flammable solvent. In some embodiments, a suitable formulation or encapsulation solution does not include ethanol.

[0122] In some embodiments, a suitable formulation or encapsulation solution may also contain a buffering agent or salt. Exemplary buffering agent may include HEPES, ammonium sulfate, sodium bicarbonate, sodium citrate, sodium acetate, potassium phosphate and sodium phosphate. Exemplary salt may include sodium chloride, magnesium chloride, and potassium chloride.

[0123] In some embodiments, ethanol, citrate buffer, and other destabilizing agents are absent during the addition of mRNA and hence the formulation does not require any further downstream processing. In some embodiments, the formulation solution comprises trehalose. The lack of destabilizing agents and the stability of trehalose solution increase the ease of scaling up the formulation and production of mRNA-encapsulated lipid nanoparticles.

[0124] In some embodiments, the lipid solution contains one or more cationic lipids, one or more non-cationic lipids, and one or more PEG lipids. In some embodiments, the lipids also contain one or more cholesterol lipids.

[0125] In some embodiments, the lipid and mRNA solutions are mixed using a pump system. In some embodiments, the pump system comprises a pulse-less flow pump. In some embodiments, the pump system is a gear pump. In some embodiments, a suitable pump is a peristaltic pump. In some embodiments, a suitable pump is a centrifugal pump. In some embodiments, the process using a pump system is performed at large scale. For example, in some embodiments, the process includes using pumps as described herein to mix a solution of at least about 1 mg, 5 mg, 10 mg, 50 mg, 100 mg, 500 mg, 1 g, 10 g, 50 g, or 100 g or more of mRNA with a lipid solution, to produce mRNA encapsulated in lipid nanoparticles. In some embodiments, the process of mixing mRNA and lipid solutions provides a composition according to the present invention that contains at least about 1 mg, 5mg, 10 mg, 50 mg, 100 mg, 500 mg, 1 g, 10 g, 50 g, or 100 g or more of encapsulated mRNA.

[0126] In some embodiments, a step of combining lipid nanoparticles encapsulating mRNA with a lipid solution is performed using a pump system. Such combining may be performed using a pump. In some embodiments, the mRNA and lipid solutions are mixed are mixed at a flow rate ranging from about 25-75 ml/minute, about 75-200 ml/minute, about 200-350 ml/minute, about 350-500 ml/minute, about 500-650 ml/minute, about 650-850 ml/minute, or about 850-1000 ml/minute. In some embodiments, an mRNA solution and a lipid solution are mixed at a flow rate of about 50 ml/minute, about 100 ml/minute, about 150 ml/minute, about 200 ml/minute, about 250 ml/minute, about 300 ml/minute, about 350 ml/minute, about 400 ml/minute, about 450 ml/minute, about 500 ml/minute, about 550 ml/minute, about 600 ml/minute, about 650 ml/minute, about 700 ml/minute, about 750 ml/minute, about 800 ml/minute, about 850 ml/minute, about 900 ml/minute, about 950 ml/minute, or about 1000 ml/minute.

[0127] In some embodiments, the mixing of an mRNA solution with a lipid solution is performed in absence of any pump.

[0128] In some embodiments, the process according to the present invention includes maintaining at ambient temperature (i.e., not applying heat from a heat source to the solution) one or more of the solution comprising the lipids, the solution comprising the mRNA and the mixed solution comprising the lipid nanoparticle encapsulated mRNA. In some embodiments, the process includes the step of maintaining at ambient temperature one or both of the mRNA

solution and the lipid solution, prior to the mixing step. In some embodiments, the process includes maintaining at ambient temperature one or more of the solution comprising the lipids and the solution comprising the mRNA during the mixing step. In some embodiments, the process includes the step of maintaining the lipid nanoparticle encapsulated mRNA at ambient temperature after the mixing step. In some embodiments, the ambient temperature at which one or more of the solutions is maintained is or is less than about 35 °C, 30 °C, 25 °C, 20 °C, or 16 °C. In some embodiments, the ambient temperature at which one or more of the solutions is maintained ranges from about 15-35 °C, about 15-30 °C, about 15-25 °C, about 15-20 °C, about 20-35 °C, about 25-35 °C, about 30-35 °C, about 20-30 °C, about 25-30 °C or about 20-25 °C. In some embodiments, the ambient temperature at which one or more of the solutions is maintained is 20-25 °C.

[0129] In some embodiments, the process according to the present invention includes performing at ambient temperature the step of mixing the mRNA and lipid solutions to form lipid nanoparticles encapsulating mRNA.

[0130] In some embodiments, greater than about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the purified nanoparticles have a size less than about 150 nm (e.g., less than about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm, about 100 nm, about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, or about 50 nm). In some embodiments, substantially all of the purified nanoparticles have a size less than 150 nm (e.g., less than about 145 nm, about 140 nm, about 135 nm, about 130 nm, about 125 nm, about 120 nm, about 115 nm, about 110 nm, about 105 nm, about 100 nm, about 95 nm, about 90 nm, about 85 nm, about 80 nm, about 75 nm, about 70 nm, about 65 nm, about 60 nm, about 55 nm, or about 50 nm). In some embodiments, greater than about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified nanoparticles have a size ranging from 50-150 nm. In some embodiments, substantially all of the purified nanoparticles have a size ranging from 50-150 nm. In some embodiments, greater than about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified nanoparticles have a size ranging from 80-150 nm. In some embodiments, substantially all of the purified nanoparticles have a size ranging from 80-150 nm.

[0131] In some embodiments, a process according to the present invention results in an encapsulation rate of greater than about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%. In some embodiments, a process according to the present invention results in

greater than about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% recovery of mRNA.

[0132] In some embodiments, the mRNA-LNP encapsulation efficiency in a formulation according to the present invention is at the same as the mRNA-LNP encapsulation efficiency in an ethanol LNP formulation.

[0133] In some embodiments, the mRNA-LNP encapsulation efficiency in a formulation according to the present invention is at least 2% higher compared to an ethanol LNP formulation. In some embodiments, the mRNA-LNP encapsulation efficiency in a formulation according to the present invention is at least 4% higher compared to an ethanol LNP formulation. In some embodiments, the mRNA-LNP encapsulation efficiency in a formulation according to the present invention is at least 5% higher compared to an ethanol LNP formulation. In some embodiments, the mRNA-LNP encapsulation efficiency in a formulation according to the present invention is at least 8% higher compared to an ethanol LNP formulation. In some embodiments, the mRNA-LNP encapsulation efficiency in a formulation according to the present invention is at least 10% higher compared to an ethanol LNP formulation. In some embodiments, the mRNA-LNP encapsulation efficiency in a formulation according to the present invention is at least 12% higher compared to an ethanol LNP formulation. In some embodiments, the mRNA-LNP encapsulation efficiency in a formulation according to the present invention is at least 15% higher compared to an ethanol LNP formulation. In some embodiments, the mRNA-LNP encapsulation efficiency in a formulation according to the present invention is at least 20% higher compared to an ethanol LNP formulation.

[0134] In some embodiments, a process according to the present invention comprises a step of incubating the mRNA-LNPs post-mixing. A step of incubating the mRNA-LNPs post-mixing is described in U.S. Provisional Application No. 62/847,837, filed May 14, 2019 and can be used to practice the present invention, all of which are incorporated herein by reference.

Purification

[0135] In some embodiments, the mRNA-LNPs are purified and/or concentrated. Various purification methods may be used. In some embodiments, the mRNA-LNPs are purified by a Tangential Flow Filtration (TFF) process. In some embodiments, the mRNA-LNPs are purified by gravity-based normal flow filtration (NFF). In some embodiments, the mRNA-LNPs are purified by any other suitable filtration process. In some embodiments, the mRNA-LNPs are purified by centrifugation. In some embodiments, the mRNA-LNPs are purified by chromatographic methods.

Delivery Vehicles

[0136] According to the present invention, mRNA encoding a protein or a peptide (*e.g.*, a full length, fragment, or portion of a protein or a peptide) as described herein may be delivered as naked RNA (unpackaged) or via delivery vehicles. As used herein, the terms “delivery vehicle,” “transfer vehicle,” “nanoparticle” or grammatical equivalent, are used interchangeably.

[0137] Delivery vehicles can be formulated in combination with one or more additional nucleic acids, carriers, targeting ligands or stabilizing reagents, or in pharmacological compositions where it is mixed with suitable excipients. For example, liposome encapsulating mRNA can be formed as described above. Techniques for formulation and administration of drugs may be found in “Remington’s Pharmaceutical Sciences,” Mack Publishing Co., Easton, Pa., latest edition. A particular delivery vehicle is selected based upon its ability to facilitate the transfection of a nucleic acid to a target cell.

[0138] In some embodiments, mRNAs encoding at least one protein or peptide may be delivered via a single delivery vehicle. In some embodiments, mRNAs encoding at least one protein or peptide may be delivered via one or more delivery vehicles each of a different composition. In some embodiments, the one or more mRNAs and/or are encapsulated within the same lipid nanoparticles. In some embodiments, the one or more mRNAs are encapsulated within separate lipid nanoparticles. In some embodiments, lipid nanoparticles are empty.

[0139] According to various embodiments, suitable delivery vehicles include, but are not limited to polymer based carriers, such as polyethyleneimine (PEI), lipid nanoparticles and liposomes, nanoliposomes, ceramide-containing nanoliposomes, proteoliposomes, both natural and synthetically-derived exosomes, natural, synthetic and semi-synthetic lamellar bodies, nanoparticulates, calcium phosphor-silicate nanoparticulates, calcium phosphate nanoparticulates, silicon dioxide nanoparticulates, nanocrystalline particulates, semiconductor nanoparticulates, poly(D-arginine), sol-gels, nanodendrimers, starch-based delivery systems, micelles, emulsions, niosomes, multi-domain-block polymers (vinyl polymers, polypropyl acrylic acid polymers, dynamic polyconjugates), dry powder formulations, plasmids, viruses, calcium phosphate nucleotides, aptamers, peptides and other vectorial tags. Also contemplated is the use of bionanocapsules and other viral capsid proteins assemblies as a suitable transfer vehicle. (Hum. Gene Ther. 2008 September; 19(9):887-95).

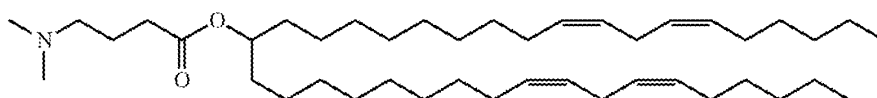
Liposomal delivery vehicles

[0140] In some embodiments, a suitable delivery vehicle is a liposomal delivery vehicle, *e.g.*, a lipid nanoparticle. As used herein, liposomal delivery vehicles, *e.g.*, lipid nanoparticles, are usually characterized as microscopic vesicles having an interior aqua space sequestered from an outer medium by a membrane of one or more bilayers. Bilayer membranes of liposomes are typically formed by amphiphilic molecules, such as lipids of synthetic or natural origin that comprise spatially separated hydrophilic and hydrophobic domains (Lasic, Trends Biotechnol., 16: 307-321, 1998). Bilayer membranes of the liposomes can also be formed by amphiphilic polymers and surfactants (*e.g.*, polymerosomes, niosomes, etc.). In the context of the present invention, a liposomal delivery vehicle typically serves to transport a desired nucleic acid (*e.g.*, mRNA) to a target cell or tissue. In some embodiments, a nanoparticle delivery vehicle is a liposome. In some embodiments, a liposome comprises one or more cationic lipids, one or more non-cationic lipids, one or more cholesterol-based lipids, or one or more PEG-modified lipids. In some embodiments, a liposome comprises no more than three distinct lipid components. In some embodiments, one distinct lipid component is a sterol-based cationic lipid.

Cationic Lipids

[0141] As used herein, the phrase “cationic lipids” refers to any of a number of lipid species that have a net positive charge at a selected pH, such as physiological pH.

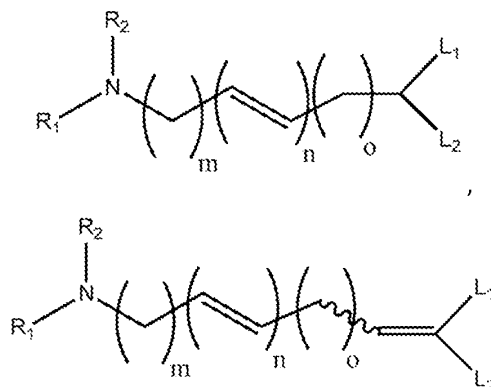
[0142] Suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO 2010/144740, which is incorporated herein by reference. In certain embodiments, the compositions and methods of the present invention include a cationic lipid, (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino) butanoate, having a compound structure of:



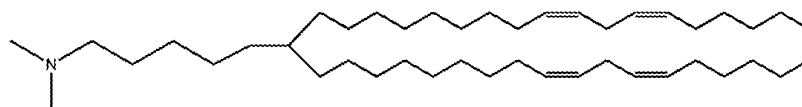
and pharmaceutically acceptable salts thereof.

[0143] Other suitable cationic lipids for use in the compositions and methods of the present invention include ionizable cationic lipids as described in International Patent Publication WO 2013/149140, which is incorporated herein by reference. In some embodiments, the

compositions and methods of the present invention include a cationic lipid of one of the following formulas:

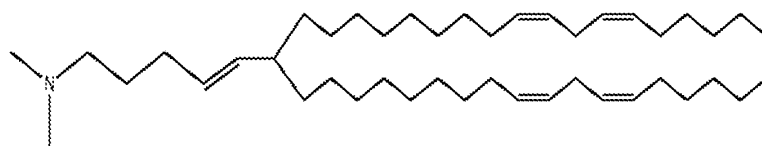


or a pharmaceutically acceptable salt thereof, wherein R_1 and R_2 are each independently selected from the group consisting of hydrogen, an optionally substituted, variably saturated or unsaturated C_1 - C_{20} alkyl and an optionally substituted, variably saturated or unsaturated C_6 - C_{20} acyl; wherein L_1 and L_2 are each independently selected from the group consisting of hydrogen, an optionally substituted C_1 - C_{30} alkyl, an optionally substituted variably unsaturated C_1 - C_{30} alkenyl, and an optionally substituted C_1 - C_{30} alkynyl; wherein m and o are each independently selected from the group consisting of zero and any positive integer (*e.g.*, where m is three); and wherein n is zero or any positive integer (*e.g.*, where n is one). In certain embodiments, the compositions and methods of the present invention include the cationic lipid (15Z, 18Z)-N,N-dimethyl-6-(9Z,12Z)-octadeca-9,12-dien-1-yl) tetracos-15,18-dien-1-amine ("HGT5000"), having a compound structure of:



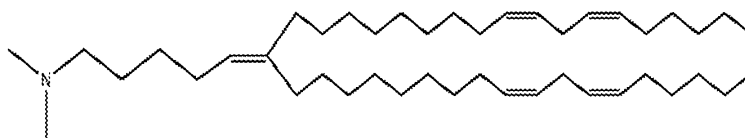
(HGT-5000)

and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include the cationic lipid (15Z, 18Z)-N,N-dimethyl-6-((9Z,12Z)-octadeca-9,12-dien-1-yl) tetracos-4,15,18-trien-1-amine ("HGT5001"), having a compound structure of:



(HGT-5001)

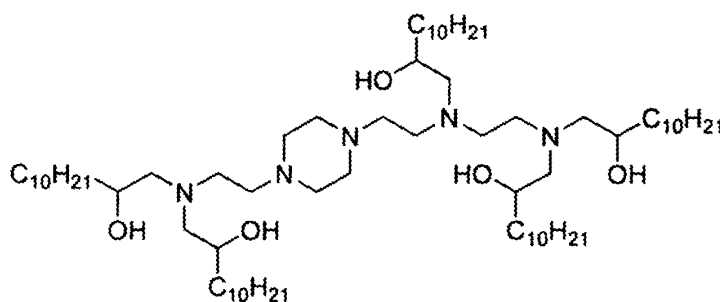
and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include the cationic lipid and (15Z,18Z)-N,N-dimethyl-6-((9Z,12Z)-octadeca-9,12-dien-1-yl) tetracos-5,15,18-trien-1-amine ("HGT5002"), having a compound structure of:



(HGT-5002)

and pharmaceutically acceptable salts thereof.

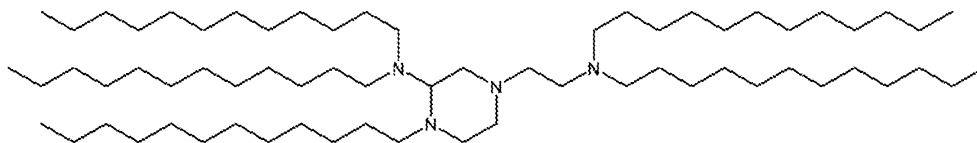
[0144] Other suitable cationic lipids for use in the compositions and methods of the invention include cationic lipids described as aminoalcohol lipidoids in International Patent Publication WO 2010/053572, which is incorporated herein by reference. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:



and pharmaceutically acceptable salts thereof.

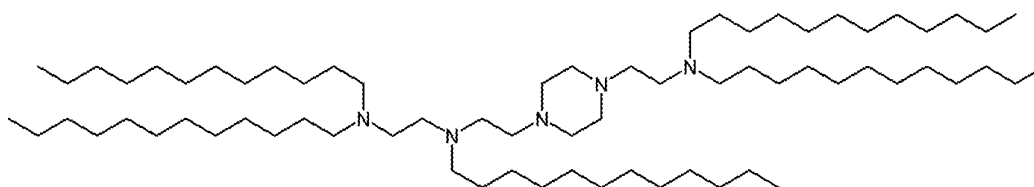
[0145] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO 2016/118725, which is incorporated herein by reference. In certain embodiments, the

compositions and methods of the present invention include a cationic lipid having a compound structure of:



and pharmaceutically acceptable salts thereof.

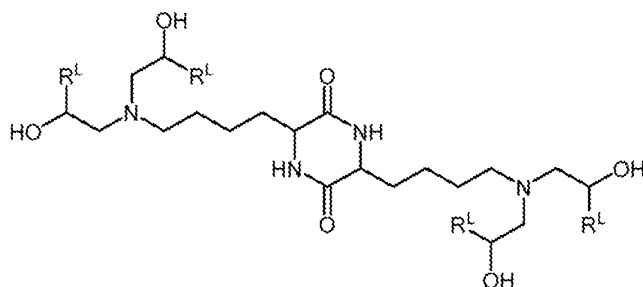
[0146] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO 2016/118724, which is incorporated herein by reference. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:



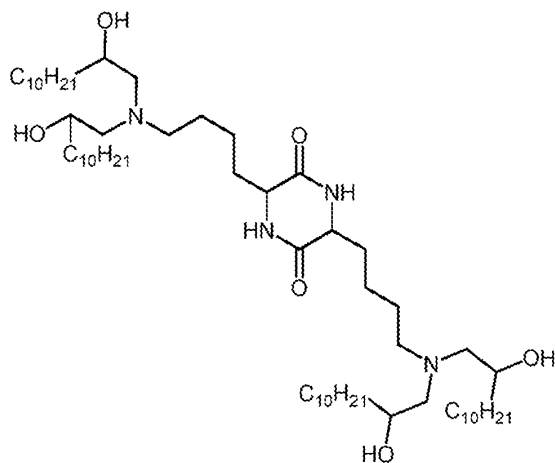
and pharmaceutically acceptable salts thereof.

[0147] Other suitable cationic lipids for use in the compositions and methods of the invention include a cationic lipid having the formula of 14,25-ditridecyl 15,18,21,24-tetraaza-octatriacontane, and pharmaceutically acceptable salts thereof.

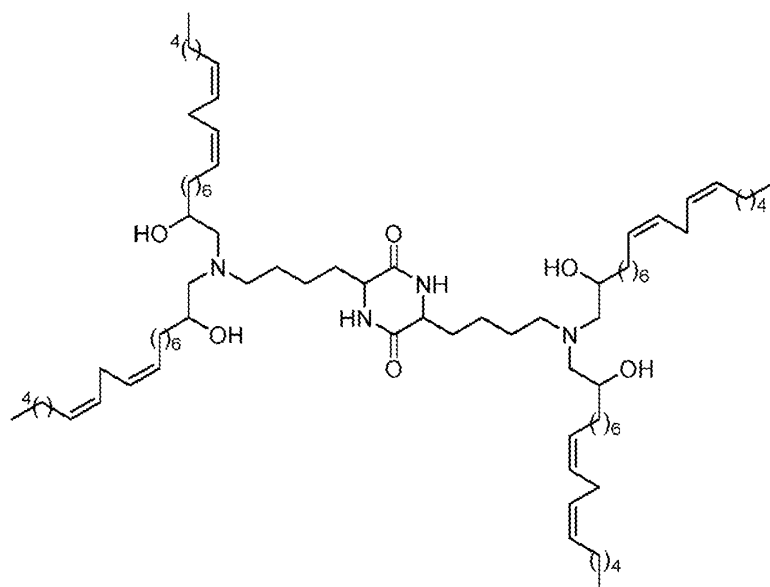
[0148] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publications WO 2013/063468 and WO 2016/205691, each of which are incorporated herein by reference. In some embodiments, the compositions and methods of the present invention include a cationic lipid of the following formula:



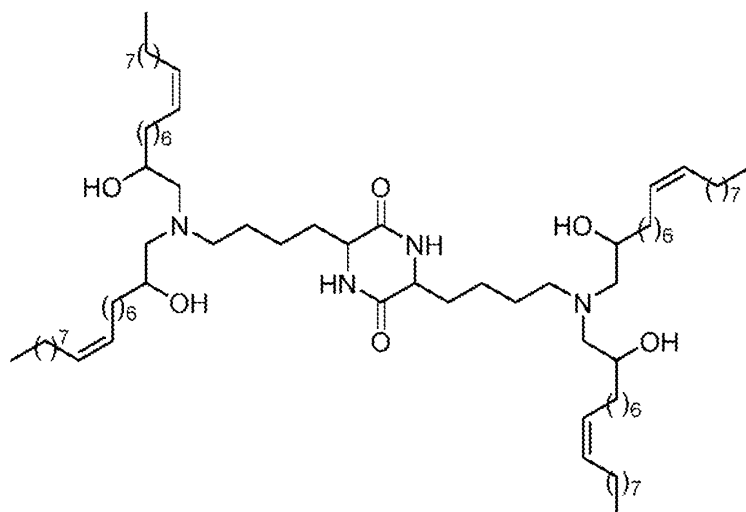
or pharmaceutically acceptable salts thereof, wherein each instance of R^L is independently optionally substituted C_6 - C_{40} alkenyl. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:



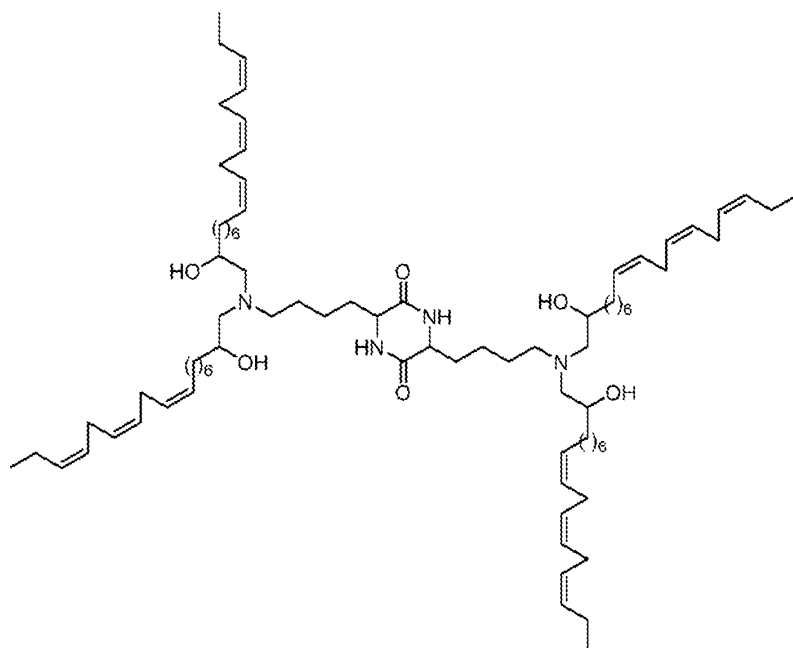
and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:



and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:

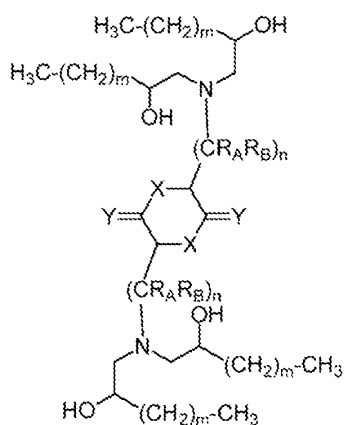


and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:



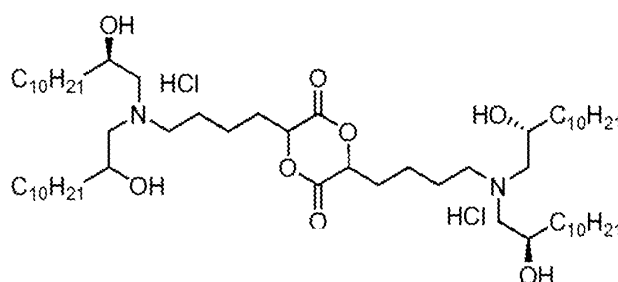
and pharmaceutically acceptable salts thereof.

[0149] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO 2015/184256, which is incorporated herein by reference. In some embodiments, the compositions and methods of the present invention include a cationic lipid of the following formula:



or a pharmaceutically acceptable salt thereof, wherein each X independently is O or S; each Y independently is O or S; each m independently is 0 to 20; each n independently is 1 to 6; each R_A is

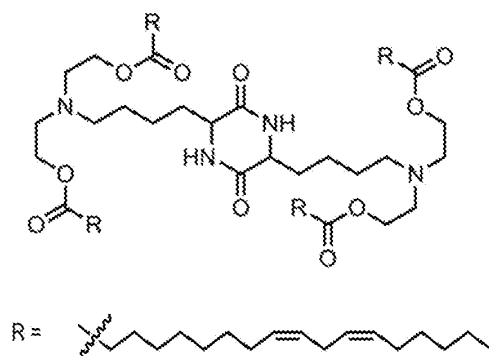
independently hydrogen, optionally substituted C1-50 alkyl, optionally substituted C2-50 alkenyl, optionally substituted C2-50 alkynyl, optionally substituted C3-10 carbocyclyl, optionally substituted 3-14 membered heterocyclyl, optionally substituted C6-14 aryl, optionally substituted 5-14 membered heteroaryl or halogen; and each R_B is independently hydrogen, optionally substituted C1-50 alkyl, optionally substituted C2-50 alkenyl, optionally substituted C2-50 alkynyl, optionally substituted C3-10 carbocyclyl, optionally substituted 3-14 membered heterocyclyl, optionally substituted C6-14 aryl, optionally substituted 5-14 membered heteroaryl or halogen. In certain embodiments, the compositions and methods of the present invention include a cationic lipid, "Target 23", having a compound structure of:



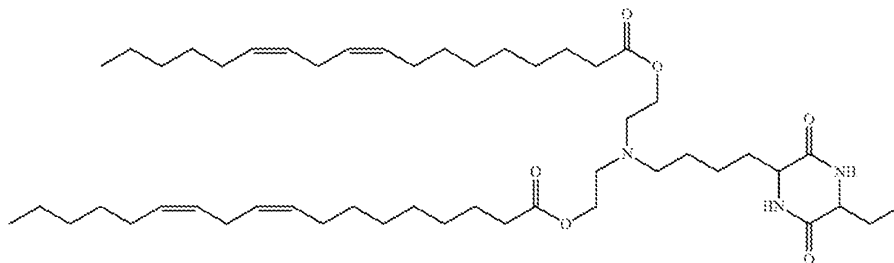
(Target 23)

and pharmaceutically acceptable salts thereof.

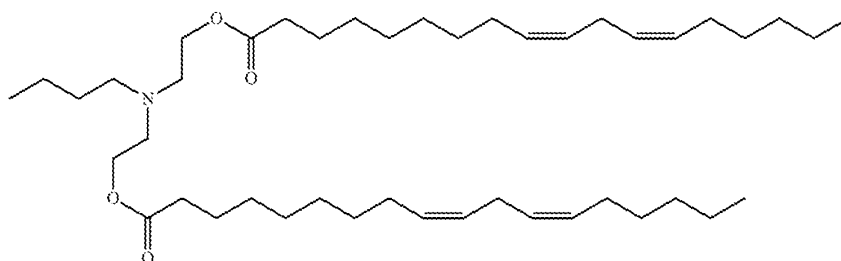
[0150] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO 2016/004202, which is incorporated herein by reference. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



or a pharmaceutically acceptable salt thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:

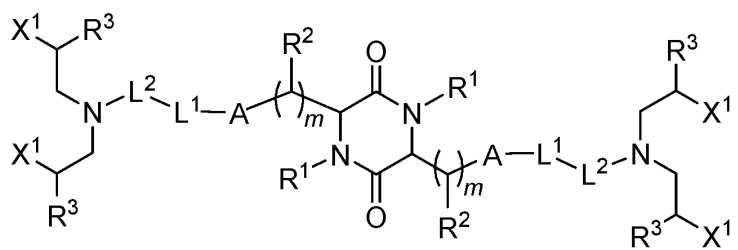


or a pharmaceutically acceptable salt thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:

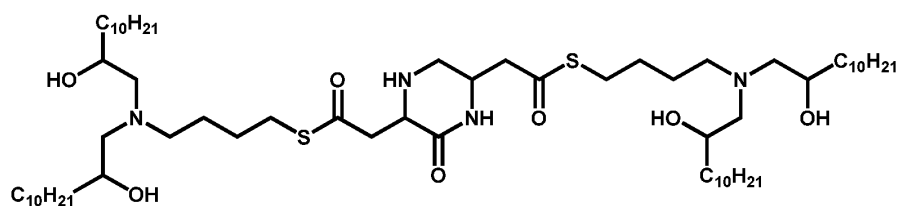


or a pharmaceutically acceptable salt thereof.

[0151] Other suitable cationic lipids for use in the compositions and methods of the present invention include cationic lipids as described in United States Provisional Patent Application Serial Number 62/758,179, which is incorporated herein by reference. In some embodiments, the compositions and methods of the present invention include a cationic lipid of the following formula:

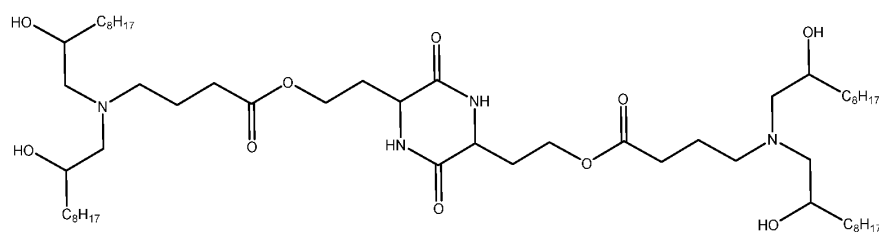


or a pharmaceutically acceptable salt thereof, wherein each R^1 and R^2 is independently H or C_1 - C_6 aliphatic; each m is independently an integer having a value of 1 to 4; each A is independently a covalent bond or arylene; each L^1 is independently an ester, thioester, disulfide, or anhydride group; each L^2 is independently C_2 - C_{10} aliphatic; each X^1 is independently H or OH; and each R^3 is independently C_6 - C_{20} aliphatic. In some embodiments, the compositions and methods of the present invention include a cationic lipid of the following formula:



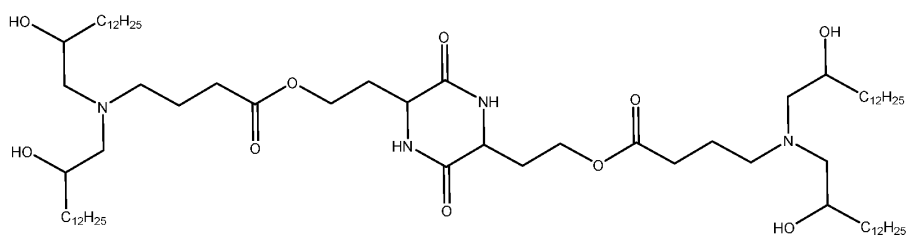
(Compound 1)

or a pharmaceutically acceptable salt thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid of the following formula:



(Compound 2)

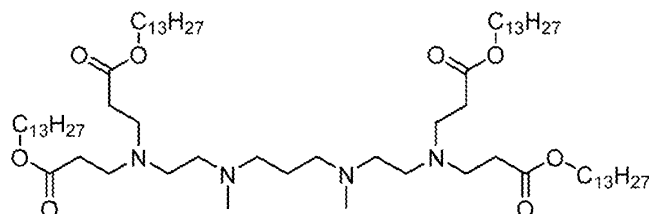
or a pharmaceutically acceptable salt thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid of the following formula:



(Compound 3)

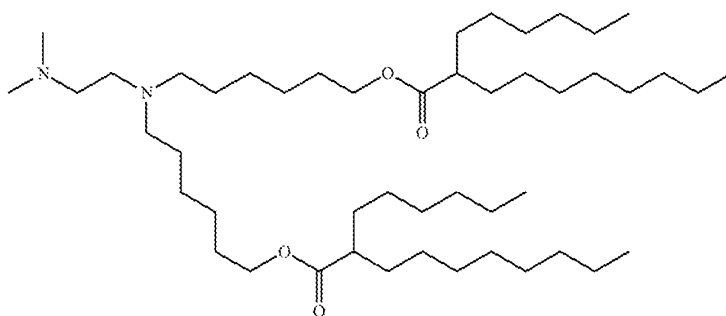
or a pharmaceutically acceptable salt thereof.

[0152] Other suitable cationic lipids for use in the compositions and methods of the present invention include the cationic lipids as described in J. McClellan, M. C. King, Cell 2010, 141, 210-217 and in Whitehead *et al.*, Nature Communications (2014) 5:4277, which is incorporated herein by reference. In certain embodiments, the cationic lipids of the compositions and methods of the present invention include a cationic lipid having a compound structure of:

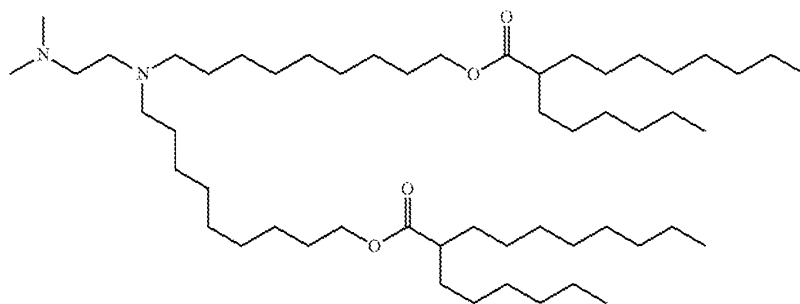


and pharmaceutically acceptable salts thereof.

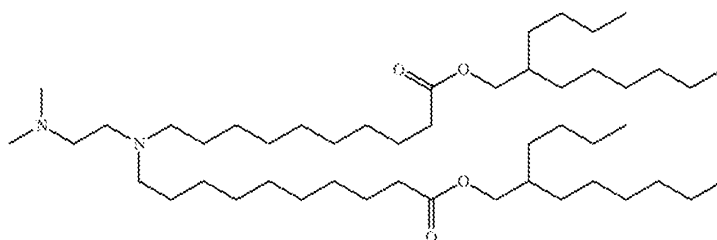
[0153] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO 2015/199952, which is incorporated herein by reference. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



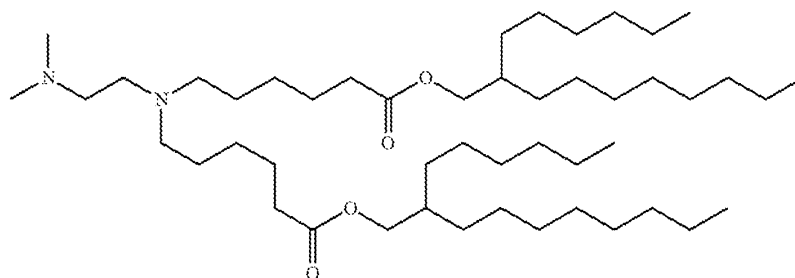
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



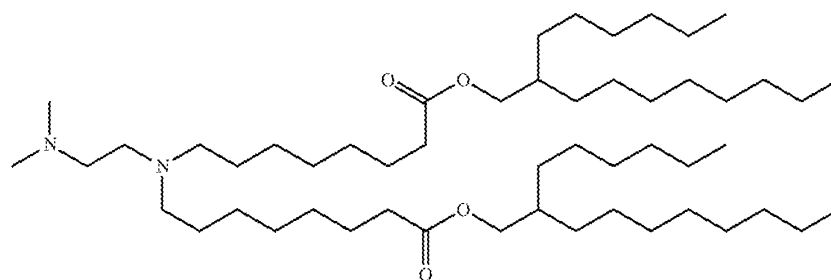
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



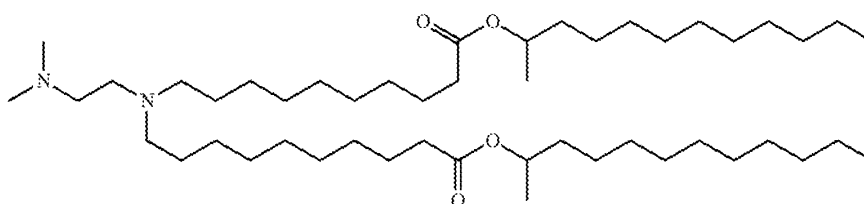
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



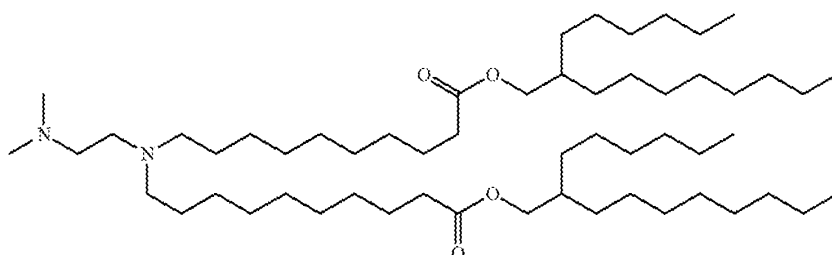
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



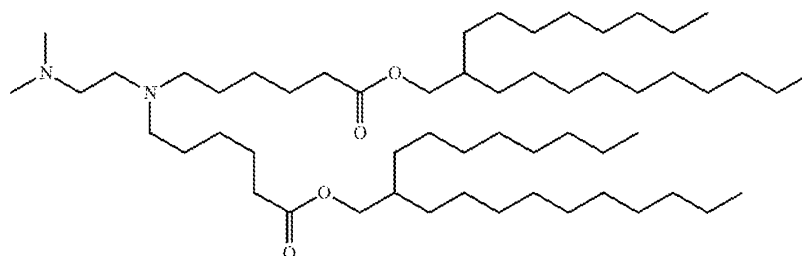
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



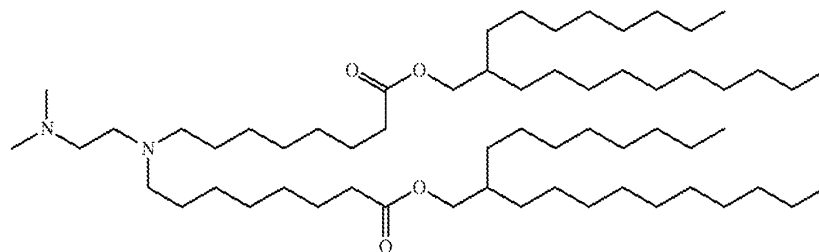
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



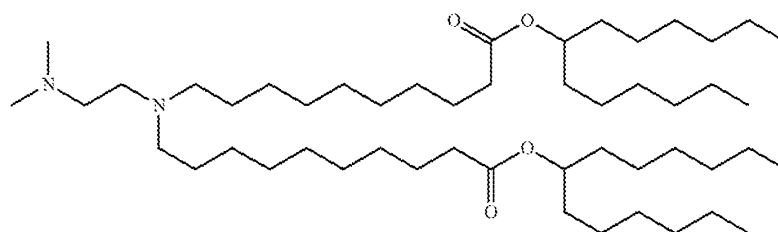
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



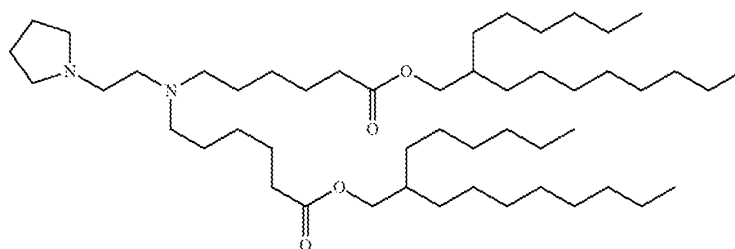
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



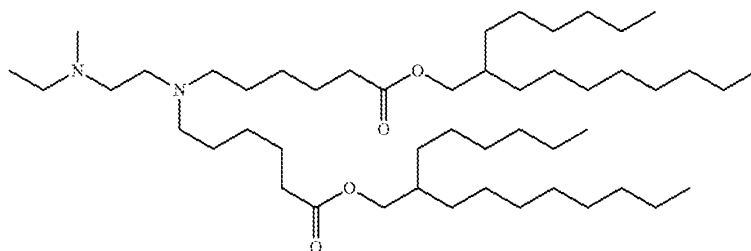
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



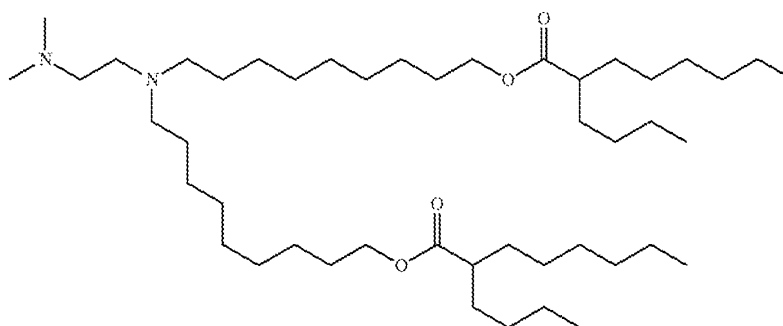
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:

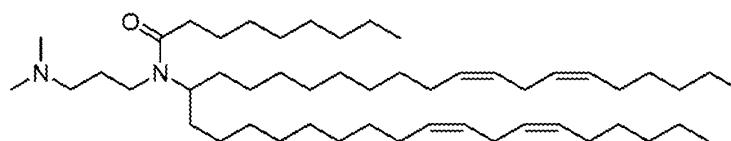


and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:

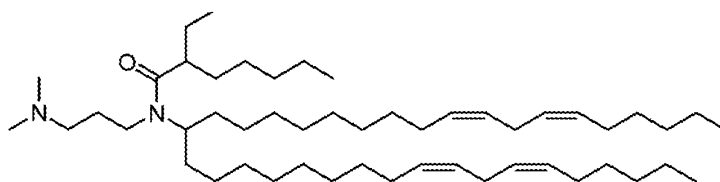


and pharmaceutically acceptable salts thereof.

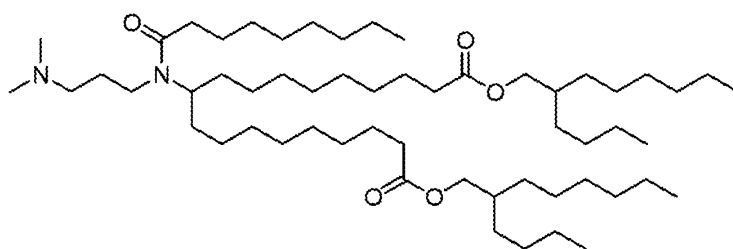
[0154] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO 2017/004143, which is incorporated herein by reference. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



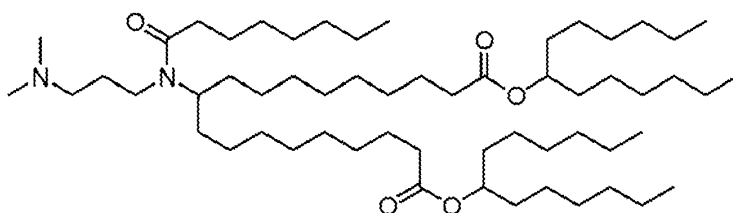
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



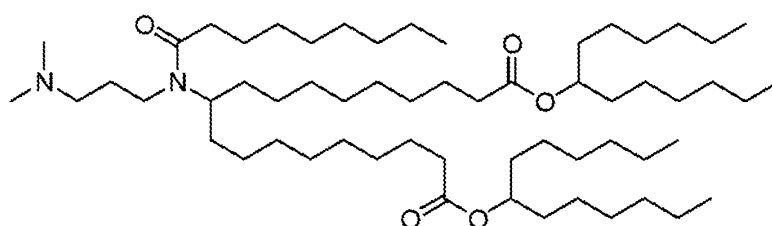
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



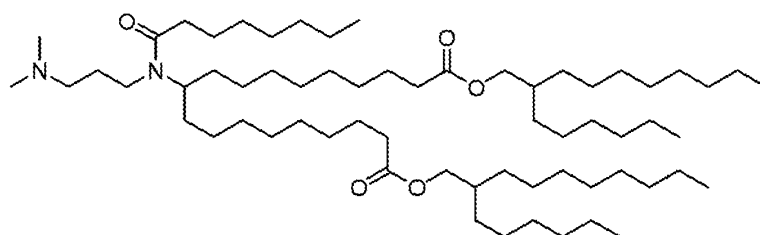
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



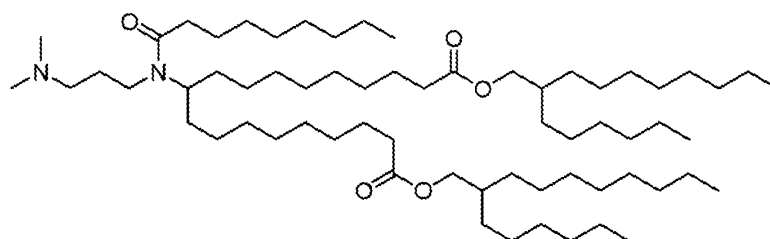
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



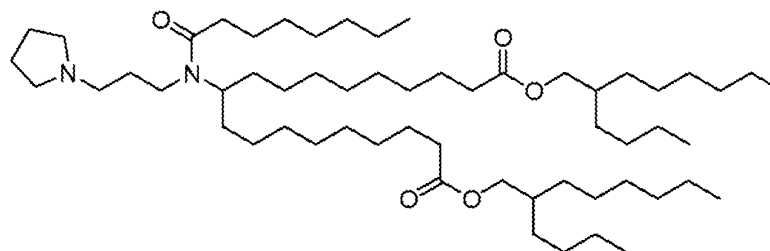
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



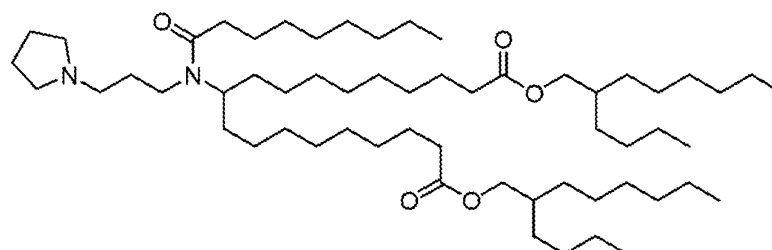
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



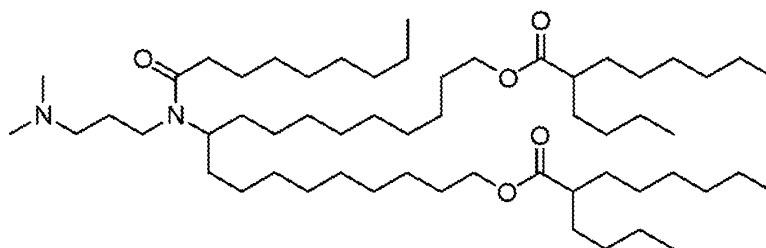
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



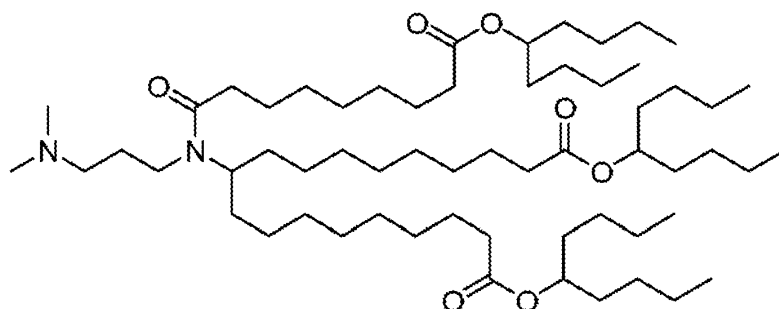
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



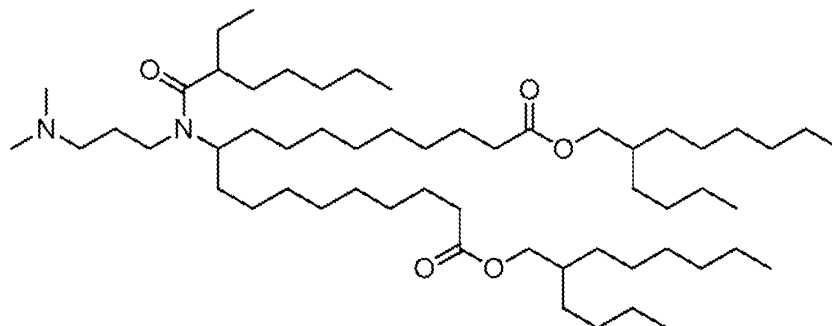
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



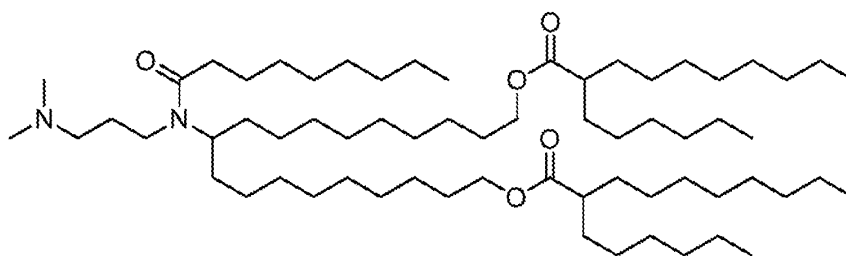
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



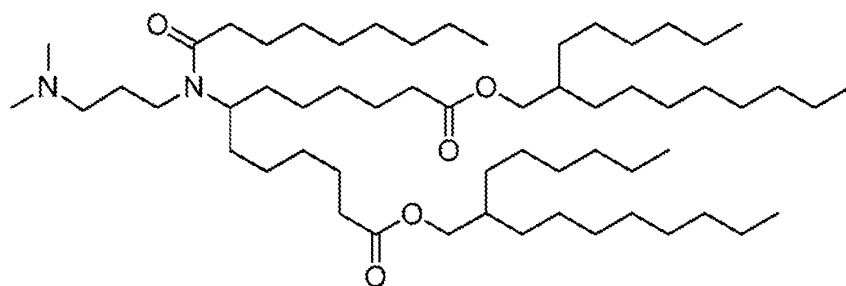
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



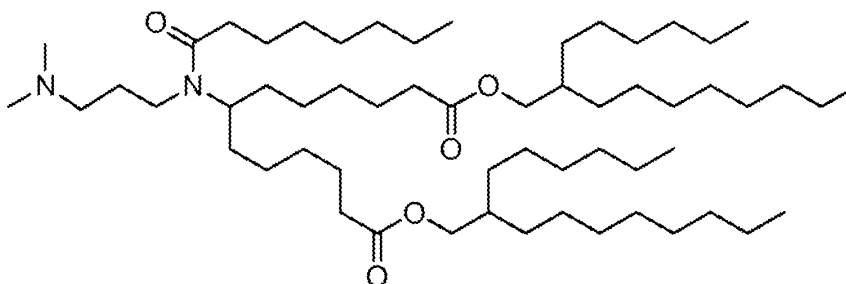
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



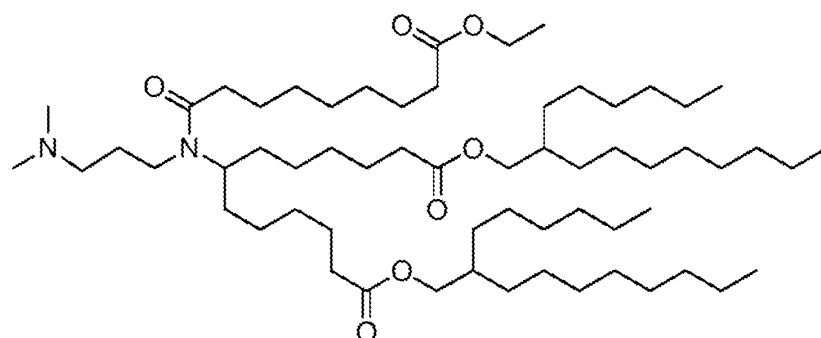
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



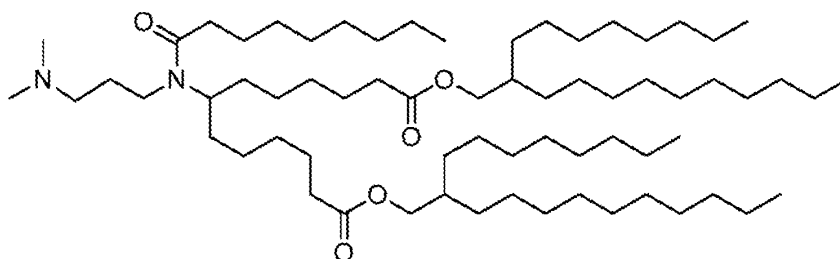
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:

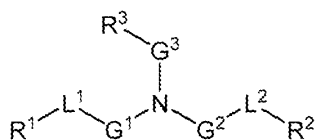


and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



and pharmaceutically acceptable salts thereof.

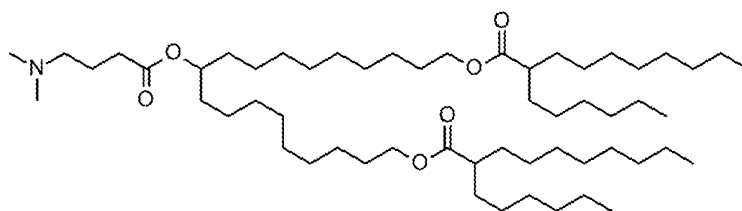
[0155] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO 2017/075531, which is incorporated herein by reference. In some embodiments, the compositions and methods of the present invention include a cationic lipid of the following formula:



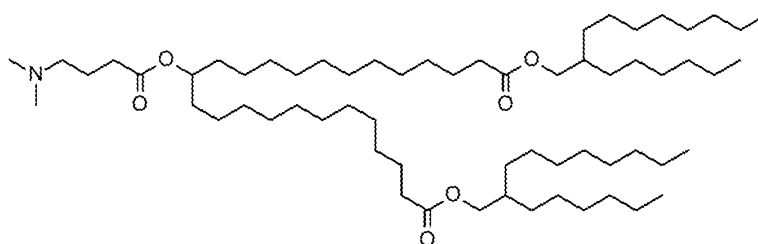
or a pharmaceutically acceptable salt thereof, wherein one of L^1 or L^2 is $-O(C=O)-$, $-(C=O)O-$, $-C(=O)-$, $-O-$, $-S(O)_x$, $-S-S-$, $-C(=O)S-$, $-SC(=O)-$, $-NR^aC(=O)-$, $-C(=O)NR^a$, $NR^aC(=O)NR^a$, $-OC(=O)NR^a$, or $-NR^aC(=O)O-$; and the other of L^1 or L^2 is $-O(C=O)-$, $-(C=O)O-$, $-C(=O)-$, $-O-$, $-S(O)_x$, $-S-S-$, $-C(=O)S-$, $SC(=O)-$, $-NR^aC(=O)-$, $-C(=O)NR^a$, $NR^aC(=O)NR^a$, $-OC(=O)NR^a$ 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^4 is C_1 - C_{12} alkyl; R^5 is H or C_1 - C_6 alkyl; and x is 0, 1 or 2.

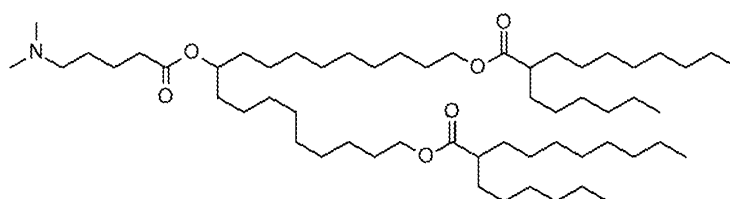
[0156] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO 2017/117528, which is incorporated herein by reference. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



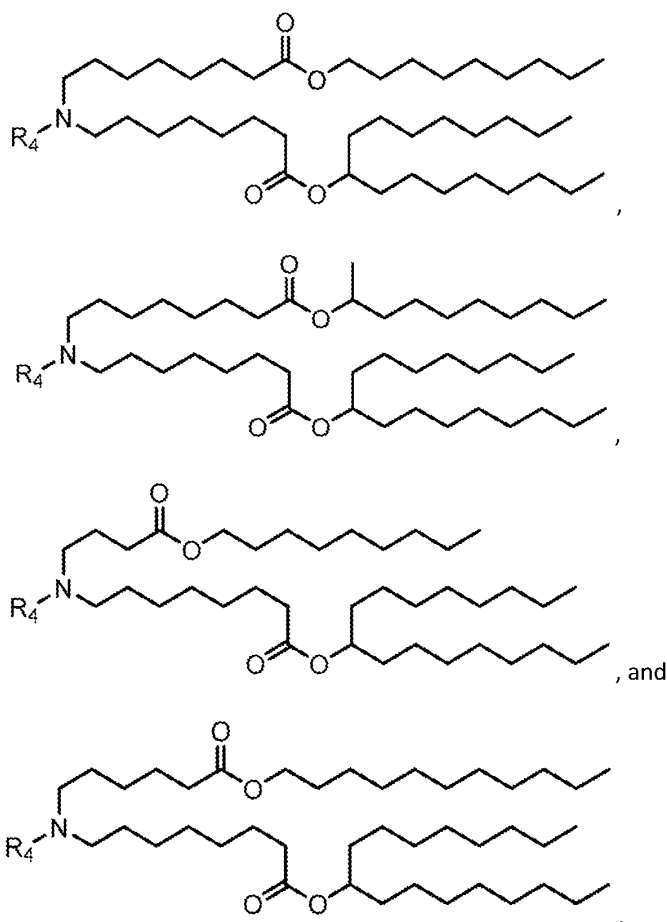
and pharmaceutically acceptable salts thereof. In some embodiments, the compositions and methods of the present invention include a cationic lipid having the compound structure:



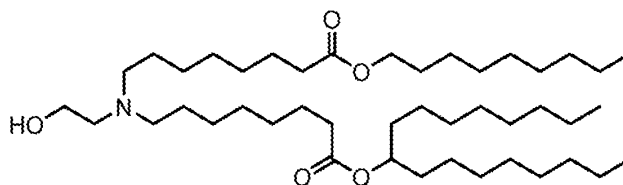
and pharmaceutically acceptable salts thereof.

[0157] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO

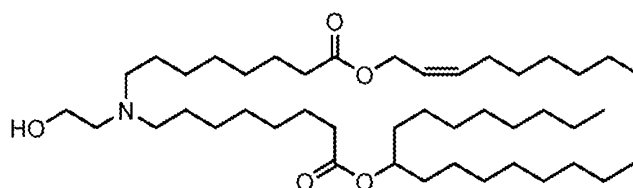
2017/049245, which is incorporated herein by reference. In some embodiments, the cationic lipids of the compositions and methods of the present invention include a compound of one of the following formulas:



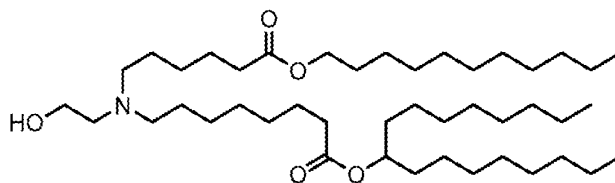
and pharmaceutically acceptable salts thereof. For any one of these four formulas, R_4 is independently selected from $-(CH_2)_nQ$ and $-(CH_2)_nCHQR$; Q is selected from the group consisting of $-OR$, $-OH$, $-O(CH_2)_nN(R)_2$, $-OC(O)R$, $-CX_3$, $-CN$, $-N(R)C(O)R$, $-N(H)C(O)R$, $-N(R)S(O)_2R$, $-N(H)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(H)C(O)N(R)_2$, $-N(H)C(O)N(H)(R)$, $-N(R)C(S)N(R)_2$, $-N(H)C(S)N(R)_2$, $-N(H)C(S)N(H)(R)$, and a heterocycle; and n is 1, 2, or 3. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:



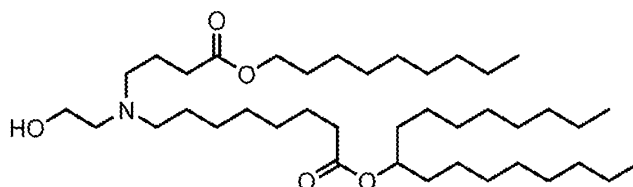
and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:



and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:

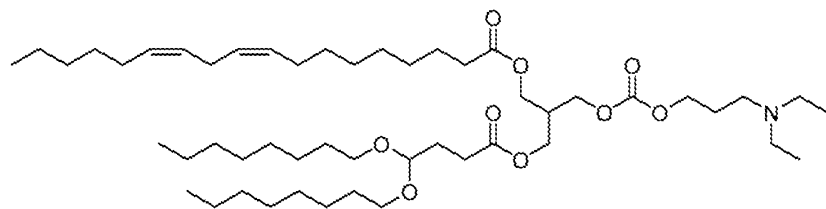


and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:

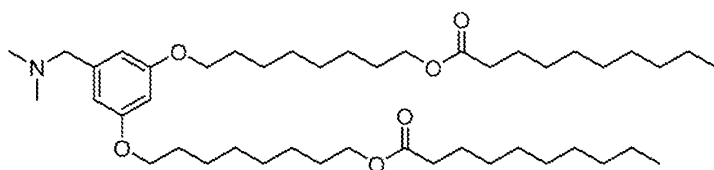


and pharmaceutically acceptable salts thereof.

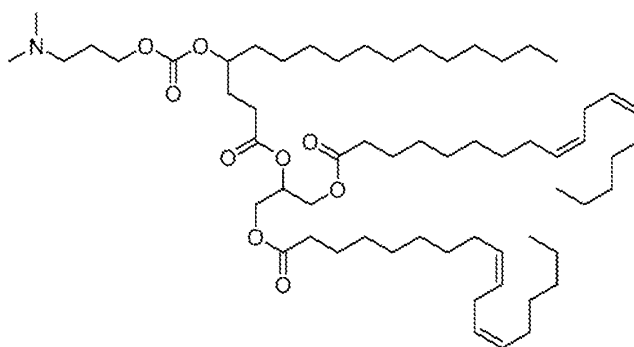
[0158] Other suitable cationic lipids for use in the compositions and methods of the invention include the cationic lipids as described in International Patent Publication WO 2017/173054 and WO 2015/095340, each of which is incorporated herein by reference. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:



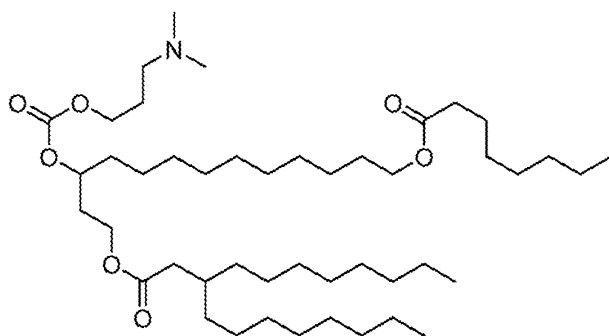
and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:



and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:

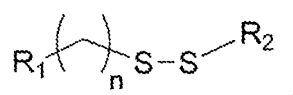


and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid having a compound structure of:

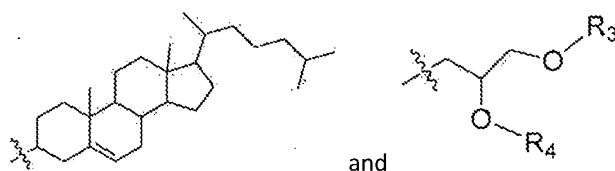


and pharmaceutically acceptable salts thereof.

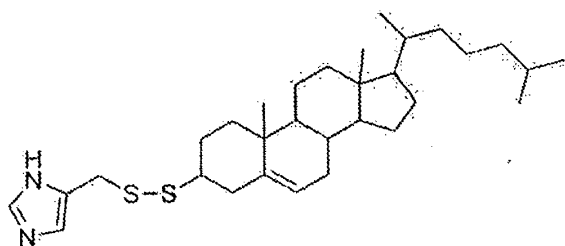
[0159] Other suitable cationic lipids for use in the compositions and methods of the present invention include cleavable cationic lipids as described in International Patent Publication WO 2012/170889, which is incorporated herein by reference. In some embodiments, the compositions and methods of the present invention include a cationic lipid of the following formula:



wherein R_1 is selected from the group consisting of imidazole, guanidinium, amino, imine, enamine, an optionally-substituted alkyl amino (*e.g.*, an alkyl amino such as dimethylamino) and pyridyl; wherein R_2 is selected from the group consisting of one of the following two formulas:

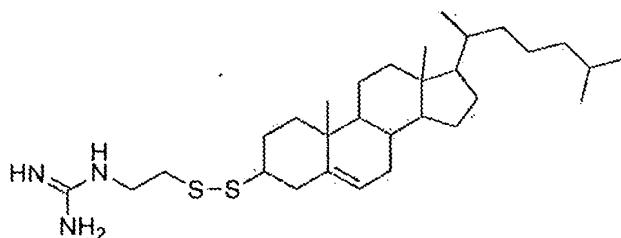


and wherein R_3 and R_4 are each independently selected from the group consisting of an optionally substituted, variably saturated or unsaturated C_6 – C_{20} alkyl and an optionally substituted, variably saturated or unsaturated C_6 – C_{20} acyl; and wherein n is zero or any positive integer (*e.g.*, one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty or more). In certain embodiments, the compositions and methods of the present invention include a cationic lipid, “HGT4001”, having a compound structure of:



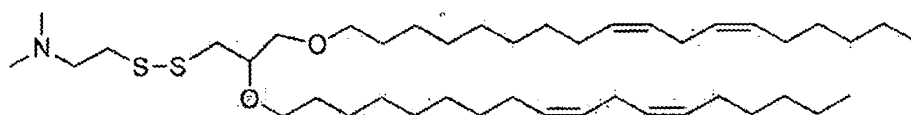
(HGT4001)

and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid, "HGT4002," having a compound structure of:



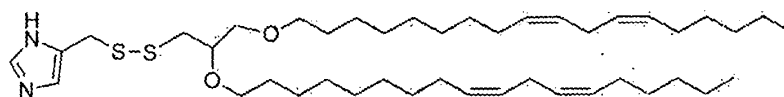
(HGT4002)

and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid, "HGT4003," having a compound structure of:



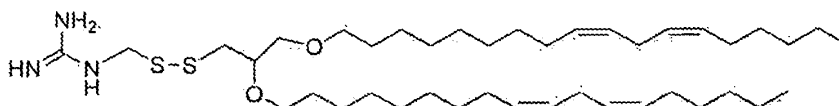
(HGT4003)

and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid, "HGT4004," having a compound structure of:



(HGT4004)

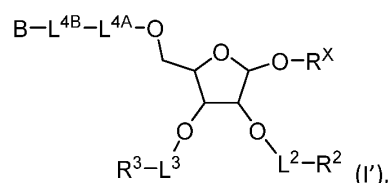
and pharmaceutically acceptable salts thereof. In certain embodiments, the compositions and methods of the present invention include a cationic lipid "HGT4005," having a compound structure of:



(HGT4005)

and pharmaceutically acceptable salts thereof.

[0160] Other suitable cationic lipids for use in the compositions and methods of the present invention include cleavable cationic lipids as described in International Application No. PCT/US2019/032522, and incorporated herein by reference. In certain embodiments, the compositions and methods of the present invention include a cationic lipid that is any of general formulas or any of structures (1a)–(21a) and (1b) – (21b) and (22)–(237) described in International Application No. PCT/US2019/032522. In certain embodiments, the compositions and methods of the present invention include a cationic lipid that has a structure according to Formula (I'),



wherein:

R^x is independently $-H$, $-L^1-R^1$, or $-L^{5A}-L^{5B}-B'$;

each of L¹, L², and L³ is independently a covalent bond, -C(O)-, -C(O)O-, -C(O)S-, or -C(O)NR^L-;

each L^{4A} and L^{5A} is independently $-C(O)-$, $-C(O)O-$, or $-C(O)NR^L-$;

each L^{4B} and L^{5B} is independently C₁-C₂₀ alkylene; C₂-C₂₀ alkenylene; or C₂-C₂₀ alkynylene;

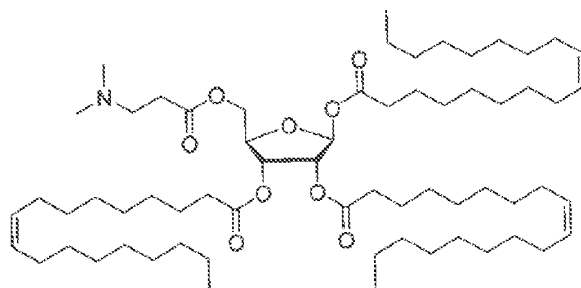
each B and B' is NR⁴R⁵ or a 5- to 10-membered nitrogen-containing heteroaryl;

each R¹, R², and R³ is independently C₆-C₃₀ alkyl, C₆-C₃₀ alkenyl, or C₆-C₃₀ alkynyl;

each R⁴ and R⁵ is independently hydrogen, C₁-C₁₀ alkyl; C₂-C₁₀ alkenyl; or C₂-C₁₀ alkynyl; and

each R^L is independently hydrogen, C₁-C₂₀ alkyl, C₂-C₂₀ alkenyl, or C₂-C₂₀ alkynyl.

In certain embodiments, the compositions and methods of the present invention include a cationic lipid that is Compound (139) of International Application No. PCT/US2019/032522, having a compound structure of:



("18:1 Carbon tail-ribose lipid").

[0161] In some embodiments, the compositions and methods of the present invention include the cationic lipid, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride ("DOTMA"). (Feigner *et al.* (Proc. Nat'l Acad. Sci. 84, 7413 (1987); U.S. Pat. No. 4,897,355, which is incorporated herein by reference). Other cationic lipids suitable for the compositions and methods of the present invention include, for example, 5-carboxyspermylglycinedioctadecylamide ("DOGS"); 2,3-dioleoyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminium ("DOSPA") (Behr *et al.* Proc. Nat'l Acad. Sci. 86, 6982 (1989), U.S. Pat. No. 5,171,678; U.S. Pat. No. 5,334,761); 1,2-Dioleoyl-3-Dimethylammonium-Propane ("DODAP"); 1,2-Dioleoyl-3-Trimethylammonium-Propane ("DOTAP").

[0162] Additional exemplary cationic lipids suitable for the compositions and methods of the present invention also include: 1,2-distearoyloxy-N,N-dimethyl-3-aminopropane ("DSDMA"); 1,2-dioleoyloxy-N,N-dimethyl-3-aminopropane ("DODMA"); 1,2-dilinoleoyloxy-N,N-dimethyl-3-aminopropane ("DLinDMA"); 1,2-dilinenoyloxy-N,N-dimethyl-3-aminopropane ("DLinDMA"); N-dioleoyl-N,N-dimethylammonium chloride ("DODAC"); N,N-distearoyl-N,N-dimethylammonium bromide ("DDAB"); N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE"); 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-1-(cis,cis-9,12-octadecadienoxy)propane ("CLinDMA"); 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxyl]-3-dimethyl-1-(cis,cis-9,12-octadecadienoxy)propane ("CpLinDMA"); N,N-dimethyl-3,4-dioleoyloxybenzylamine ("DMOBA"); 1,2-N,N'-dioleoylcarbonyl-3-dimethylaminopropane ("DOcarbDAP"); 2,3-Dilinoleoyloxy-N,N-dimethylpropylamine ("DLinDAP"); 1,2-N,N'-Dilinoleoylcarbonyl-3-dimethylaminopropane ("DLincarbDAP"); 1,2-Dilinoleoylcarbonyl-3-dimethylaminopropane ("DLinCDAP"); 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane ("DLin-K-DMA"); 2-((8-[(3P)-cholest-5-en-3-yloxy]octyl)oxy)-N,N-dimethyl-3-[(9Z, 12Z)-octadeca-9, 12-dien-1-yloxy]propane-1-amine ("Octyl-CLinDMA"); (2R)-2-((8-[(3beta)-cholest-5-en-3-yloxy]octyl)oxy)-N,N-dimethyl-3-[(9Z, 12Z)-octadeca-9, 12-dien-1-yloxy]propan-1-amine ("Octyl-

CLinDMA (2R)"); (2S)-2-((8-[(3P)-cholest-5-en-3-yloxy]octyl)oxy)-N, fsl-dimethy3-[(9Z, 12Z)-octadeca-9, 12-dien-1 -yloxy]propan-1 -amine ("Octyl-CLinDMA (2S)"); 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane ("DLin-K-UTC2-DMA"); and 2-(2,2-di((9Z,12Z)-octadeca-9,12-dien- 1-yl)-1,3-dioxolan-4-yl)-N,N-dimethylethanamine ("DLin-KC2-DMA") (see, WO 2010/042877, which is incorporated herein by reference; Semple *et al.*, Nature Biotech. 28: 172-176 (2010)). (Heyes, J., *et al.*, J Controlled Release 107: 276-287 (2005); Morrissey, DV., *et al.*, Nat. Biotechnol. 23(8): 1003-1007 (2005); International Patent Publication WO 2005/121348). In some embodiments, one or more of the cationic lipids comprise at least one of an imidazole, dialkylamino, or guanidinium moiety.

[0163] In some embodiments, one or more cationic lipids suitable for the compositions and methods of the present invention include 2,2-Dilinoleyl-4-dimethylaminoethyl-1-[1,3]-dioxolane ("UTC"); (3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine ("ALNY-100") and/or 4,7,13-tris(3-oxo-3-(undecylamino)propyl)-N1,N16-diundecyl-4,7,10,13-tetraazahexadecane-1,16-diamide ("NC98-5").

[0164] In some embodiments, the compositions of the present invention include one or more cationic lipids that constitute at least about 5%, 10%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 70%, measured by weight, of the total lipid content in the composition, *e.g.*, a lipid nanoparticle. In some embodiments, the compositions of the present invention include one or more cationic lipids that constitute at least about 5%, 10%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 70%, measured as a mol %, of the total lipid content in the composition, *e.g.*, a lipid nanoparticle. In some embodiments, the compositions of the present invention include one or more cationic lipids that constitute about 30-70 % (*e.g.*, about 30-65%, about 30-60%, about 30-55%, about 30-50%, about 30-45%, about 30-40%, about 35-50%, about 35-45%, or about 35-40%), measured by weight, of the total lipid content in the composition, *e.g.*, a lipid nanoparticle. In some embodiments, the compositions of the present invention include one or more cationic lipids that constitute about 30-70 % (*e.g.*, about 30-65%, about 30-60%, about 30-55%, about 30-50%, about 30-45%, about 30-40%, about 35-50%, about 35-45%, or about 35-40%), measured as mol %, of the total lipid content in the composition, *e.g.*, a lipid nanoparticle.

Non-Cationic/Helper Lipids

[0165] In some embodiments, the liposomes contain one or more non-cationic ("helper") lipids. As used herein, the phrase "non-cationic lipid" refers to any neutral, zwitterionic or anionic lipid. As used herein, the phrase "anionic lipid" refers to any of a number of lipid species that

carry a net negative charge at a selected pH, such as physiological pH. Non-cationic lipids include, but are not limited to, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-l-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), phosphatidylserine, sphingolipids, cerebrosides, gangliosides, 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, l-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE), or a mixture thereof.

[0166] In some embodiments, a non-cationic lipid is a neutral lipid, *i.e.*, a lipid that does not carry a net charge in the conditions under which the composition is formulated and/or administered.

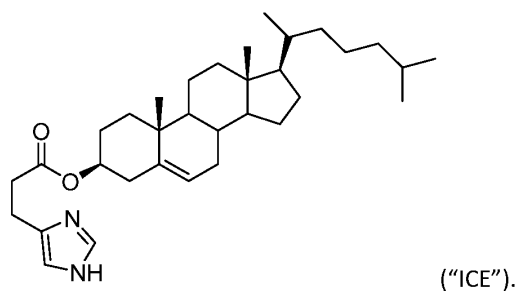
[0167] In some embodiments, such non-cationic lipids may be used alone, but are preferably used in combination with other lipids, for example, cationic lipids.

[0168] In some embodiments, a non-cationic lipid may be present in a molar ratio (mol%) of about 5% to about 90%, about 5% to about 70%, about 5% to about 50%, about 5% to about 40%, about 5% to about 30%, about 10 % to about 70%, about 10% to about 50%, or about 10% to about 40% of the total lipids present in a composition. In some embodiments, total non-cationic lipids may be present in a molar ratio (mol%) of about 5% to about 90%, about 5% to about 70%, about 5% to about 50%, about 5% to about 40%, about 5% to about 30%, about 10 % to about 70%, about 10% to about 50%, or about 10% to about 40% of the total lipids present in a composition. In some embodiments, the percentage of non-cationic lipid in a liposome may be greater than about 5 mol%, greater than about 10 mol%, greater than about 20 mol%, greater than about 30 mol%, or greater than about 40 mol%. In some embodiments, the percentage total non-cationic lipids in a liposome may be greater than about 5 mol%, greater than about 10 mol%, greater than about 20 mol%, greater than about 30 mol%, or greater than about 40 mol%. In some embodiments, the percentage of non-cationic lipid in a liposome is no more than about 5 mol%, no more than about 10 mol%, no more than about 20 mol%, no more than about 30 mol%, or no more than about 40 mol%. In some embodiments, the percentage total non-cationic lipids in a liposome may be no more than about 5 mol%, no more than about 10 mol%, no more than about 20 mol%, no more than about 30 mol%, or no more than about 40 mol%.

[0169] In some embodiments, a non-cationic lipid may be present in a weight ratio (wt%) of about 5% to about 90%, about 5% to about 70%, about 5% to about 50%, about 5% to about 40%, about 5% to about 30%, about 10 % to about 70%, about 10% to about 50%, or about 10% to about 40% of the total lipids present in a composition. In some embodiments, total non-cationic lipids may be present in a weight ratio (wt%) of about 5% to about 90%, about 5% to about 70%, about 5% to about 50%, about 5% to about 40%, about 5% to about 30%, about 10 % to about 70%, about 10% to about 50%, or about 10% to about 40% of the total lipids present in a composition. In some embodiments, the percentage of non-cationic lipid in a liposome may be greater than about 5 wt%, greater than about 10 wt%, greater than about 20 wt%, greater than about 30 wt%, or greater than about 40 wt%. In some embodiments, the percentage total non-cationic lipids in a liposome may be greater than about 5 wt%, greater than about 10 wt%, greater than about 20 wt%, greater than about 30 wt%, or greater than about 40 wt%. In some embodiments, the percentage of non-cationic lipid in a liposome is no more than about 5 wt%, no more than about 10 wt%, no more than about 20 wt%, no more than about 30 wt%, or no more than about 40 wt%. In some embodiments, the percentage total non-cationic lipids in a liposome may be no more than about 5 wt%, no more than about 10 wt%, no more than about 20 wt%, no more than about 30 wt%, or no more than about 40 wt%.

Cholesterol-Based Lipids

[0170] In some embodiments, the liposomes comprise one or more cholesterol-based lipids. For example, suitable cholesterol-based cationic lipids include, for example, DC-Choi (N,N-dimethyl-N-ethylcarboxamidocholesterol), 1,4-bis(3-N-oleylamino-propyl)piperazine (Gao, *et al.* Biochem. Biophys. Res. Comm. 179, 280 (1991); Wolf *et al.* BioTechniques 23, 139 (1997); U.S. Pat. No. 5,744,335), or imidazole cholesterol ester (ICE), which has the following structure,



[0171] In embodiments, a cholesterol-based lipid is cholesterol.

[0172] In some embodiments, the cholesterol-based lipid may comprise a molar ratio (mol %) of about 1% to about 30%, or about 5% to about 20% of the total lipids present in a liposome. In some embodiments, the percentage of cholesterol-based lipid in the lipid nanoparticle may be greater than about 5 mol%, greater than about 10 mol%, greater than about 20 mol%, greater than about 30 mol%, or greater than about 40 mol%. In some embodiments, the percentage of cholesterol-based lipid in the lipid nanoparticle may be no more than about 5 mol%, no more than about 10 mol%, no more than about 20 mol%, no more than about 30 mol%, or no more than about 40 mol%.

[0173] In some embodiments, a cholesterol-based lipid may be present in a weight ratio (wt %) of about 1% to about 30%, or about 5% to about 20% of the total lipids present in a liposome. In some embodiments, the percentage of cholesterol-based lipid in the lipid nanoparticle may be greater than about 5 wt%, greater than about 10 wt%, greater than about 20 wt%, greater than about 30 wt%, or greater than about 40 wt%. In some embodiments, the percentage of cholesterol-based lipid in the lipid nanoparticle may be no more than about 5 wt%, no more than about 10 wt%, no more than about 20 wt%, no more than about 30 wt%, or no more than about 40 wt%.

PEG-Modified Lipids

[0174] In some embodiments, the liposome comprises one or more PEGylated lipids.

[0175] For example, the use of polyethylene glycol (PEG)-modified phospholipids and derivatized lipids such as derivatized ceramides (PEG-CER), including N-Octanoyl-Sphingosine-1-[Succinyl(Methoxy Polyethylene Glycol)-2000] (C8 PEG-2000 ceramide) is also contemplated by the present invention, either alone or preferably in combination with other lipid formulations together which comprise the transfer vehicle (*e.g.*, a lipid nanoparticle).

[0176] Contemplated PEG-modified lipids include, but are not limited to, a polyethylene glycol chain of up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C₆-C₂₀ length. In some embodiments, a PEG-modified or PEGylated lipid is PEGylated cholesterol or PEG-2K. The addition of such components may prevent complex aggregation and may also provide a means for increasing circulation lifetime and increasing the delivery of the lipid-nucleic acid composition to the target tissues, (Klibanov *et al.* (1990) FEBS Letters, 268 (1): 235-237), or they may be selected to rapidly exchange out of the formulation *in vivo* (see U.S. Pat. No. 5,885,613). Particularly useful exchangeable lipids are PEG-ceramides having shorter acyl chains (*e.g.*, C₁₄ or C₁₈).

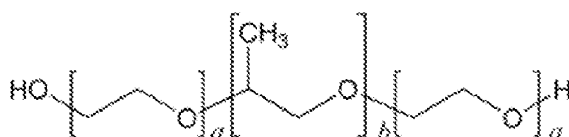
[0177] The PEG-modified phospholipid and derivitized lipids of the present invention may comprise a molar ratio from about 0% to about 20%, about 0.5% to about 20%, about 1% to about 15%, about 4% to about 10%, or about 2% of the total lipid present in the liposomal transfer vehicle. In some embodiments, one or more PEG-modified lipids constitute about 4% of the total lipids by molar ratio. In some embodiments, one or more PEG-modified lipids constitute about 5% of the total lipids by molar ratio. In some embodiments, one or more PEG-modified lipids constitute about 6% of the total lipids by molar ratio.

Amphiphilic block copolymers

[0178] In some embodiments, a suitable delivery vehicle contains amphiphilic block copolymers (e.g., poloxamers). Various amphiphilic block copolymers may be used to practice the present invention. In some embodiments, an amphiphilic block copolymer is also referred to as a surfactant or a non-ionic surfactant. In some embodiments, an amphiphilic polymer suitable for the invention is selected from poloxamers (Pluronic®), poloxamines (Tetronic®), polyoxyethylene glycol sorbitan alkyl esters (polysorbates) and polyvinyl pyrrolidones (PVPs).

Poloxamers

[0179] In some embodiments, a suitable amphiphilic polymer is a poloxamer. For example, a suitable poloxamer is of the following structure:



wherein a is an integer between 10 and 150 and b is an integer between 20 and 60. For example, a is about 12 and b is about 20, or a is about 80 and b is about 27, or a is about 64 and b is about 37, or a is about 141 and b is about 44, or a is about 101 and b is about 56.

[0180] In some embodiments, a poloxamer suitable for the invention has ethylene oxide units from about 10 to about 150. In some embodiments, a poloxamer has ethylene oxide units from about 10 to about 100.

[0181] In some embodiments, a suitable poloxamer is poloxamer 84. In some embodiments, a suitable poloxamer is poloxamer 101. In some embodiments, a suitable poloxamer is poloxamer 105. In some embodiments, a suitable poloxamer is poloxamer 108. In some embodiments, a suitable poloxamer is poloxamer 122. In some embodiments, a suitable

poloxamer is poloxamer 123. In some embodiments, a suitable poloxamer is poloxamer 124. In some embodiments, a suitable poloxamer is poloxamer 181. In some embodiments, a suitable poloxamer is poloxamer 182. In some embodiments, a suitable poloxamer is poloxamer 183. In some embodiments, a suitable poloxamer is poloxamer 184. In some embodiments, a suitable poloxamer is poloxamer 185. In some embodiments, a suitable poloxamer is poloxamer 188. In some embodiments, a suitable poloxamer is poloxamer 212. In some embodiments, a suitable poloxamer is poloxamer 215. In some embodiments, a suitable poloxamer is poloxamer 217. In some embodiments, a suitable poloxamer is poloxamer 231. In some embodiments, a suitable poloxamer is poloxamer 234. In some embodiments, a suitable poloxamer is poloxamer 235. In some embodiments, a suitable poloxamer is poloxamer 237. In some embodiments, a suitable poloxamer is poloxamer 238. In some embodiments, a suitable poloxamer is poloxamer 282. In some embodiments, a suitable poloxamer is poloxamer 284. In some embodiments, a suitable poloxamer is poloxamer 288. In some embodiments, a suitable poloxamer is poloxamer 304. In some embodiments, a suitable poloxamer is poloxamer 331. In some embodiments, a suitable poloxamer is poloxamer 333. In some embodiments, a suitable poloxamer is poloxamer 334. In some embodiments, a suitable poloxamer is poloxamer 335. In some embodiments, a suitable poloxamer is poloxamer 338. In some embodiments, a suitable poloxamer is poloxamer 401. In some embodiments, a suitable poloxamer is poloxamer 402. In some embodiments, a suitable poloxamer is poloxamer 403. In some embodiments, a suitable poloxamer is poloxamer 407. In some embodiments, a suitable poloxamer is a combination thereof.

[0182] In some embodiments, a suitable poloxamer has an average molecular weight of about 4,000 g/mol to about 20,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 1,000 g/mol to about 50,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 1,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 2,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 3,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 4,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 5,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 6,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 7,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 8,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 9,000 g/mol. In some embodiments, a suitable poloxamer has an

average molecular weight of about 10,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 20,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 25,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 30,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 40,000 g/mol. In some embodiments, a suitable poloxamer has an average molecular weight of about 50,000 g/mol.

Other amphiphilic polymers

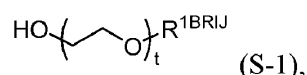
[0183] In some embodiments, an amphiphilic polymer is a poloxamine, *e.g.*, tetronic 304 or tetronic 904.

[0184] In some embodiments, an amphiphilic polymer is a polyvinylpyrrolidone (PVP), such as PVP with molecular weight of 3 kDa, 10 kDa, or 29 kDa.

[0185] In some embodiments, an amphiphilic polymer is a polyethylene glycol ether (Brij), polysorbate, sorbitan, and derivatives thereof. In some embodiments, an amphiphilic polymer is a polysorbate, such as PS 20.

[0186] In some embodiments, an amphiphilic polymer is polyethylene glycol ether (Brij), poloxamer, polysorbate, sorbitan, or derivatives thereof.

[0187] In some embodiments, an amphiphilic polymer is a polyethylene glycol ether. In some embodiments, a suitable polyethylene glycol ether is a compound of Formula (S-1):



or a salt or isomer thereof, wherein:

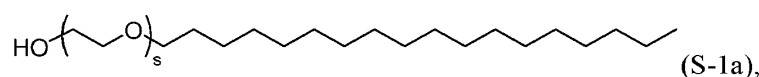
t is an integer between 1 and 100;

R^{1BRIJ} independently is C₁₀₋₄₀ alkyl, C₁₀₋₄₀ alkenyl, or C₁₀₋₄₀ alkynyl; and optionally one or more methylene groups of R^{5PEG} are independently replaced with C₃₋₁₀ carbocyclene, 4 to 10 membered heterocyclene, C₆₋₁₀ arylene, 4 to 10 membered heteroarylene, -N(R^N)-, -O-, -S-, -C(O)-, -C(O)N(R^N)-, -NR^NC(O)-, -NR C(O)N(R^N)-, -C(O)O- -OC(O)-, -OC(O)O- -OC(O)N(R^N)-, -NR^NC(O)O- -C(O)S- -SC(O)-, -C(=NR^N)-, -C(=NR^N)N(R^N)-, -NR^NC(=NR^N)N(R^N)-, -C(S)-, -C(S)N(R^N)-, -NR^NC(S)-, -NR^NC(S)N(R^N)-, -S(O)-, -OS(O)-, -S(O)O- -OS(O)O- -OS(O)₂- -S(O)₂O- -OS(O)₂O- -N(R^N)S(O)-,

-S(O)N(R^N)- -N(R^N)S(O)N(R^N)- -OS(O)N(R^N)- -N(R^N)S(O)O- -S(O)₂- -N(R^N)S(O)₂- -S(O)₂N(R^N)-, -N(R^N)S(O)₂N(R^N)- -OS(O)₂N(R^N)- or -N(R^N)S(O)₂O-; and

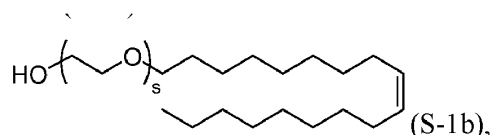
each instance of R^N is independently hydrogen, C₁₋₆ alkyl, or a nitrogen protecting group.

[0188] In some embodiment, R^{1BRU} is C is alkyl. For example, the polyethylene glycol ether is a compound of Formula (S-1a):



or a salt or isomer thereof, wherein s is an integer between 1 and 100.

[0189] In some embodiments, R^{1BRU} is C is alkenyl. For example, a suitable polyethylene glycol ether is a compound of Formula (S-1b):



or a salt or isomer thereof, wherein s is an integer between 1 and 100.

[0190] Typically, an amphiphilic polymer (*e.g.*, a poloxamer) is present in a formulation at an amount lower than its critical micelle concentration (CMC). In some embodiments, an amphiphilic polymer (*e.g.*, a poloxamer) is present in the mixture at an amount about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, or about 50% lower than its CMC. In some embodiments, an amphiphilic polymer (*e.g.*, a poloxamer) is present in the mixture at an amount about 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1% lower than its CMC. In some embodiments, an amphiphilic polymer (*e.g.*, a poloxamer) is present in the mixture at an amount about 55%, 60%, 65%, 70%, 75%, 80%, 90%, or 95% lower than its CMC.

[0191] In some embodiments, less than about 0.1%, 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, or 0.01% of the original amount of the amphiphilic polymer (*e.g.*, the poloxamer) present in the formulation remains upon removal. In some embodiments, a residual amount of the amphiphilic polymer (*e.g.*, the poloxamer) remains in a formulation upon removal. As used herein, a residual amount means a remaining amount after substantially all of the substance (an amphiphilic polymer described herein such as a poloxamer) in a composition is

removed. A residual amount may be detectable using a known technique qualitatively or quantitatively. A residual amount may not be detectable using a known technique.

[0192] In some embodiments, a suitable delivery vehicle comprises less than 5% amphiphilic block copolymers (*e.g.*, poloxamers). In some embodiments, a suitable delivery vehicle comprises less than 3% amphiphilic block copolymers (*e.g.*, poloxamers). In some embodiments, a suitable delivery vehicle comprises less than 2.5% amphiphilic block copolymers (*e.g.*, poloxamers). In some embodiments, suitable delivery vehicle comprises less than 2% amphiphilic block copolymers (*e.g.*, poloxamers). In some embodiments, a suitable delivery vehicle comprises less than 1.5% amphiphilic block copolymers (*e.g.*, poloxamers). In some embodiments, a suitable delivery vehicle comprises less than 1% amphiphilic block copolymers (*e.g.*, poloxamers). In some embodiments, a suitable delivery vehicle comprises less than 0.5% (*e.g.*, less than 0.4%, 0.3%, 0.2%, 0.1%) amphiphilic block copolymers (*e.g.*, poloxamers). In some embodiments, a suitable delivery vehicle comprises less than 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, or 0.01% amphiphilic block copolymers (*e.g.*, poloxamers). In some embodiments, a suitable delivery vehicle comprises less than 0.01% amphiphilic block copolymers (*e.g.*, poloxamers). In some embodiments, a suitable delivery vehicle contains a residual amount of amphiphilic polymers (*e.g.*, poloxamers). As used herein, a residual amount means a remaining amount after substantially all of the substance (an amphiphilic polymer described herein such as a poloxamer) in a composition is removed. A residual amount may be detectable using a known technique qualitatively or quantitatively. A residual amount may not be detectable using a known technique.

Polymers

[0193] In some embodiments, a suitable delivery vehicle is formulated using a polymer as a carrier, alone or in combination with other carriers including various lipids described herein. Thus, in some embodiments, liposomal delivery vehicles, as used herein, also encompass nanoparticles comprising polymers. Suitable polymers may include, for example, polyacrylates, polyalkycyanoacrylates, polylactide, polylactide-polyglycolide copolymers, polycaprolactones, dextran, albumin, gelatin, alginate, collagen, chitosan, cyclodextrins, protamine, PEGylated protamine, PLL, PEGylated PLL and polyethylenimine (PEI). When PEI is present, it may be branched PEI of a molecular weight ranging from 10 to 40 kDa, *e.g.*, 25 kDa branched PEI (Sigma #408727).

[0194] According to various embodiments, the selection of cationic lipids, non-cationic lipids, PEG-modified lipids, cholesterol-based lipids, and/or amphiphilic block copolymers which comprise the lipid nanoparticle, as well as the relative molar ratio of such components (lipids) to each other, is based upon the characteristics of the selected lipid(s), the nature of the intended target cells, the characteristics of the nucleic acid to be delivered. Additional considerations include, for example, the saturation of the alkyl chain, as well as the size, charge, pH, pKa, fusogenicity and tolerability of the selected lipid(s). Thus the molar ratios may be adjusted accordingly.

Use of Amphiphilic Polymers in Ethanol-free LNP Formulations

[0195] In some embodiments, amphiphilic polymers used in the methods herein comprise one or more pluronics, polyvinyl pyrrolidone, polyvinyl alcohol, polyethylene glycol (PEG), or combinations thereof. In some embodiments, the amphiphilic polymer is selected from one or more of the following: PEG triethylene glycol, tetraethylene glycol, PEG 200, PEG 300, PEG 400, PEG 600, PEG 1,000, PEG 1,500, PEG 2,000, PEG 3,000, PEG 3,350, PEG 4,000, PEG 6,000, PEG 8,000, PEG 10,000, PEG 20,000, PEG 35,000, and PEG 40,000, or combination thereof. In some embodiments, the amphiphilic polymer is triethylene glycol. In some embodiments, the amphiphilic polymer is tetraethylene glycol. In some embodiments, the amphiphilic polymer is PEG 200. In some embodiments, the amphiphilic polymer is PEG 300. In some embodiments, the amphiphilic polymer is PEG 400. In some embodiments the amphiphilic polymer is PEG 600. In some embodiments, the amphiphilic polymer is PEG 1,000. In some embodiments, the amphiphilic polymer is PEG 1,500. In some embodiments, the amphiphilic polymer is PEG 2,000. In some embodiments, the amphiphilic polymer is PEG 3,000. In some embodiments, the amphiphilic polymer is PEG 3,350. In some embodiments, the amphiphilic polymer is PEG 4,000. In some embodiments, the amphiphilic polymer is PEG 6,000. In some embodiments, the amphiphilic polymer is PEG 8,000. In some embodiments, the amphiphilic polymer is PEG 10,000. In some embodiments, the amphiphilic polymer is PEG 20,000. In some embodiments, the amphiphilic polymer is PEG 35,000. In some embodiments, the amphiphilic polymer is PEG 40,000.

[0196] In some embodiments, the amphiphilic polymer comprises a mixture of two or more kinds of molecular weight PEG polymers are used. For example, in some embodiments, two, three, four, five, six, seven, eight, nine, ten, eleven, or twelve molecular weight PEG polymers comprise the amphiphilic polymer. Accordingly, in some embodiments, the PEG solution

comprises a mixture of one or more PEG polymers. In some embodiments, the mixture of PEG polymers comprises polymers having distinct molecular weights.

[0197] In some embodiments, the lipid solution comprises one or more amphiphilic polymers. In some embodiments, the solvent in the lipid solution comprises a PEG polymer. Various kinds of PEG polymers are recognized in the art, some of which have distinct geometrical configurations. PEG polymers suitable for the methods herein include, for example, PEG polymers having linear, branched, Y-shaped, or multi-arm configuration. In some embodiments, the PEG is in a suspension comprising one or more PEG of distinct geometrical configurations. In some embodiments, the lipid solution can be achieved using PEG-6000 as a solvent. In some embodiments, the lipid solution can be achieved using PEG-400 as a solvent. In some embodiments, the lipid solution can be achieved using triethylene glycol (TEG) as a solvent. In some embodiments, the lipid solution can be achieved using triethylene glycol monomethyl ether (mTEG) as a solvent. In some embodiments, the lipid solution can be achieved using tert-butyl-TEG-O-propionate as a solvent. In some embodiments, the lipid solution can be achieved using TEG-dimethacrylate as a solvent. In some embodiments, the lipid solution can be achieved using TEG-dimethyl ether as a solvent. In some embodiments, the lipid solution can be achieved using TEG-divinyl ether as a solvent. In some embodiments, the lipid solution can be achieved using TEG-monobutyl ether as a solvent. In some embodiments, the lipid solution can be achieved using TEG-methyl ether methacrylate as a solvent. In some embodiments, the lipid solution can be achieved using TEG-monodecyl ether as a solvent. In some embodiments, the lipid solution can be achieved using TEG-dibenzoate as a solvent. Any one of these PEG or TEG based reagents can be used as solvent in the lipid solution that is mixed with the mRNA solution in an LNP formulation. The structures of each of these reagents is shown below in **Table 1**.

Table 1: Non-Organic Solvent Reagents for Lipid Solution in Lipid Nanoparticle Formulations

Reagent Name	Structure
TEG	
TEG monomethyl ether	
tert-butyl-TEG-O-propionate	
TEG-dimethylacrylate	
TEG-dimethyl ether	
TEG-divinyl ether	
TEG-monobutyl ether	
TEG-methyl ether methacrylate	
TEG-monodecyl ether	
TEG-dibenzoate	

[0198] In some embodiments, the lipid solution comprises a PEG polymer solvent, wherein the PEG polymer comprises a PEG-modified lipid. In some embodiments, the PEG-modified lipid is 1,2-dimyristoyl-sn-glycerol, methoxypolyethylene glycol (DMG-PEG-2K). In some embodiments, the PEG modified lipid is a DOPA-PEG conjugate. In some embodiments, the PEG-

modified lipid is a poloxamer-PEG conjugate. In some embodiments, the PEG-modified lipid comprises DOTAP. In some embodiments, the PEG-modified lipid comprises cholesterol.

[0199] In some embodiments, the lipid solution comprises an amphiphilic polymer. In some embodiments, the lipid solution comprises any of the aforementioned PEG reagents. In some embodiments, PEG is in the suspension at about 10% to about 100% weight/volume concentration. For example, in some embodiments, PEG is present in the suspension at about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% weight/volume concentration, and any values there between. In some embodiments, PEG is present in the suspension at about 5% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 6% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 7% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 8% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 9% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 10% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 12% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 15% weight/volume. In some embodiments, PEG is present in the suspension at about 18% weight/volume. In some embodiments, PEG is present in the suspension at about 20% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 25% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 30% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 35% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 40% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 45% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 50% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 55% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 60% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 65% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 70% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 75% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 80% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 85% weight/volume concentration. In some embodiments, PEG is present in the suspension at about

90% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 95% weight/volume concentration. In some embodiments, PEG is present in the suspension at about 100% weight/volume concentration.

[0200] In some embodiments, the formulation comprises a volume:volume ratio of PEG to total mRNA suspension volume of about 0.1 to about 5.0. For example, in some embodiments, PEG is present in the formulation at a volume:volume ratio of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75, 3.0, 3.25, 3.5, 3.75, 4.0, 4.25, 4.5, 4.75, 5.0.

Accordingly, in some embodiments, PEG is present in the formulation at a volume:volume ratio of about 0.1. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 0.2. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 0.3. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 0.4. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 0.5. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 0.6. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 0.7. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 0.8. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 0.9. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 1.0. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 1.25. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 1.5. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 1.75. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 2.0. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 2.25. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 2.5. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 2.75. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 3.0. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 3.25. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 3.5. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 3.75. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 4.0. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 4.25. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 4.50. In some embodiments, PEG is present in the formulation at a volume:volume ratio of

about 4.75. In some embodiments, PEG is present in the formulation at a volume:volume ratio of about 5.0.

[0201] In particular embodiments, the PEG is mTEG (*e.g.* about 100% or pure mTEG). In particular embodiments, the lipid solution is about 100% mTEG-lipid. A particularly suitable final concentration of mTEG in the mRNA-LNP formulation is about 55-65% weight/volume, for example about 50% weight/volume. As shown in the examples, this concentration maintains mRNA solubility and stability and allows reduced processing volumes and ease of manufacture of the formulations on a larger scale.

[0202] In some embodiments, the mRNA solution and the lipid solution (*e.g.* about 100% mTEG-lipid solution) are mixed at a ratio (v/v) of 1-8:1, for example 1-4:1. In particular embodiments, the mRNA solution and the lipid solution (*e.g.* about 100% mTEG-lipid solution) are mixed at a ratio (v/v) of about 1:1. As shown in the examples, this ratio of mRNA solution to the lipid solution maintains mRNA solubility and stability and allows reduced processing volumes and ease of manufacture of the formulations on a larger scale.

[0203] In some embodiments, the formulation is alcohol free. In some embodiments, the formulation is produced without the use of any non-aqueous solvent (*e.g.*, alcohol). In some embodiments, the solvent is free of flammable agents. In some embodiments, a solvent is free of ethanol. In some embodiments, a solvent is free of isopropyl alcohol, acetone, methyl ethyl ketone, methyl isobutyl ketone, ethanol, methanol, denatonium, and combinations thereof. In some embodiments, a solvent is free of an alcohol solvent (*e.g.*, methanol, ethanol, or isopropanol). In some embodiments, a solvent is free of a ketone solvent (*e.g.*, acetone, methyl ethyl ketone, or methyl isobutyl ketone). In some embodiments, the formulation is aqueous.

[0204] In some embodiments, the mRNA is encapsulated in the absence of ethanol. In some embodiments, the mRNA is purified in the absence of ethanol. In some embodiments, the mRNA purification, mRNA encapsulation, or both processes are in the absence of ethanol. In some embodiments, mRNA purification, mRNA encapsulation, or both processes are free of flammable agents. . In some embodiments, mRNA purification, mRNA encapsulation, or both processes are free of non-aqueous solvents.

Ratio of Distinct Lipid Components

[0205] A suitable liposome for the present invention may include one or more of any of the cationic lipids, non-cationic lipids, cholesterol lipids, PEG-modified lipids, amphiphilic block

copolymers and/or polymers described herein at various ratios. In some embodiments, a lipid nanoparticle comprises five and no more than five distinct components of nanoparticle. In some embodiments, a lipid nanoparticle comprises four and no more than four distinct components of nanoparticle. In some embodiments, a lipid nanoparticle comprises three and no more than three distinct components of nanoparticle. As non-limiting examples, a suitable liposome formulation may include a combination selected from cKK-E12 (also known as ML2), DOPE, cholesterol and DMG-PEG2K; C12-200, DOPE, cholesterol and DMG-PEG2K; HGT4003, DOPE, cholesterol and DMG-PEG2K; ICE, DOPE, cholesterol and DMG-PEG2K; or ICE, DOPE, and DMG-PEG2K.

[0206] In various embodiments, cationic lipids (*e.g.*, cKK-E12, C12-200, ICE, and/or HGT4003) constitute about 30–60 % (*e.g.*, about 30–55%, about 30–50%, about 30–45%, about 30–40%, about 35–50%, about 35–45%, or about 35–40%) of the liposome by molar ratio. In some embodiments, the percentage of cationic lipids (*e.g.*, cKK-E12, C12-200, ICE, and/or HGT4003) is or greater than about 30%, about 35%, about 40 %, about 45%, about 50%, about 55%, or about 60% of the liposome by molar ratio.

[0207] In some embodiments, the ratio of cationic lipid(s) to non-cationic lipid(s) to cholesterol-based lipid(s) to PEG-modified lipid(s) may be between about 30–60:25–35:20–30:1–15, respectively. In some embodiments, the ratio of cationic lipid(s) to non-cationic lipid(s) to cholesterol-based lipid(s) to PEG-modified lipid(s) is approximately 40:30:20:10, respectively. In some embodiments, the ratio of cationic lipid(s) to non-cationic lipid(s) to cholesterol-based lipid(s) to PEG-modified lipid(s) is approximately 40:30:25:5, respectively. In some embodiments, the ratio of cationic lipid(s) to non-cationic lipid(s) to cholesterol-based lipid(s) to PEG-modified lipid(s) is approximately 50:10:35:5, respectively. In some embodiments, the ratio of cationic lipid(s) to non-cationic lipid(s) to cholesterol-based lipid(s) to PEG-modified lipid(s) is approximately 60:35:0:5, respectively. In some embodiments, the ratio of cationic lipid(s) to non-cationic lipid(s) to cholesterol-based lipid(s) to PEG-modified lipid(s) is approximately 40:32:25:3, respectively. In some embodiments, the ratio of cationic lipid(s) to non-cationic lipid(s) to cholesterol-based lipid(s) to PEG-modified lipid(s) is approximately 50:25:20:5.

[0208] An exemplary mixture of lipids for use with the invention is composed of four lipid components: a cationic lipid (*e.g.* ML-2 or MC-3), a non-cationic lipid (*e.g.*, DSPC, DPPC, DOPE or DEPE), a cholesterol-based lipid (*e.g.*, cholesterol) and a PEG-modified lipid (*e.g.*, DMG-PEG2K). In some embodiments, the molar ratio of cationic lipid(s) (*e.g.* ML-2 or MC-3) to non-cationic lipid(s) (*e.g.* DSPC or DOPE) to cholesterol-based lipid(s) to PEG-modified lipid(s) in the LNPs may be

between about 35-55:5-35:20-40:1-15, respectively. In some embodiments, the molar ratio of cationic lipid(s) (*e.g.* ML-2) to non-cationic lipid(s) (*e.g.* DSPC or DOPE) to cholesterol-based lipid(s) to PEG-modified lipid(s) in the LNPs is 35-45:25-35:20-30:1-10. In particular embodiments, the molar ratio of cationic lipid(s) (*e.g.* ML-2) to non-cationic lipid(s) (*e.g.* DSPC or DOPE) to cholesterol-based lipid(s) to PEG-modified lipid(s) in the LNPs is about 40:30:25:5. In some embodiments, the molar ratio of cationic lipid(s) (*e.g.* MC-3) to non-cationic lipid(s) (*e.g.* DSPC or DOPE) to cholesterol-based lipid(s) to PEG-modified lipid(s) in the LNPs is 45-55:5-15:30-40:1-10. In some embodiments, the molar ratio of cationic lipid(s) (*e.g.* MC-3) to non-cationic lipid(s) (*e.g.* DSPC or DOPE) to cholesterol-based lipid(s) to PEG-modified lipid(s) in the LNPs is about 50:10:35:5. As shown in the examples, these preparations are particularly suitable for use in the formulations of the invention as they ensure suitable mRNA-LNP size and encapsulation efficacy.

[0209] In some embodiments, a mixture of lipids for use with the invention may comprise no more than three distinct lipid components. In some embodiments, one distinct lipid component in such a mixture is a cholesterol-based or imidazol-based cationic lipid. An exemplary mixture of lipids may be composed of three lipid components: a cationic lipid (*e.g.*, a cholesterol-based or imidazol-based cationic lipid such as ICE, HGT4001 or HGT4002), a non-cationic lipid (*e.g.*, DSPC, DPPC, DOPE or DEPE) and a PEG-modified lipid (*e.g.*, DMG-PEG2K). In some embodiments, the molar ratio of cationic lipid to non-cationic lipid to PEG-modified lipid may be between about 55-65:30-40:1-15, respectively. In some embodiments, the molar ratio of cationic lipid (*e.g.* ICE) to non-cationic lipid (*e.g.* DSPC) to PEG-modified lipid in the LNPs is 55-65:30-40:1-15. In particular embodiments, the molar ratio of cationic lipid (*e.g.* ICE) to non-cationic lipid (*e.g.* DSPC or DOPE) to PEG-modified lipid in the LNPs is 60:35:5. As shown in the examples, these preparations are particularly suitable for use in the formulations of the invention as they ensure suitable mRNA-LNP size and encapsulation efficacy.

[0210] In some embodiments, the concentration of the lipids and mRNA in the mRNA-LNP is such that the cationic lipid(s) (*e.g.* ML-2 or MC-3) to mRNA N/P ratio is about 2, 3, 4, 5 or 6. As shown in the examples, a particularly suitable N/P ratio is about 4, which allows efficient LNP formation and mRNA encapsulation efficacy.

[0211] In embodiments where a lipid nanoparticle comprises three and no more than three distinct components of lipids, the ratio of total lipid content (*i.e.*, the ratio of lipid component (1):lipid component (2):lipid component (3)) can be represented as x:y:z, wherein

$$(y + z) = 100 - x.$$

[0212] In some embodiments, each of “x,” “y,” and “z” represents molar percentages of the three distinct components of lipids, and the ratio is a molar ratio.

[0213] In some embodiments, each of “x,” “y,” and “z” represents weight percentages of the three distinct components of lipids, and the ratio is a weight ratio.

[0214] In some embodiments, lipid component (1), represented by variable “x,” is a sterol-based cationic lipid.

[0215] In some embodiments, lipid component (2), represented by variable “y,” is a helper lipid.

[0216] In some embodiments, lipid component (3), represented by variable “z” is a PEG lipid.

[0217] In some embodiments, variable “x,” representing the molar percentage of lipid component (1) (e.g., a sterol-based cationic lipid), is at least about 10%, about 20%, about 30%, about 40%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%.

[0218] In some embodiments, variable “x,” representing the molar percentage of lipid component (1) (e.g., a sterol-based cationic lipid), is no more than about 95%, about 90%, about 85%, about 80%, about 75%, about 70%, about 65%, about 60%, about 55%, about 50%, about 40%, about 30%, about 20%, or about 10%. In embodiments, variable “x” is no more than about 65%, about 60%, about 55%, about 50%, about 40%.

[0219] In some embodiments, variable “x,” representing the molar percentage of lipid component (1) (e.g., a sterol-based cationic lipid), is: at least about 50% but less than about 95%; at least about 50% but less than about 90%; at least about 50% but less than about 85%; at least about 50% but less than about 80%; at least about 50% but less than about 75%; at least about 50% but less than about 70%; at least about 50% but less than about 65%; or at least about 50% but less than about 60%. In embodiments, variable “x” is at least about 50% but less than about 70%; at least about 50% but less than about 65%; or at least about 50% but less than about 60%.

[0220] In some embodiments, variable “x,” representing the weight percentage of lipid component (1) (e.g., a sterol-based cationic lipid), is at least about 10%, about 20%, about 30%, about 40%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%.

[0221] In some embodiments, variable “x,” representing the weight percentage of lipid component (1) (e.g., a sterol-based cationic lipid), is no more than about 95%, about 90%, about 85%, about 80%, about 75%, about 70%, about 65%, about 60%, about 55%, about 50%, about 40%, about 30%, about 20%, or about 10%. In embodiments, variable “x” is no more than about 65%, about 60%, about 55%, about 50%, about 40%.

[0222] In some embodiments, variable “x,” representing the weight percentage of lipid component (1) (e.g., a sterol-based cationic lipid), is: at least about 50% but less than about 95%; at least about 50% but less than about 90%; at least about 50% but less than about 85%; at least about 50% but less than about 80%; at least about 50% but less than about 75%; at least about 50% but less than about 70%; at least about 50% but less than about 65%; or at least about 50% but less than about 60%. In embodiments, variable “x” is at least about 50% but less than about 70%; at least about 50% but less than about 65%; or at least about 50% but less than about 60%.

[0223] In some embodiments, variable “z,” representing the molar percentage of lipid component (3) (e.g., a PEG lipid) is no more than about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, or 25%. In embodiments, variable “z,” representing the molar percentage of lipid component (3) (e.g., a PEG lipid) is about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%. In embodiments, variable “z,” representing the molar percentage of lipid component (3) (e.g., a PEG lipid) is about 1% to about 10%, about 2% to about 10%, about 3% to about 10%, about 4% to about 10%, about 1% to about 7.5%, about 2.5% to about 10%, about 2.5% to about 7.5%, about 2.5% to about 5%, about 5% to about 7.5%, or about 5% to about 10%.

[0224] In some embodiments, variable “z,” representing the weight percentage of lipid component (3) (e.g., a PEG lipid) is no more than about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, or 25%. In embodiments, variable “z,” representing the weight percentage of lipid component (3) (e.g., a PEG lipid) is about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%. In embodiments, variable “z,” representing the weight percentage of lipid component (3) (e.g., a PEG lipid) is about 1% to about 10%, about 2% to about 10%, about 3% to about 10%, about 4% to about 10%, about 1% to about 7.5%, about 2.5% to about 10%, about 2.5% to about 7.5%, about 2.5% to about 5%, about 5% to about 7.5%, or about 5% to about 10%.

[0225] For compositions having three and only three distinct lipid components, variables “x,” “y,” and “z” may be in any combination so long as the total of the three variables sums to 100% of the total lipid content.

mRNA Synthesis

[0226] mRNAs according to the present invention may be synthesized according to any of a variety of known methods. Various methods are described in published U.S. Application No. US 2018/0258423, and can be used to practice the present invention, all of which are incorporated herein by reference. For example, mRNAs according to the present invention may be synthesized via *in vitro* transcription (IVT). Briefly, IVT is typically performed with a linear or circular DNA template containing a promoter, a pool of ribonucleotide triphosphates, a buffer system that may include DTT and magnesium ions, and an appropriate RNA polymerase (*e.g.*, T3, T7, or SP6 RNA polymerase), DNase I, pyrophosphatase, and/or RNase inhibitor. The exact conditions will vary according to the specific application.

[0227] In some embodiments, a suitable mRNA sequence is an mRNA sequence encoding a protein or a peptide. In some embodiments, a suitable mRNA sequence is codon optimized for efficient expression human cells. In some embodiments, a suitable mRNA sequence is naturally-occurring or a wild-type sequence. In some embodiments, a suitable mRNA sequence encodes a protein or a peptide that contains one or mutations in amino acid sequence.

[0228] The present invention may be used to deliver mRNAs of a variety of lengths. In some embodiments, the present invention may be used to deliver *in vitro* synthesized mRNA of or greater than about 0.5 kb, 1 kb, 1.5 kb, 2 kb, 2.5 kb, 3 kb, 3.5 kb, 4 kb, 4.5 kb, 5 kb, 6 kb, 7 kb, 8 kb, 9 kb, 10 kb, 11 kb, 12 kb, 13 kb, 14 kb, 15 kb, 20 kb, 30 kb, 40 kb, or 50 kb in length. In some embodiments, the present invention may be used to deliver *in vitro* synthesized mRNA ranging from about 1-20 kb, about 1-15 kb, about 1-10 kb, about 5-20 kb, about 5-15 kb, about 5-12 kb, about 5-10 kb, about 8-20 kb, or about 8-50 kb in length.

[0229] In some embodiments, for the preparation of mRNA according to the invention, a DNA template is transcribed *in vitro*. A suitable DNA template typically has a promoter, for example, a T3, T7 or SP6 promoter, for *in vitro* transcription, followed by desired nucleotide sequence for desired mRNA and a termination signal.

Nucleotides

[0230] Various naturally-occurring or modified nucleosides may be used to produce mRNA according to the present invention. In some embodiments, an mRNA is or comprises naturally-occurring nucleosides (or unmodified nucleotides; *e.g.*, adenosine, guanosine, cytidine, uridine); nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-

pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, pseudouridine, (*e.g.*, N-1-methyl-pseudouridine), 2-thiouridine, and 2-thiocytidine); chemically modified bases; biologically modified bases (*e.g.*, methylated bases); intercalated bases; modified sugars (*e.g.*, 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose); and/or modified phosphate groups (*e.g.*, phosphorothioates and 5'-*N*-phosphoramidite linkages).

[0231] In some embodiments, a suitable mRNA may contain backbone modifications, sugar modifications and/or base modifications. For example, modified nucleotides may include, but not be limited to, modified purines (adenine (A), guanine (G)) or pyrimidines (thymine (T), cytosine (C), uracil (U)), and as modified nucleotides analogues or derivatives of purines and pyrimidines, such as *e.g.* 1-methyl-adenine, 2-methyl-adenine, 2-methylthio-N-6-isopentenyl-adenine, N6-methyl-adenine, N6-isopentenyl-adenine, 2-thio-cytosine, 3-methyl-cytosine, 4-acetyl-cytosine, 5-methyl-cytosine, 2,6-diaminopurine, 1-methyl-guanine, 2-methyl-guanine, 2,2-dimethyl-guanine, 7-methyl-guanine, inosine, 1-methyl-inosine, pseudouracil (5-uracil), dihydro-uracil, 2-thio-uracil, 4-thio-uracil, 5-carboxymethylaminomethyl-2-thio-uracil, 5-(carboxyhydroxymethyl)-uracil, 5-fluoro-uracil, 5-bromo-uracil, 5-carboxymethylaminomethyl-uracil, 5-methyl-2-thio-uracil, 5-methyl-uracil, N-uracil-5-oxyacetic acid methyl ester, 5-methylaminomethyl-uracil, 5-methoxyaminomethyl-2-thio-uracil, 5'-methoxycarbonylmethyl-uracil, 5-methoxy-uracil, uracil-5-oxyacetic acid methyl ester, uracil-5-oxyacetic acid (v), 1-methyl-pseudouracil, queosine, .beta.-D-mannosyl-queosine, wybutoxosine, and phosphoramidates, phosphorothioates, peptide nucleotides, methylphosphonates, 7-deazaguanosine, 5-methylcytosine and inosine. The preparation of such analogues is known to a person skilled in the art *e.g.*, from the U.S. Pat. No. 4,373,071, U.S. Pat. No. 4,401,796, U.S. Pat. No. 4,415,732, U.S. Pat. No. 4,458,066, U.S. Pat. No. 4,500,707, U.S. Pat. No. 4,668,777, U.S. Pat. No. 4,973,679, U.S. Pat. No. 5,047,524, U.S. Pat. No. 5,132,418, U.S. Pat. No. 5,153,319, U.S. Pat. Nos. 5,262,530 and 5,700,642, the disclosures of which are incorporated by reference in their entirety.

[0232] In some embodiments, the mRNA comprises one or more nonstandard nucleotide residues. The nonstandard nucleotide residues may include, *e.g.*, 5-methyl-cytidine ("5mC"), pseudouridine ("ψU"), and/or 2-thio-uridine ("2sU"). See, *e.g.*, U.S. Patent No. 8,278,036 or WO 2011/012316 for a discussion of such residues and their incorporation into mRNA. The mRNA may be RNA, which is defined as RNA in which 25% of U residues are 2-thio-uridine and 25% of C

residues are 5-methylcytidine. Teachings for the use of RNA are disclosed US Patent Publication US 2012/0195936 and international publication WO 2011/012316, both of which are hereby incorporated by reference in their entirety. The presence of nonstandard nucleotide residues may render an mRNA more stable and/or less immunogenic than a control mRNA with the same sequence but containing only standard residues. In further embodiments, the mRNA may comprise one or more nonstandard nucleotide residues chosen from isocytosine, pseudoisocytosine, 5-bromouracil, 5-propynyluracil, 6-aminopurine, 2-aminopurine, inosine, diaminopurine and 2-chloro-6-aminopurine cytosine, as well as combinations of these modifications and other nucleobase modifications. Some embodiments may further include additional modifications to the furanose ring or nucleobase. Additional modifications may include, for example, sugar modifications or substitutions (*e.g.*, one or more of a 2'-O-alkyl modification, a locked nucleic acid (LNA)). In some embodiments, the RNAs may be complexed or hybridized with additional polynucleotides and/or peptide polynucleotides (PNA). In some embodiments where the sugar modification is a 2'-O-alkyl modification, such modification may include, but are not limited to a 2'-deoxy-2'-fluoro modification, a 2'-O-methyl modification, a 2'-O-methoxyethyl modification and a 2'-deoxy modification. In some embodiments, any of these modifications may be present in 0-100% of the nucleotides—for example, more than 0%, 1%, 10%, 25%, 50%, 75%, 85%, 90%, 95%, or 100% of the constituent nucleotides individually or in combination.

[0233] In some embodiments, mRNAs may contain RNA backbone modifications. Typically, a backbone modification is a modification in which the phosphates of the backbone of the nucleotides contained in the RNA are modified chemically. Exemplary backbone modifications typically include, but are not limited to, modifications from the group consisting of methylphosphonates, methylphosphoramidates, phosphoramidates, phosphorothioates (*e.g.*, cytidine 5'-O-(1-thiophosphate)), boranophosphates, positively charged guanidinium groups etc., which means by replacing the phosphodiester linkage by other anionic, cationic or neutral groups.

[0234] In some embodiments, mRNAs may contain sugar modifications. A typical sugar modification is a chemical modification of the sugar of the nucleotides it contains including, but not limited to, sugar modifications chosen from the group consisting of 2'-deoxy-2'-fluoro-oligoribonucleotide (2'-fluoro-2'-deoxycytidine 5'-triphosphate, 2'-fluoro-2'-deoxyuridine 5'-triphosphate), 2'-deoxy-2'-deamine-oligoribonucleotide (2'-amino-2'-deoxycytidine 5'-triphosphate, 2'-amino-2'-deoxyuridine 5'-triphosphate), 2'-O-alkyloligoribonucleotide, 2'-deoxy-2'-C-alkyloligoribonucleotide (2'-O-methylcytidine 5'-triphosphate, 2'-methyluridine 5'-triphosphate), 2'-C-alkyloligoribonucleotide, and isomers thereof (2'-aracytidine 5'-triphosphate,

2'-arauridine 5'-triphosphate), or azidotriphosphates (2'-azido-2'-deoxycytidine 5'-triphosphate, 2'-azido-2'-deoxyuridine 5'-triphosphate).

Post-synthesis processing

[0235] Typically, a 5' cap and/or a 3' tail may be added after the synthesis. The presence of the cap is important in providing resistance to nucleases found in most eukaryotic cells. The presence of a "tail" serves to protect the mRNA from exonuclease degradation.

[0236] A 5' cap is typically added as follows: first, an RNA terminal phosphatase removes one of the terminal phosphate groups from the 5' nucleotide, leaving two terminal phosphates; guanosine triphosphate (GTP) is then added to the terminal phosphates via a guanylyl transferase, producing a 5'5'5 triphosphate linkage; and the 7-nitrogen of guanine is then methylated by a methyltransferase. Examples of cap structures include, but are not limited to, m7G(5')ppp(5'(A,G(5')ppp(5')A and G(5')ppp(5')G. Additional cap structures are described in published U.S. Application No. US 2016/0032356 and published U.S. Application No. US 2018/0125989, which are incorporated herein by reference.

[0237] Typically, a tail structure includes a poly(A) and/or poly(C) tail. A poly-A or poly-C tail on the 3' terminus of mRNA typically includes at least 50 adenosine or cytosine nucleotides, at least 150 adenosine or cytosine nucleotides, at least 200 adenosine or cytosine nucleotides, at least 250 adenosine or cytosine nucleotides, at least 300 adenosine or cytosine nucleotides, at least 350 adenosine or cytosine nucleotides, at least 400 adenosine or cytosine nucleotides, at least 450 adenosine or cytosine nucleotides, at least 500 adenosine or cytosine nucleotides, at least 550 adenosine or cytosine nucleotides, at least 600 adenosine or cytosine nucleotides, at least 650 adenosine or cytosine nucleotides, at least 700 adenosine or cytosine nucleotides, at least 750 adenosine or cytosine nucleotides, at least 800 adenosine or cytosine nucleotides, at least 850 adenosine or cytosine nucleotides, at least 900 adenosine or cytosine nucleotides, at least 950 adenosine or cytosine nucleotides, or at least 1 kb adenosine or cytosine nucleotides, respectively. In some embodiments, a poly A or poly C tail may be about 10 to 800 adenosine or cytosine nucleotides (*e.g.*, about 10 to 200 adenosine or cytosine nucleotides, about 10 to 300 adenosine or cytosine nucleotides, about 10 to 400 adenosine or cytosine nucleotides, about 10 to 500 adenosine or cytosine nucleotides, about 10 to 550 adenosine or cytosine nucleotides, about 10 to 600 adenosine or cytosine nucleotides, about 50 to 600 adenosine or cytosine nucleotides, about 100 to 600 adenosine or cytosine nucleotides, about 150 to 600 adenosine or cytosine nucleotides, about 200 to 600 adenosine or cytosine nucleotides, about 250 to 600 adenosine or

cytosine nucleotides, about 300 to 600 adenosine or cytosine nucleotides, about 350 to 600 adenosine or cytosine nucleotides, about 400 to 600 adenosine or cytosine nucleotides, about 450 to 600 adenosine or cytosine nucleotides, about 500 to 600 adenosine or cytosine nucleotides, about 10 to 150 adenosine or cytosine nucleotides, about 10 to 100 adenosine or cytosine nucleotides, about 20 to 70 adenosine or cytosine nucleotides, or about 20 to 60 adenosine or cytosine nucleotides) respectively. In some embodiments, a tail structure includes is a combination of poly (A) and poly (C) tails with various lengths described herein. In some embodiments, a tail structure includes at least 50%, 55%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98%, or 99% adenosine nucleotides. In some embodiments, a tail structure includes at least 50%, 55%, 65%, 70%, 75%, 80%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98%, or 99% cytosine nucleotides.

[0238] As described herein, the addition of the 5' cap and/or the 3' tail facilitates the detection of abortive transcripts generated during *in vitro* synthesis because without capping and/or tailing, the size of those prematurely aborted mRNA transcripts can be too small to be detected. Thus, in some embodiments, the 5' cap and/or the 3' tail are added to the synthesized mRNA before the mRNA is tested for purity (*e.g.*, the level of abortive transcripts present in the mRNA). In some embodiments, the 5' cap and/or the 3' tail are added to the synthesized mRNA before the mRNA is purified as described herein. In other embodiments, the 5' cap and/or the 3' tail are added to the synthesized mRNA after the mRNA is purified as described herein.

[0239] mRNA synthesized may be used in the present invention without further purification. In particular, mRNA synthesized may be used according to the present invention without a step of removing shortmers. In some embodiments, mRNA synthesized may be further purified for use according to the present invention. Various methods may be used to purify mRNA synthesized. For example, purification of mRNA can be performed using centrifugation, filtration and /or chromatographic methods. In some embodiments, the synthesized mRNA is purified by ethanol precipitation or filtration or chromatography, or gel purification or any other suitable means. In some embodiments, the mRNA is purified by HPLC. In some embodiments, the mRNA is extracted in a standard phenol: chloroform : isoamyl alcohol solution, well known to one of skill in the art. In some embodiments, the mRNA is purified using Tangential Flow Filtration. Suitable purification methods include those described in published U.S. Application No. US 2016/0040154, published U.S. Application No. US 2015/0376220, published U.S. Application No. US 2018/0251755, published U.S. Application No. US 2018/0251754, U.S. Provisional Application No. 62/757,612 filed on November 8, 2018, and U.S. Provisional Application No. 62/891,781 filed on August 26, 2019,

all of which are incorporated by reference herein and may be used to practice the present invention.

[0240] In some embodiments, the mRNA is purified before capping and tailing. In some embodiments, the mRNA is purified after capping and tailing. In some embodiments, the mRNA is purified both before and after capping and tailing.

[0241] In some embodiments, the mRNA is purified either before or after or both before and after capping and tailing, by centrifugation.

[0242] In some embodiments, the mRNA is purified either before or after or both before and after capping and tailing, by filtration.

[0243] In some embodiments, the mRNA is purified either before or after or both before and after capping and tailing, by Tangential Flow Filtration (TFF).

[0244] In some embodiments, the mRNA is purified either before or after or both before and after capping and tailing by chromatography.

[0245] In some embodiments, the mRNA is purified without the use of ethanol or any other flammable solvent.

Characterization of purified mRNA

[0246] The mRNA composition described herein is substantially free of contaminants comprising short abortive RNA species, long abortive RNA species, double-stranded RNA (dsRNA), residual plasmid DNA, residual *in vitro* transcription enzymes, residual solvent and/or residual salt.

[0247] The mRNA composition described herein has a purity of about between 60% and about 100%. Accordingly, in some embodiments, the purified mRNA has a purity of about 60%. In some embodiments, the purified mRNA has a purity of about 65%. In some embodiments, the purified mRNA has a purity of about 70%. In some embodiments, the purified mRNA has a purity of about 75%. In some embodiments, the purified mRNA has a purity of about 80%. In some embodiments, the purified mRNA has a purity of about 85%. In some embodiments, the purified mRNA has a purity of about 90%. In some embodiments, the purified mRNA has a purity of about 91%. In some embodiments, the purified mRNA has a purity of about 92%. In some embodiments, the purified mRNA has a purity of about 93%. In some embodiments, the purified mRNA has a purity of about 94%. In some embodiments, the purified mRNA has a purity of about 95%. In some embodiments, the purified mRNA has a purity of about 96%. In some

embodiments, the purified mRNA has a purity of about 97%. In some embodiments, the purified mRNA has a purity of about 98%. In some embodiments, the purified mRNA has a purity of about 99%. In some embodiments, the purified mRNA has a purity of about 100%.

[0248] In some embodiments, the mRNA composition described herein has less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.5%, and/or less than 0.1% impurities other than full-length mRNA. The impurities include IVT contaminants, *e.g.*, proteins, enzymes, DNA templates, free nucleotides, residual solvent, residual salt, double-stranded RNA (dsRNA), prematurely aborted RNA sequences (“shortmers” or “short abortive RNA species”), and/or long abortive RNA species. In some embodiments, the purified mRNA is substantially free of process enzymes.

[0249] In some embodiments, the residual plasmid DNA in the purified mRNA of the present invention is less than about 1 pg/mg, less than about 2 pg/mg, less than about 3 pg/mg, less than about 4 pg/mg, less than about 5 pg/mg, less than about 6 pg/mg, less than about 7 pg/mg, less than about 8 pg/mg, less than about 9 pg/mg, less than about 10 pg/mg, less than about 11 pg/mg, or less than about 12 pg/mg. Accordingly, the residual plasmid DNA in the purified mRNA is less than about 1 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 2 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 3 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 4 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 5 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 6 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 7 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 8 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 9 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 10 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 11 pg/mg. In some embodiments, the residual plasmid DNA in the purified mRNA is less than about 12 pg/mg.

[0250] In some embodiments, a method according to the invention removes more than about 90%, 95%, 96%, 97%, 98%, 99% or substantially all prematurely aborted RNA sequences (also known as “shortmers”). In some embodiments, mRNA composition is substantially free of prematurely aborted RNA sequences. In some embodiments, mRNA composition contains less than about 5% (*e.g.*, less than about 4%, 3%, 2%, or 1%) of prematurely aborted RNA sequences.

In some embodiments, mRNA composition contains less than about 1% (*e.g.*, less than about 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1%) of prematurely aborted RNA sequences. In some embodiments, mRNA composition undetectable prematurely aborted RNA sequences as determined by, *e.g.*, high-performance liquid chromatography (HPLC) (*e.g.*, shoulders or separate peaks), ethidium bromide, Coomassie staining, capillary electrophoresis or Glyoxal gel electrophoresis (*e.g.*, presence of separate lower band). As used herein, the term “shortmers”, “short abortive RNA species”, “prematurely aborted RNA sequences” or “long abortive RNA species” refers to any transcripts that are less than full-length. In some embodiments, “shortmers”, “short abortive RNA species”, or “prematurely aborted RNA sequences” are less than 100 nucleotides in length, less than 90, less than 80, less than 70, less than 60, less than 50, less than 40, less than 30, less than 20, or less than 10 nucleotides in length. In some embodiments, shortmers are detected or quantified after adding a 5'-cap, and/or a 3'-poly A tail. In some embodiments, prematurely aborted RNA transcripts comprise less than 15 bases (*e.g.*, less than 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, or 3 bases). In some embodiments, the prematurely aborted RNA transcripts contain about 8-15, 8-14, 8-13, 8-12, 8-11, or 8-10 bases.

[0251] In some embodiments, a purified mRNA of the present invention is substantially free of enzyme reagents used in *in vitro* synthesis including, but not limited to, T7 RNA polymerase, DNase I, pyrophosphatase, and/or RNase inhibitor. In some embodiments, a purified mRNA according to the present invention contains less than about 5% (*e.g.*, less than about 4%, 3%, 2%, or 1%) of enzyme reagents used in *in vitro* synthesis. In some embodiments, a purified mRNA contains less than about 1% (*e.g.*, less than about 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, or 0.1%) of enzyme reagents used in *in vitro* synthesis including. In some embodiments, a purified mRNA contains undetectable enzyme reagents used in *in vitro* synthesis including as determined by, *e.g.*, silver stain, gel electrophoresis, high-performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), and/or capillary electrophoresis, ethidium bromide and/or Coomassie staining.

[0252] In various embodiments, a purified mRNA of the present invention maintains high degree of integrity. As used herein, the term “mRNA integrity” generally refers to the quality of mRNA after purification. mRNA integrity may be determined using methods well known in the art, for example, by RNA agarose gel electrophoresis. In some embodiments, mRNA integrity may be determined by banding patterns of RNA agarose gel electrophoresis. In some embodiments, a purified mRNA of the present invention shows little or no banding compared to reference band of RNA agarose gel electrophoresis. In some embodiments, a purified mRNA of the present invention

has an integrity greater than about 95% (*e.g.*, greater than about 96%, 97%, 98%, 99% or more). In some embodiments, a purified mRNA of the present invention has an integrity greater than 98%. In some embodiments, a purified mRNA of the present invention has an integrity greater than 99%. In some embodiments, a purified mRNA of the present invention has an integrity of approximately 100%.

[0253] In some embodiments, the purified mRNA is assessed for one or more of the following characteristics: appearance, identity, quantity, concentration, presence of impurities, microbiological assessment, pH level and activity. In some embodiments, acceptable appearance includes a clear, colorless solution, essentially free of visible particulates. In some embodiments, the identity of the mRNA is assessed by sequencing methods. In some embodiments, the concentration is assessed by a suitable method, such as UV spectrophotometry. In some embodiments, a suitable concentration is between about 90% and 110% nominal (0.9-1.1 mg/mL).

[0254] In some embodiments, assessing the purity of the mRNA includes assessment of mRNA integrity, assessment of residual plasmid DNA, and assessment of residual solvent. In some embodiments, acceptable levels of mRNA integrity are assessed by agarose gel electrophoresis. The gels are analyzed to determine whether the banding pattern and apparent nucleotide length is consistent with an analytical reference standard. Additional methods to assess RNA integrity include, for example, assessment of the purified mRNA using capillary gel electrophoresis (CGE). In some embodiments, acceptable purity of the purified mRNA as determined by CGE is that the purified mRNA composition has no greater than about 55% long abortive/degraded species. In some embodiments, residual plasmid DNA is assessed by methods in the art, for example by the use of qPCR. In some embodiments, less than 10 pg/mg (*e.g.*, less than 10 pg/mg, less than 9 pg/mg, less than 8 pg/mg, less than 7 pg/mg, less than 6 pg/mg, less than 5 pg/mg, less than 4 pg/mg, less than 3 pg/mg, less than 2 pg/mg, or less than 1 pg/mg) is an acceptable level of residual plasmid DNA. In some embodiments, acceptable residual solvent levels are not more than 10,000 ppm, 9,000 ppm, 8,000 ppm, 7,000 ppm, 6,000 ppm, 5,000 ppm, 4,000 ppm, 3,000 ppm, 2,000 ppm, 1,000 ppm. Accordingly, in some embodiments, acceptable residual solvent levels are not more than 10,000 ppm. In some embodiments, acceptable residual solvent levels are not more than 9,000 ppm. In some embodiments, acceptable residual solvent levels are not more than 8,000 ppm. In some embodiments, acceptable residual solvent levels are not more than 7,000 ppm. In some embodiments, acceptable residual solvent levels are not more than 6,000 ppm. In some embodiments, acceptable residual solvent levels are not more than 5,000 ppm. In some embodiments, acceptable residual solvent levels are not more than 4,000 ppm. In some

embodiments, acceptable residual solvent levels are not more than 3,000 ppm. In some embodiments, acceptable residual solvent levels are not more than 2,000 ppm. In some embodiments, acceptable residual solvent levels are not more than 1,000 ppm.

[0255] In some embodiments, microbiological tests are performed on the purified mRNA, which include, for example, assessment of bacterial endotoxins. In some embodiments, bacterial endotoxins are < 0.5 EU/mL, <0.4 EU/mL, <0.3 EU/mL, <0.2 EU/mL or <0.1 EU/mL. Accordingly, in some embodiments, bacterial endotoxins in the purified mRNA are < 0.5 EU/mL. In some embodiments, bacterial endotoxins in the purified mRNA are < 0.4 EU/mL. In some embodiments, bacterial endotoxins in the purified mRNA are < 0.3 EU/mL. In some embodiments, bacterial endotoxins in the purified mRNA are < 0.2 EU/mL. In some embodiments, bacterial endotoxins in the purified mRNA are < 0.2 EU/mL. In some embodiments, bacterial endotoxins in the purified mRNA are < 0.1 EU/mL. In some embodiments, the purified mRNA has not more than 1 CFU/10mL, 1 CFU/25mL, 1CFU/50mL, 1CFU/75mL, or not more than 1 CFU/100mL. Accordingly, in some embodiments, the purified mRNA has not more than 1 CFU/10 mL. In some embodiments, the purified mRNA has not more than 1 CFU/25 mL. In some embodiments, the purified mRNA has not more than 1 CFU/50 mL. In some embodiments, the purified mRNA has not more than 1 CFR/75 mL. In some embodiments, the purified mRNA has 1 CFU/100 mL.

[0256] In some embodiments, the pH of the purified mRNA is assessed. In some embodiments, acceptable pH of the purified mRNA is between 5 and 8. Accordingly, in some embodiments, the purified mRNA has a pH of about 5. In some embodiments, the purified mRNA has a pH of about 6. In some embodiments, the purified mRNA has a pH of about 7. In some embodiments, the purified mRNA has a pH of about 7.5. In some embodiments, the purified mRNA has a pH of about 8.

[0257] In some embodiments, the translational fidelity of the purified mRNA is assessed. The translational fidelity can be assessed by various methods and include, for example, transfection and Western blot analysis. Acceptable characteristics of the purified mRNA includes banding pattern on a Western blot that migrates at a similar molecular weight as a reference standard.

[0258] In some embodiments, the purified mRNA is assessed for conductance. In some embodiments, acceptable characteristics of the purified mRNA include a conductance of between about 50% and 150% of a reference standard.

[0259] The purified mRNA is also assessed for Cap percentage and for PolyA tail length. In some embodiments, an acceptable Cap percentage includes Cap1, % Area: NLT90. In some embodiments, an acceptable PolyA tail length is about 100 -1500 nucleotides (*e.g.*, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, and 1000, 1100, 1200, 1300, 1400, or 1500 nucleotides).

[0260] In some embodiments, the purified mRNA is also assessed for any residual PEG. In some embodiments, the purified mRNA has less than between 10 ng PEG/mg of purified mRNA and 1000 ng PEG/mg of mRNA. Accordingly, in some embodiments, the purified mRNA has less than about 10 ng PEG/mg of purified mRNA. In some embodiments, the purified mRNA has less than about 100 ng PEG/mg of purified mRNA. In some embodiments, the purified mRNA has less than about 250 ng PEG/mg of purified mRNA. In some embodiments, the purified mRNA has less than about 500 ng PEG/mg of purified mRNA. In some embodiments, the purified mRNA has less than about 750 ng PEG/mg of purified mRNA. In some embodiments, the purified mRNA has less than about 1000 ng PEG/mg of purified mRNA.

[0261] Various methods of detecting and quantifying mRNA purity are known in the art. For example, such methods include, blotting, capillary electrophoresis, chromatography, fluorescence, gel electrophoresis, HPLC, silver stain, spectroscopy, ultraviolet (UV), or UPLC, or a combination thereof. In some embodiments, mRNA is first denatured by a Glyoxal dye before gel electrophoresis ("Glyoxal gel electrophoresis"). In some embodiments, synthesized mRNA is characterized before capping or tailing. In some embodiments, synthesized mRNA is characterized after capping and tailing.

Therapeutic Use of Compositions

[0262] To facilitate expression of mRNA *in vivo*, delivery vehicles such as liposomes can be formulated in combination with one or more additional nucleic acids, carriers, targeting ligands or stabilizing reagents, or in pharmacological compositions where it is mixed with suitable excipients. Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., latest edition.

[0263] In some embodiments, a composition comprises mRNA encapsulated or complexed with a delivery vehicle. In some embodiments, the delivery vehicle is selected from the

group consisting of liposomes, lipid nanoparticles, solid-lipid nanoparticles, polymers, viruses, sol-gels, and nanogels.

[0264] Provided mRNA-loaded nanoparticles, and compositions containing the same, may be administered and dosed in accordance with current medical practice, taking into account the clinical condition of the subject, the site and method of administration, the scheduling of administration, the subject's age, sex, body weight and other factors relevant to clinicians of ordinary skill in the art. The "effective amount" for the purposes herein may be determined by such relevant considerations as are known to those of ordinary skill in experimental clinical research, pharmacological, clinical, and medical arts. In some embodiments, the amount administered is effective to achieve at least some stabilization, improvement or elimination of symptoms and other indicators as are selected as appropriate measures of disease progress, regression or improvement by those of skill in the art. For example, a suitable amount and dosing regimen is one that causes at least transient protein (*e.g.*, enzyme) production.

[0265] The present invention provides methods of delivering mRNA for *in vivo* protein production, comprising administering mRNA to a subject in need of delivery. In some embodiments, mRNA is administered via a route of delivery selected from the group consisting of intravenous delivery, subcutaneous delivery, oral delivery, subdermal delivery, ocular delivery, intratracheal injection pulmonary delivery (*e.g.* nebulization or instillation), intramuscular delivery, intrathecal delivery, or intraarticular delivery. Accordingly, in some embodiments, the present invention provides methods of delivering mRNA for *in vivo* protein production comprising intravenous delivery. In some embodiments, the present invention provides methods of delivering mRNA for *in vivo* protein production comprising intramuscular delivery. In some embodiments, the present invention provides methods of delivering mRNA for *in vivo* protein production comprising intratracheal injection pulmonary delivery.

[0266] The development of ethanol-free LNP formulations greatly reduces and/or eliminates fire safety concerns and also allows for bedside mixing leading to the production of low-volume formulations with 1:1 citrate-mRNA to solvent-lipid ratios that would be more suitable for dosing. Accordingly, in some embodiments, the mRNA LNP formulations are suitable for preparation and administration in various settings, including for example bedside mixing, hospital on-site mixing, and pharmacy on-site mixing.

[0267] Suitable routes of administration include, for example, oral, rectal, vaginal, transmucosal, pulmonary including intratracheal or inhaled, or intestinal administration;

parenteral delivery, including intradermal, transdermal (topical), intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, or intranasal. In some embodiments, the intramuscular administration is to a muscle selected from the group consisting of skeletal muscle, smooth muscle and cardiac muscle. In some embodiments the administration results in delivery of the mRNA to a muscle cell. In some embodiments the administration results in delivery of the mRNA to a hepatocyte (*i.e.*, liver cell). In a particular embodiment, the intramuscular administration results in delivery of the mRNA to a muscle cell.

[0268] Additional teaching of pulmonary delivery and nebulization are described in published U.S. Application No. US 2018/0125989 and published U.S. Application No. US 2018/0333457, each of which is incorporated by reference in its entirety.

[0269] Alternatively or additionally, mRNA-loaded nanoparticles and compositions of the invention may be administered in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a targeted tissue, preferably in a sustained release formulation. Local delivery can be affected in various ways, depending on the tissue to be targeted. For example, aerosols containing compositions of the present invention can be inhaled (for nasal, tracheal, or bronchial delivery); compositions of the present invention can be injected into the site of injury, disease manifestation, or pain, for example; compositions can be provided in lozenges for oral, tracheal, or esophageal application; can be supplied in liquid, tablet or capsule form for administration to the stomach or intestines, can be supplied in suppository form for rectal or vaginal application; or can even be delivered to the eye by use of creams, drops, or even injection. Formulations containing provided compositions complexed with therapeutic molecules or ligands can even be surgically administered, for example in association with a polymer or other structure or substance that can allow the compositions to diffuse from the site of implantation to surrounding cells. Alternatively, they can be applied surgically without the use of polymers or supports.

[0270] Provided methods of the present invention contemplate single as well as multiple administrations of a therapeutically effective amount of the therapeutic agents (*e.g.*, mRNA) described herein. Therapeutic agents can be administered at regular intervals, depending on the nature, severity and extent of the subject's condition. In some embodiments, a therapeutically effective amount of the therapeutic agents (*e.g.*, mRNA) of the present invention may be administered intrathecally periodically at regular intervals (*e.g.*, once every year, once every six-

months, once every five-months, once every three-months, bimonthly (once every two-months), monthly (once every month), biweekly (once every two-weeks), twice a month, once every 30-days, once every 28-days, once every 14-days, once every 10-days, once every 7-days, weekly, twice a week, daily, or continuously).

[0271] In some embodiments, provided liposomes and/or compositions are formulated such that they are suitable for extended-release of the mRNA contained therein. Such extended-release compositions may be conveniently administered to a subject at extended dosing intervals. For example, in one embodiment, the compositions of the present invention are administered to a subject twice a day, daily, or every other day. In a preferred embodiment, the compositions of the present invention are administered to a subject twice a week, once a week, once every 7-days, once every 10-days, once every 14-days, once every 28-days, once every 30-days, once every two-weeks, once every three-weeks, or more-preferably once every four-weeks, once-a-month, twice-a-month, once every six-weeks, once every eight-weeks, once every other month, once every three-months, once every four-months, once every six-months, once every eight-months, once every nine-months, or annually. Also contemplated are compositions and liposomes that are formulated for depot administration (*e.g.*, intramuscularly, subcutaneously, intravitreally) to either deliver or release therapeutic agent (*e.g.*, mRNA) over extended periods of time. Preferably, the extended-release means employed are combined with modifications made to the mRNA to enhance stability.

[0272] As used herein, the term “therapeutically effective amount” is largely determined based on the total amount of the therapeutic agent contained in the pharmaceutical compositions of the present invention. Generally, a therapeutically effective amount is sufficient to achieve a meaningful benefit to the subject (*e.g.*, treating, modulating, curing, preventing and/or ameliorating a disease or disorder). For example, a therapeutically effective amount may be an amount sufficient to achieve a desired therapeutic and/or prophylactic effect. Generally, the amount of a therapeutic agent (*e.g.*, mRNA) administered to a subject in need thereof will depend upon the characteristics of the subject. Such characteristics include the condition, disease severity, general health, age, sex and body weight of the subject. One of ordinary skill in the art will be readily able to determine appropriate dosages depending on these and other related factors. In addition, both objective and subjective assays may optionally be employed to identify optimal dosage ranges.

[0273] A therapeutically effective amount is commonly administered in a dosing regimen that may comprise multiple unit doses. For any particular therapeutic protein, a therapeutically effective amount (and/or an appropriate unit dose within an effective dosing regimen) may vary, for example, depending on route of administration, on combination with other pharmaceutical agents. Also, the specific therapeutically effective amount (and/or unit dose) for any particular patient may depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific pharmaceutical agent employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and/or rate of excretion or metabolism of the specific protein employed; the duration of the treatment; and like factors as is well known in the medical arts.

[0274] In some embodiments, the therapeutically effective dose ranges from about 0.005 mg/kg body weight to 500 mg/kg body weight, *e.g.*, from about 0.005 mg/kg body weight to 400 mg/kg body weight, from about 0.005 mg/kg body weight to 300 mg/kg body weight, from about 0.005 mg/kg body weight to 200 mg/kg body weight, from about 0.005 mg/kg body weight to 100 mg/kg body weight, from about 0.005 mg/kg body weight to 90 mg/kg body weight, from about 0.005 mg/kg body weight to 80 mg/kg body weight, from about 0.005 mg/kg body weight to 70 mg/kg body weight, from about 0.005 mg/kg body weight to 60 mg/kg body weight, from about 0.005 mg/kg body weight to 50 mg/kg body weight, from about 0.005 mg/kg body weight to 40 mg/kg body weight, from about 0.005 mg/kg body weight to 30 mg/kg body weight, from about 0.005 mg/kg body weight to 25 mg/kg body weight, from about 0.005 mg/kg body weight to 20 mg/kg body weight, from about 0.005 mg/kg body weight to 15 mg/kg body weight, from about 0.005 mg/kg body weight to 10 mg/kg body weight.

[0275] In some embodiments, the therapeutically effective dose is greater than about 0.1 mg/kg body weight, greater than about 0.5 mg/kg body weight, greater than about 1.0 mg/kg body weight, greater than about 3 mg/kg body weight, greater than about 5 mg/kg body weight, greater than about 10 mg/kg body weight, greater than about 15 mg/kg body weight, greater than about 20 mg/kg body weight, greater than about 30 mg/kg body weight, greater than about 40 mg/kg body weight, greater than about 50 mg/kg body weight, greater than about 60 mg/kg body weight, greater than about 70 mg/kg body weight, greater than about 80 mg/kg body weight, greater than about 90 mg/kg body weight, greater than about 100 mg/kg body weight, greater than about 150 mg/kg body weight, greater than about 200 mg/kg body weight, greater than about 250 mg/kg body weight, greater than about 300 mg/kg body weight, greater than about 350

mg/kg body weight, greater than about 400 mg/kg body weight, greater than about 450 mg/kg body weight, greater than about 500 mg/kg body weight. In a particular embodiment, the therapeutically effective dose is 1.0 mg/kg. In some embodiments, the therapeutically effective dose of 1.0 mg/kg is administered intramuscularly or intravenously.

[0276] Also contemplated herein are lyophilized pharmaceutical compositions comprising one or more of the liposomes disclosed herein and related methods for the use of such compositions as disclosed for example, in United States Provisional Application No. 61/494,882, filed June 8, 2011, the teachings of which are incorporated herein by reference in their entirety. For example, lyophilized pharmaceutical compositions according to the invention may be reconstituted prior to administration or can be reconstituted *in vivo*. For example, a lyophilized pharmaceutical composition can be formulated in an appropriate dosage form (*e.g.*, an intradermal dosage form such as a disk, rod or membrane) and administered such that the dosage form is rehydrated over time *in vivo* by the individual's bodily fluids.

[0277] Provided liposomes and compositions may be administered to any desired tissue. In some embodiments, the mRNA delivered by provided liposomes or compositions is expressed in the tissue in which the liposomes and/or compositions were administered. In some embodiments, the mRNA delivered is expressed in a tissue different from the tissue in which the liposomes and/or compositions were administered. Exemplary tissues in which delivered mRNA may be delivered and/or expressed include, but are not limited to the liver, kidney, heart, spleen, serum, brain, skeletal muscle, lymph nodes, skin, and/or cerebrospinal fluid.

[0278] In some embodiments, administering the provided composition results in an increased mRNA expression level in a biological sample from a subject as compared to a baseline expression level before treatment. Typically, the baseline level is measured immediately before treatment. Biological samples include, for example, whole blood, serum, plasma, urine and tissue samples (*e.g.*, muscle, liver, skin fibroblasts). In some embodiments, administering the provided composition results in an increased mRNA expression level by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% as compared to the baseline level immediately before treatment. In some embodiments, administering the provided composition results in an increased mRNA expression level as compared to an mRNA expression level in subjects who are not treated

[0279] According to various embodiments, the timing of expression of delivered mRNA can be tuned to suit a particular medical need. In some embodiments, the expression of the protein encoded by delivered mRNA is detectable 1, 2, 3, 6, 12, 24, 48, 72, and/or 96 hours after

administration of provided liposomes and/or compositions. In some embodiments, the expression of the protein encoded by delivered mRNA is detectable one-week, two-weeks, and/or one-month after administration.

[0280] The present invention also provides delivering a composition having mRNA molecules encoding a peptide or polypeptide of interest for use in the treatment of a subject, *e.g.*, a human subject or a cell of a human subject or a cell that is treated and delivered to a human subject.

EXAMPLES

[0281] While certain compounds, compositions and methods of the present invention have been described with specificity in accordance with certain embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

Example 1. Encapsulation efficiency of ethanol-free lipid nanoparticle (LNP) formulations using polymer as a solvent instead of ethanol.

[0282] This example illustrates the encapsulation efficiency achieved by using Triethylene glycol monomethyl ether (mTEG) as an exemplary solvent in dissolving various cationic lipids, including ML-2 and ICE in the production of ethanol-free LNP formulations. Development of ethanol free LNP formulations would greatly reduce and/or eliminate fire safety concerns and also allow for bedside mixing leading to the production of low-volume formulations with 1:1 citrate-mRNA to solvent-lipid ratios that would be more suitable for dosing. Such low-volume formulations are currently difficult to obtain using ethanol as a solvent.

[0283] Exemplary LNP formulations were prepared by mixing mRNA in an aqueous solution with lipids (*e.g.*, cationic lipids, non-cationic lipids, and PEG-modified lipids), dissolved in an amphiphilic polymer solution to form mRNA encapsulated within LNPs (mRNA-LNPs). In this example, the lipids were prepared in an ethanol-free mTEG solution or in an ethanol-based solution. Two different cationic lipids were assessed, ML-2 and ICE and the same ratio of PEG: Cationic lipid: Cholesterol: Non-cationic Lipid was used for each cationic lipid in both the ethanol-free polymer mixture and in the ethanol-containing mixture (see **Table 2**). Particle size, polydispersity index and encapsulation efficiency of the LNPs from the ethanol-free polymer mixture and from the ethanol-containing mixture were assessed.

Table 2. Average particle size, polydispersity index and encapsulation efficiency of mRNA-LNPs prepared using an ethanol-free mTEG mixture versus an ethanol mixture, for ML-2 and ICE cationic lipids.

Formulation Lot	mTEG or Standard	Cationic Lipid	Composition (PEG:Cat:Chol:DSPC)	Size	PDI	%EE
A	EtOH	ML-2	5:40:25:30	80	0.174	98
B	mTEG	ML-2	5:40:25:30	82	0.276	98
N	EtOH	ICE	5:60:0:35	57	0.180	70
O	mTEG	ICE	5:60:0:35	65	0.293	78

[0284] As seen in Table 2, mTEG-prepared formulations yielded LNPs of comparable size with equivalent or improved encapsulation efficiencies as compared to ethanol-prepared LNP formulations. Polydispersity indices were observed to be slightly higher in the mTEG formulations relative to ethanol formulations.

[0285] The results indicated that mTEG can be used to dissolve lipids allowing for safe manufacture of ethanol-free LNP formulations with equivalent or improved encapsulation efficiencies.

Example 2. Encapsulation efficiency of mRNA-LNPs prepared using polymer solvent for lipids relative to using ethanol solvent for lipids

[0286] This example illustrates the average particle size, polydispersity index (“PDI”) and encapsulation efficiency obtained in LNP formulations formulated using a polymer solvent for lipids relative to LNP formulations formulated using ethanol solvent for lipids. The LNPs formulated using a polymer solvent (mTEG) or ethanol, were comprised of a PEG-modified lipid, a cationic lipid of either ML-2 or MC3, cholesterol and a helper lipid (DSPC).

[0287] Exemplary mRNA-LNP formulations were prepared by dissolving the lipids for the LNP in a 100% mTEG solution or in 100% ethanol solution. The lipids included either ML-2 or MC-3 as the cationic lipid, PEG-modified lipid, cholesterol and a helper lipid (DSPC). The mTEG-lipid or ethanol-lipid solution was mixed with an aqueous solution of mRNA (in a citrate buffer) at volumetric ratio of 1 to 4 (mTEG-lipid solution to mRNA solution or ethanol-lipid solution to mRNA solution, respectively.. Prior to mixing, the mRNA in the aqueous solution was at a concentration

of 0.08 mg/mL and the lipids in the lipid solution were at a concentration needed to provide a cationic lipid (ML-2 or MC-3) to mRNA N/P ratio of 4. The PEG-modified lipids, the cholesterol and the helper lipid (DSPC) concentrations were prepared according to the target ratios (relative to cationic lipid) provided in Table 3. Particle size, polydispersity index and encapsulation efficiency of the resulting mRNA-LNPs were analyzed (Table 3).

Table 3. Average particle size, polydispersity index and encapsulation efficiency of mRNA-LNPs prepared from a 1:4 lipid solution volume to mRNA solution volume mixing, where the lipid solution was either an ethanol-free polymer solvent or an ethanol solvent.

Formulation Lot	mTEG or Standard	Cationic Lipid	Composition (PEG:Cat:Chol:DSPC)	Size	PDI	%EE
A	EtOH (1:4)	ML-2	5:40:25:30	69	0.229	86
B	mTEG (1:4)	ML-2	5:40:25:30	82	0.247	86
C	EtOH (1:4)	MC-3	5:50:35:10	55	0.152	90
D	mTEG (1:4)	MC-3	5:50:35:10	56	0.229	97

[0288] As shown in Table 3, mRNA-LNPs of comparable size were obtained in the mTEG-prepared and ethanol-prepared formulations where MC-3 was the cationic lipid. For formulations having ML-2 as the cationic lipid, the mRNA-LNPs were larger for those obtained from the mTEG-prepared lipids versus the ethanol-prepared lipids. A higher encapsulation efficiency of 97% was obtained in the mTEG-prepared MC-3 mRNA-LNP formulation as compared to 90% encapsulation efficiency obtained in the corresponding ethanol-prepared MC-3 mRNA-LNP formulations. For formulations having ML-2 as the cationic lipid, the encapsulation efficiency was comparable for mRNA-LNPs obtained from the mTEG-prepared lipids versus the ethanol-prepared lipids. There was an increase in the polydispersity index in the mTEG-prepared MC-3 mRNA-LNP formulation as compared to the ethanol-prepared mRNA-LNP formulation.

[0289] These results showed that a polymer-prepared LNP formulation, particularly an mTEG-prepared LNP formulation, provides comparable LNP size and potentially higher encapsulation efficiency as compared to an ethanol-prepared LNP formulation. Using mTEG instead of ethanol to prepare mRNA-encapsulated LNPs also can be advantageous because ethanol-free mTEG formulations can be manufactured at large scale more safely as compared to

ethanol based formulations and may require less subsequent processing to remove the solvent used for the lipid composition.

Example 3. Ethanol-free formulations require lower volumetric mixing

[0290] This example shows a significant advantage of using an mTEG solvent versus an ethanol solvent for lipids in the preparation of mRNA-LNPs, particularly with respect to lowering the needed volumes in preparing mRNA-LNP formulations, for example, for dosing. In particular, mixing 100% ethanol solution comprising lipids with an aqueous solution comprising mRNA at a 1:1 (v/v) ratio can result in unstable mRNA solubility, and in some cases mRNA precipitation, due to the high concentration (50% vol/vol) of ethanol in the resulting mixture. Accordingly, prior to the present invention, approaches to address this issue included diluting the ethanol component in the ethanol-lipid solution (which can impact lipid solubility) and/or increasing the volume of aqueous-mRNA solution and/or adding a third stream of aqueous solution, in order to yield a lower ethanol concentration in the resulting mixture and thereby avoid mRNA instability and possible mRNA precipitation. For example, mixing the ethanol-lipid solution (having 100% ethanol) with aqueous-mRNA solution at ration of 1:4 (v/v) yields a lower amount of ethanol (20% v/v) in the resulting mixture, thereby helping to avoid mRNA instability and precipitation caused by ethanol. Unfortunately, these approaches all require mixing of larger volumes than would otherwise be necessary (e.g., as might be dictated by the lowest solubility concentration and a desired N/P ratio), typically with substantially diluted amounts of lipid and mRNA in the respective solutions, which at large-scale processing levels can significantly increase time and cost, as well as require subsequent concentration steps, which further increase time and cost, in addition to ethanol removal steps.

[0291] However, when a 100% mTEG solution comprising lipids is mixed with an aqueous solution comprising mRNA at a 1:1 (v/v) ratio, to generate mRNA-LNPs, the high concentration of mTEG (50% v/v) in the resulting mixture does not appear to result in mRNA instability or precipitation. Such mRNA-LNP formulations prepared at large scale using optimized low volumes of mTEG solution comprising lipids and aqueous solution comprising mRNA, i.e., having high lipid and mRNA concentrations, respectively, can be advantageous in reducing processing volumes and thereby increasing ease of processing in manufacturing.

[0292] In order to assess the ability of mTEG solvent for lipids versus ethanol solvent for lipids to optimize volumes used in mRNA-LNP formulations, mRNA-LNP formulations were prepared at a low volume ratio (1:1 lipid volume to mRNA volume) and at a high volume ratio (1:4

lipid volume to mRNA volume) with the lipid volume comprising lipids dissolved either in 100% mTEG or in 100% ethanol. The dissolved lipids included a PEG-modified lipid, a cationic lipid of either ML-2 or MC3, cholesterol and a helper lipid (DSPC). Prior to mixing, the mRNA aqueous solution for the low volume mixing had an mRNA concentration of 0.33 mg/mL, four times the mRNA concentration of 0.08 mg/mL in the mRNA aqueous solution for high volume mixing, so that the same total amount of mRNA was mixed in each process. In the lipid solution, the lipids (in either 100% mTEG or 100% ethanol solution) were at a concentration needed to provide a cationic lipid (ML-2 or MC-3) to mRNA N/P ratio of 4, with the PEG-modified lipids, the cholesterol and the helper lipid (DSPC) concentrations prepared according to the target ratios (relative to cationic lipid) provided in Table 4. Each preparation was mixed at the low volume (1:1 lipid solution to mRNA solution) or at the high volume (1:4 lipid solution to mRNA solution) volumes and the resulting mRNA-LNPs were assessed for size, polydispersity (PDI) and percent encapsulation of mRNA (%EE).

[0293] Low-volume (1:1) mRNA-LNP formulations prepared using lipids including ML-2 as the cationic lipid and dissolved in mTEG achieved a 69% encapsulation efficiency. In contrast, low-volume (1:1) mRNA-LNP formulations prepared using lipids including ML-2 as the cationic lipid and dissolved in ethanol could not be stably produced in a low volume formulation and showed precipitation following mixing.

[0294] Low-volume (1:1) mRNA-LNP formulations prepared using lipids including MC-3 as the cationic lipid and dissolved in mTEG achieved 99% encapsulation efficiency, while low-volume (1:1) mRNA-LNP formulations prepared using lipids including MC-3 as the cationic lipid and dissolved in ethanol showed 95% encapsulation.

Table 4. Average particle size, polydispersity index and encapsulation efficiency of high-volume (1:4) and low-volume (1:1) ethanol-free mRNA-LNP formulations compared to ethanol-based mRNA-LNP formulations

Formulation Lot	mTEG or Standard	Cationic Lipid	Composition (PEG:Cat:Chol:DSPC)	Size	PDI	%EE
High-volume 1:4 Formulations						
A	EtOH (1:4)	ML-2	5:40:25:30	69	0.229	86
B	mTEG (1:4)	ML-2	5:40:25:30	82	0.247	86

C	EtOH (1:4)	MC-3	5:50:35:10	55	0.152	90
D	mTEG (1:4)	MC-3	5:50:35:10	56	0.229	97
Low-volume 1:1 Formulations						
E	EtOH (1:1)	ML-2	5:40:25:30	Precipitated after T-mix		
F	mTEG (1:1)	ML-2	5:40:25:30	101	0.197	69
G	EtOH (1:1)	MC-3	5:50:35:10	159	0.114	95
H	mTEG (1:1)	MC-3	5:50:35:10	85	0.160	99

[0295] The results indicated that mTEG was suitable for use in a low-volume, ethanol-free mRNA-LNP formulations and provided improved mRNA stability after mixing and potentially improved encapsulation efficiency relative to an ethanol-prepared LNP formulation.

Example 4. Testing ethanol-free formulations using various polymers and lipids

[0296] This example will test ethanol-free LNP formulations using various polymers and lipids.

[0297] LNP formulations will be prepared using various amphiphilic polymers, including but not limited to polyethylene glycol (PEG), mPEG, Tetraethylene glycol monomethyl ether and Pentaethylene glycol monomethyl ether.

[0298] LNP formulations will be prepared using one or more non-cationic lipids, including dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), palmitoyl-oleoylphosphatidylcholine (POPC), palmitoyl-oleoyl-phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-l-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), phosphatidylserine, sphingolipids, cerebrosides, gangliosides, 16-O-monomethyl PE, and 16-O-dimethyl PE, 18-1-trans PE, l-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE).

[0299] LNP formulations using components that do not show degradation will be further analyzed for encapsulation efficiency, LNP size and polydispersity index as described in Example 2.

[0300] LNP formulations that show a favorable encapsulation efficiency will be tested in low volume formulations as described in Example 3.

[0301] By following the steps described above, safe, cost-effective, low-volume ethanol-free LNP formulations will be prepared for mRNA delivery.

Example 5. Testing mRNA delivery in ethanol-free LNP formulations in vivo

[0302] This example illustrates measuring *in vivo* efficacy of ethanol-free LNP formulations.

[0303] Ethanol-free and ethanol LNP formulations were prepared for mRNA delivery as described in Example 1.

[0304] To test the *in vivo* efficacy of ethanol-free formulations, ethanol-free and ethanol LNP formulations comprising mRNA- encapsulated LPNs were delivered either intravenously (IV) to mice via tail vein injection or intratracheally at various doses in the range of 0.1 to 1.0 mg/kg, e.g., at 0.5 mg/kg of mouse weight.

[0305] LNP biodistribution in various organs were evaluated by bioluminescence studies and by quantitative measurements of mRNA and protein expression. The biodistribution, mRNA and protein expression results obtained with ethanol-free LNP formulations were compared with the biodistribution, mRNA and protein expression obtained with ethanol LNP formulations.

Pulmonary Delivery

[0306] Mice were administered firefly luciferase (FFL) mRNA encapsulated in LNPs which were produced using either an ethanol-free encapsulation process or an ethanol-containing encapsulation process. For these *in vivo* studies, mice were administered the mRNA containing LNPs intratracheally via a catheter, and were assessed for FFL protein expression about 24 hours post administration.

[0307] The characteristics of the FFL mRNA LNPs that were administered to the mice are shown in Table 5 below. As summarized in Table 5, low volume (1:1 lipid solution to mRNA solution) or high volume (1:4 lipid solution to mRNA solution) formulations were used in this study that either were encapsulated in an ethanol-free condition (e.g., using mTEG instead of ethanol) or were encapsulated in an ethanol-containing condition.

Table 5. Average particle size, polydispersity index and encapsulation efficiency of high-volume (1:4) and low-volume (1:1) ethanol-free mRNA-LNP formulations compared to ethanol-based mRNA-LNP formulations

Formulation Lot	mTEG or Standard	Cationic Lipid	Composition (PEG:Cat:Chol:DOPE)	Size	PDI	%EE
High-volume 1:4 Formulations						
I	EtOH (1:4)	ICE	5:60:0:35	55	0.217	93
J	mTEG (1:4)	ICE	5:60:0:35	48	0.172	88
Low-volume 1:1 Formulations						
K	EtOH (1:1)	ICE	5:60:0:35	Crashed during buffer exchange		
L	mTEG (1:1)	ICE	5:60:0:35	80	0.167	62

[0308] Table 5 shows that encapsulation of mRNA LNP formulations using ethanol-free mRNA conditions had encapsulation and size parameters that were either similar to ethanol containing mRNA-LNP formulations (1:4 lipid solution to mRNA solution; high volume conditions) or better than ethanol containing mRNA-LNP formulations (1:1 lipid solution to mRNA solution; low volume conditions).

[0309] The results of the *in vivo* pulmonary delivery study are summarized in Fig. 1. Fig. 1 shows that mice that were administered mRNA LNPs that were encapsulated using high volume conditions (1:4 lipid solution to mRNA solution) using an ethanol-free encapsulation process (e.g., mTEG) had a higher amount of protein expressed within the animal in comparison to those animals that received mRNA LNPs that were encapsulated using high volume (1:4 lipid solution to mRNA solution) ethanol-containing encapsulation process.

[0310] The results indicated the efficacy and feasibility of ethanol-free LNP formulations in mRNA delivery *in vivo*.

Intravenous Delivery

[0311] Mice were administered ornithine transcarbamylase (OTC) mRNA encapsulated in LNPs which were produced using an ethanol-free encapsulation process. For these *in vivo* studies,

mice were administered the mRNA containing LNPs intravenously via tail vein injection, and were subsequently assessed for OTC protein expression in the serum and the liver 24 hours post administration.

[0312] The characteristics of the OTC mRNA LNPs that were administered to the mice are shown in Table 6 below. As summarized in Table 6, low volume (1:1 lipid solution to mRNA solution) or high volume (1:4 lipid solution to mRNA solution) formulations were used in this study that were encapsulated in an ethanol-free condition. As a control for this study, OTC mRNA LNPs were used that was formulated using MC-3 and DOPE.

Table 6. Average particle size, polydispersity index and encapsulation efficiency of high-volume (1:4) and low-volume (1:1) ethanol-free mRNA-LNP formulations

Formulation Lot	mTEG or Standard*	Cationic Lipid	Composition (PEG:Cat:Chol:DSPC)	Size	PDI	%EE
D	mTEG (1:4)	MC-3	5:50:35:10	56	0.229	97
H	mTEG (1:1)	MC-3	5:50:35:10	85	0.160	99
M	mTEG (1:4)	ML-2	5:40:25:30	92	0.200	67
F	mTEG (1:1)	ML-2	5:40:25:30	101	0.197	69

* "Standard" refers to ethanol-based encapsulation process

[0313] The data from these studies are presented in FIG. 2. The data show that expression of OTC was present in the serum and liver in the using the following mRNA-LNP formulations: (MC-3) 1:1 mTEG; (ML-2) 1:4 mTEG; and (ML-2) 1:1 mTEG). The results indicated the efficacy and feasibility of ethanol-free LNP formulations in mRNA delivery *in vivo*.

[0314] Further stability studies of mRNA and protein expression will be compared in ethanol-free and ethanol LNP formulations by conducting measurements over several hours and days.

EQUIVALENTS AND SCOPE

[0315] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the following claims: