

IMMUNOGENIC COMPOSITIONS

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 63/588,688, filed Oct. 6, 2023, U.S. Provisional Application No. 63/562,257, filed March 6, 2024, U.S. Provisional Application No. 63/568,754, filed March 22, 2024, U.S. Provisional Application No. 63/569,082, filed March 22, 2024, and U.S. Provisional Application No. 63/699,178, filed September 26, 2024. The entire content of each of the foregoing applications is hereby incorporated by reference herein in its entirety.

REFERENCE TO SEQUENCE LISTING

This application is being filed electronically via EFS-Web and includes an electronically submitted sequence listing in .xml format. The .xml file contains a sequence listing entitled "PC073084-PRV2 Sequence Listing.xml" created on September 24, 2024, and having a size of 79 KB. The sequence listing contained in this .xml file is part of the specification and is hereby incorporated by reference herein in its entirety.

BACKGROUND

There are many challenges associated with the delivery of nucleic acids to affect a desired response in a biological system. Nucleic acid-based therapeutics have enormous potential but there remains a need for more effective delivery of nucleic acids to appropriate sites within a cell or organism in order to realize this potential. Therapeutic nucleic acids include, but are not limited to, e.g., messenger RNA (mRNA), self-amplifying RNA (saRNA), antisense oligonucleotides, ribozymes, DNAzymes, plasmids, immune stimulating nucleic acids, antagomir, antimir, mimic, supermir, and aptamers. Some nucleic acids, such as mRNA or plasmids, can be used to effect expression of specific cellular products as would be useful in the treatment of, for example, diseases related to a deficiency of a protein or enzyme, or as a vaccine. The therapeutic applications of translatable nucleotide delivery are extremely broad as constructs can be synthesized to produce any chosen protein sequence, whether or not indigenous to the system. The expression products of the nucleic acid can augment existing levels of protein, replace missing or non-functional versions of a protein, or introduce new protein and associated functionality in a cell or organism.

However, two problems currently face the use of nucleic acids in therapeutic contexts. First, free RNAs are susceptible to nuclease digestion in plasma. Second, free RNAs have limited ability to gain access to the intracellular compartment where the relevant translation machinery resides. Lipid nanoparticles formed from cholesterol with other lipid components, such as ionizable lipids, neutral lipids, polymer conjugated lipids (e.g., PEGylated lipids), and nucleic

acids have been used to block degradation of the RNAs in plasma and facilitate the cellular uptake of the oligonucleotides.

Accordingly, there remains a need for improved lipid compounds and lipid nanoparticles for the delivery of nucleic acids. Preferably, these lipid nanoparticles would provide optimal drug:lipid ratios, protect the nucleic acid from degradation and clearance in serum, be suitable for systemic or local delivery, and provide intracellular delivery of the nucleic acid. In addition, these lipid-nucleic acid particles should be well-tolerated and provide an adequate therapeutic index, such that patient treatment at an effective dose of the nucleic acid is not associated with unacceptable toxicity and/or risk to the patient. In addition, there is a need to identify formulations of lipid-containing nanoparticle compositions which promote increased RNA expression and protein yield and have improved colloidal stability which maintains RNA expression and protein yield for long-term refrigerated storage.

There is a need for improved compositions, preferably immunogenic compositions.

15 SUMMARY

The unmet needs for improved lipid nanoparticle (LNP) compositions, which promote increased RNA expression and protein yield and have improved colloidal stability which maintain RNA expression and protein yield for long-term refrigerated storage, preferably immunogenic compositions, are provided herein.

20 Described below are embodiments of the invention, where for convenience Embodiment 1 (E1) is identical to the embodiment of Formula (I) provided above. Exemplary embodiments (E) of the invention provided herein include:

E1. A lipid nanoparticle (LNP) comprising

- 25 i. an ionizable cationic lipid;
ii. a neutral lipid;
iii. a mixture of cholesterol and a cholesterol analog, wherein the molar ratio between the cholesterol and the cholesterol analog in the mixture is about 6:4, about 1:1 or about 4:6; and
30 iv. a polymer-conjugated lipid. In a preferred embodiment, the lipid nanoparticle (LNP) comprises
i. an ionizable cationic lipid;
ii. a neutral lipid;
iii. a mixture of cholesterol and a cholesterol analog, wherein the molar ratio between
35 the cholesterol and the cholesterol analog in the mixture is 6:4, 1:1 or 4:6; and
iv. a polymer-conjugated lipid.

E2. The LNP of embodiment E1, wherein the cholesterol analog is selected from sitosterol, stigmasterol, campesterol, sitostanol, campestanol, brassicasterol, fucosterol, β -sitosterol, stigmasterol, β -sitostanol, ergosterol, fecosterol, lupeol, cycloartenol, Δ^5 -avenaserol, Δ^7 -avenaserol or a Δ^7 -stigmasterol, tomatidine, ursolic acid or alpha-tocopherol, including
 5 analogs, salts or esters thereof.

E3. The LNP of embodiment E2, wherein the cholesterol analog is β -sitosterol, stigmasterol or campesterol.

E4. The LNP of embodiment E3, wherein the cholesterol analog is β -sitosterol.

10 E5. The LNP of any preceding embodiment, wherein the LNP comprises about 20-60 mol % ionizable cationic lipid, about 5-25 mol % neutral lipid, about 18-60 mol % mixture of cholesterol and a cholesterol analog, and about 0.5-10 mol % polymer-conjugated lipid.

E6. The LNP according to any one of embodiments E1-E5, wherein the lipid nanoparticle comprises about 30 mol % to about 60 mol % ionizable cationic lipids, about 0 mol % to
 15 about 30 mol % neutral lipids, about 18.5 mol % to about 48.5 mol % mixture of cholesterol and a cholesterol analog, and about 0 mol % to about 10 mol % polymer-conjugated lipid.

E7. The LNP according to any of embodiments E1-E6, wherein the LNP comprises 0.9-1.85 mg/mL ALC-0315; 0.11-0.24 mg/mL ALC-0159; 0.18 – 0.41 mg/mL neutral lipid such as DSPC; and 0.36 – 0.78 mg/mL mixture of cholesterol and a cholesterol analog.

20 E8. The LNP of any of embodiments E5-E7, wherein the LNP comprises a mol% ratio of ALC-0315:cholesterol and cholesterol analog mixture:DSPC:ALC-0159 from the group consisting of:

a) 47.5:40.7:10:1.8;

b) 30:58.2:10:1.8;

25 c) 30:48.2:20:1.8; and

d) 50:33.2:15:1.8.

E9. The LNP of embodiment E8, wherein the LNP comprises a mol% ratio of ALC-0315:cholesterol:cholesterol analog:DSPC:ALC-0159 from the group consisting of:

a) 47.5:16.3:24.4:10:1.8;

30 b) 47.5:20.4:20.4:10:1.8;

c) 47.5:24.4:16.3:10:1.8;

- d) 30:29.1:29.1:10:1.8;
- e) 30:24.1:24.1:20:1.8; and
- f) 50:16.6:16.6:15:1.8

- 5 E10. An immunogenic composition comprising at least one ribonucleic acid (RNA) polynucleotide having an open reading frame (ORF) encoding at least one antigenic polypeptide or an immunogenic fragment thereof, formulated in a lipid nanoparticle (LNP) according to any of the above listed embodiments.
- E11. The immunogenic composition of embodiment E10, wherein the RNA is modRNA or saRNA.
- 10 E12. The immunogenic composition according to any one of any of the preceding embodiments, wherein the LNP comprises an N:P ratio of from about 2:1 to about 30:1.
- E13. The immunogenic composition of embodiment E12, wherein the LNP has an N:P ratio of at least 5.
- E14. The immunogenic composition of embodiment E12, wherein the LNP has an N:P ratio of
15 at least 6.
- E15. The immunogenic composition of embodiment E12, wherein the LNP has an N:P ratio of 10.

DESCRIPTION OF THE DRAWINGS

- 20 The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1A-1C. FIG. 1A depicts 50% neutralization titers 3 weeks post dose 1 against B/Austria; FIG. 1B depicts 50% neutralization titers 2 weeks post dose 2 (0.2 ug dose). FIG. 1C depicts
25 50% neutralization titers 2 weeks post dose 2 (0.05 ug dose).

FIG. 2A-2B. FIG. 2A depicts 90% neutralization titers 3 weeks post dose 1 against B/Austria; FIG. 2B depicts 90% neutralization titers 2 weeks post dose 1 against B/Austria.

FIG. 3 depicts percentage of HA positive cells following testing with 5 different LNP formulations, listed in **Table 4**.

- 30 **FIG. 4** depicts GMFI: HA-positive cells following testing with 5 different LNP formulations, listed in **Table 4**.

FIG. 5 depicts percentage of live cells following testing with 5 different LNP formulations, listed in **Table 4**.

FIG. 6 depicts percentage of live cells following testing with 5 different LNP formulations, listed in **Table 4**.

FIG. 7A-7B. FIG. 7A depicts a comparison of LNPs having an N:P ratio of 10, LNPs having an N:P ratio of 6, and their respective fold change ($EC_{50}^{\text{cholesterol}}/EC_{50}^{\text{cholesterol analog}}$) compared to benchmark LNPs; FIG. 7B depicts a comparison of LNPs having an N:P ratio of 10, LNPs having an N:P ratio of 6, and their respective fold change ($MFI^{\text{cholesterol analog}}/MFI^{\text{cholesterol}}$).

FIG. 8A-8B. FIG. 8A depicts a comparison of LNPs having sitosterol/cholesterol in a respective 6:4 ratio, LNPs having sitosterol/cholesterol in a respective 5:5 ratio, and LNPs having sitosterol/cholesterol in a respective 4:6 ratio, and their respective fold change ($EC_{50}^{\text{cholesterol}}/EC_{50}^{\text{cholesterol analog}}$) compared to benchmark LNPs; FIG. 8B depicts a comparison of LNPs having an N:P ratio of 10, LNPs having an N:P ratio of 6, and their respective fold change ($MFI^{\text{cholesterol analog}}/MFI^{\text{cholesterol}}$).

FIG. 9A-9F depict the key attributes of various sterol-containing LNPs encapsulating RNA over a 6-month storage period at 5 °C; FIG. 9A depicts the LNP size; FIG. 9B depicts polydispersity (PDI); FIG. 9C depicts percent encapsulation of the encapsulated RNA; FIG. 9D depicts percent integrity of the encapsulated RNA; FIG. 9E depicts the EC₅₀ for total RSV F protein expression; and FIG. 9F depicts the EC₅₀ for RSV prefusion F protein expression measured in HEK293T cells.

20 DETAILED DESCRIPTION

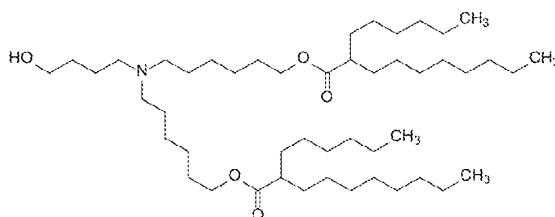
The present invention may be understood more readily by reference to the following detailed description of the embodiments of the invention and the Examples included herein. It is to be understood that this invention is not limited to specific synthetic methods of making that may of course vary. It is to be also understood that the terminology used herein is for the purpose of describing specific embodiments only and is not intended to be limiting.

Described below are embodiments of the invention, where for convenience Embodiment 1 (E1) is identical to the embodiment of Formula (I) provided above. Exemplary embodiments (E) of the invention provided herein include:

- 30 E1. A lipid nanoparticle (LNP) comprising
- i. an ionizable cationic lipid;
 - ii. a neutral lipid;
 - iii. a mixture of cholesterol and a cholesterol analog, wherein the molar ratio between the cholesterol and the cholesterol analog in the mixture is 6:4, 1:1 or 4:6; and
- 35 iv. a polymer-conjugated lipid. In a preferred embodiment, the lipid nanoparticle (LNP) comprises
- i. an ionizable cationic lipid;
 - ii. a neutral lipid;

- iii. a mixture of cholesterol and a cholesterol analog, wherein the molar ratio between the cholesterol and the cholesterol analog in the mixture is 6:4, 1:1 or 4:6; and
- iv. a polymer-conjugated lipid.

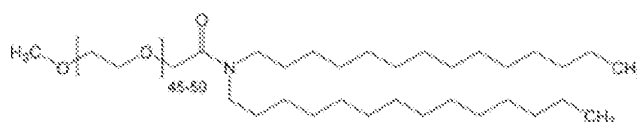
- 5 E2. The LNP of embodiment E1, wherein the cholesterol analog is selected from sitosterol, stigmasterol, campesterol, sitostanol, campestanol, brassicasterol, fucosterol, β -sitosterol, stigmasterol, β -sitostanol, ergosterol, fecosterol, lupeol, cycloartenol, Δ^5 -avenaserol, Δ^7 -avenaserol or a Δ^7 -stigmasterol, tomatidine, ursolic acid or alpha-tocopherol, including analogs, salts or esters thereof.
- 10 E3. The LNP of embodiment E2, wherein the cholesterol analog is β -sitosterol, stigmasterol or campesterol.
- E4. The LNP of embodiment E3, wherein the cholesterol analog is β -sitosterol.
- 15 E5. The LNP of embodiment E4, wherein the ionizable cationic lipid is selected from N,N-dimethyl-2,3-dioleoyloxy)propylamine (DODMA), N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dilinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), (4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) (ALC-0315), heptadecane-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy)hexyl) amino) octanoate (SM-102), or a mixture thereof.
- 20 E6. The LNP of any preceding embodiment, wherein the ionizable cationic lipid is ALC-0315 (4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) having the structure:



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- E7. The LNP of any preceding embodiment, wherein the neutral lipid is a phospholipid.
- E8. The LNP of any preceding embodiment, wherein the LNP comprises a neutral lipid selected from 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), distearoylphosphatidylcholine

- (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal),
- 5 dipalmitoylphosphatidylethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoylphosphatidylethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1-stearoyl-2-oleoylphosphatidylethanolamine (SOPE), and/or 1,2-dielaidoyl-sn-glycero-3-phosphoethanolamine (transDOPE).
- 10 E9. The LNP of embodiment E8, wherein the phospholipid is 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC).
- E10. The LNP of any preceding embodiment, wherein the polymer-conjugated lipid is a pegylated lipid.
- E11. The LNP of embodiment E10, wherein the pegylated lipid is 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159) or a PEG dialkoxypyrrolcarba PEGylated diacylglycerol (PEG-DAG), 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG), PEGylated phosphatidylethanolamine (PEG-PE), PEG succinate diacylglycerol (PEG-S-DAG), 4-O-(2',3'-di(tetradecanoyloxy)propyl)-1-O-((O-methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG), PEG-ceramide, 2-[(polyethylene
- 15 glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159) or α -(3'-[(1,2-di[myristyloxy]propanoxy)carbonylamino]propyl)- ω -methoxy, polyoxyethylene (PEG-C-DMG).
- 20 E12. The LNP of embodiment E11, wherein the pegylated lipid is ALC-0159 (2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide) having the structure:



- 25 E13. The LNP of any preceding embodiment, wherein the LNP comprises about 20-60 mol % ionizable cationic lipid, about 5-25 mol % neutral lipid, about 18-60 mol % mixture of cholesterol and a cholesterol analog, and about 0.5-10 mol % polymer-conjugated lipid.
- E14. The LNP according to any one of embodiments E1-E13, wherein the lipid nanoparticle comprises about 30 mol % to about 60 mol % ionizable cationic lipids, about 0 mol % to

about 30 mol % neutral lipids, about 18.5 mol % to about 48.5 mol % mixture of cholesterol and a cholesterol analog, and about 0 mol % to about 10 mol % polymer-conjugated lipid.

5 E15. The LNP according to any of embodiments E1-E14, wherein the LNP comprises 0.9-1.85 mg/mL ALC-0315; 0.11-0.24 mg/mL ALC-0159; 0.18 – 0.41 mg/mL neutral lipid such as DSPC; and 0.36 – 0.78 mg/mL mixture of cholesterol and a cholesterol analog.

E16. The LNP of any of embodiments E13-E15, wherein the LNP comprises a mol% ratio of ALC-0315:cholesterol and cholesterol analog mixture:DSPC:ALC-0159 from the group consisting of:

- 10 a) 47.5:40.7:10:1.8;
b) 30:58.2:10:1.8;
c) 30:48.2:20:1.8; and
d) 50:33.2:15:1.8.

E17. The LNP of embodiment E16, wherein the LNP comprises a mol% ratio of ALC-0315:cholesterol:cholesterol analog:DSPC:ALC-0159 from the group consisting of:

- 15 a) 47.5:16.3:24.4:10:1.8;
b) 47.5:20.4:20.4:10:1.8;
c) 47.5:24.4:16.3:10:1.8;
d) 30:29.1:29.1:10:1.8;
e) 30:24.1:24.1:20:1.8; and
20 f) 50:16.6:16.6:15:1.8.

E18. The LNP of any one of embodiments E13-E17, wherein the cholesterol analog is beta-sitosterol.

E19. The LNP according to any of embodiments E1-E18, wherein the LNP size is at least 40 nm.

25 E20. The LNP according to any of embodiments E1-E19, wherein the LNP size is at most 180 nm.

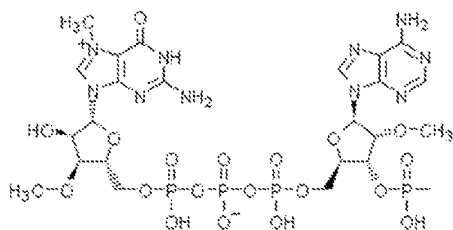
E21. An immunogenic composition comprising at least one ribonucleic acid (RNA) polynucleotide having an open reading frame (ORF) encoding at least one antigenic polypeptide or an immunogenic fragment thereof, formulated in a lipid nanoparticle (LNP)
30 according to any of embodiments E1-E20.

E22. The immunogenic composition of embodiment E21, wherein the RNA is modRNA or saRNA.

E23. The immunogenic composition of embodiments E21-E22, wherein the RNA polynucleotide comprises a 5' cap, 5' UTR, 3' UTR, and poly-A tail.

5 E24. The immunogenic composition of embodiment E23, wherein the 5' cap is a 5' cap analog.

E25. The immunogenic composition of embodiment E24, wherein the 5' cap analog comprises $m_2^{7,3'-O}Gppp(m_1^{2'-O})ApG$ or



10 E26. The immunogenic composition according to any of embodiments E21 to E25, wherein the RNA further comprises a modified nucleotide.

E27. The immunogenic composition of embodiment E26, wherein the modified nucleotide is selected from the group consisting of pseudouridine, 1-methylpseudouridine, 2-thiouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methoxyuridine, 2'-O-methyl uridine and N1-Methylpseudouridine-5'-triphosphate (m1 Ψ TP).

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20 E28. The immunogenic composition of embodiment E27, wherein the modified nucleotide comprises N1-Methylpseudouridine-5'-triphosphate (m1 Ψ TP).

E29. The immunogenic composition according to any of embodiments E21-E28, wherein the open reading frame is codon-optimized.

E30. The immunogenic composition of embodiment E21, wherein the 5' UTR comprises SEQ ID NO: 1.

25 E31. The immunogenic composition of embodiment E21, wherein the 3' UTR comprises SEQ ID NO: 2.

- E32. The immunogenic composition of embodiment E21, wherein the 3' polyadenylation tail comprises SEQ ID NO: 3.
- E33. The immunogenic composition of any preceding embodiment, wherein the RNA polynucleotide has an integrity greater than 85%.
- 5 E34. The immunogenic composition of any preceding embodiment, wherein the RNA polynucleotide has a purity of greater than 85%.
- E35. The immunogenic composition according to any one of embodiments E21-E34, wherein at least 80% of the total RNA in the immunogenic composition is encapsulated.
- 10 E36. The immunogenic composition according to any one of embodiments E21-E35, wherein the percentage of intact mRNA encapsulated in the LNP is at least 80%.
- E37. The immunogenic composition according to any one of any of the preceding embodiments, wherein the LNP comprises an N:P ratio of from about 2:1 to about 30:1.
- E38. The immunogenic composition of embodiment E37, wherein the LNP has an N:P ratio of at least 5.
- 15 E39. The immunogenic composition of embodiment E37, wherein the LNP has an N:P ratio of at least 6.
- E40. The immunogenic composition of embodiment E37, wherein the LNP has an N:P ratio of 10.
- 20 E41. The immunogenic composition according to any one of embodiments E21-E40, wherein the immunogenic composition comprises Tris.
- E42. The immunogenic composition according to any one of embodiments E21-E41, wherein the immunogenic composition comprises sucrose.
- E43. The immunogenic composition according to any one of embodiments E21-E42, wherein the immunogenic composition does not further comprise sodium chloride.
- 25 E44. The immunogenic composition according to any one of embodiments E21-E43, wherein the immunogenic composition comprises 10 mM Tris.
- E45. The immunogenic composition according to any one of embodiments E21-E44, wherein the immunogenic composition comprises 300 mM sucrose.

- E46. The immunogenic composition according to any one of embodiments E21-E45, wherein the immunogenic composition has a pH 7.4.
- E47. The immunogenic composition according to any one of embodiments E21-E46, wherein the immunogenic composition has less than or equal to 12.5 EU/mL of bacterial endotoxins.
- 5 E48. The immunogenic composition according to any one of embodiments E21-E47, wherein the immunogenic composition is stable up to about 5 °C.
- E49. The immunogenic composition according to any one of embodiments E21-E48, wherein the immunogenic composition is a vaccine.
- 10 E50. A method for delivering a nucleic acid to a subject in need thereof comprising administering to the subject an immunogenic composition of any one of embodiments E21 to E49 comprising a nucleic acid.
- E51. A method for delivering a therapeutic peptide or protein to a subject in need thereof, the method comprising administering to the subject an immunogenic composition of any one of embodiments E21 to E49 comprising a nucleic acid, wherein the nucleic acid encodes the
15 therapeutic peptide or protein.
- E52. A method for treating or preventing a disease or disorder in a subject comprising administering to the subject an immunogenic composition of any one of embodiments E21 to E49 comprising a nucleic acid, wherein administering the nucleic acid to cells of the subject is beneficial in treating or preventing the disease or disorder.
- 20 E53. A method for treating or preventing a disease or disorder in a subject comprising administering to the subject an immunogenic composition of any one of embodiments E21 to E49 comprising a nucleic acid, wherein the nucleic acid encodes a therapeutic peptide or protein and wherein delivering the therapeutic peptide or protein to the subject is beneficial in treating or preventing the disease or disorder.
- 25 E54. The method of any one of embodiments E50-E53, wherein the nucleic acid is RNA, modRNA or saRNA.
- E55. A method of eliciting an immune response against a disease comprising administering an effective amount of an immunogenic composition according to any one of embodiments E21-E49 to a subject in need thereof.
- 30 E56. The method of any one of embodiments E50 to E55, wherein the subject is a mammal.
- E57. The method of embodiment E56, wherein the mammal is a human.

E58. An immunogenic composition compound according to any one of embodiment E21 to E49 for use as a component of a medicament.

5 E59. Use of the immunogenic composition according to any one of embodiments E21-E49 for the manufacture of a medicament.

In some embodiments, the RNA polynucleotides are mixed in desired ratios in a single vessel and are subsequently formulated into lipid nanoparticles. The inventors surprisingly discovered that the initial input of different RNA polynucleotides at a known ratio to be
10 formulated in a single LNP process surprisingly resulted in LNPs encapsulating the different RNA polynucleotides in about the same ratio as the input ratio. The results were surprising in view of the potential for the manufacturing process to favor one RNA polynucleotide to another when encapsulating the RNA polynucleotides into an LNP. Such embodiments may be referred herein as "pre-mix". Accordingly, in some embodiments, first and second RNA polynucleotides
15 are formulated in a single lipid nanoparticle. In some embodiments, the first, second, third, and fourth RNA polynucleotides are formulated in a single LNP. In some embodiments, the first, second, third, fourth, and fifth RNA polynucleotides are formulated in a single LNP. In some embodiments, the first, second, third, fourth, fifth, and sixth RNA polynucleotides are formulated in a single LNP. In some embodiments, the first, second, third, fourth, fifth, sixth, and seventh
20 RNA polynucleotides are formulated in a single LNP. In some embodiments, the first, second, third, fourth, fifth, sixth, seventh, and eighth RNA polynucleotides are formulated in a single LNP.

In some embodiments, the molar ratio of the first RNA polynucleotide to the second RNA polynucleotide in the mix of RNA polynucleotides prior to formulation into LNPs is about
25 1:50, about 1:25, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, or about 5:1, about 10:1, about 25:1 or about 50:1. In some embodiments, the molar ratio of the first RNA polynucleotide to the second RNA polynucleotide is greater than 1:1.

In some embodiments, the molar ratio of the first RNA polynucleotide to the third RNA polynucleotide in the mix of RNA polynucleotides prior to formulation into LNPs is about 1:50,
30 about 1:25, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, or about 5:1, about 10:1, about 25:1 or about 50:1. In some embodiments, the molar ratio of the first RNA polynucleotide to the third RNA polynucleotide is greater than 1:1.

In some embodiments, the molar ratio of the first RNA polynucleotide to the fourth RNA polynucleotide in the mix of RNA polynucleotides prior to formulation into LNPs is about 1:50,
35 about 1:25, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, or about 5:1, about 10:1, about 25:1 or about 50:1. In some embodiments, the molar ratio of the first RNA polynucleotide to the fourth RNA polynucleotide is greater than 1:1.

In some embodiments, the molar ratio of the first RNA polynucleotide to the fifth RNA polynucleotide in the mix of RNA polynucleotides prior to formulation into LNPs is about 1:50, about 1:25, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, or about 5:1, about 10:1, about 25:1 or about 50:1. In some embodiments, the

5 molar ratio of the first RNA polynucleotide to the fifth RNA polynucleotide is greater than 1:1. In some embodiments, the molar ratio of the first RNA polynucleotide to the sixth RNA polynucleotide in the mix of RNA polynucleotides prior to formulation into LNPs is about 1:50, about 1:25, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, or about 5:1, about 10:1, about 25:1 or about 50:1. In some embodiments, the

10 molar ratio of the first RNA polynucleotide to the sixth RNA polynucleotide is greater than 1:1. In some embodiments, the molar ratio of the first RNA polynucleotide to the seventh RNA polynucleotide in the mix of RNA polynucleotides prior to formulation into LNPs is about 1:50, about 1:25, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, or about 5:1, about 10:1, about 25:1 or about 50:1. In some embodiments, the

15 molar ratio of the first RNA polynucleotide to the seventh RNA polynucleotide is greater than 1:1. In some embodiments, the molar ratio of the first RNA polynucleotide to the eighth RNA polynucleotide in the mix of RNA polynucleotides prior to formulation into LNPs is about 1:50, about 1:25, about 1:10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2:1, about 3:1, about 4:1, or about 5:1, about 10:1, about 25:1 or about 50:1. In some embodiments, the

20 molar ratio of the first RNA polynucleotide to the eighth RNA polynucleotide is greater than 1:1.

RNA

In some embodiments, the RNA molecule, such as the first RNA molecule, is an saRNA. "saRNA," "self-amplifying RNA," and "replicon" refer to RNA with the ability to replicate itself. Self-

25 amplifying RNA molecules may be produced by using replication elements derived from a virus or viruses, e.g., alphaviruses, and substituting the structural viral polypeptides with a nucleotide sequence encoding a polypeptide of interest. A self-amplifying RNA molecule is typically a positive-strand molecule that may be directly translated after delivery to a cell, and this translation provides an RNA-dependent RNA polymerase which then produces both antisense and sense

30 transcripts from the delivered RNA. The delivered RNA leads to the production of multiple daughter RNAs. These daughter RNAs, as well as collinear subgenomic transcripts, may be translated themselves to provide *in situ* expression of an encoded gene of interest, e.g., a viral antigen, or may be transcribed to provide further transcripts with the same sense as the delivered RNA which are translated to provide *in situ* expression of the protein of interest, e.g., an antigen.

35 The overall result of this sequence of transcriptions is an amplification in the number of the introduced saRNAs and so the encoded gene of interest, e.g., a viral antigen, can become a major polypeptide product of the cells.

In some embodiments, the self-amplifying RNA includes at least one or more genes selected from any one of viral replicases, viral proteases, viral helicases and other nonstructural viral proteins. In some embodiments, the self-amplifying RNA may also include 5'- and 3'-end tractive replication sequences, and optionally a heterologous sequence that encodes a desired amino acid sequence (e.g., an antigen of interest). A subgenomic promoter that directs expression of the heterologous sequence may be included in the self-amplifying RNA. Optionally, the heterologous sequence (e.g., an antigen of interest) may be fused in frame to other coding regions in the self-amplifying RNA and/or may be under the control of an internal ribosome entry site (IRES).

In some embodiments, the self-amplifying RNA molecule is not encapsulated in a virus-like particle. Self-amplifying RNA molecules described herein may be designed so that the self-amplifying RNA molecule cannot induce production of infectious viral particles. This may be achieved, for example, by omitting one or more viral genes encoding structural proteins that are necessary to produce viral particles in the self-amplifying RNA. For example, when the self-amplifying RNA molecule is based on an alphavirus, such as Sinbis virus (SIN), Semliki forest virus and Venezuelan equine encephalitis virus (VEE), one or more genes encoding viral structural proteins, such as capsid and/or envelope glycoproteins, may be omitted.

In some embodiments, a self-amplifying RNA molecule described herein encodes (i) an RNA-dependent RNA polymerase that may transcribe RNA from the self-amplifying RNA molecule and (ii) a polypeptide of interest, e.g., a viral antigen. In some embodiments, the polymerase may be an alphavirus replicase, e.g., including any one of alphavirus protein nsP1, nsP2, nsP3, nsP4, and any combination thereof. In some embodiments, the self-amplifying RNA molecules described herein may include one or more modified nucleotides (e.g., pseudouridine, N6-methyladenosine, 5-methylcytidine, 5-methyluridine). In some embodiments, the self-amplifying RNA molecules does not include a modified nucleotide (e.g., pseudouridine, N6-methyladenosine, 5-methylcytidine, 5-methyluridine).

The saRNA construct may encode at least one non-structural protein (NSP), disposed 5' or 3' of the sequence encoding at least one peptide or polypeptide of interest. In some embodiments, the sequence encoding at least one NSP is disposed 5' of the sequences encoding the peptide or polypeptide of interest. Thus, the sequence encoding at least one NSP may be disposed at the 5' end of the RNA construct. In some embodiments, at least one non-structural protein encoded by the RNA construct may be the RNA polymerase nsP4. In some embodiments, the saRNA construct encodes nsP1, nsP2, nsP3 and, nsP4. As is known in the art, nsP1 is the viral capping enzyme and membrane anchor of the replication complex (RC). nsP2 is an RNA helicase and the protease responsible for the ns polyprotein processing. nsP3 interacts with several host proteins and may modulate protein poly- and mono-ADP-ribosylation. nsP4 is the core viral RNA-dependent RNA polymerase. In some embodiments, the polymerase may be an

alphavirus replicase, e.g., comprising one or more of alphavirus proteins nsP1, nsP2, nsP3, and nsP4.

Whereas natural alphavirus genomes encode structural virion proteins in addition to the non- structural replicase polypeptide, in some embodiments, the self-amplifying RNA molecules do not encode alphavirus structural proteins. In some embodiments, the self-amplifying RNA may lead to the production of genomic RNA copies of itself in a cell, but not to the production of RNA that includes virions. Without being bound by theory or mechanism, the inability to produce these virions means that, unlike a wild-type alphavirus, the self-amplifying RNA molecule cannot perpetuate itself in infectious form. The alphavirus structural proteins which are necessary for perpetuation in wild-type viruses can be absent from self-amplifying RNAs of the present disclosure and their place can be taken by gene(s) encoding the immunogen of interest, such that the subgenomic transcript encodes the immunogen rather than the structural alphavirus virion proteins.

In some embodiments, the self-amplifying RNA molecule may have two open reading frames. The first (5') open reading frame can encode a replicase; the second (3') open reading frame can encode a polypeptide comprising an antigen of interest. In some embodiments the RNA may have additional (e.g., downstream) open reading frames, e.g., to encode further antigens or to encode accessory polypeptides.

In some embodiments, the second RNA or the saRNA molecule further includes (1) an alphavirus 5' replication recognition sequence, and (2) an alphavirus 3' replication recognition sequence. In some embodiments, the 5' sequence of the self-amplifying RNA molecule is selected to ensure compatibility with the encoded replicase.

Optionally, self-amplifying RNA molecules described herein may also be designed to induce production of infectious viral particles that are attenuated or virulent, or to produce viral particles that are capable of a single round of subsequent infection.

In some embodiments, the saRNA molecule is alphavirus-based. Alphaviruses include a set of genetically, structurally, and serologically related arthropod-borne viruses of the Togaviridae family. Exemplary viruses and virus subtypes within the alphavirus genus include Sindbis virus, Semliki Forest virus, Ross River virus, and Venezuelan equine encephalitis virus. As such, the self-amplifying RNA described herein may incorporate an RNA replicase derived from any one of semliki forest virus (SFV), sindbis virus (SIN), Venezuelan equine encephalitis virus (VEE), Ross-River virus (RRV), or other viruses belonging to the alphavirus family. In some embodiments, the self-amplifying RNA described herein may incorporate sequences derived from a mutant or wild-type virus sequence, e.g., the attenuated TC83 mutant of VEEV has been used in saRNAs.

Alphavirus-based saRNAs are (+)-stranded saRNAs that may be translated after delivery to a cell, which leads to translation of a replicase (or replicase- transcriptase). The replicase is translated as a polyprotein which auto-cleaves to provide a replication complex which creates

genomic (-)-strand copies of the (+)-strand delivered RNA. These (-)-strand transcripts may themselves be transcribed to give further copies of the (+)-stranded parent RNA and also to give a subgenomic transcript which encodes the desired gene product. Translation of the subgenomic transcript thus leads to *in situ* expression of the desired gene product by the infected cell. Suitable alphavirus saRNAs may use a replicase from a sindbis virus, a semliki forest virus, an eastern equine encephalitis virus, a Venezuelan equine encephalitis virus, or mutant variants thereof.

In some embodiments, the self-amplifying RNA molecule is derived from or based on a virus other than an alphavirus, such as a positive-stranded RNA virus, and in particular a picornavirus, flavivirus, rubivirus, pestivirus, hepacivirus, calicivirus, or coronavirus. Suitable wild-type alphavirus sequences are well-known and are available from sequence depositories, such as the American Type Culture Collection, Rockville, Md. Representative examples of suitable alphaviruses include Aura (ATCC VR-368), Bebaru virus (ATCC VR-600, ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64, ATCC VR-1241), Eastern equine encephalomyelitis virus (ATCC VR-65, ATCC VR-1242), Fort Morgan (ATCC VR-924), Getah virus (ATCC VR-369, ATCC VR-1243), Kyzylgach (ATCC VR-927), Mayaro (ATCC VR- 66), Mayaro virus (ATCC VR-1277), Middleburg (ATCC VR-370), Mucambo virus (ATCC VR-580, ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR- 372, ATCC VR-1245), Ross River virus (ATCC VR-373, ATCC VR-1246), Semliki Forest (ATCC VR-67, ATCC VR-1247), Sindbis virus (ATCC VR-68, ATCC VR-1248), Tonate (ATCC VR-925), Trinita (ATCC VR-469), Una (ATCC VR-374), Venezuelan equine encephalomyelitis (ATCC VR-69, ATCC VR-923, ATCC VR-1250 ATCC VR- 1249, ATCC VR-532), Western equine encephalomyelitis (ATCC VR-70, ATCC VR- 1251, ATCC VR-622, ATCC VR-1252), Whataroa (ATCC VR-926), and Y-62-33 (ATCC VR-375). In some aspects, one or more of the alphaviruses in the list may be excluded.

In some embodiments, the self-amplifying RNA molecules described herein are larger than other types of RNA (e.g., saRNA). Typically, the self-amplifying RNA molecules described herein include at least about 4 kb. For example, the self-amplifying RNA may be equal to any one of, at least any one of, at most any one of, or between any two of 3 kb, 4 kb, 5 kb, 6 kb, 7 kb, 8 kb, 9 kb, 10 kb, 11 kb, 12 kb, 13 kb, 14 kb, 15 kb, 16 kb. In some instances the self-amplifying RNA may include at least about 5 kb, at least about 6 kb, at least about 7 kb, at least about 8 kb, at least about 9 kb, at least about 10 kb, at least about 11 kb, at least about 12 kb, or more than 12 kb. In certain examples, the self-amplifying RNA is about 4 kb to about 12 kb, about 5 kb to about 12 kb, about 6 kb to about 12 kb, about 7 kb to about 12 kb, about 8 kb to about 12 kb, about 9 kb to about 12 kb, about 10 kb to about 12 kb, about 11 kb to about 12 kb, about 5 kb to about 11 kb, about 5 kb to about 10 kb, about 5 kb to about 9 kb, about 5 kb to about 8 kb, about 5 kb to about 7 kb, about 5 kb to about 6 kb, about 6 kb to about 12 kb, about 6 kb to about 11 kb, about 6 kb to about 10 kb, about 6 kb to about 9 kb, about 6 kb to about 8 kb, about 6 kb to about 7 kb, about 7 kb to about 11 kb, about 7 kb to about 10 kb, about 7 kb to about 9 kb, about

7 kb to about 8 kb, about 8 kb to about 11 kb, about 8 kb to about 10 kb, about 8 kb to about 9 kb, about 9 kb to about 11 kb, about 9 kb to about 10 kb, or about 10 kb to about 11 kb.

In some embodiments, the self-amplifying RNA molecule may encode a single polypeptide antigen or, optionally, two or more of polypeptide antigens linked together in a way that each of the sequences retains its identity (e.g., linked in series) when expressed as an amino acid sequence. The polypeptides generated from the self-amplifying RNA may then be produced as a fusion polypeptide or engineered in such a manner to result in separate polypeptide or peptide sequences. In some embodiments, the saRNA molecule may encode one polypeptide of interest or more, such as an antigen or more than one antigen, e.g., two, three, four, five, six, seven, eight, nine, ten, or more polypeptides. Alternatively, or in addition, one saRNA molecule may also encode more than one polypeptide of interest or more, such as an antigen, e.g., a bicistronic, or tricistronic RNA molecule that encodes different or identical antigens.

The term "linked" as used herein refers to a first amino acid sequence or polynucleotide sequence covalently or non-covalently joined to a second amino acid sequence or polynucleotide sequence, respectively. The first amino acid or polynucleotide sequence can be directly joined or juxtaposed to the second amino acid or polynucleotide sequence or alternatively an intervening sequence can covalently join the first sequence to the second sequence. The term "linked" means not only a fusion of a first RNA molecule to a RNA molecule at the 5'-end or the 3'-end, but also includes insertion of the whole first RNA molecule into any two nucleotides in the second RNA molecule. The first second RNA molecule can be linked to a second RNA molecule by a phosphodiester bond or a linker. The linker can be, e.g., a polynucleotide.

In some embodiments, the self-amplifying RNA described herein may encode one or more polypeptide antigens that include a range of epitopes. In some embodiments, the self-amplifying RNA described herein may encode epitopes capable of eliciting either a helper T-cell response or a cytotoxic T-cell response or both.

In some embodiments, the saRNA molecule is purified, e.g., such as by filtration that may occur via, e.g., ultrafiltration, diafiltration, or, e.g., tangential flow ultrafiltration/diafiltration.

Some embodiments of the disclosure are directed to a composition comprising a self-amplifying RNA molecule comprising a 5' Cap, a 5' untranslated region, a coding region comprising a sequence encoding an RNA-dependent RNA polymerase (also referred to as a "replicase"), a subgenomic promoter, such as one derived from an alphavirus, an open reading frame encoding a gene of interest (e.g., an antigen derived from a virus), a 3' untranslated region, and a 3' poly A sequence. In some embodiments, at least 5% of a total population of a particular nucleotide in the saRNA molecule has been replaced with one or more modified or unnatural nucleotides.

In some embodiments, the saRNA molecule does not include modified nucleotides, e.g., does not include modified nucleobases, and all of the nucleotides in the RNA molecule are

conventional standard ribonucleotides A, U, G and C, with the exception of an optional 5' cap that may include, for example, 7-methylguanosine, which is further described below.

In some embodiments, the saRNA molecule does not include modified nucleotides, e.g., does not include modified nucleobases, and all of the nucleotides in the RNA molecule are conventional standard ribonucleotides A, U, G and C, with the exception of an optional 5' cap that may include, for example, 7-methylguanosine, which is further described below. In some embodiments, the RNA may include a 5' cap comprising a 7'-methylguanosine, and the first 1, 2 or 3 5' ribonucleotides may be methylated at the 2' position of the ribose.

In alternative embodiments, each RNA polynucleotide encoding a particular antigen is formulated in an individual LNP, such that each LNP encapsulates an RNA polynucleotide encoding identical antigens. Such embodiments may be referred herein as "post-mix". Accordingly, in some embodiments, the first RNA polynucleotide is formulated in a first LNP; the second RNA polynucleotide is formulated in a second LNP; the third RNA polynucleotide is formulated in a third LNP; the fourth RNA polynucleotide is formulated in a fourth LNP; the fifth RNA polynucleotide is formulated in a fifth LNP; the sixth RNA polynucleotide is formulated in a sixth LNP; the seventh RNA polynucleotide is formulated in a seventh LNP; and the eighth RNA polynucleotide is formulated in an eighth LNP.

In some embodiments, the molar ratio of the first LNP to the second LNP in the mix of LNPs prior to formulation into LNPs is about 1:50, about 1:25, about 1: 10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2: 1, about 3: 1, about 4: 1, or about 5: 1, about 10: 1, about 25: 1 or about 50: 1. In some embodiments, the molar ratio of the first LNP to the second LNP is greater than 1:1.

In some embodiments, the molar ratio of the first LNP to the third LNP in the mix of LNPs prior to formulation into LNPs is about 1:50, about 1:25, about 1: 10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2: 1, about 3: 1, about 4: 1, or about 5: 1, about 10: 1, about 25: 1 or about 50: 1. In some embodiments, the molar ratio of the first LNP to the third LNP is greater than 1:1.

In some embodiments, the molar ratio of the first LNP to the fourth LNP in the mix of LNPs prior to formulation into LNPs is about 1:50, about 1:25, about 1: 10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2: 1, about 3: 1, about 4: 1, or about 5: 1, about 10: 1, about 25: 1 or about 50: 1. In some embodiments, the molar ratio of the first LNP to the fourth LNP is greater than 1:1. In some embodiments, the molar ratio of the first LNP to the fifth LNP in the mix of LNPs prior to formulation into LNPs is about 1:50, about 1:25, about 1: 10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2: 1, about 3: 1, about 4: 1, or about 5: 1, about 10: 1, about 25: 1 or about 50: 1. In some embodiments, the molar ratio of the first LNP to the fifth LNP is greater than 1:1. In some embodiments, the molar ratio of the first LNP to the sixth LNP in the mix of LNPs prior to formulation into LNPs is about 1:50, about 1:25, about 1: 10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2: 1, about 3: 1, about 4: 1, or

about 5: 1, about 10: 1, about 25: 1 or about 50: 1. In some embodiments, the molar ratio of the first LNP to the sixth LNP is greater than 1:1. In some embodiments, the molar ratio of the first LNP to the seventh LNP in the mix of LNPs prior to formulation into LNPs is about 1:50, about 1:25, about 1: 10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2: 1, about 3: 1, about 4: 1, or about 5: 1, about 10: 1, about 25: 1 or about 50: 1. In some embodiments, the molar ratio of the first LNP to the seventh LNP is greater than 1:1. In some embodiments, the molar ratio of the first LNP to the eighth LNP in the mix of LNPs prior to formulation into LNPs is about 1:50, about 1:25, about 1: 10, about 1:5, about 1:4, about 1:3, about 1:2, about 1:1, about 2: 1, about 3: 1, about 4: 1, or about 5: 1, about 10: 1, about 25: 1 or about 50: 1. In some
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embodiments, the molar ratio of the first LNP to the eighth LNP is greater than 1:1. Surprisingly, it has been discovered that regardless of the process, the resulting ratio of RNA polynucleotide is comparable whether the plurality of RNA polynucleotides are mixed prior to formulation in an LNP (pre-mixed) or whether the RNA polynucleotides encoding a particular antigen are formulated in an individual LNP and the plurality of LNPs for different antigens are mixed (post-mixed). As a result of this discovery, there may be an option for medical professionals to mix and administer different ratios of antigens depending on the need, e.g. influenza season, particularly when the individual LNPs encapsulate RNA for a single antigen. In some embodiments, the mRNAs encoding antigens are separately encapsulated (e.g., separate encapsulation of each mRNA encoding an antigen; encapsulation of mRNA(s) encoding a first antigen (e.g. influenza type A antigen) in a first population of nanoparticles (e.g., LNPs) and encapsulation of mRNA(s) encoding a second antigen (e.g. influenza type B antigen) in a second population of nanoparticles (e.g., LNPs); or encapsulation of mRNA(s) encoding a first antigen (e.g. influenza type A antigen) in a first population of nanoparticles (e.g., LNPs) and encapsulation of each mRNA encoding a second antigen (e.g. influenza type B antigen) in a separate population of nanoparticles (e.g., LNPs)). In some embodiments, the composition comprises coencapsulated mRNAs encoding an antigen. In some embodiments, a composition comprising (i) two or more different RNAs, each encoding an antigenic polypeptide (e.g., an HA protein) of a first virus (e.g., influenza type A virus), e.g. so that the two or more different RNAs, together, encode two or more different polypeptides (e.g. two or more different influenza A HA polypeptides, and (ii) two or more different RNAs, each encoding an antigenic polypeptide (e.g., an HA protein) of a second virus (e.g., an influenza type B virus), e.g. so that the two or more different RNAs, together, encode two or more different polypeptides (e.g. two or more different influenza B HA polypeptides are formulated in nanoparticles (e.g., LNPs) such that: each RNA encoding an antigenic polypeptide of a first virus (e.g. influenza type A virus) is encapsulated in a first population of nanoparticles and each RNA encoding an antigenic polypeptide of a second virus (e.g. influenza type B virus) is encapsulated in a second population of nanoparticles; each RNA is encapsulated in a separate nanoparticle; or each RNA encoding a polypeptide of a first antigen (e.g. influenza type A antigenic polypeptide) is

encapsulated in a first population of nanoparticles and the two RNAs encoding a polypeptide of second antigen (e.g. influenza type B antigenic polypeptide) are each encapsulated in separate populations of nanoparticles.

5 The present disclosure relates to mRNA vaccines in general. Several mRNA vaccine platforms are available in the prior art. The basic structure of in vitro transcribed (IVT) mRNA closely resembles "mature" eukaryotic mRNA and includes (i) a protein-encoding open reading frame (ORF), flanked by (ii) 5' and 3' untranslated regions (UTRs), and at the end sides (iii) a 7-methyl guanosine 5' cap structure and (iv) a 3' poly(A) tail. The non-coding structural features play important roles in the pharmacology of mRNA and can be individually optimized to
10 modulate the mRNA stability, translation efficiency, and immunogenicity. By incorporating modified nucleosides, mRNA transcripts referred to as "nucleoside-modified mRNA" can be produced with reduced immunostimulatory activity, and therefore an improved safety profile can be obtained. In addition, modified nucleosides allow the design of mRNA vaccines with strongly enhanced stability and translation capacity, as they can avoid the direct antiviral pathways that
15 are induced by type IFNs and are programmed to degrade and inhibit invading mRNA. For instance, the replacement of uridine with pseudouridine in IVT mRNA reduces the activity of 2'-5'-oligoadenylate synthetase, which regulates the mRNA cleavage by RNase L. In addition, lower activities are measured for protein kinase R, an enzyme that is associated with the inhibition of the mRNA translation process.

20 Besides the incorporation of modified nucleotides, other approaches have been validated to increase the translation capacity and stability of mRNA. One example is the development of "sequence-engineered mRNA". Here, mRNA expression can be strongly increased by sequence optimizations in the ORF and UTRs of mRNA, for instance by enriching the GC content, or by selecting the UTRs of natural long-lived mRNA molecules. Another
25 approach is the design of "self-amplifying mRNA" constructs. These are mostly derived from alphaviruses and contain an ORF that is replaced by the antigen of interest together with an additional ORF encoding viral replicase. The latter drives the intracellular amplification of mRNA and can therefore significantly increase the antigen expression capacity.

Also, several modifications have been implemented at the end structures of mRNA. Anti-
30 reverse cap (ARCA) modifications can ensure the correct cap orientation at the 5' end, which yields almost complete fractions of mRNA that can efficiently bind the ribosomes. Other cap modifications, such as phosphorothioate cap analogs, can further improve the affinity towards the eukaryotic translation initiation factor 4E, and increase the resistance against the RNA decapping complex.

35 Conversely, by modifying its structure, the potency of mRNA to trigger innate immune responses can be further improved, but to the detriment of translation capacity. By stabilizing the mRNA with either a phosphorothioate backbone, or by its precipitation with the cationic

protein protamine, antigen expression can be diminished, but stronger immune-stimulating capacities can be obtained.

In one aspect the invention relates to an immunogenic composition comprising an mRNA molecule that encodes one or more polypeptides or fragments thereof of an antigen.

5 In some embodiments, the mRNA molecule comprises a nucleoside-modified mRNA. mRNA useful in the disclosure typically include a first region of linked nucleosides encoding a polypeptide of interest (e.g., a coding region), a first flanking region located at the 5'-terminus of the first region (e.g., a 5'-UTR), a second flanking region located at the 3'-terminus of the first region (e.g., a 3'-UTR), at least one 5'-cap region, and a 3'-stabilizing region. In some
10 embodiments, the mRNA of the disclosure further includes a poly-A region or a Kozak sequence (e.g., in the 5'-UTR). In some cases, mRNA of the disclosure may contain one or more intronic nucleotide sequences capable of being excised from the polynucleotide. In some embodiments, mRNA of the disclosure may include a 5' cap structure, a chain terminating nucleotide, a stem loop, a poly A sequence, and/or a polyadenylation signal. Any one of the
15 regions of a nucleic acid may include one or more alternative components (e.g., an alternative nucleoside). For example, the 3'-stabilizing region may contain an alternative nucleoside such as an L-nucleoside, an inverted thymidine, or a 2'-O-methyl nucleoside and/or the coding region, 5'-UTR, 3'-UTR, or cap region may include an alternative nucleoside such as a 5-substituted uridine (e.g., 5-methoxyuridine), a 1-substituted pseudouridine (e.g., 1-methyl-pseudouridine),
20 and/or a 5-substituted cytidine (e.g., 5-methyl-cytidine).

The compositions described herein comprise at least one RNA polynucleotide, such as a mRNA (e.g., modified mRNA). mRNA, for example, is transcribed in vitro from template DNA, referred to as an "in vitro transcription template." In some embodiments, an in vitro transcription template encodes a 5' untranslated (UTR) region, contains an open reading frame, and
25 encodes a 3' UTR and a polyA tail. The particular nucleic acid sequence composition and length of an in vitro transcription template will depend on the mRNA encoded by the template.

A "5' untranslated region" (UTR) refers to a region of an mRNA that is directly upstream (i.e., 5') from the start codon (i.e., the first codon of an mRNA transcript translated by a ribosome) that does not encode a polypeptide.

30 A "3' untranslated region" (UTR) refers to a region of an mRNA that is directly downstream (i.e., 3') from the stop codon (i.e., the codon of an mRNA transcript that signals a termination of translation) that does not encode a polypeptide.

An "open reading frame" is a continuous stretch of DNA beginning with a start codon (e.g., methionine (ATG)), and ending with a stop codon (e.g., TAA, TAG or TGA) and encodes
35 a polypeptide.

A "polyA tail" is a region of mRNA that is downstream, e.g., directly downstream (i.e., 3'), from the 3' UTR that contains multiple, consecutive adenosine monophosphates. A polyA tail may contain 10 to 300 adenosine monophosphates. For example, a polyA tail may contain 10,

20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290 or 300 adenosine monophosphates. In some embodiments, a polyA tail contains 50 to 250 adenosine monophosphates. In a relevant biological setting (e.g., in cells, in vivo) the poly(A) tail functions to protect mRNA from enzymatic degradation, e.g., in the cytoplasm, and aids in transcription termination, export of the mRNA from the nucleus and translation.

In preferred embodiments, the 3' polyadenylation tail comprises SEQ ID NO: 3.

In some embodiments, a polynucleotide includes 200 to 3,000 nucleotides. For example, a polynucleotide may include 200 to 500, 200 to 1000, 200 to 1500, 200 to 3000, 500 to 1000, 500 to 1500, 500 to 2000, 500 to 3000, 1000 to 1500, 1000 to 2000, 1000 to 3000, 1500 to 3000, or 2000 to 3000 nucleotides).

In some embodiments, a LNP includes one or more RNAs, and the one or more RNAs, lipids, and amounts thereof may be selected to provide a specific N:P ratio. The N:P ratio of the composition refers to the molar ratio of nitrogen atoms in one or more lipids to the phosphate groups in an RNA. In general, a lower N:P ratio is preferred. In one embodiment, the lipid is an ionizable lipid. In another embodiment, the lipid is an ionizable cationic lipid. The one or more RNA, lipids, and amounts thereof may be selected to provide an N:P ratio from about 2: 1 to about 30:1, such as 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 12:1, 14:1, 16:1, 18:1, 20:1, 22: 1, 24: 1, 26: 1, 28: 1, or 30: 1. In certain embodiments, the N:P ratio may be from about 2: 1 to about 8: 1. In other embodiments, the N:P ratio is from about 5 : 1 to about 8: 1. For example, the N:P ratio may be about 5.0: 1, about 5.5 : 1, about 5.67: 1, about 6.0: 1, about 6.5: 1, or about 7.0: 1. For example, the N:P ratio may be about 5.67: 1.

In some embodiments, the composition comprises a second LNP, wherein the second LNP does not encapsulate a polynucleotide as described in WO2023057930, the entirety of which is incorporated herein by reference. In some aspects, the compositions and methods described herein relate to frozen or lyophilized lipid nanoparticles encapsulating or associated with RNA in the presence of a cryoprotectant, preferably a carbohydrate cryoprotectant, and/or further in the presence of lipid nanoparticles that are devoid of nucleic acid, (e.g., not encapsulating and not associated with RNA (also referred herein as "blank" LNPs), or liposomes, or a higher cryoprotectant concentration) resulting in a composition comprising LNPs encapsulating RNA or associated with RNA that is characterized by, among other things, an improved integrity of the RNA after completion of the respective freezing or lyophilization process and which is further characterized by increased storage stability, such as, for example, with respect to storage for extended periods and/or under non-cooling conditions, as compared to a composition comprising lipid nanoparticles encapsulating or associated with RNA in the absence of the blank LNPs, or liposomes, or a higher cryoprotectant concentration when assessed under identical conditions. In other words, in some aspects, the compositions include a mixture of a first lipid nanoparticle encapsulating or associated with RNA, a second lipid

nanoparticle that is devoid of nucleic acid, and a cryoprotectant that results in improved characteristics of the encapsulated RNA after freezing or lyophilization processes, preferably for use as a pharmaceutical composition, such as, for example, an immunogenic composition or vaccine. In some aspects, the compositions include a mixture of a first lipid nanoparticle encapsulating or associated with RNA and an increased cryoprotectant concentration that results in improved characteristics of the encapsulated RNA after freezing or lyophilization processes, preferably for use as a pharmaceutical composition, such as, for example, an immunogenic composition or vaccine. In some aspects, the compositions include a mixture of a first lipid nanoparticle encapsulating or associated with RNA and a second lipid nanoparticle that is devoid of nucleic acid that results in improved characteristics of the encapsulated RNA after freezing or lyophilization processes, preferably for use as a pharmaceutical composition, such as, for example, an immunogenic composition or vaccine. In some aspects, the compositions include a mixture of a first lipid nanoparticle encapsulating or associated with RNA and a liposome that results in improved characteristics of the encapsulated RNA after freezing or lyophilization processes, preferably for use as a pharmaceutical composition, such as, for example, an immunogenic composition or vaccine. In some aspects, the compositions include a mixture of a first lipid nanoparticle encapsulating or associated with RNA, a liposome, and an increased cryoprotectant concentration that result in improved characteristics of the encapsulated RNA after freezing or lyophilization processes, preferably for use as a pharmaceutical composition, such as, for example, an immunogenic composition or vaccine. Advantageously, the compositions and methods thereof described herein are suitable for use at an industrial scale. The methods described herein may be used to produce, for example, a frozen or lyophilized composition comprising LNPs encapsulating or associated with RNA having the above-mentioned properties in a reproducible and cost-effective manner. The composition comprising LNPs encapsulating or associated with RNA may advantageously be stored, shipped and applied, e.g., for example as a vaccine, without a cold chain, while the integrity and the biological activity of the RNA in the composition remain unexpectedly high.

mRNA of the disclosure may include one or more naturally occurring components, including any of the canonical nucleotides A (adenosine), G (guanosine), C (cytosine), U (uridine), or T (thymidine). In one embodiment, all or substantially all of the nucleotides comprising (a) the 5'-UTR, (b) the open reading frame (ORF), (c) the 3'-UTR, (d) the poly A tail, and any combination of (a, b, c, or d above) comprise naturally occurring canonical nucleotides A (adenosine), G (guanosine), C (cytosine), U (uridine), or T (thymidine).

mRNA of the disclosure may include one or more alternative components, as described herein, which impart useful properties including increased stability and/or the lack of a substantial induction of the innate immune response of a cell into which the polynucleotide is introduced. For example, a modRNA may exhibit reduced degradation in a cell into which the modRNA is introduced, relative to a corresponding unaltered mRNA. These alternative species

may enhance the efficiency of protein production, intracellular retention of the polynucleotides, and/or viability of contacted cells, as well as possess reduced immunogenicity.

mRNA of the disclosure may include one or more modified (e.g., altered or alternative) nucleobases, nucleosides, nucleotides, or combinations thereof. The mRNA useful in a LNP
 5 can include any useful modification or alteration, such as to the nucleobase, the sugar, or the internucleoside linkage (e.g., to a linking phosphate / to a phosphodiester linkage / to the phosphodiester backbone). In certain embodiments, alterations (e.g., one or more alterations) are present in each of the nucleobase, the sugar, and the internucleoside linkage. Alterations according to the present disclosure may be alterations of ribonucleic acids (RNAs), e.g., the
 10 substitution of the 2'-OH of the ribofuranosyl ring to 2'-H, threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs), or hybrids thereof. Additional alterations are described herein.

mRNA of the disclosure may or may not be uniformly altered along the entire length of the molecule. For example, one or more or all types of nucleotide (e.g., purine or pyrimidine, or
 15 any one or more or all of A, G, U, C) may or may not be uniformly altered in a mRNA, or in a given predetermined sequence region thereof. In some instances, all nucleotides X in a mRNA (or in a given sequence region thereof) are altered, wherein X may any one of nucleotides A, G, U, C, or any one of the combinations A+G, A+U, A+C, G+U, G+C, U+C, A+G+U, A+G+C, G+U+C or A+G+C.

Different sugar alterations and/or internucleoside linkages (e.g., backbone structures) may exist at various positions in a polynucleotide. One of ordinary skill in the art will appreciate that the nucleotide analogs or other alteration(s) may be located at any position(s) of a polynucleotide such that the function of the polynucleotide is not substantially decreased. An alteration may also be a 5'- or 3'-terminal alteration. In some embodiments, the polynucleotide
 25 includes an alteration at the 3'-terminus. The polynucleotide may contain from about 1% to about 100% alternative nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, i.e., any one or more of A, G, U or C) or any intervening percentage (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to
 30 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to
 35 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100%). It will be understood that any remaining percentage is accounted for by the presence of a canonical nucleotide (e.g., A, G, U, or C).

Polynucleotides may contain at a minimum zero and at maximum 100% alternative nucleotides, or any intervening percentage, such as at least 5% alternative nucleotides, at least 10% alternative nucleotides, at least 25% alternative nucleotides, at least 50% alternative nucleotides, at least 80% alternative nucleotides, or at least 90% alternative nucleotides. For example, polynucleotides may contain an alternative pyrimidine such as an alternative uracil or cytosine. In some embodiments, at least 5%, at least 10%, at least 25%, at least 50%, at least 80%, at least 90% or 100% of the uracil in a polynucleotide is replaced with an alternative uracil (e.g., a 5-substituted uracil). The alternative uracil can be replaced by a compound having a single unique structure or can be replaced by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures). In some instances, at least 5%, at least 10%, at least 25%, at least 50%, at least 80%, at least 90% or 100% of the cytosine in the polynucleotide is replaced with an alternative cytosine (e.g., a 5-substituted cytosine). The alternative cytosine can be replaced by a compound having a single unique structure or can be replaced by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures).

In some instances, nucleic acids do not substantially induce an innate immune response of a cell into which the polynucleotide (e.g., mRNA) is introduced. Features of an induced innate immune response include 1) increased expression of pro-inflammatory cytokines, 2) activation of intracellular PRRs (RIG-I, MDA5, etc., and/or 3) termination or reduction in protein translation.

In some embodiments, the mRNA comprises one or more alternative nucleoside or nucleotides. The alternative nucleosides and nucleotides can include an alternative nucleobase. A nucleobase of a nucleic acid is an organic base such as a purine or pyrimidine or a derivative thereof. A nucleobase may be a canonical base (e.g., adenine, guanine, uracil, thymine, and cytosine). These nucleobases can be altered or wholly replaced to provide polynucleotide molecules having enhanced properties, e.g., increased stability such as resistance to nucleases. Non-canonical or modified bases may include, for example, one or more substitutions or modifications including but not limited to alkyl, aryl, halo, oxo, hydroxyl, alkyloxy, and/or thio substitutions; one or more fused or open rings; oxidation; and/or reduction.

In some embodiments, the nucleobase is an alternative uracil. Exemplary nucleobases and nucleosides having an alternative uracil include pseudouridine (ψ), pyridin-4- one ribonucleoside, 5-aza-uracil, 6-aza-uracil, 2-thio-5-aza-uracil, 2-thio-uracil (s2U), 4-thio- uracil (s4U), 4-thio-pseudouridine, 2-thio-pseudouridine, 5 -hydroxy -uracil (ho5U), 5-aminoallyl-uracil, 5-halo-uracil (e.g., 5-iodo-uracil or 5-bromo-uracil), 3-methyl-uracil (m U), 5-methoxy-uracil (mo5U), uracil 5-oxyacetic acid (cmo5U), uracil 5-oxyacetic acid methyl ester (mcmo5U), 5-carboxymethyl-uracil (cm5U), 1 -carboxymethyl-pseudouridine, 5-carboxyhydroxymethyl-uracil (chm5U), 5-carboxyhydroxymethyl-uracil methyl ester (mchm5U), 5-methoxycarbonylmethyl-uracil (mcm5U), 5-methoxycarbonylmethyl-2-thio-uracil (mcm5s2U), 5-

aminomethyl-2-thio-uracil (nmVu), 5-methylaminomethyl-uracil (mnm5U), 5-methylaminomethyl-2-thio-uracil (mnmVu), 5-methylaminomethyl-2-seleno-uracil (mnm5se2U), 5-carbamoylmethyl-uracil (ncm5U), 5-carboxymethylaminomethyl-uracil (cmnm5U), 5-carboxymethylaminomethyl-2-thio-uracil (cmnmVu), 5-propynyl-uracil, 1-propynyl-pseudouracil, 5-taurinomethyl-uracil (xm5U), 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uracil(xm5s2U), 1-taurinomethyl-4-thio-pseudouridine, 5-methyl-uracil (m5U, i.e., having the nucleobase deoxythymine), 1-methyl-pseudouridine (mV), 5-methyl-2-thio-uracil (m5s2U), 1-methyl-4-thio-pseudouridine (m xj/), 4-thio- 1-methyl-pseudouridine, 3-methyl-pseudouridine (m \\\), 2-thio- 1-methyl-pseudouridine, 1-methyl- 1-deaza-pseudouridine, 2-thio-1-methyl- 1-deaza-pseudouridine, dihydrouracil (D), dihydropseudouridine, 5,6-dihydrouracil, 5-methyl-dihydrouracil (m5D), 2-thio-dihydrouracil, 2-thio-dihydropseudouridine, 2-methoxy-uracil, 2-methoxy-4-thio-uracil, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine, 3-(3-amino-3-carboxypropyl)uracil (acp U), 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine (acp ψ), 5-(isopentenylaminomethyl)uracil (inm5U), 5-(isopentenylaminomethyl)-2-thio-uracil (inm5s2U), 5,2'-O-dimethyl-uridine (m5Um), 2-thio-2'-O-methyl-uridine (s2Um), 5-methoxycarbonylmethyl-2'-O-methyl-uridine (mem Um), 5-carbamoylmethyl-2'-O-methyl-uridine (ncm5Um), 5-carboxymethylaminomethyl-2'-O-methyl-uridine (cmnm5Um), 3,2'-O-dimethyl-uridine (m Um), and 5-(isopentenylaminomethyl)-2'-O-methyl-uridine (inm5Um), 1-thio-uracil, deoxythymidine, 5-(2-carbomethoxyvinyl)-uracil, 5-(carbamoylhydroxymethyl)-uracil, 5-carbamoylmethyl-2-thio-uracil, 5-carboxymethyl-2-thio-uracil, 5-cyanomethyl-uracil, 5-methoxy-2-thio-uracil, and 5-[3-(1-E-propenylamino)]uracil.

In some embodiments, the nucleobase is an alternative cytosine. Exemplary nucleobases and nucleosides having an alternative cytosine include 5-aza-cytosine, 6-aza-cytosine, pseudoisocytidine, 3-methyl-cytosine (m3C), N4-acetyl-cytosine (ac4C), 5-formyl-cytosine (f5C), N4-methyl-cytosine (m4C), 5-methyl-cytosine (m5C), 5-halo-cytosine (e.g., 5-iodo-cytosine), 5-hydroxymethyl-cytosine (hm5C), 1-methyl-pseudoisocytidine, pyrrolo-cytosine, pyrrolo-pseudoisocytidine, 2-thio-cytosine (s2C), 2-thio-5-methyl-cytosine, 4-thio-pseudoisocytidine, 4-thio-1-methyl-1-pseudoisocytidine, 4-thio-1-methyl-1-deaza-pseudoisocytidine, 1-methyl-1-deaza-pseudoisocytidine, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-thio-zebularine, 2-methoxy-cytosine, 2-methoxy-5-methyl-cytosine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, lysidine (k2C), 5,2'-O-dimethyl-cytidine (m5Cm), N4-acetyl-2'-O-methyl-cytidine (ac4Cm), N4,2'-O-dimethyl-cytidine (m4Cm), 5-formyl-2'-O-methyl-cytidine (f5Cm), N4,N4,2'-O-trimethyl-cytidine (m42Cm), 1-thio-cytosine, 5-hydroxy-cytosine, 5-(3-azidopropyl)-cytosine, and 5-(2-azidoethyl)-cytosine.

In some embodiments, the nucleobase is an alternative adenine. Exemplary nucleobases and nucleosides having an alternative adenine include 2-amino-purine, 2,6-diaminopurine, 2-amino-6-halo-purine (e.g., 2-amino-6-chloro-purine), 6-halo-purine (e.g., 6-

- chloro-purine), 2-amino-6-methyl-purine, 8-azido-adenine, 7-deaza-adenine, 7-deaza-8-aza-adenine, 7-deaza-2-amino-purine, 7-deaza-8-aza-2-amino-purine, 7-deaza-2,6-diaminopurine, 7-deaza-8-aza-2,6-diaminopurine, 1-methyl-adenine (m1A), 2-methyl-adenine (m2A), N6-methyl-adenine (m6A), 2-methylthio-N6-methyl-adenine (ms2m6A), N6-isopentenyl-adenine (i6A), 2-methylthio-N6-isopentenyl-adenine (ms2i6A), N6-(cis-hydroxyisopentenyl)adenine (io6A), 2-methylthio-N6-(cis-hydroxyisopentenyl)adenine (ms2io6A), N6-glycylcarbamoyl-adenine (g6A), N6-threonylcarbamoyl-adenine (t6A), N6-methyl-N6-threonylcarbamoyl-adenine (m6t6A), 2-methylthio-N6-threonylcarbamoyl-adenine (ms2g6A), N6,N6-dimethyl-adenine (m62A), N6-hydroxynorvalylcarbamoyl-adenine (hn6A), 2-methylthio-N6-hydroxynorvalylcarbamoyl-adenine (ms2hn6A), N6-acetyl-adenine (ac6A), 7-methyl-adenine, 2-methylthio-adenine, 2-methoxy-adenine, N6,2'-O-dimethyl-adenosine (m6Am), N6,N6,2'-O-trimethyl-adenosine (m62Am), 1,2'-O-dimethyl-adenosine (m1Am), 2-amino-N6-methyl-purine, 1-thio-adenine, 8-azido-adenine, N6-(19-amino-pentaoxonadecyl)-adenine, 2,8-dimethyl-adenine, N6-formyl-adenine, and N6-hydroxymethyl-adenine.
- In some embodiments, the nucleobase is an alternative guanine. Exemplary nucleobases and nucleosides having an alternative guanine include inosine (I), 1-methyl-inosine (m1I), wyosine (imG), methylwyosine (mimG), 4-demethyl-wyosine (imG-14), isowyosine (imG2), wybutosine (yW), peroxywybutosine (o2yW), hydroxywybutosine (OHyW), undermodified hydroxywybutosine (OHyW*), 7-deaza-guanine, queuosine (Q), epoxyqueuosine (oQ), galactosyl-queuosine (galQ), mannosyl-queuosine (manQ), 7-cyano-7-deaza-guanine (preQO), 7-aminomethyl-7-deaza-guanine (preQI), archaeosine (G+), 7-deaza-8-aza-guanine, 6-thio-guanine, 6-thio-7-deaza-guanine, 6-thio-7-deaza-8-aza-guanine, 7-methyl-guanine (m7G), 6-thio-7-methyl-guanine, 7-methyl-inosine, 6-methoxy-guanine, 1-methyl-guanine (m1G), N2-methyl-guanine (m2G), N2,N2-dimethyl-guanine (m22G), N2,7-dimethyl-guanine (m2,7G), N2,N2,7-dimethyl-guanine (m2,2,7G), 8-oxo-guanine, 7-methyl-8-oxo-guanine, 1-methyl-6-thio-guanine, N2-methyl-6-thio-guanine, N2,N2-dimethyl-6-thio-guanine, N2-methyl-2'-O-methyl-guanosine (m2Gm), N2,N2-dimethyl-2'-O-methyl-guanosine (m22Gm), 1-methyl-2'-O-methyl-guanosine (m1Gm), N2,7-dimethyl-2'-O-methyl-guanosine (m2,7Gm), 2'-O-methyl-inosine (Im), 1,2'-O-dimethyl-inosine (m1Im), 1-thio-guanine, and O-6-methyl-guanine.
- The alternative nucleobase of a nucleotide can be independently a purine, a pyrimidine, a purine or pyrimidine analog. For example, the nucleobase can be an alternative to adenine, cytosine, guanine, uracil, or hypoxanthine. In another embodiment, the nucleobase can also include, for example, naturally-occurring and synthetic derivatives of a base, including pyrazolo[3,4-d]pyrimidines, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo (e.g., 8-bromo), 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxy and

other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, deazaguanine, 7-deazaguanine, 3-deazaguanine, deazaadenine, 7-deazaadenine, 3-deazaadenine, pyrazolo[3,4-d]pyrimidine, imidazo[1,5-a] 1,3,5 triazinones, 9-deazapurines, imidazo[4,5-d]pyrazines, thiazolo[4,5-d]pyrimidines, pyrazin-2-ones, 1,2,4-triazine, pyridazine; or 1,3,5 triazine. When the nucleotides are depicted using the shorthand A, G, C, T or U, each letter refers to the representative base and/or derivatives thereof, e.g., A includes adenine or adenine analogs, e.g., 7-deaza adenine).

The mRNA may include a 5'-cap structure. The 5'-cap structure of a polynucleotide is involved in nuclear export and increasing polynucleotide stability and binds the mRNA Cap Binding Protein (CBP), which is responsible for polynucleotide stability in the cell and translation competency through the association of CBP with poly-A binding protein to form the mature cyclic mRNA species. The cap further assists the removal of 5'-proximal introns removal during mRNA splicing.

Endogenous polynucleotide molecules may be 5'-end capped generating a 5'-ppp-5'-triphosphate linkage between a terminal guanosine cap residue and the 5'-terminal transcribed sense nucleotide of the polynucleotide. This 5'-guanylate cap may then be methylated to generate an N7-methyl-guanylate residue. The ribose sugars of the terminal and/or antiterminal transcribed nucleotides of the 5' end of the polynucleotide may optionally also be 2'-O-methylated. 5'-decapping through hydrolysis and cleavage of the guanylate cap structure may target a polynucleotide molecule, such as an mRNA molecule, for degradation.

Alterations to polynucleotides may generate a non-hydrolyzable cap structure preventing decapping and thus increasing polynucleotide half-life. Because cap structure hydrolysis requires cleavage of 5'-ppp-5' phosphodiester linkages, alternative nucleotides may be used during the capping reaction. For example, a Vaccinia Capping Enzyme from New England Biolabs (Ipswich, MA) may be used with a-thio-guanosine nucleotides according to the manufacturer's instructions to create a phosphorothioate linkage in the 5'-ppp-5' cap.

Additional alternative guanosine nucleotides may be used such as a-methyl-phosphonate and seleno-phosphate nucleotides. Additional alterations include, but are not limited to, 2'-O-methylation of the ribose sugars of 5'-terminal and/or 5'-antiterminal nucleotides of the polynucleotide (as mentioned above) on the 2'-hydroxy group of the sugar. Multiple distinct 5'-cap structures can be used to generate the 5'-cap of an mRNA molecule.

Cap analogs, which herein are also referred to as synthetic cap analogs, chemical caps, chemical cap analogs, or structural or functional cap analogs, differ from natural (i.e., endogenous, wild-type, or physiological) 5'-caps in their chemical structure, while retaining cap function. Cap analogs may be chemically (i.e., non-enzymatically) or enzymatically synthesized and/linked to a polynucleotide. For example, the Anti-Reverse Cap Analog (ARCA) cap contains two guanoses linked by a 5'-5'-triphosphate group, wherein one guanosine contains

an N7-methyl group as well as a 3'-O-methyl group (i.e., N7, '0-dimethyl-guanosine-5 '-triphosphate-5 '-guanosine, m7G-3'mppp-G, which may equivalently be designated 3' 0-Me-m7G(5')ppp(5')G). The 3'-O atom of the other, unaltered, guanosine becomes linked to the 5 '-terminal nucleotide of the capped polynucleotide (e.g., an mRNA). The N7- and 3'-O-methylated
5 guanosine provides the terminal moiety of the capped polynucleotide (e.g., mRNA). Another exemplary cap is mCAP, which is similar to ARCA but has a 2'-O-methyl group on guanosine (i.e., N7,2'-O-dimethyl-guanosine-5 '-triphosphate-5 '-guanosine, m7Gm- ppp-G).

A cap may be a dinucleotide cap analog. As a non-limiting example, the dinucleotide cap analog may be modified at different phosphate positions with a boranophosphate group or
10 a phosphoroselenoate group such as the dinucleotide cap analogs described in US Patent No. 8,519,110, the cap structures of which are herein incorporated by reference.

Alternatively, a cap analog may be a N7-(4-chlorophenoxy ethyl) substituted dinucleotide cap analog known in the art and/or described herein. Non-limiting examples of N7-(4-chlorophenoxy ethyl) substituted dinucleotide cap analogs include a N7-(4-
15 chlorophenoxyethyl)-G(5 ')ppp(5 ')G and a N7-(4-chlorophenoxyethyl)-m3 '-OG(5 ')ppp(5 ')G cap analog (see, e.g., the various cap analogs and the methods of synthesizing cap analogs described in Kore et al. Bioorganic & Medicinal Chemistry 2013 21 :4570-4574; the cap structures of which are herein incorporated by reference). In other instances, a cap analog useful in the polynucleotides of the present disclosure is a 4-chloro/bromophenoxy ethyl analog.

20 While cap analogs allow for the concomitant capping of a polynucleotide in an *in vitro* transcription reaction, up to 20% of transcripts remain uncapped. This, as well as the structural differences of a cap analog from endogenous 5 '-cap structures of polynucleotides produced by the endogenous, cellular transcription machinery, may lead to reduced translational competency and reduced cellular stability.

25 Alternative polynucleotides may also be capped post-transcriptionally, using enzymes, in order to generate more authentic 5'-cap structures. As used herein, the phrase "more authentic" refers to a feature that closely mirrors or mimics, either structurally or functionally, an endogenous or wild type feature. That is, a "more authentic" feature is better representative of an endogenous, wild-type, natural or physiological cellular function, and/or structure as
30 compared to synthetic features or analogs of the prior art, or which outperforms the corresponding endogenous, wild-type, natural, or physiological feature in one or more respects. Non-limiting examples of more authentic 5 '-cap structures useful in the polynucleotides of the present disclosure are those which, among other things, have enhanced binding of cap binding proteins, increased half-life, reduced susceptibility to 5'-endonucleases, and/or reduced 5'-
35 decapping, as compared to synthetic 5 '-cap structures known in the art (or to a wild-type, natural or physiological 5 '-cap structure). For example, recombinant Vaccinia Virus Capping Enzyme and recombinant 2'-O-methyltransferase enzyme can create a canonical 5 '-5 '-triphosphate linkage between the 5 '-terminal nucleotide of a polynucleotide and a guanosine

cap nucleotide wherein the cap guanosine contains an N7-methylation and the 5'-terminal nucleotide of the polynucleotide contains a 2'-O-methyl. Such a structure is termed the Cap1 structure. This cap results in a higher translational-competency, cellular stability, and a reduced activation of cellular pro-inflammatory cytokines, as compared, e.g., to other 5' cap analog structures known in the art. Other exemplary cap structures include 7mG(5')ppp(5')N,pN2p (Cap 0), 7mG(5')ppp(5')NImpNp (Cap 1), 7mG(5')-ppp(5')NImpN2mp (Cap 2), and m(7)Gpppm(3)(6,6,2')Apm(2')Apm(2')Cpm(2)(3,2')Up (Cap 4).

Because the alternative polynucleotides may be capped post-transcriptionally, and because this process is more efficient, nearly 100% of the mRNA may be capped. This is in contrast to ~80% when a cap analog is linked to a polynucleotide in the course of an *in vitro* transcription reaction. 5'-terminal caps may include endogenous caps or cap analogs. A 5'-terminal cap may include a guanosine analog. Useful guanosine analogs include inosine, N1-methyl-guanosine, 2'-fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine. In some cases, a polynucleotide contains a modified 5'-cap. A modification on the 5'-cap may increase the stability of polynucleotide, increase the half-life of the polynucleotide, and could increase the polynucleotide translational efficiency. The modified 5'-cap may include, but is not limited to, one or more of the following modifications: modification at the 2'- and/or 3'-position of a capped guanosine triphosphate (GTP), a replacement of the sugar ring oxygen (that produced the carbocyclic ring) with a methylene moiety (CH₂), a modification at the triphosphate bridge moiety of the cap structure, or a modification at the nucleobase (G) moiety.

A 5'-UTR may be provided as a flanking region to the mRNA. A 5'-UTR may be homologous or heterologous to the coding region found in a polynucleotide. Multiple 5'-UTRs may be included in the flanking region and may be the same or of different sequences. Any portion of the flanking regions, including none, may be codon optimized and any may independently contain one or more different structural or chemical alterations, before and/or after codon optimization.

In one embodiment, an ORF encoding an antigen of the disclosure is codon optimized. Codon optimization methods are known in the art. For example, an ORF of any one or more of the sequences provided herein may be codon optimized. Codon optimization, in some embodiments, may be used to match codon frequencies in target and host organisms to ensure proper folding; bias GC content to increase mRNA stability or reduce secondary structures; minimize tandem repeat codons or base runs that may impair gene construction or expression; customize transcriptional and translational control regions; insert or remove protein trafficking sequences; remove/add post translation modification sites in encoded protein (e.g., glycosylation sites); add, remove or shuffle protein domains; insert or delete restriction sites; modify ribosome binding sites and mRNA degradation sites; adjust translational rates to allow the various domains of the protein to fold properly; or reduce or eliminate problem secondary

structures within the polynucleotide. Codon optimization tools, algorithms and services are known in the art—non-limiting examples include services from GeneArt (Life Technologies), DNA2.0 (Menlo Park Calif.) and/or proprietary methods. In some embodiments, the open reading frame (ORF) sequence is optimized using optimization algorithms. To alter one or more properties of an mRNA, 5'-UTRs which are heterologous to the coding region of an mRNA may be engineered. The mRNA may then be administered to cells, tissue or organisms and outcomes such as protein level, localization, and/or half-life may be measured to evaluate the beneficial effects the heterologous 5'-UTR may have on the mRNA. Variants of the 5'-UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G. 5'-UTRs may also be codon-optimized, or altered in any manner described herein.

mRNAs may include a stem loop such as, but not limited to, a histone stem loop. The stem loop may be a nucleotide sequence that is about 25 or about 26 nucleotides in length. The histone stem loop may be located 3'-relative to the coding region (e.g., at the 3'-terminus of the coding region). As a non-limiting example, the stem loop may be located at the 3'-end of a polynucleotide described herein. In some cases, an mRNA includes more than one stem loop (e.g., two stem loops). A stem loop may be located in a second terminal region of a polynucleotide. As a non-limiting example, the stem loop may be located within an untranslated region (e.g., 3'-UTR) in a second terminal region. In some cases, a mRNA which includes the histone stem loop may be stabilized by the addition of a 3'-stabilizing region (e.g., a 3'-stabilizing region including at least one chain terminating nucleoside). Not wishing to be bound by theory, the addition of at least one chain terminating nucleoside may slow the degradation of a polynucleotide and thus can increase the half-life of the polynucleotide. In other cases, a mRNA, which includes the histone stem loop may be stabilized by an alteration to the 3'-region of the polynucleotide that can prevent and/or inhibit the addition of oligio(U). In yet other cases, a mRNA, which includes the histone stem loop may be stabilized by the addition of an oligonucleotide that terminates in a 3'-deoxynucleoside, 2',3'-dideoxynucleoside 3'-O-methylnucleosides, 3'-O- ethylnucleosides, 3'-arabinosides, and other alternative nucleosides known in the art and/or described herein. In some instances, the mRNA of the present disclosure may include a histone stem loop, a poly-A region, and/or a 5'-cap structure. The histone stem loop may be before and/or after the poly-A region. The polynucleotides including the histone stem loop and a poly-A region sequence may include a chain terminating nucleoside described herein. In other instances, the polynucleotides of the present disclosure may include a histone stem loop and a 5'-cap structure. The 5'-cap structure may include, but is not limited to, those described herein and/or known in the art. In some cases, the conserved stem loop region may include a miR sequence described herein. As a non-limiting example, the stem loop region may include the seed sequence of a miR sequence described herein. In another non-limiting example, the stem loop region may include a miR- 122 seed sequence.

mRNA may include at least one histone stem-loop and a poly-A region or polyadenylation signal. In certain cases, the polynucleotide encoding for a histone stem loop and a poly-A region or a polyadenylation signal may code for a pathogen antigen or fragment thereof. In other cases, the polynucleotide encoding for a histone stem loop and a poly-A region or a polyadenylation signal may code for a therapeutic protein. In some cases, the polynucleotide encoding for a histone stem loop and a poly-A region or a polyadenylation signal may code for a tumor antigen or fragment thereof. In other cases, the polynucleotide encoding for a histone stem loop and a poly-A region or a polyadenylation signal may code for an allergenic antigen or an autoimmune self-antigen.

10 An mRNA may include a polyA sequence and/or polyadenylation signal. A polyA sequence may be comprised entirely or mostly of adenine nucleotides or analogs or derivatives thereof. A polyA sequence may be a tail located adjacent to a 3' untranslated region of a nucleic acid. During RNA processing, a long chain of adenosine nucleotides (poly-A region) is normally added to messenger RNA (mRNA) molecules to increase the stability of the molecule.

15 Immediately after transcription, the 3'-end of the transcript is cleaved to free a 3'-hydroxy. Then poly-A polymerase adds a chain of adenosine nucleotides to the RNA. The process, called polyadenylation, adds a poly-A region that is between 100 and 250 residues long. Unique poly-A region lengths may provide certain advantages to the alternative polynucleotides of the present disclosure. Generally, the length of a poly-A region of the present disclosure is at least

20 30 nucleotides in length. In another embodiment, the poly-A region is at least 35 nucleotides in length. In another embodiment, the length is at least 40 nucleotides. In another embodiment, the length is at least 45 nucleotides. In another embodiment, the length is at least 55 nucleotides. In another embodiment, the length is at least 60 nucleotides. In another embodiment, the length is at least 70 nucleotides. In another embodiment, the length is at least

25 80 nucleotides. In another embodiment, the length is at least 90 nucleotides. In another embodiment, the length is at least 100 nucleotides. In another embodiment, the length is at least 120 nucleotides. In another embodiment, the length is at least 140 nucleotides. In another embodiment, the length is at least 160 nucleotides. In another embodiment, the length is at least 180 nucleotides. In another embodiment, the length is at least 200 nucleotides. In another

30 embodiment, the length is at least 250 nucleotides. In another embodiment, the length is at least 300 nucleotides. In another embodiment, the length is at least 350 nucleotides. In another embodiment, the length is at least 400 nucleotides. In another embodiment, the length is at least 450 nucleotides. In another embodiment, the length is at least 500 nucleotides. In another embodiment, the length is at least 600 nucleotides. In another embodiment, the length is at least 700 nucleotides. In another embodiment, the length is at least 800 nucleotides. In another embodiment, the length is at least 900 nucleotides. In another embodiment, the length is at least 1000 nucleotides. In another embodiment, the length is at least 1100 nucleotides. In another embodiment, the length is at least 1200 nucleotides. In another embodiment, the length

is at least 1300 nucleotides. In another embodiment, the length is at least 1400 nucleotides. In another embodiment, the length is at least 1500 nucleotides. In another embodiment, the length is at least 1600 nucleotides. In another embodiment, the length is at least 1700 nucleotides. In another embodiment, the length is at least 1800 nucleotides. In another embodiment, the length is at least 1900 nucleotides. In another embodiment, the length is at least 2000 nucleotides. In another embodiment, the length is at least 2500 nucleotides. In another embodiment, the length is at least 3000 nucleotides. In some instances, the poly-A region may be 80 nucleotides, 120 nucleotides, 160 nucleotides in length on an alternative polynucleotide molecule described herein. In other instances, the poly-A region may be 20, 40, 80, 100, 120, 140 or 160 nucleotides in length on an alternative polynucleotide molecule described herein. In some cases, the poly-A region is designed relative to the length of the overall alternative polynucleotide. This design may be based on the length of the coding region of the alternative polynucleotide, the length of a particular feature or region of the alternative polynucleotide (such as mRNA) or based on the length of the ultimate product expressed from the alternative polynucleotide. When relative to any feature of the alternative polynucleotide (e.g., other than the mRNA portion which includes the poly-A region) the poly-A region may be 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% greater in length than the additional feature. The poly-A region may also be designed as a fraction of the alternative polynucleotide to which it belongs. In this context, the poly-A region may be 10, 20, 30, 40, 50, 60, 70, 80, or 90% or more of the total length of the construct or the total length of the construct minus the poly-A region.

In certain cases, engineered binding sites and/or the conjugation of mRNA for poly-A binding protein may be used to enhance expression. The engineered binding sites may be sensor sequences which can operate as binding sites for ligands of the local microenvironment of the mRNA. As a non-limiting example, the mRNA may include at least one engineered binding site to alter the binding affinity of poly-A binding protein (PABP) and analogs thereof. The incorporation of at least one engineered binding site may increase the binding affinity of the PABP and analogs thereof.

Additionally, multiple distinct mRNA may be linked together to the PABP (poly-A binding protein) through the 3'-end using alternative nucleotides at the 3'-terminus of the poly-A region. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12 hours, 24 hours, 48 hours, 72 hours, and day 7 post-transfection. As a non-limiting example, the transfection experiments may be used to evaluate the effect on PABP or analogs thereof binding affinity as a result of the addition of at least one engineered binding site. In certain cases, a poly-A region may be used to modulate translation initiation. While not wishing to be bound by theory, the poly-A region recruits PABP which in turn can interact with translation initiation complex and thus may be essential for protein synthesis. In some cases, a poly-A region may also be used in the present disclosure to protect against 3'-5' exonuclease digestion. In some instances, an mRNA may include a polyA-G Quartet. The G-

quartet is a cyclic hydrogen bonded array of four guanosine nucleotides that can be formed by G-rich sequences in both DNA and RNA. In this embodiment, the G-quartet is incorporated at the end of the poly-A region. The resultant mRNA may be assayed for stability, protein production and other parameters including half-life at various time points. It has been

5 discovered that the polyA-G quartet results in protein production equivalent to at least 75% of that seen using a poly-A region of 120 nucleotides alone. In some cases, mRNA may include a poly-A region and may be stabilized by the addition of a 3'-stabilizing region. The mRNA with a poly-A region may further include a 5'-cap structure. In other cases, mRNA may include a poly-A-G Quartet. The mRNA with a poly-A-G Quartet may further include a 5'-cap structure. In

10 some cases, the 3'-stabilizing region which may be used to stabilize mRNA includes a poly-A region or poly-A-G Quartet. In other cases, the 3'-stabilizing region which may be used with the present disclosure include a chain termination nucleoside such as 3'-deoxyadenosine (cordycepin), 3'-deoxyuridine, 3'-deoxycytosine, 3'-deoxyguanosine, 3'-deoxy thymine, 2',3'-dideoxynucleosides, such as 2',3'-dideoxyadenosine, 2',3'-dideoxyuridine, 2',3'-

15 dideoxycytosine, 2', 3'-dideoxyguanosine, 2',3'-dideoxythymine, a 2'-deoxynucleoside, or an O-methylnucleoside. In other cases, mRNA which includes a polyA region or a poly-A-G Quartet may be stabilized by an alteration to the 3'-region of the polynucleotide that can prevent and/or inhibit the addition of oligo(U). In yet other instances, mRNA which includes a poly-A region or a poly-A-G Quartet may be stabilized by the addition of an oligonucleotide that

20 terminates in a 3'-deoxynucleoside, 2',3'-dideoxynucleoside 3'-O-methylnucleosides, 3'-O-ethylnucleosides, 3'-arabinosides, and other alternative nucleosides known in the art and/or described herein.

The modRNA immunogenic composition is based on a modRNA platform technology. The single stranded, 5'-capped modRNA contains an open reading frame encoding the vaccine

25 antigen of interest and features structural elements optimized for high efficacy of the RNA. The modRNA also contains a substitution of 1-methyl-pseudouridine for each uridine to decrease recognition of the vaccine RNA by innate immune sensors, such as TLRs 7 and 8, resulting in decreased innate immune activation and increased protein translation. The modRNA is encapsulated in a LNP for delivery into target cells.

30 In an embodiment, the mRNA vaccines of the disclosure comprise lipids. The lipids and mRNA can together form nanoparticles. The lipids can encapsulate the mRNA in the form of a lipid nanoparticle (LNP) to aid cell entry and stability of the RNA/lipid nanoparticles.

Lipid nanoparticles may include a lipid component and one or more additional components, such as a therapeutic and/or prophylactic. A LNP may be designed for one or

35 more specific applications or targets. The elements of a LNP may be selected based on a particular application or target, and/or based on the efficacy, toxicity, expense, ease of use, availability, or other feature of one or more elements. Similarly, the particular formulation of a

LNP may be selected for a particular application or target according to, for example, the efficacy and toxicity of particular combinations of elements. The efficacy and tolerability of a LNP formulation may be affected by the stability of the formulation.

5 Lipid nanoparticles may be designed for one or more specific applications or targets. For example, a LNP may be designed to deliver a therapeutic and/or prophylactic such as an RNA to a particular cell, tissue, organ, or system or group thereof in a mammal's body. The LNP delivery system may have adjuvant effects which enhance the immunogenicity of an encoded antigen and/or other antigens in the composition.

10 Physiochemical properties of lipid nanoparticles may be altered to increase selectivity for particular bodily targets. For instance, particle sizes may be adjusted based on the fenestration sizes of different organs. The therapeutic and/or prophylactic included in a LNP may also be selected based on the desired delivery target or targets. For example, a therapeutic and/or prophylactic may be selected for a particular indication, condition, disease, or disorder and/or for delivery to a particular cell, tissue, organ, or system or group thereof (e.g.,
15 localized or specific delivery). In certain embodiments, a LNP may include an mRNA encoding a polypeptide of interest capable of being translated within a cell to produce the polypeptide of interest. Such a composition may be designed to be specifically delivered to a particular organ. In some embodiments, a composition may be designed to be specifically delivered to a mammalian liver. In some embodiments, a composition may be designed to be specifically
20 delivered to a lymph node. In some embodiments, a composition may be designed to be specifically delivered to a mammalian spleen.

A LNP may include one or more components described herein. In some embodiments, the LNP formulation of the disclosure includes at least one lipid nanoparticle component. Lipid nanoparticles may include a lipid component and one or more additional components, such as
25 a therapeutic and/or prophylactic, such as a nucleic acid. A LNP may be designed for one or more specific applications or targets. The elements of a LNP may be selected based on a particular application or target, and/or based on the efficacy, toxicity, expense, ease of use, availability, or other feature of one or more elements. Similarly, the particular formulation of a LNP may be selected for a particular application or target according to, for example, the efficacy
30 and toxicity of particular combination of elements. The efficacy and tolerability of a LNP formulation may be affected by the stability of the formulation.

In some embodiments, for example, a polymer may be included in and/or used to encapsulate or partially encapsulate or be conjugated to a lipid (polymer-conjugated lipid or polymer lipid) in a LNP. A polymer may be biodegradable and/or biocompatible. A polymer may
35 be selected from, but is not limited to, polyamines, polyethers, polyamides, polyesters, poly carbamates, polyureas, polycarbonates, polystyrenes, polyimides, polysulfones, polyurethanes, polyacetylenes, polyethylenes, polyethyleneimines, polyisocyanates, polyacrylates, polymethacrylates, polyacrylonitriles, and polyarylates. For example, a polymer may include

poly(caprolactone) (PCL), ethylene vinyl acetate polymer (EVA), poly(lactic acid) (PLA), poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(lactic acid-co-glycolic acid) (PLGA), poly(L-lactic acid-co-glycolic acid) (PLLGA), poly(D,L-lactide) (PDLA), poly(L-lactide) (PLLA), poly(D,L-lactide-co-caprolactone), poly(D,L-lactide-co-caprolactone-co-glycolide), poly(D,L-lactide-co-PEO-co-D,L-lactide), poly(D,L-lactide-co-PPO-co-D,L-lactide), polyalkyl cyanoacrylate, polyurethane, poly-L-lysine (PLL), hydroxypropyl methacrylate (HPMA), polyethyleneglycol, poly-L-glutamic acid, poly(hydroxy acids), polyanhydrides, polyorthoesters, poly(ester amides), polyamides, poly(ester ethers), polycarbonates, polyalkylenes such as polyethylene and polypropylene, polyalkylene glycols such as poly(ethylene glycol) (PEG), polyalkylene oxides (PEO), polyalkylene terephthalates such as poly(ethylene terephthalate), polyvinyl alcohols (PVA), polyvinyl ethers, polyvinyl esters such as poly(vinyl acetate), polyvinyl halides such as poly(vinyl chloride) (PVC), polyvinylpyrrolidone (PVP), polysiloxanes, polystyrene, polyurethanes, derivatized celluloses such as alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, hydroxypropylcellulose, carboxymethylcellulose, polymers of acrylic acids, such as poly(methyl(meth)acrylate) (PMMA), poly(ethyl(meth)acrylate), poly(butyl(meth)acrylate), poly(isobutyl(meth)acrylate), poly(hexyl(meth)acrylate), poly(isodecyl(meth)acrylate), poly(lauryl(meth)acrylate), poly(phenyl(meth)acrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) and copolymers and mixtures thereof, polydioxanone and its copolymers, polyhydroxyalkanoates, polypropylene fumarate, polyoxymethylene, poloxamers, poloxamines, poly(ortho)esters, poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), trimethylene carbonate, poly(N-acryloylmorpholine) (PACM), poly(2-methyl-2-oxazoline) (PMOX), poly(2-ethyl-2-oxazoline) (PEOZ), poly(2-oxazoline)(POZ), which is a synthetic, water-soluble, and low-viscosity polymer, and polyglycerol.

In some embodiments, the amount of polymer-lipid in the lipid composition of a pharmaceutical composition disclosed herein ranges from about 0.1 mol % to about 5 mol %, from about 0.5 mol % to about 5 mol %, from about 1 mol % to about 5 mol %, from about 1.5 mol % to about 5 mol %, from about 2 mol % to about 5 mol %, from about 0.1 mol % to about 4 mol %, from about 0.5 mol % to about 4 mol %, from about 1 mol % to about 4 mol %, from about 1.5 mol % to about 4 mol %, from about 2 mol % to about 4 mol %, from about 0.1 mol % to about 3 mol %, from about 0.5 mol % to about 3 mol %, from about 1 mol % to about 3 mol %, from about 1.5 mol % to about 3 mol %, from about 2 mol % to about 3 mol %, from about 0.1 mol % to about 2 mol %, from about 0.5 mol % to about 2 mol %, from about 1 mol % to about 2 mol %, from about 1.5 mol % to about 2 mol %, from about 0.1 mol % to about 1.5 mol %, from about 0.5 mol % to about 1.5 mol %, or from about 1 mol % to about 1.5 mol %.

In some embodiments, the amount of polymer-lipid in the lipid composition disclosed herein is about 2 mol %. In some embodiments, the amount of polymer-lipid in the lipid composition disclosed herein is about 1.5 mol %. In some embodiments, the amount of

polymer-lipid in the lipid composition disclosed herein is at least about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 3, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, or 5 mol %. In a preferred embodiment, the amount of polymer-lipid (or polymer-conjugated lipid) in the lipid composition is 1.8 mol %. In some aspects, the lipid composition of the pharmaceutical compositions disclosed herein does not comprise a PEG-lipid.

Surface altering agents may include, but are not limited to, anionic proteins (e.g., bovine serum albumin), surfactants (e.g., cationic surfactants such as dimethyldioctadecyl- ammonium bromide), sugars or sugar derivatives (e.g., cyclodextrin), nucleic acids, polymers (e.g., heparin, polyethylene glycol, and poloxamer), mucolytic agents (e.g., acetylcysteine, mugwort, bromelain, papain, clerodendrum, bromhexine, carbocysteine, eprazinone, mesna, ambroxol, sobrerol, domiodol, letosteine, stepronin, tiopronin, gelsolin, thymosin β 4, dornase alfa, neltexine, and erdosteine), and DNases (e.g., rhDNase). A surface altering agent may be disposed within a nanoparticle and/or on the surface of a LNP (e.g., by coating, adsorption, covalent linkage, or other process).

A LNP may also comprise one or more functionalized lipids. For example, a lipid may be functionalized with an alkyne group that, when exposed to an azide under appropriate reaction conditions, may undergo a cycloaddition reaction. In particular, a lipid bilayer may be functionalized in this fashion with one or more groups useful in facilitating membrane permeation, cellular recognition, or imaging. The surface of a LNP may also be conjugated with one or more useful antibodies. Functional groups and conjugates useful in targeted cell delivery, imaging, and membrane permeation are well known in the art.

In addition to these components, lipid nanoparticles may include any substance useful in pharmaceutical compositions. For example, the lipid nanoparticle may include one or more pharmaceutically acceptable excipients or accessory ingredients such as, but not limited to, one or more solvents, dispersion media, diluents, dispersion aids, suspension aids, surface active agents, buffering agents, preservatives, and other species.

Surface active agents and/or emulsifiers may include, but are not limited to, natural emulsifiers (e.g., acacia, alginic acid, sodium alginate, cholesterol, and lecithin), sorbitan fatty acid esters (e.g., polyoxy ethylene sorbitan monolaurate [TWEEN®20], polyoxy ethylene sorbitan [TWEEN® 60], polyoxy ethylene sorbitan monooleate [TWEEN®80], sorbitan monopalmitate [SPAN®40], sorbitan monostearate [SPAN®60], sorbitan tristearate [SPAN®65], glyceryl monooleate, sorbitan monooleate [SPAN®80]), polyoxyethylene esters (e.g., polyoxyethylene monostearate [MYRJ® 45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and Solutol®), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g., CREMOPHOR®), polyoxyethylene ethers, (e.g., polyoxyethylene lauryl ether [BRIJ® 30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid,

ethyl laurate, sodium lauryl sulfate, PLURONIC®F 68, POLOXAMER® 188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, and/or combinations thereof.

- Examples of preservatives may include, but are not limited to, antioxidants, chelating agents, free radical scavengers, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and/or other preservatives. Examples of antioxidants include, but are not limited to, alpha tocopherol, ascorbic acid, ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxy toluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfite, sodium metabisulfite, and/or sodium sulfite. Examples of chelating agents include ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, disodium edetate, dipotassium edetate, edetic acid, fumaric acid, malic acid, phosphoric acid, sodium edetate, tartaric acid, and/or trisodium edetate. Examples of antimicrobial preservatives include, but are not limited to, benzalkonium chloride, benzethonium chloride, benzyl alcohol, bronopol, cetrimide, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxylenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and/or thimerosal. Examples of antifungal preservatives include, but are not limited to, butyl paraben, methyl paraben, ethyl paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and/or sorbic acid. Examples of alcohol preservatives include, but are not limited to, ethanol, polyethylene glycol, benzyl alcohol, phenol, phenolic compounds, bisphenol, chlorobutanol, hydroxybenzoate, and/or phenylethyl alcohol. Examples of acidic preservatives include, but are not limited to, vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroascorbic acid, ascorbic acid, sorbic acid, and/or phytic acid. Other preservatives include, but are not limited to, tocopherol, tocopherol acetate, deteroxime mesylate, cetrimide, butylated hydroxyanisole (BHA), butylated hydroxy toluene (BHT), ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium sulfite, potassium metabisulfite, GLYDANT PLUS®, PHENONIP®, methylparaben, GERMALL® 115, GERMABEN®II, NEOLONE™, KATHON™, and/or EUXYL®. An exemplary free radical scavenger includes butylated hydroxytoluene (BHT or butylhydroxytoluene) or deferoxamine.

- Examples of buffering agents include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium gluconate, calcium gluceptate, calcium gluconate, d-gluconic acid, calcium glycerophosphate, calcium lactate, calcium lactobionate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium

chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, amino-sulfonate buffers (e.g., HEPES), magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, Tris buffer, and/or combinations thereof.

- 5 In some embodiments, the formulation including a LNP may further include a salt, such as a chloride salt. In some embodiments, the formulation including a LNP may further includes a sugar such as a disaccharide. In some embodiments, the formulation further includes a sugar but not a salt, such as a chloride salt. In some embodiments, a LNP may further include one or more small hydrophobic molecules such as a vitamin (e.g., vitamin A or vitamin E) or a sterol.
- 10 Carbohydrates may include simple sugars (e.g., glucose) and polysaccharides (e.g., glycogen and derivatives and analogs thereof).

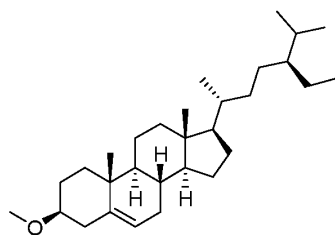
The characteristics of a LNP may depend on the components thereof. For example, a LNP including cholesterol as a structural lipid may have different characteristics than a LNP that includes a different structural lipid. As used herein, the term "structural lipid" refers to sterols and also to lipids containing sterol moieties. As defined herein, "sterols" are a subgroup of

15 steroids consisting of steroid alcohols. In some embodiments, the structural lipid is a steroid. In some embodiments, the structural lipid is cholesterol. In some embodiments, the structural lipid is an analog of cholesterol.

The lipid nanoparticle compositions may include a mixture of structural lipids.

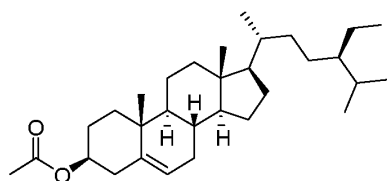
20 Incorporation of structural lipids in the lipid nanoparticle may help mitigate aggregation of other lipids in the particle. Structural lipids can be selected from the group including but not limited to, cholesterol and cholesterol analogs such as fecosterol, sitosterol, β -sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, tomatine, ursolic acid, alpha-tocopherol, hopanoids, sitostanol, phytosterols, steroids, and mixtures thereof. In some embodiments, the

25 structural lipid is a sterol. In some embodiments, the structural lipid is a steroid. In some embodiments, the structural lipid is cholesterol. In certain embodiments, the structural lipid is an analog of cholesterol. In some preferred embodiments, the sterol comprises



(SN792).

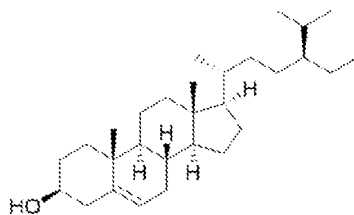
In some preferred embodiments, the sterol comprises



(CL-309557).

In some preferred embodiments, the sterol comprises stigmasterol.

5 In some preferred embodiments, the sterol comprises β -sitosterol



In some embodiments, the structural lipid is a sitosterol, a stigmasterol, a campesterol, a sitostanol, a campestanol, a brassicasterol, a fucosterol, beta-sitosterol, stigmasterol, beta-sitostanol, ergosterol, lupeol, cycloartenol, Δ^5 -avenascerol, Δ^7 -avenascerol or a Δ^7 -stigmasterol, including analogs, salts or esters thereof, alone or in combination. In some embodiments, the sterol component of a LNP of the disclosure is a single phytosterol. In some embodiments, the phytosterol component of a LNP of the disclosure is a mixture of different phytosterols (e.g. 2, 3, 4, 5 or 6 different phytosterols). In some embodiments, the phytosterol component of an LNP of the disclosure is a blend of one or more phytosterols and one or more zoosterols, such as a blend of a phytosterol (e.g., a sitosterol, such as beta-sitosterol) and cholesterol. In some embodiments, the phytosterol is β -sitosterol, campesterol, stigmasterol, or any combination thereof. In some embodiments, the phytosterol is β -sitosterol. In some embodiments, the cholesterol analog comprises β -sitosterol, campesterol, and stigmasterol. In some

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embodiments, the mixture of structural lipids comprises a mixture of β -sitosterol and cholesterol. In one embodiment, the mixture of structural lipids is a mixture of β -sitosterol and cholesterol.

In some embodiments, the mixture of structural lipids comprises about 35% to about 85% of β -sitosterol, about 5% to about 35% stigmasterol, and about 5% to about 35% of campesterol. In some embodiments, the mixture of structural lipids comprises about 40% to about 80% of β -sitosterol, about 10% to about 30% stigmasterol, and about 10% to about 30% of campesterol. In some embodiments, the mixture of structural lipids comprises about 40% to about 70% of β -sitosterol, about 10% to about 25% stigmasterol, and about 10% to about 25% of campesterol. In some embodiments, the mixture of structural lipids comprises about 40% to

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about 70% of β -sitosterol, about 15% to about 25% stigmasterol, and about 15% to about 25% of campesterol. In some embodiments, the mixture of structural lipids comprises about 35% to about 45% of β -sitosterol, about 20% to about 30% stigmasterol, and about 20% to about 30% of campesterol. In some embodiments, the the mixture of structural lipids comprises about 40%
5 to about 50% of β -sitosterol, about 25% to about 35% stigmasterol, and about 25% to about 35% of campesterol. In some embodiments, the mixture of structural lipids comprises about 65% to about 75% of β -sitosterol, about 5% to about 15% stigmasterol, and about 5% to about 15% of campesterol. In some embodiments, the mixture of structural lipids comprises about 35% to about 85% of β -sitosterol, about 5% to about 35% stigmasterol, and 0% of campesterol.
10 In some embodiments, the mixture of structural lipids comprises about 40% to about 80% of β -sitosterol, about 10% to about 30% stigmasterol, and 0% of campesterol. In some embodiments, the mixture of structural lipids comprises about 40% to about 70% of β -sitosterol, about 10% to about 25% stigmasterol, and 0% of campesterol. In some embodiments, the mixture of structural lipids comprises about 40% to about 70% of β -sitosterol, about 15% to
15 about 25% stigmasterol, and 0% of campesterol. In some embodiments, the mixture of structural lipids comprises about 35% to about 45% of β -sitosterol, about 20% to about 30% stigmasterol, and 0% of campesterol. In some embodiments, the mixture of structural lipids comprises about 40% to about 50% of β -sitosterol, about 25% to about 35% stigmasterol, and 0% of campesterol. In some embodiments, the mixture of structural lipids comprises about 65%
20 to about 75% of β -sitosterol, about 5% to about 15% stigmasterol, and 0% of campesterol. Accordingly, in some preferred embodiments, the composition does not comprise campesterol.

In some embodiments, the composition comprises a mixture of structural lipids comprising about 10% to about 30% of cholesterol, about 10% to about 30% β -sitosterol, and about 10% to about 30% stigmasterol, and 0% campesterol. See, for example, **Table 2**. In
25 some embodiments, the composition further comprises about 30-50% cationic lipid and about 5-25% phospholipid.

In some embodiments, the mixture of structural lipids is between about 30 mol% and about 60 mol%. In some embodiments, the mixture of structural lipids is between about 40 mol% and about 60 mol%. In some embodiments, the mixture of structural lipids is between
30 about 30 mol% and about 50 mol%. In some embodiments, the mixture of structural lipids is between about 40 mol% and about 50 mol%. In some embodiments, the mixture of structural lipids is about 30 mol%. In some embodiments, the mixture of structural lipids is about 40 mol%. In some embodiments, the mixture of structural lipids is about 50 mol%. In some
embodiments, the mixture of structural lipids is about 60 mol%.

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In some embodiments, the mol % of the structural lipids is between about 1% and 50% of the mol % of the compound having the structure of any of the foregoing compounds present in the lipid nanoparticle. In some embodiments, the mol % of the structural lipids is between

about 10% and 40% of the mol % of the compound having the structure of any of the foregoing compounds present in the lipid nanoparticle. In some embodiments, the mol % of the structural lipids is between about 20% and 30% of the mol % of the compound having the structure of any of the foregoing compounds present in the lipid nanoparticle. In some embodiments, the mol %
5 of the structural lipids is about 30% of the mol % of the compound having the structure of any of the foregoing compounds present in the lipid nanoparticle.

In some embodiments, the lipid nanoparticle compositions described herein can comprise about 20 mol% to about 60 mol% structural lipids. In some embodiments, the lipid nanoparticle compositions comprise about 30 mol% to about 50 mol% of structural lipids. In
10 some embodiments, the lipid nanoparticle compositions comprise about 35 mol% to about 45 mol% of structural lipids. In some embodiments, the lipid nanoparticle compositions comprise about 37 mol% to about 42 mol% of structural lipids. In some embodiments, the lipid nanoparticle compositions comprise about 35, about 36, about 37, about 38, about 39, or about 40 mol% of structural lipids. In some embodiments, the nanoparticle comprises about 39 to
15 about 40 mol% structural lipids. In some aspects, the lipid nanoparticle compositions comprising a mixture of structural lipids does not comprise a mixture of cholesterol and β -sitosterol of about 38.5 mol%.

In some embodiments, the amount of the structural lipid or mixture of structural lipids in the composition disclosed herein is at least about 30.0, 30.1, 30.2, 30.3, 30.4, 30.5, 30.6, 30.7,
20 30.8, 30.9, 31.0, 31.1, 31.2, 31.3, 31.4, 31.5, 31.6, 31.7, 31.8, 31.9, 32.0, 32.1, 32.2, 32.3, 32.4, 32.5, 32.6, 32.7, 32.8, 32.9, 33.0, 33.1, 33.2, 33.3, 33.4, 33.5, 33.6, 33.7, 33.8, 33.9, 34.0, 34.1, 34.2, 34.3, 34.4, 34.5, 34.6, 34.7, 34.8, 34.9, 35.0, 35.1, 35.2, 35.3, 35.4, 35.5, 35.6, 35.7, 35.8, 35.9, 36.0, 36.1, 36.2, 36.3, 36.4, 36.5, 36.6, 36.7, 36.8, 36.9, 37.0, 37.1, 37.2, 37.3, 37.4, 37.5, 37.6, 37.7, 37.8, 37.9, 38.0, 38.1, 38.2, 38.3, 38.4, 38.5, 38.6, 38.7, 38.8, 38.9, 39.0, 39.1, 39.2,
25 39.3, 39.4, 39.5, 39.6, 39.7, 39.8, 39.9, 40.0, 40.1, 40.2, 40.3, 40.4, 40.5, 40.6, 40.7, 40.8, 40.9, 41.0, 41.1, 41.2, 41.3, 41.4, 41.5, 41.6, 41.7, 41.8, 41.9, 42.0, 42.1, 42.2, 42.3, 42.4, 42.5, 42.6, 42.7, 42.8, 42.9, 43.0, 43.1, 43.2, 43.3, 43.4, 43.5, 43.6, 43.7, 43.8, 43.9, 44.0, 44.1, 44.2, 44.3, 44.4, 44.5, 44.6, 44.7, 44.8, 44.9, 45.0, 45.1, 45.2, 45.3, 45.4, 45.5, 45.6, 45.7, 45.8, 45.9, 46.0, 46.1, 46.2, 46.3, 46.4, 46.5, 46.6, 46.7, 46.8, 46.9, 47.0, 47.1, 47.2, 47.3, 47.4, 47.5, 47.6, 47.7,
30 47.8, 47.9, 48.0, 48.1, 48.2, 48.3, 48.4, 48.5, 48.6, 48.7, 48.8, 48.9, 49.0, 49.1, 49.2, 49.3, 49.4, 49.5, 49.6, 49.7, 49.8, 49.9, 50.0, 50.1, 50.2, 50.3, 50.4, 50.5, 50.6, 50.7, 50.8, 50.9, 51.0, 51.1, 51.2, 51.3, 51.4, 51.5, 51.6, 51.7, 51.8, 51.9, 52.0, 52.1, 52.2, 52.3, 52.4, 52.5, 52.6, 52.7, 52.8, 52.9, 53.0, 53.1, 53.2, 53.3, 53.4, 53.5, 53.6, 53.7, 53.8, 53.9, 54.0, 54.1, 54.2, 54.3, 54.4, 54.5, 54.6, 54.7, 54.8, 54.9, 55.0, 55.1, 55.2, 55.3, 55.4, 55.5, 55.6, 55.7, 55.8, 55.9, 56.0, 56.1, 56.2,
35 56.3, 56.4, 56.5, 56.6, 56.7, 56.8, 56.9, 57.0, 57.1, 57.2, 57.3, 57.4, 57.5, 57.6, 57.7, 57.8, 57.9, 58.0, 58.1, 58.2, 58.3, 58.4, 58.5, 58.6, 58.7, 58.8, 58.9, 59.0, 59.1, 59.2, 59.3, 59.4, 59.5, 59.6, 59.7, 59.8, 59.9, or 60.0 mol %. In some aspects, the composition comprising a mixture of structural lipids does not comprise a mixture of cholesterol and β -sitosterol of about 38.5 mol%.

In some embodiments, the composition comprises a mixture of structural lipids comprising cholesterol and a cholesterol analog, wherein the mixture is about 15 mol% to about 25 mol% of cholesterol and about 15 mol% to about 25 mol% cholesterol analog. See, for example, **Tables 2, 5 or 6**. In some embodiments, the amount of cholesterol in the mixture of structural lipids in the composition disclosed herein is at least about 15.0, 15.1, 15.2, 15.3, 15.4, 15.5, 15.6, 15.7, 15.8, 15.9, 16.0, 16.1, 16.2, 16.3, 16.4, 16.5, 16.6, 16.7, 16.8, 16.9, 17.0, 17.1, 17.2, 17.3, 17.4, 17.5, 17.6, 17.7, 17.8, 17.9, 18.0, 18.1, 18.2, 18.3, 18.4, 18.5, 18.6, 18.7, 18.8, 18.9, 19.0, 19.1, 19.2, 19.3, 19.4, 19.5, 19.6, 19.7, 19.8, 19.9, 20.0, 20.1, 20.2, 20.3, 20.4, 20.5, 20.6, 20.7, 20.8, 20.9, 21.0, 21.1, 21.2, 21.3, 21.4, 21.5, 21.6, 21.7, 21.8, 21.9, 22.0, 22.1, 22.2, 22.3, 22.4, 22.5, 22.6, 22.7, 22.8, 22.9, 23.0, 23.1, 23.2, 23.3, 23.4, 23.5, 23.6, 23.7, 23.8, 23.9, 24.0, 24.1, 24.2, 24.3, 24.4, 24.5, 24.6, 24.7, 24.8, 24.9, 25.0, 25.1, 25.2, 25.3, 25.4, 25.5, 25.6, 25.7, 25.8, or 25.9 mol % of cholesterol and at least about 15.0, 15.1, 15.2, 15.3, 15.4, 15.5, 15.6, 15.7, 15.8, 15.9, 16.0, 16.1, 16.2, 16.3, 16.4, 16.5, 16.6, 16.7, 16.8, 16.9, 17.0, 17.1, 17.2, 17.3, 17.4, 17.5, 17.6, 17.7, 17.8, 17.9, 18.0, 18.1, 18.2, 18.3, 18.4, 18.5, 18.6, 18.7, 18.8, 18.9, 19.0, 19.1, 19.2, 19.3, 19.4, 19.5, 19.6, 19.7, 19.8, 19.9, 20.0, 20.1, 20.2, 20.3, 20.4, 20.5, 20.6, 20.7, 20.8, 20.9, 21.0, 21.1, 21.2, 21.3, 21.4, 21.5, 21.6, 21.7, 21.8, 21.9, 22.0, 22.1, 22.2, 22.3, 22.4, 22.5, 22.6, 22.7, 22.8, 22.9, 23.0, 23.1, 23.2, 23.3, 23.4, 23.5, 23.6, 23.7, 23.8, 23.9, 24.0, 24.1, 24.2, 24.3, 24.4, 24.5, 24.6, 24.7, 24.8, 24.9, 25.0, 25.1, 25.2, 25.3, 25.4, 25.5, 25.6, 25.7, 25.8, or 25.9 mol % of cholesterol analog. In some aspects, the cholesterol analog is β -sitosterol, stigmasterol or campesterol. In a preferred embodiment, the cholesterol analog is β -sitosterol. In some embodiments, the amount of cholesterol in the mixture of structural lipids in the composition disclosed herein is at least about 15.0, 15.1, 15.2, 15.3, 15.4, 15.5, 15.6, 15.7, 15.8, 15.9, 16.0, 16.1, 16.2, 16.3, 16.4, 16.5, 16.6, 16.7, 16.8, 16.9, 17.0, 17.1, 17.2, 17.3, 17.4, 17.5, 17.6, 17.7, 17.8, 17.9, 18.0, 18.1, 18.2, 18.3, 18.4, 18.5, 18.6, 18.7, 18.8, 18.9, 19.0, 19.1, 19.2, 19.3, 19.4, 19.5, 19.6, 19.7, 19.8, 19.9, 20.0, 20.1, 20.2, 20.3, 20.4, 20.5, 20.6, 20.7, 20.8, 20.9, 21.0, 21.1, 21.2, 21.3, 21.4, 21.5, 21.6, 21.7, 21.8, 21.9, 22.0, 22.1, 22.2, 22.3, 22.4, 22.5, 22.6, 22.7, 22.8, 22.9, 23.0, 23.1, 23.2, 23.3, 23.4, 23.5, 23.6, 23.7, 23.8, 23.9, 24.0, 24.1, 24.2, 24.3, 24.4, 24.5, 24.6, 24.7, 24.8, 24.9, 25.0, 25.1, 25.2, 25.3, 25.4, 25.5, 25.6, 25.7, 25.8, or 25.9 mol % of cholesterol and at least about 15.0, 15.1, 15.2, 15.3, 15.4, 15.5, 15.6, 15.7, 15.8, 15.9, 16.0, 16.1, 16.2, 16.3, 16.4, 16.5, 16.6, 16.7, 16.8, 16.9, 17.0, 17.1, 17.2, 17.3, 17.4, 17.5, 17.6, 17.7, 17.8, 17.9, 18.0, 18.1, 18.2, 18.3, 18.4, 18.5, 18.6, 18.7, 18.8, 18.9, 19.0, 19.1, 19.2, 19.3, 19.4, 19.5, 19.6, 19.7, 19.8, 19.9, 20.0, 20.1, 20.2, 20.3, 20.4, 20.5, 20.6, 20.7, 20.8, 20.9, 21.0, 21.1, 21.2, 21.3, 21.4, 21.5, 21.6, 21.7, 21.8, 21.9, 22.0, 22.1, 22.2, 22.3, 22.4, 22.5, 22.6, 22.7, 22.8, 22.9, 23.0, 23.1, 23.2, 23.3, 23.4, 23.5, 23.6, 23.7, 23.8, 23.9, 24.0, 24.1, 24.2, 24.3, 24.4, 24.5, 24.6, 24.7, 24.8, 24.9, 25.0, 25.1, 25.2, 25.3, 25.4, 25.5, 25.6, 25.7, 25.8, or 25.9 mol % of β -sitosterol. In some aspects, the composition comprising a mixture of cholesterol and β -sitosterol is not about 38.5 mol%.

In some embodiments, the composition comprises a mixture of structural lipids comprising cholesterol and a cholesterol analog, wherein the molar ratio between the cholesterol and the cholesterol analog in the mixture is about 6:4, 1:1 or 4:6.

In some embodiments, the composition further comprises about 30-50% cationic lipid and about 5-25% phospholipid.

In some embodiments, a LNP of the invention comprises an N:P ratio of from about 2:1 to about 30:1. In some embodiments, a LNP of the invention comprises an N:P ratio of about 10:1. In some embodiments, a LNP of the invention comprises an N:P ratio of about 6:1. In some embodiments, a LNP of the invention comprises an N:P ratio of about 3:1, 4:1, or 5:1.

In some embodiments, the characteristics of a LNP may depend on the absolute or relative amounts of its components. For instance, a LNP including a higher molar fraction of a phospholipid may have different characteristics than a LNP including a lower molar fraction of a phospholipid. Characteristics may also vary depending on the method and conditions of preparation of the lipid nanoparticle. In general, phospholipids comprise a phospholipid moiety and one or more fatty acid moieties. A phospholipid moiety can be selected, for example, from the non-limiting group consisting of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl serine, phosphatidic acid, and 2-lysophosphatidyl choline.

Further examples of a phospholipid moiety for the lipid nanoparticle include a lipid that is selected from the group consisting of distearoyl-sn-glycero-phosphoethanolamine, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), monomethyl-phosphatidylethanolamine (such as 16-O-monomethyl PE), dimethyl-phosphatidylethanolamine (such as 16-O-dimethyl PE), 18-1-trans PE, 1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE), hydrogenated soy phosphatidylcholine (HSPC), egg phosphatidylcholine (EPC), dioleoylphosphatidyl serine (DOPS), sphingomyelin (SM), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), distearoylphosphatidylglycerol (DSPG), diemcoylphosphatidylcholine (DEPC), palmitoyloleoylphosphatidylglycerol (POPG), dielaidoyl-phosphatidylethanolamine (DEPE), 1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE); 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (DPHyPE); lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, lysophosphatidylcholine, dilinoleoylphosphatidylcholine, and mixtures thereof.

A fatty acid moiety can be selected, for example, from the non-limiting group consisting of lauric acid, myristic acid, myristoleic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha-linolenic acid, erucic acid, phytanoic acid, arachidic acid, arachidonic acid, eicosapentaenoic acid, behenic acid, docosapentaenoic acid, and docosahexaenoic acid.

- 5 Particular phospholipids can facilitate fusion to a membrane. In some embodiments, a cationic phospholipid can interact with one or more negatively charged phospholipids of a membrane (e.g., a cellular or intracellular membrane). Fusion of a phospholipid to a membrane can allow one or more elements (e.g., a therapeutic agent) of a lipid-containing composition (e.g., LNPs) to pass through the membrane permitting, e.g., delivery of the one or more elements to a target
- 10 tissue. Non-natural phospholipid species including natural species with modifications and substitutions including branching, oxidation, cyclization, and alkynes are also contemplated. In some embodiments, a phospholipid can be functionalized with or cross-linked to one or more alkynes (e.g., an alkenyl group in which one or more double bonds is replaced with a triple bond). Under appropriate reaction conditions, an alkyne group can undergo a copper-catalyzed
- 15 cycloaddition upon exposure to an azide. Such reactions can be useful in functionalizing a lipid bilayer of a nanoparticle composition to facilitate membrane permeation or cellular recognition or in conjugating a nanoparticle composition to a useful component such as a targeting or imaging moiety (e.g., a dye). Phospholipids include, but are not limited to, glycerophospholipids such as phosphatidylcholines, phosphatidyl-ethanolamines, phosphatidylserines,
- 20 phosphatidylinositols, phosphatidyl glycerols, and phosphatidic acids. In some embodiments, a phospholipid useful or potentially useful in the present invention is an analog or variant of DSPC.

- Lipid nanoparticles may be characterized by a variety of methods. For example, microscopy (e.g., transmission electron microscopy or scanning electron microscopy) may be
- 25 used to examine the morphology and size distribution of a LNP. Dynamic light scattering or potentiometry (e.g., potentiometric titrations) may be used to measure zeta potentials. Dynamic light scattering may also be utilized to determine particle sizes. Instruments such as the Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK) may also be used to measure multiple characteristics of a LNP, such as particle size, polydispersity index, and
- 30 zeta potential.

- The mean size of a LNP may be between 10s of nm and 100s of nm, e.g., measured by dynamic light scattering (DLS). For example, the mean size may be from about 40 nm to about 150 nm, such as about 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm. In some embodiments, the mean size of a LNP may be from about 50 nm to about 100 nm, from about 50 nm to about 90 nm, from about 50 nm to about 80 nm, from about 50 nm to about 70 nm, from about 50 nm to about 60 nm, from about 60 nm to about 100 nm, from about 60 nm to about 90 nm, from about 60 nm to about 80 nm, from about 60 nm to
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about 70 nm, from about 70 nm to about 100 nm, from about 70 nm to about 90 nm, from about 70 nm to about 80 nm, from about 80 nm to about 100 nm, from about 80 nm to about 90 nm, or from about 90 nm to about 100 nm. In certain embodiments, the mean size of a LNP may be from about 70 nm to about 100 nm. In a particular embodiment, the mean size may be about 80 nm. In other embodiments, the mean size may be about 100 nm.

A LNP may be relatively homogenous. A polydispersity index may be used to indicate the homogeneity of a LNP, e.g., the particle size distribution of the lipid nanoparticles. A small (e.g., less than 0.3) polydispersity index generally indicates a narrow particle size distribution. A LNP may have a polydispersity index from about 0 to about 0.25, such as 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.20, 0.21, 0.22, 0.23, 0.24, or 0.25. In some embodiments, the polydispersity index of a LNP may be from about 0.10 to about 0.20.

The zeta potential of a LNP may be used to indicate the electrokinetic potential of the composition. For example, the zeta potential may describe the surface charge of a LNP. Lipid nanoparticles with relatively low charges, positive or negative, are generally desirable, as more highly charged species may interact undesirably with cells, tissues, and other elements in the body. In some embodiments, the zeta potential of a LNP may be from about -10 mV to about +20 mV, from about -10 mV to about +15 mV, from about -10 mV to about +10 mV, from about -10 mV to about +5 mV, from about -10 mV to about 0 mV, from about -10 mV to about -5 mV, from about -5 mV to about +20 mV, from about -5 mV to about +15 mV, from about -5 mV to about +10 mV, from about -5 mV to about +5 mV, from about -5 mV to about 0 mV, from about 0 mV to about +20 mV, from about 0 mV to about +15 mV, from about 0 mV to about +10 mV, from about 0 mV to about +5 mV, from about +5 mV to about +20 mV, from about +5 mV to about +15 mV, or from about +5 mV to about +10 mV.

The efficiency of encapsulation of a therapeutic and/or prophylactic describes the amount of therapeutic and/or prophylactic that is encapsulated or otherwise associated with a LNP after preparation, relative to the initial amount provided. The encapsulation efficiency is desirably high (e.g., close to 100%). The encapsulation efficiency may be measured, for example, by comparing the amount of therapeutic and/or prophylactic in a solution containing the lipid nanoparticle before and after breaking up the lipid nanoparticle with one or more organic solvents or detergents. Fluorescence may be used to measure the amount of free therapeutic and/or prophylactic (e.g., RNA) in a solution. For example, if 97 mg of mRNA are encapsulated in a composition out of a total 100 mg of mRNA initially provided to the composition, the encapsulation efficiency may be given as 97%. For the lipid nanoparticles described herein, the encapsulation efficiency of a therapeutic and/or prophylactic may be at least 50%, for example 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the encapsulation efficiency

may be at least 80%. In certain embodiments, the encapsulation efficiency may be at least 90%.

5 A LNP may optionally comprise one or more coatings. For example, a LNP may be formulated in a capsule, film, or tablet having a coating. A capsule, film, or tablet including a composition described herein may have any useful size, tensile strength, hardness, or density.

Formulations comprising amphiphilic polymers and lipid nanoparticles may be formulated in whole or in part as pharmaceutical compositions. Pharmaceutical compositions may include one or more amphiphilic polymers and one or more lipid nanoparticles. For example, a pharmaceutical composition may include one or more amphiphilic polymers and one or more lipid nanoparticles including one or more different therapeutics and/or prophylactics. Pharmaceutical compositions may further include one or more pharmaceutically acceptable excipients or accessory ingredients such as those described herein. General guidelines for the formulation and manufacture of pharmaceutical compositions and agents are available, for example, in Remington's The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro; Lippincott, Williams & Wilkins, Baltimore, MD, 2006. Conventional excipients and accessory ingredients may be used in any pharmaceutical composition, except insofar as any conventional excipient or accessory ingredient may be incompatible with one or more components of a LNP or the one or more amphiphilic polymers in the formulation of the disclosure. An excipient or accessory ingredient may be incompatible with a component of a LNP or the amphiphilic polymer of the formulation if its combination with the component or amphiphilic polymer may result in any undesirable biological effect or otherwise deleterious effect.

In some embodiments, one or more excipients or accessory ingredients may make up greater than 50% of the total mass or volume of a pharmaceutical composition including a LNP. For example, the one or more excipients or accessory ingredients may make up 50%, 60%, 70%, 80%, 90%, or more of a pharmaceutical convention. In some embodiments, a pharmaceutically acceptable excipient is at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved for use in humans and for veterinary use. In some embodiments, an excipient is approved by United States Food and Drug Administration. In some embodiments, an excipient is pharmaceutical grade. In some embodiments, an excipient meets the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia. Relative amounts of the one or more amphiphilic polymers, the one or more lipid nanoparticles, the one or more pharmaceutically acceptable excipients, and/or any additional ingredients in a pharmaceutical composition in accordance with the present disclosure will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, a pharmaceutical composition may comprise between 0.1% and 100% (wt/wt) of one

or more lipid nanoparticles. As another example, a pharmaceutical composition may comprise between 0.1% and 15% (wt/vol) of one or more amphiphilic polymers (e.g., 0.5%, 1%, 2.5%, 5%, 10%, or 12.5% w/v).

In certain embodiments, the lipid nanoparticles and/or pharmaceutical compositions of the disclosure are refrigerated or frozen for storage and/or shipment (e.g., being stored at a temperature of about 5 °C or lower, such as a temperature between about -150 °C and about 5 °C or between about -150 °C and about 0 °C or between about -80 °C and about -20 °C (e.g., about 5 °C, 0 °C, -5 °C, -10 °C, -15 °C, -20 °C, -25 °C, -30 °C, -40 °C, -50 °C, -60 °C, -70 °C, -80 °C, -90 °C, -130 °C or -150 °C). For example, the pharmaceutical composition comprising one or more amphiphilic polymers and one or more lipid nanoparticles is a solution or solid (e.g., via lyophilization) that is refrigerated for storage and/or shipment at, for example, about -20 °C, -30 °C, -40 °C, -50 °C, -60 °C, -70 °C, or -80 °C. In certain embodiments, the disclosure also relates to a method of increasing stability of the lipid nanoparticles by adding an effective amount of an amphiphilic polymer and by storing the lipid nanoparticles and/or pharmaceutical compositions thereof at a temperature of 4 °C or lower, such as a temperature between about -150 °C and about 0 °C or between about -80 °C and about -20 °C, e.g., about -5 °C, -10 °C, -15 °C, -20 °C, -25 °C, -30 °C, -40 °C, -50 °C, -60 °C, -70 °C, -80 °C, -90 °C, -130 °C or -150 °C).

The chemical properties of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation of the present disclosure may be characterized by a variety of methods. In some embodiments, electrophoresis (e.g., capillary electrophoresis) or chromatography (e.g., reverse phase liquid chromatography) may be used to examine the mRNA integrity.

The efficacy of a product is dependent on expression of the delivered RNA, which requires a sufficiently intact RNA molecule. RNA integrity is a measure of RNA quality that quantitates intact RNA. The method is also capable of detecting potential degradation products. RNA integrity is preferably determined by capillary gel electrophoresis. The initial specification is set to ensure sufficient RNA integrity in drug product preparations. In some embodiments, the RNA polynucleotide has an integrity of at least about 80%, 85%, 90%, 92%, 94%, 95%, 96%, 97%, 98%, or 99%. In some embodiments, the RNA polynucleotide has an integrity of or greater than about 95%. In some embodiments, the RNA polynucleotide has an integrity of or greater than about 98%. In some embodiments, the RNA polynucleotide has an integrity of or greater than about 99%.

In preferred embodiments, the RNA polynucleotide has a clinical grade purity. In some embodiments, the purity of the RNA polynucleotide is between about 60% and about 100%. In some embodiments, the purity of the RNA polynucleotide is between about 80% and 99%. In some embodiments, the purity of the RNA polynucleotide is between about 90% and about 99%. In some embodiments, wherein the purified mRNA has a clinical grade purity without further purification. In some embodiments, the clinical grade purity is achieved through a method including tangential flow filtration (TFF) purification. In some embodiments, the clinical

grade purity is achieved without the further purification selected from high performance liquid chromatography (HPLC) purification, ligand or binding based purification, and/or ion exchange chromatography. In some embodiments, the method of producing the RNA polynucleotides removes long abortive RNA species, double-stranded RNA (dsRNA), residual plasmid DNA
 5 residual solvent and/or residual salt. In some embodiments, the short abortive transcript contaminants comprise less than 15 bases. In some embodiments, the short abortive transcript contaminants comprise about 8-12 bases. In some embodiments, the method of the invention also removes RNase inhibitor.

In some embodiments, the purified RNA polynucleotide comprises 5% or less, 4% or
 10 less, 3% or less, 2% or less, 1 % or less or is substantially free of protein contaminants as determined by capillary electrophoresis. In some embodiments, the purified RNA polynucleotide comprises less than 5%, less than 4%, less than 3%, less than 2%, less than 1 %, or is substantially free of salt contaminants determined by high performance liquid chromatography (HPLC). In some embodiments, the purified RNA polynucleotide comprises 5% or less, 4% or
 15 less, 3% or less, 2% or less, 1 % or less or is substantially free of short abortive transcript contaminants determined by known methods, such as, e.g., high performance liquid chromatography (HPLC). In some embodiments, the purified RNA polynucleotide has integrity of 60% or greater, 70% or greater, 80% or greater, 81% or greater, 82% or greater, 83% or greater, 84% or greater, 85% or greater, 86% or greater, 87% or greater, 88% or greater, 89%
 20 or greater, 90% or greater, 95% or greater, 96% or greater, 97% or greater, 98% or greater, or 99% or greater as determined by a known method, such as, e.g., capillary electrophoresis.

Modified nucleobases

Modified nucleobases which may be incorporated into modified nucleosides and
 25 nucleotides and be present in the RNA molecules include, for example, m5C (5- methylcytidine), m5U (5-methyluridine), m6A (N6-methyladenosine), s2U (2- thiouridine), Um (2'-0-methyluridine), mA (1-methyladenosine); m2A (2- methyladenosine); Am (2-1-O-methyladenosine); ms2m6A (2- methylthio-N6- methyladenosine); i6A (N6-isopentenyladenosine); ms2i6A (2-methylthio-
 30 N6isopentenyladenosine); io6A (N6-(cis-hydroxyisopentenyl)adenosine); ms2io6A (2- methylthio-N6-(cis-hydroxyisopentenyl) adenosine); g6A (N6- glycylcarbamoyladenosine); t6A (N6-threonyl carbamoyladenosine); ms2t6A (2- methylthio-N6-threonyl carbamoyladenosine); m6t6A (N6-methyl-N6- threonylcarbamoyladenosine); hn6A(N6-hydroxynorvalylcarbamoyl adenosine); ms2hn6A (2-methylthio-N6-hydroxynorvalyl carbamoyladenosine); Ar(p) (2'-0-
 35 ribosyladenosine (phosphate)); I (inosine); mil (1-methylinosine); m'Im (I,2'-0- dimethylinosine); m3C (3-methylcytidine); Cm (2T-0-methylcytidine); s2C (2- thiocytidine); ac4C (N4- acetylcytidine); £5C (5-fonnylcytidine); m5Cm (5,2-0- dimethylcytidine); ac4Cm (N4acetyl2T0methylcytidine); k2C (lysidine); m1G (1- methylguanosine); m2G (N2-

methylguanosine); m7G (7-methylguanosine); Gm (2'-O- methylguanosine); m22G (N2,N2-dimethylguanosine); m2Gm (N2,2'-O- dimethylguanosine); m22Gm (N2,N2,2'-O-trimethylguanosine); Gr(p) (2'-O- ribosylguanosine (phosphate)); yW (wybutosine); o2yW (peroxywybutosine); OHyW (hydroxywybutosine); OHyW* (undermodified hydroxywybutosine);

5 imG (wyosine); mimG (methylguanosine); Q (queuosine); oQ (epoxyqueuosine); galQ (galtactosyl- queuosine); manQ (mannosyl-queuosine); preQo (7-cyano-7-deazaguanosine); preQi (7- aminomethyl-7-deazaguanosine); G* (archaeosine); D (dihydrouridine); m5Um (5,2'-O-dimethyluridine); s4U (4-thiouridine); m5s2U (5-methyl-2-thiouridine); s2Um (2-thio-2'- O-methyluridine); acp3U (3-(3-amino-3-carboxypropyl)uridine); ho5U (5- hydroxyuridine); mo5U (5-methoxyuridine); cmo5U (uridine 5-oxyacetic acid); mcmo5U (uridine 5-oxyacetic acid methyl ester); chm5U (5-(carboxyhydroxymethyl)uridine); mchm5U (5-(carboxyhydroxymethyl)uridine methyl ester); mcm5U (5-methoxycarbonyl methyluridine); mcm5Um (S-methoxycarbonylmethyl-2-O-methyluridine); mcm5s2U (5- methoxycarbonylmethyl-2-thiouridine); nm5s2U (5-aminomethyl-2-thiouridine); mnm5U (5-methylaminomethyluridine); mnm5s2U (5-methylaminomethyl-2-thiouridine); mnm5se2U (5-methylaminomethyl-2-selenouridine); ncm5U (5-carbamoylmethyl uridine); ncm5Um (5-carbamoylmethyl-2'-O-methyluridine); cmnm5U (5-carboxymethylaminomethyluridine); cnmm5Um (5-carboxymethyl 1 aminomethyl-2-L-Omethyluridine); cmnm5s2U (5-carboxymethylaminomethyl-2-thiouridine); m62A (N6,N6-dimethyladenosine); Tm (2'-O-methylinosine); m4C (N4-methylcytidine); m4Cm (N4,2'-O-dimethylcytidine); hm5C (5-hydroxymethylcytidine); m3U (3-methyluridine); cm5U (5-carboxymethyluridine); m6Am (N6,T-0-dimethyladenosine); m62Am (N6,N6,0-2-trimethyladenosine); m2'7G (N2,7-dimethylguanosine); m2'2'7G (N2,N2,7- trimethylguanosine); m3Um (3,2T-0-dimethyluridine); m5D (5-methyldihydrouridine); f5Cm (5-formyl-2'-O-methylcytidine); mIGm (1,2'-O-dimethylguanosine); m'Am (1,2'-O- dimethyl adenosine);

25 irinomethyluridine); tm5s2U (S-taurinomethyl-2-thiouridine)); imG- 14 (4-demethyl guanosine); imG2 (isoguanosine); ac6A (N6-acetyladenosine), hypoxanthine, inosine, 8-oxo-adenine, 7-substituted derivatives thereof, dihydrouracil, pseudouracil, 2-thiouracil, 4-thiouracil, 5-aminouracil, 5-(Ci-C6)-alkyluracil, 5- methyluracil, 5-(C2-Ce)-alkenyluracil, 5-(C2-Ce)-alkynyluracil, 5-(hydroxymethyl)uracil, 5-chlorouracil, 5-fluorouracil, 5-bromouracil, 5-hydroxycytosine, 5-(Ci-C6)- alkylcytosine, 5-methylcytosine, 5-(C2-C6)-alkenylcytosine, 5-(C2-C6)-alkynylcytosine, 5- chlorocytosine, 5-fluorocytosine, 5-bromocytosine, N2-dimethylguanine, 7-deazaguanine, 8-azaguanine, 7-deaza-7-substituted guanine, 7-deaza-7-(C2-C6)alkynylguanine, 7-deaza- 8-substituted guanine, 8-hydroxyguanine, 6-thioguanine, 8-oxoguanine, 2-aminopurine, 2-amino-6-chloropurine, 2,4-diaminopurine, 2,6-diaminopurine, 8-azapurine, substituted 7-deazapurine, 7-deaza-7-substituted purine, 7-deaza-8-substituted purine, hydrogen (abasic residue), m5C, m5U, m6A, s2U, W, or 2'-O-methyl-U. In some aspects, one or more of the modified nucleosides in the list may be excluded.

Additional exemplary modified nucleotides include any one of N-1-methylpseudouridine; pseudouridine, N6-methyladenosine, 5-methylcytidine, and 5-methyluridine. In some embodiments, the modified nucleotide is N-1-methylpseudouridine.

In some embodiments, the RNA molecule may include phosphoramidate, 5 phosphorothioate, and/or methylphosphonate linkages.

In some embodiments, the RNA molecule includes a modified nucleotide selected from any one of pseudouridine, N1-methylpseudouridine, N1-ethylpseudouridine, 2-thiouridine, 4'-thiouridine, 5-methylcytosine, 5-methyluridine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 10 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methoxyuridine and 2'-O-methyl uridine. In some embodiments, the modified or unnatural nucleotides are selected from the group consisting of pseudouridine, N1-methylpseudouridine, N1-ethylpseudouridine, 2-thiouridine, 4'-thiouridine, 5-methylcytosine, 5-methyluridine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-15 dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methoxyuridine, and 2'-O-methyl uridine. In some embodiments, the modified or unnatural nucleotides are selected from the group consisting of 5-methyluridine, N1-methylpseudouridine, 5-methoxyuridine, and 5-methylcytosine. 20

In some embodiments, at least 10% of a total population of a particular nucleotide in the RNA molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 25% of a total population of a particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at 25 least 50% of a total population of a particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 75% of a total population of a particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, essentially all of the particular nucleotide population in the molecule has been replaced with one or more modified or unnatural nucleotides.

30 In some embodiments, at least a portion, or all of a total population of a particular nucleotide in the RNA molecule has been replaced with two modified or unnatural nucleotides. In some embodiments, the two modified or unnatural nucleotides are provided in a ratio equal to any one of, at least any one of, at most any one of, or between any two of 1:99 to 99:1, including 1:99; 2:98; 3:97; 4:96; 5:95; 6:94; 7:93; 8:92; 9:91; 10:90; 11:89; 12:88; 13:87; 14:86; 15:85; 16:84; 17:83; 18:82; 19:81; 20:80; 21:79; 22:78; 23:77; 24:76; 25:75; 26:74; 27:73; 28:72; 29:71; 35 30:70; 31:69; 32:68; 33:67; 34:66; 35:65; 36:64; 37:63; 38:62; 39:61; 40:60; 41:59; 42:58; 43:57; 44:56; 45:55; 46:54; 47:53; 48:52; 49:51; 50:50; 51:49; 52:48; 53:47; 54:46; 55:45; 56:44; 57:43; 58:42; 59:41; 60:40; 61:39; 62:38; 63:37; 64:36; 65:35; 66:34; 67:33; 68:32; 69:31; 70:30; 71:29;

72:28; 73:27; 74:26; 75:25; 76:24; 77:23; 78:22; 79:21; 80:20; 81:19; 82:18; 83:17; 84:16; 85:15; 86:14; 87:13; 88:12; 89:11; 90:10; 91:9; 92:8; 93:7; 94:6; 95:5; 96:4; 97:3; 98:2; and 99:1, or any range derivable therein.

5 In some embodiments, at least 10% of a total population of a first particular nucleotide in a RNA molecule as disclosed herein has been replaced with one or more modified or unnatural nucleotides, and at least 10% of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 10% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and at least 25% of a total
10 population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 10% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and at least 50% of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some
15 embodiments, at least 10% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and at least 75% of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 10% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or
20 unnatural nucleotides, and essentially all of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 25% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and at least 25% of a total population of a second particular nucleotide in the molecule has been replaced with one or more
25 modified or unnatural nucleotides. In some embodiments, at least 25% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and at least 50% of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some
30 embodiments, at least 25% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and at least 75% of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 25% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or
35 unnatural nucleotides, and essentially all of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 50% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and at least 50% of a total population of a second particular nucleotide in the molecule has been replaced with one or more

modified or unnatural nucleotides. In some embodiments, at least 50% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and at least 75% of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some

5 embodiments, at least 50% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and essentially all of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 75% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or

10 unnatural nucleotides, and at least 75% of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 75% of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and essentially all of a total population of a second particular nucleotide in the molecule has been replaced with one or more

15 modified or unnatural nucleotides. In some embodiments, essentially all of a total population of a first particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides, and essentially all of a total population of a second particular nucleotide in the molecule has been replaced with one or more modified or unnatural nucleotides.

In some embodiments, at least 25% of a total population of uridine nucleotides in the RNA

20 molecule has been replaced with N1-methylpseudouridine. In some embodiments, at least 50% of a total population of uridine nucleotides in the molecule has been replaced with N1-methylpseudouridine. In some embodiments, at least 75% of a total population of uridine nucleotides in the molecule has been replaced with N1-methylpseudouridine. In some embodiments, essentially all uridine nucleotides in the molecule have been replaced with N1-

25 methylpseudouridine. In some embodiments, at least 50% of a total population of uridine nucleotides in the molecule has been replaced with 5-methoxyuridine. In some embodiments, essentially all uridine nucleotides in the molecule have been replaced with 5-methoxyuridine. In some embodiments, at least 50% of a total population of uridine nucleotides in the molecule has been replaced with 5-methyluridine. In some embodiments, essentially all uridine nucleotides in

30 the molecule have been replaced with 5-methyluridine. In some embodiments, at least 50% of a total population of cytosine nucleotides in the molecule has been replaced with 5-methylcytosine. In some embodiments, essentially all cytosine nucleotides in the molecule have been replaced with 5-methylcytosine. In some embodiments, at least 50% of a total population of uridine nucleotides in the molecule has been replaced with 2-thiouridine. In some embodiments,

35 essentially all uridine nucleotides in the molecule have been replaced with 2-thiouridine.

In some embodiments, at least 50% of a total population of uridine nucleotides in the molecule has been replaced with N1-methylpseudouridine and essentially all cytosine nucleotides in the molecule have been replaced with 5-methylcytosine. In some embodiments,

at least 50% of a total population of uridine nucleotides in the molecule has been replaced with 5-methoxyuridine and essentially all cytosine nucleotides in the molecule have been replaced with 5-methylcytosine. In some embodiments, at least 50% of a total population of uridine nucleotides in the molecule has been replaced with 5-methyluridine and essentially all cytosine nucleotides in the molecule have been replaced with 5-methylcytosine.

In some embodiments, essentially all uridine nucleotides in the molecule have been replaced with about 50% 5-methoxyuridine and about 50% N1-methylpseudouridine. In some embodiments, essentially all uridine nucleotides in the molecule have been replaced with about 75% 5-methoxyuridine and about 25% N1-methylpseudouridine. In some embodiments, essentially all uridine nucleotides in the molecule have been replaced with about 25% 5-methoxyuridine and about 75% N1-methylpseudouridine.

UTRs

The 5' untranslated regions (UTR) is a regulatory region of DNA situated at the 5' end of a protein coding sequence that is transcribed into mRNA but not translated into protein. 5' UTRs may contain various regulatory elements, e.g., 5' cap structure, stem-loop structure, and an internal ribosome entry site (IRES), which may play a role in the control of translation initiation. The 3' UTR, situated downstream of a protein coding sequence, may be involved in regulatory processes including transcript cleavage, stability and polyadenylation, translation, and mRNA localization. In some embodiments, the UTR is derived from an mRNA that is naturally abundant in a specific tissue (e.g., lymphoid tissue), to which the mRNA expression is targeted. In some embodiments, the UTR increases protein synthesis. Without being bound by mechanism or theory, the UTR may increase protein synthesis by increasing the time that the mRNA remains in translating polysomes (message stability) and/or the rate at which ribosomes initiate translation on the message (message translation efficiency). According, the UTR sequence may prolong protein synthesis in a tissue-specific manner. In some embodiments, the 5' UTR and the 3' UTR sequences are computationally derived. In some embodiments, the 5' UTR and the 3' UTRs are derived from a naturally abundant mRNA in a tissue. The tissue may be, for example, liver, a stem cell, or lymphoid tissue. The lymphoid tissue may include, for example, any one of a lymphocyte (e.g., a B-lymphocyte, a helper T-lymphocyte, a cytotoxic T-lymphocyte, a regulatory T-lymphocyte, or a natural killer cell), a macrophage, a monocyte, a dendritic cell, a neutrophil, an eosinophil and a reticulocyte. In some embodiments, the 5' UTR and the 3' UTR are derived from an alphavirus. In some embodiments, the 5' UTR and the 3' UTR are from a wild-type alphavirus. Examples of alphaviruses are described below.

In some embodiments, the first RNA molecule includes a 5' UTR and the 3' UTR derived from a naturally abundant mRNA in a tissue. In some embodiments, the first RNA molecule includes a 5' UTR and the 3' UTR derived from an alphavirus. In some embodiments, the second RNA or the saRNA molecule includes a 5' UTR and the 3' UTR derived from an alphavirus. In

some embodiments, the second RNA or the saRNA molecule includes a 5' UTR and the 3' UTR from a wild-type alphavirus. In some embodiments, the RNA molecule includes a 5' cap.

In some embodiments, the RNA molecule includes a 5'UTR or 3'UTR as disclosed in WO2024/154061, which is hereby incorporated herein by reference in its entirety, including all sequences set forth therein.

In some embodiments, the RNA molecule comprises a 5' UTR having the sequence GAAΨAAAC ΨAGΨAΨΨCΨΨ CΨGGΨCCCCA CAGACΨCAGA GAGAACCCGC CACC (SEQ ID NO: 1) , wherein Ψ is 1-methyl-3'-pseudouridylyl.

In some embodiments, the RNA molecule comprises a 5' UTR having the sequence AATAAACTAGTATTCTTCTGGTCCCCACAGACTCAGAGAGAACCC (5' WHO UTR1) (SEQ ID NO: 4).

In some embodiments, the RNA molecule comprises a 5' UTR comprises the sequence GAGAAΨAAACΨAGΨAΨΨCΨΨ CΨGGΨCCCCA CAGACΨCAGA GAGAACCCGCCACC (SEQ ID NO: 5), wherein Ψ is 1-methyl-3'-pseudouridylyl.

In some embodiments, the RNA molecule comprises a 5' UTR comprises the sequence AGAATAAACTAGTATTCTTCTGGTCCCCACAGACTCAGAGAGAACCC (5' WHO UTR1). (SEQ ID NO: 6).

In some embodiments, the RNA molecule comprises a 3' UTR comprises the sequence C ΨCGAGCΨGGΨ ACΨGCAΨGCA CGCAAΨGCΨA GCΨGCCCCΨΨ ΨCCCGΨCCΨG GGΨACCCCGA GΨCΨCCCCCG ACCΨCGGGΨC CCAGGΨAΨGC ΨCCCACCΨCC ACCΨGCCCCA CΨCACCACCΨ CΨGCΨAGΨΨC CAGACACCΨC CCAAGCACGC AGCAAΨGCAG CΨCAAAACGC ΨΨAGCCΨAGC CACACCCCCA CGGGAAACAG CAGΨGAΨΨAA CCΨΨΨAGCAA ΨAAACGAAAG ΨΨΨAACΨAAG CΨAΨACΨAAC CCCAGGGΨΨG GΨCAAΨΨΨCG ΨGCCAGCCAC ACCCΨGGAGC ΨAGC (SEQ ID NO: 2), wherein Ψ is 1-methyl-3'-pseudouridylyl.

In some embodiments, the RNA molecule comprises a 3' UTR comprises the sequence CUCGAGCUGGUACUGCAUGCACGCAAUGCUAGCUGCCCCUUUCCCGUCCUGGGUACCC CGAGUCUCCCCCGACCUCGGGUCCCAGGU AUGCUCCACCUCACCUGCCCCACUCAC CACCUCUGCUAGUCCAGACACCUCCCAAGCACGCAGCAAUGCAGCUCAAAACGCUUAG CCUAGCCACACCCCCACGGGAAACAGCAGUGAUUAACCUUUAGCAAUAAACGAAAGUUU AACUAAGCUAUACUAACCCCAGGGUUGGUCAAUUUCGUGCCAGCCACACCCUGGAGCUA GC (3' WHO UTR2). (SEQ ID NO: 7).

In some embodiments, the RNA molecule comprises a 3' UTR comprises the sequence CΨCGAGCΨGGΨACΨGCAΨGCACGCAAΨGCΨAGCΨGCCCCΨΨΨCCCGΨCCΨGGGΨAC CCCGAGΨCΨCCCCGACCΨCGGGΨCCCAGGΨAΨGCΨCCCACCΨCCACCΨGCCCCAC ΨCACCACCΨCΨGCΨAGΨΨCCAGACACCΨCCCAAGCACGCAGCAAΨGCAGCΨCAAAAC GCΨΨAGCCΨAGCCACACCCCCACGGGAAACAGCAGΨGAΨΨAACCCΨΨΨAGCAAΨAAAC

GAAAGΨΨAACΨAAGCΨAΨACΨAACCCAGGGΨΨGGΨCAAΨΨΨCGΨGCCAGCCACA
 CCCΨGGAGCΨAGC (3' WHO ΨTR2) (SEQ ID NO: 8), wherein Ψ is 1-methyl-3'-pseudouridylyl.

Open reading frame (ORF)

5 The 5' and 3' UTRs may be operably linked to an ORF, which may be a sequence of codons derived from a gene of interest that is capable of being translated into a polypeptide of interest. As stated above, the RNA molecule may include one (monocistronic), two (bicistronic) or more (multicistronic) open reading frames (ORFs).

In some embodiments, the ORF encodes a non-structural viral gene. In some
 10 embodiments, the ORF further includes one or more subgenomic promoters. In some embodiments, the RNA molecule includes a subgenomic promoter operably linked to the ORF. In some embodiments, the subgenomic promoter comprises a cis-acting regulatory element. In some embodiments, the cis-acting regulatory element is immediately downstream (5'-3') of B². In some embodiments, the cis-acting regulatory element is immediately downstream (5'-3') of a
 15 guanine that is immediately downstream of B². In some embodiments, the cis-acting regulatory element is an AU-rich element. In some embodiments, the AU-rich element is au, auaaaagau, auaaaaagau, auag, auauauauau, auauauau, auauauauauau, augaugaugau, augau, auaaaaagaua, or auaaaaagaug. In some embodiments, the second RNA or the saRNA molecule may include (i) an ORF encoding a replicase which may transcribe RNA from the second RNA or
 20 the saRNA molecule and (ii) an ORF encoding at least one an antigen or polypeptide of interest. The polymerase may be an alphavirus replicase e.g., including any one of the non-structural alphavirus proteins nsP1, nsP2, nsP3 and nsP4, or a combination thereof. In some embodiments, the RNA molecule includes alphavirus nonstructural protein nsP1. In some embodiments, the RNA molecule includes alphavirus nonstructural protein nsP2. In some
 25 embodiments, the RNA molecule includes alphavirus nonstructural protein nsP3. In some embodiments, the RNA molecule includes alphavirus nonstructural protein nsP4. In some embodiments, the RNA molecule includes alphavirus nonstructural proteins nsP1, nsP2, and nsP3. In some embodiments, the RNA molecule includes alphavirus nonstructural proteins nsP1, nsP2, nsP3, and nsP4. In some embodiments, the RNA molecule includes any combination of
 30 nsP1, nsP2, nsP3, and nsP4. In some embodiments, the RNA molecule does not include nsP4.

In some embodiments, an open reading frame of an RNA (e.g., modified mRNA or saRNA) composition is codon-optimized. In some embodiments, the open reading frame from which the polypeptide or fragment thereof is encoded is codon-optimized.

35 Genes of Interest

The RNA molecules described herein include a gene of interest. The gene of interest encodes a polypeptide of interest selected from, e.g., biologics, antibodies, vaccines, therapeutic polypeptides or peptides, cell penetrating peptides, secreted polypeptides, plasma membrane

- polypeptides, cytoplasmic or cytoskeletal polypeptides, intracellular membrane bound polypeptides, nuclear polypeptides, polypeptides associated with human disease, targeting moieties or those polypeptides encoded by the human genome for which no therapeutic indication has been identified but which nonetheless have utility in areas of research and discovery. The
- 5 the sequence for a particular gene of interest is readily identified by one of skill in the art using public and private databases, e.g., GenBank.

In some aspects, the RNA molecule includes a coding region for an antigen preferably derived from a pathogen associated with infectious disease which are preferably selected from antigens derived from the pathogens *Acinetobacter baumannii*, *Anaplasma* genus, *Anaplasma phagocytophilum*, *Ancylostoma braziliense*, *Ancylostoma duodenale*, *Arcanobacterium haemolyticum*, *Ascaris lumbricoides*, *Aspergillus* genus, *Astroviridae*, *Babesia* genus, *Bacillus anthracis*, *Bacillus cereus*, *Bartonella henselae*, BK virus, *Blastocystis hominis*, *Blastomyces dermatitidis*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Borrelia* genus, *Borrelia* spp, *Brucella* genus, *Brugia malayi*, *Bunyaviridae* family, *Burkholderia cepacia* and other *Burkholderia* species,

10 *Burkholderia mallei*, *Burkholderia pseudomallei*, *Caliciviridae* family, *Campylobacter* genus, *Candida albicans*, *Candida* spp, *Chlamydia trachomatis*, *Chlamydophila pneumoniae*, *Chlamydophila psittaci*, CJD prion, *Clonorchis sinensis*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium perfringens*, *Clostridium* spp, *Clostridium tetani*, *Coccidioides* spp, coronaviruses, *Corynebacterium diphtheriae*, *Coxiella burnetii*, Crimean-

20 Congo hemorrhagic fever virus, *Cryptococcus neoformans*, *Cryptosporidium* genus, Cytomegalovirus (CMV), Dengue viruses (DEN-1, DEN-2, DEN-3 and DEN-4), *Dientamoeba fragilis*, Ebolavirus (EBOV), *Echinococcus* genus, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia* genus, *Entamoeba histolytica*, *Enterococcus* genus, *Enterovirus* genus, Enteroviruses, mainly Coxsackie A virus and *Enterovirus* 71 (EV71), *Epidermophyton* spp, Epstein-Barr Virus

25 (EBV), *Escherichia coli* O157:H7, O111 and O104:H4, *Escherichia coli* Fimbrial antigen H, *Fasciola hepatica* and *Fasciola gigantica*, FFI prion, Filarioidea superfamily, Flaviviruses, *Francisella tularensis*, *Fusobacterium* genus, *Geotrichum candidum*, *Giardia intestinalis*, *Gnathostoma* spp, GSS prion, Guanarito virus, *Haemophilus ducreyi*, *Haemophilus influenzae*, *Helicobacter pylori*, Henipavirus (Hendra virus Nipah virus), Hepatitis A Virus, Hepatitis B Virus

30 (HBV), Hepatitis C Virus (HCV), Hepatitis D Virus, Hepatitis E Virus, Herpes simplex virus 1 and 2 (HSV-1 and HSV-2), *Histoplasma capsulatum*, HIV (Human immunodeficiency virus), *Hortaea werneckii*, Human bocavirus (HBoV), Human herpesvirus 6 (HHV-6) and Human herpesvirus 7 (HHV-7), Human metapneumovirus (hMPV), Human papillomavirus (HPV), Human parainfluenza viruses (HPIV), Japanese encephalitis virus, JC virus, Junin virus, *Kingella kingae*, *Klebsiella granulomatis*, Kuru prion, Lassa virus, *Legionella pneumophila*, *Leishmania* genus, *Leptospira* genus, *Listeria monocytogenes*, Lymphocytic choriomeningitis virus (LCMV), Machupo virus, *Malassezia* spp, Marburg virus, Measles virus, *Metagonimus yokagawai*, Microsporidia phylum, *Molluscum contagiosum* virus (MCV), Mumps virus, *Mycobacterium leprae* and *Mycobacterium*

- lepromatosis*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycoplasma pneumoniae*, *Naegleria fowleri*, *Necator americanus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Nocardia asteroides*, *Nocardia* spp, *Onchocerca volvulus*, *Orientia tsutsugamushi*, *Orthomyxoviridae* family (Influenza), *Paracoccidioides brasiliensis*, *Paragonimus* spp, *Paragonimus westermani*,
- 5 *Parvovirus B19*, *Pasteurella* genus, *Plasmodium* genus, *Pneumocystis jirovecii*, Poliovirus, Rabies virus, Respiratory syncytial virus (RSV), Rhinovirus, rhinoviruses, *Rickettsia akari*, *Rickettsia* genus, *Rickettsia prowazekii*, *Rickettsia rickettsii*, *Rickettsia typhi*, Rift Valley fever virus, Rotavirus, Rubella virus, Sabia virus, *Salmonella* genus, *Sarcoptes scabiei*, SARS coronavirus, *Schistosoma* genus, *Shigella* genus, Sin Nombre virus, Hantavirus, *Sporothrix*
- 10 *schenckii*, *Staphylococcus* genus, *Staphylococcus* genus, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Strongyloides stercoralis*, *Taenia* genus, *Taenia solium*, Tick-borne encephalitis virus (TBEV), *Toxocara canis* or *Toxocara cati*, *Toxoplasma gondii*, *Treponema pallidum*, *Trichinella spiralis*, *Trichomonas vaginalis*, *Trichophyton* spp, *Trichuris trichiura*, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Ureaplasma*
- 15 *urealyticum*, Varicella zoster virus (VZV), Variola major or Variola minor, vCJD prion, Venezuelan equine encephalitis virus, *Vibrio cholerae*, West Nile virus, Western equine encephalitis virus, *Wuchereria bancrofti*, Yellow fever virus, *Yersinia enterocolitica*, *Yersinia pestis*, and *Yersinia pseudotuberculosis*.

5' cap

- 20 In some embodiments, the saRNA molecule described herein includes a 5' cap. In some embodiments, the 5'-cap moiety is a natural 5'-cap. A "natural 5'-cap" is defined as a cap that includes 7-methylguanosine connected to the 5' end of an mRNA molecule through a 5' to 5' triphosphate linkage. In some embodiments, the 5'-cap moiety is a 5'- cap analog. In some
- 25 embodiments, the 5' end of the RNA is capped with a modified ribonucleotide with the structure m7G (5') ppp (5') N (cap 0 structure) or a derivative thereof, which may be incorporated during RNA synthesis (e.g., co-transcriptional capping) or may be enzymatically engineered after RNA transcription (e.g., post-transcriptional capping), wherein "N" is any ribonucleotide. In some
- 30 embodiments, the 5' end of the RNA molecule is capped with a modified ribonucleotide via an enzymatic reaction after RNA transcription. In some embodiments, capping is performed after purification, e.g., tangential flow filtration, of the RNA molecule. An exemplary enzymatic reaction for capping may include use of Vaccinia Virus Capping Enzyme (VCE) that includes mRNA
- 35 triphosphatase, guanylyl- transferase, and guanine-7-methyltransferase, which catalyzes the construction of N7-monomethylated cap 0 structures. Cap 0 structure can help maintaining the stability and translational efficacy of the RNA molecule. The 5' cap of the RNA molecule may be further modified by a 2'-O-Methyltransferase which results in the generation of a cap 1 structure (m7Gppp [m2'-O] N), which may further increase translation efficacy. In some embodiments, the RNA molecule may be enzymatically capped at the 5' end using Vaccinia guanylyltransferase, guanosine triphosphate, and S-adenosyl-L-methionine to yield cap 0 structure. An inverted 7-

methylguanosine cap is added via a 5' to 5' triphosphate bridge. Alternatively, use of a 2'-methyltransferase with Vaccinia guanylyltransferase yields the cap 1 structure where in addition to the cap 0 structure, the 2'OH group is methylated on the penultimate nucleotide. S-adenosyl-L-methionine (SAM) is a cofactor utilized as a methyl transfer reagent. Non-limiting examples of

5 5' cap structures are those which, among other things, have enhanced binding of cap binding polypeptides, increased half-life, reduced susceptibility to 5' endonucleases and/or reduced 5' decapping, as compared to synthetic 5'cap structures known in the art (or to a wild-type, natural or physiological 5'cap structure). For example, recombinant Vaccinia Virus Capping Enzyme and recombinant 2'-O-methyltransferase enzyme may create a canonical 5'-5'-triphosphate linkage

10 between the 5'-terminal nucleotide of an mRNA and a guanine cap nucleotide wherein the cap guanine includes an N7 methylation and the 5'-terminal nucleotide of the mRNA includes a 2'-O-methyl. Such a structure is termed the Cap1 structure. This cap results in a higher translational-competency and cellular stability and a reduced activation of cellular pro-inflammatory cytokines, as compared, e.g., to other 5'cap analog structures known in the art. Cap structures include, but

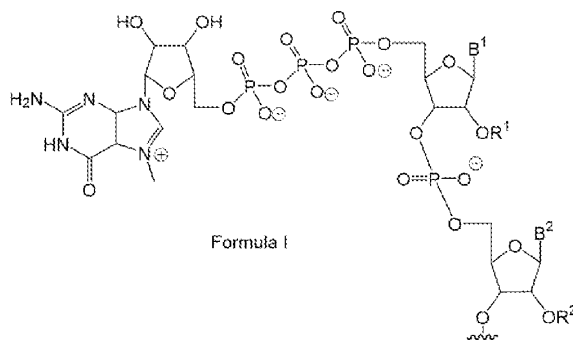
15 are not limited to, 7mG(5')ppp(5')N,pN2p (cap 0) and 7mG(5')ppp(5')N1mpNp (cap 1). Cap 0 is a N7-methyl guanosine connected to the 5' nucleotide through a 5' to 5' triphosphate linkage, typically referred to as m7G cap or m7Gppp. In the cell, the cap 0 structure can help provide for efficient translation of the mRNA that carries the cap. An additional methylation on the 2'O position of the initiating nucleotide generates Cap 1, or refers to as m7GpppNm-, wherein Nm denotes

20 any nucleotide with a 2'O methylation. In some embodiments, the 5' terminal cap includes a cap analog, for example, a 5' terminal cap may include a guanine analog. Exemplary guanine analogs include, but are not limited to, inosine, N1-methyl-guanosine, 2'fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine. In some embodiments, the capping region may include a single cap or a series of nucleotides

25 forming the cap. In this embodiment the capping region may be equal to any one of, at least any one of, at most any one of, or between any two of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or at least 2, or 10 or fewer nucleotides in length. In some embodiments, the cap is absent. In some embodiments, the first and second operational regions may be equal to any one of, at least any one of, at most any one of, or between any two of 3 to 40, e.g., 5-30, 10-20, 15, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,

30 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, or at least 4, or 30 or fewer nucleotides in length and may comprise, in addition to a Start and/or Stop codon, one or more signal and/or restriction sequences.

In some embodiments, the 5' Cap is represented by Formula I:

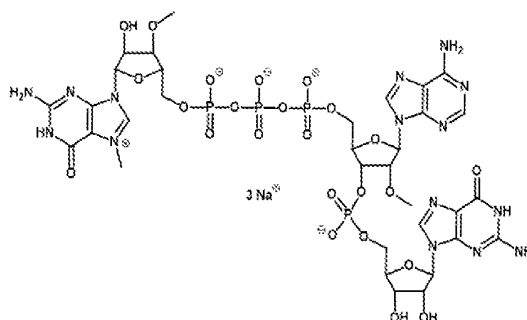


where R^1 and R^2 are each independently H or Me, and B^1 and B^2 are each independently guanine, adenine, or uracil. In some embodiments, B^1 and B^2 are naturally-occurring bases. In some embodiments, R^1 is methyl and R^2 is hydrogen. In some embodiments, B^1 is guanine. In some
 5 embodiments, B^1 is adenine. In some embodiments, B^2 is adenine. In some embodiments, B^2 is uracil. In some embodiments, B^2 is uracil and at least 5% of a total population of uracil nucleotides in the molecule that are downstream of B^2 have been replaced with one or more modified or unnatural nucleotides.

In some embodiments, the nucleotide immediately downstream (5' to 3' direction) of the
 10 5' Cap comprises guanine. In some embodiments, B^1 is adenine and B^2 is uracil. In some embodiments, B^1 is adenine, B^2 is uracil, R^1 is methyl, and R^2 is hydrogen. In some instances, the saRNA does not comprise a 5' Cap. In some instances, the 5' Cap is not represented by Formula I. In some embodiments, the nucleotide immediately downstream (5' to 3') of the 5' Cap comprises guanine, B^1 is adenine, B^2 is uracil, R^1 is methyl, and R^2 is hydrogen; this embodiment
 15 corresponds to CleanCap AU, and the inclusion of B^2 = uracil, while optionally substituting uracil nucleotides downstream of B^2 , has been shown to improve saRNA functionality in some embodiments. In some embodiments, the RNA molecule further comprises: (1) an alphavirus 5' replication recognition sequence, and (2) an alphavirus 3' replication recognition sequence. In some embodiments, the RNA molecule encodes at least one antigen. In some embodiments,
 20 the RNA molecule comprises at least 7000 nucleotides. In some embodiments, the RNA molecule comprises at least 8000 nucleotides. In some embodiments, at least 80% of the total RNA molecules are full length. In some embodiments, the alphavirus is Venezuelan equine encephalitis virus. In some embodiments, the alphavirus is Semliki Forest virus.

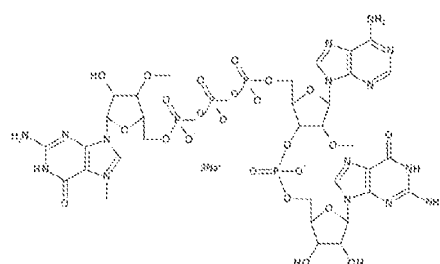
In some embodiments, the nucleotide immediately downstream (5' to 3') of the 5' Cap
 25 comprises guanine, B^1 is adenine, B^2 is uracil, R^1 is methyl, and R^2 is hydrogen, at least 50% of a total population of uridine nucleotides in the molecule has been replaced with N1-methylpseudouridine, and essentially all cytosine nucleotides in the molecule have been replaced with 5-methylcytosine. In some embodiments, the nucleotide immediately downstream (5' to 3') of the 5' Cap comprises guanine, B^1 is adenine, B^2 is uracil, R^1 is methyl, and R^2 is hydrogen, at
 30 least 50% of a total population of uridine nucleotides in the molecule has been replaced with 5-

- methoxyuridine, and essentially all cytosine nucleotides in the molecule have been replaced with 5-methylcytosine. In some embodiments, the nucleotide immediately downstream (5' to 3') of the 5' Cap comprises guanine, B¹ is adenine, B² is uracil, R¹ is methyl, and R² is hydrogen, at least 50% of a total population of uridine nucleotides in the molecule has been replaced with 5-methyluridine, and essentially all cytosine nucleotides in the molecule have been replaced with 5-methylcytosine. In some embodiments, the nucleotide immediately downstream (5' to 3') of the 5' Cap comprises guanine, B¹ is adenine, B² is uracil, R¹ is methyl, and R² is hydrogen, essentially all uridine nucleotides in the molecule have been replaced with about 50% 5-methoxyuridine and about 50% N1-methylpseudouridine. In some embodiments, the nucleotide immediately downstream (5' to 3') of the 5' Cap comprises guanine, B¹ is adenine, B² is uracil, R¹ is methyl, and R² is hydrogen, essentially all uridine nucleotides in the molecule have been replaced with about 75% 5-methoxyuridine and about 25% N1-methylpseudouridine. In some embodiments, the nucleotide immediately downstream (5' to 3') of the 5' Cap comprises guanine, B¹ is adenine, B² is uracil, R¹ is methyl, and R² is hydrogen, essentially all uridine nucleotides in the molecule have been replaced with about 25% 5-methoxyuridine and about 75% N1-methylpseudouridine.
- In some embodiments, a 5' terminal cap is 7mG(5')ppp(5')NlmpNp. In some preferred

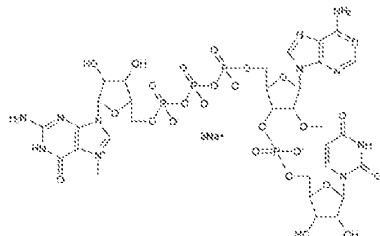


embodiments, the 5' cap comprises:

In some embodiments, the 5' cap comprises CLEANCAP® Reagent AG (3' OMe) for co-transcriptional capping of mRNA, m7(3'OMeG)(5')ppp(5')(2'OMeA)pG,



In alternative embodiments, the 5' cap comprises CLEANCAP® AU for Self-Amplifying mRNA, CLEANCAP® Reagent AU for co-transcriptional capping of mRNA, m7G(5')ppp(5')(2'OMeA)pU,



5

Poly-A tail

As used herein, "poly A tail" refers to a stretch of consecutive adenine residues, which may be attached to the 3' end of the RNA molecule. The poly-A tail may increase the half-life of the RNA molecule. Poly-A tails may play key regulatory roles in enhancing translation efficiency and regulating the efficiency of mRNA quality control and degradation. Short sequences or hyperpolyadenylation may signal for RNA degradation. Exemplary designs include a poly-A tails of about 40 adenine residues to about 80 adenine residues. In some embodiments, the RNA molecule further includes an endonuclease recognition site sequence immediately downstream of the poly A tail sequence. In some embodiments, such as for the second RNA or the saRNA molecule, the RNA molecule further includes a poly-A polymerase recognition sequence (e.g., AAUAAA) near its 3' end. A "full length" RNA molecule is one that includes a 5'-cap and a poly A tail.

In some embodiments, the poly A tail includes 5-400 adenine nucleotides in length. The poly A tail nucleotide length may be equal to any one of, at least any one of, at most any one of, or between any two of 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, and 400. In some embodiments, the RNA molecule includes a poly A tail that includes about 25 to about 400 adenosine nucleotides, a sequence of about 50 to about 400 adenosine nucleotides, a sequence of about 50 to about 300 adenosine nucleotides, a sequence of about 50 to about 250 adenosine nucleotides, a sequence of about 60 to about 250 adenosine nucleotides, or a sequence of about 40 to about 100 adenosine nucleotides. In some embodiments, the RNA molecule includes a poly A tail includes a sequence of greater than 30 adenosine nucleotides ("As"). In some embodiments, the RNA molecule includes a poly A tail that includes about 40 As. In some embodiments, the RNA molecule includes a poly A tail that includes about 80 As. As used herein, the term "about" refers to a deviation of $\pm 10\%$ of the value(s) to which it is attached. In some embodiments, the 3' poly-A tail has a stretch of at least 10 consecutive adenosine residues and at most 300 consecutive

adenosine residues. In some embodiments, the RNA molecule includes at least 20 consecutive adenosine residues and at most 40 consecutive adenosine residues. In some embodiments, the RNA molecule includes about 40 consecutive adenosine residues. In some embodiments, the RNA molecule includes about 70 consecutive adenosine residues. In some embodiments, the RNA molecule includes about 80 consecutive adenosine residues.

Compositions

In some instances, the compositions described herein include at least one RNA as described herein.

10 In some embodiments, equal to any one of, at least any one of, at most any one of, or between any two of 50%, 55%, 60%, 65%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the total RNA molecules (capped and uncapped) in the composition are capped.

15 In some embodiments, equal to any one of, at least any one of, at most any one of, or between any two of 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% of the total RNA molecules in the composition are full length RNA transcripts. Purity may be determined as described herein, e.g., via reverse
20 phase HPLC or Bioanalyzer chip-based electrophoresis and measure by, e.g., peak area of full-length RNA molecule relative to total peak. In some embodiments, a fragment analyzer (FA) may be used to quantify and purify the RNA. The fragment analyzer automates capillary electrophoresis and HPLC.

In some embodiments, the composition is substantially free of one or more impurities or
25 contaminants including the linear DNA template and/or reverse complement transcription products and, for instance, includes RNA molecules that are equal to any one of, at least any one of, at most any one of, or between any two of 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% pure; at least 98% pure, or at least 99% pure.

In some embodiments, the composition comprises an amount of the first RNA molecule
30 that is greater than the amount of the second RNA molecule. In some embodiments, the composition comprises an amount of the first RNA molecule that is at least about 1 to 2 times greater than the amount of the second RNA molecule. In some embodiments, the composition comprises an amount of the first RNA molecule that is at least about 1 to 100 times greater than the amount of the second RNA molecule.

35 In some embodiments, the composition further includes a pharmaceutically acceptable carrier. In some embodiments, the composition further includes a pharmaceutically acceptable vehicle.

In some embodiments, the composition further includes a lipid-based delivery system, which delivers an RNA molecule to the interior of a cell, where it can then replicate and/or express the encoded polypeptide of interest. The delivery system may have adjuvant effects which enhance the immunogenicity of an encoded antigen. In some embodiments, the composition

5 further includes one or more each of neutral lipids, cationic lipids, structural lipids (e.g. cholesterol or combinations thereof), and polymer conjugate lipids such as polyethylene glycol conjugated lipids (PEG-lipids), and forms nanoparticles that encompass the RNA molecules. In some

embodiments, the composition further includes any one of a cationic lipid, a liposome, a lipid nanoparticle, a polyplex, a cochleate, a virosome, an immune-stimulating complex, a

10 microparticle, a microsphere, a nanosphere, a unilamellar vesicle, a multilamellar vesicle, an oil-in-water emulsion, a water-in-oil emulsion, an emulsome, a polycationic peptide, and a cationic nanoemulsion. In some embodiments, the RNA molecule is encapsulated in, bound to or adsorbed on any one of a cationic lipid, a liposome, a lipid nanoparticle, a polyplex, a cochleate,

a virosome, an immune-stimulating complex, a microparticle, a microsphere, a nanosphere, a

15 unilamellar vesicle, a multilamellar vesicle, an oil-in-water emulsion, a water-in-oil emulsion, an emulsome, a polycationic peptide, and a cationic nanoemulsion, or a combination thereof.

In some instances, the compositions described herein include at least two RNA molecules: a first RNA molecule and a second RNA molecule as described herein. To protect against more than one strain of a virus or different viruses, a combination vaccine composition

20 may be administered that includes RNA encoding at least one antigenic polypeptide protein (or antigenic portion thereof) of a first virus or organism and further includes a second RNA molecule encoding at least one antigenic polypeptide protein (or antigenic portion thereof) of a second virus or organism. RNA can be co-formulated, for example, in a single lipid nanoparticle (LNP) or can be formulated in separate LNPs for co-administration.

25 In some embodiments, the second RNA molecule includes any one of a 5' cap, a 5' UTR, an open reading frame, a 3' UTR, and a poly A sequence, or any combination thereof. In some embodiments, the second RNA molecule includes a 5' cap moiety. In some embodiments, the second RNA molecule includes a 5' UTR and a 3'UTR. In some embodiments, the second RNA molecule includes a 5'UTR, an open reading frame, a 3'UTR, and does not further include a 5'

30 cap. In some embodiments, the second RNA molecule includes a 5' cap moiety, 5' UTR, coding region, 3' UTR, and a 3' poly A sequence. In some embodiments, the second RNA molecule includes a 5' cap moiety, 5' UTR, noncoding region, 3' UTR, and a 3' poly A sequence. In some embodiments, the second RNA molecule includes a noncoding region and does not further comprise any one of a 5' cap moiety, 5' UTR, 3' UTR, and a 3' poly A sequence. In some

35 embodiments, the second RNA molecule includes a 5' cap moiety, a 5' untranslated region (5' UTR), a modified nucleotide, an open reading frame, a 3' untranslated region (3' UTR), and a 3' poly A sequence.

Some aspects of the disclosure are directed to a composition comprising (i) first RNA molecule encoding a gene of interest; and (ii) a second RNA molecule comprising a modified or unnatural nucleotide. In some instances, the first RNA molecule is any one of the saRNA molecules described herein. In some instances, the first RNA molecule comprises a 5' Cap, a 5' untranslated region, a coding region for a nonstructural protein comprising a RNA replicase, a subgenomic promoter, an open reading frame encoding a gene of interest, a 3' untranslated region, and a 3' poly A sequence. In some instances, at least 5% of a total population of a particular nucleotide in the first RNA molecule has been replaced with one or more modified or unnatural nucleotides. In some instances, the RNA molecule comprises natural, unmodified nucleotides and does not include a modified or unnatural nucleotide. In some instances, the 5' Cap is represented by Formula I, where R¹ and R² are each independently H or Me, B¹ and B² are each independently guanine, adenine, or uracil, a 5' untranslated region, a coding region for a nonstructural protein derived from an alphavirus, a subgenomic promoter, such as one derived from an alphavirus, an open reading frame encoding a gene of interest, a 3' untranslated region, and a 3' poly A sequence. In some embodiments, B¹ and B² are naturally-occurring bases. In some embodiments, R¹ is methyl and R² is hydrogen. In some embodiments, B¹ is guanine. In some embodiments, B¹ is adenine. In some embodiments, B² is adenine. In some embodiments, B² is uracil. In some embodiments, the nucleotide immediately downstream (5' to 3' direction) of the 5' Cap comprises guanine.

In some embodiments, B¹ is adenine and B² is uracil. In some embodiments, B¹ is adenine, B² is uracil, R¹ is methyl, and R² is hydrogen. In some embodiments, the nucleotide immediately downstream (5' to 3') of the 5' Cap comprises guanine, B¹ is adenine, B² is uracil, R¹ is methyl, and R² is hydrogen; this embodiment corresponds to CLEANCAP AU (Trilink), and the inclusion of B² = uracil, while optionally substituting uracil nucleotides downstream of B², which has been shown to provide increased saRNA functionality in some embodiments.

In some embodiments, at least 10% of a total population of a particular nucleotide in the first or second RNA molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 25% of a total population of a particular nucleotide in the first or second RNA molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 50% of a total population of a particular nucleotide in the first or second RNA molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 75% of a total population of a particular nucleotide in the first or second RNA molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, essentially all of a particular nucleotide population in the first or second RNA molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, the one or more modified or unnatural replacement nucleotides comprise two modified or unnatural nucleotides provided in a ratio ranging from 1:99 to 99:1, or any derivable range therein. In some embodiments, at least 10% of a total population of a first

[illegible]

the first or second RNA molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 50% of a total population of a first particular nucleotide in the first or second RNA molecule has been replaced with one or more modified or unnatural nucleotides, and essentially all of a total population of a second particular nucleotide in the first or second RNA molecule has been replaced with one or more modified or unnatural nucleotides. In some embodiments, at least 75% of a total population of a first particular nucleotide in the first or second RNA molecule has been replaced with one or more modified or unnatural nucleotides, and essentially all of a total population of a second particular nucleotide in the first or second RNA molecule has been replaced with one or more modified or unnatural nucleotides.

In some embodiments, at least 25% of a total population of uridine nucleotides in the first RNA molecule has been replaced with N1-methylpseudouridine. In some embodiments, at least 50% of a total population of uridine nucleotides in the first RNA molecule has been replaced with N1-methylpseudouridine. In some embodiments, at least 75% of a total population of uridine nucleotides in the first RNA molecule has been replaced with N1-methylpseudouridine. In some embodiments, essentially all uridine nucleotides in the first RNA molecule have been replaced with N1-methylpseudouridine. In some embodiments, at least 50% of a total population of uridine nucleotides in the first RNA molecule has been replaced with 5-methoxyuridine. In some embodiments, essentially all uridine nucleotides in the molecule have been replaced with 5-methoxyuridine. In some embodiments, at least 50% of a total population of uridine nucleotides in the first RNA molecule has been replaced with 5-methyluridine. In some embodiments, essentially all uridine nucleotides in the first RNA molecule have been replaced with 5-methyluridine. In some embodiments, at least 50% of a total population of cytosine nucleotides in the first RNA molecule has been replaced with 5-methylcytosine. In some embodiments, essentially all cytosine nucleotides in the first RNA molecule have been replaced with 5-methylcytosine. In some embodiments, at least 50% of a total population of uridine nucleotides in the first RNA molecule has been replaced with 2-thiouridine. In some embodiments, essentially all uridine nucleotides in the first RNA molecule have been replaced with 2-thiouridine.

In some embodiments, at least 25% of a total population of uridine nucleotides in the second RNA molecule has been replaced with N1-methylpseudouridine. In some embodiments, at least 50% of a total population of uridine nucleotides in the second RNA molecule has been replaced with N1-methylpseudouridine. In some embodiments, at least 75% of a total population of uridine nucleotides in the second RNA molecule has been replaced with N1-methylpseudouridine. In some embodiments, essentially all uridine nucleotides in the second RNA molecule have been replaced with N1-methylpseudouridine. In some embodiments, at least 50% of a total population of uridine nucleotides in the second RNA molecule has been replaced with 5-methoxyuridine. In some embodiments, essentially all uridine nucleotides in the second RNA molecule have been replaced with 5-methoxyuridine. In some embodiments, at least 50%

of a total population of uridine nucleotides in the second RNA molecule has been replaced with 5-methyluridine. In some embodiments, essentially all uridine nucleotides in the second RNA molecule have been replaced with 5-methyluridine. In some embodiments, at least 50% of a total population of cytosine nucleotides in the second RNA molecule has been replaced with 5-methylcytosine. In some embodiments, essentially all cytosine nucleotides in the second RNA molecule have been replaced with 5-methylcytosine. In some embodiments, at least 50% of a total population of uridine nucleotides in the second RNA molecule has been replaced with 2-thiouridine. In some embodiments, essentially all uridine nucleotides in the second RNA molecule have been replaced with 2-thiouridine.

10 In some embodiments, at least 50% of a total population of uridine nucleotides in the second RNA molecule has been replaced with N1-methylpseudouridine and essentially all cytosine nucleotides in the second RNA molecule have been replaced with 5-methylcytosine. In some embodiments, at least 50% of a total population of uridine nucleotides in the second RNA molecule has been replaced with 5-methoxyuridine and essentially all cytosine nucleotides in the second RNA molecule have been replaced with 5-methylcytosine. In some embodiments, at least 50% of a total population of uridine nucleotides in the second RNA molecule has been replaced with 5-methyluridine and essentially all cytosine nucleotides in the second RNA molecule have been replaced with 5-methylcytosine.

20 In some embodiments, essentially all uridine nucleotides in the second RNA molecule have been replaced with about 50% 5-methoxyuridine and about 50% N1-methylpseudouridine. In some embodiments, essentially all uridine nucleotides in the second RNA molecule have been replaced with about 75% 5-methoxyuridine and about 25% N1-methylpseudouridine. In some embodiments, essentially all uridine nucleotides in the second RNA molecule have been replaced with about 25% 5-methoxyuridine and about 75% N1-methylpseudouridine.

25 In some embodiments, essentially all uridine nucleotides in the first RNA molecule have been replaced with N1-methylpseudouridine and at least 50% of a total population of uridine nucleotides in the second RNA molecule has been replaced with N1-methylpseudouridine. In some embodiments, essentially all uridine nucleotides in the first RNA molecule have been replaced with N1-methylpseudouridine and essentially all uridine nucleotides in the second RNA molecule have been replaced with N1-methylpseudouridine. In some embodiments, essentially all uridine nucleotides in the first RNA molecule have been replaced with N1-methylpseudouridine, at least 50% of a total population of uridine nucleotides in the second RNA molecule has been replaced with 5-methyluridine, and essentially all cytosine nucleotides in the second RNA molecule have been replaced with 5-methylcytosine. In some embodiments, essentially all uridine nucleotides in the first RNA molecule have been replaced with N1-methylpseudouridine and essentially all uridine

nucleotides in the second RNA molecule have been replaced with about 50% 5-methoxyuridine and about 50% N1-methylpseudouridine.

Methods of use

5 The RNA compositions may be utilized to treat and/or prevent a disease, disorder or infection comprising administering to a subject any of the RNA compositions described herein. In one embodiment, the RNA composition produces an antigen specific immune response. In some embodiments, the antigen specific immune response comprises a T cell response. In some embodiments, the antigen specific immune response comprises a B cell response. In
10 some embodiments, the antigen specific immune response comprises both a T cell response and a B cell response. In some embodiments, the method of producing an antigen specific immune response involves a single administration of the RNA composition. In some
 embodiments, the RNA composition is administered to the subject by intradermal, intramuscular injection, subcutaneous injection, intranasal inoculation, or oral administration. In some
15 embodiments, the nanoparticle has a net neutral charge at a neutral pH value. In some embodiments, the RNA (e.g., mRNA) vaccine is multivalent.

 In some embodiments, the RNA polynucleotides or portions thereof may encode one or more polypeptides or fragments thereof as an antigen.

 Some aspects of the disclosure are directed to a method of inducing an immune
20 response in a subject, comprising administering to the subject in need thereof an amount of a composition as disclosed herein that produces an immune response comprising an antibody response. In some embodiments, a composition as disclosed herein elicits an immune response comprising a T cell response. One aspect of the disclosure is directed to a method comprising administering to the subject in need thereof an effective amount of a composition as
25 disclosed herein, wherein the amount of the composition is an amount effective to produce an immune response, to treat or to prevent a disease, disorder or infection i.e. "an effective amount". Some aspects of the disclosure are directed to a method of vaccinating a subject, comprising administering to the subject in need thereof an effective amount of a composition as disclosed herein.

30 The compounds of the invention may be useful for treating or preventing a disease, disorder, or condition or delivering an active agent to treat or prevent a disease, disorder, or condition. In particular, such compositions may be useful in treating or preventing a disease, disorder, or condition characterized by missing or aberrant protein or polypeptide activity. Diseases, disorders, and/or conditions characterized by dysfunctional or aberrant protein or
35 polypeptide activity for which a composition may be administered include, but are not limited to rare diseases, infectious diseases (as both vaccines and therapeutics), cancer and proliferative diseases, genetic diseases, autoimmune diseases, diabetes, neurodegenerative diseases, cardio- and reno-vascular diseases, and metabolic diseases.

Administration and Dosing

The term "treating", "treat" or "treatment" as used herein embraces both preventative, e.g., prophylactic, and palliative treatment, e.g., relieve, alleviate, or slow the progression of the patient's disease (or condition) or any tissue damage associated with the disease.

5 As used herein, the terms, "subject", "individual" or "patient," used interchangeably, refer to any animal, including mammals. Mammals according to the invention include canine, feline, bovine, caprine, equine, ovine, porcine, rodents, lagomorphs, primates, humans and the like, and encompass mammals in utero. In an embodiment, humans are suitable subjects. Human subjects may be of any gender and at any stage of development.

10 As used herein, the phrase "therapeutically effective amount" or "effective amount" refers to the amount of active compound or pharmaceutical agent, such as a nucleic acid, that elicits the biological or medicinal response in a tissue, system, animal, individual or human that is being sought by a researcher, veterinarian, medical doctor or other clinician, which may include one or more of the following:

15 (1) preventing the disease; for example, preventing a disease, condition or disorder in an individual that may be predisposed to the disease, condition or disorder but does not yet experience or display the pathology or symptomatology of the disease;

(2) inhibiting the disease; for example, inhibiting a disease, condition or disorder in an individual that is experiencing or displaying the pathology or symptomatology of the disease, condition or disorder (e.g., arresting (or slowing) further development of the pathology or symptomatology or both); and

20

(3) ameliorating the disease; for example, ameliorating a disease, condition or disorder in an individual that is experiencing or displaying the pathology or symptomatology of the disease, condition or disorder (e.g., reversing the pathology or symptomatology or both).

25 An "effective amount" or "therapeutically effective amount" of nucleic acid is an amount sufficient to produce the desired effect, e.g. an increase or inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of the nucleic acid. An increase in expression of a target sequence is achieved when any measurable level is detected in the case of an expression product that is not present in the absence of the nucleic acid. In the case where the expression product is present at some level prior to contact with the nucleic acid, an increase in expression is achieved when the fold increase in value obtained with a nucleic acid such as mRNA relative to control is about 1.05, 1.1, 1.2, 1.3, 1.4, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 75, 100, 250, 500, 750, 1000, 5000, 10000 or greater. Inhibition of expression of a target gene or target sequence is achieved when the value

30

35 obtained with a nucleic acid such as antisense oligonucleotide relative to the control is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, e.g., examination of protein or RNA levels using techniques known to those of skill in the

art such as dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, fluorescence or luminescence of suitable reporter proteins, as well as phenotypic assays known to those of skill in the art.

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

The phrase "inhibiting expression of a target gene" refers to the ability of a nucleic acid to silence, reduce, or inhibit the expression of a target gene. To examine the extent of gene silencing, a test sample (e.g., a sample of cells in culture expressing the target gene) or a test mammal (e.g., a mammal such as a human or an animal model such as a rodent (e.g., mouse)
30 or a non-human primate (e.g., monkey) model) is contacted with a nucleic acid that silences, reduces, or inhibits expression of the target gene. Expression of the target gene in the test sample or test animal is compared to expression of the target gene in a control sample (e.g., a sample of cells in culture expressing the target gene) or a control mammal (e.g., a mammal such as a human or an animal model such as a rodent (e.g., mouse) or non-human primate (e.g., monkey)
35 model) that is not contacted with or administered the nucleic acid. The expression of the target gene in a control sample or a control mammal may be assigned a value of 100%. In particular embodiments, silencing, inhibition, or reduction of expression of a target gene is achieved when the level of target gene expression in the test sample or the test mammal relative to the level of

target gene expression in the control sample or the control mammal is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. In other words, the nucleic acids are capable of silencing, reducing, or inhibiting the expression of a target gene by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%,
5 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in a test sample or a test mammal relative to the level of target gene expression in a control sample or a control mammal not contacted with or administered the nucleic acid. Suitable assays for determining the level of target gene expression include, without limitation, examination of protein or mRNA levels using techniques known to those of skill in the art, such as, e.g., dot blots, northern blots, in situ
10 hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

Typically, a compound of the invention is administered in an amount effective to treat a condition as described herein or to deliver an active agent to treat a condition as described herein. The compounds of the invention may be administered as compound per se, or alternatively, as
15 a pharmaceutically acceptable salt. For administration and dosing purposes, the compound per se or pharmaceutically acceptable salt thereof will simply be referred to as the compounds of the invention.

The compounds of the invention are administered by any suitable route in the form of a pharmaceutical composition adapted to such a route, and in a dose effective for the treatment
20 intended. The compounds of the invention may be administered orally, rectally, vaginally, parenterally, topically, intranasally, or by inhalation.

The compounds of the invention may be administered orally. Oral administration may involve swallowing, so that the compound enters the gastrointestinal tract, or buccal or sublingual administration may be employed by which the compound enters the bloodstream directly from
25 the mouth.

In another embodiment, the compounds of the invention may also be administered parenterally, for example directly into the bloodstream, into muscle, or into an internal organ. Suitable means for parenteral administration include intravenous, intraarterial, intraperitoneal, intrathecal, intraventricular, intraurethral, intrasternal, intracranial, intramuscular and
30 subcutaneous. Suitable devices for parenteral administration include needle (including microneedle) injectors, needle-free injectors, and infusion techniques.

In another embodiment, the compounds of the invention may also be administered topically to the skin or mucosa, that is, dermally or transdermally. In another embodiment, the compounds of the invention may also be administered intranasally or by inhalation. In another
35 embodiment, the compounds of the invention may be administered rectally or vaginally. In another embodiment, the compounds of the invention may also be administered directly to the eye or ear.

The dosage regimen for the compounds of the invention or compositions containing the compounds is based on a variety of factors, including the type, age, weight, sex and medical condition of the patient; the severity of the condition; the route of administration; and the activity of the particular compound employed. Thus, the dosage regimen may vary widely. In one embodiment, the total daily dose of a compound of the invention is typically from about 0.01 to about 100 mg/kg (e.g., mg compound of the invention per kg body weight) for the treatment of the indicated conditions discussed herein or to deliver an active agent by using the compounds of the invention. In another embodiment, total daily dose of the compound of the invention is from about 0.00001 to about 50 mg/kg, and in another embodiment, from about 0.0001 to about 30 mg/kg. It is not uncommon that the administration of the compounds of the invention will be repeated a plurality of times in a day (typically no greater than 4 times). Multiple doses per day typically may be used to increase the total daily dose, if desired.

Nucleic Acids

In certain embodiments, nucleic acid sequences can exist in a variety of instances such as: isolated segments and recombinant vectors of incorporated sequences or recombinant polynucleotides encoding polypeptides, such as antigens or one or both chains of an antibody, or a fragment, derivative, mutein, or variant thereof, polynucleotides sufficient for use as hybridization probes, PCR primers or sequencing primers for identifying, analyzing, mutating or amplifying a polynucleotide encoding a polypeptide, anti-sense nucleic acids for inhibiting expression of a polynucleotide, mRNA (e.g. modified RNA), saRNA, and complementary sequences of the foregoing described herein. Nucleic acids that encode an epitope to which antibodies may bind. Nucleic acids encoding fusion proteins that include these polypeptides are also provided. The nucleic acids can be single-stranded or double-stranded and can comprise RNA and/or DNA nucleotides and artificial variants thereof (e.g., peptide nucleic acids).

The term "polynucleotide" refers to a nucleic acid molecule that can be recombinant or has been isolated from total genomic nucleic acid. Included within the term "polynucleotide" are oligonucleotides (nucleic acids 100 residues or less in length), recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like. Polynucleotides include, in certain aspects, regulatory sequences, isolated substantially away from their naturally occurring genes or protein encoding sequences. Polynucleotides may be single- stranded (coding or antisense) or double- stranded, and may be RNA, DNA (genomic, cDNA or synthetic), analogs thereof, or a combination thereof. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide.

In this respect, the term "gene" is used to refer to a nucleic acid that encodes a protein, polypeptide, or peptide (including any sequences required for proper transcription, post-translational modification, or localization). As will be understood by those in the art, this term encompasses genomic sequences, expression cassettes, cDNA sequences, and smaller

engineered nucleic acid segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides, fusion proteins, and mutants. A nucleic acid encoding all or part of a polypeptide may contain a contiguous nucleic acid sequence encoding all or a portion of such a polypeptide. It also is contemplated that a particular polypeptide may be encoded by
5 nucleic acids containing variations having slightly different nucleic acid sequences but, nonetheless, encode the same or substantially similar polypeptide.

In certain embodiments, there are polynucleotide variants having substantial identity to the sequences disclosed herein; those comprising equal to any one of, at least any one of, at most any one of, or between any two of 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or
10 99% or higher sequence identity, compared to a polynucleotide sequence provided herein using the methods described herein (e.g., BLAST analysis using standard parameters). In certain aspects, the isolated polynucleotide will comprise a nucleotide sequence encoding a polypeptide that has at least 90% identity to an amino acid sequence described herein, over the entire length of the sequence; or a nucleotide sequence complementary to said isolated polynucleotide. In
15 some embodiments, the isolated polynucleotide will comprise a nucleotide sequence encoding a polypeptide that has at least 95% identity to an amino acid sequence described herein, over the entire length of the sequence; or a nucleotide sequence complementary to said isolated polynucleotide.

The nucleic acid sequences, regardless of the length of the coding sequence itself, may
20 be combined with other nucleic acid sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. The nucleic acids can be any length. They can be, for example, equal to any one of, at least any one of, at most any one of, or between any two of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, 125, 175, 200, 250, 300, 350, 400, 450, 500,
25 750, 1000, 1500, 3000, 5000, 6000, 7000, 8000, 9000, 10000, 11000, 12000, 13000, 14000, 15000 or more nucleotides in length, and/or can comprise one or more additional sequences, for example, regulatory sequences, and/or be a part of a larger nucleic acid, for example, a vector. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length being limited by the ease of preparation and use in the intended recombinant
30 nucleic acid protocol. In some cases, a nucleic acid sequence may encode a polypeptide sequence with additional heterologous coding sequences, for example to allow for purification of the polypeptide, transport, secretion, post-translational modification, or for therapeutic benefits such as targeting or efficacy. As discussed above, a tag or other heterologous polypeptide may be added to the modified polypeptide-encoding sequence, wherein "heterologous" refers to a
35 polypeptide that is not the same as the modified polypeptide.

Lipid delivery

In some embodiments, the RNA composition comprises lipids. The lipids and RNA may together form nanoparticles. In some embodiments, the LNP integrity of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation of the present disclosure is about 5 20% or higher, about 25% or higher, about 30% or higher, about 35% or higher, about 40% or higher, about 45% or higher, about 50% or higher, about 55% or higher, about 60% or higher, about 65% or higher, about 70% or higher, about 75% or higher, about 80% or higher, about 85% or higher, about 90% or higher, about 95% or higher, about 96% or higher, about 97% or higher, about 98% or higher, or about 99% or higher.

10 In some embodiments, the LNP integrity of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation of the present disclosure is higher than the LNP integrity of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation produced by a comparable method by about 5% or higher, about 10% or more, about 15% or more, about 20% or more, about 30% or more, about 40% or more, about 50% or more, about 60% or more, 15 about 70% or more, about 80% or more, about 90% or more, about 1 folds or more, about 2 folds or more, about 3 folds or more, about 4 folds or more, about 5 folds or more, about 10 folds or more, about 20 folds or more, about 30 folds or more, about 40 folds or more, about 50 folds or more, about 100 folds or more, about 200 folds or more, about 300 folds or more, about 400 folds or more, about 500 folds or more, about 1000 folds or more, about 2000 folds or 20 more, about 3000 folds or more, about 4000 folds or more, about 5000 folds or more, or about 10000 folds or more.

In some embodiments, the T₉₀% of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation of the present disclosure is about 12 months or longer, about 15 months or longer, about 18 months or longer, about 21 months or longer, about 24 months 25 or longer, about 27 months or longer, about 30 months or longer, about 33 months or longer, about 36 months or longer, about 48 months or longer, about 60 months or longer, about 72 months or longer, about 84 months or longer, about 96 months or longer, about 108 months or longer, about 120 months or longer.

In some embodiments, the T₉₀% of the LNP, LNP suspension, lyophilized LNP 30 composition, or LNP formulation of the present disclosure is longer than the T₉₀% of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation produced by a comparable method by about 5% or higher, about 10% or more, about 15% or more, about 20% or more, about 30% or more, about 40% or more, about 50% or more, about 60% or more, about 70% or more, about 80% or more, about 90% or more, about 1 folds or more, about 2 folds or more, 35 about 3 folds or more, about 4 folds or more, about 5 folds or more.

In some embodiments, the T_{1/2} of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation of the present disclosure is about 12 months or longer, about 15 months or longer, about 18 months or longer, about 21 months or longer, about 24 months

or longer, about 27 months or longer, about 30 months or longer, about 33 months or longer, about 36 months or longer, about 48 months or longer, about 60 months or longer, about 72 months or longer, about 84 months or longer, about 96 months or longer, about 108 months or longer, about 120 months or longer.

5 In some embodiments, the T_{1/2} of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation of the present disclosure is longer than the T_{1/2} of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation produced by a comparable method by about 5% or higher, about 10% or more, about 15% or more, about 20% or more, about 30% or more, about 40% or more, about 50% or more, about 60% or more, about 70% or
10 more, about 80% or more, about 90% or more, about 1 folds or more, about 2 folds or more, about 3 folds or more, about 4 folds or more, about 5 folds or more

 As used herein, "Tx" refers to the amount of time lasted for the nucleic acid integrity (e.g., mRNA integrity) of a LNP, LNP suspension, lyophilized LNP composition, or LNP formulation to degrade to about X of the initial integrity of the nucleic acid (e.g., mRNA) used for the
15 preparation of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation. For example, "T_{80%}" refers to the amount of time lasted for the nucleic acid integrity (e.g., mRNA integrity) of a LNP, LNP suspension, lyophilized LNP composition, or LNP formulation to degrade to about 80% of the initial integrity of the nucleic acid (e.g., mRNA) used for the preparation of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation. For
20 another example, "T_{1/2}" refers to the amount of time lasted for the nucleic acid integrity (e.g., mRNA integrity) of a LNP, LNP suspension, lyophilized LNP composition, or LNP formulation to degrade to about 1/2 of the initial integrity of the nucleic acid (e.g., mRNA) used for the preparation of the LNP, LNP suspension, lyophilized LNP composition, or LNP formulation.

 Lipid nanoparticles may include a lipid component and one or more additional
25 components, such as a therapeutic and/or prophylactic, such as a nucleic acid. A LNP may be designed for one or more specific applications or targets. The elements of a LNP may be selected based on a particular application or target, and/or based on the efficacy, toxicity, expense, ease of use, availability, or other feature of one or more elements. Similarly, the particular formulation of a LNP may be selected for a particular application or target according
30 to, for example, the efficacy and toxicity of particular combination of elements. The efficacy and tolerability of a LNP formulation may be affected by the stability of the formulation.

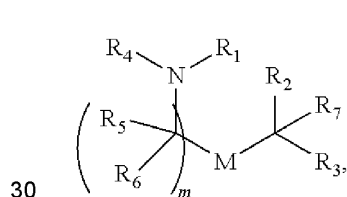
 The lipid component of a LNP may include, for example, a cationic lipid, a phospholipid (such as an unsaturated lipid, e.g., DOPE or DSPC), a PEG lipid, and a structural lipid. The elements of the lipid component may be provided in specific fractions.

35 In some embodiments, the LNP further comprises a phospholipid, a PEG lipid, a mixture of structural lipids, or any combination thereof. Suitable phospholipids, PEG lipids, and structural lipids for the methods of the present disclosure are further disclosed herein.

In some embodiments, the lipid component of a LNP includes a cationic lipid, a phospholipid, a PEG lipid, and a mixture of structural lipids. In certain embodiments, the lipid component of the lipid nanoparticle includes about 30 mol % to about 60 mol % cationic lipid, about 0 mol % to about 30 mol % phospholipid, about 18.5 mol % to about 48.5 mol % structural lipids, and about 0 mol % to about 10 mol % of PEG lipid, provided that the total mol % does not exceed 100%. In some embodiments, the lipid component of the lipid nanoparticle includes about 35 mol % to about 55 mol % compound of cationic lipid, about 5 mol % to about 25 mol % phospholipid, about 30 mol % to about 40 mol % structural lipids, and about 0 mol % to about 10 mol % of PEG lipid. In a particular embodiment, the lipid component includes about 50 mol % said cationic lipid, about 10 mol % phospholipid, about 38.5 mol % structural lipids, and about 1.5 mol % of PEG lipid. In another embodiment, the lipid component includes about 40 mol % said cationic lipid, about 20 mol % phospholipid, about 38.5 mol % structural lipids, and about 1.5 mol % of PEG lipid. In some embodiments, the phospholipid may be DOPE or DSPC. In other embodiments, the PEG lipid may be PEG-DMG and/or the structural lipids may be cholesterol.

The amount of a therapeutic and/or prophylactic in a LNP may depend on the size, composition, desired target and/or application, or other properties of the lipid nanoparticle as well as on the properties of the therapeutic and/or prophylactic. For example, the amount of an RNA useful in a LNP may depend on the size, sequence, and other characteristics of the RNA. The relative amounts of a therapeutic and/or prophylactic (i.e. pharmaceutical substance) and other elements (e.g., lipids) in a LNP may also vary. In some embodiments, the wt/wt ratio of the lipid component to a therapeutic and/or prophylactic in a LNP may be from about 5:1 to about 60:1, such as 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, and 60:1. For example, the wt/wt ratio of the lipid component to a therapeutic and/or prophylactic may be from about 10:1 to about 40:1. In certain embodiments, the wt/wt ratio is about 20:1. The amount of a therapeutic and/or prophylactic in a LNP may, for example, be measured using absorption spectroscopy (e.g., ultraviolet-visible spectroscopy).

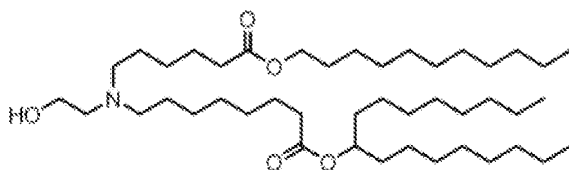
In some embodiments, the ionizable lipid is a compound of Formula (I):



or their N-oxides, or salts or isomers thereof, wherein:

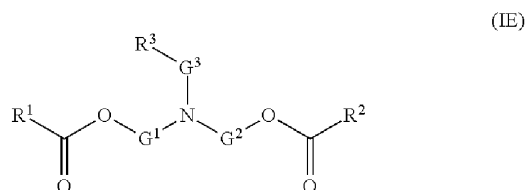
R_i is selected from the group consisting of C5-30 alkyl, C5-20 alkenyl, -R*YR", -YR", and -R"M'R'; R₂ and R₃ are independently selected from the group consisting of H, C1-14 alkyl, C2-14 alkenyl, -R*YR", -YR", and -R*OR", or R₂ and R₃, together with the atom to which they are

- attached, form a heterocycle or carbocycle; R₄ is selected from the group consisting of hydrogen, a C₃-6 carbocycle, $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, $-CHQR$, $-CQ(R)_2$, and unsubstituted C₁-6 alkyl, where Q is selected from a carbocycle, heterocycle, $-OR$, $-O(CH_2)_nN(R)_2$, $-C(O)OR$, $-OC(O)R$, $-CX_3$, $-CX_2H$, $-CXH_2$, $-CN$, $-N(R)_2$, $-C(O)N(R)_2$, $-N(R)C(O)R$, $-N(R)S(O)_2R$, $-N(R)C(O)N(R)_2$, $-N(R)C(S)N(R)_2$, $-N(R)Re$, $N(R)S(O)_2R_8$, $-O(CH_2)_nOR$, $-N(R)C(=NR_9)N(R)_2$, $-N(R)C(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, $-N(R)C(O)OR$, $-N(OR)C(O)R$, $-N(OR)S(O)_2R$, $-N(OR)C(O)OR$, $-N(OR)C(O)N(R)_2$, $-N(OR)C(S)N(R)_2$, $-N(OR)C(=NR_9)N(R)_2$, $-N(OR)C(=CHR_9)N(R)_2$, $-C(=NR_9)N(R)_2$, $-C(=NR_9)R$, $-C(O)N(R)OR$, and $-C(R)N(R)_2C(O)OR$, and each n is independently selected from 1, 2, 3, 4, and 5; each R₅ is independently selected from the group consisting of C₁-3 alkyl, C₂-3 alkenyl, and H; each Re is independently selected from the group consisting of C₁-3 alkyl, C₂-3 alkenyl, and H; M and M' are independently selected from $-C(O)O-$, $-OC(O)-$, $-OC(O)-M''-C(O)O-$, $-C(O)N(R')-$, $-N(R')C(O)-$, $-C(O)-$, $-C(S)-$, $-C(S)S-$, $-SC(S)-$, $-CH(OH)-$, $-P(O)(OR')O-$, $-S(O)_2-$, $-S-S-$, an aryl group, and a heteroaryl group, in which M'' is a bond, C₁-13 alkyl or C₂-13 alkenyl; R₇ is selected from the group consisting of C₁-3 alkyl, C₂-3 alkenyl, and H; Re is selected from the group consisting of C₃-6 carbocycle and heterocycle; R₉ is selected from the group consisting of H, CN, NO₂, C_i-6 alkyl, $-OR$, $-S(O)_2R$, $-S(O)_2N(R)_2$, C₂-6 alkenyl, C₃-6 carbocycle and heterocycle; each R is independently selected from the group consisting of C₁-3 alkyl, C₂-3 alkenyl, and H; each R' is independently selected from the group consisting of C_i-is alkyl, C₂-is alkenyl, $-R^*YR''$, $-YR''$, and H; each R'' is independently selected from the group consisting of C₃-15 alkyl and C₃-15 alkenyl; each R* is independently selected from the group consisting of C_i-i₂ alkyl and C₂-i₂ alkenyl; each Y is independently a C₃-6 carbocycle; each X is independently selected from the group consisting of F, Cl, Br, and I; and m is selected from 5, 6, 7, 8, 9, 10, 11, 12, and 13; and wherein when R₄ is $-(CH_2)_nQ$, $-(CH_2)_nCHQR$, $-CHQR$, or $-CQ(R)_2$, then (i) Q is not $-N(R)_2$ when n is 1, 2, 3, 4 or 5, or (ii) Q is not 5, 6, or 7-membered heterocycloalkyl when n is 1 or 2. In some embodiments, the ionizable lipid is SM-102. In some embodiments, the ionizable lipid is ALC-0315. In some embodiments, the ionizable lipid is:

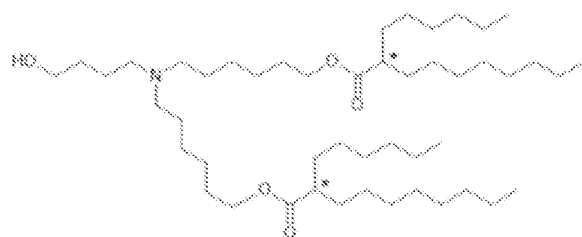


In some embodiments, the ionizable lipid is a compound having the following structure

- (I):



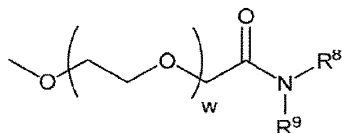
- or a pharmaceutically acceptable salt, tautomer, prodrug or stereoisomer thereof, wherein: one of L1 or L2 is $-\text{O}(\text{C}=\text{O})-$, $-(\text{C}=\text{O})\text{O}-$, $-\text{C}(\text{=O})-$, $-\text{O}-$, $-\text{S}(\text{O})_x-$, $-\text{S}-\text{S}-$, $-\text{C}(\text{=O})\text{S}-$, $\text{SC}(\text{=O})-$, $-\text{NRaC}(\text{=O})-$, $-\text{C}(\text{=O})\text{NRa}-$, $\text{NRaC}(\text{=O})\text{NRa}-$, $-\text{OC}(\text{=O})\text{NRa}-$ or $-\text{NRaC}(\text{=O})\text{O}-$, and the other of L1 or L2 is $-\text{O}(\text{C}=\text{O})-$, $-(\text{C}=\text{O})\text{O}-$, $-\text{C}(\text{=O})-$, $-\text{O}-$, $-\text{S}(\text{O})_x-$, $-\text{S}-\text{S}-$, $-\text{C}(\text{=O})\text{S}-$, $\text{SC}(\text{=O})-$, $-\text{NRaC}(\text{=O})-$, $-\text{C}(\text{=O})\text{NRa}-$, $\text{NRaC}(\text{=O})\text{NRa}-$, $-\text{OC}(\text{=O})\text{NRa}-$ or $-\text{NRaC}(\text{=O})\text{O}-$ or a direct bond; G1 and G2 are each independently unsubstituted C1-C12 alkylene or C1-C12 alkenylene; G3 is C1-C24 alkylene, C1-C24 alkenylene, C3-C8 cycloalkylene, C3-C8 cycloalkenylene; Ra is H or C1-C12 alkyl; R1 and R2 are each independently C6-C24 alkyl or C6-C24 alkenyl; R3 is H, OR5, CN, $-\text{C}(\text{=O})\text{OR4}$, $-\text{OC}(\text{=O})\text{R4}$ or $-\text{NR5C}(\text{=O})\text{R4}$; R4 is C1-C12 alkyl; R5 is H or C1-C6 alkyl; and x is 0, 1 or 2. In a preferred embodiment, the ionizable lipid is:



Asterisks (*) indicate chiral centers.

- The lipid component of a lipid nanoparticle composition may include one or more molecules comprising polyethylene glycol, such as PEG or PEG-modified lipids. Such species may be alternately referred to as PEGylated lipids. A PEG lipid is a lipid modified with polyethylene glycol. A PEG lipid may be selected from the non-limiting group including PEG-modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, PEG-modified dialkylglycerols, and mixtures thereof. In some embodiments, a PEG lipid may be PEG-c-DOMG, PEG-DMG, PEG-DLPE, PEG-DMPE, PEG-DPPC, or a PEG-DSPE lipid. As used herein, the term "PEG lipid" refers to polyethylene glycol (PEG) -modified lipids. Non-limiting examples of PEG lipids include PEG-modified phosphatidylethanolamine and phosphatidic acid, PEG-ceramide conjugates (e.g., PEG-CerC14 or PEG-CerC20), PEG-modified dialkylamines and PEG-modified 1,2-diacyloxypropan-3-amines. Such lipids are also referred to as PEGylated lipids. In some embodiments, a PEG lipid can be PEG-c-DOMG, PEG-DMG,

PEG-DLPE, PEG-DMPE, PEG-DPPC, or a PEG-DSPE lipid. In some embodiments, the PEG-modified lipids are a modified form of PEG DMG. In some embodiments, the PEG-modified lipid is PEG lipid with the formula (IV):



(IV)

- 5 wherein R⁸ and R⁹ are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 30 carbon atoms, wherein the alkyl chain is optionally interrupted by one or more ester bonds; and w has a mean value ranging from 30 to 60.

In some embodiments, a lipid nanoparticle comprises a cationic lipid, a PEG-modified lipid, a mixture of two structural lipids and a non-cationic lipid. In some embodiments, a cationic lipid is an ionizable cationic lipid and the non-cationic lipid is a neutral lipid, and the structural lipid is a sterol. In one aspect, the sterol is cholesterol. In another aspect, the sterol is a cholesterol analog. In some embodiments, a cationic lipid is selected from the group consisting of ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) (ALC-0315), 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA), dilinoleyl-methyl-4-dimethylaminobutyrate (DLin-MC3-DMA), di((Z)-non-2-en-1-yl) 9-((4-(dimethylamino)butanoyl)oxy)heptadecanedioate (L319), (12Z,15Z)—N,N-dimethyl-2-nonylhenicosa-12,15-dien-1-amine (L608), and N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]heptadecan-8-amine (L530).

In some embodiments, the nanoparticle has a mean diameter of 50-200 nm. In some embodiments, the nanoparticle is a lipid nanoparticle. In some embodiments, the lipid nanoparticle comprises a cationic lipid, a PEG-modified lipid, a mixture of cholesterol and a cholesterol analog, and a non-cationic lipid. In some embodiments, the lipid nanoparticle comprises a molar ratio of about 20-60% cationic lipid, 0.5-15% PEG-modified lipid, 25-55% mixture of structural lipids such as cholesterol and a cholesterol analog, and 25% non-cationic lipid. In some embodiments, the cationic lipid is an ionizable cationic lipid and the non-cationic lipid is a neutral lipid, and the sterol is a cholesterol.

In some embodiments, the nanoparticle has a polydispersity value of less than 0.4 (e.g., less than 0.3, 0.2 or 0.1).

In some embodiments, the nanoparticle has a net neutral charge at a neutral pH value.

In some embodiments, the composition further comprises a mixture of lipids. The mixture of lipids typically forms a lipid nanoparticle (LNP). The mRNA described herein, in some embodiments, is formulated with a lipid nanoparticle (e.g., for administration to a subject). In some embodiments, the lipid mixture, and thus the lipid nanoparticle, comprises: an ionizable amino lipid; a neutral lipid; a mixture of cholesterol and a cholesterol analog; and a PEG-modified lipid.

For example, the lipid mixture/lipid nanoparticle may comprise: 20-60 mol% ionizable amino lipid; 5-25 mol% neutral lipid; 25-55 mol% mixture of cholesterol and a cholesterol analog; and 0.5-15 mol% PEG-modified lipid. In some embodiments, the lipid nanoparticle comprises: 20-60 mol% ionizable amino lipid; 5-25 mol% neutral lipid; 25-55 mol% mixture of cholesterol and a cholesterol analog; and 0.5-15 mol% PEG-modified lipid. In some

5 embodiments, the lipid nanoparticle comprises: 40-55 mol% ionizable amino lipid; 5-15 mol% neutral lipid; 35-45 mol% mixture of cholesterol and a cholesterol analog; and 1-5 mol% PEG-modified lipid. For example, the lipid nanoparticle may comprise: (a) 47 mol% ionizable amino lipid; 11.5 mol% neutral lipid; 38.5 mol% mixture of cholesterol and a cholesterol analog; and

10 3.0 mol% PEG-modified lipid; (b) 48 mol% ionizable amino lipid; 11 mol% neutral lipid; 38.5 mol% mixture of cholesterol and a cholesterol analog ; and 2.5 mol% PEG-modified lipid; (c) 49 mol% ionizable amino lipid; 10.5 mol% neutral lipid; 38.5 mol% mixture of cholesterol and a cholesterol analog; and 2.0 mol% PEG-modified lipid; (d) 50 mol% ionizable amino lipid; 10 mol% neutral lipid; 38.5 mol% mixture of cholesterol and a cholesterol analog; and 1.5 mol%

15 PEG-modified lipid; or (e) 51 mol% ionizable amino lipid; 9.5 mol% neutral lipid; 38.5 mol% mixture of cholesterol and a cholesterol analog; and 1.0 mol% PEG-modified lipid. In some embodiments, the lipid mixture, and thus the lipid nanoparticle, comprises 20-55 mol%, 20-50 mol%, 20-45 mol%, 20-40 mol%, 25-60 mol%, 25-55 mol%, 25-50 mol%, 25-45 mol%, 25-40 mol%, 30-60 mol%, 30-55 mol%, 30-50 mol%, 30-45 mol%, 30-40 mol%, 35-60 mol%, 35-55 mol%, 35-50 mol%, 35-45 mol%, 35-40 mol%, 40-60 mol%, 40-55 mol%, 40-50 mol%, 40-45 mol%, 50-60 mol%, 50-55 mol%, or 55-60 mol% ionizable amino lipid. In some embodiments,

20 the lipid mixture, and thus the lipid nanoparticle, comprises 5-20 mol%, 5-15 mol%, 5-10 mol%, 10-25 mol%, 10-20 mol%, 10-15 mol%, 15-25 mol%, 15-20 mol%, or 20-25 mol% neutral lipid. In some embodiments, the lipid mixture, and thus the lipid nanoparticle, comprises 25-50 mol%,

25 25-45 mol%, 25-40 mol%, 25-35 mol%, 25-30 mol%, 30-55 mol%, 30-50 mol%, 30-45 mol%, 30-40 mol%, 30-35 mol%, 35-55 mol%, 35-50 mol%, 35-45 mol%, 35-40 mol%, 40-55 mol%, 40-50 mol%, 40-45 mol%, 45-55 mol%, 45-50 mol%, or 50-55 mol% mixture of cholesterol and a cholesterol analog. In some embodiments, the lipid mixture, and thus the lipid nanoparticle,

30 comprises 0.5-10 mol%, 0.5-5 mol%, 0.5-1 mol%, 1-15%, 1-10 mol%, 1-5 mol%, 1.5-15%, 1.5-10 mol%, 1.5-5 mol%, 2-15%, 2-10 mol%, 2-5 mol%, 2.5-15%, 2.5-10 mol%, 2.5-5 mol%, 3-15%, 3-10 mol%, or 3-5 mol%, PEG-modified lipid. In some embodiments, the lipid mixture comprises: 50 mol% ionizable amino lipid; 10 mol% neutral lipid; 38.5 mol% mixture of cholesterol and a cholesterol analog; and 1.5 mol% PEG-modified lipid. In some embodiments,

35 the ionizable amino lipid is heptadecan-9-yl 8 ((2 hydroxyethyl)(6 oxo 6-(undecyloxy)hexyl)amino)octanoate. In some embodiments, the neutral lipid is 1,2 distearoyl sn glycerol-3 phosphocholine (DSPC). In some embodiments, the sterol is cholesterol. In some embodiments, the PEG-modified lipid is 1-monomethoxypolyethyleneglycol-2,3-dimyristylglycerol with polyethylene glycol of average molecular weight 2000 (PEG2000 DMG).

A composition may further include a pharmaceutically-acceptable excipient, inert or active. A pharmaceutically acceptable excipient, after administered to a subject, does not cause undesirable physiological effects. The excipient in the pharmaceutical composition must be "acceptable" also in the sense that it is compatible with mRNA and can be capable of stabilizing it. One or more excipients (e.g., solubilizing agents) can be utilized as pharmaceutical carriers for delivery of the mRNA. Examples of a pharmaceutically acceptable excipients include, but are not limited to, biocompatible vehicles (e.g., LNPs), carriers, adjuvants, additives, and diluents to achieve a composition usable as a dosage form. Examples of other excipients include colloidal silicon oxide, magnesium stearate, cellulose, and sodium lauryl sulfate.

Additional suitable pharmaceutical excipients, as well as pharmaceutical necessities for their use, are described in Remington's Pharmaceutical Sciences.

In some embodiments, an mRNA is formulated using one or more excipients to: (1) increase stability; (2) increase cell transfection; (3) permit the sustained or delayed release (e.g., from a depot formulation); (4) alter the biodistribution (e.g., target to specific tissues or cell types); (5) increase the translation of encoded protein in vivo; and/or (6) alter the release profile of encoded protein (antigen) in vivo. In addition to traditional excipients such as any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, excipients can include, without limitation, lipidoids, liposomes, lipid nanoparticles, polymers, lipoplexes, core-shell nanoparticles, peptides, proteins, cells transfected with the RNA (e.g., for transplantation into a subject), hyaluronidase, nanoparticle mimics and combinations thereof. In some embodiments, a composition comprising mRNA does not include an adjuvant (the composition is adjuvant-free). Compositions may be sterile, pyrogen-free or both sterile and pyrogen-free. General considerations in the formulation and/or manufacture of pharmaceutical agents, such as compositions, may be found, for example, in Remington: The Science and Practice of Pharmacy 21st ed., Lippincott Williams & Wilkins, 2005. Formulations of the compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the mRNA into association with an excipient (e.g., a mixture of lipids and/or a lipid nanoparticle), and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-dose unit. Relative amounts of the mRNA, the pharmaceutically-acceptable excipient, and/or any additional ingredients in a composition in accordance with the disclosure may vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered.

In some embodiments, a nucleic acid containing particle (e.g., in some embodiments an LNP as described herein) comprising two or more RNA molecules, comprises each RNA molecule in the same amount (i.e., at a 1:1 ratio).

In some embodiments, a nucleic acid containing particle (e.g., in some embodiments an LNP as described herein) comprising two or more RNA molecules, comprises a different amount of each RNA molecule. For example, in some embodiments, a nucleic acid containing particle comprises a first RNA molecule and a second RNA molecule, where the first RNA molecule is present in an amount that is 0.01 to 100 times that of the second RNA molecule (e.g., wherein the amount of the first RNA molecule is 0.01 to 50, 0.01 to 4, 0.01 to 30, 0.01 to 25, 0.01 to 20, 0.01 to 15, 0.01 to 10, 0.01 to 9, 0.01 to 8, 0.01 to 7, 0.01 to 6, 0.01 to 5, 0.01 to 4, 0.01 to 3, 0.01 to 2, 0.01 to 1.5, 1 to 50, 1 to 4, 1 to 30, 1 to 25, 1 to 20, 1 to 15, 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, 1 to 2, or 1 to 1.5 times higher than the second RNA molecule). In some embodiments, a nucleic acid containing particle comprises a first RNA molecule and a second RNA molecule, wherein the concentration of the first RNA molecule is 1 to 10 times that of the second RNA molecule. In some embodiments, a nucleic acid containing particle comprises a first RNA molecule and a second RNA molecule, wherein the concentration of the first RNA molecule is 1 to 5 times that of the second RNA molecule. In some embodiments, a nucleic acid containing particle comprises a first RNA molecule and a second RNA molecule, wherein the concentration of the first RNA molecule is 1 to 3 times that of the second RNA molecule. In some embodiments, a nucleic acid containing particle comprises a first RNA molecule and a second RNA molecule, wherein the concentration of the first RNA molecule is 2 times that of the second RNA molecule. In some embodiments, a nucleic acid containing particle comprises a first RNA molecule and a second RNA molecule, wherein the concentration of the first RNA molecule is 3 times that of the second RNA molecule.

In some embodiments, a nucleic acid containing particle (e.g., in some embodiments an LNP as described herein) comprising three RNA molecules, comprises each RNA molecule in the same amount (i.e., at a 1:1:1 ratio).

In some embodiments, a nucleic acid containing particle (e.g., in some embodiments an LNP as described herein) comprising three RNA molecules, comprises a different amount of each RNA molecule. For example, in some embodiments, the ratio of first RNA molecule: second RNA molecule: third RNA molecule is 1: 0.01-100: 0.01-100 (e.g., 1: 0.01-50: 0.01-50; 1: 0.01-40: 0.01-40; 1: 0.01-30: 0.01-25; 1: 0.01-25: 0.01-25; 1: 0.01-20: 0.01-20; 1: 0.01-15: 0.01-15; 1: 0.01-10: 0.01-9; 1: 0.01-9: 0.01-9; 1: 0.01-8: 0.01-8; 1: 0.01-7: 0.01-7; 1: 0.01-6: 0.01-6; 1: 0.01-5: 0.01-5; 1: 0.01-4: 0.01-4; 1: 0.01-3: 0.01-3; 1: 0.01-2: 0.01-2; or 1: 0.01-1.5: 0.01-1.5). In some embodiments, the ratio of first RNA molecule: second RNA molecule: third RNA molecule is 1:1:3. In some embodiments, the ratio of first RNA molecule: second RNA molecule: third RNA molecule is 1:3:3.

The term "dose" as used herein refers in general to a "dose amount" which relates to the amount of RNA administered per administration, i.e., per dosing.

- The immunogenic composition including a lipid-based delivery system may further include one or more salts and/or one or more pharmaceutically acceptable surfactants, preservatives, carriers, diluents, and/or excipients, in some cases. In some aspects, the immunogenic composition including a lipid-based delivery system further include a
- 5 pharmaceutically acceptable vehicle. In some aspects, each of a buffer, stabilizing agent, and optionally a salt, may be included in the immunogenic composition including a lipid-based delivery system. In other aspects, any one or more of a buffer, stabilizing agent, salt, surfactant, preservative, and excipient may be excluded from the immunogenic composition including a lipid-based delivery system.
- 10 In a further aspect, the immunogenic composition including a lipid-based delivery system further comprises a stabilizing agent. In some aspects, the stabilizing agent comprises sucrose, mannose, sorbitol, raffinose, trehalose, mannitol, inositol, sodium chloride, arginine, lactose, hydroxyethyl starch, dextran, polyvinylpyrrolidone, glycine, or a combination thereof. In some aspects, the stabilizing agent is a disaccharide, or sugar. In one aspect, the stabilizing
- 15 agent is sucrose. In another aspect, the stabilizing agent is trehalose. In a further aspect, the stabilizing agent is a combination of sucrose and trehalose. In some aspects, the total concentration of the stabilizing agent(s) in the composition is about 5% to about 10% w/v. For example, the total concentration of the stabilizing agent may be equal to at least, at most, exactly, or between any two of 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%,
- 20 14%, 15%, 16%, 17%, 18%, 19%, or 20% w/v or any range or value derivable therein. In some aspects, the stabilizing agent concentration includes, but is not limited to, a concentration of about 10 mg/mL to about 400 mg/mL, about 100 mg/mL to about 200 mg/mL, about 100 mg/mL to about 150mg/mL, about 100 mg/mL to about 140 mg/mL, about 100 mg/mL to about 130 mg/mL, about 100 mg/mL to about 120 mg/mL, about 100 mg/mL to about 110 mg/mL, or about
- 25 100 mg/mL to about 105 mg/mL. In some aspects, the concentration of the stabilizing agent is equal to at least, at most, exactly, or between any two of 10 mg/mL, 20 mg/mL, 50 mg/mL, 100 mg/mL, 101 mg/mL, 102 mg/mL, 103 mg/mL, 104 mg/mL, 105 mg/mL, 106 mg/mL, 107 mg/mL, 108 mg/mL, 109 mg/mL, 110 mg/mL, 150 mg/mL, 200 mg/mL, 300 mg/mL, 400 mg/mL, or more.
- 30 In a further aspect, the mass amount of the stabilizing agent and the mass amount of the RNA are in a specific ratio. In one aspect, the ratio of the mass amount of the stabilizing agent and the RNA is no greater than 5000. In another aspect, the ratio of the mass amount of the stabilizing agent and the RNA is no greater than 2000. In another aspect, the ratio of the mass amount of the stabilizing agent and the RNA is no greater than 1000. In another aspect,
- 35 the ratio of the mass amount of the stabilizing agent and the RNA is no greater than 500. In another aspect, the ratio of the mass amount of the stabilizing agent and the RNA is no greater than 100. In another aspect, the ratio of the mass amount of the stabilizing agent and the

pharmaceutical substance is no greater than 50. In another aspect, the ratio of the mass amount of the stabilizing agent and the RNA is no greater than 10. In another aspect, the ratio of the mass amount of the stabilizing agent and the RNA is no greater than 1. In another aspect, the ratio of the mass amount of the stabilizing agent and the RNA is no greater than 0.5. In another aspect, the ratio of the mass amount of the stabilizing agent and the RNA is no greater than 0.1. In another aspect, the stabilizing agent and RNA comprise a mass ratio of about 200 – 2000 of the stabilizing agent : 1 of the RNA.

In some aspects, the immunogenic composition including a lipid-based delivery system further comprises a buffer. Examples of buffering agents include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium glutubionate, calcium gluceptate, calcium gluconate, d-gluconic acid, calcium glycerophosphate, calcium lactate, calcium lactobionate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, amino-sulfonate buffers (e.g., HEPES), magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, and/or combinations thereof. In some aspects, the buffer is a HEPES buffer, a Tris buffer, or a PBS buffer. Accordingly, in some embodiments, the composition further includes an adjuvant, e.g., aluminum-containing compounds, such as, for example, any of the adjuvants listed herein, including aluminum hydroxide and AlPO_4 . In one aspect, the buffer is Tris buffer. In another aspect, the buffer is a HEPES buffer. In a further aspect, the buffer is a PBS buffer. For example, the buffer concentration may be equal to at least, at most, exactly, or between any two of 1 mM, 2 mM, 3 mM, 4 mM, 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, 16 mM, 17 mM, 18 mM, 19 mM, or 20 mM, or any range or value derivable therein. The buffer may be at a neutral pH, pH 6.5 to 8.5, pH 7.0 to pH 8.0, or pH 7.2 to pH 7.6. For example, the buffer may be at least, at most, exactly, or between any two of pH 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, or 8.5, or any range or value derivable therein. In specific aspects, the buffer is at pH 7.4.

In some aspects, the immunogenic composition including a lipid-based delivery system may further comprise a salt. Examples of salts include but not limited to sodium salts and/or potassium salts. In one aspect, the salt is a sodium salt. In a specific aspect, the sodium salt is sodium chloride. In one aspect, the salt is a potassium salt. In some aspects, the potassium salt comprises potassium chloride. The concentration of the salts in the composition may be about

70 mM to about 140 mM. For example, the salt concentration may be equal to at least, at most, exactly, or between any two of 50 mM, 60 mM, 70 mM, 80 mM, 90 mM, 100 mM, 120 mM, 130 mM, 140 mM, 150 mM, 160 mM, 170 mM, 180 mM, 190 mM, or 200 mM.

In some aspects, the salt concentration includes, but is not limited to, a concentration of about 1 mg/mL to about 100 mg/mL, about 1 mg/mL to about 50 mg/mL, about 1 mg/mL to about 40 mg/mL, about 1 mg/mL to about 30 mg/mL, about 1 mg/mL to about 20 mg/mL, about 1 mg/mL to about 10 mg/mL, or about 1 mg/mL to about 15 mg/mL. In some aspects, the concentration of the salt is equal to at least, at most, exactly, or between any two of 1 mg/mL, 2 mg/mL, 3 mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 7 mg/mL, 8 mg/mL, 9 mg/mL, 10 mg/mL, 11 mg/mL, 12 mg/mL, 13 mg/mL, 14 mg/mL, 15 mg/mL, 16 mg/mL, 17 mg/mL, 18 mg/mL, 19 mg/mL, 20 mg/mL, or more. The salt may be at a neutral pH, pH 6.5 to 8.5, pH 7.0 to pH 8.0, or pH 7.2 to pH 7.6. For example, the salt may be at a pH equal to at least, at most, exactly, or between any two of 6.5, 6.6, 6.7, 6.8, 6.9, 7, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8, 8.1, 8.2, 8.3, 8.4, or 8.5.

In some aspects, the immunogenic composition including a lipid-based delivery system further comprises a surfactant, a preservative, any other excipient, or a combination thereof. As used herein, "any other excipient" includes, but is not limited to, antioxidants, glutathione, EDTA, methionine, desferal, antioxidants, metal scavengers, or free radical scavengers. In one aspect, the surfactant, preservative, excipient or combination thereof is sterile water for injection (sWFI), bacteriostatic water for injection (BWFI), saline, dextrose solution, polysorbates, poloxamers, Triton, divalent cations, Ringer's lactate, amino acids, sugars, polyols, polymers, or cyclodextrins. In some embodiments, the formulations that comprise LNPs described herein excludes Triton X-100.

Examples of excipients, which refer to ingredients in the immunogenic compositions that are not active ingredients, include but are not limited to carriers, binders, diluents, lubricants, thickeners, surface active agents, preservatives, stabilizers, emulsifiers, buffers, flavoring agents, disintegrants, coatings, plasticizers, compression agents, wet granulation agents, or colorants. Preservatives for use in the compositions disclosed herein include but are not limited to benzalkonium chloride, chlorobutanol, paraben and thimerosal. As used herein, "pharmaceutically acceptable carrier" includes any and all aqueous solvents (e.g., water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles, such as sodium chloride, Ringer's dextrose, etc.), non-aqueous solvents (e.g., propylene glycol, polyethylene glycol, vegetable oil, and injectable organic esters, such as ethyl oleate), dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial or antifungal agents, anti-oxidants, chelating agents, and inert gases), isotonic agents, absorption delaying agents, salts, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, fluid and nutrient replenishers, such like materials and combinations

thereof, as would be known to one of ordinary skill in the art. Diluents, or diluting or thinning agents, include but are not limited to ethanol, glycerol, water, sugars such as lactose, sucrose, mannitol, and sorbitol, and starches derived from wheat, corn rice, and potato; and celluloses such as microcrystalline cellulose. The amount of diluent in the composition may range from
5 about 10% to about 90% by weight of the total composition, about 25% to about 75%, about 30% to about 60% by weight, or about 12% to about 60%.

The pH and exact concentration of the various components in the immunogenic composition including a lipid-based delivery system are adjusted according to well-known parameters. The use of such media and agents for pharmaceutical active substances is well
10 known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients, its use in immunogenic, prophylactic and/or therapeutic compositions is contemplated.

It is contemplated that any embodiment discussed in this specification may be implemented with respect to any method or composition of the disclosure, and vice versa.
15 Furthermore, compositions of the disclosure may be used to achieve methods of the disclosure.

Other objects, features and advantages of the present disclosure will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the disclosure, are given by way of illustration only, since various changes and modifications within the spirit and
20 scope of the disclosure will become apparent to those skilled in the art from this detailed description.

Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the measurement or quantitation method.

The use of the word "a" or "an" when used in conjunction with the term "comprising" may
25 mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

The phrase "and/or" means "and" or "or". To illustrate, A, B, and/or C includes: A alone, B alone, C alone, a combination of A and B, a combination of A and C, a combination of B and C, or a combination of A, B, and C. In other words, "and/or" operates as an inclusive "or."

30 The words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

35 The phrase "essentially all" is defined as "at least 95%"; if essentially all members of a group have a certain property, then at least 95% of members of the group have that property. In

some instances, essentially all means equal to any one of, at least any one of, or between any two of 95, 96, 97, 98, 99, or 100 % of members of the group have that property.

The compositions and methods for their use may “comprise,” “consist essentially of,” or “consist of” any of the ingredients or steps disclosed throughout the specification. Compositions and methods “consisting essentially of” any of the ingredients or steps disclosed limits the scope of the claim to the specified materials or steps which do not materially affect the basic and novel characteristic of the claimed disclosure.

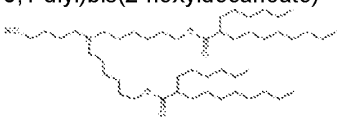
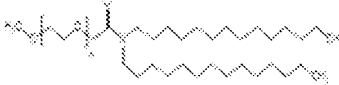
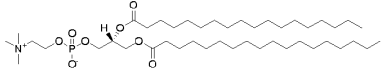
EXAMPLES

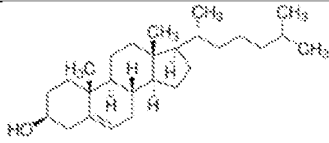
EXAMPLE 1: Lipid Nanoparticle (LNP) Composition

The benchmark LNP formulation contains a cationic lipid (ALC-0315), a PEG-lipid (ALC-0159), a neutral lipid (DSPC (1,2distearoyl-sn-glycero-3-phosphocholine)) and a Sterol (cholesterol). The physicochemical properties and the structures of the 4 lipids are shown in

Table 1 below.

Table 1. Lipids in the Benchmark Formulation

Lipid	Molecular Weight [Da]	Molecular Formula	Physical State and Storage Condition	Chemical Name (Synonyms) and Structure
ALC-0315	766	C ₄₈ H ₉₅ NO ₅	Liquid (oil) -20°C	((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) 
ALC-0159	~2400-2600	C ₃₀ H ₆₀ NO(C ₂ H ₄ O) _n OCH ₃	Solid -20°C	2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide 
DSPC	790	C ₄₄ H ₈₈ NO ₈ P	Solid -20°C	1,2-Distearoyl- <i>sn</i> -glycero-3-phosphocholine 

Cholesterol	387	C ₂₇ H ₄₆ O	Solid -20°C	
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CAS = Chemical Abstracts Service; DSPC = 1,2-Distearoyl-sn-glycero-3-phosphocholine

The LNPs of this invention further comprise an additional structural lipid including but not limited to mixtures of cholesterol and cholesterol analogs recited in the Detailed Description and the Examples set forth herein. In one embodiment, the cholesterol analog combined with

5 cholesterol is selected from sitosterol, stigmasterol, campesterol, sitostanol, campestanol, brassicasterol, fucosterol, β -sitosterol, stigmastanol, β -sitostanol, ergosterol, fecosterol, lupeol, cycloartenol, Δ^5 -avenasterol, Δ^7 -avenasterol or a Δ^7 -stigmasterol, tomatidine, ursolic acid or alpha-tocopherol, including analogs, salts or esters thereof. As used herein, the term "sitosterol" shall mean beta-sitosterol.

10 **EXAMPLE 2: Enhancing Flu B modRNA Immunogenicity with LNPs Containing Cholesterol C-24 Alkyl Derivatives**

This study was conducted to compare LNPs containing cholesterol C-24 alkyl derivatives, wherein the LNPs encompass a modified RNA encoding an influenza HA polypeptide from an influenza B strain (Austria). mRNA-LNPs containing cholesterol analogues

15 were formulated by combining an mRNA containing aqueous phase and a lipid containing organic phase using methods known in the art. The organic phase was prepared by solubilizing a mixture of ionizable lipid, DSPC, PEG-lipid, cholesterol and cholesterol analogs at various ratios in ethanol. The organic phase and aqueous phase were mixed at a flow rate ratio of 3:1 by syringe pumps. The resultant solution was dialyzed against 10 mM Tris buffer (pH 7.4). Post-

20 dialysis solution was concentrated and spiked with cryo-protectant to a final mRNA-LNP solution with RNA concentration of 0.1 mg/ml. Immunogenicity in Balb/c mice was evaluated using Neutralization and HAI assays. The test articles are listed in **Table 2**. Each dose was 50 μ l administered to mice via IM on days 0 and 28, according to the respective dose listed in Table 2. Each formulation described throughout the Examples section herein comprises ALC-

25 0159 pegylated lipid at 1.8% molar percentage in the LNP formulation. Each group were composed of 10 Balb/c mice. The mice were bled at day 21 and 42 following administration.

As shown in **FIG. 1A**, a formulation comprising sitosterol and cholesterol increased immunogenicity of modRNA-HA 11-fold at 0.2 μ g dose. With an increased N/P ratio, titers were boosted 3-fold comparing to cholesterol-only control. With a low cationic lipid formulation, titers

30 were boosted 4-fold. The campesterol-containing formulation induced titers around 2.5-fold. All cholesterol analog formulations induced a measurable response at low dose. **FIG. 1B** and **FIG. 1C** show the results 2 weeks post dose 2. 90% neutralization titers shown in **FIG. 2A** and **FIG. 2B** follow a similar trend to 50% titers.

Table 2

Gr#	Formulation description				Dose (µg)
	ALC315 (mol%)	DSPC (mol%)	Sterol (mol%)	N/P ratio	
1	n/a Saline control	n/a	n/a	n/a	n/a
2	47.5	10	Cholesterol 40.7 (benchmark)	6	0.2
3					0.05
4	47.5	10	Campesterol 40.7	6	0.2
5					0.05
6	47.5	10	Sito 24.4/Chol 16.3 (sito:chol=6:4)	6	0.2
7					0.05
8	47.5	10	Sito 24.4/Chol 16.3 (sito:chol=6:4)	10	0.2
9					0.05
10	33.5	17.5	Sito 28.3/Chol 18.9, low cat 3 (sito:chol=6:4)	10	0.2
11					0.05

EXAMPLE 3: In vitro expression of hemagglutinin (HA) from LNPs formulated with alternative sterols.

The objective of this Example was to assess *in vitro* expression (IVE) of hemagglutinin (HA) from lipid nanoparticles (LNPs) formulated with alternative cholesterol and compare this *in vitro* expression performance to LNPs formulated with novel cholesterol and/or N/P ratios. This Example helped to inform whether or not changing certain cholesterol, and/or N/P ratios, can alter the *in vitro* expression of HA from an unmodified bi-cistronic saRNA encoding HA and NA of H1N1 A/Wisconsin, in HeLa cells. An exemplary bicistronic saRNA encoding HA and NA

include the sequence set forth in SEQ ID NO: 9. Another exemplary bicistronic saRNA includes the the sequence set forth in SEQ ID NO: 10. See, for example, International patent application PCT/IB2023/057034, published as WO2024/013625, entitled, “Self-amplifying rna encoding an influenza virus antigen,” (Pfizer Inc.) filed on July 7, 2023, which is incorporated by reference

5 herein in its entirety and describes saRNA molecules and bicistronic saRNA.

Method: An 11-point, 2-fold dilution of LNPs in HeLa cell cultures was performed. Geometric mean fluorescence intensity (GMFI) of HA positive cells, the percentage of total HA positive cells and cell viability was measured. HeLa cells were plated at a density of 2.0e5 cells per well in a 12-well plate. The LPNs tested are provided in **Table 3**. Each LNP was tested in triplicate

10 and measurements were made 22 hours post LNP-addition.

Table 3

LNP #	Sterol	N/P ratio
1	Cholesterol (benchmark)	6
2	beta-Sitosterol/Cholesterol (6:4)	6
3	Campesterol	6
4	Stigmasterol/Cholesterol (1:1)	6
5	Stigmasterol/Cholesterol (1:1)	10

The sterols tested in LNP 2 through 5 increased the percentage of cells expressing the antigen of interest (HA) as shown in **FIG. 3** (HeLa IVE). The EC₅₀s for LNP 2 through 5 were

15 significantly lower than the EC₅₀ of the benchmark LNP comprising cholesterol (LNP 1) (**Table 4**). The *in vitro* activity (IVA) of the cells treated with LNPs 2 through 5 was significantly higher than the activity of the cells treated with the benchmark LNP comprising cholesterol (LNP 1).

Table 4

LNP ID #	N/P	Sample	EC ₅₀	R ² (EC ₅₀)	<i>In vitro</i> activity	R ² (IVA)
1	6	Cholesterol (benchmark)	1418	0.9339	3.6	0.9972
2	6	beta-Sitosterol / Cholesterol (6:4)	2.3	0.9865	2016.0	0.9926
3	6	Campesterol	4.6	0.9854	784.2	0.9961
4	6	Stigmasterol / Cholesterol (1:1)	5.5	0.9757	844.9	0.9932
5	10	Stigmasterol / Cholesterol (1:1)	7.8	0.9933	665.1	0.9962

Note: EC₅₀ values are approximations as a result of force fitting curves due to a lack of complete dilution curves.

Optimization of LNPs 2 through 5 using the alternative sterols increased the GMFI for HA staining for all of the sterols tested as compared to the GMFI for HA staining when the cells were treated with the benchmark LNP comprising cholesterol (LNP 1) (**FIG. 4**). Cell viability was measured, and with increasing RNA dose (DP input as ng/well), the cell viability decreased, as measured as percent live cells, in those wells using LNPs 2 through 5, as compared to the percent live cells in wells using the RNA formulated in the benchmark cholesterol LNP (LNP 1) (**FIG. 5**).

When cell viability as measured as percentage of live cells was compared to the percentage of cells that stained positive to HA, the LNP comprising beta-sitosterol/cholesterol in a 6:4 ratio exhibited the best relative percentage of antigen positive cells as compared to viability (**FIG. 6**).

These data demonstrate that changing the sterol composition of LNPs encapsulating bicistronic saRNA led to an increase in the percentage of cells expressing the target antigen of interest, HA. This was observed for all alternative LNPs tested. Both EC₅₀ and *in vitro* activity values improved with differential LNP compositions, with the b-Sitosterol/Cholesterol (6:4) LNP performing the best. GMFI from HA staining was higher for all LNPs containing alternative sterols, with the b-Sitosterol/Cholesterol (6:4) LNP performing the best. Cell viability was significantly lower for all LNPs containing alternative sterols, as compared to the benchmark LNP 1. beta-Sitosterol/Cholesterol (6:4) LNPs demonstrated the best relative ratio of live cells vs. % HA positive.

25 **EXAMPLE 4: In vitro expression of hemagglutinin (HA) from LNPs formulated with alternative sterols.**

The objective of this Example was to assess *in vitro* expression (IVE) of GFP (reporter) from lipid nanoparticles (LNPs) formulated with alternative sterols and/or N/P ratios. Each formulation described throughout the Examples section herein comprises ALC-0159 pegylated lipid at 1.8% molar percentage in the LNP formulation. Percentage of encapsulation efficiency (%EE, as used herein throughout), polydispersity index (PDI), and % of intact mRNA as measured by fragment analyzer (FA) are shown in **Table 5**.

Table 5

#	Formulation description	mol% Cationic Lipid	mol% Helper Lipid	mol% Sterol	N/P Ratio	Flow Rate	%EE	Size	PDI	FA %intact
1	Chol benchmark-NP6	ALC315 47.5	DSPC 10	Chol 40.7	6	30/10	97.2	66.2	0.109	85
2	Chol-NP10	ALC315 47.5	DSPC 10	Chol 40.7	10		98.2	74.9	0.077	85
3	sito/chol mix (6:4)-NP6	ALC315 47.5	DSPC 10	Sito 24.4/ Chol 16.3	6		94.6	95.8	0.039	86
4	sito/chol mix (6:4)-NP10	ALC315 47.5	DSPC 10	Sito 24.4/ Chol 16.3	10		94.4	95.0	0.012	84
5	sito/chol mix lc3-NP6	ALC315 33.5	DSPC 17.5	Sito 28.3/ Chol 18.9	6		98.9	104.2	0.036	84
6	sito/chol mix (6:4) lc3-NP10	ALC315 33.5	DSPC 17.5	Sito 28.3/ Chol 18.9	10		97.3	91.5	0.018	83

N/P10 LNPs (i.e., LNPs having an N:P ratio of 10 or 10:1) performed better than N/P6 LNPs (i.e., LNPs having an N:P ratio of 6) in IVE in HEK293T cells, with LC3 mix formulation showing the largest enhancement and cholesterol showing the slightest improvement. See FIG. 7A-7B.

The following is an exemplary method to assess the presence and determine the in-vitro expression of mRNA-encoded target protein. Human embryonic kidney (HEK293T) cells are seeded on one to two 12-well culture plates per assay instance and transfected with control and drug product (DP) test samples across two assay instances. After 21-24 hours, cells are harvested from the 12-well plates and transferred to 96-well assay plates. The cells are stained with fixable aqua viability dye before being permeabilized and fixed. After the fixative is washed from the cells, a fluorophore-conjugated HA antibody cocktail is added which binds to influenza HA antigens. The cells are then analyzed for HA expression via flow cytometry, which detects the fluorescent signal of the fluorophore conjugated to the strain-specific anti-HA antibodies. The in-vitro expression of the HA antigen is determined from the average percent of viable,

single cells bound with fluorophore-conjugated HA antibody. NP6 and NP10 LNPs with FluB/Austria mod-HA had similar DP analytics and IVE.

- In **Table 6**, various sterol mix ratios were tested. The LNPs encapsulated modified mRNA encoding GFP. Each formulation described throughout the Examples section herein comprises ALC-0159 pegylated lipid at 1.8% molar percentage in the LNP formulation. Decreasing sitosterol percentage in the sterol mixture appeared to result in slightly higher %EE and smaller LNP size. See also **FIG. 8A-8B**. Sito/chol 1:1 mixture showed slightly better EC50 than the other two ratios.

10 **Table 6**

#	Formulation description	mol% Cationic Lipid	mol% Helper Lipid	mol% Sterol	N/P Ratio	Flow Rate	%EE	Size	PDI	FA %intact
1	sito/chol mix (6:4) std-fast rate-NP10	ALC315 47.5	DSPC 10	Sito 24.4/ Chol 16.3	10	30/10	94.4	95.0	0.012	84
2	sito/chol mix (5:5) std-fast rate-NP10	ALC315 47.5	DSPC 10	Sito 20.4/ Chol 20.4	10	30/10	96.8	91.1	0.026	80
3	sito/chol mix (4:6) std-fast rate-NP10	ALC315 47.5	DSPC 10	Sito 16.3/ Chol 24.4	10	30/10	97.3	87.1	0.038	84

In other studies, cholesterol derivatives were observed to increase IVE (~5-10 times) of LNPs encapsulated with RMM72 NA/Wisconsin modRNA. See **Table 7**.

15 **Table 7**

Description	FA		DLS	EE	IVE	
	%Integrity	LMS	Size (nm)	%	IVE % Cells positive (125ng)	IVE EC50 (ng/well)
β-Sitosterol	88	3	152	56	89	14
Campesterol	87	3	94	87	87	23

Fucosterol (Delta 5-avenasterol)	90	3	144	80	94	15
Stigmastanol (sitostanol)	NT	NT	124	36	NT	NT
Stigmasterol	NT	NT	82	7	NT	NT
CholPC	NT	NT	110	17	NT	NT
Cholesterol	88	3	77	94	50	131

See also **Table 8** Table 1 for data showing IVE of various sterol combinations, wherein the LNP encapsulated RMM72 NA/Wisconsin modRNA. In some preferred embodiments, the sterol comprises Cholesterol : β -Sitosterol in a 4:6 ratio in the LNP.

5

Table 1

Description	FA		DLS	EE%	IVE	
	%Integrity	LMS			%Positive @ 125ng	IVE EC50 (ng/well)
Cholesterol : β -Sitosterol 0:1	90	2	150	45	93	11
Cholesterol : β -Sitosterol 1:9	87	4	176	45	95	8
Cholesterol : β -Sitosterol 2:8	89	2	159	47	85	32
Cholesterol : β -Sitosterol 3:7	88	3	116	76	83	28
Cholesterol : β -Sitosterol 4:6	92	1	91	89	94	8
Cholesterol : β -Sitosterol 10:0	91	2	91	86	83	63

10 LNP encapsulated HA/California modRNA comprising Cholesterol : β -Sitosterol in a 4:6 ratio showed the excellent combination of integrity, EE and IVE compared to other formulations. See **Table 9**, wherein the Lipid ratio tested was 47.5: 40.7:10:1.8 (ALC-0315: Cholesterol/cholesterol analog: DSPC: ALC-0159); N/P = 6; LNP Matrix had 10mM Tris, 300 mM Sucrose, pH 7.4. LNP encapsulated HA/California modRNA using Cholesterol : β -Sitosterol ratio of 4:6 showed a preferred embodiment of integrity, EE and IVE compared to other tested

15 formulations.

Table 9

Sterol type	% Integrity	LMS NMT	Size by DLS, Z-Ave(nm)	PDI	EE %	%Positive @ 125ng	IVE EC50 (ng/well)
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β -Sitosterol	79	3%	165	0.11	26	81.3	4
Cholesterol : β -Sitosterol 1:9	60	3%	166	0.08	30	84.4	3
Cholesterol : β -Sitosterol 2:8	83	3%	137	0.08	54	82.8	4
Cholesterol : β -Sitosterol 3:7	79	3%	108	0.08	86	77.5	6
Cholesterol : β -Sitosterol 4:6	85	3%	106	0.07	86	85.1	4
Cholesterol : β -Sitosterol 5:5	85	3%	104	0.1	90	79.8	6
Cholesterol : β -Sitosterol 6:4	80	3%	107	0.12	75	81.1	6
Cholesterol : β -Sitosterol 7:3	84	3%	99	0.11	88	79.1	5
Campesterol (batch 1)	86	3%	106	0.07	87	72.6	7
Campesterol (batch 2)	76	3%	111	0.13	80	81.2	4
Fucoesterol	80	3%	157	0.1	79	86.3	3
LNP control (pure cholesterol)	84	3%	94	0.17	94	84.7	5

LNP encapsulated GFP modRNA comprising Cholesterol : β -Sitosterol of 4:6 in 293T and HeLa cells were tested and EC50 observed. See Table 10, wherein Lipid ratio tested was

5 47.5: 40.7:10:1.8 (ALC-0315: Cholesterol/cholesterol analog: DSPC: ALC-0159); N/P = 6; LNP Matrix had 10mM Tris, 300 mM Sucrose, pH 7.4. LNP encapsulated modGFP using Cholesterol : β -Sitosterol of 4:6 showed a preferred embodiment of about 10 times improvement of EC50 as compared to, for example, the cholesterol formulation.

Table 10

Description	mol% Sterol	%EE	%FA	Z-Ave (d.nm)	PDI	EC50 in 293T	EC50 in Hela
Cholesterol	Chol 40.7	94	88	77	0.054	6.1	9.1
Campesterol	Cam 40.7	59	88	97	0.033	1.4	1.4
β-Sitosterol	Sito 40.7	49	87	154	0.07	1.5	1.9
Cholesterol: β-Sitosterol = 0.4:0.6	Chol 16.3 /Sito 24.4	76	88	96	0.069	0.63	0.67

EXAMPLE 5: LNP-encapsulated modified mRNA encoding GFP.

5 Various sterol mix ratios were tested. The LNPs encapsulated modified mRNA encoding GFP. mRNA-LNPs containing cholesterol analogues were formulated by combining an mRNA containing aqueous phase and a lipid containing organic phase using methods known in the art. The organic phase was prepared by solubilizing a mixture of ionizable lipid, DSPC, PEG-lipid, 10 cholesterol and cholesterol analogs at various ratios in ethanol. The organic phase and aqueous phase were mixed at a flow rate ratio of 3:1 by syringe pumps. The resulted solution was dialyzed against 10 mM Tris buffer (pH ~7.4-7.6). Post-dialysis solution was concentrated and spiked with cryo-protectant to a final mRNA-LNP solution with RNA concentration as described herein (mg/ml). The formulations described in this Example comprise ALC-0159 15 PEGylated lipid at 1.8% molar percentage in the LNP formulations. Results are shown in Table 11. Decreasing sitosterol percentage in the sterol mixture appeared to result in slightly higher %EE and smaller LNP size. Sito/chol 1:1 mixture showed slightly better EC50 than the other two ratios in human embryonic kidney (HEK293T) cells.

Table 11

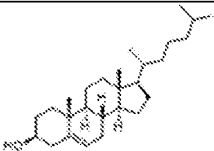
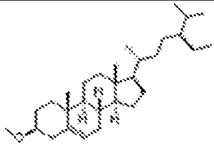
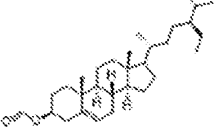
#	Formulation description	mol% Cationic Lipid	mol% Helper Lipid	mol% Sterol	N/P	%EE	Size	PDI	FA %intact
1		ALC315 47.5	DSPC 10	Sito 24.4/ Chol 16.3	10	94.4	95.0	0.012	84

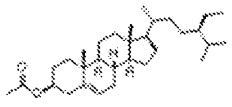
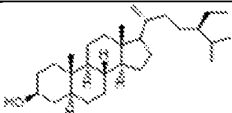
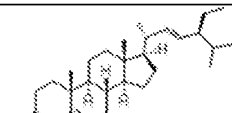
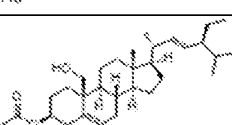
	sito/chol mix (6:4) std-fast rate- NP10								
2	sito/chol mix (5:5) std-fast rate- NP10	ALC315 47.5	DSPC 10	Sito 20.4/ Chol 20.4	10	96.8	91.1	0.026	80
3	sito/chol mix (4:6) std-fast rate- NP10	ALC315 47.5	DSPC 10	Sito 16.3/ Chol 24.4	10	97.3	87.1	0.038	84

EXAMPLE 6: Assessment on the performance of mRNA-LNP containing various sterols

The LNPs encapsulating modified mRNA encoding GFP were tested and results are set forth in **Table 12**. Each formulation described throughout this Example comprises ALC-315 at 5 47.5 mol%, DSPC at 10 mol% and ALC-0159 PEGylated lipid at 1.8% molar percentage (mol%) in the LNP formulation. The sterol component is a mixture of cholesterol and cholesterol analogues listed in the **Table 12** below at a 1:1 molar ratio.

Table 12

#	Sterol structures	Sterol compound name/ID	%EE	Size (nm)	PDI	EC50 (ng)
1		Cholesterol	98	64.58	0.082	10.3
2		SN792	89	78.37	0.032	2.977
3		SN793	74	80.70	0.011	3.702

4		CL-309557	86	83.23	0.018	2.26
5		Sitostanol	98	105.1	0.043	0.41
6		Stigmasterol (PNU-0199634)	95	93.04	0.031	0.66
7		E-429	84	76.07	0.036	18.12

EXAMPLE 7: Design of experiments (DOE) focusing on beta-sitosterol-containing formulations

5 The objective of the design of this experiment (DOE) was to identify the best sitosterol-containing LNP formulation composition and process for enhanced modRNA-LNP efficacy. This DOE allowed for efficient screening and examination of main effects, interactions, and possible second-order effects. The DOE was carried out using JMP 13 software. Duplicates were used in DOE1 (number of blocks=2). This design included 3 three-level quantitative factors (cat%,
10 phos%, and N/P ratio) and 1 two-level qualitative factor (cholesterol or sitosterol/cholesterol mix). The molar percentage of PEGylated lipid ALC-159 was kept consistent at 1.8 mol% throughout the DOE. The experiment input and output are set forth in the **Table 13** below.

Table 13

Experiment input				
Factors	Levels			Benchmark
Cat%	30	40	50	47.5
Phos%	10	15	20	10
Chol	Cholesterol		Mix Beta-Sitosterol/Cholesterol (1:1)	Cholesterol
N/P ratio	6	10	14	6

15

Table 14

Experiment output		
Critical quality attribute	Range	Analytical method
Size (nm)	50-150	DLS
PDI	0-0.3	DLS
EC50	0-500, minimize	IVE
MFI	0-100,000 maximize	IVE
%EE	70-100, maximize	Ribogreen assay

A total of 15 formulation candidates (Candidates A01-A14 and benchmark) were formulated and evaluated using the analytical methods listed. The detailed composition and critical attributes are listed in the **Table 15** below.

5

Table 15

Code	Sterol	Molar composition (%)				N/P ratio	Size (d.nm)	PDI	%EE	FA	IVE test1-96w		IVE test2-384w	
		ALC-315	DSPC	Sterol	PEG						EC50 (ng) HEK293T	MFI HEK293T @12.5 ng	EC50 (ng) HEK293T	MFI HEK293T @3.125 ng
Bench mark	Chol	47.5	10	40.7	1.8	6	67.66	0.096	97	88	15.66	3660	4.05	3481
A-01	Mix	30	10	58.2	1.8	6	92.56	0.029	99	88	2.44	15963	0.43	10406

A-02	Mix	30	20	48.2	1.8	10	90.99	0.053	98	89	1.08	37002	0.18	25757
A-03	Chol	30	15	53.2	1.8	14	79.09	0.072	99	85	6.36	12810	0.98	6506
A-04	Cho	30	10	58.2	1.8	14	84.42	0.051	99	86	5.56	13240	0.75	7670
A-05	Chol	30	20	48.2	1.8	6	74.46	0.059	98	88	12.21	5253	2.38	3735
A-06	Mix	40	20	38.2	1.8	14	92.23	0.060	94	88	1.18	25174	0.22	18035
A-07	Chol	40	15	43.2	1.8	10	69.10	0.068	98	89	10.49	4703	2.31	3889
A-08	Chol	40	10	48.2	1.8	6	63.66	0.049	98	88	20.4	3553	4.52	3686
A-09	Mix	40	15	43.2	1.8	10	90.84	0.041	97	89	1.16	30589	0.21	22077
A-10	Chol	50	20	28.2	1.8	14	68.29	0.053	95	88	100.8	3057	22.98	3598
A-11	Mix	50	15	33.2	1.8	6	95.38	0.040	92	87	1.24	30943	0.25	17898
A-12	Chol	50	10	38.2	1.8	10	67.77	0.087	97	89	17.69	3470	3.14	3520
A-13	Mix	50	10	38.2	1.8	14	85.55	0.039	96	88	0.79	21712	0.18	23519
A-14	Mix	50	20	28.2	1.8	6	97.91	0.048	83	87	0.88	34213	0.26	18473

Based on the DOE output, a predicted best formulation of

ALC315/DSPC/Sitosterol/Cholesterol/ALC159 at a molar percentage of 30/10/29.1/29.1/1.8 with a N/P ratio of 6 (formulation A-01 in **Table 15**) was achieved. Top performing sitosterol

5 LNP formulations including A-01, A-02 and A-11 were applied to modRSV formulation. The modRNA RSV formulation used was RSV F 847A which is described in International patent application PCT/IB2023/060798, filed on October 26, 2023, published as WO2024089633, which is incorporated by reference herein in its entirety and describes RSV prefusion (F) modRNA and saRNA molecules encoding RSV prefusion mutant proteins.

10 mRNA-LNPs containing cholesterol analogues were formulated by combining an mRNA containing aqueous phase and a lipid containing organic phase using methods known in the art. The organic phase was prepared by solubilizing a mixture of ionizable lipid, DSPC, PEG-lipid, cholesterol and cholesterol analogs at various ratios in ethanol. The organic phase and aqueous phase were mixed at a flow rate ratio of 3:1 by syringe pumps. The resulted solution
15 was dialyzed against 10 mM Tris buffer (pH ~7.4-7.6). Post-dialysis solution was concentrated

and spiked with cryo-protectant to a final mRNA-LNP solution with RNA concentration as described herein (mg/ml).

RSV F 847A modRNA was encapsulated into sitosterol-incorporated formulations and the in vitro expression was evaluated in HEK293T cells. As shown in **Table 16** below, over 30-fold enhancement in the total antigen expression and over 100-fold improvement in the prefusion-specific protein expression were observed in the sitosterol-containing formulations compared to the cholesterol benchmark. The four sitosterol LNP formulations exhibited comparable EC50 values for both total and preF-specific protein expression.

10 **Table 16**

Code	Sterol ratio	Molar composition (mol%)			N/P ratio	Size (d.nm)	PDI	%EE	IVE (L4-6): Total EC50 (ng)	IVE (RSV mAb-1): preF EC50 (ng)
		ALC-315	DSPC	Sterol						
Benchmark	Chol alone	47.5	10	40.7	6	66.95	0.060	98	35.40	>200
A-01	1:1 Mix	30	10	58.2	6	101.60	0.038	99	1.11	2.60
A-02	1:1 Mix	30	20	48.2	10	96.71	0.037	99	1.24	2.27
A-11	1:1 Mix	50	15	33.2	6	97.93	0.028	90	1.39	2.18
Sito/chol 6:4	6:4 Mix	47.5	10	40.7	6	94.85	0.013	94	1.36	2.13

EXAMPLE 8: Assessment on the performance of mRNA-LNP containing various sterols using RSV modRNA

15

The LNPs in this Example encapsulated modified mRNA encoding RSV F 847A modRNA (previously described in Example 6 above). Each formulation described throughout this Example comprises ALC-315, DSPC , sterol and ALC-0159 PEGylated lipidin the LNP formulation. The sterol component is a mixture of cholesterol and cholesterol analogues listed in **Table 17** at a 6:4 or 1:1 molar ratio. All sterol-candidate containing formulations showed favorable critical attributes except for SN792-formulation showing relatively low % encapsulation of the RSV preF modRNA. The in vitro expression of antigen-of-interest was measured with two antibodies in HEK293T cells, L4-6 antibody detecting total F protein and in-house mAb-1 antibody detecting prefusion-specific protein specifically. As shown in **Table 17**, all sterol candidate containing LNPs showed better or comparable in vitro expression of total F protein compared to the cholesterol benchmark formulation. Notably, the in vitro expression of the preF protein was significantly enhanced throughout the candidate panel, demonstrated by a 5.7-60.5 fold decrease in the EC50 measured with mAb-1 antibody.

20

25

Table 17

Gp #	Test articles	EE (%)	Size (nm)	PDI	FA (%)	IVE EC50 L4-6 (ng/well)	IVE EC50 mAb-1 (ng/well)
1	Cholesterol	97	72	0.06	91	16	127
2	Campesterol	94	99	0.02	91	2.7	4.2
3	Sito/Chol=6:4 [Labeled as Sito-1]	93	97	0.02	91	1.7	3.0
4	Sito/Chol=6:4 [Labeled as Sito-2]	95	93	0.04	91	2.0	2.2
5	Sito/Chol=6:4 [Labeled as Sito-3]	99	103	0.06	91	1.1	2.1
6	Sito/Chol=1:1 [Labeled as Sito-4]	98	127	0.04	91	2.2	3.0
7927	Sito/Chol=1:1 [Labeled as Sito-5]	99	106	0.1	91	3.0	2.3
8	Sito/Chol=1:1 [Labeled as Sito-6]	89	99	0.04	87	2.4	3.2
9	Stigmasterol/Chol=1:1	95	97	0.02	90	2.0	3.6
10	Sitostanol/Chol=1:1	97	98	0.03	90	3.3	5.3
11	SN792/Chol=1:1	66	83	0.03	89	15	22

5 EXAMPLE 9: LNPs encapsulating modified mRNA encoding preF RSV.

RSV modRNA was encapsulated in the LNP formulations using cholesterol analogs described in **Table 18** below (mRNA-LNPs). mRNA-LNPs containing cholesterol analogues were formulated by combining an mRNA containing aqueous phase and a lipid containing organic phase using methods known in the art. The organic phase was prepared by solubilizing

- a mixture of ionizable cationic lipid, DSPC, PEG-lipid, cholesterol and cholesterol analogs at various ratios in ethanol. The organic phase and aqueous phase were mixed at a flow rate ratio of 3:1 by syringe pumps. The resulted solution was dialyzed against 10 mM Tris buffer (pH ~7.4-7.6). Post-dialysis solution was concentrated and spiked with cryo-protectant to a final
- 5 mRNA-LNP solution with RNA concentration as described herein (mg/ml).

All the formulations showed favorable physical properties including particle size/PDI, integrity% and EE%. The formulation with Cholesterol: β -Sitosterol = 0.4:0.6, N:P = 6:1 showed highest protein expression at 4 h and 24 h compared to other formulations. The %positive cells at 500 ng/well was also same for the formulations with Cholesterol: β -Sitosterol = 0.4:0.6, N:P =

10 6:1 at 4 h and with cholesterol benchmark formulation at 22 h. It demonstrated that the formulation with Cholesterol: β -Sitosterol = 0.4:0.6, N:P = 6:1 can have a faster expression of mRNA and subsequently may lead to higher immunogenicity in vivo.

Table 18

Formulation Description	Formulation component molar ratio%	Size (nm)	PDI	Integrity %	LMP, NMT %	EE %	RSVmAb staining (pre-F trimer)				L4-6 staining (pre-F + post-F)			
							EC50 (ng/well)		%Positive cells (500 ng/well)		EC50 (ng/well)		%Positive cells (125 ng/well)	
							4 h	22 h	4 h	22 h	4 h	22 h	4 h	22 h
Cholesterol	ALC-0315/ Cholesterol/ DSPC/ ALC-0159 = 47.5: 40.7: 10: 1.8	71	0.09	91	3	96	NA*	105	35	70	68	44	56	57
Campesterol	ALC-0315/ Campesterol/ DSPC/ ALC-0159 = 47.5: 40.7: 10: 1.8	94	0.05	89	3	93	NA*	144	22	67	91	55	50	55
Cholesterol: β -Sitosterol = 0.4:0.6, N:P = 6:1	ALC-0315/ β -Sitosterol/ cholesterol/ DSPC/ ALC-0159 = 47.5: 24.4: 16.3: 10: 1.8	103	0.07	90	3	93	123	26	69	88	18	12	83	75
Cholesterol: β -Sitosterol = 0.4:0.6, N:P = 10:1	ALC-0315/ β -Sitosterol/ cholesterol/	95	0.08	91	3	95	191	36	74	87	31	16	68	68

	DSPC/ ALC-0159 = 47.5: 24.4: 16.3: 10: 1.8													
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Example 10: Assessment stability of mRNA-LNP containing various sterols at 5°C

The LNPs in this Example encapsulated modified mRNA encoding RSV F 847A modRNA (previously described in Examples 7 and 8 above). FIG. 9A-9F summarize the critical attributes of these LNPs (see Table 17 formulations) over 6-month storage at 5 °C. Sterol-containing LNPs were mostly colloiddally stable except for Sito-4, Sito-5 and stigmasterol formulations which showed a considerable increase in both size and PDI. The decrease rate of % encapsulation and integrity of the mRNA was comparable among sterol formulations and the cholesterol benchmark formulations. SN792-LNP was not further characterized due to its low encapsulation. For in vitro performance, sterol-containing LNPs continued to show significantly enhanced expression of both total F protein (measured by L4-6) and preF-specific protein (measured by mAb-1) after 6-month refrigeration, outforming cholesterol benchmark formulation that exhibited the fastest increase in the EC50 values in IVE with both antibodies. This result demonstrated improved 5 °C stability for various sterol incorporated LNPs over cholesterol formulation.

The following paragraphs describe additional aspects of the disclosure:

1. An immunogenic composition comprising at least one ribonucleic acid (RNA) polynucleotide having an open reading frame encoding at least one antigenic polypeptide or an immunogenic fragment thereof, formulated in a lipid nanoparticle comprising one or more structural lipids and/or analogs thereof. In one aspect the structural lipids comprise a mixture of cholesterol and a cholesterol analog.
2. The immunogenic composition of paragraph 1, wherein the RNA further comprises a 5' cap analog.
3. The immunogenic composition of paragraph 2, wherein the 5' cap analog comprises $m_2^{7,3'-O}Gppp(m_1^{2'-O})ApG$.
4. The immunogenic composition of paragraph 1, wherein the RNA further comprises a modified nucleotide.
5. The immunogenic composition of paragraph 4, wherein the modified nucleotide comprises N1-Methylpseudouridine-5'-triphosphate ($m_1\psi TP$).
6. The immunogenic composition of paragraph 1, wherein the antigenic polypeptide is encoded by an open reading frame (ORF) of a gene of interest.

7. The immunogenic composition according to paragraph 6, wherein the open reading frame is codon-optimized.
8. The immunogenic composition of paragraph 1, wherein the immunogenic composition further comprises a cationic lipid.
- 5 9. The immunogenic composition of paragraph 1, wherein the immunogenic composition comprises a lipid nanoparticle encompassing the mRNA molecule.
- 10 10. The immunogenic composition of paragraph 1, wherein the immunogenic composition comprises a lipid nanoparticle encompassing at least one ribonucleic acid (RNA) polynucleotide having an open reading frame derived from a gene of interest encoding at least one antigenic polypeptide.
11. The immunogenic composition of paragraph 10, wherein the lipid nanoparticle size is at least 40 nm.
12. The immunogenic composition of paragraph 10, wherein the lipid nanoparticle size is at most 180 nm.
- 15 13. The immunogenic composition of paragraph 10, wherein at least 80% of the total RNA in the immunogenic composition is encapsulated.
14. The immunogenic composition of paragraph 1, wherein the immunogenic composition comprises ALC-0315 (4-hydroxybutyl)azanediy)bis(hexane-6,1-diyl)bis(2-hexyldecanoate).
- 20 15. The immunogenic composition of paragraph 1, wherein the immunogenic composition comprises ALC-0159 (2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide).
16. The immunogenic composition of paragraph 1, wherein the immunogenic composition comprises 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC).
- 25 17. The immunogenic composition of paragraph 1, wherein the immunogenic composition comprises a structural lipid.
18. The immunogenic composition of paragraph 17, wherein the structural lipid is a sitosterol, a stigmasterol, a campesterol, a sitostanol, a campestanol, a brassicasterol, a fucosterol, beta-sitosterol, stigmastanol, beta-sitostanol, ergosterol, lupeol, cycloartenol, Δ^5 -avenaserol, Δ^7 -avenaserol or a Δ^7 -stigmasterol, including analogs, salts or esters thereof, alone or in combination.
- 30 19. The immunogenic composition of paragraph 18, wherein the the LNP comprises a structural lipid selected from the group consisting of: cholesterol, fecosterol, sitosterol, β -sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, ursolic acid, alpha-tocopherol, and mixtures thereof.
- 35 20. The immunogenic composition according to any one of any of the preceding paragraphs, wherein the LNP comprises an N:P ratio of from about 2:1 to about 30:1.

21. The immunogenic composition of paragraph 19, wherein the immunogenic composition comprises a combination of cholesterol and any one of beta-sitosterol or campesterol.
22. The immunogenic composition of paragraph 20, wherein the immunogenic composition comprises a combination of structural lipids cholesterol and beta-sitosterol.
23. The immunogenic composition of paragraph 20, wherein the immunogenic composition comprises Cholesterol: β -Sitosterol = 0.4:0.6.
24. The immunogenic composition according to any one of paragraphs 1-22, wherein the LNP comprises β -sitosterol and cholesterol having an N:P ratio of at least 5.
25. The immunogenic composition according to any one of paragraphs 1-22, wherein the LNP comprises β -sitosterol and cholesterol having an N:P ratio of at least 6.
26. The immunogenic composition according to any one of paragraphs 1-22, wherein the LNP comprises β -sitosterol and cholesterol having an N:P ratio of 10.
27. The immunogenic composition according to any one of paragraphs 1-26, wherein the LNP comprises β -sitosterol and cholesterol in a ratio of 6:4 (w/w).
28. The immunogenic composition according to any one of paragraphs 1-26, wherein the LNP comprises β -sitosterol and cholesterol in a ratio of 5:5 (w/w).
29. The immunogenic composition according to any one of paragraphs 1-26, wherein the LNP comprises β -sitosterol and cholesterol in a ratio of 4:6 (w/w).
30. The immunogenic composition according to any one of paragraphs 1-29, wherein the lipid nanoparticle comprises about 30 mol % to about 60 mol % one or more ionizable lipids, about 0 mol % to about 30 mol % one or more non-cationic helper lipids, about 18.5 mol % to about 48.5 mol % structural component, and about 0 mol % to about 10 mol % one or more PG-lipids.
31. The immunogenic composition of paragraph 30, wherein the immunogenic composition comprises 0.9-1.85 mg/mL ALC-0315; 0.11-0.24 mg/mL ALC-0159; 0.18 – 0.41 mg/mL neutral lipid such as DSPC; and 0.36 – 0.78 mg/mL structural lipid such as cholesterol or combination of cholesterol and beta-sitosterol.
32. The immunogenic composition of paragraph 1, wherein the immunogenic composition comprises Tris.
33. The immunogenic composition of paragraph 1, wherein the immunogenic composition comprises sucrose.
34. The immunogenic composition of paragraph 1, wherein the immunogenic composition does not further comprise sodium chloride.
35. The immunogenic composition of paragraph 1, wherein the immunogenic composition comprises 10 mM Tris.
36. The immunogenic composition of paragraph 1, wherein the immunogenic composition comprises 300 mM sucrose.

37. The immunogenic composition of paragraph 1, wherein the immunogenic composition has a pH 7.4.
38. The immunogenic composition of paragraph 1, wherein the immunogenic composition has less than or equal to 12.5 EU/mL of bacterial endotoxins.
- 5 39. The immunogenic composition of paragraph 1, wherein the RNA polynucleotide comprises a 5' cap, 5' UTR, 3' UTR, histone stem-loop and poly-A tail.
40. An immunogenic composition comprising: (i) a first ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a first antigen polypeptide or an immunogenic fragment thereof, and (ii) a second RNA polynucleotide having an open reading
10 frame encoding a second antigen polypeptide or an immunogenic fragment thereof, wherein the first and second RNA polynucleotides are formulated in a lipid nanoparticle (LNP).
41. The immunogenic composition of paragraph 40, wherein the first and second RNA polynucleotides are formulated in a single lipid nanoparticle.
42. The immunogenic composition of any preceding paragraph further comprising:
15 (iii) a third antigen comprising at least one antigenic polypeptide or an immunogenic fragment thereof, wherein the third antigen is from a different virus, bacteria or strain of the virus or bacteria to both the first and second antigens.
43. The immunogenic composition of paragraph 42, wherein the first, second and third RNA polynucleotides are formulated in a lipid nanoparticle.
- 20 44. The immunogenic composition of paragraph 43, wherein the first, second and third RNA polynucleotides are formulated in a single lipid nanoparticle.
45. The immunogenic composition of any preceding paragraph further comprising:
(iv) a fourth RNA polynucleotide having an open reading frame encoding a fourth antigen, said antigen comprising at least one antigenic polypeptide or an immunogenic fragment thereof,
25 wherein the fourth antigen is from a different virus, bacteria or strain of the virus or bacteria to the first, second and third antigens.
46. The immunogenic composition of paragraph 45, wherein the first, second, third, and fourth RNA polynucleotides are formulated in a lipid nanoparticle.
47. The immunogenic composition of paragraph 46, wherein the first, second, third,
30 and fourth RNA polynucleotides are formulated in a single lipid nanoparticle.
48. The immunogenic composition of any preceding paragraph further comprising:
(v) a fifth RNA polynucleotide having an open reading frame encoding a fifth antigen, said antigen comprising at least one antigenic polypeptide or an immunogenic fragment thereof,
wherein the fifth antigen is from a different virus, bacteria or strain of the virus or bacteria to the
35 first, second, third, and fourth antigens.
49. The immunogenic composition of paragraph 48, wherein the first, second, third, fourth, and fifth RNA polynucleotides are formulated in a lipid nanoparticle.

50. The immunogenic composition of paragraph 49, wherein the first, second, third, fourth, and fifth RNA polynucleotides are formulated in a single lipid nanoparticle.

51. The immunogenic composition of any preceding paragraph further comprising:
(vi) a sixth RNA polynucleotide having an open reading frame encoding a sixth antigen, said antigen comprising at least one antigenic polypeptide or an immunogenic fragment thereof, wherein the sixth antigen is from a different virus, bacteria or strain of the virus or bacteria to the first, second, third, fourth, and fifth antigens.

52. The immunogenic composition of paragraph 51, wherein the first, second, third, fourth, and fifth RNA polynucleotides are formulated in a lipid nanoparticle.

53. The immunogenic composition of paragraph 52, wherein the first, second, third, fourth, and fifth RNA polynucleotides are formulated in a single lipid nanoparticle.

54. The immunogenic composition of any preceding paragraph further comprising:
(vii) a seventh RNA polynucleotide having an open reading frame encoding a seventh antigen, said antigen comprising at least one antigenic polypeptide or an immunogenic fragment thereof, wherein the seventh antigen is from a different virus, bacteria or strain of the virus or bacteria to the first, second, third, fourth, fifth, and sixth antigens.

55. The immunogenic composition of paragraph 54, wherein the first, second, third, fourth, fifth, sixth and seventh RNA polynucleotides are formulated in a lipid nanoparticle.

56. The immunogenic composition of paragraph 55, wherein the first, second, third, fourth, fifth, sixth and seventh RNA polynucleotides are formulated in a single lipid nanoparticle.

57. The immunogenic composition of any preceding paragraph further comprising:
(viii) an eighth RNA polynucleotide having an open reading frame encoding an eighth antigen, said antigen comprising at least one antigenic polypeptide or an immunogenic fragment thereof, wherein the eighth antigen is from a different virus, bacteria or strain of the virus or bacteria to the first, second, third, fourth, fifth, sixth and seventh antigens.

58. The immunogenic composition of any one of the preceding paragraphs, wherein the ratio of the first RNA polynucleotide to the second RNA polynucleotide is 1: greater than 1.

59. The immunogenic composition of paragraph 58, wherein the RNA polynucleotides are not present in equal ratios.

60. The immunogenic composition of any one of any of the preceding paragraphs, wherein the ratio of the first RNA polynucleotide to the second RNA polynucleotide is 1:2.

61. The immunogenic composition of any one of the preceding paragraphs, wherein the ratio of the first RNA polynucleotide to the second RNA polynucleotide is 1:4.

62. The immunogenic composition of any of the preceding paragraphs, wherein the first, second, third, fourth, fifth, sixth, seventh and eighth RNA polynucleotides are formulated in a lipid nanoparticle.

63. The immunogenic composition of paragraph 62, wherein the first, second, third, fourth, fifth, sixth, seventh and eighth RNA polynucleotides are formulated in a single lipid nanoparticle.

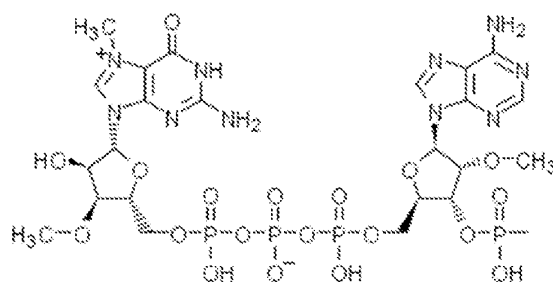
64. The immunogenic composition of any preceding paragraph, wherein the RNA polynucleotides are present in about equal ratios.

65. The immunogenic composition of any preceding paragraph, wherein each RNA polynucleotide comprises a modified nucleotide.

66. The immunogenic composition of paragraph 65, wherein the modified nucleotide is selected from the group consisting of pseudouridine, 1-methylpseudouridine, 2-thiouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methoxyuridine, and 2'-O-methyl uridine.

67. The immunogenic composition of any preceding paragraph, wherein each RNA polynucleotide comprises a 5' terminal cap, a 5' UTR, a 3'UTR, and a 3' polyadenylation tail.

68. The immunogenic composition of paragraph 67, wherein the 5' terminal cap



comprises:

69. The immunogenic composition of paragraph 67, wherein the 5' UTR comprises SEQ ID NO: 1.

70. The immunogenic composition of paragraph 67, wherein the 3' UTR comprises SEQ ID NO: 2.

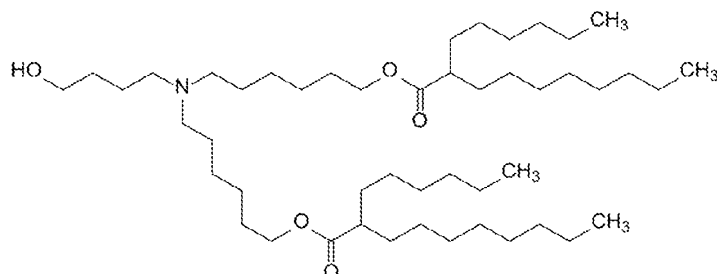
71. The immunogenic composition of paragraph 67, wherein the 3' polyadenylation tail comprises SEQ ID NO: 3.

72. The immunogenic composition of any preceding paragraph, wherein the RNA polynucleotide has an integrity greater than 85%.

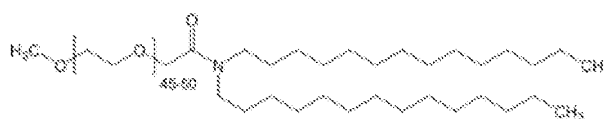
73. The immunogenic composition of any preceding paragraph, wherein the RNA polynucleotide has a purity of greater than 85%.

74. The immunogenic composition of any preceding paragraph, wherein the lipid nanoparticle comprises 20-60 mol % ionizable cationic lipid, 5-25 mol % neutral lipid, 25-55 mol % cholesterol, and 0.5-5 mol % PG-modified lipid.

75. The immunogenic composition of any preceding paragraph, wherein the cationic lipid comprises:



76. The immunogenic composition of any preceding paragraph, wherein the PEG-



5 modified lipid comprises:

77. The immunogenic composition of any preceding paragraph, wherein at least the first and second RNA polynucleotides are formulated in a single lipid nanoparticle.

78. The immunogenic composition of any preceding paragraph, wherein the first and second RNA polynucleotides are formulated in a single lipid nanoparticle.

10 79. The immunogenic composition of any preceding paragraph, wherein the first, second, and third RNA polynucleotides are formulated in a single lipid nanoparticle.

80. The immunogenic composition of any preceding paragraph, wherein the first, second, third, and fourth RNA polynucleotides are formulated in a single LNP.

15 81. The immunogenic composition of any one of paragraphs 40-80, wherein each of the RNA polynucleotides is formulated in a single LNP, wherein each single LNP encapsulates the RNA polynucleotide encoding one antigen.

82. The immunogenic composition of paragraph 76, wherein the first RNA polynucleotide is formulated in a first LNP; and the second RNA polynucleotide is formulated in a second LNP.

20 83. The immunogenic composition of paragraph 76, wherein the first RNA polynucleotide is formulated in a first LNP; the second RNA polynucleotide is formulated in a second LNP; and the third RNA polynucleotide is formulated in a third LNP.

84. The immunogenic composition of paragraph 76, wherein the first RNA polynucleotide is formulated in a first LNP; the second RNA polynucleotide is formulated in a second LNP; the third RNA polynucleotide is formulated in a third LNP; and the fourth RNA polynucleotide is formulated in a fourth LNP.

25 85. The immunogenic composition of paragraph 76, wherein the first RNA polynucleotide is formulated in a first LNP; the second RNA polynucleotide is formulated in a

second LNP; the third RNA polynucleotide is formulated in a third LNP; the fourth RNA polynucleotide is formulated in a fourth LNP; and the fifth RNA polynucleotide is formulated in a fifth LNP.

5 86. The immunogenic composition of paragraph 76, wherein the first RNA polynucleotide is formulated in a first LNP; the second RNA polynucleotide is formulated in a second LNP; the third RNA polynucleotide is formulated in a third LNP; the fourth RNA polynucleotide is formulated in a fourth LNP; the fifth RNA polynucleotide is formulated in a fifth LNP; and the sixth RNA polynucleotide is formulated in a sixth LNP.

10 87. The immunogenic composition of paragraph 76, wherein the first RNA polynucleotide is formulated in a first LNP; the second RNA polynucleotide is formulated in a second LNP; the third RNA polynucleotide is formulated in a third LNP; the fourth RNA polynucleotide is formulated in a fourth LNP; the fifth RNA polynucleotide is formulated in a fifth LNP; the sixth RNA polynucleotide is formulated in a sixth LNP; and the seventh RNA polynucleotide is formulated in a seventh LNP.

15 88. The immunogenic composition of paragraph 76, wherein the first RNA polynucleotide is formulated in a first LNP; the second RNA polynucleotide is formulated in a second LNP; the third RNA polynucleotide is formulated in a third LNP; the fourth RNA polynucleotide is formulated in a fourth LNP; the fifth RNA polynucleotide is formulated in a fifth LNP; the sixth RNA polynucleotide is formulated in a sixth LNP; the seventh RNA polynucleotide is formulated in a seventh LNP; and the eighth RNA polynucleotide is formulated in an eighth LNP.

 89. The immunogenic composition of any preceding paragraphs, for use in the eliciting an immune response against an antigen.

25 90. The immunogenic composition according to any one of paragraphs 1-89, wherein the percentage of intact mRNA encapsulated in the LNP is at least 80%.

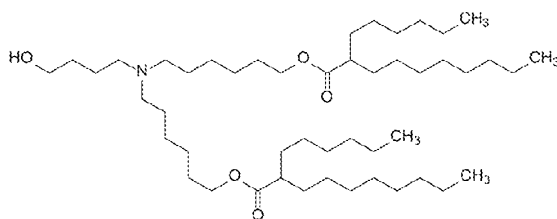
 91. The immunogenic composition according to any one of paragraphs 1-90, wherein the immunogenic composition is a vaccine.

30 All of the methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this disclosure have been described in terms of certain aspects, it will be apparent to those of skill in the art that variations may be applied to the methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the disclosure. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the disclosure as defined by the appended claims.

The contents of all cited references (including literature references, issued patents, published patent applications, and GENBANK® Accession numbers as cited throughout this application) recited in the application, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are hereby specifically and expressly
5 incorporated by reference. When definitions of terms in documents that are incorporated by reference herein conflict with those used herein, the definitions used herein govern.

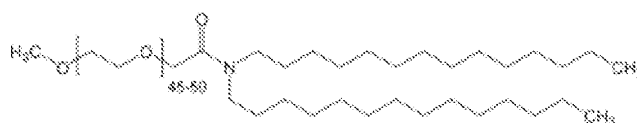
CLAIMS

1. A lipid nanoparticle (LNP) comprising
 - i. an ionizable cationic lipid;
 - ii. a neutral lipid;
 - iii. a mixture of cholesterol and a cholesterol analog, wherein the molar ratio between the cholesterol and the cholesterol analog in the mixture is 6:4, 1:1 or 4:6; and
 - iv. a polymer-conjugated lipid.
2. The LNP of claim 1, wherein the cholesterol analog is selected from sitosterol, stigmasterol, campesterol, sitostanol, campestanol, brassicasterol, fucosterol, β -sitosterol, stigmasterol, β -sitostanol, ergosterol, fecosterol, lupeol, cycloartenol, Δ^5 -avenaserol, Δ^7 -avenaserol or a Δ^7 -stigmasterol, tomatidine, ursolic acid or alpha-tocopherol, including analogs, salts or esters thereof.
3. The LNP of claim 2, wherein the cholesterol analog is β -sitosterol, stigmasterol or campesterol.
4. The LNP of claim 3, wherein the cholesterol analog is β -sitosterol.
5. The LNP of claim 4, wherein the ionizable cationic lipid is selected from N,N-dimethyl-2,3-dioleoyloxypropylamine (DODMA), N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dilinoleoyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), 2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA), (4-hydroxybutyl)azanediylbis(hexane-6,1-diyl)bis(2-hexyldecanoate) (ALC-0315), heptadecane-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy)hexyl) amino) octanoate (SM-102), or a mixture thereof.
6. The LNP of any preceding claim, wherein the ionizable cationic lipid is ALC-0315 (4-hydroxybutyl)azanediylbis(hexane-6,1-diyl)bis(2-hexyldecanoate) having the structure:



7. The LNP of any preceding claim, wherein the neutral lipid is a phospholipid.

8. The LNP of any preceding claim, wherein the LNP comprises a neutral lipid selected from 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoylphosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoylphosphatidylethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoylphosphatidylethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1-stearoyl-2-oleoylphosphatidylethanolamine (SOPE), and/or 1,2-dielaidoyl-sn-glycero-3-phosphoethanolamine (transDOPE).
9. The LNP of claim 8, wherein the phospholipid is 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC).
10. The LNP of any preceding claim, wherein the polymer-conjugated lipid is a pegylated lipid.
11. The LNP of claim 10, wherein the pegylated lipid is 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159) or a PEG dialkoxypolypropylcarba PEGylated diacylglycerol (PEG-DAG), 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG), PEGylated phosphatidylethanolamine (PEG-PE), PEG succinate diacylglycerol (PEG-S-DAG), 4-O-(2',3'-di(tetradecanoyloxy)propyl)-1-O-((O-methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG), PEG-ceramide, 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide (ALC-0159) or α -(3'-[(1,2-di[myristyloxy]propanoxy)carbonylamino]propyl)- ω -methoxy, polyoxyethylene (PEG-C-DMG).
12. The LNP of claim 11, wherein the pegylated lipid is ALC-0159 (2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide) having the structure:



25

13. The LNP of any preceding claim, wherein the LNP comprises about 20-60 mol % ionizable cationic lipid, about 5-25 mol % neutral lipid, about 18-60 mol % mixture of cholesterol and a cholesterol analog, and about 0.5-10 mol % polymer-conjugated lipid.
14. The LNP according to any one of claims 1-13, wherein the lipid nanoparticle comprises about 30 mol % to about 60 mol % ionizable cationic lipids, about 0 mol % to about 30 mol

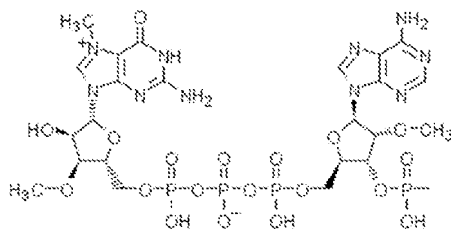
- % neutral lipids, about 18.5 mol % to about 48.5 mol % mixture of cholesterol and a cholesterol analog, and about 0 mol % to about 10 mol % polymer-conjugated lipid.
15. The LNP according to any of claims 1-14, wherein the LNP comprises 0.9-1.85 mg/mL ALC-0315; 0.11-0.24 mg/mL ALC-0159; 0.18 – 0.41 mg/mL neutral lipid such as DSPC; and 0.36 – 0.78 mg/mL mixture of cholesterol and a cholesterol analog.
16. The LNP of any of claims 13-15, wherein the LNP comprises a mol% ratio of ALC-0315:cholesterol and cholesterol analog mixture:DSPC:ALC-0159 from the group consisting of:
- a) 47.5:40.7:10:1.8;
- b) 30:58.2:10:1.8;
- c) 30:48.2:20:1.8; and
- d) 50:33.2:15:1.8.
17. The LNP of claim 16, wherein the LNP comprises a mol% ratio of ALC-0315:cholesterol:cholesterol analog:DSPC:ALC-0159 from the group consisting of:
- a) 47.5:16.3:24.4:10:1.8;
- b) 47.5:20.4:20.4:10:1.8;
- c) 47.5:24.4:16.3:10:1.8;
- d) 30:29.1:29.1:10:1.8;
- e) 30:24.1:24.1:20:1.8; and
- f) 50:16.6:16.6:15:1.8.
18. The LNP of any one of claims 13-17, wherein the cholesterol analog is beta-sitosterol.
19. The LNP according to any of claims 1-18, wherein the LNP size is at least 40 nm.
20. The LNP according to any of claims 1-19, wherein the LNP size is at most 180 nm.
21. An immunogenic composition comprising at least one ribonucleic acid (RNA) polynucleotide having an open reading frame (ORF) encoding at least one antigenic polypeptide or an immunogenic fragment thereof, formulated in a lipid nanoparticle (LNP) according to any of claims 1-20.
22. The immunogenic composition of claim 21, wherein the RNA is modRNA or saRNA.

23. The immunogenic composition of claims 21-22, wherein the RNA polynucleotide comprises a 5' cap, 5' UTR, 3' UTR, and poly-A tail.

24. The immunogenic composition of claim 23, wherein the 5' cap is a 5' cap analog.

25. The immunogenic composition of claim 24, wherein the 5' cap analog comprises $m_2^{7,3'}$ -

5 $^{\circ}\text{Gppp}(m_1^{2'-\text{O}})\text{ApG}$ or



26. The immunogenic composition according to any of claims 21 to 25, wherein the RNA further comprises a modified nucleotide.

27. The immunogenic composition of claim 26, wherein the modified nucleotide is selected from the group consisting of pseudouridine, 1-methylpseudouridine, 2-thiouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-1-methyl-pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methoxyuridine, 2'-O-methyl uridine and N1-Methylpseudouridine-5'-triphosphate ($m_1\psi\text{TP}$).

28. The immunogenic composition of claim 27, wherein the modified nucleotide comprises N1-Methylpseudouridine-5'-triphosphate ($m_1\psi\text{TP}$).

29. The immunogenic composition according to any of claims 21-28, wherein the open reading frame is codon-optimized.

30. The immunogenic composition of claim 21, wherein the 5' UTR comprises SEQ ID NO: 1.

31. The immunogenic composition of claim 21, wherein the 3' UTR comprises SEQ ID NO: 2.

32. The immunogenic composition of claim 21, wherein the 3' polyadenylation tail comprises SEQ ID NO: 3.

33. The immunogenic composition of any preceding claim, wherein the RNA polynucleotide has an integrity greater than 85%.

34. The immunogenic composition of any preceding claim, wherein the RNA polynucleotide has a purity of greater than 85%.
35. The immunogenic composition according to any one of claims 21-34, wherein at least 80% of the total RNA in the immunogenic composition is encapsulated.
- 5 36. The immunogenic composition according to any one of claims 21-35, wherein the percentage of intact mRNA encapsulated in the LNP is at least 80%.
37. The immunogenic composition according to any one of any of the preceding claims, wherein the LNP comprises an N:P ratio of from about 2:1 to about 30:1.
38. The immunogenic composition of claim 37, wherein the LNP has an N:P ratio of at least 5.
- 10 39. The immunogenic composition of claim 37, wherein the LNP has an N:P ratio of at least 6.
40. The immunogenic composition of claim 37, wherein the LNP has an N:P ratio of 10.
41. The immunogenic composition according to any one of claims 21-40, wherein the immunogenic composition comprises Tris.
42. The immunogenic composition according to any one of claims 21-41, wherein the
15 immunogenic composition comprises sucrose.
43. The immunogenic composition according to any one of claims 21-42, wherein the immunogenic composition does not further comprise sodium chloride.
44. The immunogenic composition according to any one of claims 21-43, wherein the immunogenic composition comprises 10 mM Tris.
- 20 45. The immunogenic composition according to any one of claims 21-44, wherein the immunogenic composition comprises 300 mM sucrose.
46. The immunogenic composition according to any one of claims 21-45, wherein the immunogenic composition has a pH 7.4.
47. The immunogenic composition according to any one of claims 21-46, wherein the
25 immunogenic composition has less than or equal to 12.5 EU/mL of bacterial endotoxins.
48. The immunogenic composition according to any one of claims 21-47, wherein the immunogenic composition is stable up at about 5 °C.
49. The immunogenic composition according to any one of claims 21-48, wherein the immunogenic composition is a vaccine.

50. A method for delivering a nucleic acid to a subject in need thereof comprising administering to the subject an immunogenic composition of any one of claims 21 to 49 comprising a nucleic acid.
51. A method for delivering a therapeutic peptide or protein to a subject in need thereof, the
5 method comprising administering to the subject an immunogenic composition of any one of claims 21 to 49 comprising a nucleic acid, wherein the nucleic acid encodes the therapeutic peptide or protein.
52. A method for treating or preventing a disease or disorder in a subject comprising
10 administering to the subject an immunogenic composition of any one of claims 21 to 49 comprising a nucleic acid, wherein administering the nucleic acid to cells of the subject is beneficial in treating or preventing the disease or disorder.
53. A method for treating or preventing a disease or disorder in a subject comprising
administering to the subject an immunoenic composition of any one of claims 21 to 49
15 comprising a nucleic acid, wherein the nucleic acid encodes a therapeutic peptide or protein and wherein delivering the therapeutic peptide or protein to the subject is beneficial in treating or preventing the disease or disorder.
54. The method of any one of claims 50-53, wherein the nucleic acid is RNA, modRNA or saRNA.
55. A method of eliciting an immune response against a disease comprising administering an
20 effective amount of an immunogenic composition according to any one of claims 21-49 to a subject in need thereof.
56. The method of any one of claims 50 to 55, wherein the subject is a mammal.
57. The method of claim 56, wherein the mammal is a human.
58. An immunogenic composition compound according to any one of claim 21 to 49 for use as a
25 component of a medicament.
59. Use of the immunogenic composition according to any one of claims 21-49 for the
manufacture of a medicament.

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