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(54) Title: ENCAPSULATION OF MESSENGER RNA

(57) Abrégé/Abstract:
The present invention provides an improved process for lipid nanoparticle formulation and mRNA encapsulation. In some embodiments, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising a step of mixing a mRNA solution and a lipid solution, wherein the mRNA solution and/or the lipid solution are at a pre-determined temperature greater than ambient temperature.

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(54) **Title**: ENCAPSULATION OF MESSENGER RNA

(57) **Abstract**: The present invention provides an improved process for lipid nanoparticle formulation and mRNA encapsulation. In some embodiments, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising a step of mixing a mRNA solution and a lipid solution, wherein the mRNA solution and/or the lipid solution are at a pre-determined temperature greater than ambient temperature.

ENCAPSULATION OF MESSENGER RNA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional patent application Serial No. 62/020,163, filed July 2, 2014.

BACKGROUND

[0002] Messenger RNA therapy (MRT) is becoming an increasingly important approach for the treatment of a variety of diseases. MRT involves administration of messenger RNA (mRNA) into a patient in need of the therapy for production of the protein encoded by the mRNA within the patient body. Lipid nanoparticles are commonly used to encapsulate mRNA for efficient *in vivo* delivery of mRNA. However, current methods for producing mRNA-loaded lipid nanoparticles suffer poor encapsulation efficiency, low mRNA recovery and/or heterogeneous particle sizes.

SUMMARY OF INVENTION

[0003] The present invention provides, among other things, an improved process for lipid nanoparticle formulation and mRNA encapsulation. In particular, the present invention is based on the surprising discovery that pre-heating a mRNA solution and/or a lipid solution prior to mixing resulted in significantly improved encapsulation efficiency, mRNA recovery rate, and more homogeneous and smaller particle sizes (e.g., less than 100 nm).

[0004] Thus, in some embodiments, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising a step of mixing a mRNA solution and a lipid solution, wherein the mRNA solution and/or the lipid solution are at a pre-determined temperature greater than ambient temperature. In some embodiments, a pre-determined temperature suitable for the present invention is or is greater than about 30 °C, 37 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, or 70 °C. In some embodiments, a pre-determined temperature suitable for the present invention ranges from about 25-70 °C, about 30-70 °C, about

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35-70 °C, about 40-70 °C, about 45-70 °C, about 50-70 °C, or about 60-70 °C. In particular embodiments, a pre-determined temperature suitable for the present invention is about 65 °C.

[0005] In some embodiments, the mRNA solution and the lipid solution are heated to the pre-determined temperature separately prior to the mixing. In some embodiments, the mRNA solution is heated to the pre-determined temperature and the lipid solution is at ambient temperature prior to the mixing. In some embodiments, the mRNA solution is heated to the pre-determined temperature by adding a mRNA stock solution at ambient temperature to a heated buffering solution to the pre-determined temperature. In some embodiments, the buffering solution has a pH no greater than about 4.5 (e.g., no greater than about 4.4, 4.2, 4.0 or 3.8).

[0006] In some embodiments, the mRNA solution and the lipid solution are mixed by a pulse-less flow pump. In some embodiments, a suitable pump is a gear pump. In some embodiments, a suitable pump is a peristaltic pump. In some embodiments, a suitable pump is a centrifugal pump.

[0007] 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, or 4000-5000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow rate of about 200 ml/minute, about 500 ml/minute, about 1000 ml/minute, about 2000 ml/minute, about 3000 ml/minute, about 4000 ml/minute, or about 5000 ml/minute.

[0008] 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, or about 1000 ml/minute.

[0009] In some embodiments, a process according to the present invention includes a step of first generating the mRNA solution by mixing a citrate buffer with a mRNA stock solution. In certain embodiments, a suitable citrate buffer contains about 10 mM citrate, about 150 mM NaCl, pH of about 4.5. In some embodiments, a suitable mRNA stock solution contains the mRNA at a concentration at or greater than about 0.10 mg/mL, 1 mg/ml, about 10 mg/ml, about 50 mg/ml, or about 100 mg/ml.

[0010] In some embodiments, the citrate buffer is mixed at a flow rate ranging between 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, or 4800-6000 ml/minute. In some embodiments, the citrate buffer is mixed at a flow rate of about 220 ml/minute, about 600 ml/minute, about 1200 ml/minute, about 2400 ml/minute, about 3600 ml/minute, about 4800 ml/minute, or about 6000 ml/minute.

[0011] 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.

[0012] In some embodiments, the lipid solution contains one or more cationic lipids, one or more helper lipids, one or more cholesterol-based lipids and PEG lipids in ethanol. In some embodiments, the mRNA solution and the lipid solution are mixed into a 20% ethanol, resulting in a suspension of lipid nanoparticles. In some embodiments, the lipid nanoparticles are further purified by Tangential Flow Filtration.

[0013] 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 100 nm (e.g., less than 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 100 nm (e.g., less than 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).

[0014] 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 about 40-90 nm (e.g., about 40-85 nm, about 40-80 nm, about 40-75 nm, about 40-70 nm, about 40-65 nm, or about 40-60 nm). In some embodiments, substantially all of the purified nanoparticles have a size ranging from about 40-90 nm (e.g., about 40-85 nm, about 40-80 nm, about 40-75 nm, about 40-70 nm, about 40-65 nm, or about 40-60 nm).

[0015] In some embodiments, the purified nanoparticles have an encapsulation efficiency of greater than about 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.

[0016] In some embodiments, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles, comprising (a) separately heating a mRNA solution and/or a lipid solution to a pre-determined temperature greater than ambient temperature; (b) mixing the heated mRNA solution and/or the heated lipid solution to generate a suspension of lipid nanoparticles; and (c) purifying the lipid nanoparticles.

[0017] In another aspect, the present invention provides a composition of lipid nanoparticles generated by a process described herein. In some embodiments, the present invention provides a composition comprising purified lipid nanoparticles, wherein greater than about 90% of the purified lipid nanoparticles have an individual particle size of less than about 100 nm (e.g., less than 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) and greater than about 70% of the purified lipid nanoparticles encapsulate a mRNA within each individual particle. In some embodiments, greater than about 95%, 96%, 97%, 98%, or 99% of the purified lipid nanoparticles have an individual particle size of less than about 100 nm (e.g., less than 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 lipid nanoparticles have an individual particle size of less than about 100 nm (e.g., less than 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 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the purified lipid nanoparticles encapsulate a mRNA within each individual particle. In some embodiments, substantially all of the purified lipid nanoparticles encapsulate a mRNA within each individual particle. In some embodiments, a composition according to the present invention contains at least about 1 mg, 5 mg, 10 mg, 100 mg, 500 mg, or 1000 mg of encapsulated mRNA.

[0018] In some embodiments, each individual lipid nanoparticle comprises one or more cationic lipids, one or more helper lipids, one or more cholesterol-based lipids and PEG lipids. In some embodiments, the one or more cationic lipids are selected from the group consisting of C12-200, MC3, DLinDMA, DLinkC2DMA, cKK-E12, ICE (Imidazol-based), , HGT5000, HGT5001, DODAC, DDAB, DMRIE, DOSPA, DOGS, DODAP, DODMA and DMDMA, DODAC, DLenDMA, DMRIE, CLinDMA, CpLinDMA, DMOBA, DOcarbDAP, DLinDAP, DLincarbDAP, DLinCDAP, KLin-K-DMA, DLin-K-XTC2-DMA, HGT4003, and combinations thereof.

[0019] In some embodiments, the one or more non-cationic lipids are selected from DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleyl-sn-glycero-3-phosphoethanolamine), DOPC (1,2-dioleyl-sn-glycero-3-phosphotidylcholine) DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine), DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine), DOPG (,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)).

[0020] In some embodiments, the one or more cholesterol-based lipids is cholesterol or PEGylated cholesterol. In some embodiments, the one or more PEG-modified lipids contain a poly(ethylene) glycol chain of up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C_6-C_{20} length.

[0021] In some embodiments, the present invention is used to encapsulate mRNA containing one or more modified nucleotides. In some embodiments, the present invention is used to encapsulate mRNA that is unmodified.

[0022] 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

[0023] The drawings are for illustration purposes only and not for limitation.

[0024] **Figure 1:** shows a schematic of an exemplary scaled-up lipid nanoparticle encapsulated mRNA formulation process with homogenous flow pumps.

[0025] **Figure 2:** depicts an exemplary purification and buffer exchange system for lipid nanoparticles.

[0026] **Figure 3:** depicts a schematic of an exemplary scaled-up lipid nanoparticle encapsulated mRNA formulation process with peristaltic pumps.

[0027] **Figure 4:** depicts an alternative exemplary tangential flow filtration system for purification and buffer exchange.

[0028] **Figure 5:** depicts an alternative schematic of an exemplary scaled-up lipid nanoparticle encapsulated mRNA formulation process with peristaltic pumps.

DEFINITIONS

[0029] 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.

[0030] *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 a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%,

7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0031] *Encapsulation:* As used herein, the term "encapsulation," or grammatical equivalent, refers to the process of confining an individual mRNA molecule within a nanoparticle.

[0032] *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.

[0033] *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.

[0034] *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.

[0035] *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).

[0036] *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.*).

[0037] *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.

[0038] *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 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, 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.

[0039] *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.

[0040] *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.

[0041] *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".

DETAILED DESCRIPTION

[0042] The present invention provides an improved process for lipid nanoparticle formulation and mRNA encapsulation. In some embodiments, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising a step of

mixing a mRNA solution and a lipid solution, wherein the mRNA solution and/or the lipid solution are at a pre-determined temperature greater than ambient temperature.

[0043] 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.

mRNA

[0044] The present invention may be used to encapsulate any mRNA. mRNA is typically thought of as the type of RNA that carries information from DNA to the ribosome. The existence of mRNA is typically very brief and includes processing and translation, followed by degradation. Typically, in eukaryotic organisms, mRNA processing comprises the addition of a "cap" on the N-terminal (5') end, and a "tail" on the C-terminal (3') end. A typical cap is a 7-methylguanosine cap, which is a guanosine that is linked through a 5'-5'-triphosphate bond to the first transcribed nucleotide. The presence of the cap is important in providing resistance to nucleases found in most eukaryotic cells. The tail is typically a polyadenylation event whereby a polyadenylyl moiety is added to the 3' end of the mRNA molecule. The presence of this "tail" serves to protect the mRNA from exonuclease degradation. Messenger RNA is translated by the ribosomes into a series of amino acids that make up a protein.

[0045] mRNAs may be synthesized according to any of a variety of known methods. 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.

[0046] In some embodiments, *in vitro* synthesized mRNA may be purified before formulation and encapsulation to remove undesirable impurities including various enzymes and other reagents used during mRNA synthesis.

[0047] The present invention may be used to formulate and encapsulate mRNAs of a variety of lengths. In some embodiments, the present invention may be used to formulate and encapsulate *in vitro* synthesized mRNA of or greater than about 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, or 20 kb in length. In some embodiments, the present invention may be used to formulate and encapsulate *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 8-15 kb in length.

[0048] The present invention may be used to formulate and encapsulate mRNA that is unmodified or mRNA containing one or more modifications that typically enhance stability. In some embodiments, modifications are selected from modified nucleotide, modified sugar phosphate backbones, 5' and/or 3' untranslated region.

[0049] In some embodiments, modifications of mRNA may include modifications of the nucleotides of the RNA. An modified mRNA according to the invention can include, for example, backbone modifications, sugar modifications or base modifications. In some embodiments, mRNAs may be synthesized from naturally occurring nucleotides and/or nucleotide analogues (modified nucleotides) including, but not limited to, 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 disclosure of which is included here in its full scope by reference.

[0050] Typically, mRNA synthesis includes the addition of a "cap" on the N-terminal (5') end, and a "tail" on the C-terminal (3') end. 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.

[0051] Thus, in some embodiments, mRNAs include a 5' cap structure. 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. 2'-O-methylation may also occur at the first base and/or second base following the 7-methyl guanosine triphosphate residues. Examples of cap structures include, but are not limited to, m7GpppNp-RNA , m7GpppNmp-RNA and m7GpppNmpNmp-RNA (where m indicates 2'-Omethyl residues).

[0052] In some embodiments, mRNAs include a 5' and/or 3' untranslated region. In some embodiments, a 5' untranslated region includes one or more elements that affect a mRNA's stability or translation, for example, an iron responsive element. In some embodiments, a 5' untranslated region may be between about 50 and 500 nucleotides in length.

[0053] In some embodiments, a 3' untranslated region includes one or more of a polyadenylation signal, a binding site for proteins that affect a mRNA's stability of location in a cell, or one or more binding sites for miRNAs. In some embodiments, a 3' untranslated region may be between 50 and 500 nucleotides in length or longer.

[0054] While mRNA provided from *in vitro* transcription reactions may be desirable in some embodiments, other sources of mRNA are contemplated as within the scope of the invention including mRNA produced from bacteria, fungi, plants, and/or animals.

[0055] The present invention may be used to formulate and encapsulate mRNAs encoding a variety of proteins. Non-limiting examples of mRNAs suitable for the present invention include mRNAs encoding spinal motor neuron 1 (SMN), alpha-galactosidase (GLA), argininosuccinate synthetase (ASS1), firefly luciferase, Factor IX (FIX), phenylalanine hydroxylase (PAH), and cystic fibrosis transmembrane conductance receptor (CFTR). Exemplary mRNA sequences are described in detail in the Examples section.

mRNA solution

[0056] mRNA may be provided in a solution to be mixed with a lipid solution such that the mRNA may be encapsulated in lipid nanoparticles. A suitable mRNA solution may be any aqueous solution containing mRNA to be encapsulated at various concentrations. For example, a suitable mRNA solution may contain a mRNA at a concentration of or greater than about 0.01 mg/ml, 0.05 mg/ml, 0.06 mg/ml, 0.07 mg/ml, 0.08 mg/ml, 0.09 mg/ml, 0.1 mg/ml, 0.15 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, or 1.0 mg/ml. In some embodiments, a suitable mRNA solution may contain a mRNA at a concentration ranging from about 0.01-1.0 mg/ml, 0.01-0.9 mg/ml, 0.01-0.8 mg/ml, 0.01-0.7 mg/ml, 0.01-0.6 mg/ml, 0.01-0.5 mg/ml, 0.01-0.4 mg/ml, 0.01-0.3 mg/ml, 0.01-0.2 mg/ml, 0.01-0.1 mg/ml, 0.05-1.0 mg/ml, 0.05-0.9 mg/ml, 0.05-0.8 mg/ml, 0.05-0.7 mg/ml, 0.05-0.6 mg/ml, 0.05-0.5 mg/ml, 0.05-0.4 mg/ml, 0.05-0.3 mg/ml, 0.05-0.2 mg/ml, 0.05-0.1 mg/ml, 0.1-1.0 mg/ml, 0.2-0.9 mg/ml, 0.3-0.8 mg/ml, 0.4-0.7 mg/ml, or 0.5-0.6 mg/ml. In some embodiments, a suitable mRNA solution may contain a mRNA at a concentration up to about 5.0 mg/ml, 4.0 mg/ml, 3.0 mg/ml, 2.0 mg/ml, 1.0 mg/ml, .09 mg/ml, 0.08 mg/ml, 0.07 mg/ml, 0.06 mg/ml, or 0.05 mg/ml.

[0057] 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 concentration of the buffering agent is or greater than about 0.1 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, 6 mM, 8 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, or 50 mM.

[0058] Exemplary salts can include sodium chloride, magnesium chloride, and potassium chloride. In some embodiments, suitable concentration of salts in a 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.

[0059] 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.

[0060] Various methods may be used to prepare a mRNA solution suitable for the present invention. In some embodiments, mRNA may be directly dissolved in a buffering solution described herein. In some embodiments, a mRNA solution may be generated by mixing a mRNA stock solution with a buffering solution prior to mixing with a lipid solution for encapsulation. In some embodiments, a mRNA solution may be generated by mixing a mRNA stock solution with a buffering 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.

[0061] In some embodiments, a mRNA stock solution is mixed with a buffering solution using a pump. Exemplary pumps include but are not limited to gear pumps, peristaltic pumps and centrifugal pumps.

[0062] Typically, the buffering solution is mixed at a rate greater than that of the mRNA stock solution. For example, the buffering 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 buffering 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 buffering 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.

[0063] In some embodiments, a 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, a 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.

Lipid Solution

[0064] According to the present invention, a lipid solution contains a mixture of lipids suitable to form lipid nanoparticles for encapsulation of mRNA. In some embodiments, a suitable lipid solution is ethanol based. For example, a suitable lipid solution may contain a mixture of desired lipids dissolved in pure ethanol (i.e., 100% ethanol). In another embodiment, a suitable lipid solution is isopropyl alcohol based. In another embodiment, a suitable lipid solution is dimethylsulfoxide-based. In another embodiment, a suitable lipid solution is a mixture of suitable solvents including, but not limited to, ethanol, isopropyl alcohol and dimethylsulfoxide.

[0065] 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.

[0066] Any desired lipids may be mixed at any ratios suitable for encapsulating mRNAs. In some embodiments, a suitable lipid solution contain a mixture of desired lipids including cationic lipids, helper lipids (e.g. non cationic lipids and/or cholesterol lipids) and/or PEGylated lipids. In some embodiments, a suitable lipid solution contain 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.

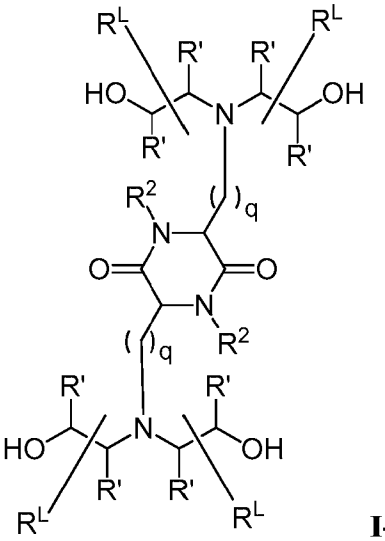
Cationic Lipids

[0067] 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. Several cationic lipids have been described in the literature, many of which are commercially available. Particularly suitable cationic lipids for use in the compositions and methods of the invention include those described in international patent publications WO 2010/053572 (and particularly, C12-200 described at paragraph [00225]) and WO 2012/170930. In certain embodiments, cationic lipids suitable for the compositions and methods of the invention include an ionizable cationic lipid, such as, e.g, (15Z, 18Z)-N,N-dimethyl-6-(9Z, 12Z)-octadeca-9, 12-dien-l -

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yl)tetracosa- 15,18-dien- 1 -amine (HGT5000), (15Z, 18Z)-N,N-dimethyl-6-((9Z, 12Z)-octadeca-9, 12-dien- 1 -yl)tetracosa- 4,15,18-trien-l -amine (HGT5001), and (15Z,18Z)-N,N-dimethyl-6-((9Z, 12Z)-octadeca-9, 12- dien- 1 -yl)tetracosa-5, 15 , 18-trien- 1 -amine (HGT5002).

[0068] In some embodiments, cationic lipids suitable for the compositions and methods of the invention include a cationic lipid described in WO 2013063468 and in U.S. provisional application entitled "Lipid Formulations for Delivery of Messenger RNA". In some embodiments, a cationic lipid comprises a compound of formula **I-cl-a:**



I-cl-a,

or a pharmaceutically acceptable salt thereof, wherein:

each R^2 independently is hydrogen or $_{C1_3}$ alkyl; each q independently is 2 to 6;

each R' independently is hydrogen or C_{1_3} alkyl; and each RL independently is $C_{8_1\ 2}$ alkyl.

[0069] In some embodiments, each R^2 independently is hydrogen, methyl or ethyl. In some embodiments, each R^2 independently is hydrogen or methyl. In some embodiments, each R^2 is hydrogen.

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[0070] In some embodiments, each q independently is 3 to 6. In some embodiments, each q independently is 3 to 5. In some embodiments, each q is 4.

[0071] In some embodiments, each R' independently is hydrogen, methyl or ethyl. In some embodiments, each R' independently is hydrogen or methyl. In some embodiments, each R' independently is hydrogen.

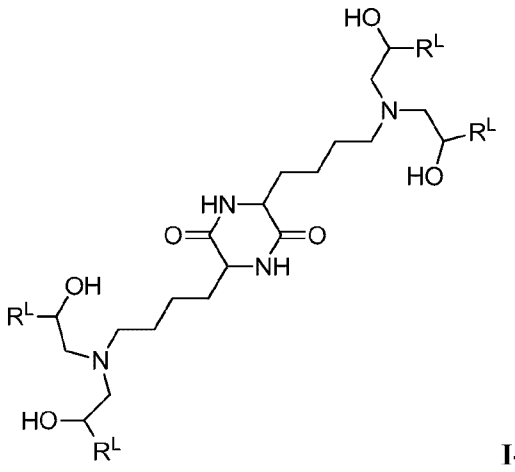
[0072] In some embodiments, each R^L independently is C_{8-12} alkyl. In some embodiments, each R^L independently is *n*-C_{8-12} alkyl. In some embodiments, each R^L independently is C_{9-11} alkyl. In some embodiments, each R^L independently is *n*-C_{9-11} alkyl. In some embodiments, each R^L independently is C_{10} alkyl. In some embodiments, each R^L independently is *n*-C_{10} alkyl.

[0073] In some embodiments, each R^2 independently is hydrogen or methyl; each q independently is 3 to 5; each R' independently is hydrogen or methyl; and each R^L independently is C_{8-12} alkyl.

[0074] In some embodiments, each R^2 is hydrogen; each q independently is 3 to 5; each R' is hydrogen; and each R^L independently is C_{8-12} alkyl.

[0075] In some embodiments, each R^2 is hydrogen; each q is 4; each R' is hydrogen; and each R^L independently is C_{8-12} alkyl.

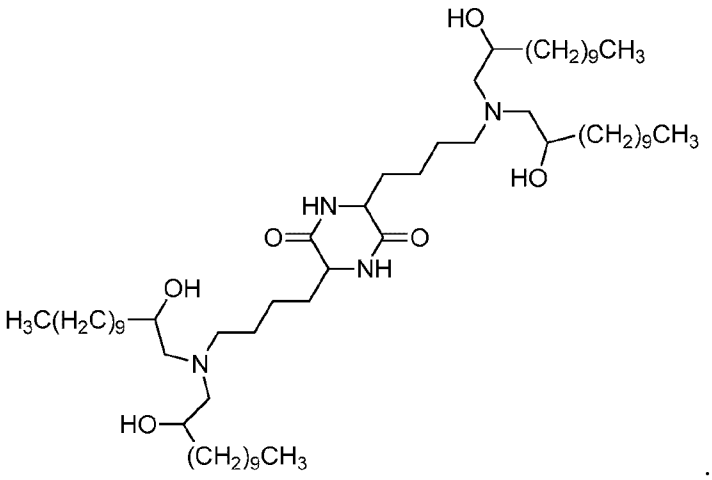
[0076] In some embodiments, a cationic lipid comprises a compound of formula **I-g**:



I-g,

or a pharmaceutically acceptable salt thereof, wherein each R^L independently is C_{8-12} alkyl. In some embodiments, each R^L independently is *n*-C_{8-12} alkyl. In some embodiments, each R^L independently is C_{9-11} alkyl. In some embodiments, each R^L independently is *n*-C_{9-11} alkyl. In some embodiments, each R^L independently is C_{10} alkyl. In some embodiments, each R^L is *n*-C_{10} alkyl.

[0077] In particular embodiments, a suitable cationic lipid is cKK-E12, or (3,6-bis(4-(bis(2-hydroxydodecyl)amino)butyl)piperazine-2,5-dione). Structure of cKK-E12 is shown below:



[0078] In some embodiments, one or more cationic lipids suitable for the present invention may be N-[l-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride or "DOTMA". (Feigner et al. (Proc. Nat'l Acad. Sci. 84, 7413 (1987); U.S. Pat. No. 4,897,355). Other suitable cationic lipids include, for example, 5-carboxyspermylglycinedioctadecylamide or "DOGS," 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-l-propanaminium or "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 or "DODAP", 1,2-Dioleoyl-3-Trimethylammonium-Propane or "DOTAP".

[0079] Additional exemplary cationic lipids also include 1,2-distearyloxy-N,N-dimethyl-3-aminopropane or "DSDMA", 1,2-dioleyloxy-N,N-dimethyl-3-aminopropane or "DODMA", 1 ,2-dilinoleyloxy-N,N-dimethyl-3-aminopropane or "DLinDMA", 1,2-dilinolenyloxy-N,N-

dimethyl-3-aminopropane or "DLenDMA", N-dioleyl-N,N-dimethylammonium chloride or "DODAC", N,N-distearyl-N,N-dimethylammonium bromide or "DDAB", N-(l,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide or "DMRIE", 3-dimethylamino-2-(cholest-5-en-3-beta-oxybutan-4-oxy)-l-(ci s,cis-9,12-octadecadienoxy)propane or "CLinDMA", 2-[5'-(cholest-5-en-3-beta-oxy)-3'-oxapentoxy)-3-dimethy 1-1-(cis,cis-9', 1-2'-octadecadienoxy)propane or "CpLinDMA", N,N-dimethyl-3,4-dioleyloxybenzylamine or "DMOBA", 1 ,2-N,N'-dioleylcarbamyl-3-dimethylaminopropane or "DOcarbDAP", 2,3-Dilinoleoyloxy-N,N-dimethylpropylamineor "DLinDAP", 1,2-N,N'-Dilinoleylcarbamyl-3-dimethylaminopropane or "DLincarbDAP", 1 ,2-Dilinoleoylcarbamyl-3-dimethylaminopropane or "DLinCDAP", 2,2-dilinoleyl-4-dimethylaminomethyl-[l,3]-dioxolane or "DLin- -DMA", 2,2-dilinoleyl-4-dimethylaminoethyl-[l,3]-dioxolaneor "DLin-K-XTC2-DMA", and 2-(2,2-di((9Z,12Z)-octadeca-9,l 2-dien- 1-yl)-1,3-dioxolan-4-yl)-N,N-dimethylethanamine (DLin-KC2-DMA)) (see, WO 2010/042877; Semple et al., Nature Biotech. 28: 172-176 (2010)), or mixtures thereof. (Heyes, J., et al., J Controlled Release 107: 276-287 (2005); Morrissey, DV., et al., Nat. Biotechnol. 23(8): 1003-1007 (2005); PCT Publication WO2005/121348Al). In some embodiments, one or more of the cationic lipids comprise at least one of an imidazole, dialkylamino, or guanidinium moiety.

[0080] In some embodiments, one or more cationic lipids may be chosen from XTC (2,2- Dilinoley1-4-dimethylaminoethy1-[1,3]-dioxolane), MC3 (((6Z,9Z,28Z,31Z)-heptatriaconta- 6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate), ALNY-100 ((3aR,5s,6aS)-N,N-dimethyl- 2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d] [1 ,3]dioxol-5-amine)), NC98-5 (4,7,13-tris(3-oxo-3-(undecylamino)propyl)-N 1,Nl 6-diundecyl-4,7,10,13- tetraazahexadecane-1,16-diamide), DODAP (l ,2-dioleyl-3-dimethylammonium propane), HGT4003 (WO 2012/170889), ICE (WO 2011/068810), HGT5000 (U.S. Provisional Patent Application No. 61/617,468) or HGT5001 (cis or trans) (Provisional Patent Application No. 61/617,468), aminoalcohol lipidoids such as those disclosed in WO2010/053572, DOTAP (l,2-dioleyl-3-trimethylammonium propane), DOTMA (l,2-di-O-octadecenyl-3-trimethylammonium propane), DLinDMA (Heyes, J.; Palmer, L.;

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Bremner, K.; MacLachlan, I. "Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids" J. Contr. Rel. 2005, 107, 276-287), DLin-KC2-DMA (Semple, S.C. et al. "Rational Design of Cationic Lipids for siRNA Delivery" Nature Biotech. 2010, 28, 172-176), C12-200 (Love, K.T. et al. "Lipid-like materials for low-dose in vivo gene silencing" PNAS 2010, 107, 1864-1869).

[0081] In some embodiments, cationic lipids constitute at least about 5%, 10%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 70% of the total lipids in a suitable lipid solution by weight or by molar. In some embodiments, cationic lipid(s) constitute(s) 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%) of the total lipid mixture by weight or by molar.

Non-cationic/Helper Lipids

[0082] 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 H, 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), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, l-stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), or a mixture thereof.

[0083] In some embodiments, non-cationic lipids may constitute at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65% or 70% of the total lipids in a suitable lipid solution by weight or by molar. In some embodiments, non-cationic lipid(s)

constitute(s) about 30-50 % (e.g., about 30-45%, about 30-40%, about 35-50%, about 35-45%, or about 35-40%) of the total lipids in a suitable lipid solution by weight or by molar.

Cholesterol-based Lipids

[0084] In some embodiments, a suitable lipid solution include 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 ICE. In some embodiments, cholesterol-based lipid(s) constitute(s) at least about 5%, 10%, 20%, 30%, 40%, 50%, 60%, or 70% of the total lipids in a suitable lipid solution by weight or by molar. In some embodiments, cholesterol-based lipid(s) constitute(s) about 30-50 % (e.g., about 30-45%, about 30-40%, about 35-50%, about 35-45%, or about 35-40%) of the total lipids in a suitable lipid solution by weight or by molar.

PEGylated Lipids

[0085] In some embodiments, a suitable lipid solution includes one or more PEGylated lipids. 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. 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 C_6-C_{20} length. In some embodiments, a PEG-modified or PEGylated lipid is PEGylated cholesterol or PEG-2K. In some embodiments, particularly useful exchangeable lipids are PEG-ceramides having shorter acyl chains (e.g., C_{14} or C_{18}).

[0086] PEG-modified phospholipid and derivatized lipids may constitute at least about 5%, 10%, 20%, 30%, 40%, 50%, 60%, or 70% of the total lipids in a suitable lipid solution by weight or by molar. In some embodiments, PEGylated lipid lipid(s) constitute(s) about 30-50 %

(e.g., about 30-45%, about 30-40%, about 35-50%, about 35-45%, or about 35-40%) of the total lipids in a suitable lipid solution by weight or by molar.

[0087] Exemplary combinations of cationic lipids, non-cationic lipids, cholesterol-based lipids, and PEG-modified lipids are described in the Examples section. For example, a suitable lipid solution may contain cKK-E12, DOPE, chol, and DMG-PEG2K; C12-200, DOPE, cholesterol, and DMG-PEG2K; HGT5000, DOPE, chol, and DMG-PEG2K; HGT5001, DOPE, chol, and DMG-PEG2K; cKK-E12, DPPC, chol, and DMG-PEG2K; C12-200, DPPC, cholesterol, and DMG-PEG2K; HGT5000, DPPC, chol, and DMG-PEG2K; or HGT5001, DPPC, chol, and DMG-PEG2K. The selection of cationic lipids, non-cationic lipids and/or PEG-modified lipids which comprise the lipid mixture as well as the relative molar ratio of such lipids to each other, is based upon the characteristics of the selected lipid(s) and the nature of the and the characteristics of the mRNA to be encapsulated. Additional considerations include, for example, the saturation of the alkyl chain, as well as the size, charge, pH, pKa, fusogenicity and toxicity of the selected lipid(s). Thus the molar ratios may be adjusted accordingly.

Mixing Process

[0088] The present invention is based on the discovery of unexpected effect of temperature on the mRNA encapsulation efficiency and recovery rate. Thus, in some embodiments, the present invention provides a process of encapsulating messenger RNA (mRNA) in lipid nanoparticles by mixing a mRNA solution and a lipid solution, described herein, wherein the mRNA solution and/or the lipid solution are heated to a pre-determined temperature greater than ambient temperature. As used herein, the term "ambient temperature" refers to the temperature in a room, or the temperature which surrounds an object of interest (e.g., a mRNA solution or lipid solution) without heating or cooling. In some embodiments, the ambient temperature refers to temperature ranging from about 20-25 °C.

[0089] Therefore, a pre-determined temperature greater than ambient temperature is typically greater than about 25 °C. In some embodiments, a pre-determined temperature suitable for the present invention is or is greater than about 30 °C, 37 °C, 40 °C, 45 °C, 50 °C, 55 °C, 60 °C, 65 °C, or 70 °C. In some embodiments, a pre-determined temperature suitable for the present

invention ranges from about 25-70 °C, about 30-70 °C, about 35-70 °C, about 40-70 °C, about 45-70 °C, about 50-70 °C, or about 60-70 °C. In particular embodiments, a pre-determined temperature suitable for the present invention is about 65 °C.

[0090] The mRNA solution, or the lipid solution, or both, may be heated to a pre-determined temperature above the ambient temperature prior to mixing. In some embodiments, the mRNA solution and the lipid solution are heated to the pre-determined temperature separately prior to the mixing. In some embodiments, the mRNA solution and the lipid solution are mixed at the ambient temperature but then heated to the pre-determined temperature after the mixing. In some embodiments, the lipid solution is heated to the pre-determined temperature and mixed with a mRNA solution at the ambient temperature. In some embodiments, the mRNA solution is heated to the pre-determined temperature and mixed with a lipid solution at ambient temperature.

[0091] In some embodiments, the mRNA solution is heated to the pre-determined temperature by adding a mRNA stock solution that is at ambient temperature to a heated buffering solution to achieve the desired pre-determined temperature.

[0092] A mRNA solution and a lipid solution may be mixed using a pump. As the encapsulation procedure can occur on a wide range of scales, different types of pumps may be used to accommodate desired scale. It is however generally desired to use a pulse-less flow pumps. As used herein, a pulse-less flow pump refers to any pump that can establish a continuous flow with a stable flow rate. Types of suitable pumps may include, but are not limited to, gear pumps and centrifugal pumps. Exemplary gear pumps include, but are not limited to, Cole-Parmer or Diener gear pumps. Exemplary centrifugal pumps include, but are not limited to, those manufactured by Grainger or Cole-Parmer.

[0093] A mRNA solution and a lipid solution may be mixed at various flow rates. Typically, the mRNA solution may be mixed at a rate greater than that of the lipid solution. For example, the mRNA 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 lipid solution.

[0094] Suitable flow rates for mixing may be determined based on the scales. In some embodiments, a mRNA solution is mixed at a flow rate ranging from about 40-400 ml/minute,

60-500 ml/minute, 70-600 ml/minute, 80-700 ml/minute, 90-800 ml/minute, 100- 900 ml/minute, 110- 1000 ml/minute, 120-1100 ml/minute, 130- 1200 ml/minute, 140-1300 ml/minute, 150-1400 ml/minute, 160- 1500 ml/minute, 170-1600 ml/minute, 180-1700 ml/minute, 150-250 ml/minute, 250-500 ml/minute, 500-1000 ml/minute, 1000-2000 ml/minute, 2000-3000 ml/minute, 3000-4000 ml/minute, or 4000-5000 ml/minute. In some embodiments, the mRNA solution is mixed at a flow rate of about 200 ml/minute, about 500 ml/minute, about 1000 ml/minute, about 2000 ml/minute, about 3000 ml/minute, about 4000 ml/minute, or about 5000 ml/minute.

[0095] In some embodiments, a lipid solution is mixed at a flow rate ranging from about 25-75 ml/minute, 20-50 ml/minute, 25-75 ml/minute, 30-90 ml/minute, 40-100 ml/minute, 50-110 ml/minute, 75-200 ml/minute, 200-350 ml/minute, 350-500 ml/minute, 500-650 ml/minute, 650-850 ml/minute, or 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, or about 1000 ml/minute.

[0096] Typically, a mRNA solution and a lipid solution are mixed into a solution such that the lipids can form nanoparticles encapsulating mRNA. Such a solution is also referred to as a formulation or encapsulation solution. A suitable formulation or encapsulation solution may be based on a solvent such as ethanol. For example, a suitable formulation or encapsulation solution may be based on about 10% ethanol, about 15% ethanol, about 20% ethanol, about 25% ethanol, about 30% ethanol, about 35% ethanol, or about 40% ethanol.

[0097] A suitable formulation or encapsulation solution may be based on a solvent such as isopropyl alcohol. For example, a suitable formulation or encapsulation solution may be based on about 10% isopropyl alcohol, about 15% isopropyl alcohol, about 20% isopropyl alcohol, about 25% isopropyl alcohol, about 30% isopropyl alcohol, about 35% isopropyl alcohol, or about 40% isopropyl alcohol.

[0098] A suitable formulation or encapsulation solution may be based on a solvent such as dimethyl sulfoxide. For example, a suitable formulation or encapsulation solution may be based on about 10% dimethyl sulfoxide, about 15% dimethyl sulfoxide, about 20% dimethyl sulfoxide, about 25% dimethyl sulfoxide, about 30% dimethyl sulfoxide, about 35% dimethyl sulfoxide, or about 40% dimethyl sulfoxide.

[0099] 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.

Purification

[0100] Typically, subsequent to formulation and encapsulation, lipid nanoparticles are purified and/or concentrated. Various purification methods may be used. In some embodiments, lipid nanoparticles are purified using Tangential Flow Filtration. Tangential flow filtration (TFF), also referred to as cross-flow filtration, is a type of filtration wherein the material to be filtered is passed tangentially *across* a filter rather than through it. In TFF, undesired permeate passes through the filter, while the desired retentate passes along the filter and is collected downstream. It is important to note that the desired material is typically contained in the retentate in TFF, which is the opposite of what one normally encounters in traditional-dead end filtration.

[0101] Depending upon the material to be filtered, TFF is usually used for either microfiltration or ultrafiltration. Microfiltration is typically defined as instances where the filter has a pore size of between 0.05 μm and 1.0 μm, inclusive, while ultrafiltration typically involves filters with a pore size of less than 0.05 μm. Pore size also determines the nominal molecular weight limits (NMWL), also referred to as the molecular weight cut off (MWCO) for a particular filter, with microfiltration membranes typically having NMWLs of greater than 1,000 kilodaltons (kDa) and ultrafiltration filters having NMWLs of between 1 kDa and 1,000 kDa.

[0102] A principal advantage of tangential flow filtration is that non-permeable particles that may aggregate in and block the filter (sometimes referred to as "filter cake") during

traditional "dead-end" filtration, are instead carried along the surface of the filter. This advantage allows tangential flow filtration to be widely used in industrial processes requiring continuous operation since down time is significantly reduced because filters do not generally need to be removed and cleaned.

[0103] Tangential flow filtration can be used for several purposes including concentration and diafiltration, among others. Concentration is a process whereby solvent is removed from a solution while solute molecules are retained. In order to effectively concentrate a sample, a membrane having a NMWL or MWCO that is substantially lower than the molecular weight of the solute molecules to be retained is used. Generally, one of skill may select a filter having a NMWL or MWCO of three to six times below the molecular weight of the target molecule(s).

[0104] Diafiltration is a fractionation process whereby small undesired particles are passed through a filter while larger desired nanoparticles are maintained in the retentate without changing the concentration of those nanoparticles in solution. Diafiltration is often used to remove salts or reaction buffers from a solution. Diafiltration may be either continuous or discontinuous. In continuous diafiltration, a diafiltration solution is added to the sample feed at the same rate that filtrate is generated. In discontinuous diafiltration, the solution is first diluted and then concentrated back to the starting concentration. Discontinuous diafiltration may be repeated until a desired concentration of nanoparticles is reached.

[0105] Purified and/or concentrated lipid nanoparticles may be formulated in a desired buffer such as, for example, PBS.

Provided Nanoparticles Encapsulating mRNA

[0106] A process according to the present invention results in more homogeneous and smaller particle sizes (e.g., less than 100 nm), as well as significantly improved encapsulation efficiency and/or mRNA recovery rate as compared to a prior art process.

[0107] Thus, the present invention provides a composition comprising purified nanoparticles described herein. In some embodiments, majority of purified nanoparticles in a composition, i.e., 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 100 nm (e.g., less than 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 100 nm (e.g., less than 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).

[0108] In addition, more homogeneous nanoparticles with narrow particle size range are achieved by a process of the present invention. For example, greater than about 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the purified nanoparticles in a composition provided by the present invention have a size ranging from about 40-90 nm (e.g., about 40-85 nm, about 40-80 nm, about 40-75 nm, about 40-70 nm, about 40-65 nm, or about 40-60 nm). In some embodiments, substantially all of the purified nanoparticles have a size ranging from about 40-90 nm (e.g., about 40-85 nm, about 40-80 nm, about 40-75 nm, about 40-70 nm, about 40-65 nm, or about 40-60 nm).

[0109] In some embodiments, the dispersity , or measure of heterogeneity in size of molecules (PDI), of nanoparticles in a composition provided by the present invention is less than about 0.16 (e.g., less than about 0.15, 0.14, 0.13, 0.12, 0.11, 0.10, 0.09, or 0.08).

[0110] In some embodiments, greater than about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the purified lipid nanoparticles in a composition provided by the present invention encapsulate a mRNA within each individual particle. In some embodiments, substantially all of the purified lipid nanoparticles in a composition encapsulate a mRNA within each individual particle.

[0111] In some embodiments, a composition according to the present invention contains at least about 1 mg, 5 mg, 10 mg, 100 mg, 500 mg, or 1000 mg of encapsulated mRNA. 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.

EXAMPLES

[0112] 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 compounds of the invention and are not intended to limit the same.

Example 1. Effect of temperature on nanoparticle encapsulation process

[0113] This example demonstrates that an increase in temperature during nanoparticle encapsulation process results in increased yield and/or encapsulation efficiency.

Lipid Materials

[0114] The formulations described in the following Examples, unless otherwise specified, contain a multi-component lipid mixture of varying ratios employing one or more cationic lipids, helper lipids (e.g., non-cationic lipids and/or cholesterol lipids) and PEGylated lipids designed to encapsulate various nucleic acid materials. Cationic lipids for the process can include but are not limited to DOTAP (1,2-dioleyl-3-trimethylammonium propane), DODAP (1,2-dioleyl-3-dimethylammonium propane), DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane), DLinDMA (Heyes, J.; Palmer, L.; Bremner, K.; MacLachlan, I. "Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids" J. Contr. Rel. 2005, 107, 276-287), DLin-KC2-DMA (Semple, S.C. et al. "Rational Design of Cationic Lipids for siRNA Delivery" Nature Biotech. 2010, 28, 172-176), C12-200 (Love, K.T. et al. "Lipid-like materials for low-dose in vivo gene silencing" PNAS 2010, 107, 1864-1869), cKK-E12 (3,6-bis(4-(bis(2-hydroxydodecyl)amino)butyl)piperazine-2,5-dione), HGT5000, HGT5001, HGT4003, ICE, dialkylamino-based, imidazole-based, guanidinium-based, etc. Helper lipids can include but are not limited to DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleyl-sn-glycero-3-phosphoethanolamine), DOPC (1,2-dioleyl-sn-glycero-3-phosphotidylcholine) DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine), DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine), DOPG (,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)), cholesterol,

etc. The PEGylated lipids can include but are not limited to a poly(ethylene) glycol chain of up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C6-C20 length.

Messenger RNA Material

[0115] Codon-optimized human spinal motor neuron 1 (SMN) messenger RNA, argininosuccinate synthetase (ASS1) messenger RNA, modified cystic fibrosis transmembrane conductance regulator (SNIM® CFTR, 25% pseudouridine, 25% 5-methyl-cytidine) messenger RNA, firefly luciferase (FFL) messenger RNA, Factor IX (FIX) messenger RNA, phelyalanine hydroxylase (PAH) messenger RNA and alpha-galactosidase (GLA) messenger RNA was synthesized by in vitro transcription from a plasmid DNA template encoding the gene, which was followed by the addition of a 5' cap structure (Cap 1) (Fechter, P.; Brownlee, G.G. "Recognition of mRNA cap structures by viral and cellular proteins" J. Gen. Virology 2005, 86, 1239-1249) and a 3' poly(A) tail of approximately 250 nucleotides in length as determined by gel electrophoresis. 5' and 3' untranslated regions present in each mRNA product are represented as X and Y, respectively and defined as stated (vide infra).

Codon-Optimized Human Spinal Motor Neuron 1(SMN) mRNA:

XAUGGCCAUGAGCAGCGGAGGCAGCGGCGGAGGAGUGCCCGAGCAGGAGGACAG
CGUGCUGUUCAGGAGAGGCACCGGCCAGAGCGAUGACAGCGAUAUCUGGGACGA
UACCGCUCUGAUCAAGGCCUACGACAAGGCCGUGGCCAGCUUCAAGCACGCCCUG
AAAAACGGCGACAUCUGCGAGACCAGCGGCAAGCCCAAGACAACCCCCAAGAGAA
AGCCCGCCAAGAAGAAUAAGAGCCAGAAAAAGAACACCGCCGCCAGCCUGCAGCA
GUGGAAGGUGGGCGACAAGUGCAGCGCCAUCUGGAGCGAGGACGGCUGCAUCUA
CCCCGCCACCAUCGCCAGCAUCGACUUCAAGAGAGAGACCUGCGUGGUCGUGUAC
ACCGGCUACGGCAACAGAGAGGAGCAGAACCUGAGCGACCUGCUGAGCCCCAUUU
GUGAGGUGGCCAAUAACAUCGAACAGAACGCCCAGGAGAACGAGAAUGAAAGCC
AGGUGAGCACCGACGAGAGCGAGAACAGCAGAUCUCCUGGCAACAAGAGCGACAA
CAUCAAGCCUAAGUCUGCCCCUUGGAACAGCUUCCUGCCCCCUCCUCCACCCAUG
CCCGGACCCAGACUGGGACCCGGAAAACCUGGCCUGAAGUUCAACGGACCACCUC
CCCCUCCACCUCCUCCCCCACCUCAUCUCCUGAGCUGCUGGCUGCCACCCUUCCCC
AGCGGACCCCCUAUCAUCCCACCACCCCCUCCCAUCUGCCCCGACAGCCUGGACGA
CGCCGAUGCCCUGGGCAGCAUGCUGAUCAGCUGGUACAUGAGCGGCUACCACACA
GGAUACUACAUGGGCUUCAGACAGAACCAGAAGGAGGGCAGAUGCUCCCACUCCC
UGAACUGA**Y**

Human alpha-galactosidase (GLA) mRNA:

XAUGCAGCUGAGGAACCCAGAACUACAUCUGGGCUGCGCGCUUGCGCUUCGCUUC
CUGGCCCUCGUUUCCUGGGACAUCCCUGGGGCUAGAGCACUGGACAAUGGAUUGG
CAAGGACGCCUACCAUGGGCUGGCUGCACUGGGAGCGCUUCAUGUGCAACCUUGA
CUGCCAGGAAGAGCCAGAUUCCUGCAUCAGUGAGAAGCUCUUCAUGGAGAUGGC
AGAGCUCAUGGUCUCAGAAGGCUGGAAGGAUGCAGGUUAUGAGUACCUCUGCAU
UGAUGACUGUUGGAUGGCUCCCCAAAGAGAUUCAGAAGGCAGACUUCAGGCAGA
CCCUCAGCGCUUUCCUCAUGGGAUUCGCCAGCUAGCUAAUUAUGUUCACAGCAAA
GGACUGAAGCUAGGGAUUUAUGCAGAUGUUGGAAAUAAAACCUGCGCAGGCUUC
CCUGGGAGUUUUGGAUACUACGACAUUGAUGCCCAGACCUUUGCUGACUGGGGA
GUAGAUCUGCUAAAAUUUGAUGGUUGUUACUGUGACAGUUUGGAAAAUUUGGCA
GAUGGUUAUAAGCACAUGUCCUUGGCCCUGAAUAGGACUGGCAGAAGCAUUGUG
UACUCCUGUGAGUGGCCUCUUUAUAUGUGGCCCUUUCAAAAGCCCAAUUAUACAG
AAAUCCGACAGUACUGCAAUCACUGGCGAAAUUUUGCUGACAUUGAUGAUUCCU
GGAAAAGUAUAAAGAGUAUCUUGGACUGGACAUCUUUUAACCAGGAGAGAAUUG
UUGAUGUUGCUGGACCAGGGGGUUGGAAUGACCCAGAUAUGUUAGUGAUUGGCA
ACUUUGGCCUCAGCUGGAAUCAGCAAGUAACUCAGAUGGCCCUCUGGGCUAUCAU
GGCUGCUCCUUUAUUCAUGUCUAAUGACCUCCGACACAUCAGCCCUCAAGCCAAA
GCUCUCCUUCAGGAUAAGGACGUAAUUGCCAUCAAUCAGGACCCCUUGGGCAAGC
AAGGGUACCAGCUUAGACAGGGAGACAACUUUGAAGUGUGGGAACGACCUCUCU
CAGGCUUAGCCUGGGCUGUAGCUAUGAUAAACCGGCAGGAGAUUGGUGGACCUC
GCUCUUAUACCAUCGCAGUUGCUUCCCUGGGUAAAGGAGUGGCCUGUAAUCCUGC
CUGCUUCAUCACACAGCUCCUCCCUGUGAAAAGGAAGCUAGGGUUCUAUGAAUGG
ACUUCAAGGUUAAGAAGUCACAUAAAUCCCACAGGCACUGUUUUGCUUCAGCUA
GAAAAUACAAUGCAGAUGUCAUUAAAAGACUUACUUUAA**Y**

Codon-Optimized Human Argininosuccinate Synthetase (ASS1) mRNA:

XAUGAGCAGCAAGGGCAGCGUGGUGCUGGCCUACAGCGGCGGCCUGGACACCAGC
UGCAUCCUGGUGUGGCUGAAGGAGCAGGGCUACGACGUGAUCGCCUACCUGGCCA
ACAUCGGCCAGAAGGAGGACUUCGAGGAGGCCCGCAAGAAGGCCCUGAAGCUGGG
CGCCAAGAAGGUGUUCAUCGAGGACGUGAGCCGCGAGUUCGUGGAGGAGUUCAU
CUGGCCCGCCAUCCAGAGCAGCGCCCUGUACGAGGACCGCUACCUGCUGGGCACC
AGCCUGGCCCGCCCCUGCAUCGCCCGCAAGCAGGUGGAGAUCGCCCAGCGCGAGG
GCGCCAAGUACGUGAGCCACGGCGCCACCGGCAAGGGCAACGACCAGGUGCGCUU
CGAGCUGAGCUGCUACAGCCUGGCCCCCCAGAUCAAGGUGAUCGCCCCCUGGCGC
AUGCCCGAGUUCUACAACCGCUUCAAGGGCCGCAACGACCUGAUGGAGUACGCCA
AGCAGCACGGCAUCCCCAUCCCCGUGACCCCCAAGAACCCCUGGAGCAUGGACGA
GAACCUGAUGCACAUCAGCUACGAGGCCGGCAUCCUGGAGAACCCCAAGAACCAG
GCCCCCCCCCGGCCUGUACACCAAGACCCAGGACCCCGCCAAGGCCCCCAACACCCC
CGACAUCCUGGAGAUCGAGUUCAAGAAGGGCGUGCCCGUGAAGGUGACCAACGU
GAAGGACGGCACCACCCACCAGACCAGCCUGGAGCUGUUCAUGUACCUGAACGAG
GUGGCCGGCAAGCACGGCGUGGGCCGCAUCGACAUCGUGGAGAACCGCUUCAUCG
GCAUGAAGAGCCGCGGCAUCUACGAGACCCCCGCCGGCACCAUCCUGUACCACGC
CCACCUGGACAUCGAGGCCUUCACCAUGGACCGCGAGGUGCGCAAGAUCAAGCAG

GGCCUGGGCCUGAAGUUCGCCGAGCUGGUGUACACCGGCUUCUGGCACAGCCCCG
AGUGCGAGUUCGUGCGCCACUGCAUCGCCAAGAGCCAGGAGCGCGUGGAGGGCAA
GGUGCAGGUGAGCGUGCUGAAGGGCCAGGUGUACAUCCUGGGCCGCGAGAGCCCC
CUGAGCCUGUACAACGAGGAGCUGGUGAGCAUGAACGUGCAGGGCGACUACGAG
CCCACCGACGCCACCGGCUUCAUCAACAUCAACAGCCUGCGCCUGAAGGAGUACC
ACCGCCUGCAGAGCAAGGUGACCGCCAAGUGA**Y**

Codon-Optimized Firefly Luciferase mRNA:

XAUGGAAGAUGCCAAAAACAUUAAGAAGGGCCCAGCGCCAUUCUACCCACUCGAA
GACGGGACCGCCGGCGAGCAGCUGCACAAAGCCAUGAAGCGCUACGCCCUGGUGC
CCGGCACCAUCGCCUUUACCGACGCACAUAUCGAGGUGGACAUUACCUACGCCGA
GUACUUCGAGAUGAGCGUUCGGCUGGCAGAAGCUAUGAAGCGCUAUGGGCUGAA
UACAAACCAUCGGAUCGUGGUGUGCAGCGAGAAUAGCUUGCAGUUCUUCAUGCCC
GUGUUGGGUGCCCUGUUCAUCGGUGUGGCUGUGGCCCCAGCUAACGACAUCUACA
ACGAGCGCGAGCUGCUGAACAGCAUGGGCAUCAGCCAGCCCACCGUCGUAUUCGU
GAGCAAGAAAGGGCUGCAAAAGAUCCUCAACGUGCAAAAGAAGCUACCGAUCAU
ACAAAAGAUCAUCAUCAUGGAUAGCAAGACCGACUACCAGGGCUUCCAAAGCAUG
UACACCUUCGUGACUUCCCAUUUGCCACCCGGCUUCAACGAGUACGACUUCGUGC
CCGAGAGCUUCGACCGGGACAAAACCAUCGCCCUGAUCAUGAACAGUAGUGGCAG
UACCGGAUUGCCCAAGGGCGUAGCCCUACCGCACCGCACCGCUUGUGUCCGAUUC
AGUCAUGCCCGCGACCCCAUCUUCGGCAACCAGAUCAUCCCCGACACCGCUAUCC
UCAGCGUGGUGCCAUUUCACCACGGCUUCGGCAUGUUCACCACGCUGGGCUACUU
GAUCUGCGGCUUUCGGGUCGUGCUCAUGUACCGCUUCGAGGAGGAGCUAUUCUU
GCGCAGCUUGCAAGACUAUAAGAUUCAAUCUGCCCUGCUGGUGCCCACACUAUUU
AGCUUCUUCGCUAAGAGCACUCUCAUCGACAAGUACGACCUAAGCAACUUGCACG
AGAUCGCCAGCGGCGGGGCGCCGCUCAGCAAGGAGGUAGGUGAGGCCGUGGCCAA
ACGCUUCCACCUACCAGGCAUCCGCCAGGGCUACGGCCUGACAGAAACAACCAGC
GCCAUUCUGAUCACCCCCGAAGGGGACGACAAGCCUGGCGCAGUAGGCAAGGUGG
UGCCCUUCUUCGAGGCUAAGGUGGUGGACUUGGACACCGGUAAGACACUGGGUG
UGAACCAGCGCGGCGAGCUGUGCGUCCGUGGCCCCAUGAUCAUGAGCGGCUACGU
UAACAACCCCGAGGCUACAAACGCUCUCAUCGACAAGGACGGCUGGCUGCACAGC
GGCGACAUCGCCUACUGGGACGAGGACGAGCACUUCUUCAUCGUGGACCGGCUGA
AGAGCCUGAUCAAAUACAAGGGCUACCAGGUAGCCCCAGCCGAACUGGAGAGCAU
CCUGCUGCAACACCCCAACAUCUUCGACGCCGGGGUCGCCGGCCUGCCCGACGAC
GAUGCCGGCGAGCUGCCCGCCGCAGUCGUCGUGCUGGAACACGGUAAAACCAUGA
CCGAGAAGGAGAUCGUGGACUAUGUGGCCAGCCAGGUUACAACCGCCAAGAAGCU
GCGCGGUGGUGUUGUGUUCGUGGACGAGGUGCCUAAAGGACUGACCGGCAAGUU
GGACGCCCGCAAGAUCCGCGAGAUUCUCAUUAAGGCCAAGAAGGGCGGCAAGAUC
GCCGUGUAA**Y**

Human Factor IX (FIX) mRNA:

XAUGCAGCGCGUGAACAUGAUCAUGGCAGAAUCACCAGGCCUCAUCACCAUCUGC
CUUUUAGGAUAUCUACUCAGUGCUGAAUGUACAGUUUUUCUUGAUCAUGAAAAC
GCCAACAAAAUUCUGAGGCGGAGAAGGAGGUAUAAUUCAGGUAAAUUGGAAGAG
UUUGUUCAAGGGAACCUUGAGAGAGAAUGUAUGGAAGAAAAGUGUAGUUUUGAA
GAAGCACGAGAAGUUUUUGAAAACACUGAAAGAACAACUGAAUUUUGGAAGCAG
UAUGUUGAUGGAGAUCAGUGUGAGUCCAAUCCAUGUUUAAAUGGCGGCAGUUGC
AAGGAUGACAUUAAUUCCUAUGAAUGUUGGUGUCCCUUUGGAUUUGAAGGAAAG
AACUGUGAAUUAGAUGUAACAUGUAACAUUAAGAAUGGCAGAUGCGAGCAGUUU
UGUAAAAAUAGUGCUGAUAACAAGGUGGUUUGCUCCUGUACUGAGGGAUAUCGA
CUUGCAGAAAACCAGAAGUCCUGUGAACCAGCAGUGCCAUUUCCAUGUGGAAGA
GUUUCUGUUUCACAAACUUCUAAGCUCACCCGUGCUGAGGCUGUUUUUCCUGAUG
UGGACUAUGUAAAUUCUACUGAAGCUGAAACCAUUUUGGAUAACAUCACUCAAA
GCACCCAAUCAUUUAAUGACUUCACUCGGGUUGUUGGUGGAGAAGAUGCCAAAC
CAGGUCAAUUCCCUUGGCAGGUUGUUUUGAAUGGUAAAGUUGAUGCAUUCUGUG
GAGGCUCUAUCGUUAAUGAAAAAUGGAUUGUAACUGCUGCCCACUGUGUUGAAA
CUGGUGUUAAAAUUACAGUUGUCGCAGGUGAACAUAAUAUUGAGGAGACAGAAC
AUACAGAGCAAAAGCGAAAUGUGAUUCGAAUUAUUCCUCACCACAACUACAAUG
CAGCUAUUAAUAAGUACAACCAUGACAUUGCCCUUCUGGAACUGGACGAACCCUU
AGUGCUAAACAGCUACGUUACACCUAUUUGCAUUGCUGACAAGGAAUACACGAA
CAUCUUCCUCAAAUUUGGAUCUGGCUAUGUAAGUGGCUGGGGAAGAGUCUUCCA
CAAAGGGAGAUCAGCUUUAGUUCUUCAGUACCUUAGAGUUCCACUUGUUGACCG
AGCCACAUGUCUUCGAUCUACAAAGUUCACCAUCUAUAACAACAUGUUCUGUGCU
GGCUUCCAUGAAGGAGGUAGAGAUUCAUGUCAAGGAGAUAGUGGGGGACCCCAU
GUUACUGAAGUGGAAGGGACCAGUUUCUUAACUGGAAUUAUUAGCUGGGGUGAA
GAGUGUGCAAUGAAAGGCAAAUAUGGAAUAUAUACCAAGGUAUCCCGGUAUGUC
AACUGGAUUAAGGAAAAAACAAAGCUCACUUAA**Y**

Codon-Optimized Human Phenylalanine Hydroxylase (PAH) mRNA:

XAUGAGCACCGCCGUGCUGGAGAACCCCGGCCUGGGCCGCAAGCUGAGCGACUUC
GGCCAGGAGACCAGCUACAUCGAGGACAACUGCAACCAGAACGGCGCCAUCAGCC
UGAUCUUCAGCCUGAAGGAGGAGGUGGGCGCCCUGGCCAAGGUGCUGCGCCUGUU
CGAGGAGAACGACGUGAACCUGACCCACAUCGAGAGCCGCCCCAGCCGCCUGAAG
AAGGACGAGUACGAGUUCUUCACCCACCUGGACAAGCGCAGCCUGCCCGCCCUGA
CCAACAUCAUCAAGAUCCUGCGCCACGACAUCGGCGCCACCGUGCACGAGCUGAG
CCGCGACAAGAAGAAGGACACCGUGCCCUGGUUCCCCCGCACCAUCCAGGAGCUG
GACCGCUUCGCCAACCAGAUCCUGAGCUACGGCGCCGAGCUGGACGCCGACCACC
CCGGCUUCAAGGACCCCGUGUACCGCGCCCGCCGCAAGCAGUUCGCCGACAUCGC
CUACAACUACCGCCACGGCCAGCCCAUCCCCCGCGUGGAGUACAUGGAGGAGGAG
AAGAAGACCUGGGGCACCGUGUUCAAGACCCUGAAGAGCCUGUACAAGACCCACG
CCUGCUACGAGUACAACCACAUCUUCCCCCUGCUGGAGAAGUACUGCGGCUUCCA
CGAGGACAACAUCCCCCAGCUGGAGGACGUGAGCCAGUUCCUGCAGACCUGCACC
GGCUUCCGCCUGCGCCCCGUGGCCGGCCUGCUGAGCAGCCGCGACUUCCUGGGCG
GCCUGGCCUUCCGCGUGUUCCACUGCACCCAGUACAUCCGCCACGGCAGCAAGCC

CAUGUACACCCCCGAGCCCGACAUCUGCCACGAGCUGCUGGGCCACGUGCCCCUG
UUCAGCGACCGCAGCUUCGCCCAGUUCAGCCAGGAGAUCGGCCUGGCCAGCCUGG
GCGCCCCCGACGAGUACAUCGAGAAGCUGGCCACCAUCUACUGGUUCACCGUGGA
GUUCGGCCUGUGCAAGCAGGGCGACAGCAUCAAGGCCUACGGCGCCGGCCUGCUG
AGCAGCUUCGGCGAGCUGCAGUACUGCCUGAGCGAGAAGCCCAAGCUGCUGCCCC
UGGAGCUGGAGAAGACCGCCAUCCAGAACUACACCGUGACCGAGUUCCAGCCCCU
GUACUACGUGGCCGAGAGCUUCAACGACGCCAAGGAGAAGGUGCGCAACUUCGCC
GCCACCAUCCCCCGCCCCUUCAGCGUGCGCUACGACCCCUACACCCAGCGCAUCGA
GGUGCUGGACAACACCCAGCAGCUGAAGAUCCUGGCCGACAGCAUCAACAGCGAG
AUCGGCAUCCUGUGCAGCGCCCUGCAGAAGAUCAAGUAA**Y**

Codon-Optimized Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) mRNA:

AUGCAGCGGUCCCCGCUCGAAAAGGCCAGUGUCGUGUCCAAACUCUUCUUCUCAU
GGACUCGGCCUAUCCUUAGAAAGGGGUAUCGGCAGAGGCUUGAGUUGUCUGACA
UCUACCAGAUCCCCUCGGUAGAUUCGGCGGAUAACCUCUCGGAGAAGCUCGAACG
GGAAUGGGACCGCGAACUCGCGUCUAAGAAAAACCCGAAGCUCAUCAACGCACUG
AGAAGGUGCUUCUUCUGGCGGUUCAUGUUCUACGGUAUCUUCUUGUAUCUCGGG
GAGGUCACAAAAGCAGUCCAACCCCUGUUGUUGGGUCGCAUUAUCGCCUCGUACG
ACCCCGAUAACAAAGAAGAACGGAGCAUCGCGAUCUACCUCGGGAUCGGACUGUG
UUUGCUUUUCAUCGUCAGAACACUUUUGUUGCAUCCAGCAAUCUUCGGCCUCCAU
CACAUCGGUAUGCAGAUGCGAAUCGCUAUGUUUAGCUUGAUCUACAAAAAGACA
CUGAAACUCUCGUCGCGGGUGUUGGAUAAGAUUUCCAUCGGUCAGUUGGUGUCC
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GUGUGGAUUGCCCCGUUGCAAGUCGCCCUUUUGAUGGGCCUUAUUUGGGAGCUG
UUGCAGGCAUCUGCCUUUUGUGGCCUGGGAUUUCUGAUUGUGUUGGCAUUGUUU
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AUCUCGGAAAGACUCGUCAUCACUUCGGAAAUGAUCGAAAACAUCCAGUCGGUCA
AAGCCUAUUGCUGGGAAGAAGCUAUGGAGAAGAUGAUUGAAAACCUCCGCCAAA
CUGAGCUGAAACUGACCCGCAAGGCGGCGUAUGUCCGGUAUUUCAAUUCGUCAGC
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AUUAAGGGGAUUAUCCUCCGCAAGAUUUUCACCACGAUUUCGUUCUGCAUUGUA
UUGCGCAUGGCAGUGACACGGCAAUUUCCGUGGGCCGUGCAGACAUGGUAUGAC
UCGCUUGGAGCGAUCAACAAAAUCCAAGACUUCUUGCAAAAGCAAGAGUACAAG
ACCCUGGAGUACAAUCUUACUACUACGGAGGUAGUAAUGGAGAAUGUGACGGCU
UUUUGGGAAGAGGGUUUUGGAGAACUGUUUGAGAAAGCAAAGCAGAAUAACAAC
AACCGCAAGACCUCAAAUGGGGACGAUUCCCUGUUUUUCUCGAACUUCUCCCUGC
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GCAAUUUUGGAUGACCUUCUGCCCCUGACGAUCUUCGACUUCAUCCAGUUGUUGC
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GCAUCUUGUGACCAGUUUGAAGGGAUUGUGGACGUUGCGCGCCUUUGGCAGGCA
GCCCUACUUUGAAACACUGUUCCACAAAGCGCUGAAUCUCCAUACGGCAAAUUGG
UUUUUGUAUUUGAGUACCCUCCGAUGGUUUCAGAUGCGCAUUGAGAUGAUUUUU
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GAGGGACGGGUCGGUAUUAUCCUGACACUCGCCAUGAACAUUAUGAGCACUUUG
CAGUGGGCAGUGAACAGCUCGAUUGAUGUGGAUAGCCUGAUGAGGUCCGUUUCG
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ACGGCAAAAUACACCGAGGGAGGGAACGCAAUCCUUGAAAACAUCUCGUUCAGCA
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UAAUCCCCCAAAAGGUCUUUAUCUUUAGCGGAACCUUCCGAAAGAAUCUCGAUCC
UUAUGAACAGUGGUCAGAUCAAGAGAUUUGGAAAGUCGCGGACGAGGUUGGCCU
UCGGAGUGUAAUCGAGCAGUUUCCGGGAAAACUCGACUUUGUCCUUGUAGAUGG
GGGAUGCGUCCUGUCGCAUGGGCACAAGCAGCUCAUGUGCCUGGCGCGAUCCGUC
CUCUCUAAAGCGAAAAUUCUUCUCUUGGAUGAACCUUCGGCCCAUCUGGACCCGG

UAACGUAUCAGAUCAUCAGAAGGACACUUAAGCAGGCGUUUGCCGACUGCACGG
UGAUUCUCUGUGAGCAUCGUAUCGAGGCCAUGCUCGAAUGCCAGCAAUUUCUUG
UCAUCGAAGAGAAUAAGGUCCGCCAGUACGACUCCAUCCAGAAGCUGCUUAAUGA
GAGAUCAUUGUUCCGGCAGGCGAUUUCACCAUCCGAUAGGGUGAAACUUUUUCC
ACACAGAAAUUCGUCGAAGUGCAAGUCCAAACCGCAGAUCGCGGCCUUGAAAGAA
GAGACUGAAGAAGAAGUUCAAGACACGCGUCUUUAA

5' and 3' UTR Sequences

X =

GGACAGAUCGCCUGGAGACGCCAUCCACGCUGUUUUGACCUCCAUAGAAGACACC
GGGACCGAUCCAGCCUCCGCGGCCGGGAACGGUGCAUUGGAACGCGGAUUCCCCG
UGCCAAGAGUGACUCACCGUCCUUGACACG

Y =

CGGGUGGCAUCCCUGUGACCCCUCCCCAGUGCCUCUCCUGGCCCUGGAAGUUGCC
ACUCCAGUGCCCACCAGCCUUGUCCUAAUAAAAUUAAGUUGCAUCAAGCU

Lipid Nanoparticle Formulations

[0116] Ethanolic solution of mixture of lipids (cationic lipid, helper lipids, zwitterionic lipids, PEG lipids etc.) was prepared to the reported volume and heated to the selected temperature. Separately, an aqueous buffered solution (10 mM citrate/150 mM NaCl, pH 4.5) of mRNA was prepared from a 1 mg/mL stock and heated to the selected temperature for 5-10 minutes.

[0117] For small scale formulations, the lipid solution was injected rapidly into the aqueous mRNA solution using a syringe pump (3.71 mL/sec) and the resulting suspension was shaken to yield the lipid nanoparticles in 20% ethanol. The resulting nanoparticle suspension was dia-filtrated with 1x PBS (pH 7.4), concentrated and stored at 2-8°C

Representative Example at 25 °C

[0118] Aliquots of 50 mg/mL ethanolic solutions of cKK-E12, DOPE, Chol and DMG-PEG2K were mixed and diluted with ethanol to 3 mL final volume. Separately, an aqueous buffered solution (10 mM citrate/150 mM NaCl, pH 4.5) of FFL mRNA was prepared from a 1 mg/mL stock. The lipid solution was injected rapidly into the aqueous mRNA solution and shaken to yield a final suspension in 20% ethanol. The resulting nanoparticle suspension was filtered, diafiltrated with 1x PBS (pH 7.4), concentrated and stored at 2-8°C. Final concentration = 0.20 mg/mL FFL mRNA (encapsulated). Z_{ave} = 91 nm PDI (0.16).

Formulation at 37 °C

[0119] Aliquots of 50 mg/mL ethanolic solutions of cKK-E12, DOPE, Chol and DMG-PEG2K were mixed and diluted with ethanol to 3 mL final volume. Separately, an aqueous buffered solution (10 mM citrate/150 mM NaCl, pH 4.5) of FIX mRNA was prepared from a 1 mg/mL stock. The lipid solution was injected rapidly into the aqueous mRNA solution and shaken to yield a final suspension in 20% ethanol. The resulting nanoparticle suspension was filtered, diafiltrated with 1x PBS (pH 7.4), concentrated and stored at 2-8°C. Final concentration = 0.20 mg/mL FIX mRNA (encapsulated). Z_{ave} = 64 nm; PDI (0.12).

Formulation at 65 °C

[0120] Aliquots of 50 mg/mL ethanolic solutions of cKK-E12, DOPE, Chol and DMG-PEG2K were mixed and diluted with ethanol to 3 mL final volume. Separately, an aqueous buffered solution (10 mM citrate/150 mM NaCl, pH 4.5) of FIX mRNA was prepared from a 1 mg/mL stock. The lipid solution was injected rapidly into the aqueous mRNA solution and shaken to yield a final suspension in 20% ethanol. The resulting nanoparticle suspension was filtered, diafiltrated with 1x PBS (pH 7.4), concentrated and stored at 2-8°C. Final concentration = 0.20 mg/mL FIX mRNA (encapsulated). Z_{ave} = 73 nm; PDI (0.13).

Effect of Temperature on the Nanoparticle Encapsulation Process

[0121] Both the ethanol lipid solution and the aqueous buffered solution of mRNA (10 mM citrate/150 mM NaCl, pH 4.5) were heated at different selected temperatures before the formulation process to determine the effect of temperature on the final yield and the encapsulation efficiency of the formulation.

[0122] The effect of temperature on the nanoparticle formulation process was evaluated for size, size dispersity, encapsulation efficiency and yield (or recovery). Exemplary data are shown in Table 1. As can be seen, an increase in temperature (e.g., above the ambient temperature) results in increased encapsulation efficiency and/or yield/recover, as well as reduced particle size and/or size dispersity.

Table 1. Effect of Temperature on Nanoparticle Formation and mRNA Encapsulation

Formulation process at ambient temperature (25°C):						
Formulation #	**mRNA**	**Temperature**	**Size**	**PDI**	**Encapsulation**	**Recovery**
1	FFL	25	91	0.16	71%	30%
2	FFL	25	88	0.14	76%	33%
Formulation process at 37 °C:						
Formulation #	mRNA	Temperature	Size	PDI	Encapsulation	Recovery
3	FIX	37	77	0.13	57%	29%
4	FIX	37	80	0.12	68%	36%
5	FIX	37	64	0.12	69%	37%
6	FIX	37	63	0.12	65%	51%
Formulation process at 65 °C:						
Formulation #	mRNA	Temperature	Size	PDI	Encapsulation	Recovery
7	ASS1	65	86	0.12	85%	64%
8	ASS1	65	84	0.11	96%	98%

9	ASS1	65	81	0.16	86%	64%
10	ASS1	65	84	0.11	96%	98%
11	PAH	65	79	0.12	82%	77%
12	FIX	65	73	0.13	81%	77%
13	FIX	65	79	0.14	92%	82%
14	FIX	65	85	0.13	95%	70%
15	FFL	65	68	0.10	92%	78%
16	FFL	65	83	0.12	91%	77%
17	FFL	65	80	0.11	91%	75%
18	FFL	65	83	0.11	88%	72%
19	FFL	65	80	0.16	90%	75%
20	FFL	65	78	0.11	81%	77%
21	FFL	65	86	0.12	82%	75%

Example 2. Scaled-up formulation process

[0123] This example illustrates an exemplary scaled-up formulation process for encapsulating mRNA at an increased temperature.

[0124] An exemplary scaled-up formulation process is shown in Figure 1. Ismatec programmable digital drive pumps (Cole Parmer Model # CP 78008-10) were used. Micropump A-mount Suction Shoe Pump Head 316 SS body/graphite gears/PTFE seals, 0.084 mL/rev, w/out internal bypass (Cole Parmer Model # 07002-27) and Pharma Pure Tubing Size 14, 0.06" ID, 1/16" (Spectrum labs Part # ACTU-P14-25N) were used.

[0125] Nanoparticle formulation and encapsulation of mRNA is prepared by mixing an ethanol lipid solution with mRNA in citrate buffer (10 mM citrate buffer, 150 mM NaCl, pH 4.5) using a 'T' junction (or "Y" junction). Exemplary flow rates for the mRNA in citrate buffer and lipids in ethanol solution are 200 mL/minute and 50 mL/minute respectively. During this

process, both pumps are started simultaneously. Both the starting and the end fractions of the formulations are discarded, only the intermediate formulation is collected. Accurate flow rates and pulse less flow are two important parameters of this process.

Purification and Buffer Exchange

[0126] Purification and buffer exchange of the formulation from the above step is performed with KrosFlo ® Research IIi Tangential Flow Filtration system from Spectrum labs using the modified polyethersulfone hollow fiber filter modules. Buffer exchange is performed with 6x volumes of sterile PBS (pH 7.4) in a continuous diafiltration form. See Figure 2. Formulation is analyzed for Size (PDI) and encapsulation (yield). Exemplary data is presented in Table 2.

Table 2. Examples of scaled-up formulation

Formulation	Batch Size (mg)	N/P	mRNA	Size (nm)	PDI	Encapsulation
22	5	4	ASS1	59	0.09	88%
23	5	4	ASS1	60	0.12	81%
24	5	4	ASS1	59	0.11	92%
25	5	4	ASS1	62	0.12	91%
26	5	4	ASS1	59	0.11	89%
27	5	4	ASS1	62	0.07	97%
28	5	4	ASS1	57	0.12	91%
29	5	4	ASS1	62	0.07	97%
30	5	4	ASS1	67	0.12	88%
31	5	4	ASS1	60	0.15	82%
32	5	4	ASS1	75	0.09	92%
33	5	4	ASS1	67	0.12	91%
34	5	4	ASS1	71	0.13	92%
35	5	4	ASS1	69	0.11	92%
36	5	4	ASS1	66	0.13	94%
37	5	4	ASS1	72	0.11	94%
38	5	4	ASS1	82	0.13	96%
39	5	4	ASS1	62	0.12	90%
40	5	4	ASS1	60	0.11	86%
41	5	4	ASS1	67	0.15	91%
42	5	4	ASS1	69	0.14	94%
43	5	4	ASS1	65	0.16	90%
44	5	4	ASS1	63	0.12	89%
45	5	4	ASS1	65	0.08	86%
46	5	4	GLA	62	0.11	95%
47	5	4	GLA	57	0.16	89%
48	5	4	GLA	54	0.08	95%
49	5	4	GLA	62	0.12	88%

50	5	2	SMN	61	0.14	81%
51	5	4	25%s2U, 25%5mC CFTR	60	0.13	96%
52	20	2	ASS1	72	0.10	90%
53	20	4	ASS1	75	0.12	92%
54	20	4	ASS1	81	0.11	82%
55	20	6	FFluc	82	0.11	94%
56	20	4	FFLuc	78	0.11	94%
57	20	4	CFTR	80	0.12	98%
58	30	4	CFTR	75	0.12	85%
59	50	4	CFTR	69	0.17	92%
60	50	2	ASS1	73	0.15	82%
61	60	4	ASS1	71	0.13	95%
62	300	2	ASS1	59	0.18	95%
63	300	4	ASS1	64	0.11	96%
64	1000	2	ASS1	51	0.18	89%
65	1000	4	ASS1	61	0.19	91%
66	1000	2	ASS1	56	0.18	81%
67	1000	4	ASS1	71	0.08	92%
68	1000	2	ASS1	51	0.12	90%
69	1000	4	ASS1	73	0.13	89%
AVERAGE				**65.8**	**0.12**	**91%**

[0127] Using this process, very narrow particle size range is achieved as well as high encapsulation efficiency (e.g., >90% average).

[0128] To test the importance of pulse-less homogeneous flow, peristaltic pumps that have some degree of pulsating flow were used for the formulation process. See Figure 3. mRNA in citrate buffer and lipids in pure ethanol were mixed at flow rate of 200 mL/minute and 50 mL/minute respectively. Exemplary results were shown in Table 3. As can be seen, the use of peristaltic pumps within this process results in the formulation of nanoparticles with larger size. This is likely due to non-homogeneous mixing due to pulsating flow.

Table 3. Examples of formulations with peristaltic pumps

Formulation #	mRNA	Size(nm)	PDI
70	CFTR	112	0.19
71	CFTR	116	0.17
72	FFL	128	0.14
73	FFL	134	0.16

EQUIVALENTS AND SCOPE

[0129] 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:

CLAIMS

We claim:

1. A process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising a step of (i) generating an mRNA solution by mixing a citrate buffer with an mRNA stock solution, (ii) mixing the mRNA solution and a lipid solution, such that the mRNA is encapsulated in lipid nanoparticles, wherein the mRNA solution and/or the lipid solution are at a pre-determined temperature greater than ambient temperature, and wherein the mRNA solution and the lipid solution are mixed by a pulse-less flow pump.

2. The process of claim 1, wherein the pre-determined temperature ranges from 25-70 °C.

3. The process of claim 1 or 2, wherein the mRNA solution and the lipid solution are heated to the pre-determined temperature separately prior to the mixing.

4. The process of claim 1 or 2, wherein the mRNA solution is heated to the predetermined temperature and the lipid solution is at ambient temperature prior to the mixing.

5. The process of any one of claims 1-4, wherein the mRNA solution is heated to the pre-determined temperature by adding a mRNA stock solution at ambient temperature to a heated buffering solution to the pre-determined temperature.

6. The process of claim 5, wherein the buffering solution has a pH no greater than about 4.5.

7. The process of any one of claims 1-6, wherein the pump is a gear pump.

8. The process of any one of claims 1-6, wherein the pump is a centrifugal pump.

9. The process of any one of claims 1-8, wherein the mRNA solution is mixed at a flow rate of about 200 ml/minute.

10. The process of any one of claims 1-8, wherein the lipid solution is mixed at a flow rate of about 50 ml/minute.

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11. The process of any one of claims 1-10, wherein the citrate buffer comprises about 10 mM citrate, about 150 mM NaCl, pH of about 4.5.

12. The process of any one of claims 1-11, wherein the mRNA stock solution comprises the mRNA at a concentration at or greater than about 1 mg/ml.

13. The process of any one of claims 1-12, wherein the citrate buffer is mixed at a flow rate ranging between about 100-300 ml/minute to about 4800-6000 ml/minute.

14. The process of any one claims 1-13, wherein the citrate buffer is mixed at a flow rate of about 220 ml/minute to about 6000 ml/minute.

15. The process of any one of claims 1-14, wherein the mRNA stock solution is mixed at a flow rate ranging between about 10-30 ml/minute to about 480-600 ml/minute.

16. A process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising steps of: generating an mRNA solution by mixing an mRNA stock solution at a flow rate of about 20 ml/minute to about 600 ml/minute with a citrate buffer; mixing the mRNA solution and a lipid solution by a pulse-less flow pump, wherein the mRNA and/or the lipid solution are at a pre-determined temperature greater than ambient temperature.

17. A process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising steps of: generating an mRNA solution by mixing a citrate buffer at a flow rate of about 220 ml/minute to about 6000 ml/minute with an mRNA stock solution; and mixing the mRNA solution and a lipid solution by a pulse-less flow pump, wherein the mRNA solution and/or the lipid solution are at a pre-determined temperature greater than ambient temperature.

18. The process of any one of claims 1-17, wherein the lipid solution comprises one or more cationic lipids, one or more non-cationic lipids, , one or more cholesterol-based lipids, and one or more PEG-modified lipids in ethanol.

19. The process of any one of claims 1-18, wherein the mRNA solution and the lipid solution are mixed into a 20% ethanol, resulting in a suspension of lipid nanoparticles.

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20. The process of any one of claims 1, 16 or 17, wherein the lipid nanoparticles are further purified by Tangential Flow Filtration.

21. The process of any one of claims 1-20, comprising
a. separately heating an mRNA solution and/or a lipid solution to a pre-determined temperature greater than ambient temperature;
b. mixing the heated mRNA solution and/or the heated lipid solution to generate a suspension of lipid nanoparticles; and
c. purifying the lipid nanoparticles.

22. A process of encapsulating messenger RNA (mRNA) in lipid nanoparticles comprising mixing by a pulse-less flow pump an mRNA solution and a lipid solution at a N/P ratio above 1, wherein the N/P ratio is maintained constant throughout the mixing, such that the mixing results in encapsulation of mRNA in lipid nanoparticles at an encapsulation efficiency of 80% or greater, and

wherein the lipid nanoparticles have an average size of 100 nm or less and a dispersity measurement (PDI) of 0.19 or less.

23. The process of claim 22, wherein the lipid nanoparticle comprises one or more cationic lipids, one or more non-cationic lipids, one or more cholesterol-based lipids, and one or more PEG-modified lipids.

24. The process of claim 23, wherein the one or more cationic lipids are selected from the group consisting of C12-200, MC3, DLinDMA, DLinkC2DMA, cKK-E12, ICE (Imidazol-based), HGT5000, HGT5001, DODAC, DDAB, DMRIE, DOSPA, DOGS, DODAP, DODMA and DMDMA, DODAC, DLenDMA, DMRIE, CLinDMA, CpLinDMA, DMOBA, DOcarbDAP, DLinDAP, DLincarbDAP, DLinCDAP, KLin-K-DMA, DLin-K-XTC2-DMA, HGT4003, and combinations thereof.

25. The process of claim 23, wherein the one or more non-cationic lipids are selected from DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleyl-sn-glycero-3-phosphoethanolamine), DOPC (1,2-dioleyl-sn-glycero-3-phosphotidylcholine) DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine),

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DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine), DOPG (1,2-dioleoyl-sn-glycero-3-phospho-(1-rac-glycerol)).

26. The process of claim 23, wherein the one or more cholesterol-based lipids is cholesterol or PEGylated cholesterol.

27. The process of claim 23, wherein the one or more PEG-modified lipids comprise a poly(ethylene) glycol chain of up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C_6-C_{20} length.

28. The process of any one of claims 1-27, wherein the mRNA comprises one or more modified nucleotides.

29. The process of any one of claims 1-27, wherein the mRNA is unmodified.

30. A composition of lipid nanoparticles generated by a process of any one of claims 1-29.

31. The composition of claim 30, wherein greater than about 95% of the purified lipid nanoparticles have an individual particle size of less than about 100 nm.

32. The composition of any one of claims 30 or 31, wherein greater than about 80% of the purified lipid nanoparticles encapsulate a mRNA within each individual particle.

33. The composition of any one of claims 30-32, wherein the composition comprises at least 1 mg of encapsulated mRNA.

34. The composition of any one of claims 30-33, wherein the lipid nanoparticle comprises one or more cationic lipids, one or more non-cationic lipids, one or more cholesterol-based lipids and one or more PEG-modified lipids.

35. The composition of claim 34, wherein the one or more cationic lipids are selected from the group consisting of C12-200, MC3, DLinDMA, DLinkC2DMA, CK-E12, ICE (Imidazol-based), HGT5000, HGT5001, DODAC, DDAB, DMRIE, DOSPA, DOGS, DODAP, DODMA and DMDMA, DODAC, DLenDMA, DMRIE, CLinDMA, CpLinDMA, DMOBA, DOcarbDAP,

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DLinDAP, DLincarbDAP, DLinCDAP, KLin-K-DMA, DLin-K-XTC2-DMA, HGT4003, and combinations thereof.

36. The composition of claim 34, wherein the one or more non-cationic lipids are selected from DSPC (l,2-distearoyl-sn-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleyl-sn-glycero-3-phosphoethanolamine), DOPC (l,2-dioleyl-sn-glycero-3-phosphotidylcholine) DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine), DMPE (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine), DOPG (1,2-dioleoyl-sn-glycero-3-phospho-(l ac-glycerol)).

37. The composition of claim 34, wherein the one or more cholesterol-based lipids is cholesterol or PEGylated cholesterol.

38. The composition of claim 34, wherein the one or more PEG-modified lipids comprise a poly(ethylene) glycol chain of up to 5 kDa in length covalently attached to a lipid with alkyl chain(s) of C_6-C_{20} length.

39. The composition of any one of claims 30-38, wherein the mRNA comprises one or more modified nucleotides.

40. The composition of any one of claims 30-38, wherein the mRNA is unmodified.

41. A nanoparticle formulation comprising lipid nanoparticles encapsulating mRNA encoding cystic fibrosis conductance regulator protein,

wherein the mRNA encoding cystic fibrosis conductance regulator protein has a length greater than 4 kb and is encapsulated in the lipid nanoparticles at an encapsulation efficiency of 80% or greater, and

wherein the lipid nanoparticles have an average size of 80 nm or less and a dispersity measurement (PDI) of 0.19 or less.

Date Reçue/Date Received 2022-09-09

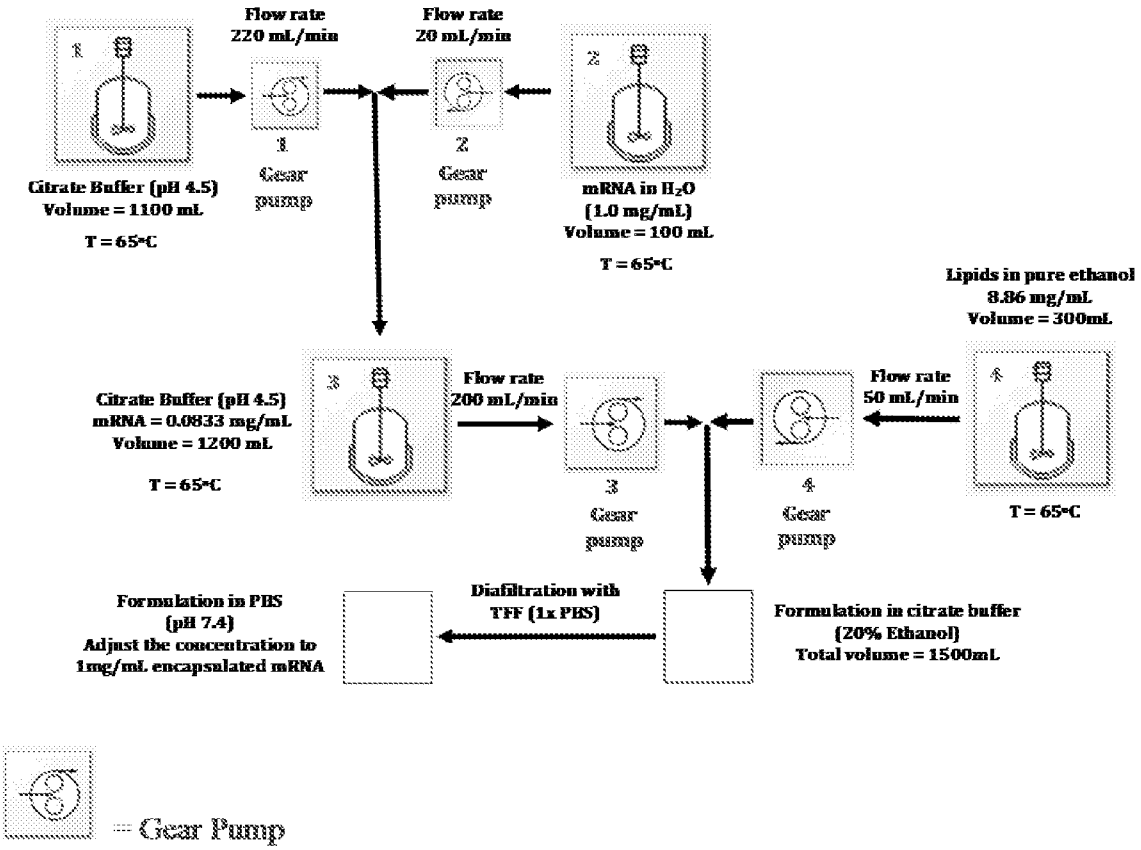


Figure 1. Scaled-Up Formulation Process with Homogenous Flow Pumps

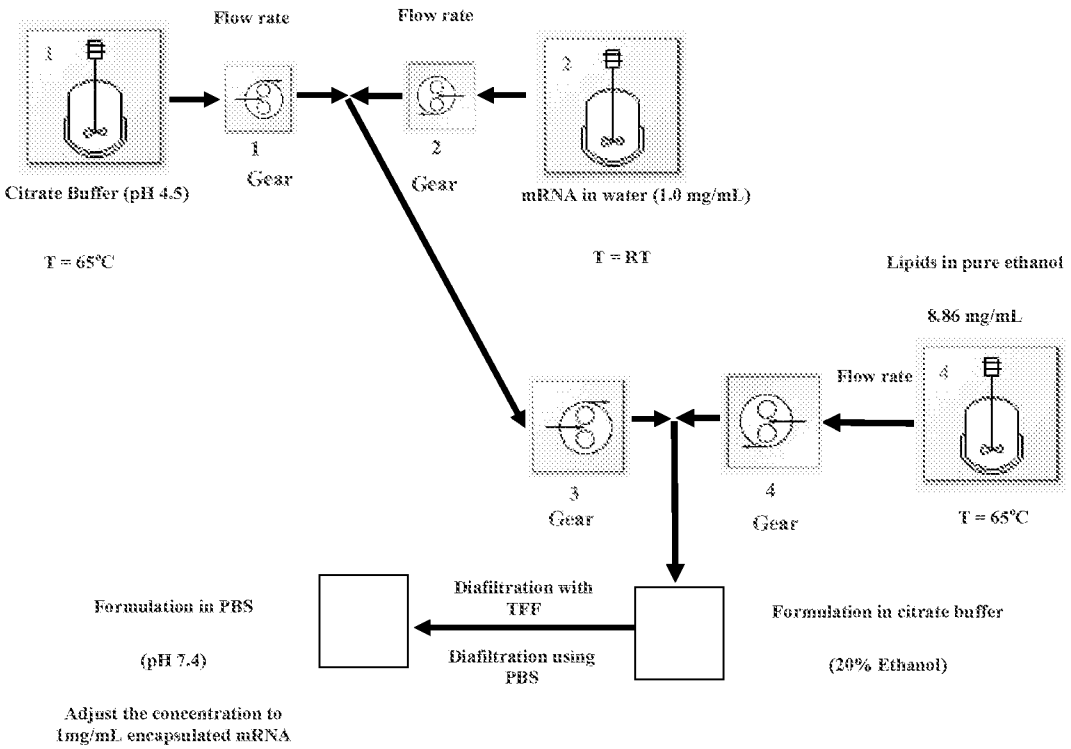


Figure 2. Scaled-Up Formulation Process with Homogenous Flow Pumps

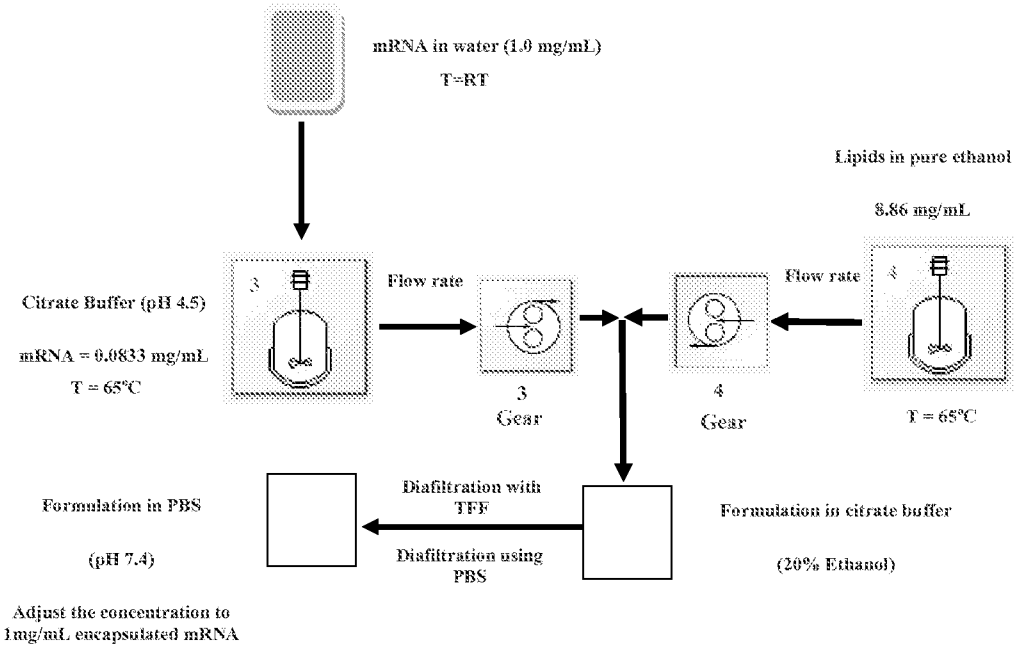


Figure 3. Scaled-Up Formulation Process with Homogenous Flow Pumps.

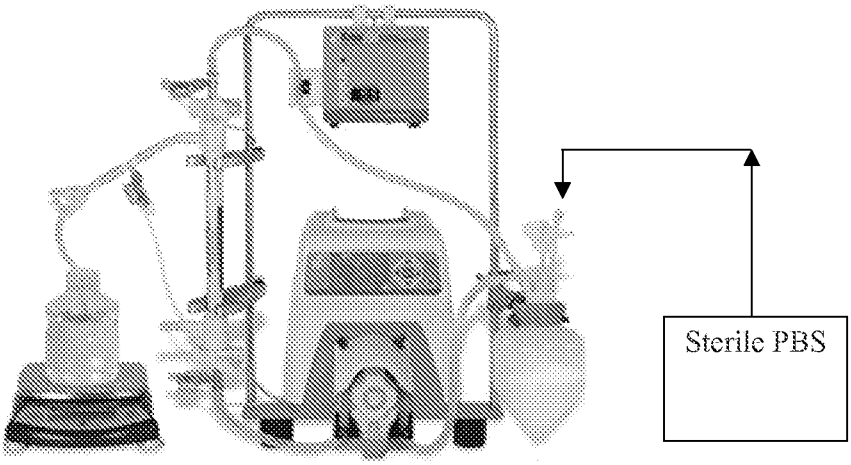


Figure 4

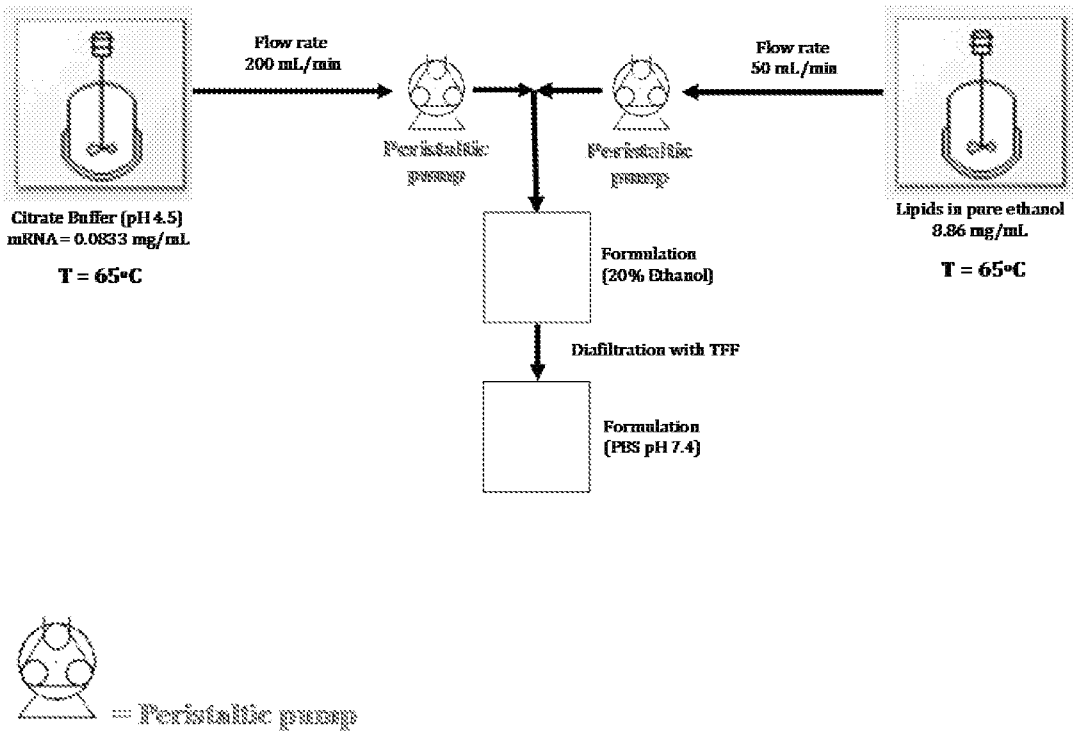


Figure 5 Scaled-Up Formulation Process with Peristaltic Pumps