



(51) International Patent Classification:

C07K 16/12 (2006.01) A61K 47/24 (2006.01)
 A61K 9/127 (2006.01) A61P 31/00 (2006.01)
 A61K 31/7105 (2006.01) A61P 35/00 (2006.01)
 A61K 39/40 (2006.01) C07K 16/08 (2006.01)
 A61K 39/42 (2006.01) C07K 16/30 (2006.01)
 A61K 47/14 (2017.01) C12N 15/88 (2006.01)

(21) International Application Number:

PCT/US2022/021895

(22) International Filing Date:

25 March 2022 (25.03.2022)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/169,792 01 April 2021 (01.04.2021) US

(71) Applicant: MODERNATX, INC. [US/US]; 200 Technology Square, Cambridge, MA 02139 (US).

(72) Inventors: DEAL, Cailin; 200 Technology Square, Cambridge, MA 02139 (US). PLANTE, Obadiah; 200 Technology Square, Cambridge, MA 02139 (US).

(74) Agent: LOCKHART, Helen C. et al.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210-2206 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

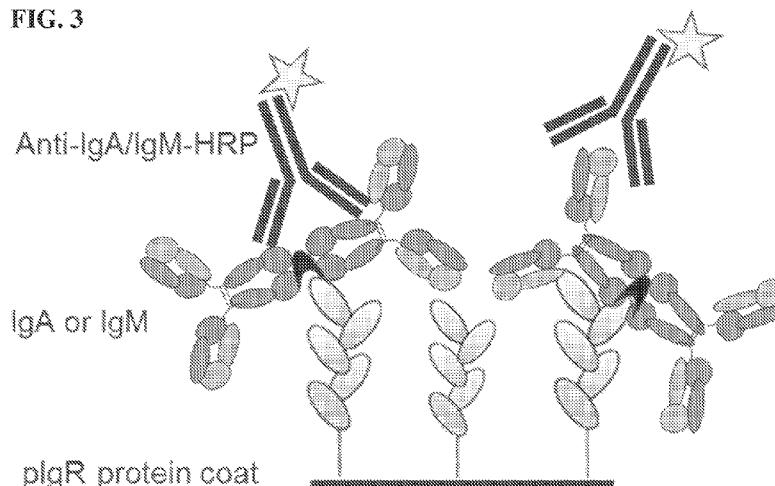
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: MUCOSAL EXPRESSION OF ANTIBODY STRUCTURES AND ISOTYPES BY MRNA

FIG. 3



(57) Abstract: The present application is directed to compositions comprising RNA having an open reading frame encoding an antibody heavy chain, an RNA having an open reading frame encoding an antibody light chain and an RNA having an open reading frame encoding an antibody J-chain, wherein the ratio of the RNAs is effective in producing an assembled antibody capable of forming a multimer. Compositions comprising RNA having an open reading frame encoding an IgG3 heavy chain, an RNA having an open reading frame encoding an antibody light chain, wherein the ratio of RNAs is effective in producing an assembled antibody are defined. Methods of producing therapeutic levels of IgA or IgM or IgG3 *in vivo* are also defined. The use of a lipid nanoparticle delivery vehicle is disclosed to administer the RNAs *in vivo*.



MUCOSAL EXPRESSION OF ANTIBODY STRUCTURES AND ISOTYPES BY MRNA**RELATED APPLICATIONS**

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional
5 Application serial number 63/169,792, filed April 1, 2021, entitled “Mucosal Expression of
Antibody Structures and Isotypes by mRNA,” which is incorporated by reference herein in its
entirety.

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS A TEXT FILE

10 The instant application contains a Sequence Listing which has been submitted in ASCII
format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on
March 23, 2022, is named M137870149WO00-SEQ-EAS and is 28,852 bytes in size.

BACKGROUND

15 Infectious diseases kill over 10 million people per year globally and are the fourth
leading cause of death in the United States. They also represent a \$68 billion dollar
pharmaceutical market. Antibiotics and anti-virals overlook millennia of nature’s learnings by
failing to utilize the immune system. And while intravenous immunoglobulin heeds nature’s
example, its applications in infectious disease are few and its limitations numerous (e.g., cost,
20 cumbersome manufacturing, risk of infection).

Efforts to develop monoclonal antibodies for infectious disease are underway, but are
limited to individual targets, given the high cost of existing approaches to antibody generation.
Further, the development of specific antibody isotypes (e.g., IgA and IgM) has been limited
because several characteristics (e.g., high molecular weight, protein instability upon expression,
25 aggregation at high concentration, non-specific binding yielding difficulty in purification
processes, shorter half-lives relative to IgG isotypes, and low affinities) make the isotypes
problematic to work with in a recombinant manner. Engineered mRNA antibodies may
overcome limitations of expression, purification, and affinity and additionally may provide
therapeutic applications not currently available.

SUMMARY

30 Aspects of the application relate to compositions of polynucleotides present in a specific
effective ratio for producing an assembled antibody. In some aspects, a ribonucleic acid (RNA)
composition is provided herein that builds on the knowledge that RNA (e.g., messenger RNA
35 (mRNA)) can safely direct the body’s cellular machinery to produce nearly any protein of
interest, from native proteins to antibodies and other entirely novel protein constructs that can
have therapeutic activity inside and outside of cells.

The RNA (*e.g.*, mRNA) compositions of the disclosure may be utilized in various settings depending on the prevalence of the infection or the degree or level of unmet medical need. The RNA compositions may be utilized to treat and/or prevent an infection by bacteria or virus of various genotypes, strains, and isolates. In some aspects, the disclosure relates to RNA compositions that may be utilized to provide passive immunization against an infectious disease. In some aspects, the disclosure relates to methods of treating and/or preventing infectious disease or cancer in a subject.

In some aspects the invention is a composition, comprising: (i) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody heavy chain (HC); (ii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody light chain (LC), and (iii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody joining (J) chain, wherein (i), (ii), and (iii) are present in the composition in a specific ratio effective for producing an assembled antibody and wherein the assembled antibody is capable of forming a multimer.

In some embodiments, the antibody heavy chain is an IgA heavy chain and wherein the specific ratio of (i), (ii), and (iii) encompasses a range of specific effective ratios including 1:1:1, 2:2:1, 4:4:1, 6:6:1, and 8:8:1. In some embodiments, the specific effective ratio of (i), (ii), and (iii) is 8:8:1. In some embodiments, the specific effective ratio of (i), (ii), and (iii) is 6:6:1.

In some embodiments, the IgA heavy chain further comprises a variable region, wherein the variable region comprises three heavy chain CDRs and four framework regions. In some embodiments, the IgA heavy chain further comprises a variable region, wherein the heavy chain variable region (HCVR) comprises: (a) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 1 (CDR1); (b) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 2 (CDR2); and (c) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 3 (CDR3).

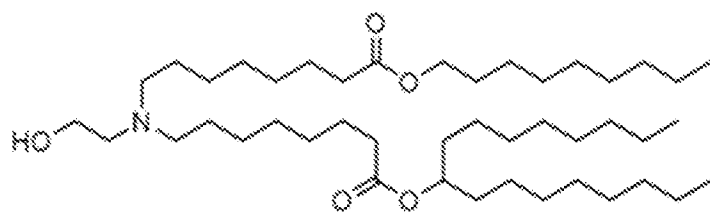
In some embodiments, the IgA light chain further comprises a variable region, wherein the light chain variable region (LCVR) comprises: (a) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 1 (CDR1); (b) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 2 (CDR2); and (c) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 3 (CDR3).

In some embodiments, the antibody heavy chain is an IgM heavy chain and wherein the specific ratio of (i), (ii), and (iii) encompasses a range of specific effective ratios including 1:1:1, 2:2:1, 4:4:1, 6:6:1, 8:8:1, and 10:10:1. In some embodiments, the specific effective ratio of (i), (ii), and (iii) is 4:4:1. In some embodiments, the specific effective ratio of (i), (ii), and (iii) is 8:8:1. In some embodiments, the specific effective ratio of (i), (ii), and (iii) is 10:10:1.

In some embodiments, the IgM heavy chain further comprises a variable region, wherein the variable region comprises three heavy chain CDRs and four framework regions. In some embodiments, the IgM heavy chain further comprises a variable region, wherein the heavy chain variable region (HCVR) comprises: (a) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 1 (CDR1); (b) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 2 (CDR2); and (c) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 3 (CDR3). In some embodiments, the IgM light chain further comprises a variable region, wherein the light chain variable region (LCVR) comprises: (a) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 1 (CDR1); (b) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 2 (CDR2); and (c) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 3 (CDR3).

In some embodiments, the ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody J chain comprises a protein sequence at least 90% identical to SEQ ID NO: 10 or 11. In some embodiments, the ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody J chain comprises SEQ ID NO: 10 or 11.

In some embodiments, the composition further comprises a lipid nanoparticle (LNP). In some embodiments, the lipid nanoparticle comprises a mixture of lipids, wherein the mixture of lipids comprises an ionizable amino lipid, a PEG-modified lipid, a structural lipid and a phospholipid. In some embodiments, the mixture of lipids comprises 0.5-15 mol% PEG-modified lipid; 5-25 mol% non-cationic lipid; 25-55 mol% sterol; and 20-60 mol% ionizable amino lipid. In some embodiments, the PEG-modified lipid is 1,2 dimyristoyl-sn-glycerol, methoxypolyethyleneglycol (PEG2000 DMG), the non-cationic lipid is 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC), the sterol is cholesterol; and the ionizable amino lipid has the structure of Compound 1:



Compound 1.

In some embodiments, the assembled antibody is capable of binding polymeric immunoglobulin receptor (pIgR).

In some embodiments, the assembled antibody is capable of being incorporated into one or more mucosal regions. In some embodiments, the mucosal region is one or more of: salivary glands, ocular tissues, mammary glands, gut-associated lymphoid tissue, gastrointestinal tract, bronchus-associated lymphoid tissue, respiratory tract, and/or urogenital tract. In some embodiments, the assembled antibody is capable of being incorporated into mucosal secretions. In some embodiments, the mucosal secretions are one or more of: tears, saliva, sweat, gastrointestinal fluid, respiratory tract secretions, and/or breast milk.

In some embodiments, the assembled antibody specifically binds a bacterial antigen.

In some embodiments, the assembled antibody specifically binds a viral antigen.

In some embodiments, the assembled antibody specifically binds a tumor antigen.

In some embodiments, the assembled antibody specifically binds a parasitic antigen.

In some aspects the invention is a method producing a therapeutic level of an IgA antibody in a human subject *in vivo*, comprising administering to the subject an LNP-formulated mRNA, the mRNA encoding the IgA antibody, in a dose effective to produce a functional oligomeric antibody in a mucosal tissue of the subject.

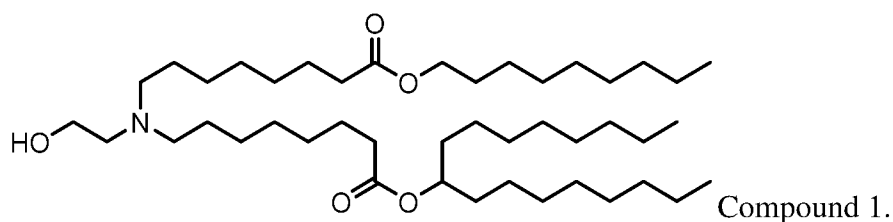
In some aspects the invention is a method producing a therapeutic level of an IgM antibody in a human subject *in vivo*, the method comprising administering to the subject an LNP-formulated mRNA, the mRNA encoding the IgA antibody, in a dose effective to produce a functional oligomeric antibody in a mucosal tissue of the subject.

In some embodiments, the IgA or IgM antibody are encoded by mRNA formulated in an LNP comprising: (i) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody heavy chain (HC); (ii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody light chain (LC), and (iii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody joining (J) chain, wherein (i), (ii), and (iii) are present in the LNP in a specific ratio effective for producing an assembled antibody and wherein the assembled antibody is capable of forming a multimer. In some embodiments, the specific ratio of (i), (ii), and (iii) encompasses a range of specific effective ratios including 1:1:1, 2:2:1, 4:4:1, 6:6:1, 8:8:1, and 10:10:1. In some embodiments, the specific effective ratio of (i), (ii), and (iii) is 4:4:1. In some embodiments, the specific effective ratio of

(i), (ii), and (iii) is 8:8:1. In some embodiments, the specific effective ratio of (i), (ii), and (iii) is 10:10:1.

In some embodiments, the antibody is administered either IV, SC or IM for systemic circulation and distribution to mucosal tissues.

In some embodiments, the LNP comprises a mixture of lipids, wherein the mixture of lipids comprises an ionizable amino lipid, a PEG-modified lipid, a structural lipid and a phospholipid. In some embodiments, the mixture of lipids comprises 0.5-15 mol% PEG-modified lipid; 5-25 mol% non-cationic lipid; 25-55 mol% sterol; and 20-60 mol% ionizable amino lipid. In some embodiments, the PEG-modified lipid is 1,2 dimyristoyl-sn-glycerol, methoxypolyethyleneglycol (PEG2000 DMG), the non-cationic lipid is 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC), the sterol is cholesterol; and the ionizable cationic lipid has the structure of Compound 1:

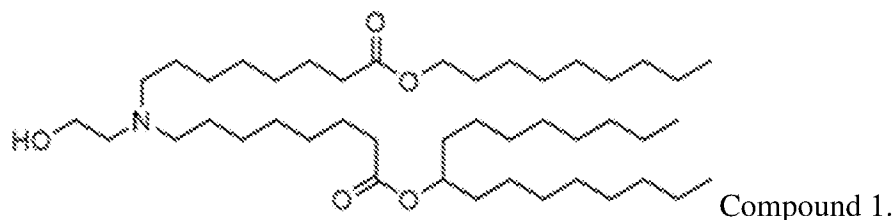


In some aspects the invention is a composition comprising: (i) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody IgG3 heavy chain (HC) and (ii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody light chain (LC), wherein (i) and (ii) are present in the composition in a specific ratio effective for producing an assembled antibody. In some embodiments, the composition does not comprise a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody joining (J) chain.

In some embodiments, the antibody heavy chain is an IgG3 heavy chain and wherein the specific ratio of (i) and (ii) encompasses a range of specific effective ratios including 1:1 and 2:1. In some embodiments, the specific effective ratio of (i) and (ii) is 2:1. In some embodiments, the IgG3 heavy chain further comprises a variable region, wherein the variable region comprises three heavy chain CDRs and four framework regions. In some embodiments, the IgG3 heavy chain further comprises a variable region, wherein the heavy chain variable region (HCVR) comprises: (a) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 1 (CDR1); (b) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 2 (CDR2); and (c) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 3 (CDR3). In some embodiments, the IgG3 light chain further comprises a variable region, wherein the light chain

variable region (LCVR) comprises: (a) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 1 (CDR1); (b) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 2 (CDR2); and (c) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 3 (CDR3).

In some embodiments, the composition further comprises a lipid nanoparticle (LNP). In some embodiments, the lipid nanoparticle comprises a mixture of lipids, wherein the mixture of lipids comprises an ionizable amino lipid, a PEG-modified lipid, a structural lipid and aphospholipid. In some embodiments, the mixture of lipids comprises 0.5-15 mol% PEG-modified lipid; 5-25 mol% non-cationic lipid; 25-55 mol% sterol; and 20-60 mol% ionizable amino lipid. In some embodiments, the PEG-modified lipid is 1,2 dimyristoyl-sn-glycerol, methoxypolyethyleneglycol (PEG2000 DMG), the non-cationic lipid is 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC), the sterol is cholesterol; and the ionizable amino lipid has the structure of Compound 1:



In some embodiments, the assembled antibody is capable of being incorporated into one or more mucosal regions. In some embodiments, the mucosal region is one or more of: salivary glands, ocular tissues, mammary glands, gut-associated lymphoid tissue, gastrointestinal tract, bronchus-associated lymphoid tissue, respiratory tract, and/or urogenital tract. In some embodiments, the assembled antibody is capable of being incorporated into mucosal secretions. In some embodiments, the mucosal secretions are one or more of: tears, saliva, sweat, gastrointestinal fluid, respiratory tract secretions, and/or breast milk.

In some embodiments, the assembled antibody specifically binds a bacterial antigen.

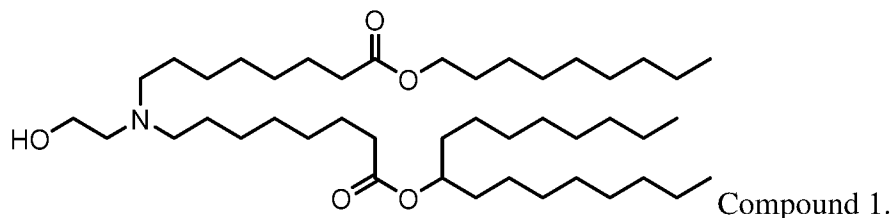
In some aspects the invention is a method of producing a therapeutic level of an IgG3 antibody in a human subject *in vivo*, comprising administering to the subject an LNP-formulated mRNA, the mRNA encoding the IgG3 antibody, in a dose effective to produce a functional antibody in a mucosal tissue of the subject. In some embodiments, the IgG3 antibody is encoded by mRNA formulated in an LNP comprising: (i) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody heavy chain (HC), and (ii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody light chain (LC), wherein (i) and (ii) are present in the LNP in a specific ratio effective for producing an assembled antibody.

In some embodiments, the LNP does not comprise a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody joining (J) chain.

In some embodiments, the specific ratio of (i) and (ii) encompasses a range of specific effective ratios including 1:1 and 2:1. In some embodiments, the specific effective ratio of (i) and (ii) is 2:1.

In some embodiments, the antibody is administered either IV, SC or IM for systemic circulation and distribution to mucosal tissues.

In some embodiments, the LNP comprises a mixture of lipids, wherein the mixture of lipids comprises an ionizable amino lipid, a PEG-modified lipid, a structural lipid and a phospholipid. In some embodiments, the mixture of lipids comprises 0.5-15 mol% PEG-modified lipid; 5-25 mol% non-cationic lipid; 25-55 mol% sterol; and 20-60 mol% ionizable amino lipid. In some embodiments, the PEG-modified lipid is 1,2 dimyristoyl-sn-glycerol, methoxypolyethyleneglycol (PEG2000 DMG), the non-cationic lipid is 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC), the sterol is cholesterol; and the ionizable cationic lipid has the structure of Compound 1:



Each of the limitations of the disclosure can encompass various embodiments of the disclosure. It is, therefore, anticipated that each of the limitations of the disclosure involving any one element or combinations of elements can be included in each aspect of the disclosure. This disclosure is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The disclosure is capable of other embodiments and of being practiced or of being carried out in various ways.

BRIEF DESCRIPTION OF DRAWINGS

The accompanying drawings are not intended to be drawn to scale. In the drawings, each identical or nearly identical component that is illustrated in various figures is represented by a like numeral. For purposes of clarity, not every component may be labeled in every drawing. In the drawings:

FIG. 1 is a table depicting various ratios of heavy chain to light chain to J chain (H:L:J) used in an *in vitro* study analyzing IgG, IgG3, IgA (IgA1, IgA2m1, and IgA2m2) and IgM antibodies class switched with either CAM003, a *Pseudomonas aeruginosa* mAb targeting Psl, or Hu605, a *Pseudomonas aeruginosa* mAb targeting LPS core.

FIGs. 2A-2B are graphs depicting class switched CAM003 and Hu605 antibodies in an isotype specific ELISA. The ratio of 2:1, without a third component refers to J chain equal to zero or not present. **FIG. 2A** is a graph depicting class switched CAM003 IgA1 and IgM detected in the supernatant. **FIG. 2B** is a graph depicting class switched Hu605 IgA2m1 and IgM detected in the supernatant.

FIG. 3 is a schematic of a polymeric immunoglobulin receptor (pIgR) binding ELISA. Plates were coated with recombinant human pIgR and then blocked to reduce non-specific binding. Antibodies were added and detected with anti-human IgA or human IgM HRP antibody. As shown in this illustration, only antibodies with J chain incorporated are able to bind pIgR.

FIGs. 4A-4E are graphs depicting IgA and IgM antibodies expressed from mRNAs encoding H:L:J chains on separate mRNAs encapsulated in the indicated ratios in LNPs using pIgR binding ELISA. The ratio of 2:1, without a third component refers to J chain equal to zero or not present. **FIG. 4A** is a graph depicting class switched CAM003 IgA1 with different ratios of H:L:J binding to pIgR. **FIG. 4B** is a graph depicting CAM003 IgA1 binding to pIgR quantitated for different H:L:J ratios and compared to total IgA1. **FIG. 4C** is a graph depicting class switched CAM003 IgM with different ratios of H:L:J binding to pIgR. **FIG. 4D** is a graph depicting class switched Hu605 IgA2m1 with different ratios of H:L:J binding to pIgR. **FIG. 4E** is a graph depicting class switched Hu605 IgM with different ratios of H:L:J binding to pIgR.

FIGs. 5A-5D are graphs depicting IgA and IgM antibodies expressed from mRNAs encoding H:L:J chains on separate mRNAs encapsulated in the indicated ratios in LNPs that retained the ability to bind *Pseudomonas aeruginosa*. The ratio of 2:1, without a third component refers to J chain equal to zero or not present. **FIG. 5A** depicts antigen binding via optical measurements of absorbance 450 nm across serial dilutions of CAM003 IgA1. **FIG. 5B** depicts antigen binding via optical measurements of absorbance 450 nm across serial dilutions of Hu605 IgA2m1. **FIG. 5C** depicts antigen binding via optical measurements of absorbance 450 nm across serial dilutions of CAM003 IgM. **FIG. 5D** depicts antigen binding via optical measurements of absorbance 450 nm across serial dilutions of Hu605 IgM.

FIG. 6 is a graph depicting the secretion of class switched CAM003 isotypes from IgG to IgG3, all IgA subtypes (IgA1, IgA2m1 and IgA2m2), and IgM isotypes. IgA1 and IgA2m1 subtypes increased antibody expression significantly with incorporation of J chain.

FIG. 7 is a graph depicting that the CAM003 isotype class switched antibodies retained antigen binding capabilities with and without incorporation of J chain. Antigen binding was detected via optical measurements of absorbance 450 nm across serial dilutions of CAM003 isotype class switched antibodies.

FIGs. 8A-C are graphs depicting the ability of class switched IgA antibody subtypes with and without J chain incorporated to bind pIgR. **FIG. 8A** is a graph depicting binding curves of class-switched IgA antibody subtypes to pIgR with and without J chain. **FIG. 8B** is a graph depicting the amount of an IgA subtype with J chain that binds to pIgR as compared to total IgA. **FIG. 8C** is a graph depicting the percent of pIgR binding IgA of total IgA.

FIG. 9 is a graph depicting *in vivo* CAM003 IgA expression with different H:L:J ratios. CAM003 IgA expression was measured in serum of C57Bl/6 mice 6, 12, 24, and 48 hours post administration of intravenous injection. The ratio of 2:1, without a third component refers to J chain equal to zero or not present.

FIGs. 10A-10B are graphs depicting *in vivo* CAM003 IgA expression in mouse lung tissue and correlation between serum and lungs. The ratio of 2:1, without a third component refers to J chain equal to zero or not present. **FIG. 10A** is a graph depicting expression of CAM003 IgA expression with different H:L:J ratios in the lungs of C57Bl/6 mice 6, 12, 24, and 48 hours post administration of intravenous injection. **FIG. 10B** is a graph depicting the correlation between serum and lung antibody expression.

FIG. 11 is a graph depicting *in vivo* CAM003 IgA expression is elevated in liver as compared to IgG. The ratio of 2:1, without a third component refers to J chain equal to zero or not present.

FIG. 12 is a graph depicting IgA antibody in serum and liver of C57Bl/6 mice retains functional binding to antigen over time in a *Pseudomonas aeruginosa* whole cell ELISA. Expression levels of IgA and IgG antibody were measured at 6 hours and 24 hours post administration of intravenous injection. The ratio of 2:1, without a third component refers to J chain equal to zero or not present.

FIG. 13 is a graph depicting human IgA antibodies in the serum binding to human pIgR at 6 hours post administration of intravenous injection, demonstrating J chain incorporation.

FIG. 14 is a graph depicting *in vivo* CAM003 IgM expression with different H:L:J ratios. CAM003 IgM expression was measured in serum of C57Bl/6 mice 6, 24, 48, and 96 hours post administration of intravenous injection. The ratio of 2:1, without a third component refers to J chain equal to zero or not present.

FIG. 15 is a graph depicting expression of CAM003 IgM expression with different H:L:J ratios in the lungs of C57Bl/6 mice 6, 24, 48, and 96 hours post administration of intravenous injection. The ratio of 2:1, without a third component refers to J chain equal to zero or not present.

FIG. 16 is a graph depicting *in vivo* CAM003 IgM is quickly cleared from the liver as compared to IgG. The ratio of 2:1, without a third component refers to J chain equal to zero or not present.

FIG. 17 is a graph depicting CAM003 IgM antibody in serum of C57Bl/6 mice at 24 hours post intravenous administration retains functional binding to antigen over time in a *Pseudomonas aeruginosa* whole cell ELISA.

FIG. 18 is a graph depicting CAM003 IgG1 and IgG3 antibody in serum of C57Bl/6 mice following intravenous (IV) or intraperitoneal (IP) injection at 6, 24, 48, and 96 hours post injection.

FIG. 19 is a graph depicting CAM003 IgA1, IgA2m1, and IgA2m2 antibody in serum of C57Bl/6 mice following intravenous (IV) or intraperitoneal (IP) injection at 6, 24, 48, and 96 hours post injection.

FIG. 20 is a graph depicting CAM003 IgM antibody in serum of C57Bl/6 mice following intravenous (IV) or intraperitoneal (IP) injection at 6, 24, 48, and 96 hours post injection.

FIG. 21 is a graph depicting 5J8 and EM4C04 IgG1 and IgA1 antibody in serum of SD-rats following intravenous injection at 6, 24, 48, 96, and 168 hours post injection.

FIG. 22 is a graph depicting Sal4 IgG with and without mRNA stabilizing elements, Sa4 IgAm1 with and without mRNA stabilizing elements, Sal4 IgG protein, and Sal4 IgA2m1 protein in serum of C57Bl/6 mice following intravenous injection at 6, 24, 48, 96, and 168 hours post injection.

FIG. 23 is a graph depicting Sal4 IgG with and without mRNA stabilizing elements, Sa4 IgAm1 with and without mRNA stabilizing elements, Sal4 IgG protein, and Sal4 IgA2m1 protein in feces of C57Bl/6 mice following intravenous injection at 6, 24, 48, 96, and 168 hours post injection.

FIG. 24 is a graph depicting Sal4 IgG with and without mRNA stabilizing elements, Sa4 IgAm1 with and without mRNA stabilizing elements, Sal4 IgG protein, and Sal4 IgA2m1 protein in intestine of C57Bl/6 mice following intravenous injection at 168 hours post injection.

FIG. 25 is a graph depicting Actoxumab IgG, Actoxumab IgA2m1, Actoxumab IgG protein, and Actoxumab IgA2m1 protein in serum of SD-rats following intravenous injection at 6, 24, 48, 96, and 168 hours post-treatment.

FIG. 26 is a graph depicting Bezlotuxumab IgG, Bezlotuxumab IgA2m1, Bezlotuxumab IgG protein, and Bezlotuxumab IgA2m1 protein in serum of SD-rats following intravenous injection at 6, 24, 48, 96, and 168 hours post-treatment.

FIG. 27 is a graph depicting percent neutralization of Toxin A relative to concentration of Actoxumab IgG, Actoxumab IgA2m1, Actoxumab IgG protein, and Actoxumab IgA2m1 protein.

FIG. 28 is a graph depicting competitive index of *Salmonella enterica serovar typhimurium* (AR05) relative to *Salmonella enterica serovar typhimurium* (AR04) in intestine of mice following intravenous injection of Sal4 IgG mRNA with mRNA stabilizing elements, Sal4 IgA2m1 mRNA with mRNA stabilizing elements, Sal4 IgG protein, and Sal4 IgA2m1 protein and subsequent oral challenge with equal amounts of AR05 and AR04. Statistical significance determined by Kruskal-Wallis test with Dunn's multiple comparison test (***) = $p < 0.001$.

FIG. 29 are graphs depicting fecal isotype concentration (left) and serum isotype concentration (right) in mice 24 hours after intravenous injection of Sal4 IgG mRNA with mRNA stabilizing elements, Sal4 IgA2m1 mRNA with mRNA stabilizing elements, Sal4 IgG protein and Sal4 IgA2m1 protein.

FIG. 30 is a schematic showing the experimental design for a *C. difficile* hamster challenge model.

FIG. 31 is a schematic showing the experimental design for a *P. aeruginosa* mouse challenge model.

DETAILED DESCRIPTION

It is of great interest in the fields of therapeutics, diagnostics, reagents and for biological assays to be able design, synthesize and deliver a nucleic acid, e.g., a ribonucleic acid (RNA) inside a cell, whether in vitro, in vivo, in situ or ex vivo, such as to effect physiologic outcomes which are beneficial to the cell, tissue or organ and ultimately to an organism. One beneficial outcome is to cause intracellular translation of the nucleic acid and production of at least one encoded peptide or polypeptide of interest.

Antibodies, also known as immunoglobulins, are glycoproteins produced by B cells. Using a unique and highly evolved system of recognition, antibodies can recognize a target and tag a target epitope, foreign entity or invading microbe for attack by the immune system thereby neutralizing its effect. The production of antibodies is the main function of the humoral immune system. Antibodies are secreted by a plasma cell which is a type of white blood cell.

Antibodies occur in two physical forms, a soluble form that is secreted from the cell, and a membrane-bound form that is attached to the surface of a B cell and is referred to as the B cell receptor (BCR). Soluble antibodies are released into the blood and tissue fluids, as well as many secretions to continue to survey for invading microorganisms.

The majority of antibodies comprise two heavy chains and two light chains. There are several different types of antibody heavy chains, and several different kinds of antibodies, which

are grouped into different isotypes based on which heavy chain they possess. Five different antibody isotypes (IgA, IgD, IgE, IgG and IgM) are known in mammals and trigger a different immune response for each different type of foreign object, epitope or microbe they encounter.

5 Frequently the binding of an antibody to an antigen has no direct biological effect. Rather, the significant biological effects are a consequence of secondary "effector functions" of antibodies. The immunoglobulins mediate a variety of these effector functions. These functions include fixation of complement, binding of phagocytic cells, lymphocytes, platelets, mast cells, and basophils which have immunoglobulin receptors. This binding can activate the cells to
10 perform some function. Some antibodies or immunoglobulins (*e.g.*, IgM isotypes) have privileged killing activity against bacterial pathogens, which are often too large to neutralize. Some antibodies or immunoglobulins also bind to receptors on placental trophoblasts, which results in transfer of the immunoglobulin across the placenta. As a result, the transferred maternal antibodies provide immunity to the fetus and newborn. Some antibodies or
15 immunoglobulins (*e.g.*, IgA isotypes) have privileged access to mucosal regions and can be found in secretions such as tears, saliva, sweat, gastrointestinal fluid, and breast milk. As a result, some antibodies or immunoglobulins may be especially effective for mitigating diseases and/or infections relating to mucosal regions.

Currently, the majority of antibodies are generated using recombinant or cloning
20 strategies and product heterogeneity is common to monoclonal antibody and other recombinant biological production. Such heterogeneity is typically introduced either upstream during expression or downstream during manufacturing. Recombinant antibody engineering involves the use of viruses or yeast to create antibodies, rather than mice which are used in cloning strategies. All of these however, suffer from drawbacks associated with the systems used for
25 generation including degree of purity, speed of development, cross reactivity, low affinity and variable specificity.

Described herein are compositions of antibodies where the components of the antibody are encoded by one or more polynucleotides. As such the present invention is directed, in part, to polynucleotides, specifically IVT polynucleotides, chimeric polynucleotides and/or circular
30 polynucleotides encoding one or more antibodies and/or components thereof. In some embodiments, the polynucleotides encode an antibody heavy chain. In some embodiments, the polynucleotides encode an antibody light chain. In some embodiments, the polynucleotides encode an antibody joining chain (J chain).

According to the present invention, the polynucleotides are described in compositions
35 wherein the polynucleotides encoding one or more antibodies and/or components thereof are

present in a specific ratio effective for producing an assembled antibody. In some embodiments, the assembled antibody is capable of forming a multimer. In some embodiments, the polynucleotides, and the resulting assembled antibodies, are preferably modified in a manner as to avoid the deficiencies of or provide improvements over other antibody molecules of the art.

5 Provided herein, therefore, are antibodies or portions thereof encoded by polynucleotide(s) and antibody compositions comprising at least one polynucleotide which have been designed to produce assembled antibodies that may provide a therapeutic utility and optionally may improve one or more of the stability and/or clearance in tissues, receptor uptake and/or kinetics, cellular access, engagement with translational machinery, mRNA half-life, translation efficiency, protein production capacity, secretion efficiency (when applicable), accessibility to circulation, protein half-life and/or modulation of a cell's status, antibody target affinity and/or specificity, reduction of antibody cross reactivity, increase of antibody purity, increase or alteration of antibody effector function and/or antibody activity.

15 In some embodiments, the polynucleotides are designed to produce one or more antibodies, or combinations of antibodies selected from the group consisting of IgA, IgG, IgM, IgE, and IgD. In some embodiments, the polynucleotides are designed to produce IgA antibodies. In some embodiments, the polynucleotides are designed to produce IgM antibodies.

20 An antibody (interchangeably used in plural form) is an immunoglobulin molecule capable of specific binding to a target, such as a carbohydrate, polynucleotide, lipid, polypeptide, through at least one antigen recognition site, located in the variable region of the immunoglobulin molecule.

25 The antibodies described herein can be derived from murine, rat, human, or any other origin. As used herein, the term "antibody" encompasses not only intact (*i.e.*, full-length) antibodies, but also antigen-binding fragments thereof (such as Fab, Fab', F(ab')₂, Fv), single chain (scFv), mutants thereof, fusion proteins comprising an antibody portion, humanized antibodies, chimeric antibodies, diabodies, nanobodies, linear antibodies, single chain antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), single domain antibodies such as heavy-chain antibodies, and any other modified configuration of the immunoglobulin molecule that comprises an antigen recognition site of the required specificity, including glycosylation variants of antibodies, amino acid sequence variants of antibodies, and covalently modified antibodies.

30 An antibody includes an antibody of any class, such as IgD, IgE, IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody's amino acid sequence of the constant domain of its heavy chains (if applicable), immunoglobulins can be assigned to different classes. There are five major classes of naturally-occurring

35 immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into

subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. As used herein, the term “assembled antibody” encompasses both intact (*i.e.*, full-length) antibodies, and antibodies that have oligomerized (*e.g.*,
5 formed multimers). As used herein, “oligomers” and “multimers” are used interchangeably. “Oligomers” or “multimers” refer to antibodies including, but not limited to, dimers, trimers, tetramers, pentamers, and hexamers. Depending on an antibody’s class or subclass, in addition to the heavy chain and light chain domains, an antibody may further comprise a joining chain or J chain (*e.g.*, IgA and IgM). Some antibodies are capable of forming a multimer without the
10 inclusion of a J chain (*e.g.*, IgM). The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

An antibody described herein may comprise a heavy chain variable region (V_H), a light chain variable region (V_L), a joining chain (J chain), or a combination thereof. Optionally, the antibody may further comprise an antibody constant region or a portion thereof (*e.g.*, C_{H1} , C_{H2} ,
15 C_{H3} , or a combination thereof). The heavy chain constant region can be of any suitable class as described herein and of any suitable origin, *e.g.*, human, mouse, rat, or rabbit. In one specific example, the heavy chain constant region is derived from a human IgG (a gamma heavy chain). In another specific example, the heavy chain constant region is derived from a human IgA (an alpha heavy chain). In yet another specific example, the heavy chain constant region is derived
20 from a human IgM. The light chain constant region can be a kappa chain or a lambda chain from a suitable origin. Antibody heavy and light chain constant regions are well known in the art, *e.g.*, those provided in the IMGT database (www.imgt.org) or at www.vbase2.org/vbstat.php., both of which are incorporated by reference herein.

When needed, the antibody as described herein may comprise a modified constant region.
25 For example, it may comprise a modified constant region that is immunologically inert, *e.g.*, does not trigger complement mediated lysis, or does not stimulate antibody-dependent cell mediated cytotoxicity (ADCC). ADCC activity can be assessed using methods disclosed in U.S. Pat. No. 5,500,362. Alternatively, the constant region may be modified such that it has an elevated effector activity, for example, enhanced ADCC activity. In some embodiments, the
30 constant region can be modified as described in Eur. J. Immunol. (1999) 29:2613-2624; PCT Application No. PCT/GB99/01441; and/or UK Patent Application No. 9809951.8.

In some embodiments, the heavy chain constant region used in the antibodies described herein may comprise mutations (*e.g.*, amino acid residue substitutions) to enhance a desired characteristic of the antibody, for example, increasing the binding activity to the neonatal Fc
35 receptor (FcRn) and thus the serum half-life of the antibodies. It was known that binding to

FcRn is critical for maintaining antibody homeostasis and regulating the serum half-life of antibodies. One or more (*e.g.*, 1, 2, 3, 4, 5, or more) mutations (*e.g.*, amino acid residue substitutions) may be introduced into the constant region at suitable positions (*e.g.*, in C_H2 region) to enhance FcRn binding and enhance the half-life of the antibody. See, *e.g.*,

- 5 Dall'Acqua et al., J.B.C., 2006, 281:23514-23524; Robbie et al., Antimicrob. Agents Chemother, 2013, 57(12):6147; and Dall'Acqua et al., J. Immunol. 2002 169:5171-5180.

In some embodiments, the antibodies described herein specifically bind to the corresponding target antigen or an epitope thereof. An antibody that “specifically binds” to an antigen or an epitope or is “specific for” a target antigen or epitope is a term well understood in
10 the art. A molecule is said to exhibit “specific binding” if it reacts more frequently, more rapidly, with greater duration and/or with greater affinity with a particular target antigen than it does with alternative targets. An antibody “specifically binds” to a target antigen or epitope or is “specific for” a target antigen or epitope if it binds with greater affinity, avidity, more readily, and/or with greater duration than it binds to other substances. For example, an antibody that
15 specifically (or preferentially) binds to an antigen (*e.g.*, HA of a specific influenza virus strain) or an antigenic epitope therein is an antibody that binds this target antigen with greater affinity, avidity, more readily, and/or with greater duration than it binds to other antigens or other epitopes in the same antigen. It is also understood with this definition that, for example, an antibody that specifically binds to a first target antigen may or may not specifically or
20 preferentially bind to a second target antigen. As such, “specific binding” or “preferential binding” does not necessarily require (although it can include) exclusive binding. In some examples, an antibody that “specifically binds” to a target antigen or an epitope thereof may not bind to other antigens or other epitopes in the same antigen.

In some embodiments, an antibody as described herein has a suitable binding affinity for
25 the target antigen or antigenic epitopes thereof. As used herein, “binding affinity” refers to the apparent association constant or K_A . The K_A is the reciprocal of the dissociation constant (K_D). The antibody described herein may have a binding affinity (K_D) of at least 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} M, or lower for the target antigen or antigenic epitope. An increased binding affinity corresponds to a decreased K_D . Higher affinity binding of an antibody for a first antigen relative
30 to a second antigen can be indicated by a higher K_A (or a smaller numerical value K_D) for binding the first antigen than the K_A (or numerical value K_D) for binding the second antigen. In such cases, the antibody has specificity for the first antigen (*e.g.*, a first protein in a first conformation or mimic thereof) relative to the second antigen (*e.g.*, the same first protein in a second conformation or mimic thereof; or a second protein).

For example, in some embodiments, the antibodies described herein have a higher binding affinity (a higher K_A or smaller K_D) to a specific bacterial, viral, or parasitic pathogen as compared to the binding affinity to second specific bacterial, viral, or parasitic pathogen. Differences in binding affinity (*e.g.*, for specificity or other comparisons) can be at least 1.5, 2, 3, 4, 5, 10, 15, 20, 37.5, 50, 70, 80, 91, 100, 500, 1000, 10,000 or 10^5 fold. In some embodiments, any of the antibodies may be further affinity matured to increase the binding affinity of the antibody to the target antigen or antigenic epitope thereof.

Binding affinity (or binding specificity) can be determined by a variety of methods including equilibrium dialysis, equilibrium binding, gel filtration, ELISA, surface plasmon resonance, or spectroscopy (*e.g.*, using a fluorescence assay). Exemplary conditions for evaluating binding affinity are in HBS-P buffer (10 mM HEPES pH7.4, 150 mM NaCl, 0.005% (v/v) Surfactant P20). These techniques can be used to measure the concentration of bound binding protein as a function of target protein concentration. The concentration of bound binding protein ([Bound]) is generally related to the concentration of free target protein ([Free]) by the following equation:

$$[\text{Bound}] = [\text{Free}] / (K_d + [\text{Free}])$$

It is not always necessary to make an exact determination of K_A or K_D though, since sometimes it is sufficient to obtain a quantitative measurement of affinity, *e.g.*, determined using a method such as ELISA or FACS analysis, is proportional to K_A or K_D , and thus can be used for comparisons, such as determining whether a higher affinity is, *e.g.*, 2-fold higher, to obtain a qualitative measurement of affinity, or to obtain an inference of affinity, *e.g.*, by activity in a functional assay, *e.g.*, an *in vitro* or *in vivo* assay.

In one example, the antibody described herein is a humanized antibody. Humanized antibodies refer to forms of non-human (*e.g.*, murine) antibodies that are specific chimeric immunoglobulins, immunoglobulin chains, or antigen-binding fragments thereof that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity and/or affinity. In some instances, one or more Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences, but are included to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at

least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence (*e.g.*, a germline sequence or a consensus sequence). The humanized antibody optimally may also comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin. Antibodies may have Fc regions modified as described in WO 99/58572. Other forms of humanized antibodies have one or more CDRs (one, two, three, four, five, and/or six) which are altered with respect to the original antibody (termed one or more CDRs “derived from” one or more CDRs from the original antibody). Humanized antibodies may also involve optimized antibodies derived from affinity maturation.

In another example, the antibody as described herein is a chimeric antibody, which can include a heavy constant region and optionally a light constant region from a human antibody. Chimeric antibodies refer to antibodies having a variable region or part of variable region from a first species and a constant region from a second species. Typically, in these chimeric antibodies, the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals (*e.g.*, a non-human mammal such as mouse, rabbit, and rat), while the constant portions are homologous to the sequences in antibodies derived from another mammal such as human. In some embodiments, amino acid modifications can be made in the variable region and/or the constant region.

In yet another example, the antibody described herein can be a single-domain antibody, which interacts with the target antigen via only one single variable domain such as a single heavy chain domain (as opposed to traditional antibodies, which interact with the target antigen via heavy chain and light chain variable domains). A single-domain antibody can be a heavy-chain antibody (VHH) which contains only an antibody heavy chain and is devoid of light chain. In addition to a variable region (for example, a V_H), a single-domain antibody may further comprise a constant region, for example, C_{H1} , C_{H2} , C_{H3} , C_{H4} , or a combination thereof.

In another example, the antibody described herein may be a bispecific antibody. Naturally occurring IgG antibodies are bivalent and monospecific. Bispecific antibodies having binding specificities for two different antigens can be produced using recombinant technologies and have broad clinical applications. The antibody described herein can be either a two variable-region bispecific antibody or a four-variable region antibody. Four variable domain antibodies are described in U.S. Pat. No. 7,612,181 which describes a dual-variable-domain IgG (DVD-IgG) bispecific antibody that is based on the dual-Fv format described in U.S. Pat. No. 5,989,830. An alternative format for four variable domain antibodies is the cross-over dual variable region antibodies as described in US 9,181,349.

In some embodiments, the antibodies and antigen binding fragments thereof comprises a heavy chain variable region having an amino acid sequence sharing at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with any of the amino acid sequences provided herein. In some embodiments, the amino acid sequence of the antibody comprises an amino acid sequence provided herein.

In some examples, the antibody binds the same epitope as an antibody comprising any of the VH chains known in the art and/or exemplified herein and/or competes against such an antibody from binding to the antigen. Such an antibody may comprise the same heavy chain CDRs as those known in the art and/or exemplified herein. An antibody having the same CDR (*e.g.*, CDR3) as a reference antibody means that the two antibodies have the same amino acid sequence in that CDR region as determined by the same methodology (*e.g.*, the Kabat definition, the Chothia definition, the AbM definition, or the contact definition).

Alternatively, an antibody described herein may comprise up to 5 (*e.g.*, 4, 3, 2, or 1) amino acid residue variations in one or more of the CDR regions of one of the antibodies known in the art and/or exemplified herein and binds the same epitope of antigen with substantially similar affinity (*e.g.*, having a K_D value in the same order). In one example, the amino acid residue variations are conservative amino acid residue substitutions. As used herein, a “conservative amino acid substitution” refers to an amino acid substitution that does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, *e.g.*, Molecular Cloning: A Laboratory Manual, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or Current Protocols in Molecular Biology, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

In some instances, the antibody may be a germline variant of any of the exemplary antibodies disclosed herein. A germline variant contains one or more mutations in the framework regions as relative to its parent antibody towards the corresponding germline sequence. To make a germline variant, the heavy or light chain variable region sequence of the parent antibody or a portion thereof (*e.g.*, a framework sequence) can be used as a query against an antibody germline sequence database (*e.g.*, <http://www.bioinfo.org.uk/abs/>, <http://www.vbase2.org>, or <http://www.imgt.org/>) to identify the corresponding germline sequence used by the parent antibody and amino acid residue variations in one or more of the

framework regions between the germline sequence and the parent antibody. One or more amino acid substitutions can then be introduced into the parent antibody based on the germline sequence to produce a germline variant.

In some examples, the antibody is a single chain antibody, which may comprise only one variable region (*e.g.*, V_H) or comprise both a V_H and a V_L. Such an antibody can be encoded by a single RNA molecule. In other examples, the antibody described herein is a multi-chain antibody comprising an independent heavy chain and an independent light chain. In other examples, the antibody described herein is a multi-chain antibody comprising an independent heavy chain, an independent light chain, and an independent J chain. Such a multi-chain antibody may be encoded by a single ribonucleic acid (RNA) molecule, which can be a bicistronic molecule encoding two separate polypeptide chains or can be a polycistronic molecule encoding three separate polypeptide chains. Such an RNA molecule may contain a signal sequence between the two or three coding sequences such that two or three separate polypeptides would be produced in the translation process. Alternatively, the RNA molecule may include a sequence coding for a cleavage site (*e.g.*, a protease cleavage site) in between the heavy and light chains such that it produces a single precursor polypeptide, which can be processed via cleavage at the cleavage site to produce the two separate heavy and light chains. Alternatively, the heavy and light antibody chains may be encoded by two or three separate RNA molecules.

Aspects of the disclosure provide compositions comprising RNA polynucleotides encoding one or more assembled antibodies and antigen binding fragments thereof. In some embodiments, the antibodies and antigen binding fragments thereof encoded by an RNA polynucleotide of the present application comprises a heavy chain antibody comprising a variable (V_H) domain. In some embodiments, the heavy chain antibody comprising a variable (V_H) domain is an IgA antibody. In some embodiments, the heavy chain antibody comprising a variable (V_H) domain is an IgM antibody.

In some embodiments, the antibodies and antigen binding fragments thereof encoded by an RNA polynucleotide of the present application comprises a fragment crystallizable (Fc) region. The Fc region is the tail region of an antibodies and antigen binding fragments thereof which contains constant domains (*e.g.*, CH2 and CH3); the other region of the antibodies and antigen binding fragments thereof being the Fab region which contains a variable domain (*e.g.*, V_H) and a constant domain (*e.g.*, CH1), the former of which defines binding specificity.

As described herein, antibodies can comprise a V_H domain. In some embodiments, the V_H domain further comprises one or more constant domains (*e.g.*, CH2 and/or CH3) of an Fc region and/or one or more constant domains (*e.g.*, CH1) of a Fab region. In some embodiments,

each of the one or more constant domains (*e.g.*, CH1, CH2, and/or CH3) can comprise or consist of portions of a constant domain. For example, in some embodiments, the constant domain comprises 99% or less, 98% or less, 97% or less, 96% or less, 95% or less, 90% or less, 80% or less, 70% or less, 60% or less, 50% or less, 40% or less, 30% or less, 20% or less, or 10% or less of the corresponding full sequence.

Targets and Compositions of the Invention

Targets of the Invention

The polynucleotides, constructs, and/or compositions of the present invention are useful in targeting or binding to polypeptides or proteins. In some embodiments polynucleotides encode one or more antibodies or fragments thereof which bind to an infective agent such as a bacteria, virus or biomolecules thereof, cell surface molecules or cancer antigens, and or parasitic antigens. Examples of infectious agents which may be targeted or bound by the peptides or proteins encoded by the polynucleotides of the present invention include both bacteria and viruses.

Examples of viruses which may be targeted using the compositions or constructs of the present invention include, but are not limited to, adenovirus; chikungunya, Herpes simplex, type 1; Herpes simplex, type 2; Varicella-zoster virus; Epstein-barr virus; Human cytomegalovirus; Human herpesvirus, type 8; Human papillomavirus; BK virus; JC virus; Smallpox; Hepatitis B virus; Human bocavirus; Parvovirus B19; Human astrovirus; Norwalk virus; coxsackievirus; hepatitis A virus; poliovirus; rhinovirus; Severe acute respiratory syndrome virus; Hepatitis C virus; yellow fever virus; dengue virus; West Nile virus; Rubella virus; Hepatitis E virus; Human immunodeficiency virus (HIV); Influenza virus; Guanarito virus; Junin virus; Lassa virus; Machupo virus; Sabiá virus; Crimean-Congo hemorrhagic fever virus; Ebola virus; Marburg virus; Measles virus; Mumps virus; Parainfluenza virus; Respiratory syncytial virus; Human metapneumovirus; Hendra virus; Nipah virus; Rabies virus; Hepatitis D; Rotavirus; Orbivirus; Coltivirus; or Banna virus.

Examples of pathogenic bacteria which may be targeted using the compositions or constructs of the present invention include, but are not limited to, *Acinetobacter baumannii*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia pneumoniae*, *Chlamydia trachomatis*, *Chlamydophila psittaci*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Francisella tularensis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Legionella pneumophila*, *Leptospira interrogans*, *Listeria monocytogenes*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycoplasma pneumoniae*,

Neisseria gonorrhoeae, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Rickettsia rickettsii*,
Salmonella typhi, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*,
Staphylococcus epidermidis, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*,
Streptococcus pneumoniae, *Streptococcus pyogenes*, *Treponema pallidum*, *Vibrio cholerae*,
5 *Yersinia pestis*, *Yersinia enterocolitica*, and/or *Yersinia pseudotuberculosis*.

The compositions of the present disclosure may be designed as a single therapeutic that treats a variety of pathogenic strains of seasonal flu and pandemic flu where the polynucleotides encode IgG against hemagglutinins associated with emerging strains with pandemic potential.

According to the present invention, polynucleotides or constructs and their associated
10 compositions may be designed to produce a commercially available antibody, a variant or a portion thereof *in vivo*.

In some embodiments, the polynucleotides of the invention may be used to protect an infant against infection or disease, including but not limited to diseases caused by respiratory syncytial virus (RSV) alone or in combination with *B. pertussis*, by administering the
15 polynucleotides to a pregnant female with a gestational infant. While not wishing to be bound by theory, the antibody encoded by the polynucleotide of interest may be transferred via the placenta to the gestational infant, protecting the infant against infection or disease. In some embodiments, the antibody encoded by the polynucleotides of the invention may be secreted into the breast milk, protecting the infant against infection or disease. The polynucleotides of the
20 invention may be administered alone or in combination with an immunogenic composition as described in WO2014/024024 and WO2014/024026, the contents of each which is herein incorporated by reference in its entirety.

Hepatitis C is a contagious liver disease that results from infection with the hepatitis C virus, one of the most common viral liver infections with approximately 150 million people have
25 chronic infections with risk of liver cirrhosis and/or liver cancer, 3-4 million people infected yearly and about 350,000 deaths every year. It can range in severity from a mild illness lasting a few weeks to a serious, lifelong illness. HCV infection and associated liver cirrhosis is the most common indication for orthotopic liver transplantation among adults and HCV infection remains a problem after transplantation and recurrent hepatic infection is the leading cause of graft
30 failure.

Chronic hepatitis C is characterized by a high turnover of infected cells and continuous de novo infection of target cells. Due to the vital role of de novo infection in maintenance of HCV infection, blocking of de novo infection is a potential target for antiviral therapy. The viral envelope glycoproteins, E1 and E2, are the major components of the HCV particle and hence

play a pivotal role in the entry process and hypervariable region 1 (HVR1), consisting of the first 27 amino acids of E2 (aa 384–410), is a major target for neutralizing antibodies.

Another approach that is currently promoted for the treatment and prevention of HCV infection/re-infection is blocking a pathway preventing the immune system from recognizing and fighting cancer cells and pathogens. Current treatment involves a combination of IFN- α and ribavirin.

In one embodiment, the polynucleotides of the invention may be used in the treatment and/or prevention of hepatitis C virus (HCV) infection. As used herein “hepatitis C virus” or “HCV” means a viral disease that can lead to swelling of the liver. In one embodiment, the polynucleotides of the invention may encode at least one antibody, fragment or variant thereof which may be used for prognosing, diagnosing, and/or treating of HCV in a subject. In another embodiment, the polynucleotides of the invention may be used to protect a subject from or inhibit HCV-mediated morbidity or mortality in a subject.

In one embodiment, the polynucleotides of the current invention may be used in combination with ribavirin, IFN- α and/or pegylated (peg) IFN- α to treat and/or prevent HCV.

Rabies is a widespread viral disease that is transmitted from animals to humans. Around 60,000 people die annually from rabies, and the disease threatens over 3 billion people in rural areas of Asia and Africa where human vaccines and immunoglobulin are not readily available or accessible. Rabies is an RNA virus that belongs to the order Mononegavirales. The viral genome encodes 5 proteins designated as N, P, M, G, and L. Currently, lethal rabies is prevented by administering a rabies virus vaccine and rabies virus immunoglobulin (RIG) post-exposure. Two types of RIG are employed, Human RIG (HRIG) and Equine RIG (ERIG).

In one embodiment, the polynucleotides of the invention may be used in the treatment or prevention of rabies virus infection. As used herein “rabies virus” is a virus normally spread to people from the saliva of infected animals and infects nerve cells. In one embodiment, the polynucleotides of the invention may encode at least one antibody, fragment or variant thereof which may be used for the treatment or prevention of rabies.

The human immunodeficiency virus (HIV) is a lentivirus that causes the acquired immunodeficiency syndrome (AIDS). HIV infects cells of the human immune system such as helper cells expressing the CD4 receptor on their surface, macrophages, and dendritic cells, compromising cell-mediated immunity and allowing opportunistic infections and cancers to thrive.

In one embodiment, the polynucleotides of the invention may be used in the treatment or prevention of human immunodeficiency virus (HIV). As used herein “human immunodeficiency virus” or “HIV” means a variable retrovirus that invades and inactivates helper T cells of the

immune system and is a cause of AIDS and AIDS-related complex. In one embodiment, the polynucleotides of the invention may encode at least one antibody, fragment or variant thereof which may be used for the treatment or prevention of HIV.

5 In one embodiment, the polynucleotides may encode at least one neutralizing HIV antibody, which may target the HIV-1 viral spike.

Staphylococcus is a genus of Gram-positive bacteria. Staphylococci, gram positive bacteria including coagulase-negative staphylococci (CONS) and *Staphylococcus aureus*, are responsible for between 56 and >75% of hospital-acquired, late-onset neonatal sepsis.

10 In one embodiment, the polynucleotides of the invention may be used in the treatment or prevention Staphylococcus infection. As used herein “Staphylococcus” means a bacteria that can cause sepsis. In one embodiment, the polynucleotides of the invention may encode at least one antibody, fragment or variant thereof which may be used for the treatment or prevention of sepsis caused by Staphylococcus.

15 Anthrax is a serious infectious disease caused by gram-positive bacteria known as *Bacillus anthracis* (*B. anthracis*). Although rare, human can get infected with anthrax if they come in contact with infected animals or contaminated animal products. *Bacillus anthracis* has also long been considered a potential biological warfare agent.

20 Anthrax toxin is composed of a cell-binding protein, known as protective antigen (PA), and two enzyme components, called edema factor (EF) and lethal factor (LF). These three protein components act together to impart their physiological effects. PA is a protein component of the toxins produced by the bacterium. It initiates the activity of the toxins by attaching to cells in the infected person, and then facilitates the entry of additional destructive factors – LF and EF into the cells. PA comprises a protein having a weight of about 83 kD (PA83) that is cleaved into a protein having a weight of about 63 kD (PA63).

25 Three forms of human anthrax disease exist based on their portal of entry: cutaneous (most common), causes a localized inflammatory necrotic lesion; pulmonary (highly fatal), causes sudden, massive chest edema succeeded by cardiovascular shock; gastrointestinal, a (rare but also fatal) from ingestion of spores.

30 In one embodiment, the polynucleotides of the invention may be used in the treatment or prevention of *Bacillus anthracis* (*B. anthracis*) infection and anthrax. As used herein “*Bacillus anthracis*” or “*B. anthracis*” is the bacterium that causes anthrax. In one embodiment, the polynucleotides of the invention may encode at least one antibody, fragment or variant thereof which may be used for the treatment or prevention of anthrax.

35 Shiga toxin (Stx)-producing *Escherichia coli* (STEC) causes hemorrhagic colitis and hemolytic-uremic syndrome (HUS). Diarrhea-associated HUS is a common cause of acute renal

failure and up to 50% of patients with HUS develop some degree of renal impairment. The Shiga toxins produced by *E. coli* are majorly Stx1 and Stx2. (Thorpe, *Clinical Infectious Disease*, vol.38(9), 1298-1303 (2004), the contents of which are incorporated herein by reference in their entirety).

5 In one embodiment, the polynucleotides of the invention may encode any antibody that targets a Shiga toxin, including, but not limited to the shigamabs antibody, for the prevention or treatment of STEC and HUS. The polynucleotides may be used in combination with antibiotic therapies in the prevention and/or treatment of STEC and HUS.

10 Clostridium difficile (*C. difficile*) is a bacterium that can cause symptoms ranging from diarrhea to life-threatening inflammation of the colon. *C. difficile* most commonly affects older adults in hospitals or in long-term care facilities and often occurs after the use of antibiotics. The most well-understood toxins produced by pathogenic *C. difficile* strains are enterotoxin (Clostridium difficile toxin A) and cytotoxin (Clostridium difficile toxin B), both of which can produce diarrhea and inflammation in infected patients.

15 In one embodiment, the polynucleotides of the invention may be used in the treatment or prevention of Clostridium difficile (*C. difficile*) infection. In one embodiment, the polynucleotides of the invention may encode at least one antibody, fragment or variant thereof which may be used for the treatment or prevention of *C. difficile* infection.

20 In one embodiment, the polynucleotides described herein encode a monoclonal antibody that is directed to toxin A and/or toxin B for *C. difficile*, which may be used for the treatment or prevention of *C. difficile* infection.

Besides *Pseudomonas aeruginosa*, there are other genera of Gram-negative bacteria, such as the *Acinetobacter* species, that often produce multidrug-resistant and even pan-resistant strains.

25 *Acinetobacter baumannii* is a Gram-negative bacterium that has been isolated from water and soil samples. *A. baumannii* affects people with compromised immune systems, and is becoming increasingly more frequent as a hospital-derived infection. Due to its ability to form a biofilm, it can persist on artificial surfaces and infect new patients. It is thought that the ability of *A. baumannii* to form biofilms is correlated with multi drug resistance (MDR). *A. baumannii* also
30 forms protective capsules composed of polysaccharides around each individual cell, further providing additional protection from antibiotics and antibacterial agents. In a 2009 study, *A. baumannii* was found to be responsible for 19.1% of ventilator-associated pneumonia (VAP) cases in European intensive care units.

35 Although the passive immunization approach provides only temporary immunity, it may be sufficient to clear an acute *A. baumannii* infection, alone or in combination with other

antimicrobials. Passive immunization may therefore become an important therapeutic approach, in particular given the high incidence of multi-drug resistant strains.

In one embodiment, the antibodies encoded by the polynucleotides of the present invention bind or target one or more proteins or peptides of *Acinetobacter baumannii*.

5 Hepatitis B virus (HBV) causes an infectious illness of the liver and has caused epidemics in parts of Asia and Africa, and is still endemic in China. The virus is transmitted by exposure to infectious blood or body fluids such as semen and vaginal fluids. Perinatal infection is a major route of infection in developing countries. The acute illness causes liver inflammation, vomiting, jaundice. HBV results in one million deaths annually, primarily due to cirrhosis and
10 liver cancer.

The hepatitis B virus is a partially double stranded DNA virus composed of a 27 nm nucleocapsid core (HBcAg), surrounded by an outer lipoprotein coat (envelope) containing the surface antigen (HBsAg). The nucleocapsid has been found to be very immunogenic and a number of antibodies with nucleocapsid epitopes have been described. The nucleocapsid is
15 dimorphic and is comprised of either 90 or 120 dimers arranged such that the four-helix bundles project from the surface as 25 Å-long spike. Together these two capsid forms are known as core antigen (HBcAg). The principal antigenic determinant of HBcAg, the immunodominant loop, is located at the apices of the capsid spikes (Watts et al, Non-Canonical Binding Of An Antibody Resembling A Naïve B Cell Receptor Immunoglobulin To Hepatitis B Virus Capsids J Mol Biol.
20 Jun 20, 2008; 379(5): 1119–1129, the contents of which are herein incorporated by reference in its entirety).

In some embodiments, the polynucleotides of the invention may encode at least one antibody that binds to the nucleocapsid of HBV.

In some embodiments, the polynucleotides of the invention may encode at least one
25 antibody that binds to the nucleocapsid of HBV for the prevention, management, or treatment of HBV infections.

In some embodiments, the polynucleotides of the invention may encode at least one antibody that binds to the nucleocapsid of HBV that can be used in combination with other HBV treatments, including existing HBV vaccines.

30 Cancer is one of the leading causes of death in the United States. Conventional methods of cancer treatment like chemotherapy, surgery or radiation therapy, can be limited in their efficacy since they are often nonspecific to the cancer. In many cases tumors, however, can specifically express genes whose products are required for inducing or maintaining the malignant state. These proteins may serve as antigen markers for the development and establishment of

efficient anti-cancer treatments. The polynucleotides of the invention may encode anti-cancer antibodies. Such antibodies may be used to target cancer cells by binding cancer antigens.

Cancer antigens can elicit an immune response. These antigens can be either proteins, polysaccharides, lipids, or glycolipids, which can be recognized as foreign by immune cells, such as T cells and B cells. Exposure of immune cells to one or more of these antigens can elicit a rapid cell division and differentiation response resulting in the formation of clones of the exposed T cells and B cells. B cells can differentiate into plasma cells which in turn can produce antibodies which selectively bind to the antigens.

In cancer, there are four general groups of tumor antigens: (i) viral tumor antigens which can be identical for any viral tumor of this type, (ii) carcinogenic tumor antigens which can be specific for patients and for the tumors, (iii) isoantigens of the transplantation type or tumor-specific transplantation antigens which can be different in all individual types of tumor but can be the same in different tumors caused by the same virus; and (iv) embryonic antigens. The polynucleotides of the invention encode any type of cancer antigen including any of these 4 classes of antigens.

In addition to the development of antibodies against tumor antigens for cancer treatment, antibodies that target immune cells to boost the immune response have also been developed. For example, an anti-CD40 antibody that is a CD40 agonist can be used to activate dendritic cells to enhance the immune response.

Additionally, antibodies may function as immune checkpoint modulators. In some preferred embodiments of the invention the polynucleotides encode an antibody that is a T cell activator such as an immune checkpoint modulator. Immune checkpoint modulators include both stimulatory checkpoint molecules and inhibitory checkpoint molecules i.e., an anti-CTLA4 and anti-PD1 antibody.

Stimulatory checkpoint inhibitors function by promoting the checkpoint process. Several stimulatory checkpoint molecules are members of the tumor necrosis factor (TNF) receptor superfamily - CD27, CD40, OX40, GITR and CD137, while others belong to the B7-CD28 superfamily - CD28 and ICOS. OX40 (CD134), is involved in the expansion of effector and memory T cells. Anti-OX40 monoclonal antibodies have been shown to be effective in treating advanced cancer. MEDI0562 is a humanized OX40 agonist. GITR, Glucocorticoid-Induced TNFR family Related gene, is involved in T cell expansion. Several antibodies to GITR have been shown to promote an anti-tumor response. ICOS, Inducible T-cell costimulator, is important in T cell effector function. CD27 supports antigen-specific expansion of naïve T cells and is involved in the generation of T and B cell memory. Several agonistic anti-CD27

antibodies are in development. CD122 is the Interleukin-2 receptor beta sub-unit. NKTR-214 is a CD122-biased immune-stimulatory cytokine.

Inhibitory checkpoint molecules include but are not limited to PD-1, TIM-3, VISTA, A2AR, B7-H3, B7-H4, BTLA, CTLA-4, IDO, KIR and LAG3. CTLA-4, PD-1 and its ligands are members of the CD28-B7 family of co-signaling molecules that play important roles throughout all stages of T-cell function and other cell functions. CTLA-4, Cytotoxic T-Lymphocyte-Associated protein 4 (CD152), is involved in controlling T cell proliferation.

The PD-1 receptor is expressed on the surface of activated T cells (and B cells) and, under normal circumstances, binds to its ligands (PD-L1 and PD-L2) that are expressed on the surface of antigen-presenting cells, such as dendritic cells or macrophages. This interaction sends a signal into the T cell and inhibits it. Cancer cells take advantage of this system by driving high levels of expression of PD-L1 on their surface. This allows them to gain control of the PD-1 pathway and switch off T cells expressing PD-1 that may enter the tumor microenvironment, thus suppressing the anticancer immune response. Pembrolizumab (formerly MK-3475 and lambrolizumab, trade name Keytruda) is a human antibody used in cancer immunotherapy. It targets the PD-1 receptor.

The checkpoint inhibitor is a molecule such as a monoclonal antibody, a humanized antibody, a fully human antibody, a fusion protein or a combination thereof or a small molecule. For instance, the checkpoint inhibitor inhibits a checkpoint protein which may be CTLA-4, PDL1, PDL2, PD1, B7-H3, B7-H4, BTLA, HVEM, TIM3, GAL9, LAG3, VISTA, KIR, 2B4, CD160, CGEN-15049, CHK 1, CHK2, A2aR, B-7 family ligands or a combination thereof. Ligands of checkpoint proteins include but are not limited to CTLA-4, PDL1, PDL2, PD1, B7-H3, B7-H4, BTLA, HVEM, TIM3, GAL9, LAG3, VISTA, KIR, 2B4, CD160, CGEN-15049, CHK 1, CHK2, A2aR, and B-7 family ligands. In some embodiments the anti-PD-1 antibody is BMS-936558 (nivolumab). In other embodiments the anti-CTLA-4 antibody is ipilimumab (trade name Yervoy, formerly known as MDX-010 and MDX-101).

The RNA polynucleotide of the invention may encode an antibody against any cancer antigen. As used herein, the terms “cancer antigen” and “tumor antigen” are used interchangeably to refer to antigens which are differentially expressed by cancer cells and can thereby be exploited in order to target cancer cells. Cancer antigens are antigens which can potentially stimulate apparently tumor-specific immune responses. Some of these antigens are encoded, although not necessarily expressed, by normal cells. These antigens can be characterized as those which are normally silent (i.e., not expressed) in normal cells, those that are expressed only at certain stages of differentiation and those that are temporally expressed such as embryonic and fetal antigens. Other cancer antigens are encoded by mutant cellular

genes, such as oncogenes (e.g., activated ras oncogene), suppressor genes (e.g., mutant p53), fusion proteins resulting from internal deletions or chromosomal translocations. Still other cancer antigens can be encoded by viral genes such as those carried on RNA and DNA tumor viruses.

5 An antibody specific for a cell surface antigen of, for example, a cancer cell, may promote an immune response resulting in antibody dependent cellular cytotoxicity (ADCC). In one embodiment, the antibody may be selected from the group consisting of Ributaxin, Herceptin, Quadramet, Panorex, IDEC-Y2B8, BEC2, C225, Oncolym, SMART M195, ATRAGEN, Ovarex, Bexxar, LDP-03, ior t6, MDX-210, MDX-11, MDX-22, OV103,
 10 3622W94, anti-VEGF, Zenapax, MDX-220, MDX-447, MELIMMUNE-2, MELIMMUNE-1, CEACIDE, Pretarget, NovoMAb-G2, TNT, Gliomab-H, GNI-250, EMD-72000, LymphoCide, CMA 676, Monopharm-C, 4B5, ior egf.r3, ior c5, BABS, anti-FLK-2, MDX-260, ANA Ab, SMART 1D10 Ab, SMART ABL 364 Ab and ImmuRAIT-CEA.

Examples of parasites and/or parasitic infections which may be targeted using the
 15 compositions or constructs of the present invention include, but are not limited to, any one or more of (or any combination of) Malaria (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*), *Schistosomes*, *Trypanosomes*, *Leishmania*, Filarial nematodes (e.g., *Wuchereria bancrofti*, *Brugia malayi*), Trichomoniasis (e.g., *Trichomonas vaginalis*), *Sarcosporidiasis*, *Taenia* (e.g., *Taenia saginata*, *Taenia solium*, *Taenia asiatica*), *Toxoplasma*
 20 *gondii*, Trichinellosis (*Trichinella spiralis*) or Coccidiosis (e.g., *Eimeria* species). In some embodiments, the RNA polynucleotide of the invention may encode an antibody that specifically binds antigens derived from one or more of the organisms listed above.

In some embodiments, the RNA polynucleotide of the invention may encode an antibody that binds one or more antigens derived from a parasite that causes Malarira (e.g., *Plasmodium*
 25 *falciparum*, *Plasmodium vivax*, *Plasmodium malariae*).

In some embodiments, the RNA polynucleotide of the invention may encode an antibody that binds one or more antigens derived from a parasite that causes Trichomoniasis (e.g., *Trichomonas vaginalis*).

In some embodiments, the RNA polynucleotide of the invention may encode an antibody
 30 that binds one or more antigens derived from a parasite that causes Coccidiosis (e.g., *Eimeria* species).

Intrabody Constructs

According to the present invention, an intrabody construct is a polynucleotide which has been modified for expression inside a target cell and where the expression product binds an
 35 intracellular protein. Such constructs may have sub picomolar binding affinities and may be

formulated for targeting to particular sites or tissues. For example, intrabody constructs may be formulated in any of the lipid nanoparticle formulations disclosed herein.

Bicistronic, Pseudo-bicistronic, and/or Tricistronic Constructs

According to the present invention, a bicistronic or tricistronic construct is a polynucleotide encoding a two- or three-protein chain antibody on a single polynucleotide strand. A pseudo-bicistronic construct is a polynucleotide encoding a single chain antibody discontinuously on a single polynucleotide strand. For bicistronic or tricistronic constructs, the encoded two or three strands or two or three portions/regions and/or domains (as is the case with pseudo-bicistronic) are separated by at least one nucleotide not encoding the strands or domains. More often the separation comprises a cleavage signal or site or a non-coding region of nucleotides. Such cleavage sites include, for example, furin cleavage sites encoded as an “RKR” site in the resultant polypeptide.

Single Domain Constructs

According to the present invention, a single domain construct comprises one or two polynucleotides encoding a single monomeric variable antibody domain. Typically single domain antibodies comprise one variable domain (VH) of a heavy-chain antibody.

Single chain Fv Constructs

According to the present invention, a single chain Fv constructs is a polynucleotide encoding at least two coding regions and a linker region. The scFv construct may encode a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a short linker peptide of ten to about 25 amino acids. See Fig. 3A for an example. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. Other linkers include those known in the art and disclosed herein.

Single chain antibodies may be camelid antibodies. They may also be human heavy chain only antibodies such as those made by Crescendo Biologics.

Bispecific Constructs

According to the present invention, a bispecific construct is a polynucleotide encoding portions or regions of two different antibodies. Bispecific constructs encode polypeptides which may bind two different antigens. Polynucleotides of the present invention may also encode trispecific antibodies having an affinity for three antigens.

IgA Antibodies

In some embodiments of the present invention, the antibodies encoded by the polynucleotides are IgA antibodies. In some embodiments, the IgA antibody of the present invention is encoded by a ribonucleic acid (RNA) polynucleotide encoding an antibody heavy

chain, a ribonucleic acid (RNA) polynucleotide encoding an antibody light chain, and a ribonucleic acid (RNA) polynucleotide encoding an antibody joining (J) chain. In some embodiments, the antibody heavy chain, antibody light chain, and the antibody J chain of the present invention are present in the composition in a specific ratio effective for producing an IgA assembled antibody. As used herein, a “specific effective ratio” is based on mass ratios and describes the proportion of heavy chain to light chain to J chain (H:L:J) relative to one another that is capable of producing an assembled antibody in a composition of the present invention. In some embodiments, the specific ratio effective for producing an IgA assembled antibody encompasses a range of ratios, including but not limited to, 1:1:1, 2:2:1, 4:4:1, 6:6:1, and 8:8:1.

One example of a specific effective ratio of an IgA assembled antibody of the present invention is 8:8:1.

In some embodiments, the incorporation of the J chain into an IgA antibody, produces an assembled antibody capable of polymerizing to form dimers, trimers, tetramers, pentamers and/or multimers. In some embodiments, the IgA assembled antibody is a multimer. In some embodiments, the IgA assembled antibody is capable of binding polymeric immunoglobulin receptor (pIgR). In some embodiments, the IgA assembled antibody is capable of being incorporated into mucosal regions (*e.g.*, salivary glands, ocular tissues, mammary glands, gut-associated lymphoid tissue, gastrointestinal tract, bronchus-associated lymphoid tissue, respiratory tract, and/or urogenital tract). In some embodiments, the IgA assembled antibody is capable of being incorporated into mucosal secretions. For example, the IgA of the present invention may be a secretory IgA (SIgA) and may be secreted into tears, saliva, sweat, gastrointestinal fluid, and/or breast milk.

In some embodiments of the present invention, the antibodies comprise an IgA heavy chain wherein the IgA heavy chain further comprises a variable region. In some embodiments, the heavy chain variable region comprises three heavy chain CDRs (CDR1, CDR2, and CDR3) and four framework regions (FR1, FR2, FR3, and FR4).

In some embodiments of the present invention, an IgA assembled antibody comprises an IgA isotype. One example of an IgA isotype of the present invention comprises a VH antigen binding domain and an IgA2 backbone which can provide protection from bacterial IgA1 protease.

In some embodiments, an IgA assembled antibody of the present invention can specifically bind a bacterial, viral, or parasitic antigen.

IgM Antibodies

In some embodiments of the present invention, the antibodies encoded by the polynucleotides are IgM antibodies. In some embodiments, the IgM antibody of the present

invention is encoded by a a ribonucleic acid (RNA) polynucleotide encoding an antibody heavy chain, a ribonucleic acid (RNA) polynucleotide encoding an antibody light chain, and a ribonucleic acid (RNA) polynucleotide encoding an antibody joining (J) chain. In some embodiments, the antibody heavy chain, antibody light chain, and the antibody J chain of the present invention are present in the composition in a specific ratio effective for producing an IgM assembled antibody. In some embodiments, the specific ratio effective for producing an IgM assembled antibody encompasses a range of ratios, including but not limited to, 1:1:1, 2:2:1, 4:4:1, 6:6:1, 8:8:1, and 10:10:1.

One example of a specific effective ratio of an IgM assembled antibody of the present invention is 8:8:1. Another example of a specific effective ratio of an IgM assembled antibody of the present invention is 10:10:1.

In some embodiments, the incorporation of the J chain into an IgM antibody, produces an assembled antibody capable of polymerizing to form dimers, pentamers, hexamers and/or multimers. In one example of the present invention, the IgM assembled antibody is a pentamer and the J chain is incorporated. In another example of the present invention, the IgM assembled antibody is a hexamer and the J chain is incorporated. In some embodiments, the IgM assembled antibody is capable of binding polymeric immunoglobulin receptor (pIgR). In some embodiments, the IgM assembled antibody is capable of being incorporated into mucosal regions (*e.g.*, salivary glands, ocular tissues, mammary glands, gut-associated lymphoid tissue, gastrointestinal tract, bronchus-associated lymphoid tissue, respiratory tract, and/or urogenital tract). In some embodiments, the IgM assembled antibody is capable of being incorporated into mucosal secretions. For example, the IgM of the present invention may be a secretory IgM (SIgM) and may be secreted into tears, saliva, sweat, gastrointestinal fluid, and/or breast milk.

In some embodiments of the present invention, the antibodies comprise an IgM heavy chain wherein the IgM heavy chain further comprises a variable region. In some embodiments, the heavy chain variable region comprises three heavy chain CDRs (CDR1, CDR2, and CDR3) and four framework regions (FR1, FR2, FR3, and FR4).

In some embodiments of the present disclosure, an IgM assembled antibody does not comprise a J chain.

In some embodiments of the present disclosure, the IgM assembled antibody can activate the complement system. For example, the IgM assembled antibody has increased complement mediated killing relative to the other antibody isotypes. In some embodiments, an IgM assembled antibody of the present invention can specifically bind a bacterial, viral, or parasitic antigen.

In one embodiment, the polynucleotides of the present invention is or functions as a messenger RNA (mRNA). As used herein, the term “messenger RNA” (mRNA) refers to any polynucleotide which encodes at least one peptide or polypeptide of interest and which is capable of being translated to produce the encoded peptide polypeptide of interest *in vitro*, *in vivo*, *in situ* or *ex vivo*.

The basic components of an mRNA molecule typically include at least one coding region, a 5' untranslated region (UTR), a 3' UTR, a 5' cap, and a poly-A tail. Polynucleotides of the present disclosure may function as mRNA but can be distinguished from wild-type mRNA in their functional and/or structural design features which serve to overcome existing problems of effective polypeptide expression using nucleic-acid based therapeutics.

In some embodiments, an RNA polynucleotide encodes 2-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 2-3, 3-10, 3-9, 3-8, 3-7, 3-6, 3-5, 3-4, 4-10, 4-9, 4-8, 4-7, 4-6, 4-5, 5-10, 5-9, 5-8, 5-7, 5-6, 6-10, 6-9, 6-8, 6-7, 7-10, 7-9, 7-8, 8-10, 8-9 or 9-10 antibodies and antigen binding fragment polypeptides. In some embodiments, an RNA polynucleotide encodes at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 antibodies and antigen binding fragment polypeptides. In some embodiments, an RNA polynucleotide encodes at least 100 or at least 200 antibodies and antigen binding fragment polypeptides. In some embodiments, an RNA polynucleotide encodes 1-10, 5-15, 10-20, 15-25, 20-30, 25-35, 30-40, 35-45, 40-50, 1-50, 1-100, 2-50 or 2-100 antibodies and antigen binding fragment polypeptides.

Polynucleotides of the present disclosure, in some embodiments, are codon optimized. Codon optimization methods are known in the art and may be used as provided herein. 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 to 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 CA), and/or proprietary methods. In some embodiments, the open reading frame (ORF) sequence is optimized using optimization algorithms.

In some embodiments, a codon optimized sequence shares less than 95% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type

mRNA sequence encoding a polypeptide or protein of interest, such as an antibodies and antigen binding fragments thereof). In some embodiments, a codon optimized sequence shares less than 90% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest, such as an antibodies and antigen binding fragments thereof). In some embodiments, a codon optimized sequence shares less than 85% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest, such as an antibodies and antigen binding fragments thereof). In some embodiments, a codon optimized sequence shares less than 80% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest, such as an antibodies and antigen binding fragments thereof). In some embodiments, a codon optimized sequence shares less than 75% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest, such as an antibodies and antigen binding fragments thereof).

In some embodiments, a codon optimized sequence shares between 65% and 85% (*e.g.*, between about 67% and about 85% or between about 67% and about 80%) sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest, such as an HCAb). In some embodiments, a codon optimized sequence shares between 65% and 75 or about 80% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a polypeptide or protein of interest, such as an HCAb).

In some embodiments a codon optimized RNA may, for instance, be one in which the levels of G/C are enhanced. The G/C-content of nucleic acid molecules may influence the stability of the RNA. RNA having an increased amount of guanine (G) and/or cytosine (C) residues may be functionally more stable than nucleic acids containing a large amount of adenine (A) and thymine (T) or uracil (U) nucleotides. WO02/098443 discloses a pharmaceutical composition containing an mRNA stabilized by sequence modifications in the translated region. Due to the degeneracy of the genetic code, the modifications work by substituting existing codons for those that promote greater RNA stability without changing the resulting amino acid. The approach is limited to coding regions of the RNA.

In some embodiments, an influenza virus binding polypeptide is longer than 4 amino acids and shorter than 30 amino acids. Thus, polypeptides include gene products, naturally occurring polypeptides, synthetic polypeptides, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing. A polypeptide may be a single

molecule or may be a multi-molecular complex such as a dimer, trimer or tetramer. Polypeptides may also comprise single chain or multichain polypeptides such as antibodies and may be associated or linked. Most commonly, disulfide linkages are found in multichain polypeptides. The term polypeptide may also apply to amino acid polymers in which at least one amino acid residue is an artificial chemical analogue of a corresponding naturally-occurring amino acid.

The term “polypeptide variant” refers to molecules which differ in their amino acid sequence from a native or reference sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence, as compared to a native or reference sequence. Ordinarily, variants possess at least 50% identity to a native or reference sequence. In some embodiments, variants share at least 80%, or at least 90% identity with a native or reference sequence.

In some embodiments “variant mimics” are provided. As used herein, the term “variant mimic” is one which contains at least one amino acid that would mimic an activated sequence. For example, glutamate may serve as a mimic for phosphoro-threonine and/or phosphoro-serine. Alternatively, variant mimics may result in deactivation or in an inactivated product containing the mimic, for example, phenylalanine may act as an inactivating substitution for tyrosine; or alanine may act as an inactivating substitution for serine.

“Orthologs” refers to genes in different species that evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function in the course of evolution. Identification of orthologs is critical for reliable prediction of gene function in newly sequenced genomes.

“Analog” is meant to include polypeptide variants which differ by one or more amino acid alterations, for example, substitutions, additions or deletions of amino acid residues that still maintain one or more of the properties of the parent or starting polypeptide.

The present disclosure provides several types of compositions that are polynucleotide or polypeptide based, including variants and derivatives. These include, for example, substitutional, insertional, deletion and covalent variants and derivatives. The term “derivative” is used synonymously with the term “variant” but generally refers to a molecule that has been modified and/or changed in any way relative to a reference molecule or starting molecule.

As such, polynucleotides encoding peptides or polypeptides containing substitutions, insertions and/or additions, deletions and covalent modifications with respect to reference sequences, in particular the polypeptide sequences disclosed herein, are included within the scope of this disclosure. For example, sequence tags or amino acids, such as one or more lysines, can be added to peptide sequences (*e.g.*, at the N-terminal or C-terminal ends). Sequence tags can be used for peptide detection, purification or localization. Lysines can be used to

increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (*e.g.*, C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support. In some embodiments, sequences for (or encoding) signal sequences, termination sequences, transmembrane domains, linkers, multimerization domains (such as, *e.g.*, foldon regions) and the like may be substituted with alternative sequences that achieve the same or a similar function. In some embodiments, cavities in the core of proteins can be filled to improve stability, *e.g.*, by introducing larger amino acids. In other embodiments, buried hydrogen bond networks may be replaced with hydrophobic residues to improve stability. In yet other embodiments, glycosylation sites may be removed and replaced with appropriate residues. Such sequences are readily identifiable to one of skill in the art.

“Substitutional variants” when referring to polypeptides are those that have at least one amino acid residue in a native or starting sequence removed and a different amino acid inserted in its place at the same position. Substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

As used herein, the term “conservative amino acid substitution” refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine, and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

“Features” when referring to polypeptide or polynucleotide are defined as distinct amino acid sequence-based or nucleotide-based components of a molecule respectively. Features of the polypeptides encoded by the polynucleotides include surface manifestations, local

conformational shape, folds, loops, half-loops, domains, half-domains, sites, termini, or any combination thereof.

As used herein, when referring to polypeptides, the term “domain” refers to a motif of a polypeptide having one or more identifiable structural or functional characteristics or properties (e.g., binding capacity, serving as a site for protein-protein interactions).

As used herein, when referring to polypeptides, the terms “site” as it pertains to amino acid based embodiments is used synonymously with “amino acid residue” and “amino acid side chain.” As used herein, when referring to polynucleotides, the terms “site” as it pertains to nucleotide based embodiments is used synonymously with “nucleotide.” A site represents a position within a peptide or polypeptide or polynucleotide that may be modified, manipulated, altered, derivatized or varied within the polypeptide or polynucleotide based molecules.

As used herein, the terms “termini” or “terminus” when referring to polypeptides or polynucleotides refers to an extremity of a polypeptide or polynucleotide, respectively. Such extremity is not limited only to the first or final site of the polypeptide or polynucleotide, but may include additional amino acids or nucleotides in the terminal regions. Polypeptide-based molecules may be characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH₂)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)). Proteins are in some cases made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (e.g., multimers, oligomers). These proteins have multiple N- and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide based moiety such as an organic conjugate.

As recognized by those skilled in the art, protein fragments, functional protein domains, and homologous proteins are also considered to be within the scope of polypeptides of interest. For example, provided herein is any protein fragment (meaning a polypeptide sequence at least one amino acid residue shorter than a reference polypeptide sequence but otherwise identical) of a reference protein 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or greater than 100 amino acids in length. In another example, any protein that includes a stretch of 20, 30, 40, 50, or 100 amino acids which are 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% identical to any of the sequences described herein can be utilized in accordance with the disclosure. In some embodiments, a polypeptide includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations as shown in any of the sequences provided or referenced herein. In another example, any protein that includes a stretch of 20, 30, 40, 50, or 100 amino acids that are greater than 80%, 90%, 95%, or 100% identical to any of the sequences described herein, wherein the protein has a stretch of 5, 10, 15,

20, 25, or 30 amino acids that are less than 80%, 75%, 70%, 65% or 60% identical to any of the sequences described herein can be utilized in accordance with the disclosure.

Polypeptide or polynucleotide molecules of the present disclosure may share a certain degree of sequence similarity or identity with the reference molecules (*e.g.*, reference polypeptides or reference polynucleotides), for example, with art-described molecules (*e.g.*, engineered or designed molecules or wild-type molecules). The term “identity” as known in the art, refers to a relationship between the sequences of two or more polypeptides or polynucleotides, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between them as determined by the number of matches between strings of two or more amino acid residues or nucleic acid residues. Identity measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (*e.g.*, “algorithms”). Identity of related peptides can be readily calculated by known methods. “% identity” as it applies to polypeptide or polynucleotide sequences is defined as the percentage of residues (amino acid residues or nucleic acid residues) in the candidate amino acid or nucleic acid sequence that are identical with the residues in the amino acid sequence or nucleic acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent identity. Methods and computer programs for the alignment are well known in the art. It is understood that identity depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation. Generally, variants of a particular polynucleotide or polypeptide have at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. Such tools for alignment include those of the BLAST suite (Stephen F. Altschul, et al (1997), “Gapped BLAST and PSI-BLAST: a new generation of protein database search programs”, *Nucleic Acids Res.* 25:3389-3402). Another popular local alignment technique is based on the Smith-Waterman algorithm (Smith, T.F. & Waterman, M.S. (1981) “Identification of common molecular subsequences.” *J. Mol. Biol.* 147:195-197). A general global alignment technique based on dynamic programming is the Needleman–Wunsch algorithm (Needleman, S.B. & Wunsch, C.D. (1970) “A general method applicable to the search for similarities in the amino acid sequences of two proteins.” *J. Mol. Biol.* 48:443-453.). More recently, a Fast Optimal Global Sequence Alignment Algorithm (FOGSAA) has been developed that purportedly produces global alignment of nucleotide and protein sequences faster than other optimal global

alignment methods, including the Needleman–Wunsch algorithm. Other tools are described herein, specifically in the definition of “identity” below.

As used herein, the term “homology” refers to the overall relatedness between polymeric molecules, *e.g.* between nucleic acid molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Polymeric molecules (*e.g.*, nucleic acid molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or polypeptide molecules) that share a threshold level of similarity or identity determined by alignment of matching residues are termed homologous. Homology is a qualitative term that describes a relationship between molecules and can be based upon the quantitative similarity or identity. Similarity or identity is a quantitative term that defines the degree of sequence match between two compared sequences. In some embodiments, polymeric molecules are considered to be “homologous” to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical or similar. The term “homologous” necessarily refers to a comparison between at least two sequences (polynucleotide or polypeptide sequences). Two polynucleotide sequences are considered homologous if the polypeptides they encode are at least 50%, 60%, 70%, 80%, 90%, 95%, or even 99% for at least one stretch of at least 20 amino acids. In some embodiments, homologous polynucleotide sequences are characterized by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. For polynucleotide sequences less than 60 nucleotides in length, homology is determined by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. Two protein sequences are considered homologous if the proteins are at least 50%, 60%, 70%, 80%, or 90% identical for at least one stretch of at least 20 amino acids.

Homology implies that the compared sequences diverged in evolution from a common origin. The term “homolog” refers to a first amino acid sequence or nucleic acid sequence (*e.g.*, gene (DNA or RNA) or protein sequence) that is related to a second amino acid sequence or nucleic acid sequence by descent from a common ancestral sequence. The term “homolog” may apply to the relationship between genes and/or proteins separated by the event of speciation or to the relationship between genes and/or proteins separated by the event of genetic duplication. “Orthologs” are genes (or proteins) in different species that evolved from a common ancestral gene (or protein) by speciation. Typically, orthologs retain the same function in the course of evolution. “Paralogs” are genes (or proteins) related by duplication within a genome. Orthologs retain the same function in the course of evolution, whereas paralogs evolve new functions, even if these are related to the original one.

The term “identity” refers to the overall relatedness between polymeric molecules, for example, between polynucleotide molecules (*e.g.* DNA molecules and/or RNA molecules)

and/or between polypeptide molecules. Calculation of the percent identity of two polynucleic acid sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleic acid sequences can be determined using methods such as those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; each of which is incorporated herein by reference. For example, the percent identity between two nucleic acid sequences can be determined using the algorithm of Meyers and Miller (CABIOS, 1989, 4:11-17), which has been incorporated into the ALIGN program (version 2.0) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleic acid sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix. Methods commonly employed to determine percent identity between sequences include, but are not limited to those disclosed in Carillo, H., and Lipman, D., SIAM J Applied Math., 48:1073 (1988); incorporated herein by reference. Techniques for determining identity are codified in publicly available computer programs. Exemplary computer software to determine homology between two sequences include, but are not limited to, GCG program package, Devereux, J., et al., Nucleic Acids Research, 12(1), 387 (1984)), BLASTP, BLASTN, and FASTA Altschul, S. F. et al., J. Molec. Biol., 215, 403 (1990)).

RNA (*e.g.*, mRNA) treatments of the present disclosure comprise at least one ribonucleic acid (RNA) polynucleotide having an open reading frame encoding at least one influenza virus protein binding polypeptide that comprises at least one chemical modification.

The terms “chemical modification” and “chemically modified” refer to modification with respect to adenosine (A), guanosine (G), uridine (U), thymidine (T) or cytidine (C) ribonucleosides or deoxyribnucleosides in at least one of their position, pattern, percent or population. Generally, these terms do not refer to the ribonucleotide modifications in naturally occurring 5'-terminal mRNA cap moieties. With respect to a polypeptide, the term “modification” refers to a modification relative to the canonical set 20 amino acids.

Polypeptides, as provided herein, are also considered “modified” if they contain amino acid substitutions, insertions, or a combination of substitutions and insertions.

Polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides), in some embodiments, comprise various (more than one) different modifications. In some embodiments, a particular region of a polynucleotide contains one, two or more (optionally different) nucleoside or nucleotide modifications. In some embodiments, a modified RNA polynucleotide (*e.g.*, a modified mRNA polynucleotide), introduced to a cell or organism, exhibits reduced degradation in the cell or organism, respectively, relative to an unmodified polynucleotide. In some embodiments, a modified RNA polynucleotide (*e.g.*, a modified mRNA polynucleotide), introduced into a cell or organism, may exhibit reduced immunogenicity in the cell or organism, respectively (*e.g.*, a reduced innate response).

Modifications of polynucleotides include, without limitation, those described herein. Polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) may comprise modifications that are naturally-occurring, non-naturally-occurring, or the polynucleotide may comprise a combination of naturally-occurring and non-naturally-occurring modifications. Polynucleotides may include any useful modification, for example, of a sugar, a nucleobase, or an internucleoside linkage (*e.g.*, to a linking phosphate, to a phosphodiester linkage or to the phosphodiester backbone).

Polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides), in some embodiments, comprise non-natural modified nucleotides that are introduced during synthesis or post-synthesis of the polynucleotides to achieve desired functions or properties. The modifications may be present on internucleotide linkages, purine or pyrimidine bases, or sugars. The modification may be introduced with chemical synthesis or with a polymerase enzyme at the terminal of a chain or anywhere else in the chain. Any of the regions of a polynucleotide may be chemically modified.

The present disclosure provides for modified nucleosides and nucleotides of a polynucleotide (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides). A “nucleoside” refers to a compound containing a sugar molecule (*e.g.*, a pentose or ribose) or a derivative thereof in combination with an organic base (*e.g.*, a purine or pyrimidine) or a derivative thereof (also referred to herein as “nucleobase”). A “nucleotide” refers to a nucleoside comprising one or more phosphate groups. Modified nucleotides may be synthesized by any useful method, such as, for example, chemically, enzymatically, or recombinantly, to include one or more modified or non-natural nucleosides. Polynucleotides may comprise a region or regions of linked nucleosides. Such regions may have variable backbone linkages. The linkages may be standard phosphodiester linkages, in which case the polynucleotides would comprise regions of nucleotides.

Modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine, or uracil. Any combination of base/sugar or linker may be incorporated into polynucleotides of the present disclosure.

The skilled artisan will appreciate that, except where otherwise noted, polynucleotide sequences set forth in the instant application will recite “T”s in a representative DNA sequence but where the sequence represents RNA, the “T”s would be substituted for “U”s.

Modifications of polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) that are useful in the compositions, methods and synthetic processes of the present disclosure of the present disclosure include, but are not limited to the following: 2-methylthio-N6-(*cis*-hydroxyisopentenyl)adenosine; 2-methylthio-N6-methyladenosine; 2-methylthio-N6-threonyl carbamoyladenosine; N6-glyciny carbamoyladenosine; N6-isopentenyladenosine; N6-methyladenosine; N6-threonyl carbamoyladenosine; 1,2'-O-dimethyladenosine; 1-methyladenosine; 2'-O-methyladenosine; 2'-O-ribosyladenosine (phosphate); 2-methyladenosine; 2-methylthio-N6 isopentenyladenosine; 2-methylthio-N6-hydroxynorvalyl carbamoyladenosine; 2'-O-methyladenosine; 2'-O-ribosyladenosine (phosphate); Isopentenyladenosine; N6-(*cis*-hydroxyisopentenyl)adenosine; N6,2'-O-dimethyladenosine; N6,2'-O-dimethyladenosine; N6,N6,2'-O-trimethyladenosine; N6,N6-dimethyladenosine; N6-acetyladenosine; N6-hydroxynorvalyl carbamoyladenosine; N6-methyl-N6-threonyl carbamoyladenosine; 2-methyladenosine; 2-methylthio-N6-isopentenyladenosine; 7-

deaza-adenosine; N1-methyl-adenosine; N6, N6 (dimethyl)adenine; N6-cis-hydroxy-isopentenyl-adenosine; α -thio-adenosine; 2 (amino)adenine; 2 (aminopropyl)adenine; 2 (methylthio) N6 (isopentenyl)adenine; 2-(alkyl)adenine; 2-(aminoalkyl)adenine; 2-(aminopropyl)adenine; 2-(halo)adenine; 2-(halo)adenine; 2-(propyl)adenine; 2'-Amino-2'-deoxy-ATP; 2'-Azido-2'-deoxy-ATP; 2'-Deoxy-2'-a-aminoadenosine TP; 2'-Deoxy-2'-a-azidoadenosine TP; 6 (alkyl)adenine; 6 (methyl)adenine; 6-(alkyl)adenine; 6-(methyl)adenine; 7 (deaza)adenine; 8 (alkenyl)adenine; 8 (alkynyl)adenine; 8 (amino)adenine; 8 (thioalkyl)adenine; 8-(alkenyl)adenine; 8-(alkyl)adenine; 8-(alkynyl)adenine; 8-(amino)adenine; 8-(halo)adenine; 8-(hydroxyl)adenine; 8-(thioalkyl)adenine; 8-(thiol)adenine; 8-azido-adenosine; aza adenine; deaza adenine; N6 (methyl)adenine; N6-(isopentyl)adenine; 7-deaza-8-aza-adenosine; 7-methyladenine; 1-Deazaadenosine TP; 2'-Fluoro-N6-Bz-deoxyadenosine TP; 2'-OMe-2-Amino-ATP; 2'-O-methyl-N6-Bz-deoxyadenosine TP; 2'-a-Ethynyladenosine TP; 2-aminoadenine; 2-Aminoadenosine TP; 2-Amino-ATP; 2'-a-Trifluoromethyladenosine TP; 2-Azidoadenosine TP; 2'-b-Ethynyladenosine TP; 2-Bromoadenosine TP; 2'-b-Trifluoromethyladenosine TP; 2-Chloroadenosine TP; 2'-Deoxy-2',2'-difluoroadenosine TP; 2'-Deoxy-2'-a-mercaptopadenosine TP; 2'-Deoxy-2'-a-thiomethoxyadenosine TP; 2'-Deoxy-2'-b-aminoadenosine TP; 2'-Deoxy-2'-b-azidoadenosine TP; 2'-Deoxy-2'-b-bromoadenosine TP; 2'-Deoxy-2'-b-chloroadenosine TP; 2'-Deoxy-2'-b-fluoroadenosine TP; 2'-Deoxy-2'-b-iodoadenosine TP; 2'-Deoxy-2'-b-mercaptopadenosine TP; 2'-Deoxy-2'-b-thiomethoxyadenosine TP; 2-Fluoroadenosine TP; 2-Iodoadenosine TP; 2-Mercaptopadenosine TP; 2-methoxy-adenine; 2-methylthio-adenine; 2-Trifluoromethyladenosine TP; 3-Deaza-3-bromoadenosine TP; 3-Deaza-3-chloroadenosine TP; 3-Deaza-3-fluoroadenosine TP; 3-Deaza-3-iodoadenosine TP; 3-Deazaadenosine TP; 4'-Azidoadenosine TP; 4'-Carbocyclic adenosine TP; 4'-Ethynyladenosine TP; 5'-Homo-adenosine TP; 8-Aza-ATP; 8-bromo-adenosine TP; 8-Trifluoromethyladenosine TP; 9-Deazaadenosine TP; 2-aminopurine; 7-deaza-2,6-diaminopurine; 7-deaza-8-aza-2,6-diaminopurine; 7-deaza-8-aza-2-aminopurine; 2,6-diaminopurine; 7-deaza-8-aza-adenine, 7-deaza-2-aminopurine; 2-thiocytidine; 3-methylcytidine; 5-formylcytidine; 5-hydroxymethylcytidine; 5-methylcytidine; N4-acetylcytidine; 2'-O-methylcytidine; 2'-O-methylcytidine; 5,2'-O-dimethylcytidine; 5-formyl-2'-O-methylcytidine; Lysidine; N4,2'-O-dimethylcytidine; N4-acetyl-2'-O-methylcytidine; N4-methylcytidine; N4,N4-Dimethyl-2'-OMe-Cytidine TP; 4-methylcytidine; 5-aza-cytidine; Pseudo-iso-cytidine; pyrrolo-cytidine; α -thio-cytidine; 2-(thio)cytosine; 2'-Amino-2'-deoxy-CTP; 2'-Azido-2'-deoxy-CTP; 2'-Deoxy-2'-a-aminocytidine TP; 2'-Deoxy-2'-a-azidocytidine TP; 3 (deaza) 5 (aza)cytosine; 3 (methyl)cytosine; 3-(alkyl)cytosine; 3-(deaza) 5 (aza)cytosine; 3-(methyl)cytidine; 4,2'-O-dimethylcytidine; 5 (halo)cytosine; 5 (methyl)cytosine; 5 (propynyl)cytosine; 5 (trifluoromethyl)cytosine; 5-(alkyl)cytosine; 5-(alkynyl)cytosine; 5-

(halo)cytosine; 5-(propynyl)cytosine; 5-(trifluoromethyl)cytosine; 5-bromo-cytidine; 5-iodo-cytidine; 5-propynyl cytosine; 6-(azo)cytosine; 6-aza-cytidine; aza cytosine; deaza cytosine; N4 (acetyl)cytosine; 1-methyl-1-deaza-pseudoisocytidine; 1-methyl-pseudoisocytidine; 2-methoxy-5-methyl-cytidine; 2-methoxy-cytidine; 2-thio-5-methyl-cytidine; 4-methoxy-1-methyl-pseudoisocytidine; 4-methoxy-pseudoisocytidine; 4-thio-1-methyl-1-deaza-pseudoisocytidine; 4-thio-1-methyl-pseudoisocytidine; 4-thio-pseudoisocytidine; 5-aza-zebularine; 5-methyl-zebularine; pyrrolo-pseudoisocytidine; Zebularine; (E)-5-(2-Bromo-vinyl)cytidine TP; 2,2'-anhydro-cytidine TP hydrochloride; 2'Fluor-N4-Bz-cytidine TP; 2'Fluoro-N4-Acetyl-cytidine TP; 2'-O-Methyl-N4-Acetyl-cytidine TP; 2'-O-methyl-N4-Bz-cytidine TP; 2'-a-Ethynylcytidine TP; 2'-a-Trifluoromethylcytidine TP; 2'-b-Ethynylcytidine TP; 2'-b-Trifluoromethylcytidine TP; 2'-Deoxy-2',2'-difluorocytidine TP; 2'-Deoxy-2'-a-mercaptopcytidine TP; 2'-Deoxy-2'-a-thiomethoxycytidine TP; 2'-Deoxy-2'-b-aminocytidine TP; 2'-Deoxy-2'-b-azidocytidine TP; 2'-Deoxy-2'-b-bromocytidine TP; 2'-Deoxy-2'-b-chlorocytidine TP; 2'-Deoxy-2'-b-fluorocytidine TP; 2'-Deoxy-2'-b-iodocytidine TP; 2'-Deoxy-2'-b-mercaptopcytidine TP; 2'-Deoxy-2'-b-thiomethoxycytidine TP; 2'-O-Methyl-5-(1-propynyl)cytidine TP; 3'-Ethynylcytidine TP; 4'-Azidocytidine TP; 4'-Carbocyclic cytidine TP; 4'-Ethynylcytidine TP; 5-(1-Propynyl)aracytidine TP; 5-(2-Chloro-phenyl)-2-thiocytidine TP; 5-(4-Amino-phenyl)-2-thiocytidine TP; 5-Aminoallyl-CTP; 5-Cyanocytidine TP; 5-Ethynylara-cytidine TP; 5-Ethynylcytidine TP; 5'-Homo-cytidine TP; 5-Methoxycytidine TP; 5-Trifluoromethyl-Cytidine TP; N4-Amino-cytidine TP; N4-Benzoyl-cytidine TP; Pseudoisocytidine; 7-methylguanosine; N2,2'-O-dimethylguanosine; N2-methylguanosine; Wyosine; 1,2'-O-dimethylguanosine; 1-methylguanosine; 2'-O-methylguanosine; 2'-O-ribosylguanosine (phosphate); 2'-O-methylguanosine; 2'-O-ribosylguanosine (phosphate); 7-aminomethyl-7-deazaguanosine; 7-cyano-7-deazaguanosine; Archaeosine; Methylwyosine; N2,7-dimethylguanosine; N2,N2,2'-O-trimethylguanosine; N2,N2,7-trimethylguanosine; N2,N2-dimethylguanosine; N2,7,2'-O-trimethylguanosine; 6-thio-guanosine; 7-deaza-guanosine; 8-oxo-guanosine; N1-methyl-guanosine; α -thio-guanosine; 2 (propyl)guanine; 2-(alkyl)guanine; 2'-Amino-2'-deoxy-GTP; 2'-Azido-2'-deoxy-GTP; 2'-Deoxy-2'-a-aminoguanosine TP; 2'-Deoxy-2'-a-azidoguanosine TP; 6 (methyl)guanine; 6-(alkyl)guanine; 6-(methyl)guanine; 6-methyl-guanosine; 7 (alkyl)guanine; 7 (deaza)guanine; 7 (methyl)guanine; 7-(alkyl)guanine; 7-(deaza)guanine; 7-(methyl)guanine; 8 (alkyl)guanine; 8 (alkynyl)guanine; 8 (halo)guanine; 8 (thioalkyl)guanine; 8-(alkenyl)guanine; 8-(alkyl)guanine; 8-(alkynyl)guanine; 8-(amino)guanine; 8-(halo)guanine; 8-(hydroxyl)guanine; 8-(thioalkyl)guanine; 8-(thiol)guanine; aza guanine; deaza guanine; N (methyl)guanine; N-(methyl)guanine; 1-methyl-6-thio-guanosine; 6-methoxy-guanosine; 6-thio-7-deaza-8-azaguanosine; 6-thio-7-deaza-guanosine; 6-thio-7-methyl-guanosine; 7-deaza-8-aza-guanosine; 7-

methyl-8-oxo-guanosine; N2,N2-dimethyl-6-thio-guanosine; N2-methyl-6-thio-guanosine; 1-Me-GTP; 2'Fluoro-N2-isobutyl-guanosine TP; 2'O-methyl-N2-isobutyl-guanosine TP; 2'-a-Ethynylguanosine TP; 2'-a-Trifluoromethylguanosine TP; 2'-b-Ethynylguanosine TP; 2'-b-Trifluoromethylguanosine TP; 2'-Deoxy-2',2'-difluoroguanosine TP; 2'-Deoxy-2'-a-mercaptoguanosine TP; 2'-Deoxy-2'-a-thiomethoxyguanosine TP; 2'-Deoxy-2'-b-aminoguanosine TP; 2'-Deoxy-2'-b-azidoguanosine TP; 2'-Deoxy-2'-b-bromoguanosine TP; 2'-Deoxy-2'-b-chloroguanosine TP; 2'-Deoxy-2'-b-fluoroguanosine TP; 2'-Deoxy-2'-b-iodoguanosine TP; 2'-Deoxy-2'-b-mercaptoguanosine TP; 2'-Deoxy-2'-b-thiomethoxyguanosine TP; 4'-Azidoguanosine TP; 4'-Carbocyclic guanosine TP; 4'-Ethynylguanosine TP; 5'-Homoguanosine TP; 8-bromo-guanosine TP; 9-Deazaguanosine TP; N2-isobutyl-guanosine TP; 1-methylinosine; Inosine; 1,2'-O-dimethylinosine; 2'-O-methylinosine; 7-methylinosine; 2'-O-methylinosine; Epoxyqueuosine; galactosyl-queuosine; Mannosylqueuosine; Queuosine; allyamino-thymidine; aza thymidine; deaza thymidine; deoxy-thymidine; 2'-O-methyluridine; 2-thiouridine; 3-methyluridine; 5-carboxymethyluridine; 5-hydroxyuridine; 5-methyluridine; 5-taurinomethyl-2-thiouridine; 5-taurinomethyluridine; Dihydrouridine; Pseudouridine; (3-(3-amino-3-carboxypropyl)uridine; 1-methyl-3-(3-amino-5-carboxypropyl)pseudouridine; 1-methylpseudouridine; 1-methyl-pseudouridine; 2'-O-methyluridine; 2'-O-methylpseudouridine; 2'-O-methyluridine; 2-thio-2'-O-methyluridine; 3-(3-amino-3-carboxypropyl)uridine; 3,2'-O-dimethyluridine; 3-Methyl-pseudo-Uridine TP; 4-thiouridine; 5-(carboxyhydroxymethyl)uridine; 5-(carboxyhydroxymethyl)uridine methyl ester; 5,2'-O-dimethyluridine; 5,6-dihydro-uridine; 5-aminomethyl-2-thiouridine; 5-carbamoylmethyl-2'-O-methyluridine; 5-carbamoylmethyluridine; 5-carboxyhydroxymethyluridine; 5-carboxyhydroxymethyluridine methyl ester; 5-carboxymethylaminomethyl-2'-O-methyluridine; 5-carboxymethylaminomethyl-2-thiouridine; 5-carboxymethylaminomethyl-2-thiouridine; 5-carboxymethylaminomethyluridine; 5-carboxymethylaminomethyluridine; 5-Carbamoylmethyluridine TP; 5-methoxycarbonylmethyl-2'-O-methyluridine; 5-methoxycarbonylmethyl-2-thiouridine; 5-methoxycarbonylmethyluridine; 5-methyluridine.), 5-methoxyuridine; 5-methyl-2-thiouridine; 5-methylaminomethyl-2-selenouridine; 5-methylaminomethyl-2-thiouridine; 5-methylaminomethyluridine; 5-Methyldihydrouridine; 5-Oxyacetic acid- Uridine TP; 5-Oxyacetic acid-methyl ester-Uridine TP; N1-methyl-pseudo-uridine, N1-ethylpseudouridine; uridine 5-oxyacetic acid; uridine 5-oxyacetic acid methyl ester; 3-(3-Amino-3-carboxypropyl)-Uridine TP; 5-(iso-Pentenylaminomethyl)- 2-thiouridine TP; 5-(iso-Pentenylaminomethyl)-2'-O-methyluridine TP; 5-(iso-Pentenylaminomethyl)uridine TP; 5-propynyl uracil; α -thio-uridine; 1 (aminoalkylamino-carbonylethylenyl)-2(thio)-pseudouracil; 1 (aminoalkylaminocarbonylethylenyl)-2,4-(dithio)pseudouracil; 1 (aminoalkylaminocarbonylethylenyl)-4 (thio)pseudouracil; 1

(aminoalkylaminocarbonylethyl)enyl)-pseudouracil; 1 (aminocarbonylethyl)enyl)-2(thio)-pseudouracil; 1 (aminocarbonylethyl)enyl)-2,4-(dithio)pseudouracil; 1 (aminocarbonylethyl)enyl)-4 (thio)pseudouracil; 1 (aminocarbonylethyl)enyl)-pseudouracil; 1 substituted 2(thio)-pseudouracil; 1 substituted 2,4-(dithio)pseudouracil; 1 substituted 4 (thio)pseudouracil; 1 substituted pseudouracil; 1-(aminoalkylamino-carbonylethyl)enyl)-2-(thio)-pseudouracil; 1-Methyl-3-(3-amino-3-carboxypropyl) pseudouridine TP; 1-Methyl-3-(3-amino-3-carboxypropyl)pseudo-UTP; 1-Methyl-pseudo-UTP; 2 (thio)pseudouracil; 2' deoxy uridine; 2' fluorouridine; 2-(thio)uracil; 2,4-(dithio)pseudouracil; 2' methyl, 2' amino, 2' azido, 2' fluoro-guanosine; 2'-Amino-2'-deoxy-UTP; 2'-Azido-2'-deoxy-UTP; 2'-Azido-deoxyuridine TP; 2'-O-methylpseudouridine; 2' deoxy uridine; 2' fluorouridine; 2'-Deoxy-2'-a-aminouridine TP; 2'-Deoxy-2'-a-azidouridine TP; 2-methylpseudouridine; 3 (3 amino-3 carboxypropyl)uracil; 4 (thio)pseudouracil; 4-(thio)pseudouracil; 4-(thio)uracil; 4-thiouracil; 5 (1,3-diazole-1-alkyl)uracil; 5 (2-aminopropyl)uracil; 5 (aminoalkyl)uracil; 5 (dimethylaminoalkyl)uracil; 5 (guanidiniumalkyl)uracil; 5 (methoxycarbonylmethyl)-2-(thio)uracil; 5 (methoxycarbonylmethyl)uracil; 5 (methyl) 2 (thio)uracil; 5 (methyl) 2,4 (dithio)uracil; 5 (methyl) 4 (thio)uracil; 5 (methylaminomethyl)-2 (thio)uracil; 5 (methylaminomethyl)-2,4 (dithio)uracil; 5 (methylaminomethyl)-4 (thio)uracil; 5 (propynyl)uracil; 5 (trifluoromethyl)uracil; 5-(2-aminopropyl)uracil; 5-(alkyl)-2-(thio)pseudouracil; 5-(alkyl)-2,4 (dithio)pseudouracil; 5-(alkyl)-4 (thio)pseudouracil; 5-(alkyl)pseudouracil; 5-(alkyl)uracil; 5-(alkynyl)uracil; 5-(allylamino)uracil; 5-(cyanoalkyl)uracil; 5-(dialkylaminoalkyl)uracil; 5-(dimethylaminoalkyl)uracil; 5-(guanidiniumalkyl)uracil; 5-(halo)uracil; 5-(1,3-diazole-1-alkyl)uracil; 5-(methoxy)uracil; 5-(methoxycarbonylmethyl)-2-(thio)uracil; 5-(methoxycarbonylmethyl)uracil; 5-(methyl) 2(thio)uracil; 5-(methyl) 2,4 (dithio)uracil; 5-(methyl) 4 (thio)uracil; 5-(methyl)-2-(thio)pseudouracil; 5-(methyl)-2,4 (dithio)pseudouracil; 5-(methyl)-4 (thio)pseudouracil; 5-(methyl)pseudouracil; 5-(methylaminomethyl)-2 (thio)uracil; 5-(methylaminomethyl)-2,4(dithio)uracil; 5-(methylaminomethyl)-4-(thio)uracil; 5-(propynyl)uracil; 5-(trifluoromethyl)uracil; 5-aminoallyl-uridine; 5-bromo-uridine; 5-iodo-uridine; 5-uracil; 6 (azo)uracil; 6-(azo)uracil; 6-aza-uridine; allylamino-uracil; aza uracil; deaza uracil; N3 (methyl)uracil; P pseudo-UTP-1-2-ethanoic acid; Pseudouracil; 4-Thio-pseudo-UTP; 1-carboxymethyl-pseudouridine; 1-methyl-1-deaza-pseudouridine; 1-propynyl-uridine; 1-taurinomethyl-1-methyl-uridine; 1-taurinomethyl-4-thio-uridine; 1-taurinomethyl-pseudouridine; 2-methoxy-4-thio-pseudouridine; 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; ($\pm$)1-(2-

Hydroxypropyl)pseudouridine TP; (2R)-1-(2-Hydroxypropyl)pseudouridine TP; (2S)-1-(2-Hydroxypropyl)pseudouridine TP; (E)-5-(2-Bromo-vinyl)ara-uridine TP; (E)-5-(2-Bromo-vinyl)uridine TP; (Z)-5-(2-Bromo-vinyl)ara-uridine TP; (Z)-5-(2-Bromo-vinyl)uridine TP; 1-(2,2,2-Trifluoroethyl)-pseudo-UTP; 1-(2,2,3,3,3-Pentafluoropropyl)pseudouridine TP; 1-(2,2-Diethoxyethyl)pseudouridine TP; 1-(2,4,6-Trimethylbenzyl)pseudouridine TP; 1-(2,4,6-Trimethyl-benzyl)pseudo-UTP; 1-(2,4,6-Trimethyl-phenyl)pseudo-UTP; 1-(2-Amino-2-carboxyethyl)pseudo-UTP; 1-(2-Amino-ethyl)pseudo-UTP; 1-(2-Hydroxyethyl)pseudouridine TP; 1-(2-Methoxyethyl)pseudouridine TP; 1-(3,4-Bis-trifluoromethoxybenzyl)pseudouridine TP; 1-(3,4-Dimethoxybenzyl)pseudouridine TP; 1-(3-Amino-3-carboxypropyl)pseudo-UTP; 1-(3-Amino-propyl)pseudo-UTP; 1-(3-Cyclopropyl-prop-2-ynyl)pseudouridine TP; 1-(4-Amino-4-carboxybutyl)pseudo-UTP; 1-(4-Amino-benzyl)pseudo-UTP; 1-(4-Amino-butyl)pseudo-UTP; 1-(4-Amino-phenyl)pseudo-UTP; 1-(4-Azidobenzyl)pseudouridine TP; 1-(4-Bromobenzyl)pseudouridine TP; 1-(4-Chlorobenzyl)pseudouridine TP; 1-(4-Fluorobenzyl)pseudouridine TP; 1-(4-Iodobenzyl)pseudouridine TP; 1-(4-Methanesulfonylbenzyl)pseudouridine TP; 1-(4-Methoxybenzyl)pseudouridine TP; 1-(4-Methoxy-benzyl)pseudo-UTP; 1-(4-Methoxy-phenyl)pseudo-UTP; 1-(4-Methylbenzyl)pseudouridine TP; 1-(4-Methyl-benzyl)pseudo-UTP; 1-(4-Nitrobenzyl)pseudouridine TP; 1-(4-Nitro-benzyl)pseudo-UTP; 1-(4-Nitro-phenyl)pseudo-UTP; 1-(4-Thiomethoxybenzyl)pseudouridine TP; 1-(4-Trifluoromethoxybenzyl)pseudouridine TP; 1-(4-Trifluoromethylbenzyl)pseudouridine TP; 1-(5-Amino-pentyl)pseudo-UTP; 1-(6-Amino-hexyl)pseudo-UTP; 1,6-Dimethyl-pseudo-UTP; 1-[3-(2-{2-[2-(2-Aminoethoxy)-ethoxy]-ethoxy}-ethoxy)-propionyl]pseudouridine TP; 1-{3-[2-(2-Aminoethoxy)-ethoxy]-propionyl } pseudouridine TP; 1-Acetylpsudouridine TP; 1-Alkyl-6-(1-propynyl)-pseudo-UTP; 1-Alkyl-6-(2-propynyl)-pseudo-UTP; 1-Alkyl-6-allyl-pseudo-UTP; 1-Alkyl-6-ethynyl-pseudo-UTP; 1-Alkyl-6-homoallyl-pseudo-UTP; 1-Alkyl-6-vinyl-pseudo-UTP; 1-Allylpsudouridine TP; 1-Aminomethyl-pseudo-UTP; 1-Benzoylpsudouridine TP; 1-Benzoyloxymethylpsudouridine TP; 1-Benzyl-pseudo-UTP; 1-Biotinyl-PEG2-pseudouridine TP; 1-Biotinylpsudouridine TP; 1-Butyl-pseudo-UTP; 1-Cyanomethylpsudouridine TP; 1-Cyclobutylmethyl-pseudo-UTP; 1-Cyclobutyl-pseudo-UTP; 1-Cycloheptylmethyl-pseudo-UTP; 1-Cycloheptyl-pseudo-UTP; 1-Cyclohexylmethyl-pseudo-UTP; 1-Cyclohexyl-pseudo-UTP; 1-Cyclooctylmethyl-pseudo-UTP; 1-Cyclooctyl-pseudo-UTP; 1-Cyclopentylmethyl-pseudo-UTP; 1-Cyclopentyl-pseudo-UTP; 1-Cyclopropylmethyl-pseudo-UTP; 1-Cyclopropyl-pseudo-UTP; 1-Ethyl-pseudo-UTP; 1-Hexyl-pseudo-UTP; 1-Homoallylpsudouridine TP; 1-Hydroxymethylpsudouridine TP; 1-iso-propyl-pseudo-UTP; 1-Me-2-thio-pseudo-UTP; 1-Me-4-thio-pseudo-UTP; 1-Me-alpha-thio-pseudo-UTP; 1-Methanesulfonylmethylpsudouridine TP; 1-Methoxymethylpsudouridine TP; 1-

Methyl-6-(2,2,2-Trifluoroethyl)pseudo-UTP; 1-Methyl-6-(4-morpholino)-pseudo-UTP; 1-Methyl-6-(4-thiomorpholino)-pseudo-UTP; 1-Methyl-6-(substituted phenyl)pseudo-UTP; 1-Methyl-6-amino-pseudo-UTP; 1-Methyl-6-azido-pseudo-UTP; 1-Methyl-6-bromo-pseudo-UTP; 1-Methyl-6-butyl-pseudo-UTP; 1-Methyl-6-chloro-pseudo-UTP; 1-Methyl-6-cyano-pseudo-UTP; 1-Methyl-6-dimethylamino-pseudo-UTP; 1-Methyl-6-ethoxy-pseudo-UTP; 1-Methyl-6-ethylcarboxylate-pseudo-UTP; 1-Methyl-6-ethyl-pseudo-UTP; 1-Methyl-6-fluoro-pseudo-UTP; 1-Methyl-6-formyl-pseudo-UTP; 1-Methyl-6-hydroxyamino-pseudo-UTP; 1-Methyl-6-hydroxy-pseudo-UTP; 1-Methyl-6-iodo-pseudo-UTP; 1-Methyl-6-iso-propyl-pseudo-UTP; 1-Methyl-6-methoxy-pseudo-UTP; 1-Methyl-6-methylamino-pseudo-UTP; 1-Methyl-6-phenyl-pseudo-UTP; 1-Methyl-6-propyl-pseudo-UTP; 1-Methyl-6-tert-butyl-pseudo-UTP; 1-Methyl-6-trifluoromethoxy-pseudo-UTP; 1-Methyl-6-trifluoromethyl-pseudo-UTP; 1-Morpholinomethylpseudouridine TP; 1-Pentyl-pseudo-UTP; 1-Phenyl-pseudo-UTP; 1-Pivaloylpseudouridine TP; 1-Propargylpseudouridine TP; 1-Propyl-pseudo-UTP; 1-propynylpseudouridine; 1-p-tolyl-pseudo-UTP; 1-tert-Butyl-pseudo-UTP; 1-Thiomethoxymethylpseudouridine TP; 1-Thiomorpholinomethylpseudouridine TP; 1-Trifluoroacetylpsudouridine TP; 1-Trifluoromethyl-pseudo-UTP; 1-Vinylpseudouridine TP; 2,2'-anhydro-uridine TP; 2'-bromo-deoxyuridine TP; 2'-F-5-Methyl-2'-deoxy-UTP; 2'-OMe-5-Me-UTP; 2'-OMe-pseudo-UTP; 2'-a-Ethynyluridine TP; 2'-a-Trifluoromethyluridine TP; 2'-b-Ethynyluridine TP; 2'-b-Trifluoromethyluridine TP; 2'-Deoxy-2',2'-difluorouridine TP; 2'-Deoxy-2'-a-mercaptopuridine TP; 2'-Deoxy-2'-a-thiomethoxyuridine TP; 2'-Deoxy-2'-b-aminouridine TP; 2'-Deoxy-2'-b-azidouridine TP; 2'-Deoxy-2'-b-bromouridine TP; 2'-Deoxy-2'-b-chlorouridine TP; 2'-Deoxy-2'-b-fluorouridine TP; 2'-Deoxy-2'-b-iodouridine TP; 2'-Deoxy-2'-b-mercaptopuridine TP; 2'-Deoxy-2'-b-thiomethoxyuridine TP; 2-methoxy-4-thio-uridine; 2-methoxyuridine; 2'-O-Methyl-5-(1-propynyl)uridine TP; 3-Alkyl-pseudo-UTP; 4'-Azidouridine TP; 4'-Carbocyclic uridine TP; 4'-Ethynyluridine TP; 5-(1-Propynyl)ara-uridine TP; 5-(2-Furanyl)uridine TP; 5-Cyanouridine TP; 5-Dimethylaminouridine TP; 5'-Homo-uridine TP; 5-iodo-2'-fluoro-deoxyuridine TP; 5-Phenylethynyluridine TP; 5-Trideuteromethyl-6-deuterouridine TP; 5-Trifluoromethyl-Uridine TP; 5-Vinylarauridine TP; 6-(2,2,2-Trifluoroethyl)-pseudo-UTP; 6-(4-Morpholino)-pseudo-UTP; 6-(4-Thiomorpholino)-pseudo-UTP; 6-(Substituted-Phenyl)-pseudo-UTP; 6-Amino-pseudo-UTP; 6-Azido-pseudo-UTP; 6-Bromo-pseudo-UTP; 6-Butyl-pseudo-UTP; 6-Chloro-pseudo-UTP; 6-Cyano-pseudo-UTP; 6-Dimethylamino-pseudo-UTP; 6-Ethoxy-pseudo-UTP; 6-Ethylcarboxylate-pseudo-UTP; 6-Ethyl-pseudo-UTP; 6-Fluoro-pseudo-UTP; 6-Formyl-pseudo-UTP; 6-Hydroxyamino-pseudo-UTP; 6-Hydroxy-pseudo-UTP; 6-Iodo-pseudo-UTP; 6-iso-Propyl-pseudo-UTP; 6-Methoxy-pseudo-UTP; 6-Methylamino-pseudo-UTP; 6-Methyl-pseudo-UTP; 6-Phenyl-pseudo-UTP; 6-Phenyl-

pseudo-UTP; 6-Propyl-pseudo-UTP; 6-tert-Butyl-pseudo-UTP; 6-Trifluoromethoxy-pseudo-UTP; 6-Trifluoromethyl-pseudo-UTP; Alpha-thio-pseudo-UTP; Pseudouridine 1-(4-methylbenzenesulfonic acid) TP; Pseudouridine 1-(4-methylbenzoic acid) TP; Pseudouridine TP 1-[3-(2-ethoxy)]propionic acid; Pseudouridine TP 1-[3-{2-(2-[2-(2-ethoxy)-ethoxy]-ethoxy)-ethoxy}]propionic acid; Pseudouridine TP 1-[3-{2-(2-[2-{2(2-ethoxy)-ethoxy}-ethoxy]-ethoxy)-ethoxy}]propionic acid; Pseudouridine TP 1-[3-{2-(2-[2-ethoxy]-ethoxy)-ethoxy}]propionic acid; Pseudouridine TP 1-[3-{2-(2-ethoxy)-ethoxy}] propionic acid; Pseudouridine TP 1-methylphosphonic acid; Pseudouridine TP 1-methylphosphonic acid diethyl ester; Pseudo-UTP-N1-3-propionic acid; Pseudo-UTP-N1-4-butanoic acid; Pseudo-UTP-N1-5-pentanoic acid; Pseudo-UTP-N1-6-hexanoic acid; Pseudo-UTP-N1-7-heptanoic acid; Pseudo-UTP-N1-methyl-p-benzoic acid; Pseudo-UTP-N1-p-benzoic acid; Wybutosine; Hydroxywybutosine; Isowyosine; Peroxywybutosine; undermodified hydroxywybutosine; 4-demethylwyosine; 2,6-(diamino)purine; 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl; 1,3-(diaz)-2-(oxo)-phenthiazin-1-yl; 1,3-(diaz)-2-(oxo)-phenoxazin-1-yl; 1,3,5-(triaz)-2,6-(diox)-naphthalene; 2(amino)purine; 2,4,5-(trimethyl)phenyl; 2' methyl, 2' amino, 2' azido, 2' fluoro-cytidine; 2' methyl, 2' amino, 2' azido, 2' fluoro-adenine; 2' methyl, 2' amino, 2' azido, 2' fluoro-uridine; 2'-amino-2'-deoxyribose; 2-amino-6-Chloro-purine; 2-aza-inosinyl; 2'-azido-2'-deoxyribose; 2'fluoro-2'-deoxyribose; 2'-fluoro-modified bases; 2'-O-methyl-ribose; 2-oxo-7-aminopyridopyrimidin-3-yl; 2-oxo-pyridopyrimidine-3-yl; 2-pyridinone; 3 nitropyrrole; 3-(methyl)-7-(propynyl)isocarbostyryl; 3-(methyl)isocarbostyryl; 4-(fluoro)-6-(methyl)benzimidazole; 4-(methyl)benzimidazole; 4-(methyl)indolyl; 4,6-(dimethyl)indolyl; 5 nitroindole; 5 substituted pyrimidines; 5-(methyl)isocarbostyryl; 5-nitroindole; 6-(aza)pyrimidine; 6-(azo)thymine; 6-(methyl)-7-(aza)indolyl; 6-chloro-purine; 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl; 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl; 7-(aminoalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenoxazin-1-yl; 7-(aminoalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenthiazin-1-yl; 7-(aminoalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenoxazin-1-yl; 7-(aza)indolyl; 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl; 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl; 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl; 7-(guanidiniumalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenoxazin-1-yl; 7-(guanidiniumalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenthiazin-1-yl; 7-(guanidiniumalkylhydroxy)-1,3-(diaz)-2-(oxo)-phenoxazin-1-yl; 7-(propynyl)isocarbostyryl; 7-(propynyl)isocarbostyryl, propynyl-7-(aza)indolyl; 7-deaza-inosinyl; 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl; 7-substituted 1,3-(diaz)-2-(oxo)-phenoxazin-1-yl; 9-(methyl)-imidizopyridinyl; Aminoindolyl; Anthracenyl; bis-ortho-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; bis-ortho-

substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; Difluorotolyl; Hypoxanthine; Imidizopyridinyl; Inosinyl; Isocarbostyryl; Isoguanisine; N2-substituted purines; N6-methyl-2-amino-purine; N6-substituted purines; N-alkylated derivative; Napthalenyl; Nitrobenzimidazolyl; Nitroimidazolyl; Nitroindazolyl; Nitropyrazolyl; Nubularine; O6-substituted purines; O-alkylated derivative; ortho-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; Oxoformycin TP; para-(aminoalkylhydroxy)-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; para-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl; Pentacenyl; Phenanthracenyl; Phenyl; propynyl-7-(aza)indolyl; Pyrenyl; pyridopyrimidin-3-yl; pyridopyrimidin-3-yl, 2-oxo-7-amino-pyridopyrimidin-3-yl; pyrrolo-pyrimidin-2-on-3-yl; Pyrrolopyrimidinyl; Pyrrolopyrizinyl; Stilbenzyl; substituted 1,2,4-triazoles; Tetracenyl; Tubercidine; Xanthine; Xanthosine-5'-TP; 2-thio-zebularine; 5-aza-2-thio-zebularine; 7-deaza-2-amino-purine; pyridin-4-one ribonucleoside; 2-Amino-riboside-TP; Formycin A TP; Formycin B TP; Pyrrolosine TP; 2'-OH-ara-adenosine TP; 2'-OH-ara-cytidine TP; 2'-OH-ara-uridine TP; 2'-OH-ara-guanosine TP; 5-(2-carbomethoxyvinyl)uridine TP; and N6-(19-Amino-pentaoxanonadecyl)adenosine TP.

In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) include a combination of at least two (*e.g.*, 2, 3, 4 or more) of the aforementioned modified nucleobases.

In some embodiments, modified nucleobases in polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) are selected from the group consisting of pseudouridine (ψ), N1-methylpseudouridine (m1 ψ), N1-ethylpseudouridine, 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-methyluridine, 5-methoxyuridine and 2'-O-methyl uridine. In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) include a combination of at least two (*e.g.*, 2, 3, 4 or more) of the aforementioned modified nucleobases.

In some embodiments, modified nucleobases in polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) are selected from the group consisting of 1-methyl-pseudouridine (m1 ψ), 5-methoxy-uridine (mo5U), 5-methyl-cytidine (m5C), pseudouridine (ψ), α -thio-guanosine, and α -thio-adenosine. In some embodiments, polynucleotides includes a combination of at least two (*e.g.*, 2, 3, 4 or more) of the aforementioned modified nucleobases.

In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise pseudouridine (ψ) and 5-methyl-cytidine (m5C). In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise 1-methyl-pseudouridine (m1 ψ). In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise 1-methyl-pseudouridine (m1 ψ) and 5-methyl-cytidine (m5C). In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise 2-thiouridine (s2U). In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise 2-thiouridine and 5-methyl-cytidine (m5C). In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise methoxy-uridine (mo5U). In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise 5-methoxy-uridine (mo5U) and 5-methyl-cytidine (m5C). In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise 2'-O-methyl uridine. In some embodiments polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise 2'-O-methyl uridine and 5-methyl-cytidine (m5C). In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise N6-methyl-adenosine (m6A). In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) comprise N6-methyl-adenosine (m6A) and 5-methyl-cytidine (m5C).

In some embodiments, polynucleotides (*e.g.*, RNA polynucleotides, such as mRNA polynucleotides) are uniformly modified (*e.g.*, fully modified, modified throughout the entire sequence) for a particular modification. For example, a polynucleotide can be uniformly modified with 5-methyl-cytidine (m5C), meaning that all cytosine residues in the mRNA sequence are replaced with 5-methyl-cytidine (m5C). Similarly, a polynucleotide can be uniformly modified for any type of nucleoside residue present in the sequence by replacement with a modified residue such as those set forth above.

Exemplary nucleobases and nucleosides having a modified cytosine include N4-acetyl-cytidine (ac4C), 5-methyl-cytidine (m5C), 5-halo-cytidine (*e.g.*, 5-iodo-cytidine), 5-hydroxymethyl-cytidine (hm5C), 1-methyl-pseudoisocytidine, 2-thio-cytidine (s2C), and 2-thio-5-methyl-cytidine.

In some embodiments, a modified nucleobase is a modified uridine. Exemplary nucleobases and nucleosides having a modified uridine include 5-cyano uridine, and 4'-thio uridine.

In some embodiments, a modified nucleobase is a modified adenine. Exemplary nucleobases and nucleosides having a modified adenine include 7-deaza-adenine, 1-methyl-adenosine (m1A), 2-methyl-adenine (m2A), and N6-methyl-adenosine (m6A).

In some embodiments, a modified nucleobase is a modified guanine. Exemplary nucleobases and nucleosides having a modified guanine include inosine (I), 1-methyl-inosine (m1I), wyosine (imG), methylwyosine (mimG), 7-deaza-guanosine, 7-cyano-7-deaza-guanosine (preQ0), 7-aminomethyl-7-deaza-guanosine (preQ1), 7-methyl-guanosine (m7G), 1-methyl-guanosine (m1G), 8-oxo-guanosine, 7-methyl-8-oxo-guanosine.

The polynucleotides of the present disclosure may be partially or fully modified along the entire length of the molecule. For example, one or more or all or a given type of nucleotide (*e.g.*, purine or pyrimidine, or any one or more or all of A, G, U, C) may be uniformly modified in a polynucleotide of the disclosure, or in a given predetermined sequence region thereof (*e.g.*, in the mRNA including or excluding the polyA tail). In some embodiments, all nucleotides X in a polynucleotide of the present disclosure (or in a given sequence region thereof) are modified nucleotides, 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.

The polynucleotide may contain from about 1% to about 100% modified 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 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 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 unmodified A, G, U, or C.

The polynucleotides may contain at a minimum 1% and at maximum 100% modified nucleotides, or any intervening percentage, such as at least 5% modified nucleotides, at least 10% modified nucleotides, at least 25% modified nucleotides, at least 50% modified nucleotides, at least 80% modified nucleotides, or at least 90% modified nucleotides. For example, the polynucleotides may contain a modified pyrimidine such as a modified 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 the polynucleotide is replaced with a modified uracil (*e.g.*, a 5-substituted uracil). The modified 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 embodiments, 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 a modified cytosine (*e.g.*, a 5-substituted cytosine). The modified 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).

Thus, in some embodiments, the RNA treatments comprise a 5'UTR element, an optionally codon optimized open reading frame, and a 3'UTR element, a poly(A) sequence and/or a polyadenylation signal wherein the RNA is not chemically modified.

In some embodiments, the modified nucleobase is a modified uracil. Exemplary nucleobases and nucleosides having a modified uracil include pseudouridine (ψ), pyridin-4-one ribonucleoside, 5-aza-uridine, 6-aza-uridine, 2-thio-5-aza-uridine, 2-thio-uridine (s2U), 4-thio-uridine (s4U), 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxy-uridine (ho5U), 5-aminoallyl-uridine, 5-halo-uridine (*e.g.*, 5-iodo-uridine or 5-bromo-uridine), 3-methyl-uridine (m3U), 5-methoxy-uridine (mo5U), uridine 5-oxyacetic acid (cmo5U), uridine 5-oxyacetic acid methyl ester (mcmo5U), 5-carboxymethyl-uridine (cm5U), 1-carboxymethyl-pseudouridine, 5-carboxyhydroxymethyl-uridine (chm5U), 5-carboxyhydroxymethyl-uridine methyl ester (mchm5U), 5-methoxycarbonylmethyl-uridine (mcm5U), 5-methoxycarbonylmethyl-2-thio-uridine (mcm5s2U), 5-aminomethyl-2-thio-uridine (nm5s2U), 5-methylaminomethyl-uridine (mnm5U), 5-methylaminomethyl-2-thio-uridine (mnm5s2U), 5-methylaminomethyl-2-seleno-uridine (mnm5se2U), 5-carbamoylmethyl-uridine (ncm5U), 5-carboxymethylaminomethyl-uridine (cmnm5U), 5-carboxymethylaminomethyl-2-thio-uridine (cmnm5s2U), 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-taurinomethyl-uridine (τ m5U), 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uridine (τ m5s2U), 1-taurinomethyl-4-thio-pseudouridine, 5-methyl-uridine (m5U, *i.e.*, having the nucleobase deoxythymine), 1-methyl-pseudouridine (m1 ψ), 5-methyl-2-thio-uridine (m5s2U), 1-methyl-4-thio-pseudouridine (m1s4 ψ), 4-thio-1-methyl-pseudouridine, 3-methyl-pseudouridine (m3 ψ), 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouridine (D), dihydropseudouridine, 5,6-dihydrouridine, 5-methyl-dihydrouridine (m5D), 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 2-methoxy-uridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine, N1-ethylpseudouridine, 3-(3-amino-3-carboxypropyl)uridine (acp3U), 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine (acp3 ψ), 5-(isopentenylaminomethyl)uridine (inm5U), 5-

(isopentenylaminomethyl)-2-thio-uridine (inm5s2U), α -thio-uridine, 2'-O-methyl-uridine (Um), 5,2'-O-dimethyl-uridine (m5Um), 2'-O-methyl-pseudouridine (ψ m), 2-thio-2'-O-methyl-uridine (s2Um), 5-methoxycarbonylmethyl-2'-O-methyl-uridine (mcm5Um), 5-carbamoylmethyl-2'-O-methyl-uridine (ncm5Um), 5-carboxymethylaminomethyl-2'-O-methyl-uridine (cmnm5Um), 3,2'-O-dimethyl-uridine (m3Um), and 5-(isopentenylaminomethyl)-2'-O-methyl-uridine (inm5Um), 1-thio-uridine, deoxythymidine, 2'-F-ara-uridine, 2'-F-uridine, 2'-OH-ara-uridine, 5-(2-carbomethoxyvinyl) uridine, and 5-[3-(1-E-propenylamino)]uridine.

In some embodiments, the modified nucleobase is a modified cytosine. Exemplary nucleobases and nucleosides having a modified cytosine include 5-aza-cytidine, 6-aza-cytidine, pseudoisocytidine, 3-methyl-cytidine (m3C), N4-acetyl-cytidine (ac4C), 5-formyl-cytidine (f5C), N4-methyl-cytidine (m4C), 5-methyl-cytidine (m5C), 5-halo-cytidine (*e.g.*, 5-iodo-cytidine), 5-hydroxymethyl-cytidine (hm5C), 1-methyl-pseudoisocytidine, pyrrolo-cytidine, pyrrolo-pseudoisocytidine, 2-thio-cytidine (s2C), 2-thio-5-methyl-cytidine, 4-thio-pseudoisocytidine, 4-thio-1-methyl-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-cytidine, 2-methoxy-5-methyl-cytidine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, lysidine (k2C), α -thio-cytidine, 2'-O-methyl-cytidine (Cm), 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-cytidine, 2'-F-ara-cytidine, 2'-F-cytidine, and 2'-OH-ara-cytidine.

In some embodiments, the modified nucleobase is a modified adenine. Exemplary nucleobases and nucleosides having a modified 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-adenosine, 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-adenosine (m1A), 2-methyl-adenine (m2A), N6-methyl-adenosine (m6A), 2-methylthio-N6-methyl-adenosine (ms2m6A), N6-isopentenyl-adenosine (i6A), 2-methylthio-N6-isopentenyl-adenosine (ms2i6A), N6-(*cis*-hydroxyisopentenyl)adenosine (io6A), 2-methylthio-N6-(*cis*-hydroxyisopentenyl)adenosine (ms2io6A), N6-glycinylocarbamoyl-adenosine (g6A), N6-threonylocarbamoyl-adenosine (t6A), N6-methyl-N6-threonylocarbamoyl-adenosine (m6t6A), 2-methylthio-N6-threonylocarbamoyl-adenosine (ms2g6A), N6,N6-dimethyl-adenosine (m62A), N6-hydroxynorvalylcarbamoyl-adenosine (hn6A), 2-methylthio-N6-hydroxynorvalylcarbamoyl-adenosine (ms2hn6A), N6-acetyl-adenosine (ac6A), 7-methyl-adenine, 2-methylthio-adenine, 2-methoxy-adenine, α -thio-

adenosine, 2'-O-methyl-adenosine (Am), N6,2'-O-dimethyl-adenosine (m6Am), N6,N6,2'-O-trimethyl-adenosine (m62Am), 1,2'-O-dimethyl-adenosine (m1Am), 2'-O-ribosyladenosine (phosphate) (Ar(p)), 2-amino-N6-methyl-purine, 1-thio-adenosine, 8-azido-adenosine, 2'-F-ara-adenosine, 2'-F-adenosine, 2'-OH-ara-adenosine, and N6-(19-amino-pentaoxanonadecyl)-adenosine.

In some embodiments, the modified nucleobase is a modified guanine. Exemplary nucleobases and nucleosides having a modified 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-guanosine, queuosine (Q), epoxyqueuosine (oQ), galactosyl-queuosine (galQ), mannosyl-queuosine (manQ), 7-cyano-7-deaza-guanosine (preQ0), 7-aminomethyl-7-deaza-guanosine (preQ1), archaeosine (G+), 7-deaza-8-aza-guanosine, 6-thio-guanosine, 6-thio-7-deaza-guanosine, 6-thio-7-deaza-8-aza-guanosine, 7-methyl-guanosine (m7G), 6-thio-7-methyl-guanosine, 7-methyl-inosine, 6-methoxy-guanosine, 1-methyl-guanosine (m1G), N2-methyl-guanosine (m2G), N2,N2-dimethyl-guanosine (m22G), N2,7-dimethyl-guanosine (m2,7G), N2, N2,7-dimethyl-guanosine (m2,2,7G), 8-oxo-guanosine, 7-methyl-8-oxo-guanosine, 1-methyl-6-thio-guanosine, N2-methyl-6-thio-guanosine, N2,N2-dimethyl-6-thio-guanosine, α -thio-guanosine, 2'-O-methyl-guanosine (Gm), 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), 2'-O-ribosylguanosine (phosphate) (Gr(p)), 1-thio-guanosine, O6-methyl-guanosine, 2'-F-ara-guanosine, and 2'-F-guanosine.

Antibodies and antigen binding fragments thereof of the present disclosure comprise at least one RNA polynucleotide, such as an 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 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.

Sequence Optimization

In some embodiments, 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 CA) and/or proprietary methods. In some embodiments, the open reading frame (ORF) sequence is optimized using optimization algorithms.

In some embodiments, a codon optimized sequence shares less than 95% sequence identity to a naturally-occurring or wild-type sequence ORF (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a coronavirus antigen). In some embodiments, a codon optimized sequence shares less than 90% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a coronavirus antigen). In some embodiments, a codon optimized sequence shares less than 85% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a coronavirus antigen). In some embodiments, a codon optimized sequence shares less than 80% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a coronavirus antigen). In some embodiments, a codon optimized sequence shares less than 75% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a coronavirus antigen).

In some embodiments, a codon optimized sequence shares between 65% and 85% (*e.g.*, between about 67% and about 85% or between about 67% and about 80%) sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a coronavirus antigen). In some embodiments, a codon optimized sequence shares between 65% and 75% or about 80% sequence identity to a naturally-occurring or wild-type sequence (*e.g.*, a naturally-occurring or wild-type mRNA sequence encoding a coronavirus antigen).

In some embodiments, a codon-optimized sequence encodes an antigen that is as immunogenic as, or more immunogenic than (*e.g.*, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 100%, or at least 200% more), than a coronavirus antigen encoded by a non-codon-optimized sequence.

When transfected into mammalian host cells, the modified mRNAs have a stability of between 12-18 hours, or greater than 18 hours, *e.g.*, 24, 36, 48, 60, 72, or greater than 72 hours and are capable of being expressed by the mammalian host cells.

In some embodiments, a codon optimized RNA may be one in which the levels of G/C are enhanced. The G/C-content of nucleic acid molecules (*e.g.*, mRNA) may influence the stability of the RNA. RNA having an increased amount of guanine (G) and/or cytosine (C) residues may be functionally more stable than RNA containing a large amount of adenine (A) and thymine (T) or uracil (U) nucleotides. As an example, WO02/098443 discloses a pharmaceutical composition containing an mRNA stabilized by sequence modifications in the translated region. Due to the degeneracy of the genetic code, the modifications work by substituting existing codons for those that promote greater RNA stability without changing the resulting amino acid. The approach is limited to coding regions of the RNA.

Chemically Unmodified Nucleotides

In some embodiments, an RNA (*e.g.*, mRNA) is not chemically modified and comprises the standard ribonucleotides consisting of adenosine, guanosine, cytosine and uridine. In some embodiments, nucleotides and nucleosides of the present disclosure comprise standard nucleoside residues such as those present in transcribed RNA (*e.g.* A, G, C, or U). In some embodiments, nucleotides and nucleosides of the present disclosure comprise standard deoxyribonucleosides such as those present in DNA (*e.g.* dA, dG, dC, or dT).

Chemical Modifications

The compositions of the present disclosure comprise, in some embodiments, an RNA having an open reading frame encoding a coronavirus antigen, wherein the nucleic acid comprises nucleotides and/or nucleosides that can be standard (unmodified) or modified as is known in the art. In some embodiments, nucleotides and nucleosides of the present disclosure comprise modified nucleotides or nucleosides. Such modified nucleotides and nucleosides can be naturally-occurring modified nucleotides and nucleosides or non-naturally occurring modified nucleotides and nucleosides. Such modifications can include those at the sugar, backbone, or nucleobase portion of the nucleotide and/or nucleoside as are recognized in the art.

In some embodiments, a naturally-occurring modified nucleotide or nucleoside of the disclosure is one as is generally known or recognized in the art. Non-limiting examples of such naturally occurring modified nucleotides and nucleosides can be found, *inter alia*, in the widely recognized MODOMICS database.

In some embodiments, a non-naturally occurring modified nucleotide or nucleoside of the disclosure is one as is generally known or recognized in the art. Non-limiting examples of such

non-naturally occurring modified nucleotides and nucleosides can be found, inter alia, in published US application Nos. PCT/US2012/058519; PCT/US2013/075177; PCT/US2014/058897; PCT/US2014/058891; PCT/US2014/070413; PCT/US2015/36773; PCT/US2015/36759; PCT/US2015/36771; or PCT/IB2017/051367 all of which are incorporated
5 by reference herein.

Hence, nucleic acids of the disclosure (*e.g.*, DNA nucleic acids and RNA nucleic acids, such as mRNA nucleic acids) can comprise standard nucleotides and nucleosides, naturally-occurring nucleotides and nucleosides, non-naturally-occurring nucleotides and nucleosides, or any combination thereof.

10 Nucleic acids of the disclosure (*e.g.*, DNA nucleic acids and RNA nucleic acids, such as mRNA nucleic acids), in some embodiments, comprise various (more than one) different types of standard and/or modified nucleotides and nucleosides. In some embodiments, a particular region of a nucleic acid contains one, two or more (optionally different) types of standard and/or modified nucleotides and nucleosides.

15 In some embodiments, a modified RNA nucleic acid (*e.g.*, a modified mRNA nucleic acid), introduced to a cell or organism, exhibits reduced degradation in the cell or organism, respectively, relative to an unmodified nucleic acid comprising standard nucleotides and nucleosides.

In some embodiments, a modified RNA nucleic acid (*e.g.*, a modified mRNA nucleic acid), introduced into a cell or organism, may exhibit reduced immunogenicity in the cell or organism, respectively (*e.g.*, a reduced innate response) relative to an unmodified nucleic acid comprising standard nucleotides and nucleosides.
20

Nucleic acids (*e.g.*, RNA nucleic acids, such as mRNA nucleic acids), in some embodiments, comprise non-natural modified nucleotides that are introduced during synthesis or post-synthesis of the nucleic acids to achieve desired functions or properties. The modifications may be present on internucleotide linkages, purine or pyrimidine bases, or sugars. The modification may be introduced with chemical synthesis or with a polymerase enzyme at the terminal of a chain or anywhere else in the chain. Any of the regions of a nucleic acid may be chemically modified.
25

30 The present disclosure provides for modified nucleosides and nucleotides of a nucleic acid (*e.g.*, RNA nucleic acids, such as mRNA nucleic acids). A “nucleoside” refers to a compound containing a sugar molecule (*e.g.*, a pentose or ribose) or a derivative thereof in combination with an organic base (*e.g.*, a purine or pyrimidine) or a derivative thereof (also referred to herein as “nucleobase”). A “nucleotide” refers to a nucleoside, including a phosphate
35 group. Modified nucleotides may be synthesized by any useful method, such as, for example,

chemically, enzymatically, or recombinantly, to include one or more modified or non-natural nucleosides. Nucleic acids can comprise a region or regions of linked nucleosides. Such regions may have variable backbone linkages. The linkages can be standard phosphodiester linkages, in which case the nucleic acids would comprise regions of nucleotides.

5 Modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard
10 base structures, such as, for example, in those nucleic acids having at least one chemical modification. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine or uracil. Any combination of base/sugar or linker may be incorporated into nucleic acids of the present disclosure.

In some embodiments, modified nucleobases in nucleic acids (*e.g.*, RNA nucleic acids, such as mRNA nucleic acids) comprise 1-methyl-pseudouridine (m1ψ), 1-ethyl-pseudouridine (e1ψ), 5-methoxy-uridine (mo5U), 5-methyl-cytidine (m5C), and/or pseudouridine (ψ). In some
15 embodiments, modified nucleobases in nucleic acids (*e.g.*, RNA nucleic acids, such as mRNA nucleic acids) comprise 5-methoxymethyl uridine, 5-methylthio uridine, 1-methoxymethyl pseudouridine, 5-methyl cytidine, and/or 5-methoxy cytidine. In some embodiments, the
20 polyribonucleotide includes a combination of at least two (*e.g.*, 2, 3, 4 or more) of any of the aforementioned modified nucleobases, including but not limited to chemical modifications.

In some embodiments, a mRNA of the disclosure comprises 1-methyl-pseudouridine (m1ψ) substitutions at one or more or all uridine positions of the nucleic acid.

In some embodiments, a mRNA of the disclosure comprises 1-methyl-pseudouridine
25 (m1ψ) substitutions at one or more or all uridine positions of the nucleic acid and 5-methyl cytidine substitutions at one or more or all cytidine positions of the nucleic acid.

In some embodiments, a mRNA of the disclosure comprises pseudouridine (ψ) substitutions at one or more or all uridine positions of the nucleic acid.

In some embodiments, a mRNA of the disclosure comprises pseudouridine (ψ)
30 substitutions at one or more or all uridine positions of the nucleic acid and 5-methyl cytidine substitutions at one or more or all cytidine positions of the nucleic acid..

In some embodiments, a mRNA of the disclosure comprises uridine at one or more or all uridine positions of the nucleic acid.

In some embodiments, mRNAs are uniformly modified (*e.g.*, fully modified, modified
35 throughout the entire sequence) for a particular modification. For example, a nucleic acid can be

uniformly modified with 1-methyl-pseudouridine, meaning that all uridine residues in the mRNA sequence are replaced with 1-methyl-pseudouridine. Similarly, a nucleic acid can be uniformly modified for any type of nucleoside residue present in the sequence by replacement with a modified residue such as those set forth above.

5 The nucleic acids of the present disclosure may be partially or fully modified along the entire length of the molecule. For example, one or more or all or a given type of nucleotide (*e.g.*, purine or pyrimidine, or any one or more or all of A, G, U, C) may be uniformly modified in a nucleic acid of the disclosure, or in a predetermined sequence region thereof (*e.g.*, in the mRNA including or excluding the poly(A) tail). In some embodiments, all nucleotides X in a nucleic
10 acid of the present disclosure (or in a sequence region thereof) are modified nucleotides, wherein X may be 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.

 The nucleic acid may contain from about 1% to about 100% modified nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, *i.e.*,
15 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 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%,
20 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 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 unmodified A, G,
25 U, or C.

 The mRNAs may contain at a minimum 1% and at maximum 100% modified nucleotides, or any intervening percentage, such as at least 5% modified nucleotides, at least 10% modified nucleotides, at least 25% modified nucleotides, at least 50% modified nucleotides, at least 80% modified nucleotides, or at least 90% modified nucleotides. For example, the
30 nucleic acids may contain a modified pyrimidine such as a modified 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 the nucleic acid is replaced with a modified uracil (*e.g.*, a 5-substituted uracil). The modified 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
35 unique structures). 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 cytosine in the nucleic acid is replaced with a modified cytosine (*e.g.*, a 5-substituted cytosine). The modified 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).

5

Untranslated Regions (UTRs)

The mRNAs of the present disclosure may comprise one or more regions or parts which act or function as an untranslated region. Where mRNAs are designed to encode at least one antigen of interest, the nucleic acid may comprise one or more of these untranslated regions (UTRs).

10 Wild-type untranslated regions of a nucleic acid are transcribed but not translated. In mRNA, the 5' UTR starts at the transcription start site and continues to the start codon but does not include the start codon; whereas, the 3' UTR starts immediately following the stop codon and continues until the transcriptional termination signal. There is growing body of evidence about the regulatory roles played by the UTRs in terms of stability of the nucleic acid molecule and translation. The regulatory features of a UTR can be incorporated into the polynucleotides of the present disclosure to, among other things, enhance the stability of the molecule. The specific features can also be incorporated to ensure controlled down-regulation of the transcript in case they are misdirected to undesired organs sites. A variety of 5'UTR and 3'UTR sequences are known and available in the art.

20 A 5' UTR is region of an mRNA that is directly upstream (5') from the start codon (the first codon of an mRNA transcript translated by a ribosome). A 5' UTR does not encode a protein (is non-coding). Natural 5'UTRs have features that play roles in translation initiation. They harbor signatures like Kozak sequences which are commonly known to be involved in the process by which the ribosome initiates translation of many genes. Kozak sequences have the consensus CCR(A/G)CCAUGG (SEQ ID NO: 15), where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. 5'UTR also have been known to form secondary structures which are involved in elongation factor binding.

In some embodiments of the disclosure, a 5' UTR is a heterologous UTR, *i.e.*, is a UTR found in nature associated with a different ORF. In another embodiment, a 5' UTR is a synthetic UTR, *i.e.*, does not occur in nature. Synthetic UTRs include UTRs that have been mutated to improve their properties, *e.g.*, which increase gene expression as well as those which are completely synthetic. Exemplary 5' UTRs include *Xenopus* or human derived α -globin or β -globin (8278063; 9012219), human cytochrome b-245 a polypeptide, and hydroxysteroid (17 β) dehydrogenase, and Tobacco etch virus (US8278063, 9012219). CMV immediate-early 1 (IE1) gene (US20140206753, WO2013/185069), the sequence GGGAUCCUACC (SEQ ID NO: 16)

35

(WO2014144196) may also be used. In another embodiment, 5' UTR of a TOP gene is a 5' UTR of a TOP gene lacking the 5' TOP motif (the oligopyrimidine tract) (*e.g.*, WO/2015101414, WO2015101415, WO/2015/062738, WO2015024667, WO2015024667; 5' UTR element derived from ribosomal protein Large 32 (L32) gene (WO/2015101414, WO2015101415, WO/2015/062738), 5' UTR element derived from the 5'UTR of an hydroxysteroid (17- β) dehydrogenase 4 gene (HSD17B4) (WO2015024667), or a 5' UTR element derived from the 5' UTR of ATP5A1 (WO2015024667) can be used. In some embodiments, an internal ribosome entry site (IRES) is used instead of a 5' UTR.

In some embodiments, a 5' UTR of the present disclosure comprises a sequence selected from SEQ ID NO: 4 and SEQ ID NO: 6.

A 3' UTR is region of an mRNA that is directly downstream (3') from the stop codon (the codon of an mRNA transcript that signals a termination of translation). A 3' UTR does not encode a protein (is non-coding). Natural or wild type 3' UTRs are known to have stretches of adenosines and uridines embedded in them. These AU rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU rich elements (AREs) can be separated into three classes (Chen et al, 1995): Class I AREs contain several dispersed copies of an AUUUA motif within U-rich regions. C-Myc and MyoD contain class I AREs. Class II AREs possess two or more overlapping UUAUUUA(U/A)(U/A) nonamers. Molecules containing this type of AREs include GM-CSF and TNF- α . Class III AREs are less well defined. These U rich regions do not contain an AUUUA motif. c-Jun and Myogenin are two well-studied examples of this class. Most proteins binding to the AREs are known to destabilize the messenger, whereas members of the ELAV family, most notably HuR, have been documented to increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3' UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message *in vivo*.

Introduction, removal or modification of 3' UTR AU rich elements (AREs) can be used to modulate the stability of nucleic acids (*e.g.*, RNA) of the disclosure. When engineering specific nucleic acids, one or more copies of an ARE can be introduced to make nucleic acids of the disclosure less stable and thereby curtail translation and decrease production of the resultant protein. Likewise, AREs can be identified and removed or mutated to increase the intracellular stability and thus increase translation and production of the resultant protein. Transfection experiments can be conducted in relevant cell lines, using nucleic acids of the disclosure and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different ARE-engineering molecules and by using an ELISA kit to the

relevant protein and assaying protein produced at 6 hour, 12 hour, 24 hour, 48 hour, and 7 days post-transfection.

3' UTRs may be heterologous or synthetic. With respect to 3' UTRs, globin UTRs, including *Xenopus* β -globin UTRs and human β -globin UTRs are known in the art (8278063, 9012219, US20110086907). A modified β -globin construct with enhanced stability in some cell types by cloning two sequential human β -globin 3'UTRs head to tail has been developed and is well known in the art (US2012/0195936, WO2014/071963). In addition α 2-globin, α 1-globin, UTRs and mutants thereof are also known in the art (WO2015101415, WO2015024667). Other 3' UTRs described in the mRNA constructs in the non-patent literature include CYBA (Ferizi et al., 2015) and albumin (Thess et al., 2015). Other exemplary 3' UTRs include that of bovine or human growth hormone (wild type or modified) (WO2013/185069, US20140206753, WO2014152774), rabbit β globin and hepatitis B virus (HBV), α -globin 3' UTR and Viral VEEV 3' UTR sequences are also known in the art. In some embodiments, the sequence UUUGAAUU (WO2014144196) is used. In some embodiments, 3' UTRs of human and mouse ribosomal protein are used. Other examples include rps9 3'UTR (WO2015101414), FIG4 (WO2015101415), and human albumin 7 (WO2015101415).

In some embodiments, a 3' UTR of the present disclosure comprises a sequence selected from SEQ ID NO: 5 and SEQ ID NO: 7.

Those of ordinary skill in the art will understand that 5'UTRs that are heterologous or synthetic may be used with any desired 3' UTR sequence. For example, a heterologous 5'UTR may be used with a synthetic 3'UTR with a heterologous 3' UTR.

Non-UTR sequences may also be used as regions or subregions within a nucleic acid. For example, introns or portions of introns sequences may be incorporated into regions of nucleic acid of the disclosure. Incorporation of intronic sequences may increase protein production as well as nucleic acid levels.

Combinations of features may be included in flanking regions and may be contained within other features. For example, the ORF may be flanked by a 5' UTR which may contain a strong Kozak translational initiation signal and/or a 3' UTR which may include an oligo(dT) sequence for templated addition of a poly-A tail. 5' UTR may comprise a first polynucleotide fragment and a second polynucleotide fragment from the same and/or different genes such as the 5' UTRs described in US Patent Application Publication No. 20100293625 and PCT/US2014/069155, herein incorporated by reference in its entirety.

It should be understood that any UTR from any gene may be incorporated into the regions of a nucleic acid. Furthermore, multiple wild-type UTRs of any known gene may be utilized. It is also within the scope of the present disclosure to provide artificial UTRs which are

not variants of wild type regions. These UTRs or portions thereof may be placed in the same orientation as in the transcript from which they were selected or may be altered in orientation or location. Hence a 5' or 3' UTR may be inverted, shortened, lengthened, made with one or more other 5' UTRs or 3' UTRs. As used herein, the term "altered" as it relates to a UTR sequence, means that the UTR has been changed in some way in relation to a reference sequence. For example, a 3' UTR or 5' UTR may be altered relative to a wild-type or native UTR by the change in orientation or location as taught above or may be altered by the inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of nucleotides. Any of these changes producing an "altered" UTR (whether 3' or 5') comprise a variant UTR.

In some embodiments, a double, triple or quadruple UTR such as a 5' UTR or 3' UTR may be used. As used herein, a "double" UTR is one in which two copies of the same UTR are encoded either in series or substantially in series. For example, a double beta-globin 3' UTR may be used as described in US Patent publication 20100129877, the contents of which are incorporated herein by reference in its entirety.

It is also within the scope of the present disclosure to have patterned UTRs. As used herein "patterned UTRs" are those UTRs which reflect a repeating or alternating pattern, such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than 3 times. In these patterns, each letter, A, B, or C represent a different UTR at the nucleotide level.

In some embodiments, flanking regions are selected from a family of transcripts whose proteins share a common function, structure, feature or property. For example, polypeptides of interest may belong to a family of proteins which are expressed in a particular cell, tissue or at some time during development. The UTRs from any of these genes may be swapped for any other UTR of the same or different family of proteins to create a new polynucleotide. As used herein, a "family of proteins" is used in the broadest sense to refer to a group of two or more polypeptides of interest which share at least one function, structure, feature, localization, origin, or expression pattern.

The untranslated region may also include translation enhancer elements (TEE). As a non-limiting example, the TEE may include those described in US Application No.20090226470, herein incorporated by reference in its entirety, and those known in the art.

The scope of the present disclosure includes embodiments wherein the 3' UTR is chemically modified. In some embodiments, the 3' UTR is modified with mRNA stabilizing elements. In some embodiments, a 3' UTR comprising mRNA stabilizing elements stabilizes mRNA and allows the mRNA to persist longer. In some embodiments, a 3'UTR comprising mRNA stabilizing elements results in an increased mRNA half life.

***In vitro* Transcription of RNA**

cDNA encoding the polynucleotides described herein may be transcribed using an *in vitro* transcription (IVT) system. *In vitro* transcription of RNA is known in the art and is described in International Publication WO 2014/152027, which is incorporated by reference herein in its entirety. In some embodiments, the RNA of the present disclosure is prepared in accordance with any one or more of the methods described in WO 2018/053209 and WO 2019/036682, each of which is incorporated by reference herein.

In some embodiments, the RNA transcript is generated using a non-amplified, linearized DNA template in an *in vitro* transcription reaction to generate the RNA transcript. In some embodiments, the template DNA is isolated DNA. In some embodiments, the template DNA is cDNA. In some embodiments, the cDNA is formed by reverse transcription of a RNA polynucleotide, for example, but not limited to coronavirus mRNA. In some embodiments, cells, *e.g.*, bacterial cells, *e.g.*, *E. coli*, *e.g.*, DH-1 cells are transfected with the plasmid DNA template. In some embodiments, the transfected cells are cultured to replicate the plasmid DNA which is then isolated and purified. In some embodiments, the DNA template includes a RNA polymerase promoter, *e.g.*, a T7 promoter located 5' to and operably linked to the gene of interest.

In some embodiments, an *in vitro* transcription template encodes a 5' untranslated (UTR) region, contains an open reading frame, and encodes a 3' UTR and a poly(A) 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. When RNA transcripts are being generated, the 5' UTR may comprise a promoter sequence. Such promoter sequences are known in the art. It should be understood that such promoter sequences will not be present in a vaccine of the disclosure.

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 a polypeptide.

A "poly(A) 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 poly(A) tail may contain 10 to 300 adenosine monophosphates. For example, a poly(A) 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 poly(A) 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, and/or export of the mRNA from the nucleus and translation.

In some embodiments, a nucleic acid includes 200 to 3,000 nucleotides. For example, a nucleic acid 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).

An *in vitro* transcription system typically comprises a transcription buffer, nucleotide triphosphates (NTPs), an RNase inhibitor and a polymerase.

The NTPs may be manufactured in house, may be selected from a supplier, or may be synthesized as described herein. The NTPs may be selected from, but are not limited to, those described herein including natural and unnatural (modified) NTPs.

Any number of RNA polymerases or variants may be used in the method of the present disclosure. The polymerase may be selected from, but is not limited to, a phage RNA polymerase, *e.g.*, a T7 RNA polymerase, a T3 RNA polymerase, a SP6 RNA polymerase, and/or mutant polymerases such as, but not limited to, polymerases able to incorporate modified nucleic acids and/or modified nucleotides, including chemically modified nucleic acids and/or nucleotides. Some embodiments exclude the use of DNase.

In some embodiments, the RNA transcript is capped via enzymatic capping. In some embodiments, the RNA comprises 5' terminal cap, for example, 7mG(5')ppp(5')NlmpNp.

Chemical Synthesis

Solid-phase chemical synthesis. Nucleic acids the present disclosure may be manufactured in whole or in part using solid phase techniques. Solid-phase chemical synthesis of nucleic acids is an automated method wherein molecules are immobilized on a solid support and synthesized step by step in a reactant solution. Solid-phase synthesis is useful in site-specific introduction of chemical modifications in the nucleic acid sequences.

Liquid Phase Chemical Synthesis. The synthesis of nucleic acids of the present disclosure by the sequential addition of monomer building blocks may be carried out in a liquid phase.

Combination of Synthetic Methods. The synthetic methods discussed above each has its own advantages and limitations. Attempts have been conducted to combine these methods to overcome the limitations. Such combinations of methods are within the scope of the present

disclosure. The use of solid-phase or liquid-phase chemical synthesis in combination with enzymatic ligation provides an efficient way to generate long chain nucleic acids that cannot be obtained by chemical synthesis alone.

5 **Ligation of Nucleic Acid Regions or Subregions**

Assembling nucleic acids by a ligase may also be used. DNA or RNA ligases promote intermolecular ligation of the 5' and 3' ends of polynucleotide chains through the formation of a phosphodiester bond. Nucleic acids such as chimeric polynucleotides and/or circular nucleic acids may be prepared by ligation of one or more regions or subregions. DNA fragments can be
10 joined by a ligase catalyzed reaction to create recombinant DNA with different functions. Two oligodeoxynucleotides, one with a 5' phosphoryl group and another with a free 3' hydroxyl group, serve as substrates for a DNA ligase.

Purification

15 Purification of the nucleic acids described herein may include, but is not limited to, nucleic acid clean-up, quality assurance and quality control. Clean-up may be performed by methods known in the arts such as, but not limited to, AGENCOURT® beads (Beckman Coulter Genomics, Danvers, MA), poly-T beads, LNATM oligo-T capture probes (EXIQON® Inc, Vedbaek, Denmark) or HPLC based purification methods such as, but not limited to, strong
20 anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC). The term "purified" when used in relation to a nucleic acid such as a "purified nucleic acid" refers to one that is separated from at least one contaminant. A "contaminant" is any substance that makes another unfit, impure or inferior. Thus, a purified nucleic acid (*e.g.*, DNA and RNA) is present in a form or setting different from
25 that in which it is found in nature, or a form or setting different from that which existed prior to subjecting it to a treatment or purification method.

A quality assurance and/or quality control check may be conducted using methods such as, but not limited to, gel electrophoresis, UV absorbance, or analytical HPLC.

30 In some embodiments, the nucleic acids may be sequenced by methods including, but not limited to reverse-transcriptase-PCR.

Quantification

In some embodiments, the nucleic acids of the present disclosure may be quantified in exosomes or when derived from one or more bodily fluid. Bodily fluids include peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial
35 fluid, aqueous humor, amniotic fluid, cerumen, breast milk, bronchoalveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, sweat, fecal matter, hair, tears, cyst

fluid, pleural and peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, blastocyl cavity fluid, and umbilical cord blood. Alternatively, exosomes may be retrieved from an organ selected from the group consisting of lung, heart, pancreas, stomach, intestine, bladder, kidney, ovary, testis, skin, colon, breast, prostate, brain, esophagus, liver, and placenta.

Assays may be performed using construct specific probes, cytometry, qRT-PCR, real-time PCR, PCR, flow cytometry, electrophoresis, mass spectrometry, or combinations thereof while the exosomes may be isolated using immunohistochemical methods such as enzyme linked immunosorbent assay (ELISA) methods. Exosomes may also be isolated by size exclusion chromatography, density gradient centrifugation, differential centrifugation, nanomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof.

These methods afford the investigator the ability to monitor, in real time, the level of nucleic acids remaining or delivered. This is possible because the nucleic acids of the present disclosure, in some embodiments, differ from the endogenous forms due to the structural or chemical modifications.

In some embodiments, the nucleic acid may be quantified using methods such as, but not limited to, ultraviolet visible spectroscopy (UV/Vis). A non-limiting example of a UV/Vis spectrometer is a NANODROP® spectrometer (ThermoFisher, Waltham, MA). The quantified nucleic acid may be analyzed in order to determine if the nucleic acid may be of proper size, check that no degradation of the nucleic acid has occurred. Degradation of the nucleic acid may be checked by methods such as, but not limited to, agarose gel electrophoresis, HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC), liquid chromatography-mass spectrometry (LCMS), capillary electrophoresis (CE) and capillary gel electrophoresis (CGE).

Lipid Nanoparticle Formulations

In some embodiments, the RNA (e.g., mRNA) of the disclosure is formulated in a lipid nanoparticle (LNP). This is referred to herein as a LNP-formulated mRNA vaccine (fLNP). As used herein, “lipid nanoparticles” typically comprise ionizable cationic lipid, non-cationic lipid, sterol and PEG lipid components along with the nucleic acid cargo of interest. In the lipid nanoparticles used herein, the ionizable cationic lipid is an ionizable amino lipid. The lipid nanoparticles of the invention can be generated using components, compositions, and methods as are generally known in the art, see for example PCT/US2016/052352; PCT/US2016/068300;

PCT/US2017/037551; PCT/US2015/027400; PCT/US2016/047406; PCT/US2016000129; PCT/US2016/014280; PCT/US2016/014280; PCT/US2017/038426; PCT/US2014/027077; PCT/US2014/055394; PCT/US2016/52117; PCT/US2012/069610; PCT/US2017/027492; PCT/US2016/059575; PCT/US2016/069491; PCT/US2016/069493; and

5 PCT/US2014/066242, all of which are incorporated by reference herein in their entirety.

Vaccines of the present disclosure are typically formulated in lipid nanoparticle. In some embodiments, the lipid nanoparticle comprises at least one ionizable amino lipid, at least one non-cationic lipid, at least one sterol, and/or at least one polyethylene glycol (PEG)-modified lipid.

10 In some embodiments, the lipid nanoparticle comprises a molar ratio of 20-60% amino lipid relative to the other lipid components. For example, the lipid nanoparticle may comprise a molar ratio of 20-50%, 20-40%, 20-30%, 30-60%, 30-50%, 30-40%, 40-60%, 40-50%, or 50-60% amino lipid. In some embodiments, the lipid nanoparticle comprises a molar ratio of 20%, 30%, 40%, 50, or 60% amino lipid.

15 In some embodiments, the lipid nanoparticle comprises a molar ratio of 5-25% phospholipid relative to the other lipid components. For example, the lipid nanoparticle may comprise a molar ratio of 5-30%, 5-15%, 5-10%, 10-25%, 10-20%, 10-25%, 15-25%, 15-20%, 20-25%, or 25-30% phospholipid. In some embodiments, the lipid nanoparticle comprises a molar ratio of 5%, 10%, 15%, 20%, 25%, or 30% non-cationic lipid.

20 In some embodiments, the lipid nanoparticle comprises a molar ratio of 25-55% structural lipid relative to the other lipid components. For example, the lipid nanoparticle may comprise a molar ratio of 10- 55%, 25-50%, 25-45%, 25-40%, 25-35%, 25-30%, 30-55%, 30-50%, 30-45%, 30-40%, 30-35%, 35-55%, 35-50%, 35-45%, 35-40%, 40-55%, 40-50%, 40-45%, 45-55%, 45-50%, or 50-55% structural lipid. In some embodiments, the lipid nanoparticle

25 comprises a molar ratio of 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, or 55% structural lipid.

In some embodiments, the lipid nanoparticle comprises a molar ratio of 0.5-15% PEG lipid relative to the other lipid components. For example, the lipid nanoparticle may comprise a molar ratio of 0.5-10%, 0.5-5%, 1-15%, 1-10%, 1-5%, 2-15%, 2-10%, 2-5%, 5-15%, 5-10%, or

30 10-15% PEG lipid. In some embodiments, the lipid nanoparticle comprises a molar ratio of 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, or 15% PEG- lipid.

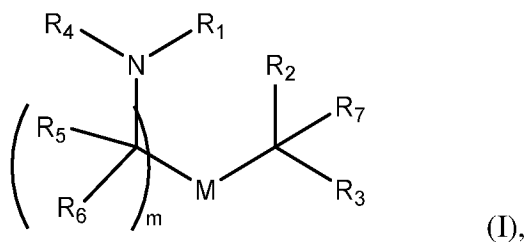
In some embodiments, the lipid nanoparticle comprises a molar ratio of 20-60% amino lipid, 5-25% phospholipid, 25-55% structural lipid, and 0.5-15% PEG lipid.

In some embodiments, the lipid nanoparticle comprises a molar ratio of 20-60% amino

35 lipid, 5-30% phospholipid, 10-55% structural lipid, and 0.5-15% PEG lipid.

Amino lipids

In some aspects, the amino lipids of the present disclosure may be one or more of compounds of Formula (I):



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

R₁ is selected from the group consisting of C₅₋₃₀ alkyl, C₅₋₂₀ alkenyl, -R*YR'', -YR'', and -R''M'R';

10 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, -(CH₂)_nQ, -(CH₂)_nCHQR, -CHQR, -CQ(R)₂, and unsubstituted C₁₋₆ alkyl, where Q is selected from a carbocycle, heterocycle, -OR, -O(CH₂)_nN(R)₂, -C(O)OR, -OC(O)R, -CX₃, -CX₂H, -CXH₂, -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)R₈, -N(R)S(O)₂R₈, -O(CH₂)_nOR, -N(R)C(=NR₉)N(R)₂, -N(R)C(=CHR₉)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(=NR₉)N(R)₂, -N(OR)C(=CHR₉)N(R)₂, -C(=NR₉)N(R)₂, -C(=NR₉)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;

20

each R₅ is independently selected from the group consisting of C₁₋₃ alkyl, C₂₋₃ alkenyl, and H;

each R₆ is independently selected from the group consisting of C₁₋₃ alkyl, C₂₋₃ alkenyl, and H;

25 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;

R₈ is selected from the group consisting of C₃₋₆ carbocycle and heterocycle;

30 R₉ is selected from the group consisting of H, CN, NO₂, C₁₋₆ 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 C₁₋₁₈ alkyl, C₂₋₁₈ alkenyl, -R*YR'', -YR'', and H;

5 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 C₁₋₁₂ alkyl and C₂₋₁₂ alkenyl;

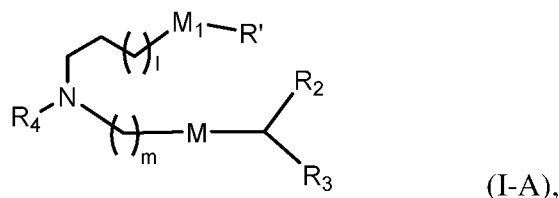
each Y is independently a C₃₋₆ carbocycle;

10 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 -(CH₂)_nQ, -(CH₂)_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.

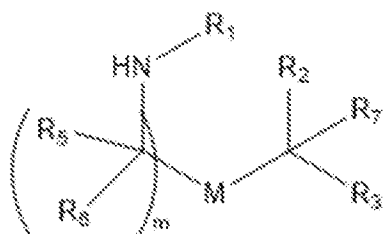
In certain embodiments, a subset of compounds of Formula (I) includes those of Formula

15 (I-A):



or its N-oxide, or a salt or isomer thereof, wherein l is selected from 1, 2, 3, 4, and 5; m is selected from 5, 6, 7, 8, and 9; M₁ is a bond or M'; R₄ is hydrogen, unsubstituted C₁₋₃ alkyl, or -(CH₂)_nQ, in which Q is OH, -NHC(S)N(R)₂, -NHC(O)N(R)₂, -N(R)C(O)R, -N(R)S(O)₂R, -N(R)R₈, -NHC(=NR₉)N(R)₂, -NHC(=CHR₉)N(R)₂, -OC(O)N(R)₂, -N(R)C(O)OR, heteroaryl or heterocycloalkyl; M and M' are independently selected from -C(O)O-, -OC(O)-, -OC(O)-M''-C(O)O-, -C(O)N(R')-, -P(O)(OR')O-, -S-S-, an aryl group, and a heteroaryl group; and R₂ and R₃ are independently selected from the group consisting of H, C₁₋₁₄ alkyl, and C₂₋₁₄ alkenyl. For example, m is 5, 7, or 9. For example, Q is OH, -NHC(S)N(R)₂, or -NHC(O)N(R)₂. For example, Q is -N(R)C(O)R, or -N(R)S(O)₂R.

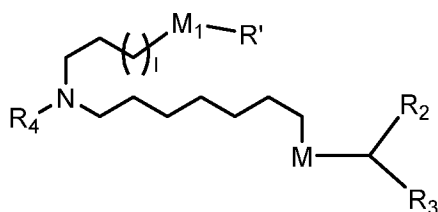
In certain embodiments, a subset of compounds of Formula (I) includes those of Formula (I-B):



(I-B), or its N-oxide, or a salt or isomer thereof in

which all variables are as defined herein. For example, m is selected from 5, 6, 7, 8, and 9; R_4 is hydrogen, unsubstituted C_{1-3} alkyl, or $-(CH_2)_nQ$, in which Q is OH, $-NHC(S)N(R)_2$, $-NHC(O)N(R)_2$, $-N(R)C(O)R$, $-N(R)S(O)_2R$, $-N(R)R_8$, $-NHC(=NR_9)N(R)_2$, $-NHC(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, $-N(R)C(O)OR$, heteroaryl or heterocycloalkyl; M and M' are independently selected from $-C(O)O-$, $-OC(O)-$, $-OC(O)-M''-C(O)O-$, $-C(O)N(R')-$, $-P(O)(OR')O-$, $-S-S-$, an aryl group, and a heteroaryl group; and R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, and C_{2-14} alkenyl. For example, m is 5, 7, or 9. For example, Q is OH, $-NHC(S)N(R)_2$, or $-NHC(O)N(R)_2$. For example, Q is $-N(R)C(O)R$, or $-N(R)S(O)_2R$.

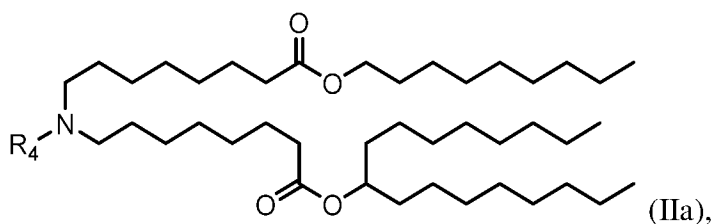
In certain embodiments, a subset of compounds of Formula (I) includes those of Formula (II):



(II), or its N-oxide, or a salt or isomer

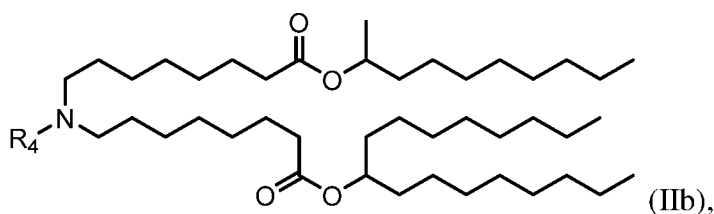
thereof, wherein l is selected from 1, 2, 3, 4, and 5; M_1 is a bond or M' ; R_4 is hydrogen, unsubstituted C_{1-3} alkyl, or $-(CH_2)_nQ$, in which n is 2, 3, or 4, and Q is OH, $-NHC(S)N(R)_2$, $-NHC(O)N(R)_2$, $-N(R)C(O)R$, $-N(R)S(O)_2R$, $-N(R)R_8$, $-NHC(=NR_9)N(R)_2$, $-NHC(=CHR_9)N(R)_2$, $-OC(O)N(R)_2$, $-N(R)C(O)OR$, heteroaryl or heterocycloalkyl; M and M' are independently selected from $-C(O)O-$, $-OC(O)-$, $-OC(O)-M''-C(O)O-$, $-C(O)N(R')-$, $-P(O)(OR')O-$, $-S-S-$, an aryl group, and a heteroaryl group; and R_2 and R_3 are independently selected from the group consisting of H, C_{1-14} alkyl, and C_{2-14} alkenyl.

In one embodiment, the compounds of Formula (I) are of Formula (IIa),



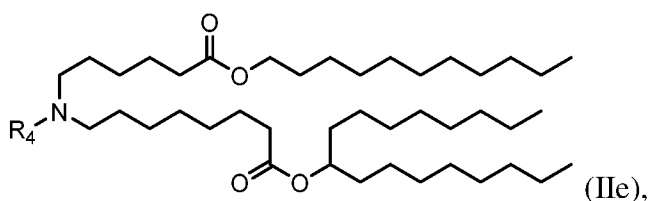
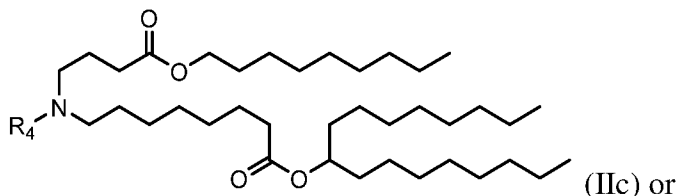
or their N-oxides, or salts or isomers thereof, wherein R_4 is as described herein.

In another embodiment, the compounds of Formula (I) are of Formula (IIb),



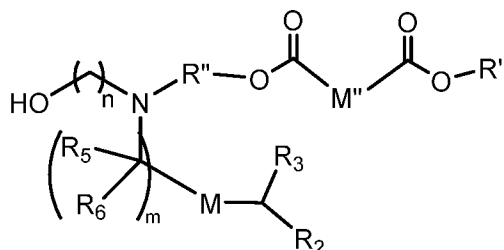
or their N-oxides, or salts or isomers thereof, wherein R_4 is as described herein.

In another embodiment, the compounds of Formula (I) are of Formula (IIc) or (Ile):



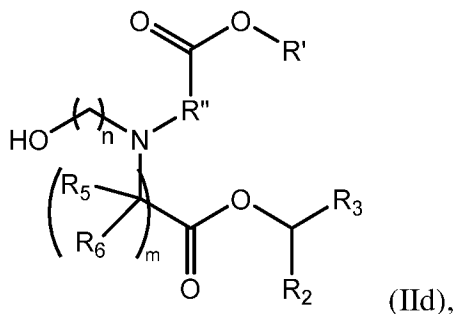
or their N-oxides, or salts or isomers thereof, wherein R_4 is as described herein.

In another embodiment, the compounds of Formula (I) are of Formula (IIIf):



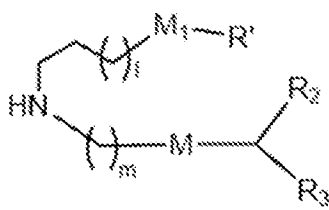
wherein M is $-C(O)O-$ or $-OC(O)-$, M'' is C_{1-6} alkyl or C_{2-6} alkenyl, R_2 and R_3 are independently selected from the group consisting of C_{5-14} alkyl and C_{5-14} alkenyl, and n is selected from 2, 3, and 4.

In a further embodiment, the compounds of Formula (I) are of Formula (IIId),



or their N-oxides, or salts or isomers thereof, wherein n is 2, 3, or 4; and m , R' , R'' , and R_2 through R_6 are as described herein. For example, each of R_2 and R_3 may be independently selected from the group consisting of C_{5-14} alkyl and C_{5-14} alkenyl.

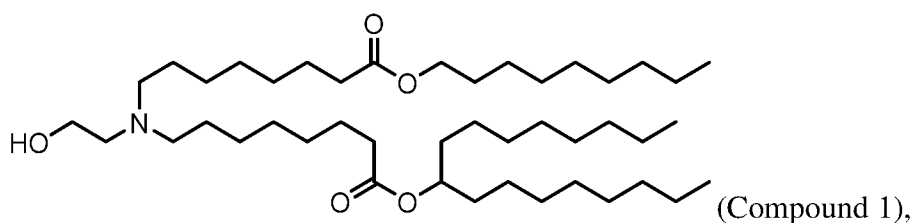
In a further embodiment, the compounds of Formula (I) are of Formula (IIg),



(IIg), or their N-oxides, or salts or isomers thereof, wherein l is selected from 1, 2, 3, 4, and 5; m is selected from 5, 6, 7, 8, and 9; M₁ is a bond or M'; M and M' are independently selected from -C(O)O-, -OC(O)-, -OC(O)-M''-C(O)O-, -C(O)N(R')-, -P(O)(OR')O-, -S-S-, an aryl group, and a heteroaryl group; and R₂ and R₃ are independently selected from the group consisting of H, C₁₋₁₄ alkyl, and C₂₋₁₄ alkenyl. For example, M'' is C₁₋₆ alkyl (e.g., C₁₋₄ alkyl) or C₂₋₆ alkenyl (e.g., C₂₋₄ alkenyl). For example, R₂ and R₃ are independently selected from the group consisting of C₅₋₁₄ alkyl and C₅₋₁₄ alkenyl.

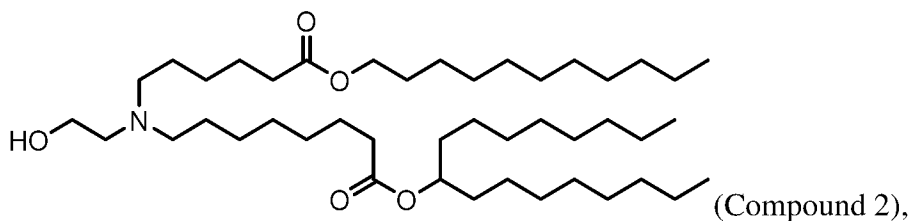
In some embodiments, the amino lipids are one or more of the compounds described in U.S. Application Nos. 62/220,091, 62/252,316, 62/253,433, 62/266,460, 62/333,557, 62/382,740, 62/393,940, 62/471,937, 62/471,949, 62/475,140, and 62/475,166, and PCT Application No. PCT/US2016/052352.

In some embodiments, the amino lipid is Compound 1:



or a salt thereof.

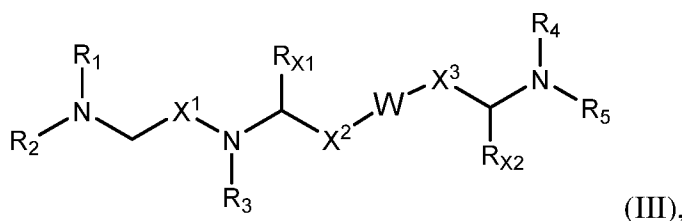
In some embodiments, the amino lipid is Compound 2:



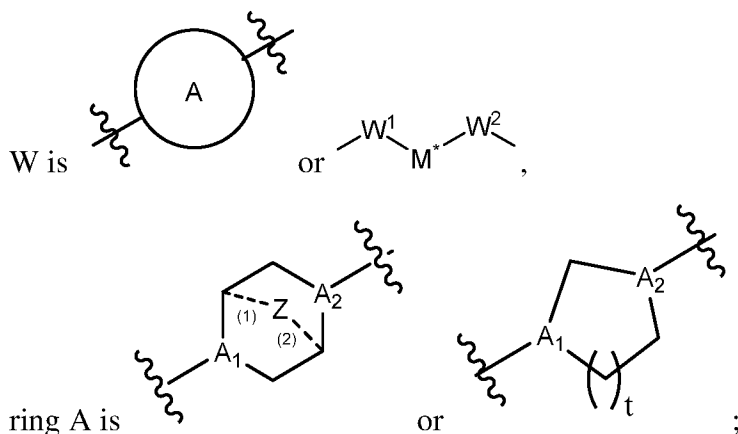
or a salt thereof.

The central amine moiety of a lipid according to Formula (I), (I-A), (I-B), (II), (IIa), (IIb), (IIc), (IId), (IIe), (IIIf), or (IIg) may be protonated at a physiological pH. Thus, a lipid may have a positive or partial positive charge at physiological pH. Such amino lipids may be referred to as cationic lipids, ionizable lipids, cationic amino lipids, or ionizable amino lipids. Amino lipids may also be zwitterionic, i.e., neutral molecules having both a positive and a negative charge.

In some aspects, the amino lipids of the present disclosure may be one or more of compounds of formula (III),



or salts or isomers thereof, wherein



t is 1 or 2;

A₁ and A₂ are each independently selected from CH or N;

Z is CH₂ or absent wherein when Z is CH₂, the dashed lines (1) and (2) each represent a single bond; and when Z is absent, the dashed lines (1) and (2) are both absent;

R₁, R₂, R₃, R₄, and R₅ are independently selected from the group consisting of C₅₋₂₀ alkyl, C₅₋₂₀ alkenyl, -R''MR', -R*YR'', -YR'', and -R*OR'';

R_{X1} and R_{X2} are each independently H or C₁₋₃ alkyl;

each M is independently selected from the group consisting of -C(O)O-, -OC(O)-, -OC(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-, -C(O)S-, -SC(O)-, an aryl group, and a heteroaryl group;

M* is C₁₋₆ alkyl,

W¹ and W² are each independently selected from the group consisting of -O- and -N(R₆)-;

each R₆ is independently selected from the group consisting of H and C₁₋₅ alkyl;

X¹, X², and X³ are independently selected from the group consisting of a bond, -CH₂-, -(CH₂)₂-, -CHR-, -CHY-, -C(O)-, -C(O)O-, -OC(O)-, -(CH₂)_n-C(O)-, -C(O)-(CH₂)_n-, -(CH₂)_n-C(O)O-, -OC(O)-(CH₂)_n-, -(CH₂)_n-OC(O)-, -C(O)O-(CH₂)_n-, -CH(OH)-, -C(S)-, and -CH(SH)-;

each Y is independently a C₃₋₆ carbocycle;

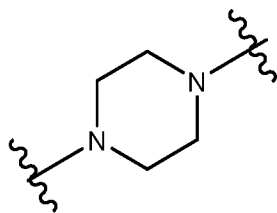
each R* is independently selected from the group consisting of C₁₋₁₂ alkyl and C₂₋₁₂ alkenyl;

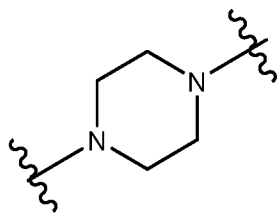
each R is independently selected from the group consisting of C₁₋₃ alkyl and a C₃₋₆ carbocycle;

each R' is independently selected from the group consisting of C₁₋₁₂ alkyl, C₂₋₁₂ alkenyl, and H;

each R'' is independently selected from the group consisting of C₃₋₁₂ alkyl, C₃₋₁₂ alkenyl and -R*MR'; and

5 n is an integer from 1-6;

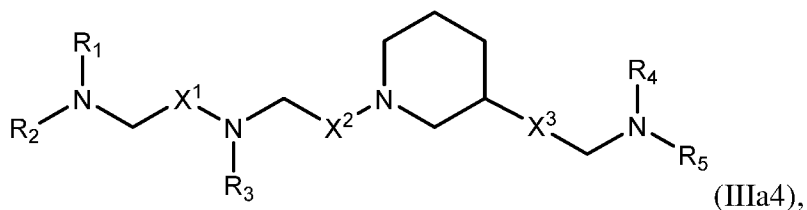
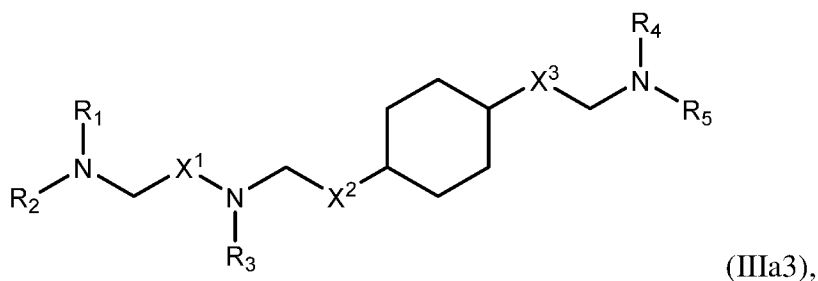
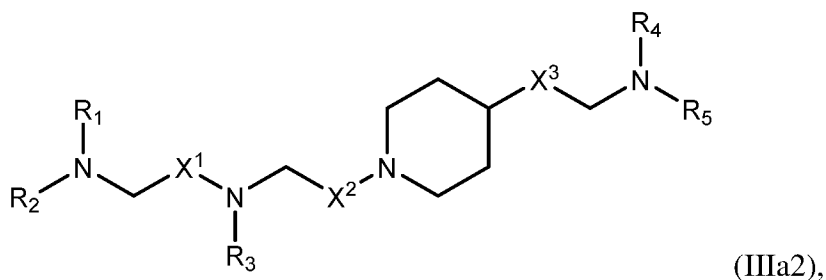
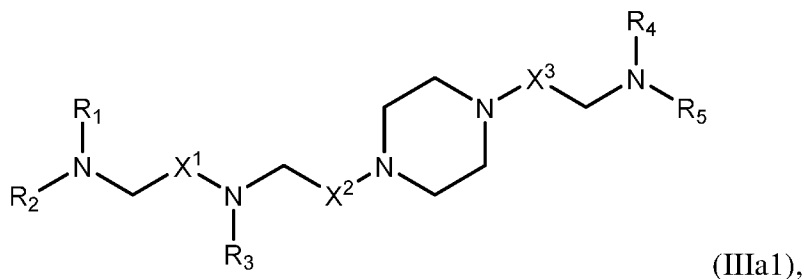


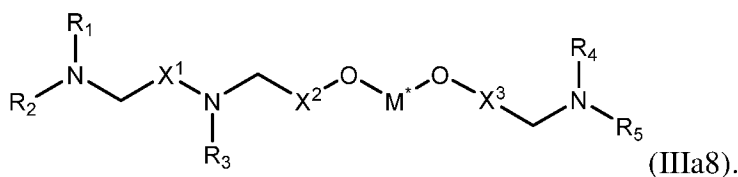
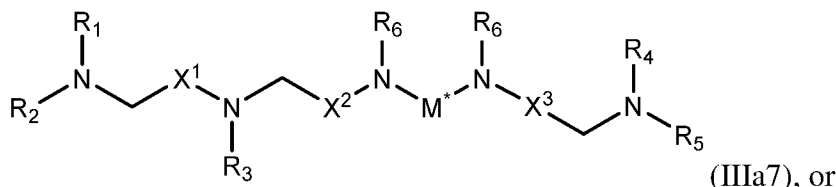
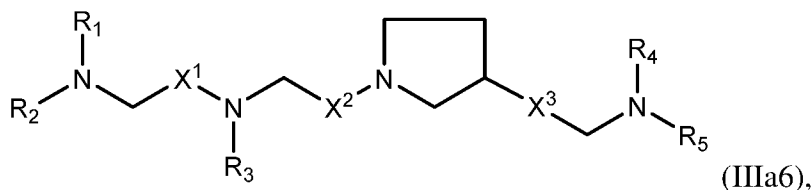
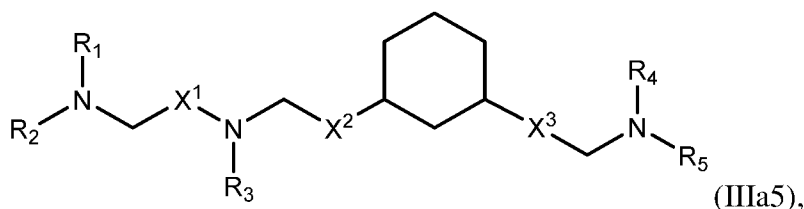
wherein when ring A is , then

i) at least one of X¹, X², and X³ is not -CH₂-; and/or

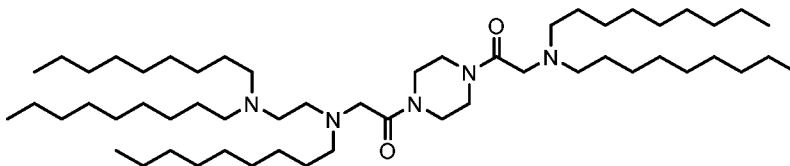
ii) at least one of R₁, R₂, R₃, R₄, and R₅ is -R''MR'.

In some embodiments, the compound is of any of formulae (IIIa1)-(IIIa8):





5 In some embodiments, the amino lipid is



, or a salt thereof.

The central amine moiety of a lipid according to Formula (III), (IIIa1), (IIIa2), (IIIa3), (IIIa4), (IIIa5), (IIIa6), (IIIa7), or (IIIa8) may be protonated at a physiological pH. Thus, a lipid may have a positive or partial positive charge at physiological pH.

10

Phospholipids

The lipid composition of the lipid nanoparticle composition disclosed herein can comprise one or more phospholipids, for example, one or more saturated or (poly)unsaturated phospholipids or a combination thereof. In general, phospholipids comprise a phospholipid moiety and one or more fatty acid moieties.

15

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, 2-lysophosphatidyl choline, and a sphingomyelin.

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.

20

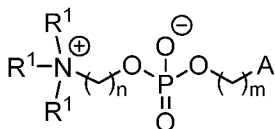
Particular phospholipids can facilitate fusion to a membrane. For example, a cationic phospholipid can interact with one or more negatively charged phospholipids of a membrane (e.g., a cellular or intracellular membrane). Fusion of a phospholipid to a membrane can allow one or more elements (e.g., a 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 tissue.

Non-natural phospholipid species including natural species with modifications and substitutions including branching, oxidation, cyclization, and alkynes are also contemplated. For example, 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 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, phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols, phosphatidyl glycerols, and phosphatidic acids. Phospholipids also include phosphosphingolipid, such as sphingomyelin.

In some embodiments, a phospholipid of the invention comprises 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-diundecanoyl-sn-glycero-phosphocholine (DUPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (18:0 Diether PC), 1-oleoyl-2-cholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (OChemPC), 1-hexadecyl-sn-glycero-3-phosphocholine (C16 Lyso PC), 1,2-dilinolenoyl-sn-glycero-3-phosphocholine, 1,2-diarachidonoyl-sn-glycero-3-phosphocholine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphocholine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine (ME 16.0 PE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine, 1,2-dilinolenoyl-sn-glycero-3-phosphoethanolamine, 1,2-diarachidonoyl-sn-glycero-3-phosphoethanolamine, 1,2-didocosahexaenoyl-sn-glycero-3-phosphoethanolamine, 1,2-dioleoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt (DOPG), sphingomyelin, and mixtures thereof.

In certain embodiments, a phospholipid useful or potentially useful in the present invention is an analog or variant of DSPC. In certain embodiments, a phospholipid useful or potentially useful in the present invention is a compound of Formula (IV):



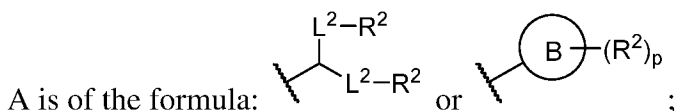
(IV),

or a salt thereof, wherein:

each R^1 is independently optionally substituted alkyl; or optionally two R^1 are joined together with the intervening atoms to form optionally substituted monocyclic carbocyclyl or optionally substituted monocyclic heterocyclyl; or optionally three R^1 are joined together with the intervening atoms to form optionally substituted bicyclic carbocyclyl or optionally substituted bicyclic heterocyclyl;

n is 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;

m is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;



each instance of L^2 is independently a bond or optionally substituted C_{1-6} alkylene, wherein one methylene unit of the optionally substituted C_{1-6} alkylene is optionally replaced with O, $\text{N}(\text{R}^N)$, S, $\text{C}(\text{O})$, $\text{C}(\text{O})\text{N}(\text{R}^N)$, $\text{NR}^N\text{C}(\text{O})$, $\text{C}(\text{O})\text{O}$, $\text{OC}(\text{O})$, $\text{OC}(\text{O})\text{O}$, $\text{OC}(\text{O})\text{N}(\text{R}^N)$, $\text{NR}^N\text{C}(\text{O})\text{O}$, or $\text{NR}^N\text{C}(\text{O})\text{N}(\text{R}^N)$;

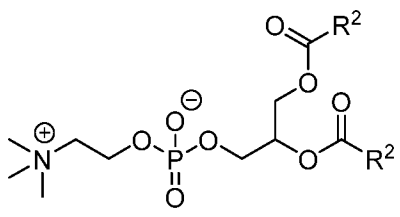
each instance of R^2 is independently optionally substituted C_{1-30} alkyl, optionally substituted C_{1-30} alkenyl, or optionally substituted C_{1-30} alkynyl; optionally wherein one or more methylene units of R^2 are independently replaced with optionally substituted carbocyclylene, optionally substituted heterocyclylene, optionally substituted arylene, optionally substituted heteroarylene, $\text{N}(\text{R}^N)$, O, S, $\text{C}(\text{O})$, $\text{C}(\text{O})\text{N}(\text{R}^N)$, $\text{NR}^N\text{C}(\text{O})$, $\text{NR}^N\text{C}(\text{O})\text{N}(\text{R}^N)$, $\text{C}(\text{O})\text{O}$, $\text{OC}(\text{O})$, - $\text{OC}(\text{O})\text{O}$, $\text{OC}(\text{O})\text{N}(\text{R}^N)$, $\text{NR}^N\text{C}(\text{O})\text{O}$, $\text{C}(\text{O})\text{S}$, $\text{SC}(\text{O})$, $\text{C}(\text{=NR}^N)$, $\text{C}(\text{=NR}^N)\text{N}(\text{R}^N)$, $\text{NR}^N\text{C}(\text{=NR}^N)$, $\text{NR}^N\text{C}(\text{=NR}^N)\text{N}(\text{R}^N)$, $\text{C}(\text{S})$, $\text{C}(\text{S})\text{N}(\text{R}^N)$, $\text{NR}^N\text{C}(\text{S})$, $\text{NR}^N\text{C}(\text{S})\text{N}(\text{R}^N)$, $\text{S}(\text{O})$, $\text{OS}(\text{O})$, $\text{S}(\text{O})\text{O}$, - $\text{OS}(\text{O})\text{O}$, $\text{OS}(\text{O})_2$, $\text{S}(\text{O})_2\text{O}$, $\text{OS}(\text{O})_2\text{O}$, $\text{N}(\text{R}^N)\text{S}(\text{O})$, $\text{S}(\text{O})\text{N}(\text{R}^N)$, $\text{N}(\text{R}^N)\text{S}(\text{O})\text{N}(\text{R}^N)$, $\text{OS}(\text{O})\text{N}(\text{R}^N)$, $\text{N}(\text{R}^N)\text{S}(\text{O})\text{O}$, $\text{S}(\text{O})_2$, $\text{N}(\text{R}^N)\text{S}(\text{O})_2$, $\text{S}(\text{O})_2\text{N}(\text{R}^N)$, $\text{N}(\text{R}^N)\text{S}(\text{O})_2\text{N}(\text{R}^N)$, $\text{OS}(\text{O})_2\text{N}(\text{R}^N)$, or - $\text{N}(\text{R}^N)\text{S}(\text{O})_2\text{O}$;

each instance of R^N is independently hydrogen, optionally substituted alkyl, or a nitrogen protecting group;

Ring B is optionally substituted carbocyclyl, optionally substituted heterocyclyl, optionally substituted aryl, or optionally substituted heteroaryl; and

p is 1 or 2;

provided that the compound is not of the formula:



wherein each instance of R² is independently unsubstituted alkyl, unsubstituted alkenyl,
 5 or unsubstituted alkynyl.

In some embodiments, the phospholipids may be one or more of the phospholipids described in PCT Application No. PCT/US2018/037922.

Structural Lipids

10 The lipid composition of a pharmaceutical composition disclosed herein can comprise one or more structural lipids. As used herein, the term “structural lipid” refers to sterols and also to lipids containing sterol moieties.

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
 15 limited to, cholesterol, fecosterol, sitosterol, ergosterol, campesterol, stigmasterol, brassicasterol, tomatidine, tomatine, ursolic acid, alpha-tocopherol, hopanoids, phytosterols, steroids, and mixtures thereof. In some embodiments, the structural lipid is a sterol. As defined herein, “sterols” are a subgroup of steroids consisting of steroid alcohols. In certain embodiments, the structural lipid is a steroid. In certain embodiments, the structural lipid is cholesterol. In certain
 20 embodiments, the structural lipid is an analog of cholesterol. In certain embodiments, the structural lipid is alpha-tocopherol.

In some embodiments, the structural lipids may be one or more of the structural lipids described in U.S. Application No. 16/493,814.

Polyethylene Glycol (PEG)-Lipids

25 The lipid composition of a pharmaceutical composition disclosed herein can comprise one or more polyethylene glycol (PEG) lipids.

As used herein, the term “PEG-lipid” refers to polyethylene glycol (PEG)-modified lipids. Non-limiting examples of PEG-lipids include PEG-modified phosphatidylethanolamine
 30 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. For example, 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-lipid includes, but not limited to 1,2-dimyristoyl-sn-glycerol methoxypolyethylene glycol (PEG-DMG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)] (PEG-DSPE), PEG-disteryl glycerol (PEG-DSG), PEG-dipalmitoleyl, PEG-dioleoyl, PEG-distearoyl, PEG-diacylglyceramide (PEG-DAG), PEG-dipalmitoyl phosphatidylethanolamine (PEG-DPPE), or PEG-1,2-dimyristyloxylpropyl-3-amine (PEG-c-DMA).

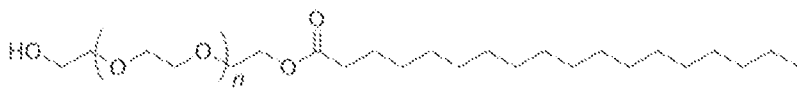
In one embodiment, the PEG-lipid is selected from the group consisting of a PEG-modified phosphatidylethanolamine, a PEG-modified phosphatidic acid, a PEG-modified ceramide, a PEG-modified dialkylamine, a PEG-modified diacylglycerol, a PEG-modified dialkylglycerol, and mixtures thereof. In some embodiments, the PEG-modified lipid is PEG-DMG, PEG-c-DOMG (also referred to as PEG-DOMG), PEG-DSG and/or PEG-DPG.

In some embodiments, the lipid moiety of the PEG-lipids includes those having lengths of from about C₁₄ to about C₂₂, preferably from about C₁₄ to about C₁₆. In some embodiments, a PEG moiety, for example an mPEG-NH₂, has a size of about 1000, 2000, 5000, 10,000, 15,000 or 20,000 daltons. In one embodiment, the PEG-lipid is PEG_{2k}-DMG.

In one embodiment, the lipid nanoparticles described herein can comprise a PEG lipid which is a non-diffusible PEG. Non-limiting examples of non-diffusible PEGs include PEG-DSG and PEG-DSPE.

PEG-lipids are known in the art, such as those described in U.S. Patent No. 8158601 and International Publ. No. WO 2015/130584 A2, which are incorporated herein by reference in their entirety.

In some embodiments, PEG-lipid comprises 134-hydroxy-3,6,9,12,15,18,21,24,27,30,33,36,39,42,45,48,51,54,57,60,63,66,69,72,75,78,81,84,87,90,93,96,99,102,105,108,111,114,117,120,123,126,129,132-tetratetracontaoxatetratriacontahexyl

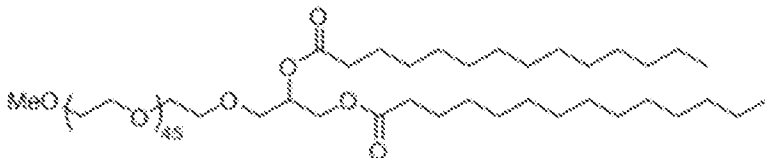
stearate: , wherein n = 45 (Compound 3).

In general, some of the other lipid components (e.g., PEG lipids) of various formulae, described herein may be synthesized as described International Patent Application No. PCT/US2016/000129, filed December 10, 2016, entitled “Compositions and Methods for Delivery of Therapeutic Agents,” which is incorporated by reference in its entirety.

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-

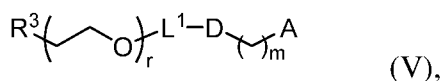
modified phosphatidylethanolamines, PEG-modified phosphatidic acids, PEG-modified ceramides, PEG-modified dialkylamines, PEG-modified diacylglycerols, PEG-modified dialkylglycerols, and mixtures thereof. For example, a PEG lipid may be PEG-c-DOMG, PEG-DMG, PEG-DLPE, PEG-DMPE, PEG-DPPC, or a PEG-DSPE lipid.

In some embodiments the PEG-modified lipids are a modified form of PEG DMG. PEG-DMG has the following structure:



In one embodiment, PEG lipids useful in the present invention can be PEGylated lipids described in International Publication No. WO2012099755, the contents of which is herein incorporated by reference in its entirety. Any of these exemplary PEG lipids described herein may be modified to comprise a hydroxyl group on the PEG chain. In certain embodiments, the PEG lipid is a PEG-OH lipid. As generally defined herein, a “PEG-OH lipid” (also referred to herein as “hydroxy-PEGylated lipid”) is a PEGylated lipid having one or more hydroxyl (–OH) groups on the lipid. In certain embodiments, the PEG-OH lipid includes one or more hydroxyl groups on the PEG chain. In certain embodiments, a PEG-OH or hydroxy-PEGylated lipid comprises an –OH group at the terminus of the PEG chain. Each possibility represents a separate embodiment of the present invention.

In certain embodiments, a PEG lipid useful in the present invention is a compound of Formula (II). Provided herein are compounds of Formula (V):



or salts thereof, wherein:

R^3 is ---OR^O ;

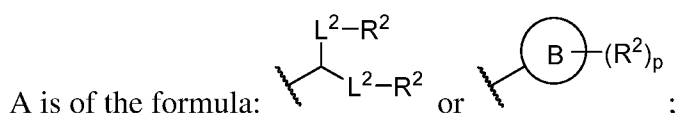
R^O is hydrogen, optionally substituted alkyl, or an oxygen protecting group;

r is an integer between 1 and 100, inclusive;

L^1 is optionally substituted C_{1-10} alkylene, wherein at least one methylene of the optionally substituted C_{1-10} alkylene is independently replaced with optionally substituted carbocyclylene, optionally substituted heterocyclylene, optionally substituted arylene, optionally substituted heteroarylene, O, $N(R^N)$, S, C(O), $C(O)N(R^N)$, $NR^NC(O)$, $C(O)O$, $OC(O)$, $OC(O)O$, $OC(O)N(R^N)$, $NR^NC(O)O$, or $NR^NC(O)N(R^N)$;

D is a moiety obtained by click chemistry or a moiety cleavable under physiological conditions;

m is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10;



each instance of L^2 is independently a bond or optionally substituted C_{1-6} alkylene, wherein one methylene unit of the optionally substituted C_{1-6} alkylene is optionally replaced with O, $N(R^N)$, S, C(O), C(O) $N(R^N)$, NR^N C(O), C(O)O, OC(O), OC(O)O, OC(O) $N(R^N)$, NR^N C(O)O, or NR^N C(O) $N(R^N)$;

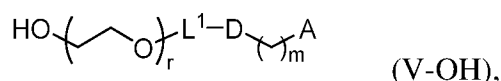
each instance of R^2 is independently optionally substituted C_{1-30} alkyl, optionally substituted C_{1-30} alkenyl, or optionally substituted C_{1-30} alkynyl; optionally wherein one or more methylene units of R^2 are independently replaced with optionally substituted carbocyclylene, optionally substituted heterocyclylene, optionally substituted arylene, optionally substituted heteroarylene, $N(R^N)$, O, S, C(O), C(O) $N(R^N)$, NR^N C(O), NR^N C(O) $N(R^N)$, C(O)O, OC(O), -OC(O)O, OC(O) $N(R^N)$, NR^N C(O)O, C(O)S, SC(O), C(=NR^N), C(=NR^N) $N(R^N)$, NR^N C(=NR^N), NR^N C(=NR^N) $N(R^N)$, C(S), C(S) $N(R^N)$, NR^N C(S), NR^N C(S) $N(R^N)$, S(O), OS(O), S(O)O, -OS(O)O, OS(O)₂, S(O)₂O, OS(O)₂O, $N(R^N)$ S(O), S(O) $N(R^N)$, $N(R^N)$ S(O) $N(R^N)$, OS(O) $N(R^N)$, $N(R^N)$ S(O)O, S(O)₂, $N(R^N)$ S(O)₂, S(O)₂ $N(R^N)$, $N(R^N)$ S(O)₂ $N(R^N)$, OS(O)₂ $N(R^N)$, or - $N(R^N)$ S(O)₂O;

each instance of R^N is independently hydrogen, optionally substituted alkyl, or a nitrogen protecting group;

Ring B is optionally substituted carbocyclyl, optionally substituted heterocyclyl, optionally substituted aryl, or optionally substituted heteroaryl; and

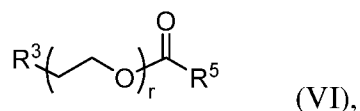
p is 1 or 2.

In certain embodiments, the compound of Formula (V) is a PEG-OH lipid (*i.e.*, R^3 is -OR^O, and R^O is hydrogen). In certain embodiments, the compound of Formula (V) is of Formula (V-OH):



or a salt thereof.

In certain embodiments, a PEG lipid useful in the present invention is a PEGylated fatty acid. In certain embodiments, a PEG lipid useful in the present invention is a compound of Formula (VI). Provided herein are compounds of Formula (VI):



or a salts thereof, wherein:

R^3 is -OR^O;

R^O is hydrogen, optionally substituted alkyl or an oxygen protecting group;

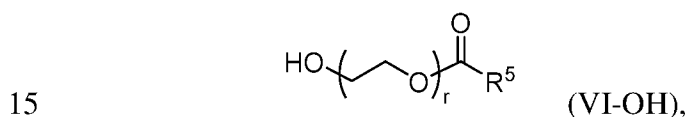
r is an integer between 1 and 100, inclusive;

R^5 is optionally substituted C_{10-40} alkyl, optionally substituted C_{10-40} alkenyl, or optionally substituted C_{10-40} alkynyl; and optionally one or more methylene groups of R^5 are replaced with

optionally substituted carbocyclylene, optionally substituted heterocyclylene, optionally substituted arylene, optionally substituted heteroarylene, $N(R^N)$, O, S, C(O), C(O) $N(R^N)$, -
 $NR^NC(O)$, $NR^NC(O)N(R^N)$, C(O)O, OC(O), OC(O)O, OC(O) $N(R^N)$, $NR^NC(O)O$, C(O)S, SC(O),
 $C(=NR^N)$, $C(=NR^N)N(R^N)$, $NR^NC(=NR^N)$, $NR^NC(=NR^N)N(R^N)$, C(S), C(S) $N(R^N)$, $NR^NC(S)$, -
 $NR^NC(S)N(R^N)$, S(O), OS(O), S(O)O, OS(O)O, OS(O)₂, S(O)₂O, OS(O)₂O, $N(R^N)S(O)$, -
 $S(O)N(R^N)$, $N(R^N)S(O)N(R^N)$, OS(O) $N(R^N)$, $N(R^N)S(O)O$, S(O)₂, $N(R^N)S(O)_2$, S(O)₂ $N(R^N)$, -
 $N(R^N)S(O)_2N(R^N)$, OS(O)₂ $N(R^N)$, or $N(R^N)S(O)_2O$; and

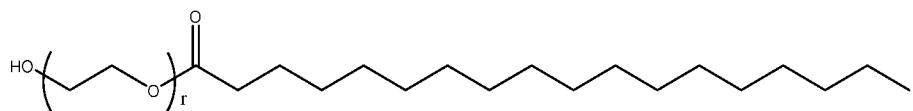
each instance of R^N is independently hydrogen, optionally substituted alkyl, or a nitrogen protecting group.

In certain embodiments, the compound of Formula (VI) is of Formula (VI-OH):



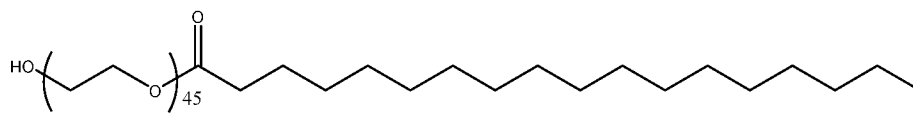
or a salt thereof. In some embodiments, r is 40-50.

In yet other embodiments the compound of Formula (VI) is:



or a salt thereof.

20 In one embodiment, the compound of Formula (VI) is



In some aspects, the lipid composition of the pharmaceutical compositions disclosed herein does not comprise a PEG-lipid.

25 In some embodiments, the PEG-lipids may be one or more of the PEG lipids described in U.S. Application No. US15/674,872.

In some embodiments, a LNP of the invention comprises an amino lipid of any of Formula I, II or III, a phospholipid comprising DSPC, a structural lipid, and a PEG lipid comprising PEG-DMG.

30 In some embodiments, a LNP of the invention comprises an amino lipid of any of Formula I, II or III, a phospholipid comprising DSPC, a structural lipid, and a PEG lipid comprising a compound having Formula VI.

In some embodiments, a LNP of the invention comprises an amino lipid of Formula I, II or III, a phospholipid comprising a compound having Formula IV, a structural lipid, and the PEG lipid comprising a compound having Formula V or VI.

5 In some embodiments, a LNP of the invention comprises an amino lipid of Formula I, II or III, a phospholipid comprising a compound having Formula IV, a structural lipid, and the PEG lipid comprising a compound having Formula V or VI.

In some embodiments, a LNP of the invention comprises an amino lipid of Formula I, II or III, a phospholipid having Formula IV, a structural lipid, and a PEG lipid comprising a compound having Formula VI.

10 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 6:1.

In some embodiments, a LNP of the invention comprises an N:P ratio of about 3:1, 4:1, or 5:1.

15 In some embodiments, a LNP of the invention comprises a wt/wt ratio of the amino lipid component to the RNA of from about 10:1 to about 100:1.

In some embodiments, a LNP of the invention comprises a wt/wt ratio of the amino lipid component to the RNA of about 20:1.

20 In some embodiments, a LNP of the invention comprises a wt/wt ratio of the amino lipid component to the RNA of about 10:1.

In some embodiments, a LNP of the invention has a mean diameter from about 30nm to about 150nm.

In some embodiments, a LNP of the invention has a mean diameter from about 60nm to about 120nm.

25 As disclosed herein, a lipid nanoparticle (LNP) refers to a nanoscale construct (*e.g.*, a nanoparticle, typically less than 100 nm in diameter) comprising lipid molecules arranged in a substantially spherical (*i.e.*, spheroid) geometry, sometimes encapsulating one or more additional molecular species. A LNP may comprise one or more types of lipids, including but not limited to amino lipids (*e.g.*, ionizable amino lipids), neutral lipids, non-cationic lipids, charged lipids, 30 PEG-modified lipids, phospholipids, structural lipids and sterols. In some embodiments, a LNP may further comprise one or more cargo molecules, including but not limited to nucleic acids (*e.g.*, mRNA, plasmid DNA, DNA or RNA oligonucleotides, siRNA, shRNA, snRNA, snoRNA, lncRNA, etc.), small molecules, proteins and peptides. A LNP may have a unilamellar structure (*i.e.*, having a single lipid layer or lipid bilayer surrounding a central region) or a multilamellar 35 structure (*i.e.*, having more than one lipid layer or lipid bilayer surrounding a central region). In

some embodiments, a lipid nanoparticle may be a liposome. A liposome is a nanoparticle comprising lipids arranged into one or more concentric lipid bilayers around a central region. The central region of a liposome may comprise an aqueous solution, suspension, or other aqueous composition.

5 In some embodiments, a lipid nanoparticle may comprise two or more components (e.g., amino lipid and nucleic acid, PEG-lipid, phospholipid, structural lipid). For instance, a lipid nanoparticle may comprise an amino lipid and a nucleic acid. Compositions comprising the lipid nanoparticles, such as those described herein, may be used for a wide variety of applications, including the stealth delivery of therapeutic payloads with minimal adverse innate immune
10 response.

Effective *in vivo* delivery of nucleic acids represents a continuing medical challenge. Exogenous nucleic acids (*i.e.*, originating from outside of a cell or organism) are readily degraded in the body, *e.g.*, by the immune system. Accordingly, effective delivery of nucleic acids to cells often requires the use of a particulate carrier (*e.g.*, lipid nanoparticles). The
15 particulate carrier should be formulated to have minimal particle aggregation, be relatively stable prior to intracellular delivery, effectively deliver nucleic acids intracellularly, and illicit no or minimal immune response. To achieve minimal particle aggregation and pre-delivery stability, many conventional particulate carriers have relied on the presence and/or concentration of certain components (*e.g.*, PEG-lipid). However, it has been discovered that certain components
20 may decrease the stability of encapsulated nucleic acids (*e.g.*, mRNA molecules). The reduced stability may limit the broad applicability of the particulate carriers. As such, there remains a need for methods by which to improve the stability of nucleic acid (*e.g.*, mRNA) encapsulated within lipid nanoparticles.

In some embodiments, the lipid nanoparticles comprise one or more of ionizable
25 molecules, polynucleotides, and optional components, such as structural lipids, sterols, neutral lipids, phospholipids and a molecule capable of reducing particle aggregation (*e.g.*, polyethylene glycol (PEG), PEG-modified lipid), such as those described above.

In some embodiments, a LNP described herein may include one or more ionizable molecules (*e.g.*, amino lipids or ionizable lipids). The ionizable molecule may comprise a
30 charged group and may have a certain pKa. In certain embodiments, the pKa of the ionizable molecule may be greater than or equal to about 6, greater than or equal to about 6.2, greater than or equal to about 6.5, greater than or equal to about 6.8, greater than or equal to about 7, greater than or equal to about 7.2, greater than or equal to about 7.5, greater than or equal to about 7.8, greater than or equal to about 8. In some embodiments, the pKa of the ionizable molecule may
35 be less than or equal to about 10, less than or equal to about 9.8, less than or equal to about 9.5,

less than or equal to about 9.2, less than or equal to about 9.0, less than or equal to about 8.8, or less than or equal to about 8.5. Combinations of the above referenced ranges are also possible (e.g., greater than or equal to 6 and less than or equal to about 8.5). Other ranges are also possible. In embodiments in which more than one type of ionizable molecule are present in a particle, each type of ionizable molecule may independently have a pKa in one or more of the ranges described above.

In general, an ionizable molecule comprises one or more charged groups. In some embodiments, an ionizable molecule may be positively charged or negatively charged. For instance, an ionizable molecule may be positively charged. For example, an ionizable molecule may comprise an amine group. As used herein, the term “ionizable molecule” has its ordinary meaning in the art and may refer to a molecule or matrix comprising one or more charged moiety. As used herein, a “charged moiety” is a chemical moiety that carries a formal electronic charge, e.g., monovalent (+1, or -1), divalent (+2, or -2), trivalent (+3, or -3), etc. The charged moiety may be anionic (*i.e.*, negatively charged) or cationic (*i.e.*, positively charged). Examples of positively-charged moieties include amine groups (e.g., primary, secondary, and/or tertiary amines), ammonium groups, pyridinium group, guanidine groups, and imidazolium groups. In a particular embodiment, the charged moieties comprise amine groups. Examples of negatively-charged groups or precursors thereof, include carboxylate groups, sulfonate groups, sulfate groups, phosphonate groups, phosphate groups, hydroxyl groups, and the like. The charge of the charged moiety may vary, in some cases, with the environmental conditions, for example, changes in pH may alter the charge of the moiety, and/or cause the moiety to become charged or uncharged. In general, the charge density of the molecule and/or matrix may be selected as desired.

In some cases, an ionizable molecule (e.g., an amino lipid or ionizable lipid) may include one or more precursor moieties that can be converted to charged moieties. For instance, the ionizable molecule may include a neutral moiety that can be hydrolyzed to form a charged moiety, such as those described above. As a non-limiting specific example, the molecule or matrix may include an amide, which can be hydrolyzed to form an amine, respectively. Those of ordinary skill in the art will be able to determine whether a given chemical moiety carries a formal electronic charge (for example, by inspection, pH titration, ionic conductivity measurements, etc.), and/or whether a given chemical moiety can be reacted (e.g., hydrolyzed) to form a chemical moiety that carries a formal electronic charge.

The ionizable molecule (e.g., amino lipid or ionizable lipid) may have any suitable molecular weight. In certain embodiments, the molecular weight of an ionizable molecule is less than or equal to about 2,500 g/mol, less than or equal to about 2,000 g/mol, less than or equal to

about 1,500 g/mol, less than or equal to about 1,250 g/mol, less than or equal to about 1,000 g/mol, less than or equal to about 900 g/mol, less than or equal to about 800 g/mol, less than or equal to about 700 g/mol, less than or equal to about 600 g/mol, less than or equal to about 500 g/mol, less than or equal to about 400 g/mol, less than or equal to about 300 g/mol, less than or equal to about 200 g/mol, or less than or equal to about 100 g/mol. In some instances, the molecular weight of an ionizable molecule is greater than or equal to about 100 g/mol, greater than or equal to about 200 g/mol, greater than or equal to about 300 g/mol, greater than or equal to about 400 g/mol, greater than or equal to about 500 g/mol, greater than or equal to about 600 g/mol, greater than or equal to about 700 g/mol, greater than or equal to about 1000 g/mol, greater than or equal to about 1,250 g/mol, greater than or equal to about 1,500 g/mol, greater than or equal to about 1,750 g/mol, greater than or equal to about 2,000 g/mol, or greater than or equal to about 2,250 g/mol. Combinations of the above ranges (e.g., at least about 200 g/mol and less than or equal to about 2,500 g/mol) are also possible. In embodiments in which more than one type of ionizable molecules are present in a particle, each type of ionizable molecule may independently have a molecular weight in one or more of the ranges described above.

In some embodiments, the percentage (e.g., by weight, or by mole) of a single type of ionizable molecule (e.g., amino lipid or ionizable lipid) and/or of all the ionizable molecules within a particle may be greater than or equal to about 15%, greater than or equal to about 16%, greater than or equal to about 17%, greater than or equal to about 18%, greater than or equal to about 19%, greater than or equal to about 20%, greater than or equal to about 21%, greater than or equal to about 22%, greater than or equal to about 23%, greater than or equal to about 24%, greater than or equal to about 25%, greater than or equal to about 30%, greater than or equal to about 35%, greater than or equal to about 40%, greater than or equal to about 42%, greater than or equal to about 45%, greater than or equal to about 48%, greater than or equal to about 50%, greater than or equal to about 52%, greater than or equal to about 55%, greater than or equal to about 58%, greater than or equal to about 60%, greater than or equal to about 62%, greater than or equal to about 65%, or greater than or equal to about 68%. In some instances, the percentage (e.g., by weight, or by mole) may be less than or equal to about 70%, less than or equal to about 68%, less than or equal to about 65%, less than or equal to about 62%, less than or equal to about 60%, less than or equal to about 58%, less than or equal to about 55%, less than or equal to about 52%, less than or equal to about 50%, or less than or equal to about 48%. Combinations of the above referenced ranges are also possible (e.g., greater than or equal to 20% and less than or equal to about 60%, greater than or equal to 40% and less than or equal to about 55%, etc.). In embodiments in which more than one type of ionizable molecule is present in a particle, each type of ionizable molecule may independently have a percentage (e.g., by weight, or by mole) in

one or more of the ranges described above. The percentage (e.g., by weight, or by mole) may be determined by extracting the ionizable molecule(s) from the dried particles using, e.g., organic solvents, and measuring the quantity of the agent using high pressure liquid chromatography (i.e., HPLC), liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance (NMR), or mass spectrometry (MS). Those of ordinary skill in the art would be knowledgeable of techniques to determine the quantity of a component using the above-referenced techniques. For example, HPLC may be used to quantify the amount of a component, by, e.g., comparing the area under the curve of a HPLC chromatogram to a standard curve.

It should be understood that the terms “charged” or “charged moiety” does not refer to a “partial negative charge” or “partial positive charge” on a molecule. The terms “partial negative charge” and “partial positive charge” are given their ordinary meaning in the art. A “partial negative charge” may result when a functional group comprises a bond that becomes polarized such that electron density is pulled toward one atom of the bond, creating a partial negative charge on the atom. Those of ordinary skill in the art will, in general, recognize bonds that can become polarized in this way.

As described herein, in some embodiments, mRNA is formulated with LNP, such that the mRNA is at least partially encompassed within the LNP. LNP-formulated mRNA vaccines (fLNPs) comprise a structure that protects the RNA from environmental components that may lead to mRNA degradation.

MicroRNA (miRNA) Binding Sites

Polynucleotides of the present disclosure can include regulatory elements, for example, microRNA (miRNA) binding sites, transcription factor binding sites, structured mRNA sequences and/or motifs, artificial binding sites engineered to act as pseudo-receptors for endogenous nucleic acid binding molecules, and combinations thereof. In some embodiments, polynucleotides including such regulatory elements are referred to as including “sensor sequences”. Non-limiting examples of sensor sequences are described in U.S. Publication 2014/0200261, the contents of which are incorporated herein by reference in their entirety.

In some embodiments, a polynucleotide (e.g., a ribonucleic acid (RNA), e.g., a messenger RNA (mRNA)) of the present disclosure comprises an open reading frame (ORF) encoding a polypeptide of interest and further comprises one or more miRNA binding site(s). Inclusion or incorporation of miRNA binding site(s) provides for regulation of polynucleotides of the present disclosure, and in turn, of the polypeptides encoded therefrom, based on tissue-specific and/or cell-type specific expression of naturally-occurring miRNAs.

A miRNA, e.g., a natural-occurring miRNA, is a 19-25 nucleotide long noncoding RNA that binds to a polynucleotide and down-regulates gene expression either by reducing stability or

by inhibiting translation of the polynucleotide. A miRNA sequence comprises a “seed” region, i.e., a sequence in the region of positions 2-8 of the mature miRNA. A miRNA seed can comprise positions 2-8 or 2-7 of the mature miRNA. In some embodiments, a miRNA seed can comprise 7 nucleotides (e.g., nucleotides 2-8 of the mature miRNA), wherein the seed-complementary site in the corresponding miRNA binding site is flanked by an adenosine (A) opposed to miRNA position 1. In some embodiments, a miRNA seed can comprise 6 nucleotides (e.g., nucleotides 2-7 of the mature miRNA), wherein the seed-complementary site in the corresponding miRNA binding site is flanked by an adenosine (A) opposed to miRNA position 1. See, for example, Grimson A, Farh KK, Johnston WK, Garrett-Engle P, Lim LP, Bartel DP; Mol Cell. 2007 Jul 6;27(1):91-105. miRNA profiling of the target cells or tissues can be conducted to determine the presence or absence of miRNA in the cells or tissues. In some embodiments, a polynucleotide (e.g., a ribonucleic acid (RNA), e.g., a messenger RNA (mRNA)) of the present disclosure comprises one or more microRNA binding sites, microRNA target sequences, microRNA complementary sequences, or microRNA seed complementary sequences. Such sequences can correspond to, e.g., have complementarity to, any known microRNA such as those taught in US Publication US2005/0261218 and US Publication US2005/0059005, the contents of each of which are incorporated herein by reference in their entirety.

As used herein, the term “microRNA (miRNA or miR) binding site” refers to a sequence within a polynucleotide, e.g., within a DNA or within an RNA transcript, including in the 5'UTR and/or 3'UTR, that has sufficient complementarity to all or a region of a miRNA to interact with, associate with or bind to the miRNA. In some embodiments, a polynucleotide of the present disclosure comprising an ORF encoding a polypeptide of interest and further comprises one or more miRNA binding site(s). In exemplary embodiments, a 5'UTR and/or 3'UTR of the polynucleotide (e.g., a ribonucleic acid (RNA), e.g., a messenger RNA (mRNA)) comprises the one or more miRNA binding site(s).

A miRNA binding site having sufficient complementarity to a miRNA refers to a degree of complementarity sufficient to facilitate miRNA-mediated regulation of a polynucleotide, e.g., miRNA-mediated translational repression or degradation of the polynucleotide. In exemplary aspects of the present disclosure, a miRNA binding site having sufficient complementarity to the miRNA refers to a degree of complementarity sufficient to facilitate miRNA-mediated degradation of the polynucleotide, e.g., miRNA-guided RNA-induced silencing complex (RISC)-mediated cleavage of mRNA. The miRNA binding site can have complementarity to, for example, a 19-25 nucleotide miRNA sequence, to a 19-23 nucleotide miRNA sequence, or to a 22 nucleotide miRNA sequence. A miRNA binding site can be complementary to only a

portion of a miRNA, e.g., to a portion less than 1, 2, 3, or 4 nucleotides of the full length of a naturally-occurring miRNA sequence. Full or complete complementarity (e.g., full complementarity or complete complementarity over all or a significant portion of the length of a naturally-occurring miRNA) is preferred when the desired regulation is mRNA degradation.

5 In some embodiments, a miRNA binding site includes a sequence that has complementarity (e.g., partial or complete complementarity) with an miRNA seed sequence. In some embodiments, the miRNA binding site includes a sequence that has complete complementarity with a miRNA seed sequence. In some embodiments, a miRNA binding site includes a sequence that has complementarity (e.g., partial or complete complementarity) with an
10 miRNA sequence. In some embodiments, the miRNA binding site includes a sequence that has complete complementarity with a miRNA sequence. In some embodiments, a miRNA binding site has complete complementarity with a miRNA sequence but for 1, 2, or 3 nucleotide substitutions, terminal additions, and/or truncations.

In some embodiments, the miRNA binding site is the same length as the corresponding
15 miRNA. In other embodiments, the miRNA binding site is one, two, three, four, five, six, seven, eight, nine, ten, eleven or twelve nucleotide(s) shorter than the corresponding miRNA at the 5' terminus, the 3' terminus, or both. In still other embodiments, the microRNA binding site is two nucleotides shorter than the corresponding microRNA at the 5' terminus, the 3' terminus, or both. The miRNA binding sites that are shorter than the corresponding miRNAs are still capable of
20 degrading the mRNA incorporating one or more of the miRNA binding sites or preventing the mRNA from translation.

In some embodiments, the miRNA binding site binds the corresponding mature miRNA that is part of an active RISC containing Dicer. In another embodiment, binding of the miRNA binding site to the corresponding miRNA in RISC degrades the mRNA containing the miRNA
25 binding site or prevents the mRNA from being translated. In some embodiments, the miRNA binding site has sufficient complementarity to miRNA so that a RISC complex comprising the miRNA cleaves the polynucleotide comprising the miRNA binding site. In other embodiments, the miRNA binding site has imperfect complementarity so that a RISC complex comprising the miRNA induces instability in the polynucleotide comprising the miRNA binding site. In another
30 embodiment, the miRNA binding site has imperfect complementarity so that a RISC complex comprising the miRNA represses transcription of the polynucleotide comprising the miRNA binding site.

In some embodiments, the miRNA binding site has one, two, three, four, five, six, seven, eight, nine, ten, eleven or twelve mismatch(es) from the corresponding miRNA.

In some embodiments, the miRNA binding site has at least about ten, at least about eleven, at least about twelve, at least about thirteen, at least about fourteen, at least about fifteen, at least about sixteen, at least about seventeen, at least about eighteen, at least about nineteen, at least about twenty, or at least about twenty-one contiguous nucleotides complementary to at least about ten, at least about eleven, at least about twelve, at least about thirteen, at least about fourteen, at least about fifteen, at least about sixteen, at least about seventeen, at least about eighteen, at least about nineteen, at least about twenty, or at least about twenty-one, respectively, contiguous nucleotides of the corresponding miRNA.

By engineering one or more miRNA binding sites into a polynucleotide of the present disclosure, the polynucleotide can be targeted for degradation or reduced translation, provided the miRNA in question is available. This can reduce off-target effects upon delivery of the polynucleotide. For example, if a polynucleotide of the present disclosure is not intended to be delivered to a tissue or cell but ends up in said tissue or cell, then a miRNA abundant in the tissue or cell can inhibit the expression of the gene of interest if one or multiple binding sites of the miRNA are engineered into the 5'UTR and/or 3'UTR of the polynucleotide.

Conversely, miRNA binding sites can be removed from polynucleotide sequences in which they naturally occur in order to increase protein expression in specific tissues. For example, a binding site for a specific miRNA can be removed from a polynucleotide to improve protein expression in tissues or cells containing the miRNA.

In one embodiment, a polynucleotide of the present disclosure can include at least one miRNA-binding site in the 5'UTR and/or 3'UTR in order to regulate cytotoxic or cytoprotective mRNA therapeutics to specific cells such as, but not limited to, normal and/or cancerous cells. In another embodiment, a polynucleotide of the present disclosure can include two, three, four, five, six, seven, eight, nine, ten, or more miRNA-binding sites in the 5'-UTR and/or 3'-UTR in order to regulate cytotoxic or cytoprotective mRNA therapeutics to specific cells such as, but not limited to, normal and/or cancerous cells.

Regulation of expression in multiple tissues can be accomplished through introduction or removal of one or more miRNA binding sites, e.g., one or more distinct miRNA binding sites. The decision whether to remove or insert a miRNA binding site can be made based on miRNA expression patterns and/or their profilings in tissues and/or cells in development and/or disease. Identification of miRNAs, miRNA binding sites, and their expression patterns and role in biology have been reported (e.g., Bonauer et al., *Curr Drug Targets* 2010 11:943-949; Anand and Cheresch *Curr Opin Hematol* 2011 18:171-176; Contreras and Rao *Leukemia* 2012 26:404-413 (2011 Dec 20. doi: 10.1038/leu.2011.356); Bartel *Cell* 2009 136:215-233; Landgraf et al, *Cell*,

2007 129:1401-1414; Gentner and Naldini, Tissue Antigens. 2012 80:393-403 and all references therein; each of which is incorporated herein by reference in its entirety).

miRNAs and miRNA binding sites can correspond to any known sequence, including non-limiting examples described in U.S. Publication Nos. 2014/0200261, 2005/0261218, and 2005/0059005, each of which are incorporated herein by reference in their entirety.

Examples of tissues where miRNA are known to regulate mRNA, and thereby protein expression, include, but are not limited to, liver (miR-122), muscle (miR-133, miR-206, miR-208), endothelial cells (miR-17-92, miR-126), myeloid cells (miR-142-3p, miR-142-5p, miR-16, miR-21, miR-223, miR-24, miR-27), adipose tissue (let-7, miR-30c), heart (miR-1d, miR-149), kidney (miR-192, miR-194, miR-204), and lung epithelial cells (let-7, miR-133, miR-126).

Specifically, miRNAs are known to be differentially expressed in immune cells (also called hematopoietic cells), such as antigen presenting cells (APCs) (e.g., dendritic cells and macrophages), macrophages, monocytes, B lymphocytes, T lymphocytes, granulocytes, natural killer cells, etc. Immune cell specific miRNAs are involved in immunogenicity, autoimmunity, the immune-response to infection, inflammation, as well as unwanted immune response after gene therapy and tissue/organ transplantation. Immune cells specific miRNAs also regulate many aspects of development, proliferation, differentiation and apoptosis of hematopoietic cells (immune cells). For example, miR-142 and miR-146 are exclusively expressed in immune cells, particularly abundant in myeloid dendritic cells. It has been demonstrated that the immune response to a polynucleotide can be shut-off by adding miR-142 binding sites to the 3'-UTR of the polynucleotide, enabling more stable gene transfer in tissues and cells. miR-142 efficiently degrades exogenous polynucleotides in antigen presenting cells and suppresses cytotoxic elimination of transduced cells (e.g., Annoni A et al., blood, 2009, 114, 5152-5161; Brown BD, et al., Nat med. 2006, 12(5), 585-591; Brown BD, et al., blood, 2007, 110(13): 4144-4152, each of which is incorporated herein by reference in its entirety).

An antigen-mediated immune response can refer to an immune response triggered by foreign antigens, which, when entering an organism, are processed by the antigen presenting cells and displayed on the surface of the antigen presenting cells. T cells can recognize the presented antigen and induce a cytotoxic elimination of cells that express the antigen.

Introducing a miR-142 binding site into the 5'UTR and/or 3'UTR of a polynucleotide of the present disclosure can selectively repress gene expression in antigen presenting cells through miR-142 mediated degradation, limiting antigen presentation in antigen presenting cells (e.g., dendritic cells) and thereby preventing antigen-mediated immune response after the delivery of the polynucleotide. The polynucleotide is then stably expressed in target tissues or cells without triggering cytotoxic elimination.

In one embodiment, binding sites for miRNAs that are known to be expressed in immune cells, in particular, antigen presenting cells, can be engineered into a polynucleotide of the present disclosure to suppress the expression of the polynucleotide in antigen presenting cells through miRNA mediated RNA degradation, subduing the antigen-mediated immune response.

Expression of the polynucleotide is maintained in non-immune cells where the immune cell specific miRNAs are not expressed. For example, in some embodiments, to prevent an immunogenic reaction against a liver specific protein, any miR-122 binding site can be removed and a miR-142 (and/or mirR-146) binding site can be engineered into the 5'UTR and/or 3'UTR of a polynucleotide of the present disclosure.

To further drive the selective degradation and suppression in APCs and macrophage, a polynucleotide of the present disclosure can include a further negative regulatory element in the 5'UTR and/or 3'UTR, either alone or in combination with miR-142 and/or miR-146 binding sites. As a non-limiting example, the further negative regulatory element is a Constitutive Decay Element (CDE).

Immune cell specific miRNAs include, but are not limited to, hsa-let-7a-2-3p, hsa-let-7a-3p, hsa-7a-5p, hsa-let-7c, hsa-let-7e-3p, hsa-let-7e-5p, hsa-let-7g-3p, hsa-let-7g-5p, hsa-let-7i-3p, hsa-let-7i-5p, miR-10a-3p, miR-10a-5p, miR-1184, hsa-let-7f-1--3p, hsa-let-7f-2--5p, hsa-let-7f-5p, miR-125b-1-3p, miR-125b-2-3p, miR-125b-5p, miR-1279, miR-130a-3p, miR-130a-5p, miR-132-3p, miR-132-5p, miR-142-3p, miR-142-5p, miR-143-3p, miR-143-5p, miR-146a-3p, miR-146a-5p, miR-146b-3p, miR-146b-5p, miR-147a, miR-147b, miR-148a-5p, miR-148a-3p, miR-150-3p, miR-150-5p, miR-151b, miR-155-3p, miR-155-5p, miR-15a-3p, miR-15a-5p, miR-15b-5p, miR-15b-3p, miR-16-1-3p, miR-16-2-3p, miR-16-5p, miR-17-5p, miR-181a-3p, miR-181a-5p, miR-181a-2-3p, miR-182-3p, miR-182-5p, miR-197-3p, miR-197-5p, miR-21-5p, miR-21-3p, miR-214-3p, miR-214-5p, miR-223-3p, miR-223-5p, miR-221-3p, miR-221-5p, miR-23b-3p, miR-23b-5p, miR-24-1-5p, miR-24-2-5p, miR-24-3p, miR-26a-1-3p, miR-26a-2-3p, miR-26a-5p, miR-26b-3p, miR-26b-5p, miR-27a-3p, miR-27a-5p, miR-27b-3p, miR-27b-5p, miR-28-3p, miR-28-5p, miR-2909, miR-29a-3p, miR-29a-5p, miR-29b-1-5p, miR-29b-2-5p, miR-29c-3p, miR-29c-5p, miR-30e-3p, miR-30e-5p, miR-331-5p, miR-339-3p, miR-339-5p, miR-345-3p, miR-345-5p, miR-346, miR-34a-3p, miR-34a-5p, , miR-363-3p, miR-363-5p, miR-372, miR-377-3p, miR-377-5p, miR-493-3p, miR-493-5p, miR-542, miR-548b-5p, miR-548c-5p, miR-548i, miR-548j, miR-548n, miR-574-3p, miR-598, miR-718, miR-935, miR-99a-3p, miR-99a-5p, miR-99b-3p, and miR-99b-5p. Furthermore, novel miRNAs can be identified in immune cell through micro-array hybridization and microtome analysis (e.g., Jima DD et al, Blood, 2010, 116:e118-e127; Vaz C et al., BMC Genomics, 2010, 11,288, the content of each of which is incorporated herein by reference in its entirety.)

miRNAs that are known to be expressed in the liver include, but are not limited to, miR-107, miR-122-3p, miR-122-5p, miR-1228-3p, miR-1228-5p, miR-1249, miR-129-5p, miR-1303, miR-151a-3p, miR-151a-5p, miR-152, miR-194-3p, miR-194-5p, miR-199a-3p, miR-199a-5p, miR-199b-3p, miR-199b-5p, miR-296-5p, miR-557, miR-581, miR-939-3p, and miR-939-5p.

- 5 MiRNA binding sites from any liver specific miRNA can be introduced to or removed from a polynucleotide of the present disclosure to regulate expression of the polynucleotide in the liver. Liver specific miRNA binding sites can be engineered alone or further in combination with immune cell (e.g., APC) miRNA binding sites in a polynucleotide of the present disclosure.

- 10 miRNAs that are known to be expressed in the lung include, but are not limited to, let-7a-2-3p, let-7a-3p, let-7a-5p, miR-126-3p, miR-126-5p, miR-127-3p, miR-127-5p, miR-130a-3p, miR-130a-5p, miR-130b-3p, miR-130b-5p, miR-133a, miR-133b, miR-134, miR-18a-3p, miR-18a-5p, miR-18b-3p, miR-18b-5p, miR-24-1-5p, miR-24-2-5p, miR-24-3p, miR-296-3p, miR-296-5p, miR-32-3p, miR-337-3p, miR-337-5p, miR-381-3p, and miR-381-5p. miRNA binding sites from any lung specific miRNA can be introduced to or removed from a polynucleotide of the present disclosure to regulate expression of the polynucleotide in the lung. Lung specific miRNA binding sites can be engineered alone or further in combination with immune cell (e.g., APC) miRNA binding sites in a polynucleotide of the present disclosure.
- 15

- miRNAs that are known to be expressed in the heart include, but are not limited to, miR-1, miR-133a, miR-133b, miR-149-3p, miR-149-5p, miR-186-3p, miR-186-5p, miR-208a, miR-208b, miR-210, miR-296-3p, miR-320, miR-451a, miR-451b, miR-499a-3p, miR-499a-5p, miR-499b-3p, miR-499b-5p, miR-744-3p, miR-744-5p, miR-92b-3p, and miR-92b-5p. miRNA binding sites from any heart specific microRNA can be introduced to or removed from a polynucleotide of the present disclosure to regulate expression of the polynucleotide in the heart. Heart specific miRNA binding sites can be engineered alone or further in combination with immune cell (e.g., APC) miRNA binding sites in a polynucleotide of the present disclosure.
- 20
- 25

- miRNAs that are known to be expressed in the nervous system include, but are not limited to, miR-124-5p, miR-125a-3p, miR-125a-5p, miR-125b-1-3p, miR-125b-2-3p, miR-125b-5p, miR-1271-3p, miR-1271-5p, miR-128, miR-132-5p, miR-135a-3p, miR-135a-5p, miR-135b-3p, miR-135b-5p, miR-137, miR-139-5p, miR-139-3p, miR-149-3p, miR-149-5p, miR-153, miR-181c-3p, miR-181c-5p, miR-183-3p, miR-183-5p, miR-190a, miR-190b, miR-212-3p, miR-212-5p, miR-219-1-3p, miR-219-2-3p, miR-23a-3p, miR-23a-5p, miR-30a-5p, miR-30b-3p, miR-30b-5p, miR-30c-1-3p, miR-30c-2-3p, miR-30c-5p, miR-30d-3p, miR-30d-5p, miR-329, miR-342-3p, miR-3665, miR-3666, miR-380-3p, miR-380-5p, miR-383, miR-410, miR-425-3p, miR-425-5p, miR-454-3p, miR-454-5p, miR-483, miR-510, miR-516a-3p, miR-548b-5p, miR-548c-5p, miR-571, miR-7-1-3p, miR-7-2-3p, miR-7-5p, miR-802, miR-922, miR-9-3p, and miR-
- 30
- 35

9-5p. miRNAs enriched in the nervous system further include those specifically expressed in neurons, including, but not limited to, miR-132-3p, miR-132-5p, miR-148b-3p, miR-148b-5p, miR-151a-3p, miR-151a-5p, miR-212-3p, miR-212-5p, miR-320b, miR-320e, miR-323a-3p, miR-323a-5p, miR-324-5p, miR-325, miR-326, miR-328, miR-922 and those specifically
5 expressed in glial cells, including, but not limited to, miR-1250, miR-219-1-3p, miR-219-2-3p, miR-219-5p, miR-23a-3p, miR-23a-5p, miR-3065-3p, miR-3065-5p, miR-30e-3p, miR-30e-5p, miR-32-5p, miR-338-5p, and miR-657. miRNA binding sites from any CNS specific miRNA can be introduced to or removed from a polynucleotide of the present disclosure to regulate expression of the polynucleotide in the nervous system. Nervous system specific miRNA
10 binding sites can be engineered alone or further in combination with immune cell (e.g., APC) miRNA binding sites in a polynucleotide of the present disclosure.

miRNAs that are known to be expressed in the pancreas include, but are not limited to, miR-105-3p, miR-105-5p, miR-184, miR-195-3p, miR-195-5p, miR-196a-3p, miR-196a-5p, miR-214-3p, miR-214-5p, miR-216a-3p, miR-216a-5p, miR-30a-3p, miR-33a-3p, miR-33a-5p,
15 miR-375, miR-7-1-3p, miR-7-2-3p, miR-493-3p, miR-493-5p, and miR-944. MiRNA binding sites from any pancreas specific miRNA can be introduced to or removed from a polynucleotide of the present disclosure to regulate expression of the polynucleotide in the pancreas. Pancreas specific miRNA binding sites can be engineered alone or further in combination with immune cell (e.g. APC) miRNA binding sites in a polynucleotide of the present disclosure.

20 miRNAs that are known to be expressed in the kidney include, but are not limited to, miR-122-3p, miR-145-5p, miR-17-5p, miR-192-3p, miR-192-5p, miR-194-3p, miR-194-5p, miR-20a-3p, miR-20a-5p, miR-204-3p, miR-204-5p, miR-210, miR-216a-3p, miR-216a-5p, miR-296-3p, miR-30a-3p, miR-30a-5p, miR-30b-3p, miR-30b-5p, miR-30c-1-3p, miR-30c-2-3p, miR-30c-5p, miR-324-3p, miR-335-3p, miR-335-5p, miR-363-3p, miR-363-5p, and miR-562.
25 miRNA binding sites from any kidney specific miRNA can be introduced to or removed from a polynucleotide of the present disclosure to regulate expression of the polynucleotide in the kidney. Kidney specific miRNA binding sites can be engineered alone or further in combination with immune cell (e.g., APC) miRNA binding sites in a polynucleotide of the present disclosure.

miRNAs that are known to be expressed in the muscle include, but are not limited to, let-
30 7g-3p, let-7g-5p, miR-1, miR-1286, miR-133a, miR-133b, miR-140-3p, miR-143-3p, miR-143-5p, miR-145-3p, miR-145-5p, miR-188-3p, miR-188-5p, miR-206, miR-208a, miR-208b, miR-25-3p, and miR-25-5p. MiRNA binding sites from any muscle specific miRNA can be introduced to or removed from a polynucleotide of the present disclosure to regulate expression of the polynucleotide in the muscle. Muscle specific miRNA binding sites can be engineered

alone or further in combination with immune cell (e.g., APC) miRNA binding sites in a polynucleotide of the present disclosure.

miRNAs are also differentially expressed in different types of cells, such as, but not limited to, endothelial cells, epithelial cells, and adipocytes.

5 miRNAs that are known to be expressed in endothelial cells include, but are not limited to, let-7b-3p, let-7b-5p, miR-100-3p, miR-100-5p, miR-101-3p, miR-101-5p, miR-126-3p, miR-126-5p, miR-1236-3p, miR-1236-5p, miR-130a-3p, miR-130a-5p, miR-17-5p, miR-17-3p, miR-18a-3p, miR-18a-5p, miR-19a-3p, miR-19a-5p, miR-19b-1-5p, miR-19b-2-5p, miR-19b-3p, miR-20a-3p, miR-20a-5p, miR-217, miR-210, miR-21-3p, miR-21-5p, miR-221-3p, miR-221-
10 5p, miR-222-3p, miR-222-5p, miR-23a-3p, miR-23a-5p, miR-296-5p, miR-361-3p, miR-361-5p, miR-421, miR-424-3p, miR-424-5p, miR-513a-5p, miR-92a-1-5p, miR-92a-2-5p, miR-92a-3p, miR-92b-3p, and miR-92b-5p. Many novel miRNAs are discovered in endothelial cells from deep-sequencing analysis (e.g., Voellenkle C et al., RNA, 2012, 18, 472-484, herein incorporated by reference in its entirety). miRNA binding sites from any endothelial cell specific miRNA can
15 be introduced to or removed from a polynucleotide of the present disclosure to regulate expression of the polynucleotide in the endothelial cells.

miRNAs that are known to be expressed in epithelial cells include, but are not limited to, let-7b-3p, let-7b-5p, miR-1246, miR-200a-3p, miR-200a-5p, miR-200b-3p, miR-200b-5p, miR-200c-3p, miR-200c-5p, miR-338-3p, miR-429, miR-451a, miR-451b, miR-494, miR-802 and
20 miR-34a, miR-34b-5p, miR-34c-5p, miR-449a, miR-449b-3p, miR-449b-5p specific in respiratory ciliated epithelial cells, let-7 family, miR-133a, miR-133b, miR-126 specific in lung epithelial cells, miR-382-3p, miR-382-5p specific in renal epithelial cells, and miR-762 specific in corneal epithelial cells. miRNA binding sites from any epithelial cell specific miRNA can be introduced to or removed from a polynucleotide of the present disclosure to regulate expression
25 of the polynucleotide in the epithelial cells.

In addition, a large group of miRNAs are enriched in embryonic stem cells, controlling stem cell self-renewal as well as the development and/or differentiation of various cell lineages, such as neural cells, cardiac, hematopoietic cells, skin cells, osteogenic cells and muscle cells (e.g., Kuppusamy KT et al., Curr. Mol Med, 2013, 13(5), 757-764; Vidigal JA and Ventura A,
30 Semin Cancer Biol. 2012, 22(5-6), 428-436; Goff LA et al., PLoS One, 2009, 4:e7192; Morin RD et al., Genome Res, 2008, 18, 610-621; Yoo JK et al., Stem Cells Dev. 2012, 21(11), 2049-2057, each of which is herein incorporated by reference in its entirety). MiRNAs abundant in embryonic stem cells include, but are not limited to, let-7a-2-3p, let-a-3p, let-7a-5p, let7d-3p, let-7d-5p, miR-103a-2-3p, miR-103a-5p, miR-106b-3p, miR-106b-5p, miR-1246, miR-1275, miR-
35 138-1-3p, miR-138-2-3p, miR-138-5p, miR-154-3p, miR-154-5p, miR-200c-3p, miR-200c-5p,

miR-290, miR-301a-3p, miR-301a-5p, miR-302a-3p, miR-302a-5p, miR-302b-3p, miR-302b-5p, miR-302c-3p, miR-302c-5p, miR-302d-3p, miR-302d-5p, miR-302e, miR-367-3p, miR-367-5p, miR-369-3p, miR-369-5p, miR-370, miR-371, miR-373, miR-380-5p, miR-423-3p, miR-423-5p, miR-486-5p, miR-520c-3p, miR-548e, miR-548f, miR-548g-3p, miR-548g-5p, miR-548i, miR-548k, miR-548l, miR-548m, miR-548n, miR-548o-3p, miR-548o-5p, miR-548p, miR-664a-3p, miR-664a-5p, miR-664b-3p, miR-664b-5p, miR-766-3p, miR-766-5p, miR-885-3p, miR-885-5p, miR-93-3p, miR-93-5p, miR-941, miR-96-3p, miR-96-5p, miR-99b-3p and miR-99b-5p. Many predicted novel miRNAs are discovered by deep sequencing in human embryonic stem cells (e.g., Morin RD et al., *Genome Res*, 2008, 18, 610-621; Goff LA et al., *PLoS One*, 2009, 4:e7192; Bar M et al., *Stem cells*, 2008, 26, 2496-2505, the content of each of which is incorporated herein by reference in its entirety).

In one embodiment, the binding sites of embryonic stem cell specific miRNAs can be included in or removed from the 3'UTR of a polynucleotide of the present disclosure to modulate the development and/or differentiation of embryonic stem cells, to inhibit the senescence of stem cells in a degenerative condition (e.g. degenerative diseases), or to stimulate the senescence and apoptosis of stem cells in a disease condition (e.g. cancer stem cells).

As a non-limiting example, miRNA binding sites for miRNAs that are over-expressed in certain cancer and/or tumor cells can be removed from the 3'UTR of a polynucleotide of the present disclosure, restoring the expression suppressed by the over-expressed miRNAs in cancer cells, thus ameliorating the responsive biological function, for instance, transcription stimulation and/or repression, cell cycle arrest, apoptosis and cell death. Normal cells and tissues, wherein miRNAs expression is not up-regulated, will remain unaffected.

miRNA can also regulate complex biological processes such as angiogenesis (e.g., miR-132) (Anand and Cheresch *Curr Opin Hematol* 2011 18:171-176). In the polynucleotides of the present disclosure, miRNA binding sites that are involved in such processes can be removed or introduced, in order to tailor the expression of the polynucleotides to biologically relevant cell types or relevant biological processes. In this context, the polynucleotides of the present disclosure are defined as auxotrophic polynucleotides.

In some embodiments, a polynucleotide of the present disclosure comprises a miRNA binding site, wherein the miRNA binding site comprises one or more nucleotide sequences selected from Table 2, including one or more copies of any one or more of the miRNA binding site sequences. In some embodiments, a polynucleotide of the present disclosure further comprises at least one, two, three, four, five, six, seven, eight, nine, ten, or more of the same or different miRNA binding sites selected from Table 2, including any combination thereof. In some embodiments, the miRNA binding site binds to miR-142 or is complementary to miR-142.

In some embodiments, the miR-142 comprises SEQ ID NO: 12. In some embodiments, the miRNA binding site binds to miR-142-3p or miR-142-5p. In some embodiments, the miR-142-3p binding site comprises SEQ ID NO: 14. In some embodiments, the miR-142-5p binding site comprises SEQ ID NO: 18. In some embodiments, the miRNA binding site comprises a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, or 100% identical to SEQ ID NO: 14 or SEQ ID NO: 18.

Table 2. miR-142 and miR-142 binding sites

SEQ ID NO.	Description	Sequence
12	miR-142	GACAGUGCAGUCACCCAUAAGUAGA AAGCACUACUACAGCACUGGAGGGU GUAGUGUUUCCUACUUUAUGGAUGAG UGUACUGUG
13	miR-142-3p	UGUAGUGUUUCCUACUUUAUGGA
14	miR-142-3p binding site	UCCAUAAGUAGGAAACACUACA
17	miR-142-5p	CAUAAGUAGAAAGCACUACU
18	miR-142-5p binding site	AGUAGUGCUUUCUACUUUAUG

In some embodiments, a miRNA binding site is inserted in the polynucleotide of the present disclosure in any position of the polynucleotide (e.g., the 5'UTR and/or 3'UTR). In some embodiments, the 5'UTR comprises a miRNA binding site. In some embodiments, the 3'UTR comprises a miRNA binding site. In some embodiments, the 5'UTR and the 3'UTR comprise a miRNA binding site. The insertion site in the polynucleotide can be anywhere in the polynucleotide as long as the insertion of the miRNA binding site in the polynucleotide does not interfere with the translation of a functional polypeptide in the absence of the corresponding miRNA; and in the presence of the miRNA, the insertion of the miRNA binding site in the polynucleotide and the binding of the miRNA binding site to the corresponding miRNA are capable of degrading the polynucleotide or preventing the translation of the polynucleotide.

In some embodiments, a miRNA binding site is inserted in at least about 30 nucleotides downstream from the stop codon of an ORF in a polynucleotide of the present disclosure comprising the ORF. In some embodiments, a miRNA binding site is inserted in at least about 10 nucleotides, at least about 15 nucleotides, at least about 20 nucleotides, at least about 25 nucleotides, at least about 30 nucleotides, at least about 35 nucleotides, at least about 40 nucleotides, at least about 45 nucleotides, at least about 50 nucleotides, at least about 55 nucleotides, at least about 60 nucleotides, at least about 65 nucleotides, at least about 70 nucleotides, at least about 75 nucleotides, at least about 80 nucleotides, at least about 85 nucleotides, at least about 90 nucleotides, at least about 95 nucleotides, or at least about 100 nucleotides downstream from the stop codon of an ORF in a polynucleotide of the present

disclosure. In some embodiments, a miRNA binding site is inserted in about 10 nucleotides to about 100 nucleotides, about 20 nucleotides to about 90 nucleotides, about 30 nucleotides to about 80 nucleotides, about 40 nucleotides to about 70 nucleotides, about 50 nucleotides to about 60 nucleotides, about 45 nucleotides to about 65 nucleotides downstream from the stop codon of an ORF in a polynucleotide of the present disclosure.

miRNA gene regulation can be influenced by the sequence surrounding the miRNA such as, but not limited to, the species of the surrounding sequence, the type of sequence (e.g., heterologous, homologous, exogenous, endogenous, or artificial), regulatory elements in the surrounding sequence and/or structural elements in the surrounding sequence. The miRNA can be influenced by the 5'UTR and/or 3'UTR. As a non-limiting example, a non-human 3'UTR can increase the regulatory effect of the miRNA sequence on the expression of a polypeptide of interest compared to a human 3'UTR of the same sequence type.

In one embodiment, other regulatory elements and/or structural elements of the 5'UTR can influence miRNA mediated gene regulation. One example of a regulatory element and/or structural element is a structured IRES (Internal Ribosome Entry Site) in the 5'UTR, which is necessary for the binding of translational elongation factors to initiate protein translation. EIF4A2 binding to this secondarily structured element in the 5'-UTR is necessary for miRNA mediated gene expression (Meijer HA et al., Science, 2013, 340, 82-85, herein incorporated by reference in its entirety). The polynucleotides of the present disclosure can further include this structured 5'UTR in order to enhance microRNA mediated gene regulation.

At least one miRNA binding site can be engineered into the 3'UTR of a polynucleotide of the present disclosure. In this context, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, or more miRNA binding sites can be engineered into a 3'UTR of a polynucleotide of the present disclosure. For example, 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, 2, or 1 miRNA binding sites can be engineered into the 3'UTR of a polynucleotide of the present disclosure. In one embodiment, miRNA binding sites incorporated into a polynucleotide of the present disclosure can be the same or can be different miRNA sites. A combination of different miRNA binding sites incorporated into a polynucleotide of the present disclosure can include combinations in which more than one copy of any of the different miRNA sites are incorporated. In another embodiment, miRNA binding sites incorporated into a polynucleotide of the present disclosure can target the same or different tissues in the body. As a non-limiting example, through the introduction of tissue-, cell-type-, or disease-specific miRNA binding sites in the 3'-UTR of a polynucleotide of the present disclosure, the degree of expression in specific cell types (e.g., hepatocytes, myeloid cells, endothelial cells, cancer cells, etc.) can be reduced.

In one embodiment, a miRNA binding site can be engineered near the 5' terminus of the 3'UTR, about halfway between the 5' terminus and 3' terminus of the 3'UTR and/or near the 3' terminus of the 3'UTR in a polynucleotide of the present disclosure. As a non-limiting example, a miRNA binding site can be engineered near the 5' terminus of the 3'UTR and about halfway
5 between the 5' terminus and 3' terminus of the 3'UTR. As another non-limiting example, a miRNA binding site can be engineered near the 3' terminus of the 3'UTR and about halfway between the 5' terminus and 3' terminus of the 3'UTR. As yet another non-limiting example, a miRNA binding site can be engineered near the 5' terminus of the 3'UTR and near the 3' terminus of the 3'UTR.

10 In another embodiment, a 3'UTR can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 miRNA binding sites. The miRNA binding sites can be complementary to a miRNA, miRNA seed sequence, and/or miRNA sequences flanking the seed sequence.

In one embodiment, a polynucleotide of the present disclosure can be engineered to include more than one miRNA site expressed in different tissues or different cell types of a
15 subject. As a non-limiting example, a polynucleotide of the present disclosure can be engineered to include miR-192 and miR-122 to regulate expression of the polynucleotide in the liver and kidneys of a subject. In another embodiment, a polynucleotide of the present disclosure can be engineered to include more than one miRNA site for the same tissue.

In some embodiments, the expression of a polynucleotide of the present disclosure can be
20 controlled by incorporating at least one miR binding site in the polynucleotide and formulating the polynucleotide for administration. As a non-limiting example, a polynucleotide of the present disclosure can be targeted to a tissue or cell by incorporating a miRNA binding site and formulating the polynucleotide in a lipid nanoparticle comprising an ionizable *e.g.*, an ionizable amino lipid, sometimes referred to in the prior art as an “ionizable cationic lipid”, including any
25 of the lipids described herein.

A polynucleotide of the present disclosure can be engineered for more targeted expression in specific tissues, cell types, or biological conditions based on the expression patterns of miRNAs in the different tissues, cell types, or biological conditions. Through introduction of tissue-specific miRNA binding sites, a polynucleotide of the present disclosure
30 can be designed for optimal protein expression in a tissue or cell, or in the context of a biological condition.

In some embodiments, a polynucleotide of the present disclosure can be designed to incorporate miRNA binding sites that either have 100% identity to known miRNA seed sequences or have less than 100% identity to miRNA seed sequences. In some embodiments, a
35 polynucleotide of the present disclosure can be designed to incorporate miRNA binding sites that

have at least: 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to known miRNA seed sequences. The miRNA seed sequence can be partially mutated to decrease miRNA binding affinity and as such result in reduced downmodulation of the polynucleotide. In essence, the degree of match or mis-match between the miRNA binding site and the miRNA seed can act as a rheostat to more finely tune the ability of the miRNA to modulate protein expression. In addition, mutation in the non-seed region of a miRNA binding site can also impact the ability of a miRNA to modulate protein expression.

In one embodiment, a miRNA sequence can be incorporated into the loop of a stem loop.

In another embodiment, a miRNA seed sequence can be incorporated in the loop of a stem loop and a miRNA binding site can be incorporated into the 5' or 3' stem of the stem loop.

In one embodiment, a translation enhancer element (TEE) can be incorporated on the 5' end of the stem of a stem loop and a miRNA seed can be incorporated into the stem of the stem loop. In another embodiment, a TEE can be incorporated on the 5' end of the stem of a stem loop, a miRNA seed can be incorporated into the stem of the stem loop and a miRNA binding site can be incorporated into the 3' end of the stem or the sequence after the stem loop. The miRNA seed and the miRNA binding site can be for the same and/or different miRNA sequences.

In one embodiment, the incorporation of a miRNA sequence and/or a TEE sequence changes the shape of the stem loop region which can increase and/or decrease translation. (see e.g., Kedde et al., "A Pumilio-induced RNA structure switch in p27-3'UTR controls miR-221 and miR-22 accessibility." *Nature Cell Biology*. 2010, incorporated herein by reference in its entirety).

In one embodiment, the 5'-UTR of a polynucleotide of the present disclosure can comprise at least one miRNA sequence. The miRNA sequence can be, but is not limited to, a 19 or 22 nucleotide sequence and/or a miRNA sequence without the seed.

In one embodiment the miRNA sequence in the 5'UTR can be used to stabilize a polynucleotide of the present disclosure described herein.

In another embodiment, a miRNA sequence in the 5'UTR of a polynucleotide of the present disclosure can be used to decrease the accessibility of the site of translation initiation such as, but not limited to a start codon. See, e.g., Matsuda et al., *PLoS One*. 2010 11(5):e15057; incorporated herein by reference in its entirety, which used antisense locked nucleic acid (LNA) oligonucleotides and exon-junction complexes (EJCs) around a start codon (-4 to +37 where the A of the AUG codons is +1) in order to decrease the accessibility to the first start codon (AUG). Matsuda showed that altering the sequence around the start codon with an LNA or EJC affected the efficiency, length and structural stability of a polynucleotide. A

polynucleotide of the present disclosure can comprise a miRNA sequence, instead of the LNA or EJC sequence described by Matsuda et al, near the site of translation initiation in order to decrease the accessibility to the site of translation initiation. The site of translation initiation can be prior to, after or within the miRNA sequence. As a non-limiting example, the site of translation initiation can be located within a miRNA sequence such as a seed sequence or binding site. As another non-limiting example, the site of translation initiation can be located within a miR-122 sequence such as the seed sequence or the mir-122 binding site.

In some embodiments, a polynucleotide of the present disclosure can include at least one miRNA in order to dampen the antigen presentation by antigen presenting cells. The miRNA can be the complete miRNA sequence, the miRNA seed sequence, the miRNA sequence without the seed, or a combination thereof. As a non-limiting example, a miRNA incorporated into a polynucleotide of the present disclosure can be specific to the hematopoietic system. As another non-limiting example, a miRNA incorporated into a polynucleotide of the present disclosure to dampen antigen presentation is miR-142-3p.

In some embodiments, a polynucleotide of the present disclosure can include at least one miRNA in order to dampen expression of the encoded polypeptide in a tissue or cell of interest. As a non-limiting example, a polynucleotide of the present disclosure can include at least one miR-122 binding site in order to dampen expression of an encoded polypeptide of interest in the liver. As another non-limiting example a polynucleotide of the present disclosure can include at least one miR-142-3p binding site, miR-142-3p seed sequence, miR-142-3p binding site without the seed, miR-142-5p binding site, miR-142-5p seed sequence, miR-142-5p binding site without the seed, miR-146 binding site, miR-146 seed sequence and/or miR-146 binding site without the seed sequence.

In some embodiments, a polynucleotide of the present disclosure can comprise at least one miRNA binding site in the 3'UTR in order to selectively degrade mRNA therapeutics in the immune cells to subdue unwanted immunogenic reactions caused by therapeutic delivery. As a non-limiting example, the miRNA binding site can make a polynucleotide of the present disclosure more unstable in antigen presenting cells. Non-limiting examples of these miRNAs include mir-142-5p, mir-142-3p, mir-146a-5p, and mir-146-3p.

In one embodiment, a polynucleotide of the present disclosure comprises at least one miRNA sequence in a region of the polynucleotide that can interact with a RNA binding protein.

In some embodiments, the polynucleotide of the present disclosure (e.g., a RNA, e.g., an mRNA) comprising (i) a sequence-optimized nucleotide sequence (e.g., an ORF) encoding an antibody (e.g., the wild-type sequence, functional fragment, or variant thereof) and (ii) a miRNA binding site (e.g., a miRNA binding site that binds to miR-142).

In some embodiments, the polynucleotide of the present disclosure comprises a uracil-modified sequence encoding an antibody disclosed herein and a miRNA binding site disclosed herein, e.g., a miRNA binding site that binds to miR-142. In some embodiments, the uracil-modified sequence encoding an antibody comprises at least one chemically modified nucleobase, e.g., 5-methoxyuracil. In some embodiments, at least 95% of a type of nucleobase (e.g., uracil) in a uracil-modified sequence encoding an antibody of the present disclosure are modified nucleobases. In some embodiments, at least 95% of uracil in a uracil-modified sequence encoding an antibody is 5-methoxyuridine. In some embodiments, the polynucleotide comprising a nucleotide sequence encoding an antibody disclosed herein and a miRNA binding site is formulated with a delivery agent, e.g., a compound having the Formula (I), (IA), (II), (IIa), (IIb), (IIc), (IId) or (IIE), e.g., any of Compounds 1-232.

3' UTRs

In certain embodiments, a polynucleotide of the present disclosure (e.g., a polynucleotide comprising a nucleotide sequence encoding an antibody of the present disclosure) further comprises a 3' UTR.

3'-UTR is the section of mRNA that immediately follows the translation termination codon and often contains regulatory regions that post-transcriptionally influence gene expression. Regulatory regions within the 3'-UTR can influence polyadenylation, translation efficiency, localization, and stability of the mRNA. In one embodiment, the 3'-UTR useful for the present disclosure comprises a binding site for regulatory proteins or microRNAs.

Regions having a 5' Cap

The present disclosure also includes a polynucleotide that comprises both a 5' Cap and a polynucleotide of the present disclosure (e.g., a polynucleotide comprising a nucleotide sequence encoding an antibody).

The 5' cap structure of a natural mRNA is involved in nuclear export, increasing mRNA stability and binds the mRNA Cap Binding Protein (CBP), which is responsible for mRNA 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 during mRNA splicing.

Endogenous mRNA molecules can 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 mRNA molecule. This 5'-guanylate cap can 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 mRNA can optionally also be 2'-O-methylated. 5'-decapping

through hydrolysis and cleavage of the guanylate cap structure can target a nucleic acid molecule, such as an mRNA molecule, for degradation.

In some embodiments, the polynucleotides of the present disclosure (e.g., a polynucleotide comprising a nucleotide sequence encoding an antibody) incorporate a cap moiety.

In some embodiments, polynucleotides of the present disclosure (e.g., a polynucleotide comprising a nucleotide sequence encoding an antibody) comprise a non-hydrolyzable cap structure preventing decapping and thus increasing mRNA half-life. Because cap structure hydrolysis requires cleavage of 5'-ppp-5' phosphodiester linkages, modified nucleotides can be used during the capping reaction. For example, a Vaccinia Capping Enzyme from New England Biolabs (Ipswich, MA) can be used with α -thio-guanosine nucleotides according to the manufacturer's instructions to create a phosphorothioate linkage in the 5'-ppp-5' cap. Additional modified guanosine nucleotides can be used such as α -methyl-phosphonate and seleno-phosphate nucleotides.

Additional modifications 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'-hydroxyl group of the sugar ring. Multiple distinct 5'-cap structures can be used to generate the 5'-cap of a nucleic acid molecule, such as a polynucleotide that functions as 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 can be chemically (*i.e.*, non-enzymatically) or enzymatically synthesized and/or linked to the polynucleotides of the present disclosure.

For example, the Anti-Reverse Cap Analog (ARCA) cap contains two guanines linked by a 5'-5'-triphosphate group, wherein one guanine contains an N7 methyl group as well as a 3'-O-methyl group (*i.e.*, N7,3'-O-dimethyl-guanosine-5'-triphosphate-5'-guanosine (m⁷G-3'mppp-G; which can equivalently be designated 3' O-Me-m⁷G(5')ppp(5')G). The 3'-O atom of the other, unmodified, guanine becomes linked to the 5'-terminal nucleotide of the capped polynucleotide. The N7- and 3'-O-methylated guanine provides the terminal moiety of the capped polynucleotide.

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).

In some embodiments, the cap is a dinucleotide cap analog. As a non-limiting example, the dinucleotide cap analog can be modified at different phosphate positions with a boranophosphate group or a phosphoroselenoate group such as the dinucleotide cap analogs

described in U.S. Patent No. US 8,519,110, the contents of which are herein incorporated by reference in its entirety.

In another embodiment, the cap is a cap analog is a N7-(4-chlorophenoxyethyl) substituted dicucleotide form of a cap analog known in the art and/or described herein. Non-limiting examples of a N7-(4-chlorophenoxyethyl) substituted dicucleotide form of a cap analog include a N7-(4-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 contents of which are herein incorporated by reference in its entirety). In another embodiment, a cap analog of the present disclosure is a 4-chloro/bromophenoxyethyl analog.

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

Polynucleotides of the present disclosure (e.g., a polynucleotide comprising a nucleotide sequence encoding an antibody) can also be capped post-manufacture (whether IVT or chemical synthesis), 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 compared to synthetic features or analogs, etc., 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 of the present disclosure are those that, among other things, have enhanced binding of cap binding proteins, 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 can create a canonical 5'-5'-triphosphate linkage between the 5'-terminal nucleotide of a polynucleotide and a guanine cap nucleotide wherein the cap guanine contains an N7 methylation and the 5'-terminal nucleotide of the mRNA contains 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 are not limited to, 7mG(5')ppp(5')N_pN₂p (cap 0), 7mG(5')ppp(5')NlmpN_p (cap 1), and 7mG(5')-ppp(5')NlmpN₂mp (cap 2).

As a non-limiting example, capping chimeric polynucleotides post-manufacture can be more efficient as nearly 100% of the chimeric polynucleotides can be capped. This is in contrast to ~80% when a cap analog is linked to a chimeric polynucleotide in the course of an *in vitro* transcription reaction.

According to the present disclosure, 5' terminal caps can include endogenous caps or cap analogs. According to the present disclosure, a 5' terminal cap can comprise a guanine analog. Useful 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.

Poly-A Tails

In some embodiments, the polynucleotides of the present disclosure (e.g., a polynucleotide comprising a nucleotide sequence encoding an antibody) further comprise a poly-A tail. In further embodiments, terminal groups on the poly-A tail can be incorporated for stabilization. In other embodiments, a poly-A tail comprises des-3' hydroxyl tails.

During RNA processing, a long chain of adenine nucleotides (poly-A tail) can be added to a polynucleotide such as an mRNA molecule in order to increase stability. Immediately after transcription, the 3' end of the transcript can be cleaved to free a 3' hydroxyl. Then poly-A polymerase adds a chain of adenine nucleotides to the RNA. The process, called polyadenylation, adds a poly-A tail that can be between, for example, approximately 80 to approximately 250 residues long, including approximately 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240 or 250 residues long.

PolyA tails can also be added after the construct is exported from the nucleus.

According to the present disclosure, terminal groups on the poly A tail can be incorporated for stabilization. Polynucleotides of the present disclosure can include des-3' hydroxyl tails. They can also include structural moieties or 2'-O-methyl modifications as taught by Junjie Li, et al. (Current Biology, Vol. 15, 1501–1507, August 23, 2005, the contents of which are incorporated herein by reference in its entirety).

The polynucleotides of the present disclosure can be designed to encode transcripts with alternative polyA tail structures including histone mRNA. According to Norbury, "Terminal uridylation has also been detected on human replication-dependent histone mRNAs. The turnover of these mRNAs is thought to be important for the prevention of potentially toxic histone accumulation following the completion or inhibition of chromosomal DNA replication. These mRNAs are distinguished by their lack of a 3' poly(A) tail, the function of which is

instead assumed by a stable stem-loop structure and its cognate stem-loop binding protein (SLBP); the latter carries out the same functions as those of PABP on polyadenylated mRNAs" (Norbury, "Cytoplasmic RNA: a case of the tail wagging the dog," Nature Reviews Molecular Cell Biology; AOP, published online 29 August 2013; doi:10.1038/nrm3645) the contents of which are incorporated herein by reference in its entirety.

Unique poly-A tail lengths provide certain advantages to the polynucleotides of the present disclosure. Generally, the length of a poly-A tail, when present, is greater than 30 nucleotides in length. In another embodiment, the poly-A tail is greater than 35 nucleotides in length (*e.g.*, at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000 nucleotides).

In some embodiments, the polynucleotide or region thereof includes from about 30 to about 3,000 nucleotides (*e.g.*, from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 750, from 30 to 1,000, from 30 to 1,500, from 30 to 2,000, from 30 to 2,500, from 50 to 100, from 50 to 250, from 50 to 500, from 50 to 750, from 50 to 1,000, from 50 to 1,500, from 50 to 2,000, from 50 to 2,500, from 50 to 3,000, from 100 to 500, from 100 to 750, from 100 to 1,000, from 100 to 1,500, from 100 to 2,000, from 100 to 2,500, from 100 to 3,000, from 500 to 750, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 2,500, from 500 to 3,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 2,500, from 1,000 to 3,000, from 1,500 to 2,000, from 1,500 to 2,500, from 1,500 to 3,000, from 2,000 to 3,000, from 2,000 to 2,500, and from 2,500 to 3,000).

In some embodiments, the poly-A tail is designed relative to the length of the overall polynucleotide or the length of a particular region of the polynucleotide. This design can be based on the length of a coding region, the length of a particular feature or region or based on the length of the ultimate product expressed from the polynucleotides.

In this context, the poly-A tail can be 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% greater in length than the polynucleotide or feature thereof. The poly-A tail can also be designed as a fraction of the polynucleotides to which it belongs. In this context, the poly-A tail can be 10, 20, 30, 40, 50, 60, 70, 80, or 90% or more of the total length of the construct, a construct region or the total length of the construct minus the poly-A tail. Further, engineered binding sites and conjugation of polynucleotides for Poly-A binding protein can enhance expression.

Additionally, multiple distinct polynucleotides can be linked together via the PABP (Poly-A binding protein) through the 3'-end using modified nucleotides at the 3'-terminus of the poly-A tail. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12hr, 24hr, 48hr, 72hr and day 7 post-transfection.

In some embodiments, the polynucleotides of the present disclosure are designed to include a polyA-G Quartet region. The G-quartet is a cyclic hydrogen bonded array of four guanine 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 tail. The resultant polynucleotide is assayed for stability, protein production and other parameters including half-life at various time points. It has been discovered that the polyA-G quartet results in protein production from an mRNA equivalent to at least 75% of that seen using a poly-A tail of 120 nucleotides alone.

Start codon region

The present disclosure also includes a polynucleotide that comprises both a start codon region and the polynucleotide described herein (e.g., a polynucleotide comprising a nucleotide sequence encoding an antibody). In some embodiments, the polynucleotides of the present disclosure can have regions that are analogous to or function like a start codon region.

In some embodiments, the translation of a polynucleotide can initiate on a codon that is not the start codon AUG. Translation of the polynucleotide can initiate on an alternative start codon such as, but not limited to, ACG, AGG, AAG, CTG/CUG, GTG/GUG, ATA/AUA, ATT/AUU, TTG/UUG (see Touriol et al. *Biology of the Cell* 95 (2003) 169-178 and Matsuda and Mauro *PLoS ONE*, 2010 5:11; the contents of each of which are herein incorporated by reference in its entirety).

As a non-limiting example, the translation of a polynucleotide begins on the alternative start codon ACG. As another non-limiting example, polynucleotide translation begins on the alternative start codon CTG or CUG. As yet another non-limiting example, the translation of a polynucleotide begins on the alternative start codon GTG or GUG.

Nucleotides flanking a codon that initiates translation such as, but not limited to, a start codon or an alternative start codon, are known to affect the translation efficiency, the length and/or the structure of the polynucleotide. (*See, e.g.,* Matsuda and Mauro *PLoS ONE*, 2010 5:11; the contents of which are herein incorporated by reference in its entirety). Masking any of the nucleotides flanking a codon that initiates translation can be used to alter the position of translation initiation, translation efficiency, length and/or structure of a polynucleotide.

In some embodiments, a masking agent can be used near the start codon or alternative start codon in order to mask or hide the codon to reduce the probability of translation initiation at the masked start codon or alternative start codon. Non-limiting examples of masking agents include antisense locked nucleic acids (LNA) polynucleotides and exon-junction complexes (EJC) (*See, e.g.,* Matsuda and Mauro describing masking agents LNA polynucleotides and EJC

(PLoS ONE, 2010 5:11); the contents of which are herein incorporated by reference in its entirety).

In another embodiment, a masking agent can be used to mask a start codon of a polynucleotide in order to increase the likelihood that translation will initiate on an alternative start codon. In some embodiments, a masking agent can be used to mask a first start codon or alternative start codon in order to increase the chance that translation will initiate on a start codon or alternative start codon downstream to the masked start codon or alternative start codon.

In some embodiments, a start codon or alternative start codon can be located within a perfect complement for a miR binding site. The perfect complement of a miR binding site can help control the translation, length and/or structure of the polynucleotide similar to a masking agent. As a non-limiting example, the start codon or alternative start codon can be located in the middle of a perfect complement for a miRNA binding site. The start codon or alternative start codon can be located after the first nucleotide, second nucleotide, third nucleotide, fourth nucleotide, fifth nucleotide, sixth nucleotide, seventh nucleotide, eighth nucleotide, ninth nucleotide, tenth nucleotide, eleventh nucleotide, twelfth nucleotide, thirteenth nucleotide, fourteenth nucleotide, fifteenth nucleotide, sixteenth nucleotide, seventeenth nucleotide, eighteenth nucleotide, nineteenth nucleotide, twentieth nucleotide or twenty-first nucleotide.

In another embodiment, the start codon of a polynucleotide can be removed from the polynucleotide sequence in order to have the translation of the polynucleotide begin on a codon that is not the start codon. Translation of the polynucleotide can begin on the codon following the removed start codon or on a downstream start codon or an alternative start codon. In a non-limiting example, the start codon ATG or AUG is removed as the first 3 nucleotides of the polynucleotide sequence in order to have translation initiate on a downstream start codon or alternative start codon. The polynucleotide sequence where the start codon was removed can further comprise at least one masking agent for the downstream start codon and/or alternative start codons in order to control or attempt to control the initiation of translation, the length of the polynucleotide and/or the structure of the polynucleotide.

Stop Codon Region

The present disclosure also includes a polynucleotide that comprises both a stop codon region and the polynucleotide described herein (e.g., a polynucleotide comprising a nucleotide sequence encoding an antibody). In some embodiments, the polynucleotides of the present disclosure can include at least two stop codons before the 3' untranslated region (UTR). The stop codon can be selected from TGA, TAA and TAG in the case of DNA, or from UGA, UAA and UAG in the case of RNA. In some embodiments, the polynucleotides of the present disclosure include the stop codon TGA in the case of DNA, or the stop codon UGA in the case of RNA,

and one additional stop codon. In a further embodiment the addition stop codon can be TAA or UAA. In another embodiment, the polynucleotides of the present disclosure include three consecutive stop codons, four stop codons, or more.

Insertions and Substitutions

5 The present disclosure also includes a polynucleotide of the present disclosure that further comprises insertions and/or substitutions.

 In some embodiments, the 5'UTR of the polynucleotide can be replaced by the insertion of at least one region and/or string of nucleosides of the same base. The region and/or string of nucleotides can include, but is not limited to, at least 3, at least 4, at least 5, at least 6, at least 7
10 or at least 8 nucleotides and the nucleotides can be natural and/or unnatural. As a non-limiting example, the group of nucleotides can include 5-8 adenine, cytosine, thymine, a string of any of the other nucleotides disclosed herein and/or combinations thereof.

 In some embodiments, the 5'UTR of the polynucleotide can be replaced by the insertion of at least two regions and/or strings of nucleotides of two different bases such as, but not limited
15 to, adenine, cytosine, thymine, any of the other nucleotides disclosed herein and/or combinations thereof. For example, the 5'UTR can be replaced by inserting 5-8 adenine bases followed by the insertion of 5-8 cytosine bases. In another example, the 5'UTR can be replaced by inserting 5-8 cytosine bases followed by the insertion of 5-8 adenine bases.

 In some embodiments, the polynucleotide can include at least one substitution and/or
20 insertion downstream of the transcription start site that can be recognized by an RNA polymerase. As a non-limiting example, at least one substitution and/or insertion can occur downstream of the transcription start site by substituting at least one nucleic acid in the region just downstream of the transcription start site (such as, but not limited to, +1 to +6). Changes to region of nucleotides just downstream of the transcription start site can affect initiation rates,
25 increase apparent nucleotide triphosphate (NTP) reaction constant values, and increase the dissociation of short transcripts from the transcription complex curing initial transcription (Briebe et al, Biochemistry (2002) 41: 5144-5149; herein incorporated by reference in its entirety). The modification, substitution and/or insertion of at least one nucleoside can cause a silent mutation of the sequence or can cause a mutation in the amino acid sequence.

30 In some embodiments, the polynucleotide can include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12 or at least 13 guanine bases downstream of the transcription start site.

 In some embodiments, the polynucleotide can include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5 or at least 6 guanine bases in the region just downstream of
35 the transcription start site. As a non-limiting example, if the nucleotides in the region are

GGGAGA, the guanine bases can be substituted by at least 1, at least 2, at least 3 or at least 4 adenine nucleotides. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases can be substituted by at least 1, at least 2, at least 3 or at least 4 cytosine bases. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases can be substituted by at least 1, at least 2, at least 3 or at least 4 thymine, and/or any of the nucleotides described herein.

In some embodiments, the polynucleotide can include at least one substitution and/or insertion upstream of the start codon. For the purpose of clarity, one of skill in the art would appreciate that the start codon is the first codon of the protein coding region whereas the transcription start site is the site where transcription begins. The polynucleotide can include, but is not limited to, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7 or at least 8 substitutions and/or insertions of nucleotide bases. The nucleotide bases can be inserted or substituted at 1, at least 1, at least 2, at least 3, at least 4 or at least 5 locations upstream of the start codon. The nucleotides inserted and/or substituted can be the same base (*e.g.*, all A or all C or all T or all G), two different bases (*e.g.*, A and C, A and T, or C and T), three different bases (*e.g.*, A, C and T or A, C and T) or at least four different bases.

As a non-limiting example, the guanine base upstream of the coding region in the polynucleotide can be substituted with adenine, cytosine, thymine, or any of the nucleotides described herein. In another non-limiting example the substitution of guanine bases in the polynucleotide can be designed so as to leave one guanine base in the region downstream of the transcription start site and before the start codon (see Esvelt *et al.* Nature (2011) 472(7344):499-503; the contents of which is herein incorporated by reference in its entirety). As a non-limiting example, at least 5 nucleotides can be inserted at 1 location downstream of the transcription start site but upstream of the start codon and the at least 5 nucleotides can be the same base type.

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.

Provided herein are compositions (*e.g.*, pharmaceutical compositions), methods, kits and reagents for prevention and/or treatment of disease in humans and other mammals. The antibodies can be used as therapeutic or prophylactic agents. For example, when the antibody is an anti-influenza virus antibody the RNA encoding such an antibody is used to provide prophylactic or therapeutic protection from influenza virus infection. Prophylactic protection from influenza virus infection can be achieved following administration of a composition (*e.g.*, a composition comprising one or more polynucleotides encoding one or more HCABs) of the

present disclosure. In some embodiments, the influenza virus is a type A influenza virus. In some embodiments, the influenza virus is any influenza virus comprising HA proteins of subtype H1 and/or H3 (*e.g.*, H1N1, H3N2). Compositions can be administered once, twice, three times, four times or more. In some aspects, the compositions can be administered to an infected individual to achieve a therapeutic response. Dosing may need to be adjusted accordingly.

It is envisioned that there may be situations where persons are at risk for infection with more than one strain of type of infectious agent. RNA (mRNA) therapeutic treatments are particularly amenable to combination vaccination approaches due to a number of factors including, but not limited to, speed of manufacture, ability to rapidly tailor treatments to accommodate perceived geographical threat, and the like. To protect against more than one strain of influenza virus, a combination treatment can be administered that includes RNA encoding at least one polypeptide (or portion thereof) of a first influenza HA-binding HCAb and further includes RNA encoding at least one polypeptide (or portion thereof) of a second influenza-HA binding HCAb. RNAs (mRNAs) can be co-formulated, for example, in a single lipid nanoparticle (LNP) or can be formulated in separate LNPs destined for co-administration. In some embodiments, the influenza virus is a type A influenza virus. In some embodiments, the influenza virus is any influenza virus comprising HA proteins of subtype H1 and/or H3 (*e.g.*, H1N1, H3N2).

A prophylactically effective dose is a therapeutically effective dose that prevents infection with the virus at a clinically acceptable level. In some embodiments, the therapeutically effective dose is a dose listed in a package insert for the treatment. A prophylactic therapy as used herein refers to a therapy that prevents, to some extent, the infection from increasing. The infection may be prevented completely or partially.

The methods of the invention involve, in some aspects, passively immunizing a mammalian subject against an influenza virus infection. The method involves administering to the subject a composition comprising at least one RNA polynucleotide having an open reading frame encoding at least one HCAb that targets (*e.g.*, binds to) an influenza virus protein. In some aspects, methods of the present disclosure provide prophylactic treatments against an influenza virus infection. In some embodiments, the influenza virus is a type A influenza virus. In some embodiments, the influenza virus is any influenza virus comprising HA proteins of subtype H1 and/or H3 (*e.g.*, H1N1, H3N2).

Therapeutic methods of treatment are also included within the invention. Methods of treating an influenza virus infection in a subject are provided in aspects of the disclosure. The method involves administering to the subject having an influenza virus infection a composition comprising at least one RNA polynucleotide having an open reading frame encoding at least one

HCAb that targets (*e.g.*, binds to) an influenza virus protein. In some embodiments, the influenza virus protein is an HA protein. In some embodiments, the influenza virus is a type A influenza virus. In some embodiments, the influenza virus is any influenza virus comprising HA proteins of subtype H1 and/or H3 (*e.g.*, H1N1, H3N2).

5 In some embodiments, the polynucleotide encodes an amino acid sequence of an antibody that binds Ebola virus (EBOV) protein.

Aspects of the disclosure provide compositions comprising RNA polynucleotides encoding single-domain antibodies. In some embodiments, the single domain antibody encoded by an RNA polynucleotide of the present application is a heavy chain antibody such as found in
10 camelidae (*e.g.*, camels and llamas). The binding elements of such heavy chain antibodies consist of a single polypeptide domain, known as the variable domain of heavy chain antibodies (V_HH). These antibody fragments are naturally devoid of light-chains, with the V_HH forming the entirety of the antigen-binding site. In contrast, classical antibodies (*e.g.*, murine, human) have binding elements comprising two polypeptide domains: the variable regions of the heavy chain
15 (V_H) and the light chain (V_L). The lack of dependence on interaction with a light chain variable domain for maintaining structural and functional integrity gives V_HH domains a substantial advantage over other small antibody fragments, in terms of ease of production and behavior in solution. For example, V_HH domains are the preferred types of molecules for immuno-affinity purification due to their high stability and ability to refold efficiently after complete denaturation,
20 which frequently occurs during elution of antigen. Additionally, the smaller size and single domain make V_HH domains optimal for cellular transformation.

Exemplary polynucleotides, *e.g.*, polynucleotide constructs, include antibody-encoding mRNA polynucleotides. In some embodiments, the RNA treatment of the disclosure is a polynucleotide encoding an antibody that binds to Ebola virus (EBOV) protein. There are five
25 Ebola viruses within the genus Ebolavirus. Four of the five known ebolaviruses cause a severe and often fatal hemorrhagic fever in humans and other mammals, known as Ebola virus disease (EVD). The Ebola glycoprotein (GP) is the only virally expressed protein on the virion surface, where it is essential for the attachment to host cells and catalyzes membrane fusion. As a result, the Ebola GP is a critical component of vaccines, as well as a target of neutralizing antibodies
30 and inhibitors of attachment and fusion. Pre-GP is cleaved by furin at a multi-basic motif into two subunits, GP1 and GP2, which remain associated through a disulfide linkage between Cys53 of GP1 and Cys609 of GP2. The heterodimer (GP1 and GP2) then assembles into a 450-kDa trimer (3 GP1 and 3 GP2) at the surface of nascent virions, where it exerts its functions.

In some embodiments, the single-domain antibody encoded by the nucleic acid
35 composition targets (*e.g.*, binds to) an EBOV glycoprotein (GP). In some embodiments, the

single-domain antibody targets (*e.g.*, binds to) surface GP. In some embodiments, the single-domain antibody targets (*e.g.*, binds to) secreted GP (sGP). In some embodiments, the single-domain antibody targets (*e.g.*, binds to) small sGP (ssGP). In some embodiments, the single-domain antibody targets (*e.g.*, binds to) shed GP. In some embodiments, the single-domain antibody encoded by the nucleic acid composition targets (*e.g.*, binds to) an EBOV nucleoprotein. In some embodiments, the single-domain antibody encoded by the nucleic acid composition targets (*e.g.*, binds to) an EBOV matrix protein.

As used herein, the terms treat, treated, or treating when used with respect to a disorder such as a viral infection, refers to a treatment which increases the resistance of a subject to development of the disease or, in other words, decreases the likelihood that the subject will develop the disease in response to infection with the virus as well as a treatment after the subject has developed the disease in order to fight the infection or prevent the infection from becoming worse.

An “effective amount” of an antibody RNA treatment is provided based, at least in part, on the target tissue, target cell type, means of administration, physical characteristics of the polynucleotide (*e.g.*, size, and extent of modified nucleosides), and other components of the RNA treatment, and other determinants. Increased antibody production may be demonstrated by increased cell transfection (the percentage of cells transfected with the RNA treatment), increased protein translation from the polynucleotide, decreased nucleic acid degradation (as demonstrated, for example, by increased duration of protein translation from a modified polynucleotide), or altered response of the host cell.

In some embodiments, RNA treatments (including polynucleotides and their encoded polypeptides) in accordance with the present disclosure may be used for treatment of the disease.

RNA treatments may be administered prophylactically or therapeutically as part of an active immunization scheme to healthy individuals or early in infection during the incubation phase or during active infection after onset of symptoms. In some embodiments, the amount of RNA treatments of the present disclosure provided to a cell, a tissue or a subject may be an amount effective for immune prophylaxis.

RNA treatments may be administered with other prophylactic or therapeutic compounds. As a non-limiting example, a prophylactic or therapeutic compound may be a vaccine containing an virus treatment with or without an adjuvant or a booster. As used herein, when referring to a prophylactic composition, such as a treatment or vaccine, the term “booster” refers to an extra administration of the prophylactic composition. A booster (or booster vaccine) may be given after an earlier administration of the prophylactic composition. The time of administration between the initial administration of the prophylactic composition and the booster may be, but is

not limited to, 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 6 minutes, 7 minutes, 8 minutes, 9 minutes, 10 minutes, 15 minutes, 20 minutes, 35 minutes, 40 minutes, 45 minutes, 50 minutes, 55 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 1 day, 36 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 10 days, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, 18 months, 2 years, 3 years, 4 years, 5 years, 6 years, 7 years, 8 years, 9 years, 10 years, 11 years, 12 years, 13 years, 14 years, 15 years, 16 years, 17 years, 18 years, 19 years, 20 years, 25 years, 30 years, 35 years, 40 years, 45 years, 50 years, 55 years, 60 years, 65 years, 70 years, 75 years, 80 years, 85 years, 90 years, 95 years, or more than 99 years. In exemplary embodiments, the time of administration between the initial administration of the prophylactic composition and the booster may be, but is not limited to, 1 week, 2 weeks, 3 weeks, 1 month, 2 months, 3 months, 6 months, or 1 year.

In some embodiments, RNA treatments may be administered subcutaneously, intraocularly, intravitreally, parenterally, subcutaneously, intravenously, intracerebro-ventricularly, intramuscularly, intrathecally, orally, intraperitoneally, by oral or nasal inhalation, or by direct injection to one or more cells, tissues, or organs.

Provided herein are pharmaceutical compositions including RNA treatments and RNA compositions and/or complexes optionally in combination with one or more pharmaceutically acceptable excipients.

RNA treatments may be formulated or administered in combination with one or more pharmaceutically-acceptable excipients. In some embodiments, compositions comprise at least one additional active substance, such as, for example, a therapeutically-active substance, a prophylactically-active substance, or a combination of both. Treatment 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 treatment compositions, may be found, for example, in Remington: The Science and Practice of Pharmacy 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference in its entirety).

In some embodiments, RNA treatments are administered to humans, human patients, or subjects. In some embodiments, the subject is a mammal. In some embodiments, the subject is a human.

Some aspects of the application provide methods of passively immunizing a mammalian subject against a bacterial, viral, or parasitic antigen comprising administering to the subject a composition described herein, wherein the subject is at risk of having or being exposed to a

bacterial, viral, or parasitic infection. In some embodiments, the mammalian subject is a human. In some embodiments, the subject is a non-human primate. Non-limiting examples of non-human primate subjects include macaques, marmosets, tamarins, spider monkeys, owl monkeys, vervet monkeys, squirrel monkeys, baboons, gorillas, chimpanzees, and orangutans. Other exemplary subjects include domesticated animals such as dogs and cats; livestock such as horses, cattle, pigs, sheep, goats, and chickens; and other animals such as mice, rats, guinea pigs, and hamsters.

For the purposes of the present disclosure, the phrase “active ingredient” generally refers to the RNA treatments or the polynucleotides contained therein, for example, RNA polynucleotides (*e.g.*, mRNA polynucleotides) encoding HCAb polypeptides.

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 active ingredient (*e.g.*, mRNA polynucleotide) into association with an excipient and/or one or more other accessory ingredients, 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 active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the 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, the composition may comprise between 0.1% and 100%, *e.g.*, between 0.5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

RNA treatments can be 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 (*e.g.*, HCAb) *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 RNA treatments (*e.g.*, for transplantation into a subject), hyaluronidase, nanoparticle mimics, and combinations thereof.

Naturally-occurring eukaryotic mRNA molecules have been found to contain stabilizing elements, including, but not limited to untranslated regions (UTR) at their 5'-end (5'UTR) and/or at their 3'-end (3'UTR), in addition to other structural features, such as a 5'-cap structure or a 3'-

poly(A) tail. Both the 5'UTR and the 3'UTR are typically transcribed from the genomic DNA and are elements of the premature mRNA. Characteristic structural features of mature mRNA, such as the 5'-cap and the 3'-poly(A) tail are usually added to the transcribed (premature) mRNA during mRNA processing. The 3'-poly(A) tail is typically a stretch of adenine nucleotides added to the 3'-end of the transcribed mRNA. It can comprise up to about 400 adenine nucleotides. In some embodiments the length of the 3'-poly(A) tail may be an essential element with respect to the stability of the individual mRNA.

In some embodiments, the RNA treatment may include one or more stabilizing elements. Stabilizing elements may include, for instance, a histone stem-loop. A stem-loop binding protein (SLBP), a 32 kDa protein has been identified. It is associated with the histone stem-loop at the 3'-end of the histone messages in both the nucleus and the cytoplasm. Its expression level is regulated by the cell cycle; it peaks during the S-phase, when histone mRNA levels are also elevated. The protein has been shown to be essential for efficient 3'-end processing of histone pre-mRNA by the U7 snRNP. SLBP continues to be associated with the stem-loop after processing, and then stimulates the translation of mature histone mRNAs into histone proteins in the cytoplasm. The RNA binding domain of SLBP is conserved through metazoa and protozoa; its binding to the histone stem-loop depends on the structure of the loop. The minimum binding site includes at least three nucleotides 5' and two nucleotides 3' relative to the stem-loop.

In some embodiments, the RNA treatments include a coding region, at least one histone stem-loop, and optionally, a poly(A) sequence or polyadenylation signal. The poly(A) sequence or polyadenylation signal generally should enhance the expression level of the encoded protein. The encoded protein, in some embodiments, is not a histone protein, a reporter protein (*e.g.* Luciferase, GFP, EGFP, β -Galactosidase, EGFP), or a marker or selection protein (*e.g.* alpha-Globin, Galactokinase and Xanthine:guanine phosphoribosyl transferase (GPT)).

In some embodiments, the combination of a poly(A) sequence or polyadenylation signal and at least one histone stem-loop, even though both represent alternative mechanisms in nature, acts synergistically to increase the protein expression beyond the level observed with either of the individual elements. It has been found that the synergistic effect of the combination of poly(A) and at least one histone stem-loop does not depend on the order of the elements or the length of the poly(A) sequence.

In some embodiments, the polynucleotides described herein can be formulated in lipid nanoparticles having a diameter from about 1 nm to about 100 nm such as, but not limited to, about 1 nm to about 20 nm, from about 1 nm to about 30 nm, from about 1 nm to about 40 nm, from about 1 nm to about 50 nm, from about 1 nm to about 60 nm, from about 1 nm to about 70 nm, from about 1 nm to about 80 nm, from about 1 nm to about 90 nm, from about 5 nm to about

from 100 nm, from about 5 nm to about 10 nm, about 5 nm to about 20 nm, from about 5 nm to about 30 nm, from about 5 nm to about 40 nm, from about 5 nm to about 50 nm, from about 5 nm to about 60 nm, from about 5 nm to about 70 nm, from about 5 nm to about 80 nm, from about 5 nm to about 90 nm, about 10 to about 20 nm, about 10 to about 30 nm, about 10 to about 40 nm, about 10 to about 50 nm, about 10 to about 60 nm, about 10 to about 70 nm, about 10 to about 80 nm, about 10 to about 90 nm, about 20 to about 30 nm, about 20 to about 40 nm, about 20 to about 50 nm, about 20 to about 60 nm, about 20 to about 70 nm, about 20 to about 80 nm, about 20 to about 90 nm, about 20 to about 100 nm, about 30 to about 40 nm, about 30 to about 50 nm, about 30 to about 60 nm, about 30 to about 70 nm, about 30 to about 80 nm, about 30 to about 90 nm, about 30 to about 100 nm, about 40 to about 50 nm, about 40 to about 60 nm, about 40 to about 70 nm, about 40 to about 80 nm, about 40 to about 90 nm, about 40 to about 100 nm, about 50 to about 60 nm, about 50 to about 70 nm, about 50 to about 80 nm, about 50 to about 90 nm, about 50 to about 100 nm, about 60 to about 70 nm, about 60 to about 80 nm, about 60 to about 90 nm, about 60 to about 100 nm, about 70 to about 80 nm, about 70 to about 90 nm, about 70 to about 100 nm, about 80 to about 90 nm, about 80 to about 100 nm and/or about 90 to about 100 nm.

In some embodiments, the lipid nanoparticles can have a diameter from about 10 to 500 nm. In one embodiment, the lipid nanoparticle can have a diameter greater than 100 nm, greater than 150 nm, greater than 200 nm, greater than 250 nm, greater than 300 nm, greater than 350 nm, greater than 400 nm, greater than 450 nm, greater than 500 nm, greater than 550 nm, greater than 600 nm, greater than 650 nm, greater than 700 nm, greater than 750 nm, greater than 800 nm, greater than 850 nm, greater than 900 nm, greater than 950 nm or greater than 1000 nm.

In some embodiments, the polynucleotides can be delivered using smaller LNPs. Such particles can comprise a diameter from below 0.1 μm up to 100 nm such as, but not limited to, less than 0.1 μm , less than 1.0 μm , less than 5 μm , less than 10 μm , less than 15 μm , less than 20 μm , less than 25 μm , less than 30 μm , less than 35 μm , less than 40 μm , less than 50 μm , less than 55 μm , less than 60 μm , less than 65 μm , less than 70 μm , less than 75 μm , less than 80 μm , less than 85 μm , less than 90 μm , less than 95 μm , less than 100 μm , less than 125 μm , less than 150 μm , less than 175 μm , less than 200 μm , less than 225 μm , less than 250 μm , less than 275 μm , less than 300 μm , less than 325 μm , less than 350 μm , less than 375 μm , less than 400 μm , less than 425 μm , less than 450 μm , less than 475 μm , less than 500 μm , less than 525 μm , less than 550 μm , less than 575 μm , less than 600 μm , less than 625 μm , less than 650 μm , less than 675 μm , less than 700 μm , less than 725 μm , less than 750 μm , less than 775 μm , less than 800 μm , less than 825 μm , less than 850 μm , less than 875 μm , less than 900 μm , less than 925 μm , less than 950 μm , or less than 975 μm .

The nanoparticles and microparticles described herein can be geometrically engineered to modulate macrophage and/or the immune response. The geometrically engineered particles can have varied shapes, sizes and/or surface charges to incorporate the polynucleotides described herein for targeted delivery such as, but not limited to, pulmonary delivery (see, e.g., Intl. Pub. No. WO2013/082111, herein incorporated by reference in its entirety). Other physical features the geometrically engineering particles can include, but are not limited to, fenestrations, angled arms, asymmetry and surface roughness, charge that can alter the interactions with cells and tissues.

RNA treatments may be administered by any route which results in a therapeutically effective outcome. These include, but are not limited, to intradermal, intramuscular, and/or subcutaneous administration. The present disclosure provides methods comprising administering RNA treatments to a subject in need thereof. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like. RNA treatments compositions are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of RNA treatments compositions may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective, prophylactically effective, or appropriate imaging dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

In some embodiments, RNA treatments compositions may be administered at dosage levels sufficient to deliver 0.0001 mg/kg to 100 mg/kg, 0.001 mg/kg to 0.05 mg/kg, 0.005 mg/kg to 0.05 mg/kg, 0.001 mg/kg to 0.005 mg/kg, 0.05 mg/kg to 0.5 mg/kg, 0.01 mg/kg to 50 mg/kg, 0.1 mg/kg to 40 mg/kg, 0.5 mg/kg to 30 mg/kg, 0.01 mg/kg to 10 mg/kg, 0.1 mg/kg to 10 mg/kg, or 1 mg/kg to 25 mg/kg, of subject body weight per day, one or more times a day, per week, per month, etc. to obtain the desired therapeutic, diagnostic, prophylactic, or imaging effect (see e.g., the range of unit doses described in International Publication No WO2013/078199, herein incorporated by reference in its entirety). The desired dosage may be delivered three times a day, two times a day, once a day, every other day, every third day, every week, every two weeks, every three weeks, every four weeks, every 2 months, every three months, every 6 months, etc..

In certain embodiments, the desired dosage may be delivered using multiple administrations (*e.g.*, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations). When multiple administrations are employed, split dosing regimens such as those described herein may be used. In exemplary embodiments, RNA treatment compositions may be administered at dosage levels sufficient to deliver 0.0005 mg/kg to 0.01 mg/kg, *e.g.*,
5 about 0.0005 mg/kg to about 0.0075 mg/kg, *e.g.*, about 0.0005 mg/kg, about 0.001 mg/kg, about 0.002 mg/kg, about 0.003 mg/kg, about 0.004 mg/kg or about 0.005 mg/kg.

In some embodiments, RNA treatment compositions may be administered once or twice (or more) at dosage levels sufficient to deliver 0.025 mg/kg to 0.250 mg/kg, 0.025 mg/kg to
10 0.500 mg/kg, 0.025 mg/kg to 0.750 mg/kg, or 0.025 mg/kg to 1.0 mg/kg.

In some embodiments, RNA treatment compositions may be administered twice (*e.g.*, Day 0 and Day 7, Day 0 and Day 14, Day 0 and Day 21, Day 0 and Day 28, Day 0 and Day 60, Day 0 and Day 90, Day 0 and Day 120, Day 0 and Day 150, Day 0 and Day 180, Day 0 and 3 months later, Day 0 and 6 months later, Day 0 and 9 months later, Day 0 and 12 months later,
15 Day 0 and 18 months later, Day 0 and 2 years later, Day 0 and 5 years later, or Day 0 and 10 years later) at a total dose of or at dosage levels sufficient to deliver a total dose of 0.0100 mg, 0.025 mg, 0.050 mg, 0.075 mg, 0.100 mg, 0.125 mg, 0.150 mg, 0.175 mg, 0.200 mg, 0.225 mg, 0.250 mg, 0.275 mg, 0.300 mg, 0.325 mg, 0.350 mg, 0.375 mg, 0.400 mg, 0.425 mg, 0.450 mg, 0.475 mg, 0.500 mg, 0.525 mg, 0.550 mg, 0.575 mg, 0.600 mg, 0.625 mg, 0.650 mg, 0.675 mg,
20 0.700 mg, 0.725 mg, 0.750 mg, 0.775 mg, 0.800 mg, 0.825 mg, 0.850 mg, 0.875 mg, 0.900 mg, 0.925 mg, 0.950 mg, 0.975 mg, or 1.0 mg. Higher and lower dosages and frequency of administration are encompassed by the present disclosure. For example, an RNA treatment composition may be administered three or four times.

In some embodiments, RNA treatment compositions may be administered twice (*e.g.*,
25 Day 0 and Day 7, Day 0 and Day 14, Day 0 and Day 21, Day 0 and Day 28, Day 0 and Day 60, Day 0 and Day 90, Day 0 and Day 120, Day 0 and Day 150, Day 0 and Day 180, Day 0 and 3 months later, Day 0 and 6 months later, Day 0 and 9 months later, Day 0 and 12 months later, Day 0 and 18 months later, Day 0 and 2 years later, Day 0 and 5 years later, or Day 0 and 10 years later) at a total dose of or at dosage levels sufficient to deliver a total dose of 0.010 mg, 0.025 mg, 0.100 mg or 0.400 mg.
30

In some embodiments, the RNA for use in a method of treating a subject is administered to the subject in a single dosage of between 10 µg/kg and 400 µg/kg of the nucleic acid treatment in an effective amount to treat the subject. In some embodiments, the RNA treatment for use in a method of treating a subject is administered to the subject in a single dosage of between 10 µg
35 and 400 µg of the nucleic acid treatment in an effective amount to treat the subject.

An RNA pharmaceutical composition described herein can be formulated into a dosage form described herein, such as an intranasal, intratracheal, or injectable (*e.g.*, intravenous, intraocular, intravitreal, intramuscular, intradermal, intracardiac, intraperitoneal, and subcutaneous).

This disclosure is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The disclosure is capable of other embodiments and of being practiced or of being carried out in various ways. Also, the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of “including,” “comprising,” or “having,” “containing,” “involving,” and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

EXAMPLES

Example 1: Effective J chain Ratio in Class Switched CAM003 Antibodies Assembled from mRNA

The studies below required IgA and IgM antibodies where the antibody isotype was class switched from an IgG isotype. To class-switch antibodies to a different isotype, only the heavy chain constant region is changed. The end of the heavy chain variable region and beginning of the heavy chain constant region is identified. This can be denoted by looking for amino acids VSS, VSA or TVSS to mark the end of the variable region or amino acids AST, ASP or GSA to mark the beginning of the heavy chain constant region of IgG, IgA or IgM, respectively. The entire sequence of the heavy chain constant region is swapped with that of the new isotype heavy chain constant region sequence.

The number of natural allelic differences in heavy chain constant regions is not known. For IgG1, there are at least 5 and at least 15 for IgG3. Despite this, there are common heavy chain constant region sequences including but not limited to SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, and SEQ ID NO: 9 and all have been tested for functionality.

Varying the ratios of light chain to heavy chain to J chain can increase or decrease monomer, dimer, and polymer formation for IgA1, IgA2m1, and IgA2m2 (*e.g.*, Lombana et al., In MAbs 2019 Aug 18:Vol. 11, No. 6, pp. 1122-1138). To investigate the specific ratio effective for proper antibody assembly from mRNA, an *in vitro* assay was designed. CAM003, a monoclonal antibody targeting exopolysaccharide Psl from *Pseudomonas aeruginosa* was class-switched from an IgG1 to an IgG3, IgM, IgA1, IgA2m1 or IgA2m2 and prepared as mRNA. Similarly, Hu605, a monoclonal antibody targeting LPS core from *Pseudomonas aeruginosa* was class-switched from an IgG1 to an IgM and IgA2m1 and were prepared as mRNA. 293-F cells

grown in 15 mL of suspension culture were transfected with 15µg total of mRNA with the ratios of heavy chain to light chain to J chain (H:L:J) as shown in **FIG. 1**. Following transfection, cells were allowed to express mRNA for 48 hours after which supernatant was collected.

Quantitation of expression was determined by isotype specific enzyme-linked immunosorbent assay (ELISA). Plates were coated with anti IgG Fc, anti-IgM or anti-IgA antibodies and blocked to reduce non-specific binding. Serial dilutions of supernatants were added to the plates and binding was detected with anti-IgM, IgA, or IgG. Standard binding curves were generated using CAM003 IgA, Hu605 IgA, CAM003 IgG, or purified IgM.

CAM003 IgA1 and IgM expressed to comparable amounts as the native IgG1 (**FIG. 2A**). Varying the ratios of heavy chain to light chain to J chain resulted in different levels of antibody secretion for both IgM and IgA1. For CAM003, in this example, inclusion of the J chain generally led to more antibody secretion than without the J chain. Both CAM003 and Hu605 were successfully expressed in the supernatant when class-switched to IgA and IgM isotypes (**FIG. 2B**).

Example 2: J chain Influences Oligomeric Structure of Class Switched CAM003 Antibodies Assembled from mRNA

The amount of J chain influences oligomeric antibody structure. To evaluate the effective specific ratio of heavy chain to light chain to J chain (H:L:J) for antibody oligomerization, the ratio of polymeric immunoglobulin receptor (pIgR) binding to total antibody isotype was analyzed using an assay design shown in **FIG. 3**. Supernatant from the 293-F cell transfections was concentrated using Amicon filters with a 50kDa cutoff. Unbound J chain, at ~15-20 kDa and unbound light chain at ~25-30 kDa passed through the filter. Heavy chains and fully assembled and oligomerized antibodies were retained by the filter.

Quantitation of binding to pIgR was determined by ELISA as described in **FIG. 3**. Plates were coated with recombinant human pIgR and blocked to reduce non-specific binding. Concentrated supernatant was added to plates and bound antibody was detected using an isotype specific secondary conjugated to HRP (*e.g.*, IgA or IgM HRP antibody). Only antibodies with J chain incorporated were able to bind to pIgR. Standard binding curves were generated using CAM003 IgA1 and Hu605 IgA2m1 dimers that were isolated using size exclusion chromatography (SEC). Standard binding curves for IgM were generated using SEC separated fractions of CAM003 IgM and Hu605 IgM that contain the J chain.

An effective ratio of H:L:J for CAM003 IgA was 8:8:1, which expressed well, had similar levels of pIgR binding as compared to a 4:4:1 ratio, and had higher levels of total IgA (**FIGs. 4A-4B**). Effective ratios for CAM003 IgM were 6:6:1, 8:8:1 or 10:10:1 (**FIG. 4C**),

which also enhanced amount of J chain incorporated. Effective ratios for Hu605 IgA2m1 and IgM were 6:6:1 and 8:8:1 for IgA2m1 (**FIG. 4D**) and 10:10:1 for IgM (**FIG. 4E**).

Example 3: Class Switched CAM003 Antibodies Assembled from mRNA with J Chain

Incorporated Retain Ability to Bind Antigen

To investigate whether class switched antibodies assembled from mRNA retain the ability to bind antigen, a whole cell *Pseudomonas aeruginosa* ELISA was designed.

Quantitation of antigen binding was determined by *Pseudomonas aeruginosa* ELISA. Plates were coated with whole cell *Pseudomonas aeruginosa* and blocked to reduce non-specific binding. Serial dilutions of concentrated supernatant from IgA and IgM with different H:L:J ratios were added to the plates and optical measurements using an absorbance of 450 nm were taken to detect binding. IgG with a H:L ratio of 2:1 was used as a control.

Class switched CAM003 IgA1 (**FIG. 5A**) and CAM003 IgM (**FIG. 5C**) along with Hu605 IgA2m1 (**FIG. 5B**) and Hu605 IgM (**FIG. 5D**) with different H:L:J ratios retained the ability to bind antigen.

Example 4: CAM003 Can Be Class Switched to All Isotypes

To investigate the ability of an IgG to be class-switched to all 3 major subtypes of IgA, an isotype specific ELISA was used to analyze isotype specific binding with and without J chain.

Quantitation of expression was determined by isotype specific ELISA. 293-F cells were transfected with mRNA encoding different isotypes of class switched CAM003 using the method described above in Example 1. All transfections without J chain were performed using a 2:1 heavy to light (H:L) ratio. All IgA transfections with J chain were performed using a 8:8:1 H:L:J ratio. All IgM transfections with J chain were performed using a 10:10:1 H:L:J ratio. Supernatant was collected from all antibody isotypes with and without J chain. CAM003 IgG, CAM003 IgG3, CAM003 IgA and polyclonal human IgM were used as standards.

For all IgA subtypes, the incorporation of J chain led to increased secretion of antibody in the supernatant as shown in **FIG. 6**. IgA1 expressed the best followed closely by IgA2m1 and then IgA2m2, all including the J chain. IgM expressed to equal levels with and without the J chain. Importantly, IgA1, IgA2m1 and IgM expressed and secreted in similar levels as IgG and IgG3 in the supernatant as shown in **FIG. 6**.

Example 5: Ability to Bind Antigen is Not Altered by Class Switched CAM003 Antibodies Assembled from mRNA

To investigate whether class switched antibodies assembled from mRNA impacted the ability of the antibody to bind antigen, a whole cell *Pseudomonas aeruginosa* ELISA was designed.

Quantitation of antigen binding was determined by *Pseudomonas aeruginosa* ELISA. Plates were coated with whole cell *Pseudomonas aeruginosa* and blocked to reduce non-specific binding. Serial dilutions of concentrated supernatant with and without J chain were added to the plates and optical measurements using an absorbance of 450 nm were taken to detect binding.

Isotype class switched antibodies with and without the incorporation of J chain were able to bind the antigen, indicating that antigen recognition was not altered by class switching to an alternative isotype or by the addition of J chain as shown in **FIG. 7**.

Example 6: Incorporation of J chain in CAM003 Antibodies Class-Switched to IgA and Assembled from mRNA Allows for Binding to pIgR

To investigate the ability of antibodies class-switched to IgA subtypes to bind pIgR, a pIgR ELISA was used.

Supernatant from the 293-F cell transfections was concentrated using Amicon filters with a 50kDa cutoff. Unbound J chain, at ~15-20 kDa and unbound light chain at ~25-30 kDa passed through the filter. Heavy chain and assembled antibodies were retained in the filter.

Quantitation of binding to pIgR was determined by ELISA. Plates were coated with recombinant human pIgR and blocked to reduce non-specific binding. Serial dilutions of concentrated supernatant was added to plates and bound antibody was detected using an isotype specific secondary conjugated to HRP (*e.g.*, IgA or IgM HRP antibody). Optical measurements were taken at an absorbance of 450 nm.

Only antibodies with J chain incorporated (*e.g.*, IgA1 + J, IgA2m1 + J, and IgA2m2 + J,) were able to bind to pIgR as shown in **FIG. 8A**. While total IgA expressed in the supernatant was highest for IgA1, a smaller fraction of the total IgA was capable of binding pIgR (**FIGs. 8B** and **8C**). In contrast, IgA2m2 subtype expressed poorly when quantitated for total IgA however over 80% of total IgA bound to pIgR (**FIGs. 8B** and **8C**), demonstrating the different properties of the IgA subtypes.

Example 7: CAM003 IgA Expression *in vivo*

To investigate whether antibodies class switched to IgA could be expressed *in vivo* from mRNA, CAM003 antibody concentration was measured in various mouse tissues after intravenous delivery of mRNA formulated into LNPs. 8-10 week old female C57Bl/6 mice (n = 5) were treated with 0.5 mg/kg Compound 1 formulated mRNA LNPs intravenously. CAM003 IgA H:L:J chain ratios of 2:1, 4:4:1, and 8:8:1 were evaluated. PBS, CAM003 IgG protein, and CAM003 IgG mRNA were used as controls. Serum, lungs, and liver were collected from mice at 6, 12, 24, and 48 hours post-treatment. Lung and liver tissue were homogenized using a bead homogenizer at 300 mg/mL and 1000 mg/mL, respectively.

A H:L:J chain ratio of 4:4:1 resulted in increased CAM003 IgA antibody concentration relative to 2:1 and 8:8:1 in serum (**FIG. 9**) and in lung (**FIG. 10A**) of treated mice. CAM003 IgA antibody expression was also found to correlate between serum and lung (**FIG. 10B**). A H:L:J chain ratio of 4:4:1 resulted in retention of CAM003 IgA antibody for longer time relative to 2:1 and 8:8:1 in liver (**FIG. 11**).

Example 8: CAM003 IgA Antigen Binding *in vivo*

To investigate whether incorporation of J chain in class switched antibodies assembled from mRNA impacted the ability of the isotypes to bind antigen, samples containing CAM003 IgA or IgG antibodies were evaluated for binding in a whole cell *Pseudomonas aeruginosa* ELISA. 8-10 week old female C57Bl/6 mice (n = 5) were treated with 0.5 mg/kg Compound 1 formulated mRNA LNPs intravenously. CAM003 IgA H:L:J chain ratios of 2:1, 4:4:1, and 8:8:1 were evaluated. CAM003 IgG protein and CAM003 IgG mRNA LNPs were used as controls. Serum, lungs, and liver were collected from mice at 6 and 24 hours post-treatment. Lung and liver tissue were homogenized using a bead homogenizer at 300 mg/mL and 1000 mg/mL, respectively.

CAM003 IgA antibody in the serum and liver retained binding to antigen over time in a *Pseudomonas aeruginosa* whole cell ELISA (**FIG. 12**).

Example 9: CAM003 IgA Expressed from mRNA LNPs *in vivo* Bind to Human pIgR

To investigate whether mRNA encoding IgA heavy, light and J chains expressed functional IgA class switched antibodies, a pIgR ELISA was used. In the example, 8-10 week old female C57Bl/6 mice (n = 4) were treated with 0.5 mg/kg Compound 1 formulated mRNA LNPs intravenously. CAM003 IgA H:L:J chain ratios of 4:4:1 and 8:8:1 were evaluated. Serum was collected from mice 6 hours post-treatment and measured by pIgR ELISA.

The amount of CAM003 IgA antibody in mouse serum able to bind human pIgR was measured relative to total CAM0003 IgA antibody (**FIG. 13**), suggesting successful incorporation of the J chain.

Example 10: CAM003 IgM Expression *in vivo*

To investigate whether antibodies IgG class switched to IgM could be expressed *in vivo* from mRNA LNPs, CAM003 antibody concentration was measured in various mouse tissues after intravenous mRNA LNP delivery. In this example, 8-10 week old female C57Bl/6 mice (n = 5) were treated with 0.5 mg/kg Compound 1 formulated mRNA LNPs intravenously.

CAM003 IgM H:L:J chain ratios of 2:1, 6:6:1, and 10:10:1 were evaluated. PBS, CAM003 IgM protein (2.2 mg/kg), and CAM003 IgG mRNA (at a H:L:J chain ratio of 2:1) were used as controls. Serum, lungs, and liver were collected from mice at 6, 24, 48, and 96 hours post-

treatment. Lung and liver tissue were homogenized using a bead homogenizer at 300 mg/mL and 1000 mg/mL, respectively.

CAM003 IgM expressed from mRNA resulted in higher levels of expression and persisted for longer duration of time in mouse serum relative to CAM003 IgM protein (**FIG. 14**).

5 CAM003 IgM did not appear to localize to lung tissue (**FIG. 15**), and CAM003 IgM was found to clear quickly from liver tissue relative to recombinant IgG protein (**FIG. 16**).

Example 11: CAM003 IgM Antigen Binding *in vivo*

To investigate whether incorporation of J chain in class switched IgA and IgM antibodies
10 assembled from mRNA LNPs impacted the ability of the isotypes to bind antigen, samples containing CAM003 IgM antibodies were evaluated for binding in a whole cell *Pseudomonas aeruginosa* ELISA. 8-10 week old female C57Bl/6 mice (n = 5) were treated with 0.5 mg/kg Compound 1 formulated mRNA LNPs intravenously. CAM003 IgM H:L:J chain ratios of 2:1:0, 6:6:1, and 10:10:1 were evaluated. CAM003 IgM protein was used as a control. Serum, lungs,
15 and liver were collected from mice at 6 and 24 hours post-treatment. Lung and liver tissue were homogenized using a bead homogenizer at 300 mg/mL and 1000 mg/mL, respectively.

Both 6:6:1 and 10:10:1 H:L:J chain ratios of CAM003 IgM antibody in the serum retained binding to antigen in a *Pseudomonas aeruginosa* whole cell ELISA (**FIG. 17**).

Example 12: CAM003 Isotype Expression *in vivo* Following Intravenous or Intraperitoneal Injection

To investigate whether antibodies IgG class switched to IgM or IgA isotypes could be expressed *in vivo* from mRNA LNPs, CAM003 antibody concentration was measured in mouse serum after either intravenous or intraperitoneal mRNA LNP delivery. In this example, 8-10
25 week old female C57Bl/6 mice (n = 5) were treated with 0.5 mg/kg Compound 1 formulated mRNA LNPs intravenously or intraperitoneally. CAM003 IgM mRNA with H:L:J chain ratios of 8:8:1 and CAM003 IgA isotypes (IgA1 and IgA2m1) mRNA with chain ratios of H:L:J of 4:4:1 were evaluated. CAM003 IgG isotypes (IgG1 and IgG3) mRNA with H:L:J chain ratios of 2:1:0 were evaluated. PBS was used as a controls. Serum was collected from mice at 6, 24, 48,
30 and 96 hours post-treatment.

CAM003 IgG1 and IgG3 encoded by mRNA LNPs were able to be expressed when injected intravenously or intraperitoneally (**FIG. 18**). CAM003 IgA1, IgA2m1, and IgA2m2 encoded by mRNA LNPs were able to be expressed when injected intravenously or intraperitoneally (**FIG. 19**). CAM003 IgM encoded by mRNA LNPs were able to be expressed
35 when injected intravenously or intraperitoneally (**FIG. 20**).

Example 13: H1 HA influenza Antibodies 5J8 and EM4CO4 Expression *in vivo*

To investigate whether antibodies class switched to IgA1 could be expressed *in vivo* from mRNA, H1 binding, HA influenza antibodies 5J8 and EM4CO4 antibody concentration were measured in rat serum after intravenous delivery of mRNA formulated into LNPs. See

FIG 21. 60-65 day old male SD rats (n = 5) were treated with 0.5 mg/kg Compound 1 and Compound 3 co-formulated mRNA LNPs intravenously. 5J8 IgA1 mRNA and EM4CO4 IgA1 mRNA with H:L:J chain ratios of 4:4:1 were evaluated. 5J8 IgG1 mRNA and EM4CO4 IgG1 mRNA with H:L:J chain ratios of 2:1:0 were used as controls. Serum was collected from rats at 6, 24, 48, 96, and 168 hours post-treatment.

Class switched antibodies from both 5J8 and EM4CO4 encoded by mRNA LNPs were able to be expressed *in vivo* (**FIG. 21**).

Example 14: Sal4 IgG and IgA2m1 Expression *in vivo*

Sal4 is an O5 serotype specific antibody that recognizes the LPS of *Salmonella enterica serovar typhimurium* and is well documented for protection from Salmonella challenge. To investigate whether Sal4 antibodies class switched to IgA2m1 could be expressed *in vivo* from mRNA LNPs, Sal4 antibody concentration was measured in mouse serum, feces, and intestine after intravenous mRNA LNP delivery.

In this example, 8-10 week old female C57Bl/6 mice (n = 5) were treated with 1.0 mg/kg Compound 1 formulated mRNA LNPs intravenously. Sal4 IgA2m1 mRNA with H:L:J chain ratios of 4:4:1 with and without mRNA stabilizing elements and Sal4 IgG mRNA with a H:L:J chain ratio of 2:1:0 with and without mRNA stabilizing elements were evaluated. Sal4 IgG protein, and Sal4 IgA2m1 protein were used as controls. Serum and fecal pellets were collected from mice at 6, 24, 48, 96, and 168 hours post-treatment. 2-3 fecal pellets were weighed and homogenized at 200mg/mL. Supernatant was run on isotype specific ELISAs. Washed intestinal tissue was collected from mice at 168 hours post injection. Intestinal tissues were weighed and homogenized at 2g/mL and supernatant was run on isotype specific ELISAs.

mRNA stabilizing elements, a 3' UTR chemical modification that stabilizes mRNA and allows the mRNA to persist longer, resulted in an increased mRNA half life. Sal4 IgG mRNA mRNA stabilizing elements and Sal4 IgA2m1 mRNA mRNA stabilizing elements both exhibited greater expression in serum of mice relative to mRNA with no addition of mRNA stabilizing elements and relative to protein (**FIG. 22**). Higher levels of IgA2m1 relative to IgG was found in feces (**FIG. 23**), which is indicative of transcytosis of IgA2m1 to mucosal sites via the polymeric Ig receptor (pIgR). Higher IgA2m1 levels in feces were not due to leakage or blood contamination because higher levels of IgG relative to IgA2m1 were found in serum (**FIG. 22**). High levels of IgG antibody were also found in the intestinal tissue at 168 hours post injection

(FIG. 24) further indicating that unlike IgA, IgG can get into the intestinal tissue but cannot be transported into the mucosa due to lack of binding to pIgR. Expression levels in intestine correlated with serum levels at the 168 hour time point.

5 **Example 15: Actoxumab and Bezlotuxumab IgA2m1 Expression *in vivo***

To investigate whether monoclonal antibodies class switched to IgA isotypes could be expressed *in vivo* from mRNA LNPs, Actoxumab, a monoclonal antibody that targets *Clostridium difficile* toxin A and Bezlotuxumab, an FDA approved monoclonal antibody that targets *Clostridium difficile* toxin B, were evaluated. Actoxumab and Bezlotuxumab
10 concentration was measured in rat serum after intravenous mRNA LNP delivery. Antibody in the serum was measured using anti-idiotypic ELISA (Coat: anti human IgG or anti human IgA; detection: anti human kappa HRP or anti human IgA HRP).

60-65 day old male SD rats (n = 5) were treated with 0.5 mg/kg Compound 1 and Compound 3 co-formulated mRNA LNPs intravenously. Actoxumab IgA2m1 mRNA and
15 Bezlotuxumab IgA2m1 mRNA with H:L:J chain ratios of 4:4:1 were evaluated. Actoxumab IgG mRNA and Bezlotuxumab IgG mRNA with H:L:J chain ratios of 2:1:0 were also evaluated. Actoxumab IgG protein, Actoxumab IgA2m1 protein, Bezlotuxumab IgG protein, and Bezlotuxumab IgA2m1 protein were used as controls. Serum was collected from rats at 6, 24, 48, 96, and 168 hours post-treatment.

Both Actoxumab and Bezlotuxumab are able to be expressed *in vivo* as IgA2m1 and IgG1 (FIG. 25 and FIG. 26). Importantly, Actoxumab and Bezlotuxumab expressed as IgA2m1 from mRNA resulted in higher serum expression and longer serum half-life than their analogous recombinant protein.

25 **Example 16: *C. difficile* toxin A neutralization by Actoxumab**

To investigate if Actoxumab IgG or IgA2m1 encoded by mRNA LNPs could neutralize *C. difficile* toxin A, an *in vitro* neutralization assay was conducted. Purified Toxin A 8x 50% tissue culture infective dose (8x TCID₅₀) was premixed with antibody protein or mRNA transfected cell concentrated supernatant and incubated for 1 hour at 37°C, then added to Vero
30 cells, then incubated for 72 hours at 37°C, 5% CO₂. Cells were washed and incubated with MEM with 10% alamarBlue for 4 hours prior to reading at 600 nm. Percent neutralization of toxin was calculated as follows: 1-(average absorbance of antibody sample/average absorbance of toxin only sample) * 100%.

Antibody collected from supernatant of EXPI293 cells transfected with mRNA encoding
35 for Actoxumab IgA2m1 at a H:L:J chain ratio of 4:4:1 or Actoxumab IgG1 at a H:L:J chain ratio

2:1:0 were evaluated. Actoxumab IgG or IgA2m1 recombinant antibody protein was used as a control.

Actoxumab as an IgA2m1 has a ~200 fold better potency (lower IC50) than IgG (**FIG. 27**) for both the mRNA and the recombinant antibody protein. This indicates that Actoxumab is more potent as an IgA2m1 isotype and that antibody made from mRNA transfection is equivalent to purified recombinant antibody protein in terms of functionality. This data further demonstrates that mRNA encoded IgA is functional.

Example 17: Evaluation of mRNA encoded Sal4 IgG and IgA in *Salmonella enterica* serovar typhimurium Challenge Model

To investigate whether mRNA encoded Sal4 IgG or IgA can provide protection against *Salmonella enterica* serovar typhimurium (AR05) infection, challenge studies in mice are conducted as follows.

To test mRNA encoded Sal4 as IgG or IgA, mice (n = 8) are given one of Sal4 IgG protein, Sal4 IgA protein, Sal4 IgG mRNA with mRNA stabilizing elements, Sal4 IgA mRNA with mRNA stabilizing elements, or saline (negative control).

Balb/c mice received antibody treatment intravenously on day (-1) before being challenged with oral challenge of equal amounts of O5 serotype *Salmonella enterica* serovar typhimurium (AR05) and O4 serotype specific *Salmonella enterica* serovar typhimurium (AR04) on day 0. Sal4 only binds the AR05 serotype and not the AR04 serotype. The AR04 serotype serves as a loading control. If Sal4 antibody has a protective impact, the amount of AR05 serotype will decrease relative to AR04 serotype in subject mice. If Sal4 antibody has no effect, AR05 serotype will have approximately the same colony counts as AR04 serotype.

Animals were euthanized 24 hours post infection. Peyer's patches were isolated from small intestine and homogenized. Homogenates were serially diluted and plated on LB agar containing kanamycin and X-gal and incubated overnight. Blue colonies were indicative of AR04 growth and white colonies were indicative of AR05 growth. Competitive indices of AR04 and AR05 were calculated as follows: $CI = [(\% \text{strain AR05 recovered} / \% \text{AR04 recovered}) / (\% \text{ARO5 inoculated} / \% \text{AR04 inoculated})]$.

A decrease in AR05 colonies in mice given mRNA encoded Sal4 IgA as compared to saline control demonstrated the functionality of IgA *in vivo* (**FIG. 28**).

Antibody isotype concentration of Sal4 IgG, Sal4 IgA, Sal4 IgG protein and Sal4 IgA protein was measured in serum and feces in mice 24 hours after intravenous injection (**FIG. 29**).

Example 18: Evaluation of mRNA encoded Sal4 IgG and IgA in *Salmonella enterica* serovar typhimurium

To investigate whether antibodies class switched to IgA2m1 could be expressed *in vivo* from mRNA LNPs, Sal4 antibody concentration is measured in mouse serum, lung, liver, feces, large intestinal scraping, large intestine tissue, small intestinal scraping, and small intestinal tissue after intravenous mRNA LNP delivery. Antibody concentration is measured using anti-human IgG and anti-human IgA via Luminex.

In this example, 8-10 week old female Balb/c mice (n = 65) are treated with 1.0 mg/kg Compound 1 formulated mRNA LNPs intravenously. Sal4 IgA2m1 mRNA with H:L:J chain ratios of 4:4:1 are evaluated. Sal4 IgG mRNA with H:L:J chain ratios of 2:1:0, Sal4 IgG protein (5 mg/kg), and Sal4 IgA2m1 protein (5mg/kg) are used as controls. Serum, lung, liver, feces, large intestinal scraping, large intestine tissue, small intestinal scraping, and small intestinal tissue are collected from mice at 6, 24, 48, and 96 hours post treatment. Tissues are collected post perfusion of the mouse with PBS. Number of mice sacked per timepoint is as follows: 6 hours (20 mice), 24 hours (15 mice), 48 hours (15 mice), and 96 hours (15 mice). Serum is obtained and supernatant is collected. Liver tissue is weighed, homogenized at 1mg/mL and supernatant is collected. Lung tissue is weighted, homogenized at 300mg/mL and supernatant is collected. Intestinal squeezing and/or scraping is collected in 500uL of PBS. Washed large and small intestinal tissue are weighed and homogenized at 1g/mL and 2g/mL, respectively, and supernatant is collected. 2-3 fecal pellets are weighed and homogenized at 200mg/mL and supernatant is collected. Supernatant from all samples are evaluated for human IgG and human IgA via Luminex.

It is predicted that increased amounts of mRNA encoded Sal4 IgA2m1 relative to mRNA encoded Sal4 IgG1 will be detectable across mucosal membranes (e.g, in lung tissue, feces) due to the ability of IgA to transcytosis across cells to mucosal membranes by binding to pIgR.

Example 19: Evaluation of mRNA encoded Actoxumab and Bezlotuxumab in *C. difficile* hamster challenge model

To investigate whether mRNA encoded Actoxumab as IgA2m1 or IgG and/or mRNA encoded as Bezlotuxumab as IgA2m1 or IgG can provide protection against *C. difficile* infection, two separate primary hamster challenge studies are conducted as follows.

To test mRNA encoded Actoxumab as IgA2m1 or IgG, hamsters (n = 8-10 pre group) are given Actoxumab IgG1 protein, Actoxumab IgA2m1 protein, Actoxumab IgG1 mRNA, Actoxumab IgA2m1 mRNA, negative control protein, or a negative control mRNA.

To test mRNA encoded Bezlotuxumab as IgA2m1 or IgG, hamsters (n = 8-10 pre group) are given Bezlotuxumab IgG1 protein, Bezlotuxumab IgA2m1 protein, Bezlotuxumab IgG1 mRNA, Bezlotuxumab IgA2m1 mRNA, negative control protein, or a negative control mRNA.

Hamsters will receive antibody treatment intravenously on day (-2) before being challenged with *C. difficile* spore by oral gavage (day 0). Hamsters will be bled for serum samples on the following days: day (-1), day 0, day 3, day 6, day 10, day 10, and day 21 (**FIG. 30**). Fecal samples will also be collected on days serum is collected.

Study readouts will include serum antibody level quantitation, hamster weight, mortality and/or time to mortality, toxin A and toxin B quantitation in feces, and fecal toxin A/B per CFU. It is predicted that a delay in time to mortality may be observed when animals are treated with antibodies against one toxin rather than an increased survival rate due to the presence of both toxin A and B in hamsters challenged by *C. difficile*. It is predicted that mRNA delivered antibodies class-switched to IgA2m1 will have a longer delay in time to mortality than recombinant antibody protein (IgA2m1 or IgG isotype) or mRNA encoded IgG due to the ability of IgA to transcytose to the site of infection.

Example 20: *Pseudomonas aeruginosa* acute pneumonia lung challenge model

To investigate whether mRNA encoded CAM003 antibody isotypes and mRNA encoded Hu605 antibody isotypes can provide protection against *P. aeruginosa* infection, a *P. aeruginosa* acute pneumonia lung challenge model in mice is conducted. 6-8 week old female Balb/c mice are treated with 1.0 mg/kg Compound 1 formulated mRNA LNPs intravenously.

To test CAM003 antibody isotypes, CAM003 IgG mRNA with H:L:J chain ratios of 2:1:0, CAM003 IgG3 mRNA with H:L:J chain ratios of 2:1:0, CAM003 IgA1 mRNA with H:L:J chain ratios of 4:4:1, CAM003 IgA2m1 mRNA with H:L:J chain ratios of 4:4:1, and CAM003 IgM mRNA with H:L:J chain ratios of 8:8:1 are evaluated. CAM003 IgG protein, CAM003 IgG3 protein, CAM003 IgA2m2 protein, and CAM003 IgM protein are used as controls.

To test Hu605 antibody isotypes, Hu605 IgG mRNA with H:L:J chain ratios of 2:1:0, Hu605 IgG3 mRNA with H:L:J chain ratios of 2:1:0, Hu605 IgA2m1 mRNA with H:L:J chain ratios of 4:4:1, and Hu605 IgM mRNA with H:L:J chain ratios of 8:8:1 are evaluated. Hu605 IgG protein, Hu605 IgG3 protein, CAM003 IgA2m2 protein, and CAM003 IgM protein are used as controls.

Mice will receive antibody treatment intravenously on day (-1) before being challenged with *P. aeruginosa* by intranasal delivery (day 0). Mice will be bled for serum samples on D0 (**FIG. 31**). Weight, morbidity, and mortality will be evaluated.

Table 1: Antibody Constant Region Sequences

Antibody Region	Amino Acid Sequence
Light chain (kappa)	RTVAAPS VFIFPPSDEQLKSGTASV VCLNNFYPREAKVQWKVDNALQSGNSQESVTEQD SKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO: 1)
Light chain (lambda)	GQPKAAPS VTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQ SNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTVAPTECS (SEQ ID NO: 2)
Heavy chain (IgG1)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSV FLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQG NVFSCSV MHEALHNHYTQKSLSLSPGK (SEQ ID NO: 3)
Heavy chain (IgG1 LS mutation)	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSV FLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTK NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQG NVFSCSVLHEALHSHYTQKSLSLSPGK (SEQ ID NO: 4)
Heavy chain (IgA1)	ASPTSPKVFPLSLCSTQPDGNVVIACLVQGFFPQEPLSVTWSESGQGV TARNFPPSQDASGD LYTSSQLTLPATQCLAGKSVTCHVKHYTNPSQDVTVPVPCVPSTPPTSPSTPPTSPSCCHP RLSLHRPALEDLLLGSEANLTCTLTGLRDASGVTFWTPSSGKSAVQGPPERDLCGCYSVS SVLPGCAEPWNHGTFTCTAAYPEKSTPLTATLSKSGNTFRPEVHLLPPPSEELALNELVTL TCLARGFSPKDVLRWLQGSQELPREKYL TWASRQEPSQGTTFATVSILRVA AEDWKKG DTFSCMVGHEALPLAFTQKTIDRLAGKPTHVNVSVVMAEVDGTCY (SEQ ID NO: 5)
Heavy chain (IgA2m1)	ASPTSPKVFPLSLDSTPQDGNVVVACLQVGFPPQEPLSVTWSESGQNV TARNFPPSQDASG DLYTTSSQLTLPATQCPDGKSVTCHVKHYTNPSQDVTVPVPPPPPCCHPRLSLHRPALE DLLLGSEANLTCTLTGLRDASGATFTWTPSSGKSAVQGPPERDLCGCYSVS SVLPGCAQP WNHGETFTCTAAHPELKTPLTANITKSGNTFRPEVHLLPPPSEELALNELVTLTCLARGFSP KDVLRWLQGSQELPREKYL TWASRQEPSQGTTFATVSILRVA AEDWKKGDTFSCMVG HEALPLAFTQKTIDRLAGKPTHVNVSVVMAEVDGTCY (SEQ ID NO: 6)
Heavy chain (IgA2m2)	ASPTSPKVFPLSLDSTPQDGNVVVACLQVGFPPQEPLSVTWSESGQNV TARNFPPSQDASG DLYTTSSQLTLPATQCPDGKSVTCHVKHYTNSSQDVTVPVPPPPPCCHPRLSLHRPALE DLLLGSEANLTCTLTGLRDASGATFTWTPSSGKSAVQGPPERDLCGCYSVS SVLPGCAQP WNHGETFTCTAAHPELKTPLTANITKSGNTFRPEVHLLPPPSEELALNELVTLTCLARGFSP KDVLRWLQGSQELPREKYL TWASRQEPSQGTTFATVSILRVA AEDWKKGETFSCMVG HEALPLAFTQKTIDRLAGKPTHINVSVM A EADGTCY (SEQ ID NO: 7)
Heavy chain (IgM)	GSASAPTLFPLVSCENSPTSSTSSVAVGCLAQDFLPDSITFSWKYKNNSDISSTRGFPSVLRGG KYAATSQVLLPSKDVMQGTDEHVCKVQHPNGNKEKNVPLPVIAELPPKVS VFVPPRDGF FGNPRKSKLICQATGFSPRQIQVSWLREGKQVSGSVTTDQVQAEAKESGPTTYKVTSTLTI KESDWLGQSMFTCRVDHRLTFQQNASSMCVPDQDTAIRVFAIPPSFASIFLTKSTKLTLCL VTDLTTYDSVTISWTRQNGEAVKTHTNISESHPNATFSAVGEASICEDDWSNGERFTCTVT HTDLPSPKQTISRPKGVALHRPDVYLLPPAREQLNLRESATITCLVTGFSPADVFVQWMQ RGQPLSPEKYVTSAPMPEPQAPGRYFAHSILTVSEEEWNTGETYTCVVAHEALPNRV TERT VDKSTGKPTLYNVSLVMSDTAGTCY (SEQ ID NO: 8)
Heavy chain (IgG3)	ASTKGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSG LYSLSVVTVPSSSLGTQTYTCNVNHKPSNTKVDKRVELKTPLGDTTHTCPRCPEPKSCDT PPPCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPAPELLGGPSVFLFPPKPKDTLMISRT PEVTCVVDVSHEDPEVQFKWYVDGVEVHNAKTKPREEQYNSTFRVSVLTVLHQDWL NGKEYKCKVSNKALPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPS DIAVEWESSGQPENNYNTTPMLDSDGSFFLYSKLTVDKSRWQQGNIFSCSV MHEALHNR FTQKSLSLSPGK (SEQ ID NO: 9)
J chain (human), with secretion signal 1	<u>MKNHLLFWGVLA FIKAVHVKA</u> QEDERIVLVDNKCKCARITSRIIRSSDPNEDIVERNIR IIVPLNNRENISDPTSPLRTRFVYHLSDLCKKCDPTEVELDNQIVTATQSNICDEDSATETCY TYDRNKCYTAVVPLVYGGETK MVETALTPDACYPD (SEQ ID NO: 10)

J chain (human), with secretion signal 2	<u>MGWSCILFLVATATGVHS</u> QEDERIVLVDNKCKCARITSRIIRSSDPNEDIVERNIRIIVPL NNRENISDPTSPLRTRFVYHLSDLCKKCDPTEVELDNQIVTATQSNICDEDSATETCYTYDR NKCYTAVVPLVYGGETKMMVETALTPDACYPD (SEQ ID NO: 11)
--	---

EQUIVALENTS

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, *i.e.*, elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with “and/or” should be construed in the same fashion, *i.e.*, “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically

identified by the “and/or” clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, *i.e.*, the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (*i.e.* “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” “composed of,” and the like are to be understood to be open-ended, *i.e.*, to mean including but not limited to. Only the transitional phrases “consisting of” and “consisting essentially of” shall be closed or semi-closed

5 transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03. It should be appreciated that embodiments described in this document using an open-ended transitional phrase (*e.g.*, “comprising”) are also contemplated, in alternative embodiments, as “consisting of” and “consisting essentially of” the feature described by the open-ended transitional phrase. For example, if the disclosure describes

10 “a composition comprising A and B”, the disclosure also contemplates the alternative embodiments “a composition consisting of A and B” and “a composition consisting essentially of A and B”.

CLAIMS

1. A composition comprising:

(i) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody heavy chain (HC);

5 (ii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody light chain (LC), and

(iii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody joining (J) chain,

10 wherein (i), (ii), and (iii) are present in the composition in a specific ratio effective for producing an assembled antibody and wherein the assembled antibody is capable of forming a multimer.

2. The composition of claim 1, wherein the antibody heavy chain is an IgA heavy chain and wherein the specific ratio of (i), (ii), and (iii) encompasses a range of specific effective ratios
15 including 1:1:1, 2:2:1, 4:4:1, 6:6:1, and 8:8:1.

3. The composition of claim 2, wherein the specific effective ratio of (i), (ii), and (iii) is 8:8:1.

20 4. The composition of claim 2, wherein the specific effective ratio of (i), (ii), and (iii) is 6:6:1.

5. The composition of any one of claims 2-4, wherein the IgA heavy chain further comprises a variable region, wherein the variable region comprises three heavy chain CDRs and
25 four framework regions.

6. The composition of any one of claims 2-5, wherein the IgA heavy chain further comprises a variable region, wherein the heavy chain variable region (HCVR) comprises:

30 (a) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 1 (CDR1);

(b) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 2 (CDR2); and

(c) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 3 (CDR3).

35

7. The composition of any one of claims 2-6, wherein the IgA light chain further comprises a variable region, wherein the light chain variable region (LCVR) comprises:

- (a) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 1 (CDR1);
- 5 (b) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 2 (CDR2); and
- (c) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 3 (CDR3).

10 8. The composition of claim 1, wherein the antibody heavy chain is an IgM heavy chain and wherein the specific ratio of (i), (ii), and (iii) encompasses a range of specific effective ratios including 1:1:1, 2:2:1, 4:4:1, 6:6:1, 8:8:1, and 10:10:1.

9. The composition of claim 8, wherein the specific effective ratio of (i), (ii), and (iii) is
15 4:4:1.

10. The composition of claim 8, wherein the specific effective ratio of (i), (ii), and (iii) is 8:8:1.

20 11. The composition of claim 8, wherein the specific effective ratio of (i), (ii), and (iii) is 10:10:1.

12. The composition of any one of claims 8-11, wherein the IgM heavy chain further comprises a variable region, wherein the variable region comprises three heavy chain CDRs and
25 four framework regions.

13. The composition of any one of claims 8-12, wherein the IgM heavy chain further comprises a variable region, wherein the heavy chain variable region (HCVR) comprises:

- 30 (d) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 1 (CDR1);
- (e) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 2 (CDR2); and
- (f) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 3 (CDR3).

35

14. The composition of any one of claims 8-13, wherein the IgM light chain further comprises a variable region, wherein the light chain variable region (LCVR) comprises:

- (d) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 1 (CDR1);
- 5 (e) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 2 (CDR2); and
- (f) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 3 (CDR3).

10 15. The composition of any one of claims 1-14, wherein the ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody J chain comprises a protein sequence at least 90% identical to SEQ ID NO: 10 or 11.

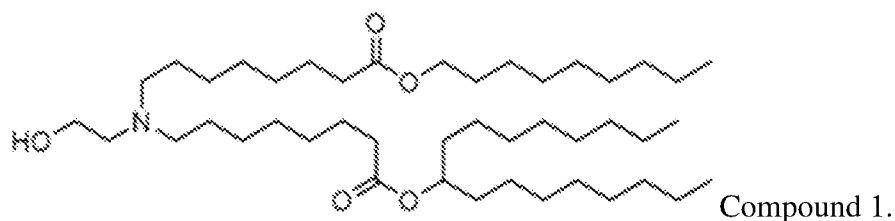
16. The composition of any one of claims 1-15, wherein the ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody J chain comprises SEQ ID NO: 10 or 11.

17. The composition of any one of claims 1-16, wherein the composition further comprises a lipid nanoparticle (LNP).

20 18. The composition of claim 17, wherein the lipid nanoparticle comprises a mixture of lipids, wherein the mixture of lipids comprises an ionizable amino lipid, a PEG-modified lipid, a structural lipid and aphospholipid.

25 19. The composition of claim 18, wherein the mixture of lipids comprises 0.5-15 mol% PEG-modified lipid; 5-25 mol% non-cationic lipid; 25-55 mol% sterol; and 20-60 mol% ionizable amino lipid.

30 20. The composition of claim 19, wherein the PEG-modified lipid is 1,2 dimyristoyl-sn-glycerol, methoxypolyethyleneglycol (PEG2000 DMG), the non-cationic lipid is 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC), the sterol is cholesterol; and the ionizable amino lipid has the structure of Compound 1:



21. The composition of any one of claims 1-20, wherein the assembled antibody is capable of binding polymeric immunoglobulin receptor (pIgR).

5

22. The composition of any one of claims 1-21, wherein the assembled antibody is capable of being incorporated into one or more mucosal regions.

23. The composition of claim 22, wherein the mucosal region is one or more of: salivary glands, ocular tissues, mammary glands, gut-associated lymphoid tissue, gastrointestinal tract, bronchus-associated lymphoid tissue, respiratory tract, and/or urogenital tract.

10

24. The composition of any one of claims 1-23, wherein the assembled antibody is capable of being incorporated into mucosal secretions.

15

25. The composition of claim 24, wherein the mucosal secretions are one or more of: tears, saliva, sweat, gastrointestinal fluid, respiratory tract secretions, and/or breast milk.

26. The composition of any one of claims 1-25, wherein the assembled antibody specifically binds a bacterial antigen.

20

27. The composition of any one of claims 1-26, wherein the assembled antibody specifically binds a viral antigen.

28. The composition of any one of claims 1-27, wherein the assembled antibody specifically binds a tumor antigen.

25

29. The composition of any one of claims 1-28, wherein the assembled antibody specifically binds a parasitic antigen.

30

30. A method of producing a therapeutic level of an IgA antibody in a human subject *in vivo*, the method comprising administering to the subject an LNP-formulated mRNA, the mRNA

encoding the IgA antibody, in a dose effective to produce a functional oligomeric antibody in a mucosal tissue of the subject.

31. A method of producing a therapeutic level of an IgM antibody in a human subject *in vivo*,
the method comprising administering to the subject an LNP-formulated mRNA, the mRNA
encoding the IgA antibody, in a dose effective to produce a functional oligomeric antibody in a
mucosal tissue of the subject.

32. The method of claims 30 and 31, wherein the IgA or IgM antibody are encoded by
mRNA formulated in an LNP comprising:

(i) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an
antibody heavy chain (HC);

(ii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an
antibody light chain (LC), and

(iii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an
antibody joining (J) chain,

wherein (i), (ii), and (iii) are present in the LNP in a specific ratio effective for producing
an assembled antibody and wherein the assembled antibody is capable of forming a multimer.

33. The method of claim 32, wherein the specific ratio of (i), (ii), and (iii) encompasses a
range of specific effective ratios including 1:1:1, 2:2:1, 4:4:1, 6:6:1, 8:8:1, and 10:10:1.

34. The method of claim 33, wherein the specific effective ratio of (i), (ii), and (iii) is 4:4:1.

35. The method of claim 33, wherein the specific effective ratio of (i), (ii), and (iii) is 8:8:1.

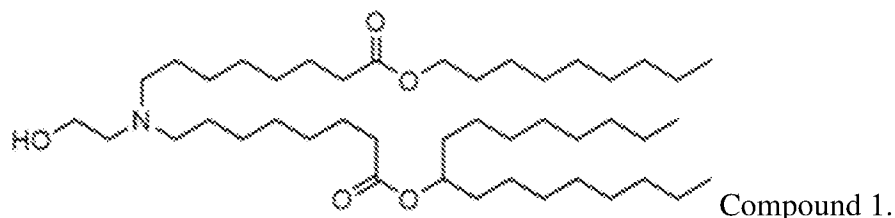
36. The method of claim 33, wherein the specific effective ratio of (i), (ii), and (iii) is
10:10:1.

37. The method of any one of claims 30-36, wherein the antibody is administered either IV,
SC or IM for systemic circulation and distribution to mucosal tissues.

38. The method of any one of claims 30-37, wherein the LNP comprises an ionizable amino
lipid, a PEG-modified lipid, a structural lipid and a phospholipid.

39. The method of claim 38, wherein the LNP comprises a mixture of lipids, wherein the mixture of lipids comprises an ionizable amino lipid, a PEG-modified lipid, a structural lipid and a phospholipid.

40. The method of claim 39, wherein the PEG-modified lipid is 1,2 dimyristoyl-sn-glycerol, methoxypolyethyleneglycol (PEG2000 DMG), the non-cationic lipid is 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC), the sterol is cholesterol; and the ionizable amino lipid has the structure of Compound 1:



41. A composition comprising:

(i) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody IgG3 heavy chain (HC) and

(ii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody light chain (LC),

wherein (i) and (ii) are present in the composition in a specific ratio effective for producing an assembled antibody.

42. The composition of claim 41, wherein the composition does not comprise a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody joining (J) chain.

43. The composition of claim 41 or 42, wherein the antibody heavy chain is an IgG3 heavy chain and wherein the specific ratio of (i) and (ii) encompasses a range of specific effective ratios including 1:1 and 2:1.

44. The composition of claim 43, wherein the specific effective ratio of (i) and (ii) is 2:1.

45. The composition of claim 43 or 44, wherein the IgG3 heavy chain further comprises a variable region, wherein the variable region comprises three heavy chain CDRs and four framework regions.

46. The composition of any one of claims 43-45, wherein the IgG3 heavy chain further comprises a variable region, wherein the heavy chain variable region (HCVR) comprises:

- (g) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 1 (CDR1);
- 5 (h) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 2 (CDR2); and
- (i) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a heavy chain complementarity determining region 3 (CDR3).

10 47. The composition of any one of claims 43-46, wherein the IgG3 light chain further comprises a variable region, wherein the light chain variable region (LCVR) comprises:

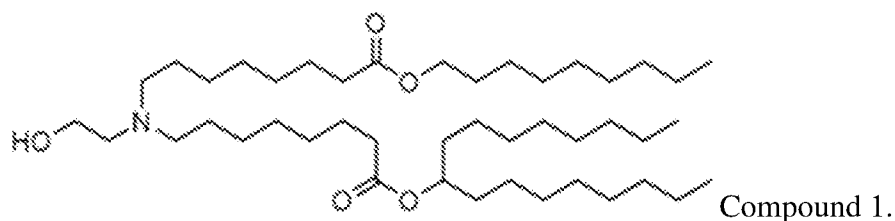
- (g) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 1 (CDR1);
- (h) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 2 (CDR2); and
- 15 (i) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding a light chain complementarity determining region 3 (CDR3).

48. The composition of any one of claims 41-47, wherein the composition further comprises
20 a lipid nanoparticle (LNP).

49. The composition of claim 48, wherein the lipid nanoparticle comprises a mixture of lipids, wherein the mixture of lipids comprises an ionizable amino lipid, a PEG-modified lipid, a structural lipid and a phospholipid.

25 50. The composition of claim 49, wherein the mixture of mixture of lipids comprises 0.5-15 mol% PEG-modified lipid; 5-25 mol% non-cationic lipid; 25-55 mol% sterol; and 20-60 mol% ionizable amino lipid.

30 51. The composition of claim 50, wherein the PEG-modified lipid is 1,2 dimyristoyl-sn-glycerol, methoxypolyethyleneglycol (PEG2000 DMG), the non-cationic lipid is 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC), the sterol is cholesterol; and the ionizable amino lipid has the structure of Compound 1:



52. The composition of any one of claims 41-51, wherein the assembled antibody is capable of being incorporated into one or more mucosal regions.

5

53. The composition of claim 52, wherein the mucosal region is one or more of: salivary glands, ocular tissues, mammary glands, gut-associated lymphoid tissue, gastrointestinal tract, bronchus-associated lymphoid tissue, respiratory tract, and/or urogenital tract.

10 54. The composition of any one of claims 41-53, wherein the assembled antibody is capable of being incorporated into mucosal secretions.

55. The composition of claim 54, wherein the mucosal secretions are one or more of: tears, saliva, sweat, gastrointestinal fluid, respiratory tract secretions, and/or breast milk.

15

56. The composition of any one of claims 41-55, wherein the assembled antibody specifically binds a bacterial antigen.

57. A method of producing a therapeutic level of an IgG3 antibody in a human subject *in vivo*, the method comprising administering to the subject an LNP-formulated mRNA, the mRNA encoding the IgG3 antibody, in a dose effective to produce a functional antibody in a mucosal tissue of the subject.

20

58. The method of claim 57, wherein the IgG3 antibody is encoded by mRNA formulated in an LNP comprising:

25

(i) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody heavy chain (HC), and

(ii) a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody light chain (LC),

30 wherein (i) and (ii) are present in the LNP in a specific ratio effective for producing an assembled antibody.

59. The method of claim 58, wherein the LNP does not comprise a ribonucleic acid (RNA) polynucleotide having an open reading frame encoding an antibody joining (J) chain.

60. The method of claim 58 or 59, wherein the specific ratio of (i) and (ii) encompasses a range of specific effective ratios including 1:1 and 2:1.

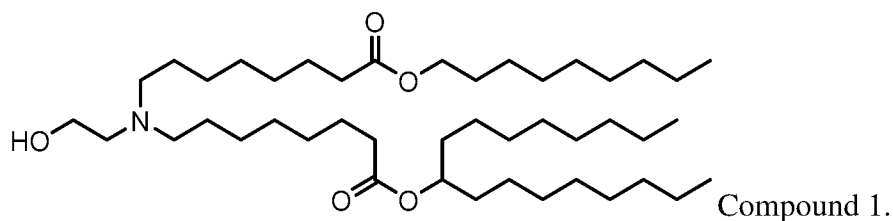
61. The method of claim 60, wherein the specific effective ratio of (i) and (ii) is 2:1.

62. The method of any one of claims 58-61 wherein the antibody is administered either IV, SC or IM for systemic circulation and distribution to mucosal tissues.

63. The method of any one of claims 57-62, wherein the LNP comprises a mixture of lipids, wherein the mixture of lipids comprises an ionizable amino lipid, a PEG-modified lipid, a structural lipid and a phospholipid.

64. The method of claim 63, wherein the mixture of lipids comprises 0.5-15 mol% PEG-modified lipid; 5-25 mol% non-cationic lipid; 25-55 mol% sterol; and 20-60 mol% ionizable amino lipid.

65. The method of claim 64, wherein the PEG-modified lipid is 1,2 dimyristoyl-sn-glycerol, methoxypolyethyleneglycol (PEG2000 DMG), the non-cationic lipid is 1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC), the sterol is cholesterol; and the ionizable cationic lipid has the structure of Compound 1:



Ratios (H:L:J)	IgG	IgG3	IgA	IgM
2:1:0	X	X	X	X
1:1:1			X	X
2:2:1			X	X
4:4:1			X	X
6:6:1			X	X
8:8:1			X	X
10:10:1				X

FIG. 1

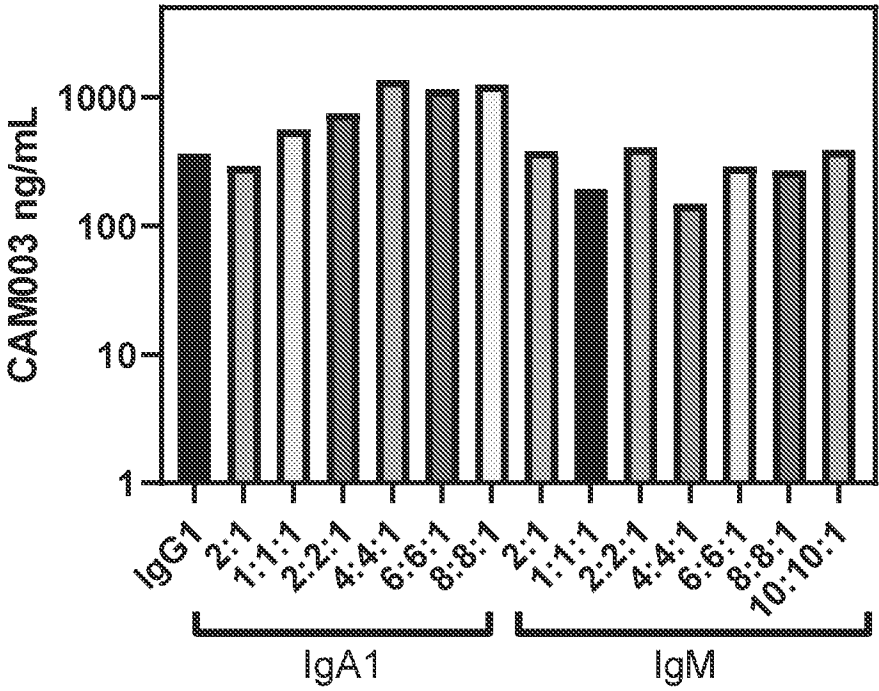


FIG. 2A

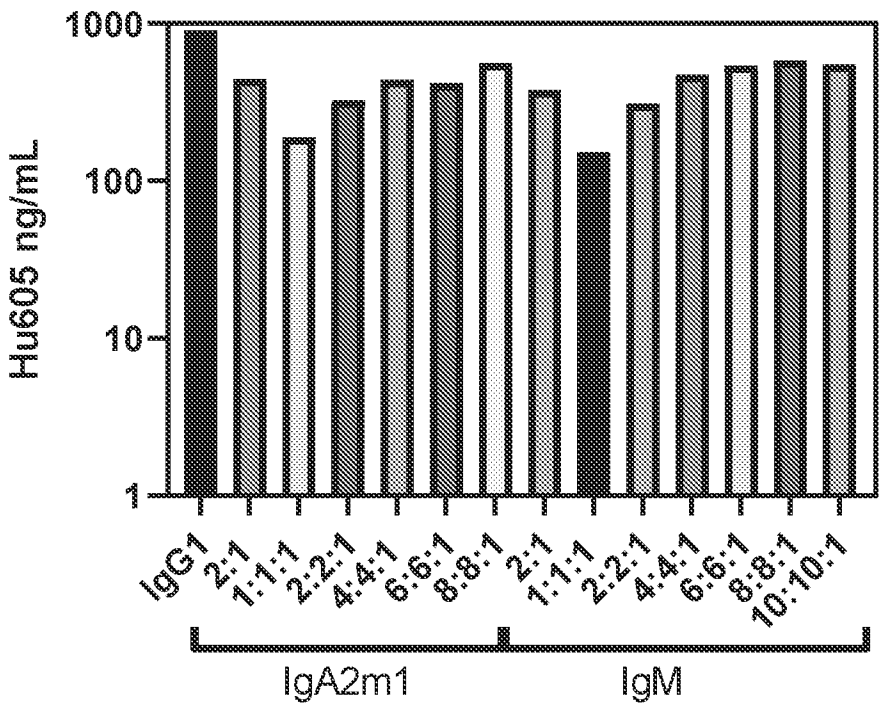


FIG. 2B

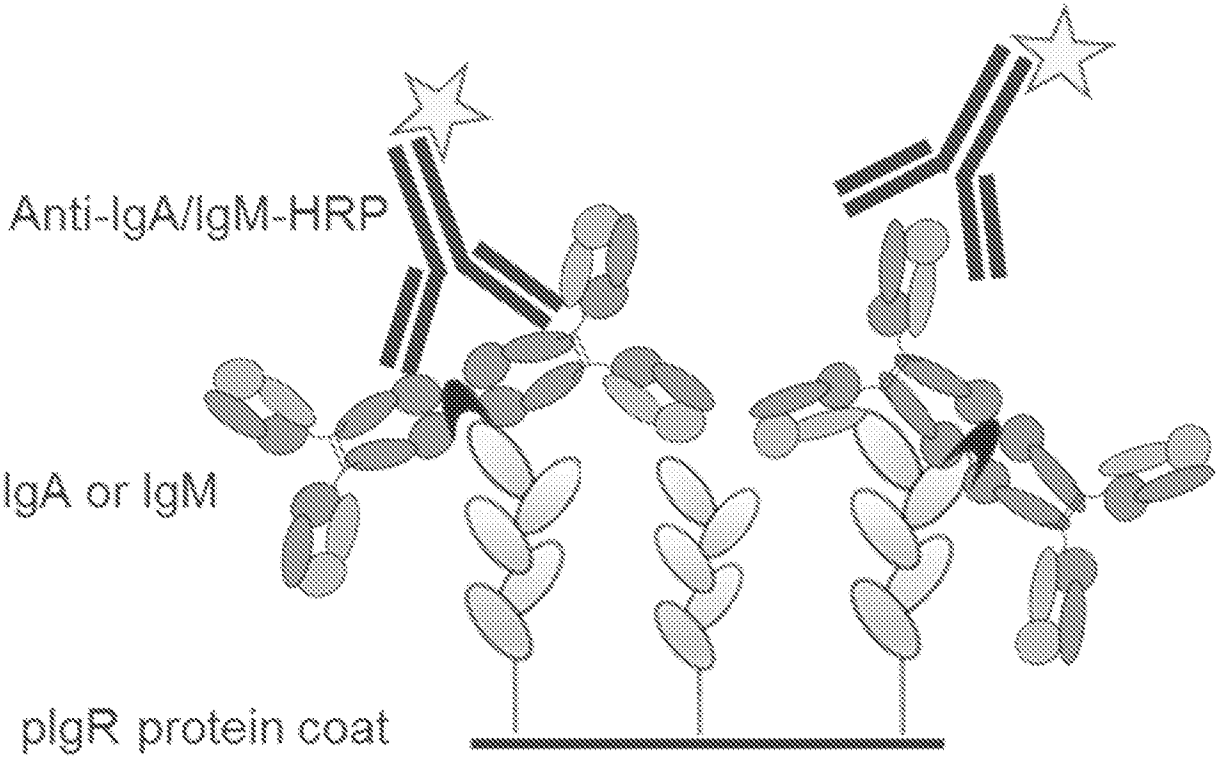


FIG. 3

4/23

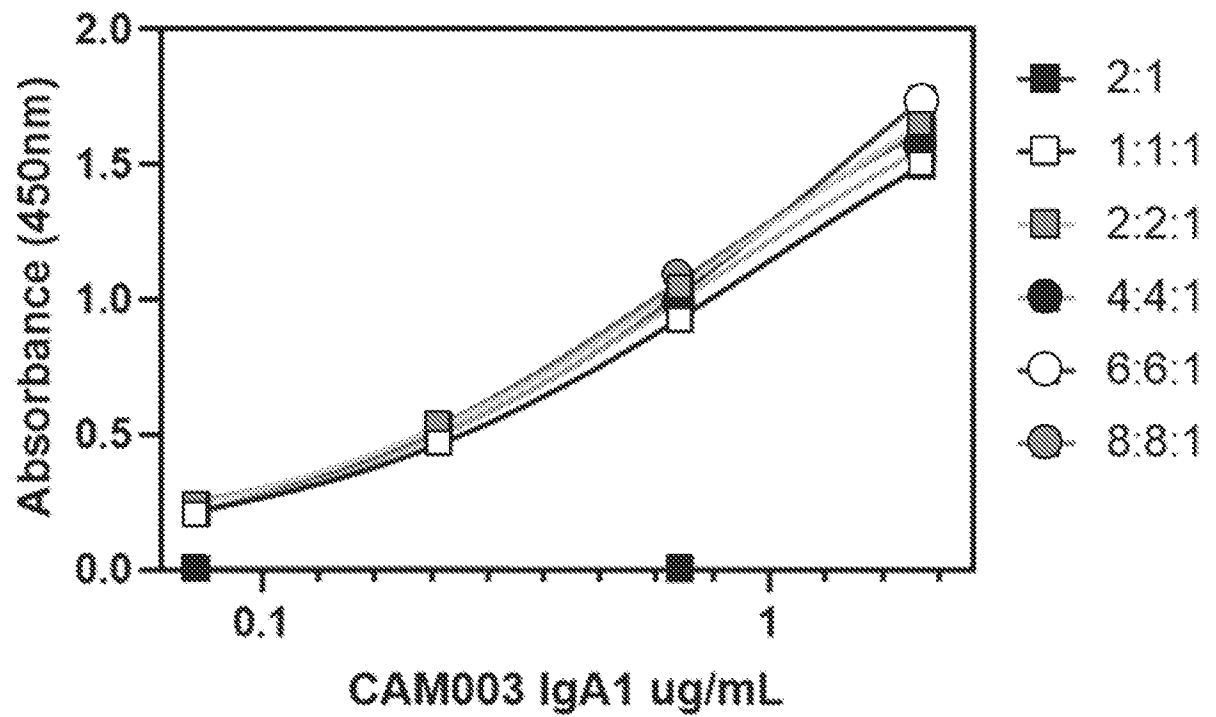


FIG. 4A

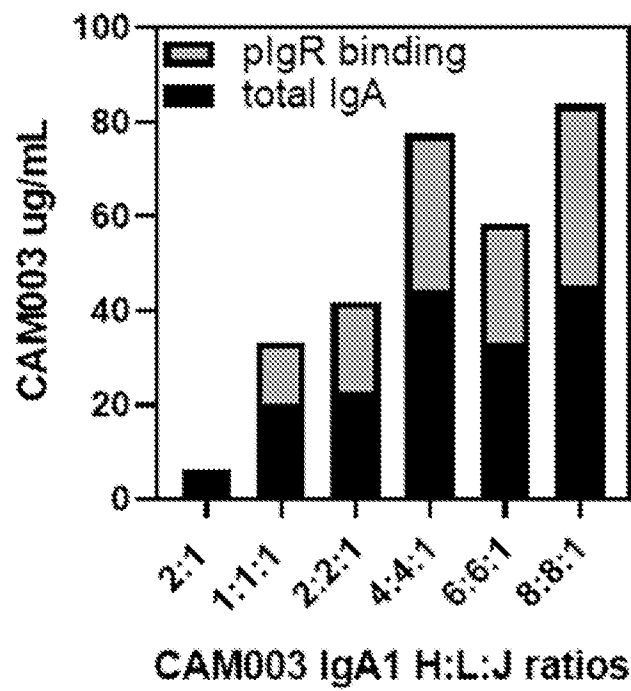


FIG. 4B

5/23

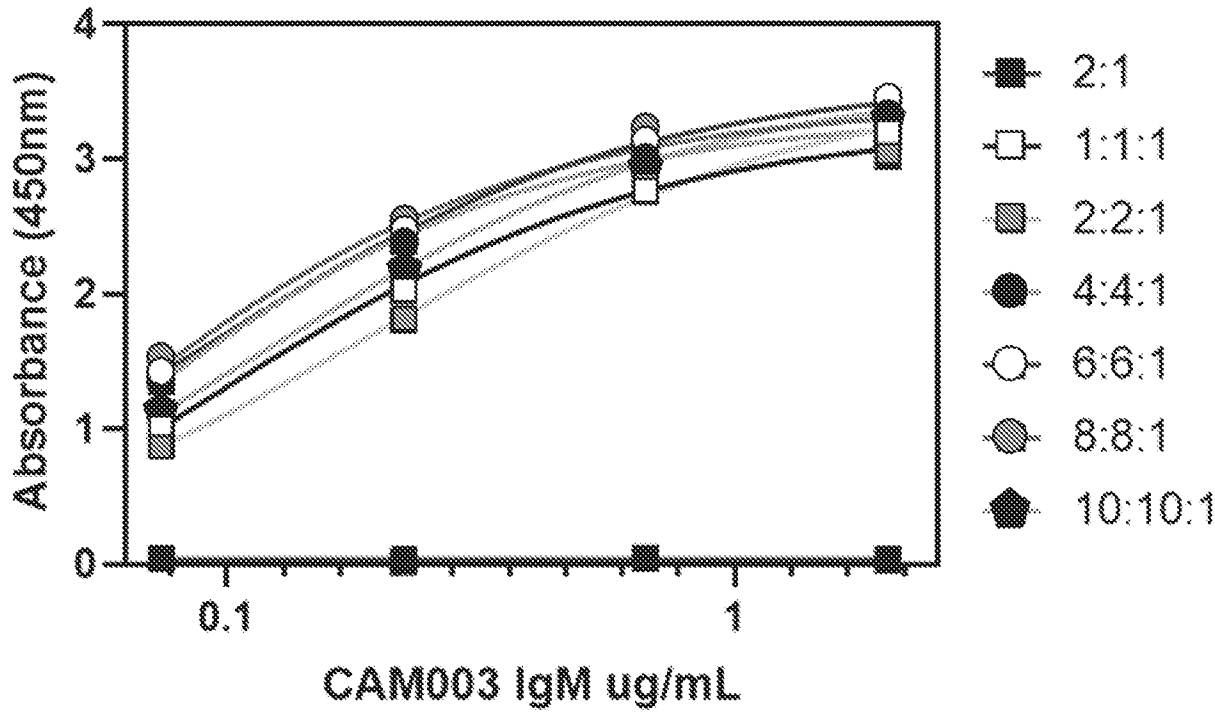


FIG. 4C

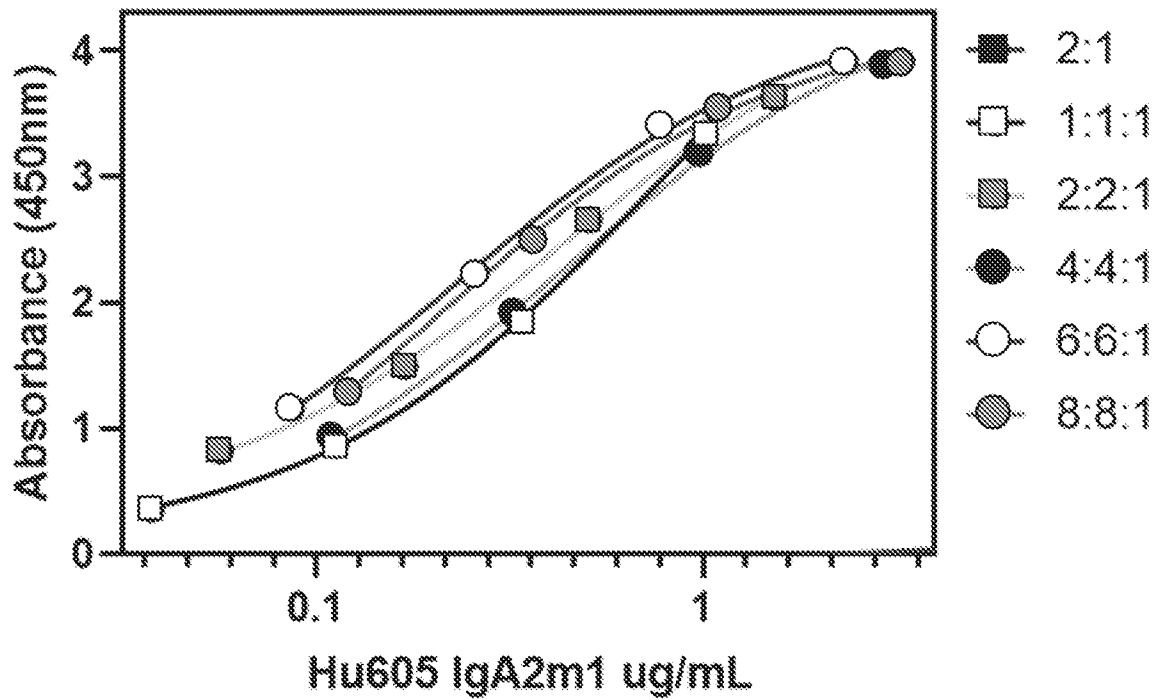


FIG. 4D

6/23

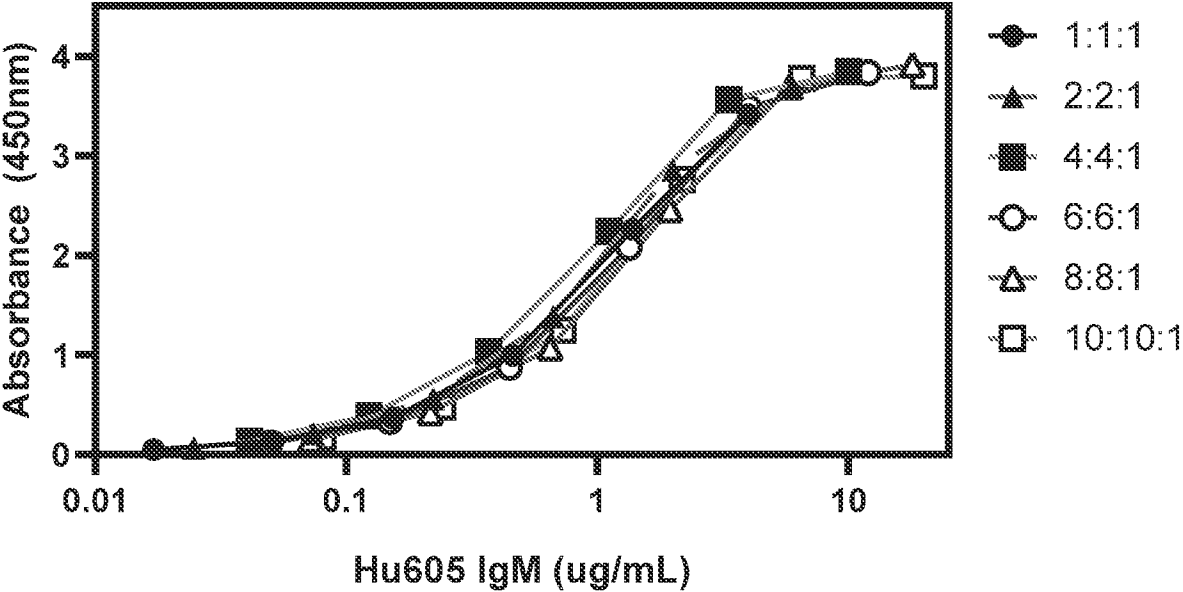


FIG. 4E

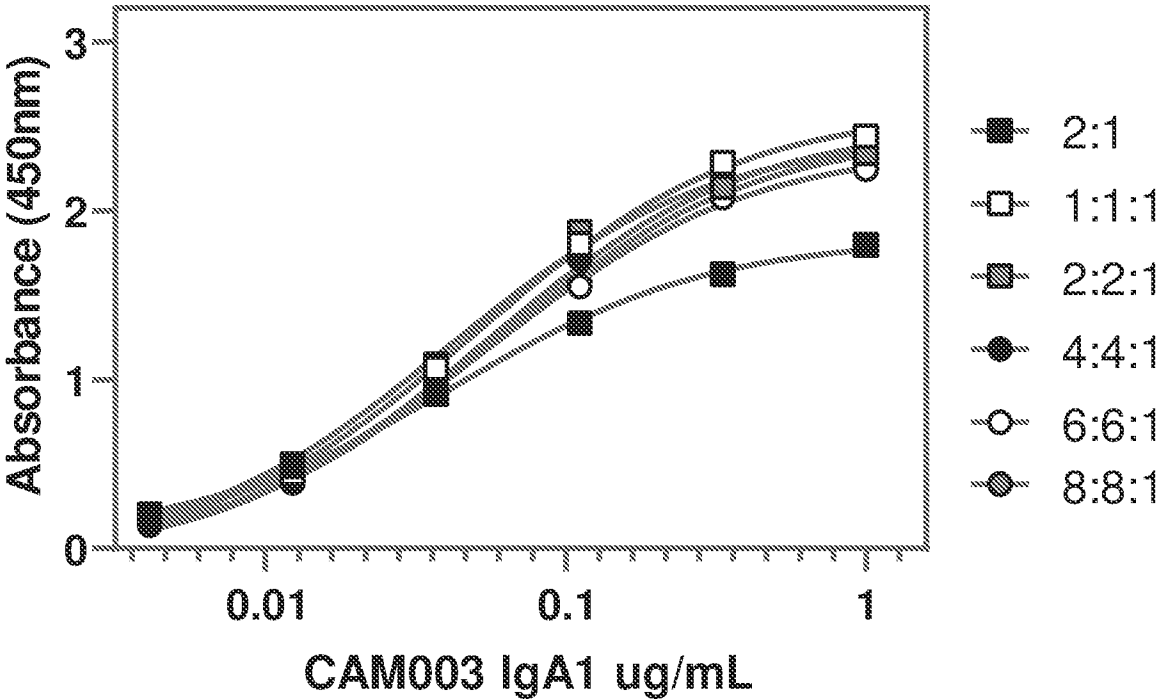


FIG. 5A

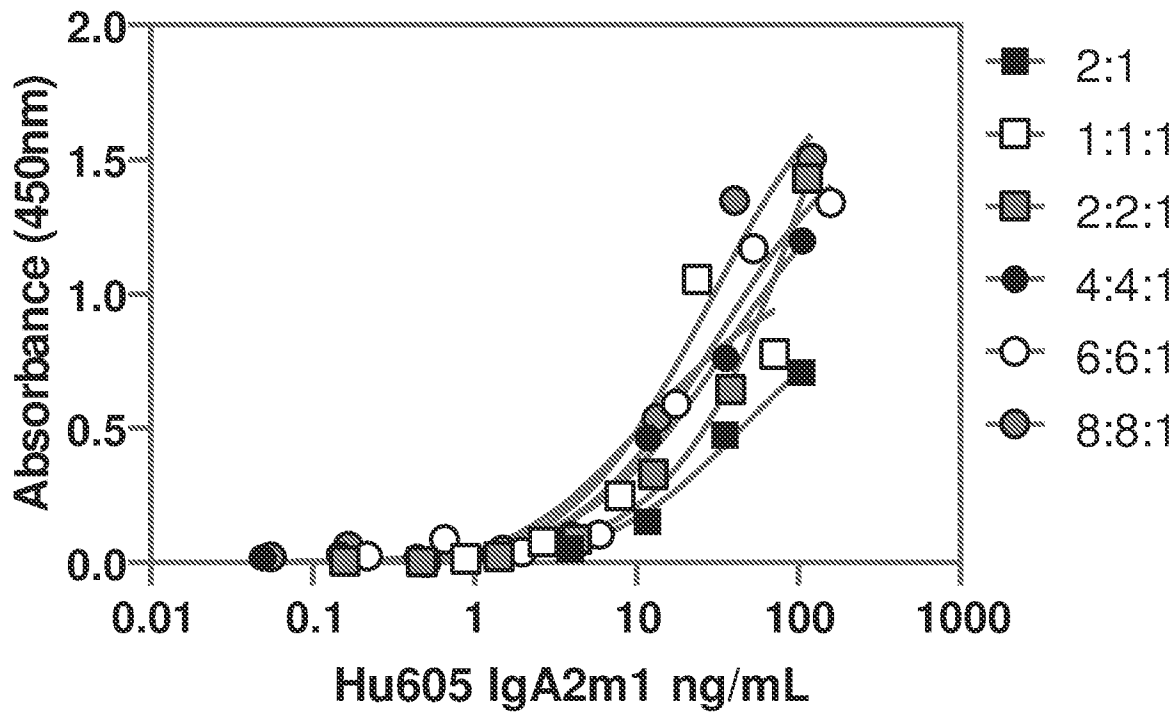


FIG. 5B

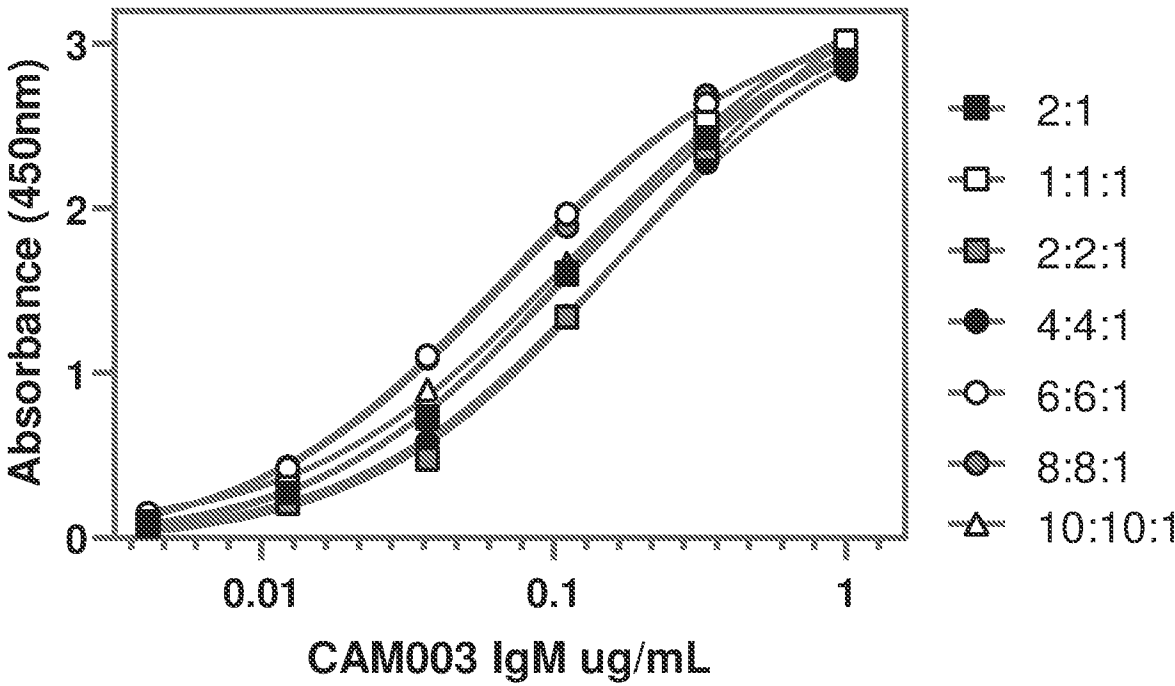


FIG. 5C

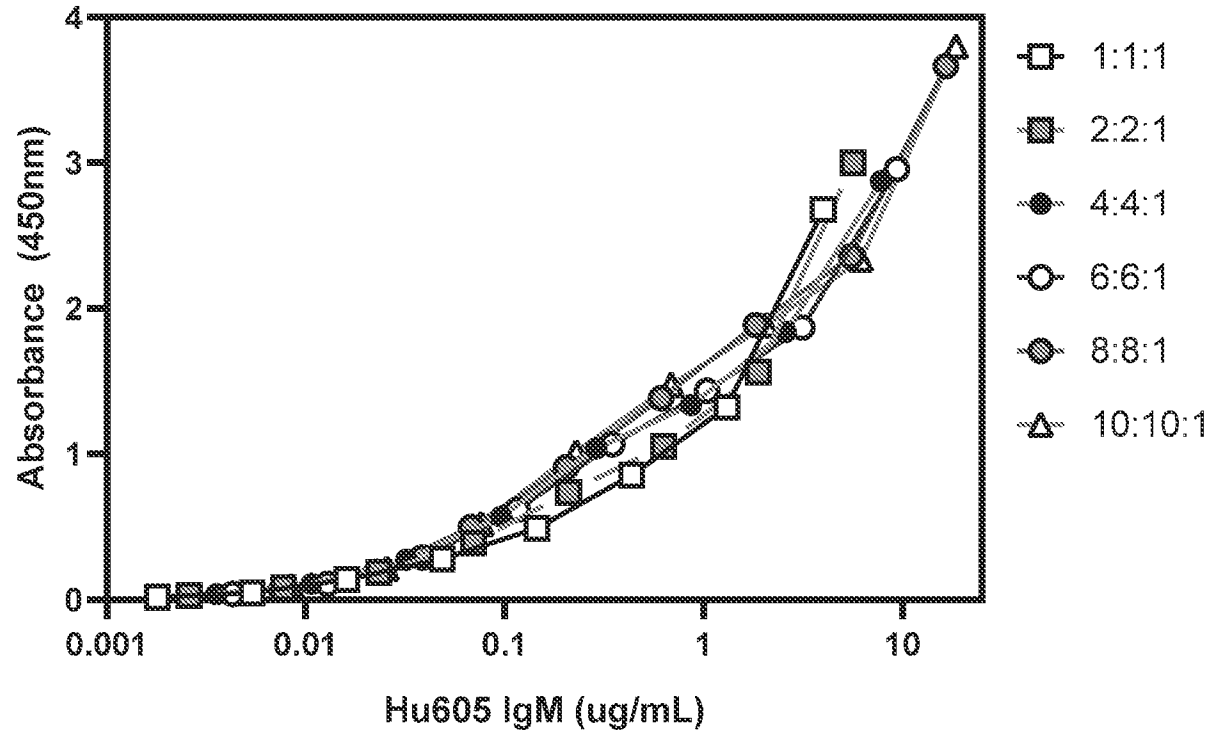


FIG. 5D

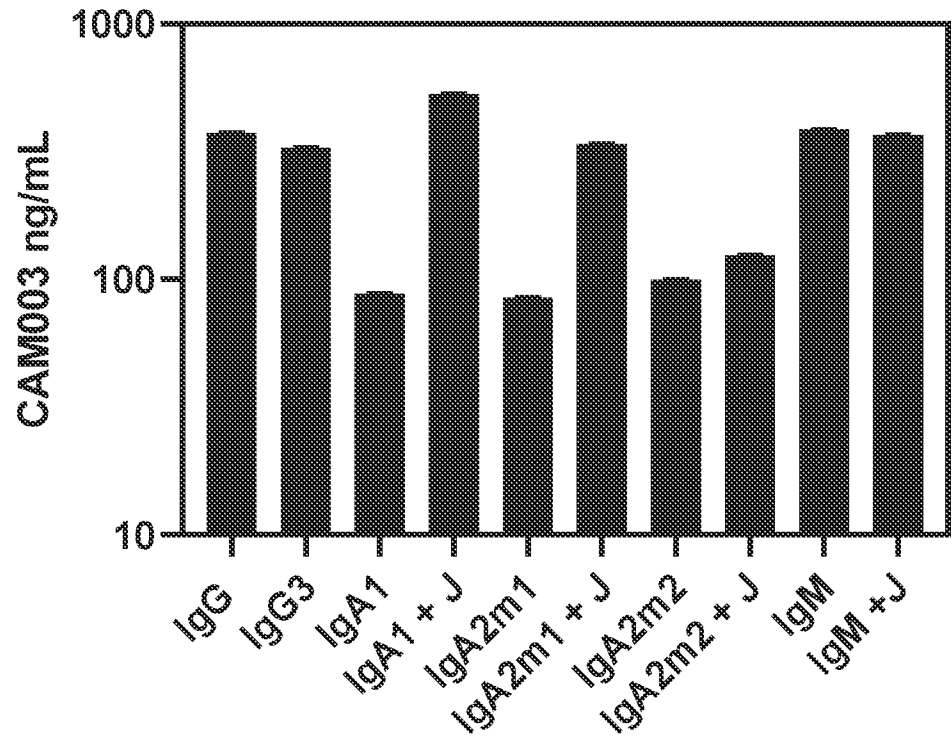


FIG. 6

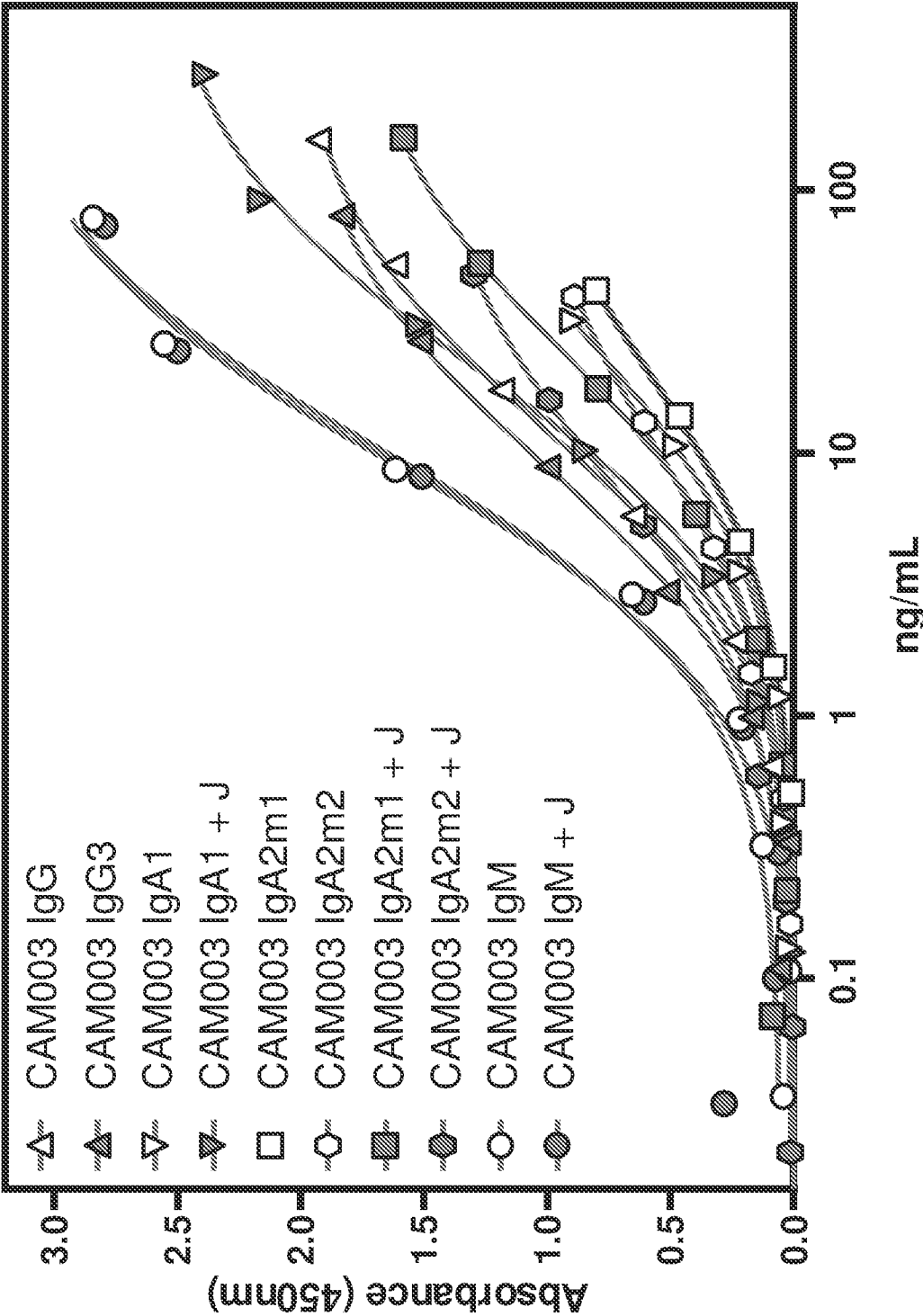


FIG. 7

10/23

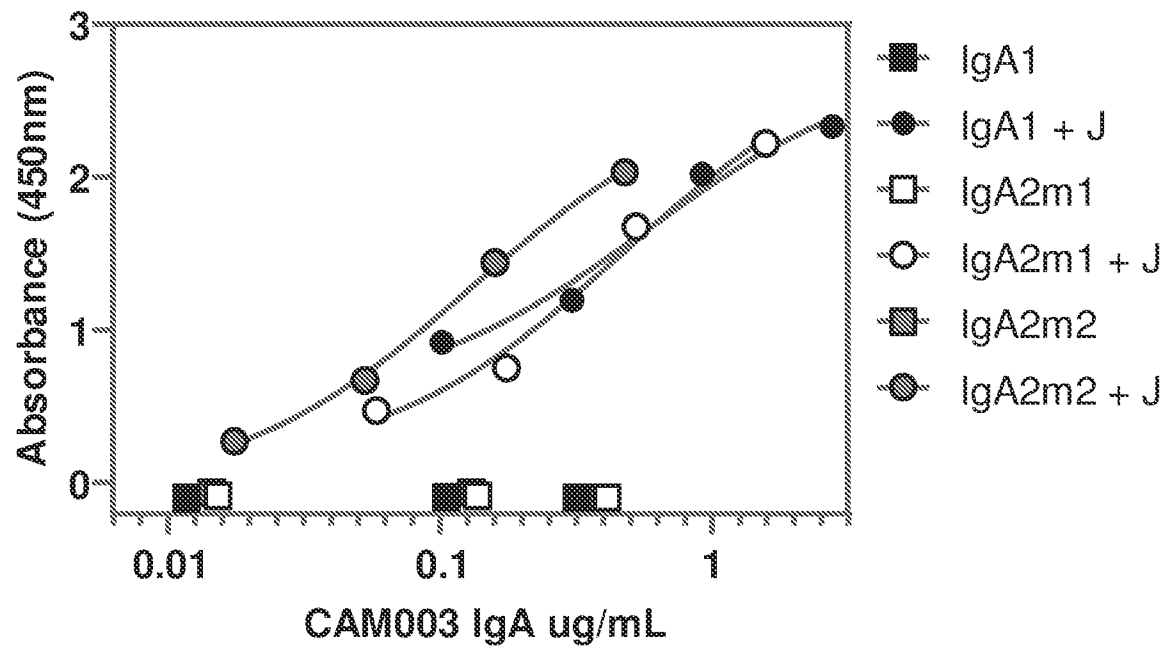


FIG. 8A

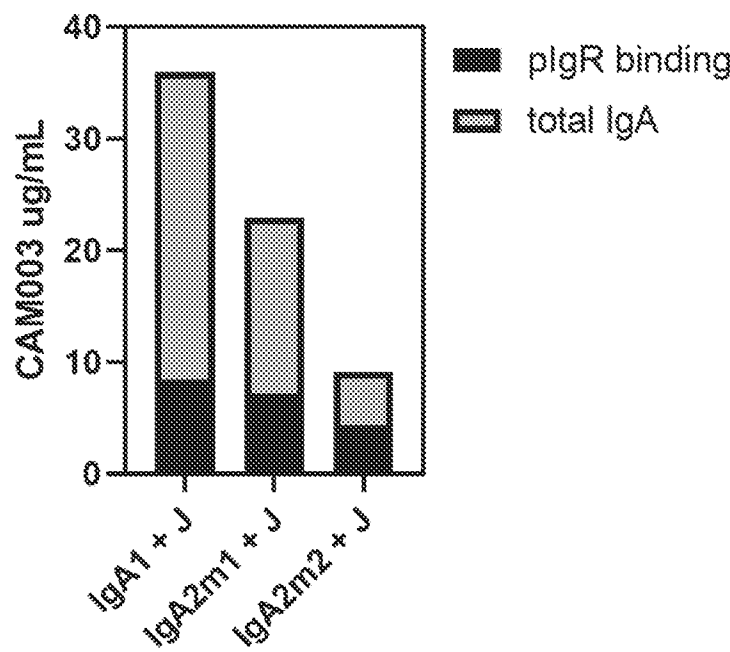


FIG. 8B

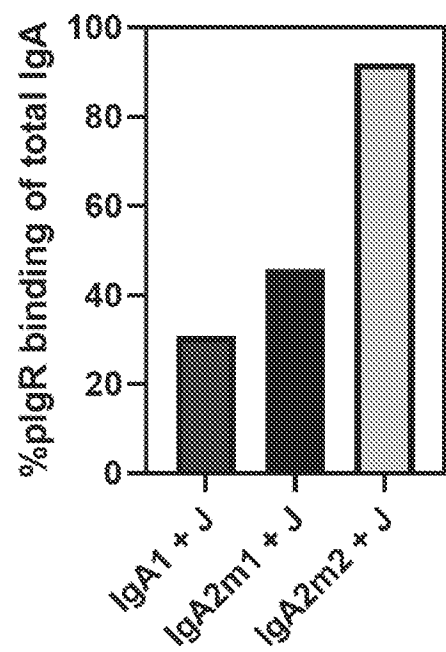


FIG. 8C

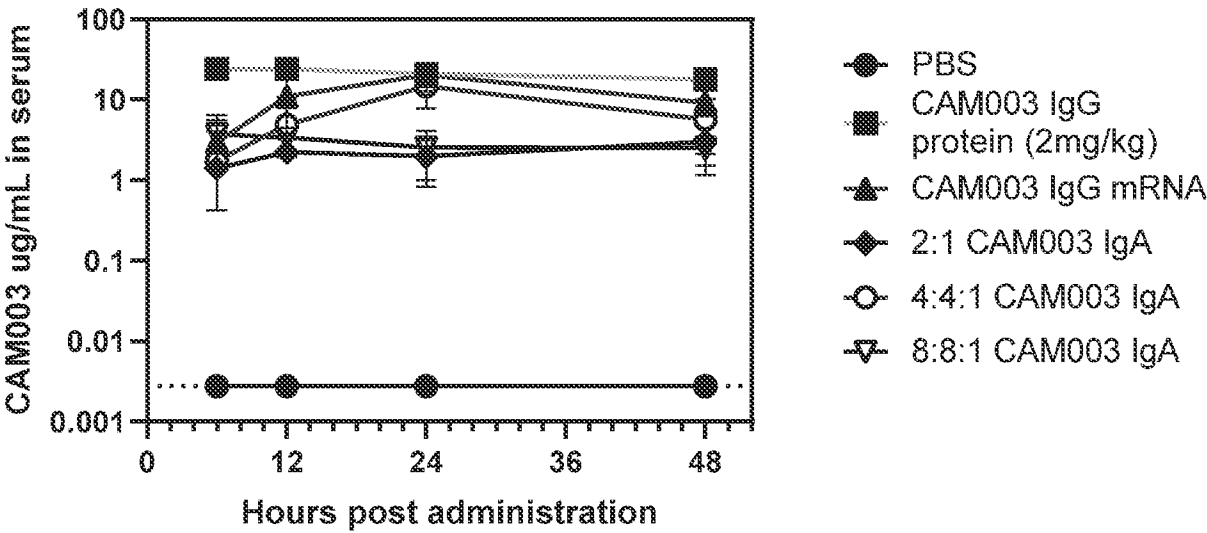


FIG. 9

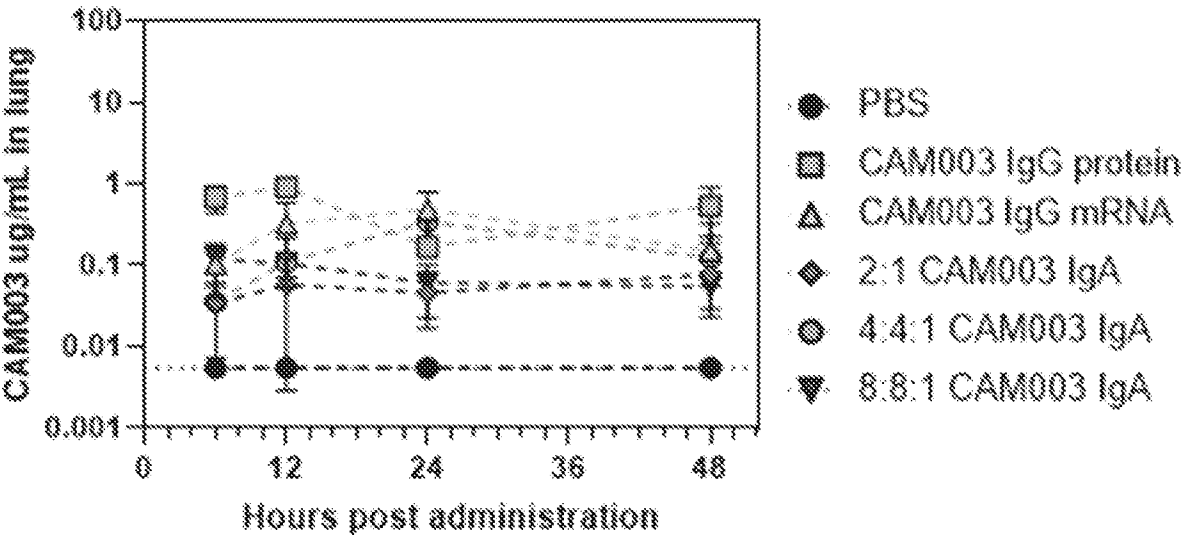


FIG. 10A

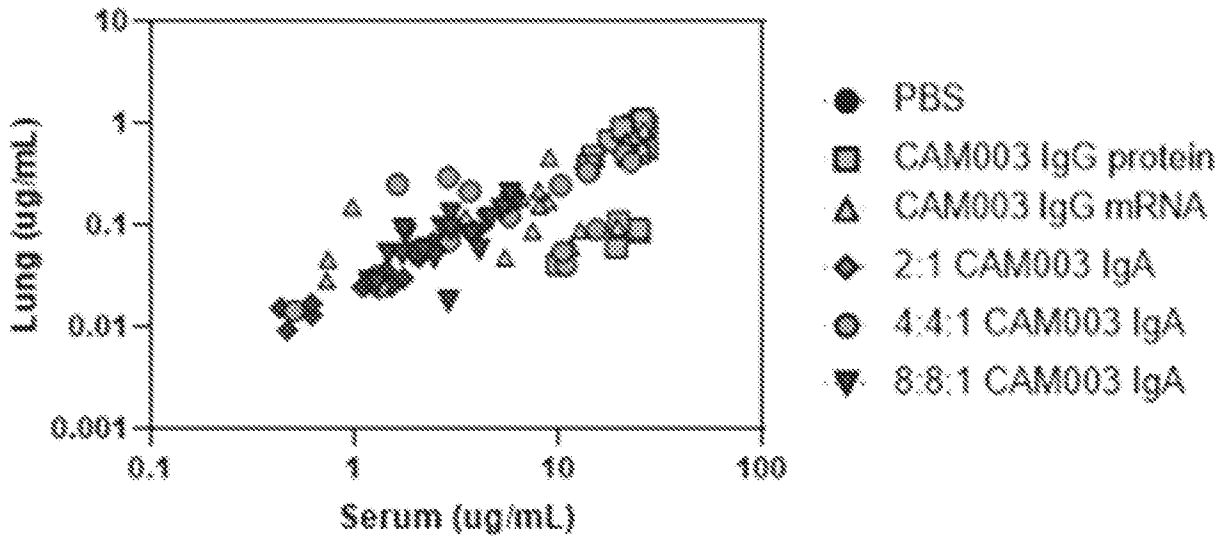


FIG. 10B

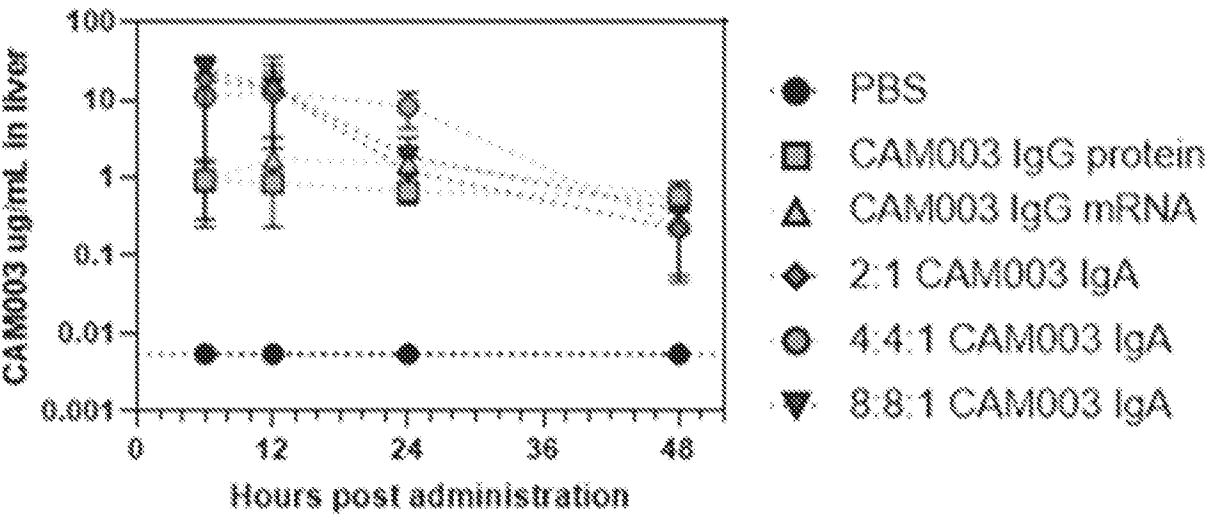


FIG. 11

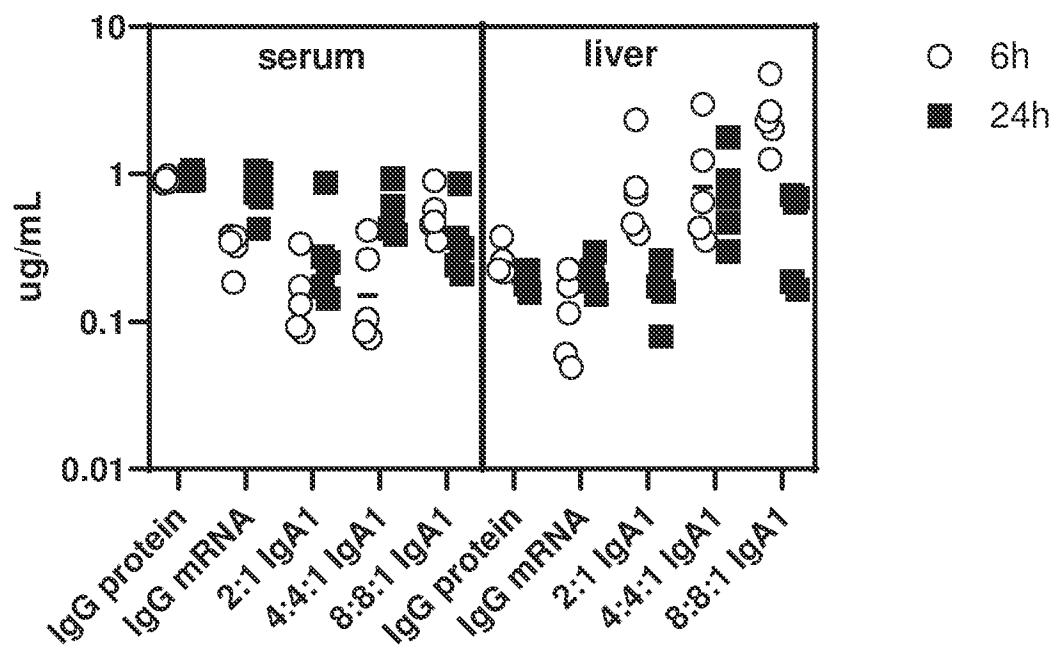


FIG. 12

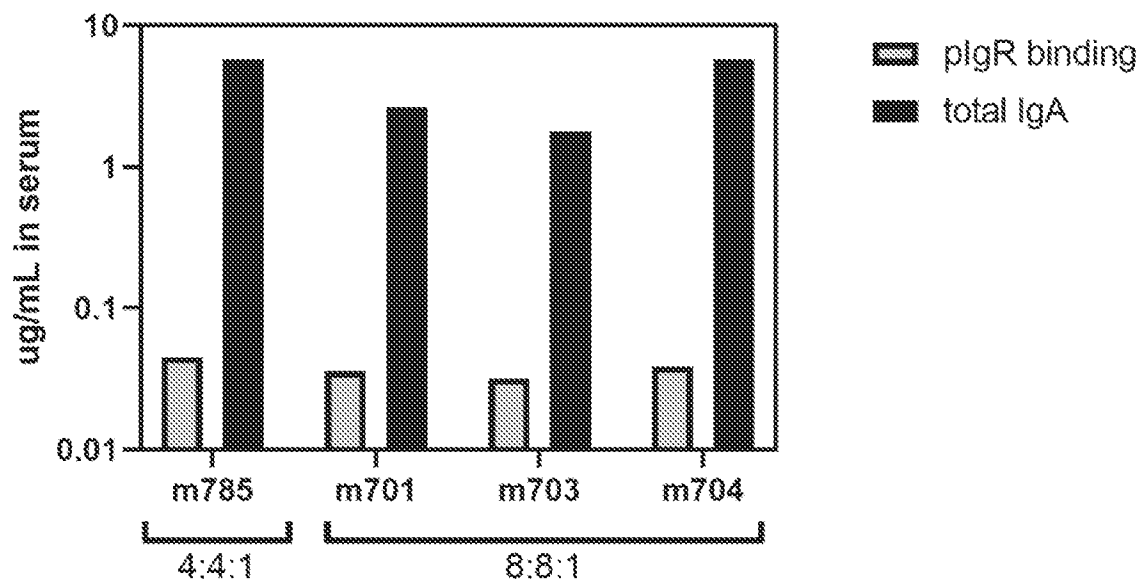


FIG. 13

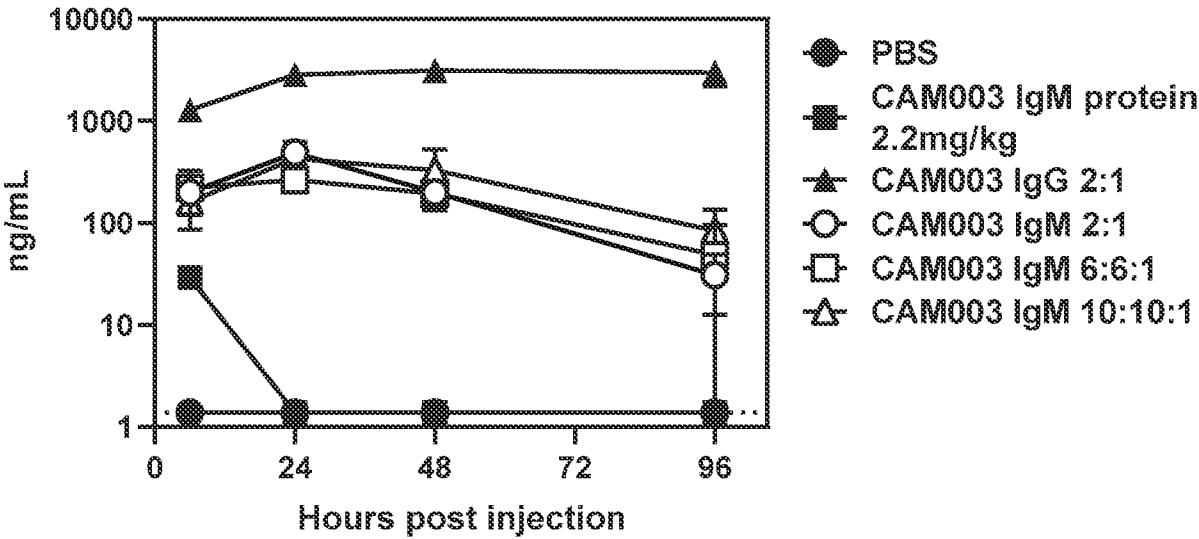


FIG. 14

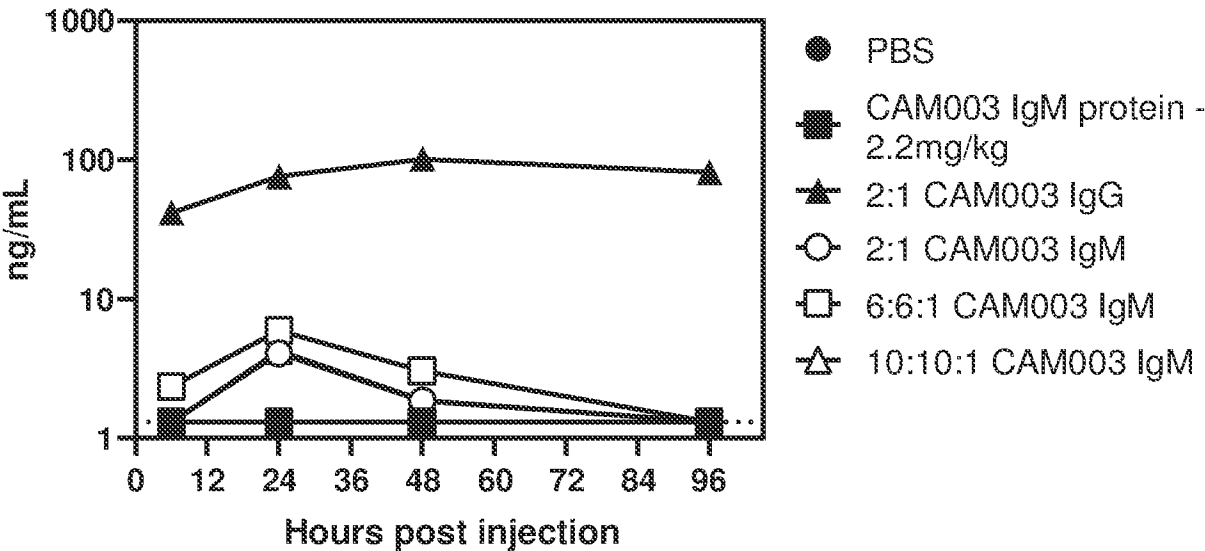


FIG. 15

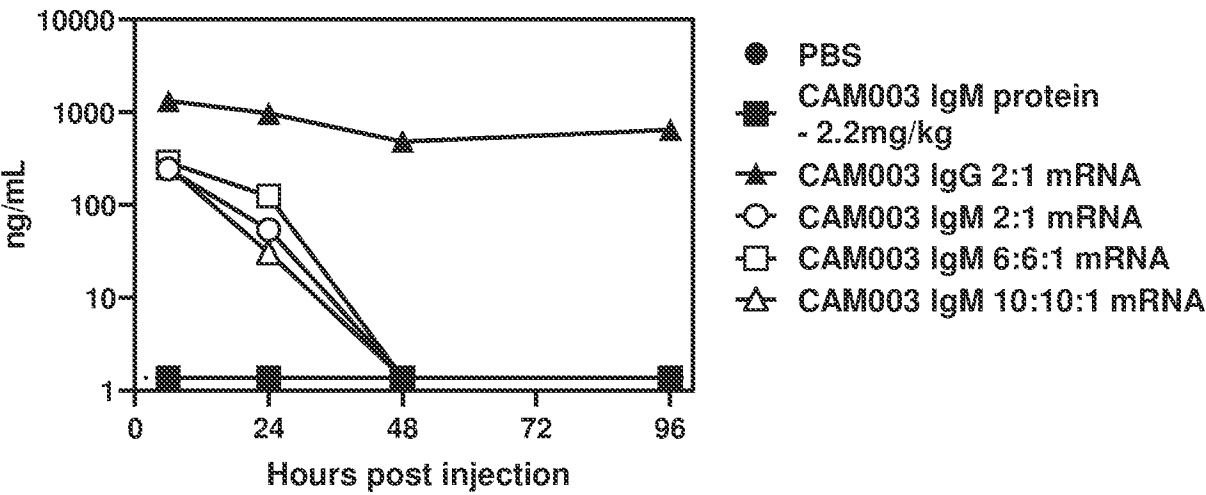


FIG. 16

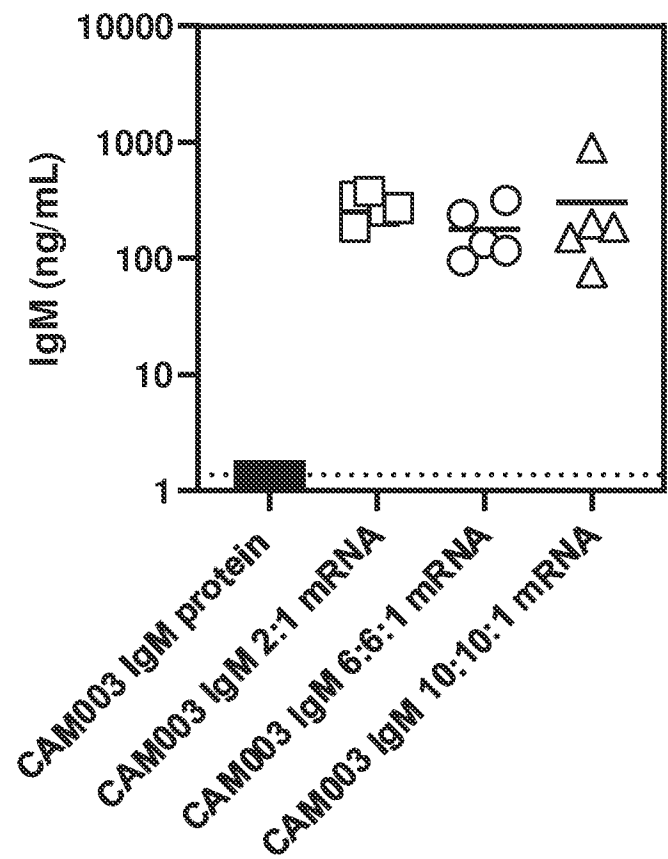


FIG. 17

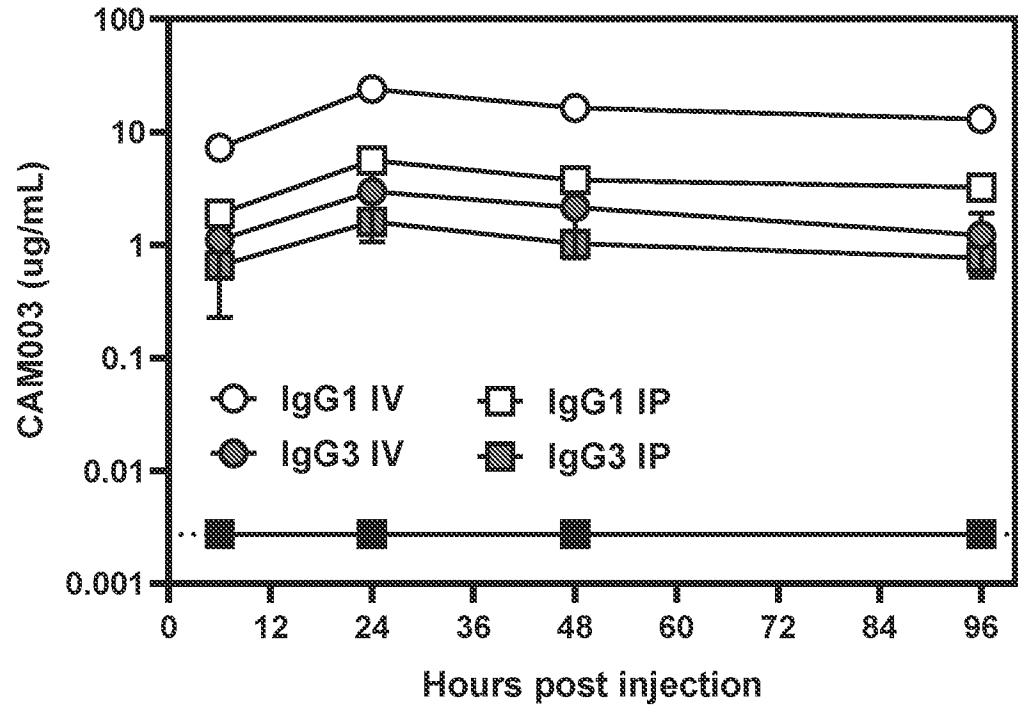


FIG. 18

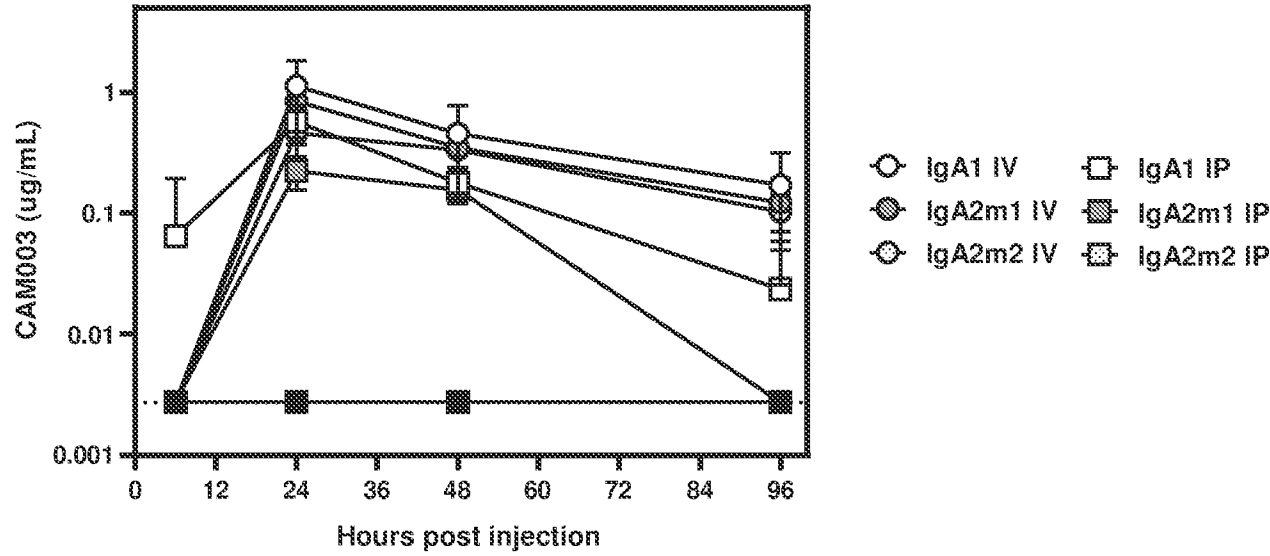


FIG. 19

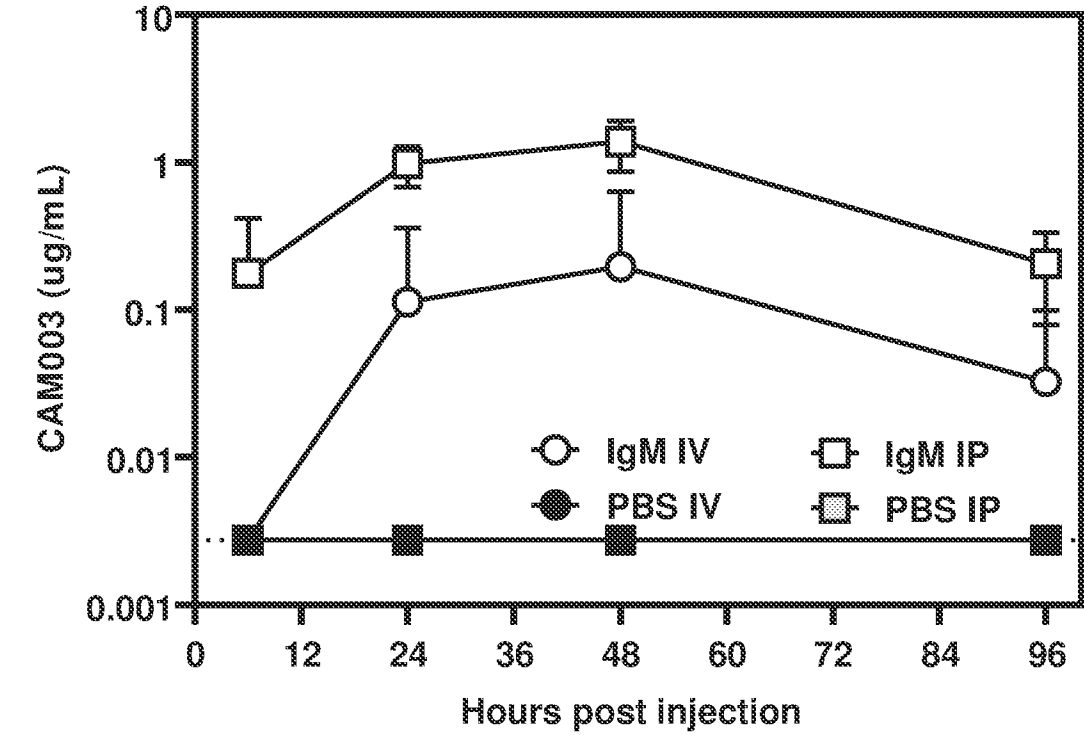


FIG. 20

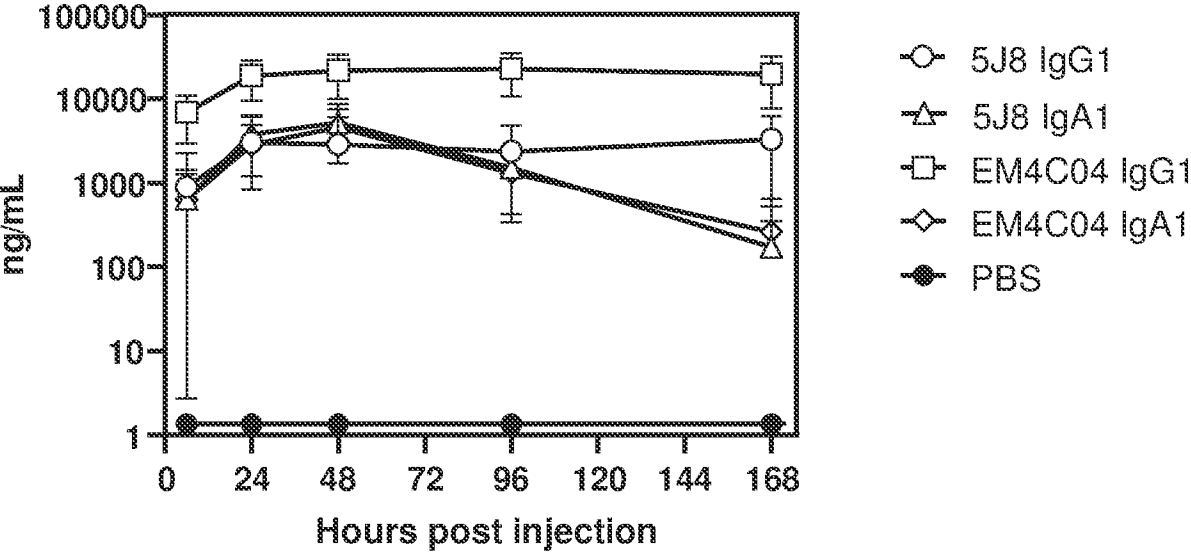


FIG. 21

18/23

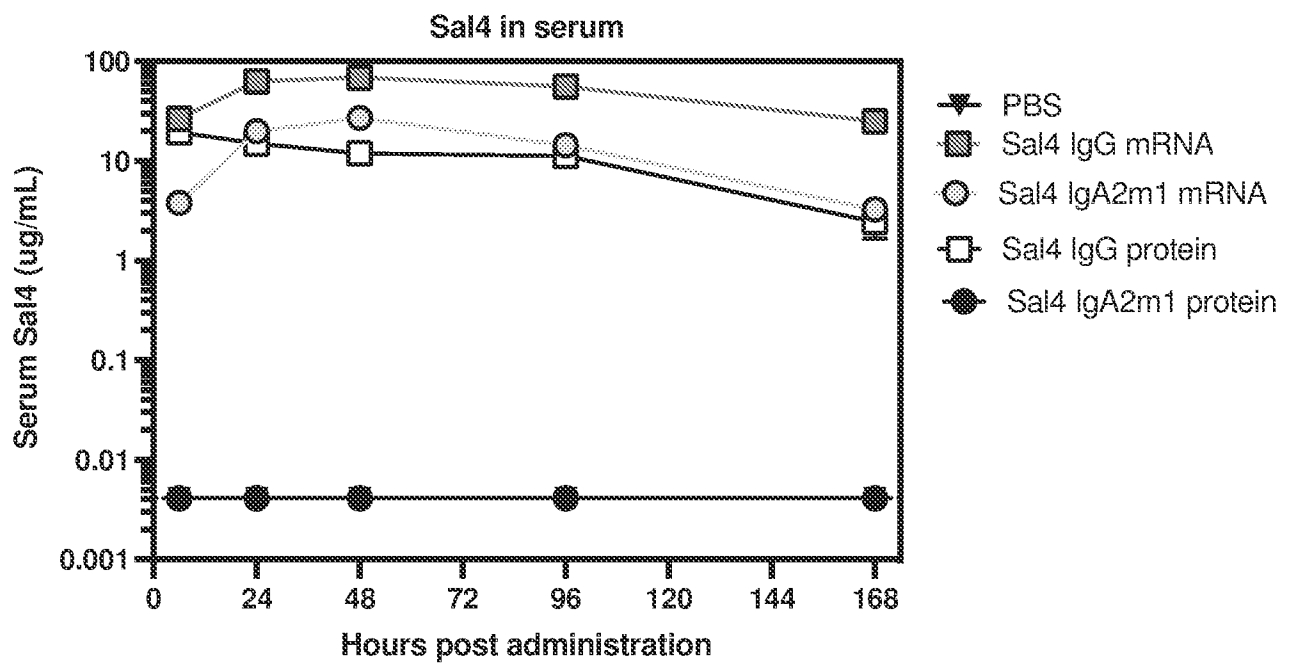


FIG. 22

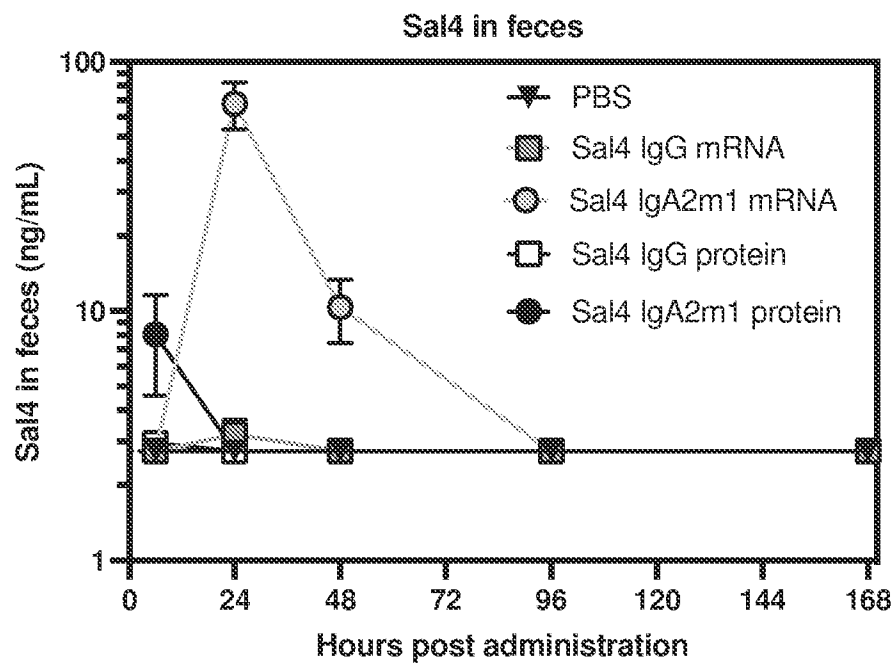


FIG. 23

19/23



FIG. 24

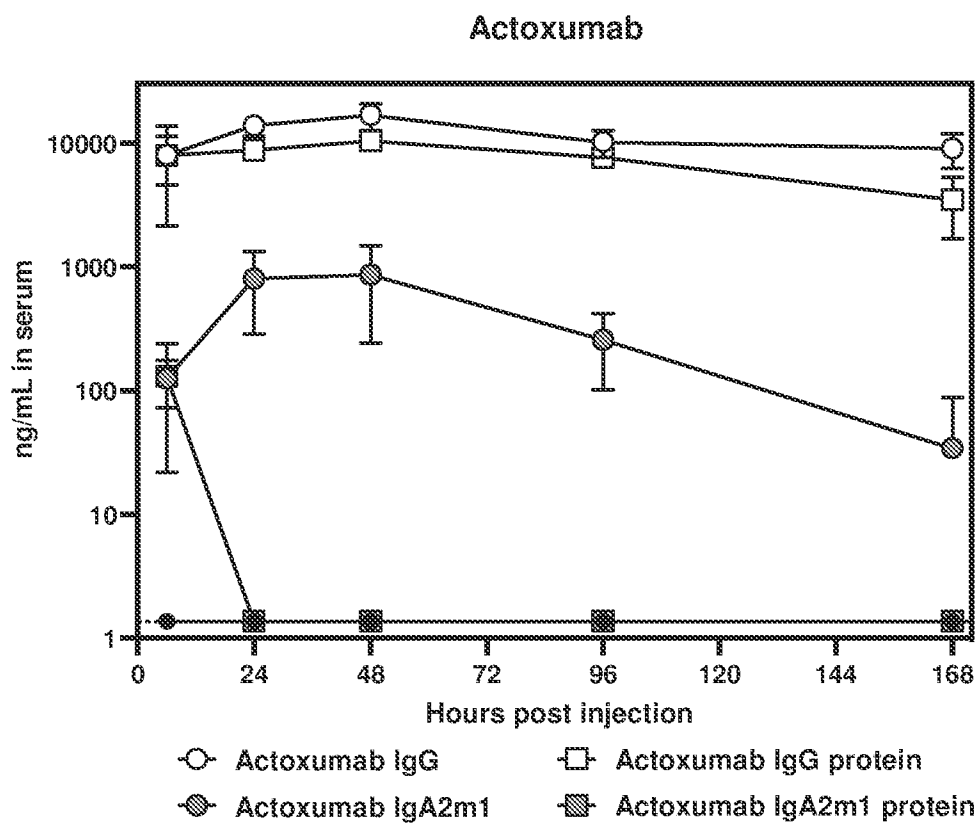


FIG. 25

20/23

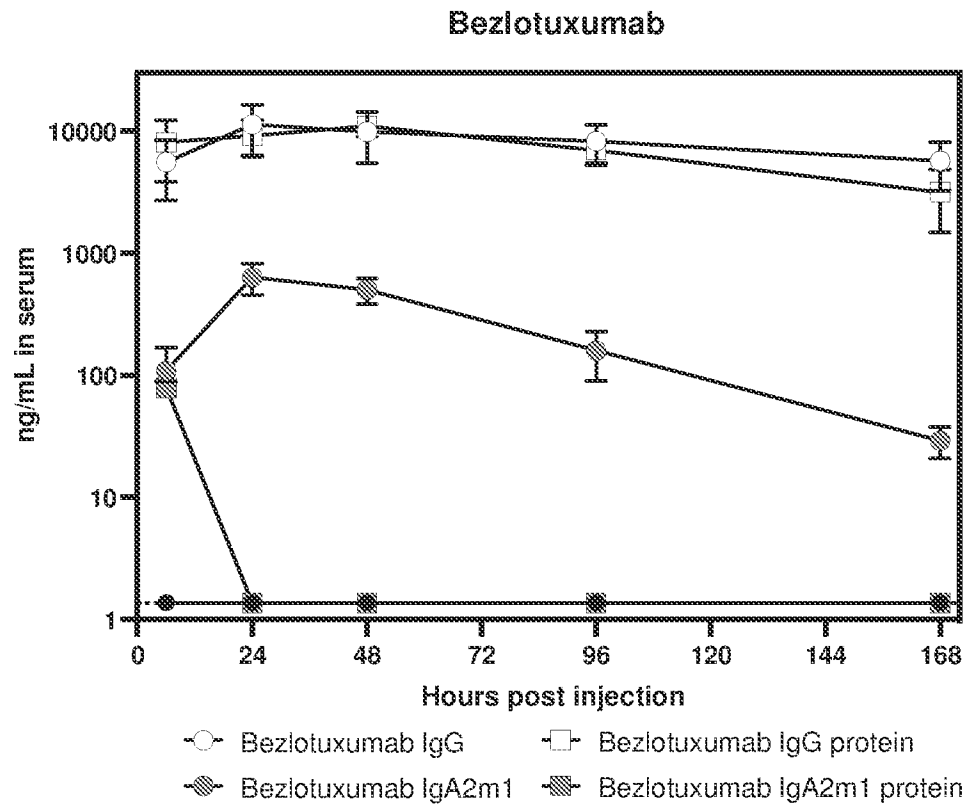


FIG. 26

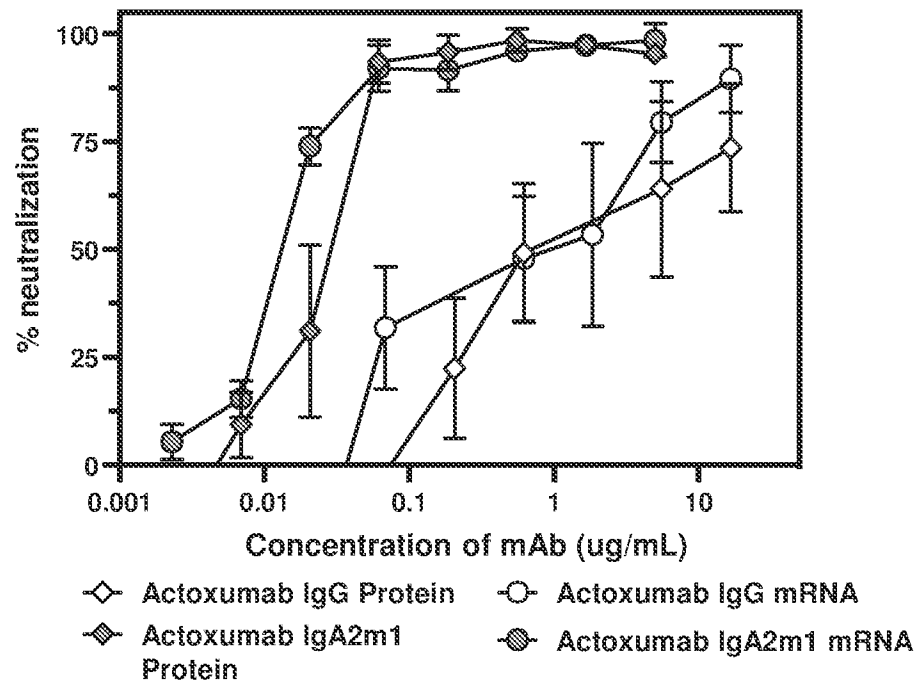


FIG. 27

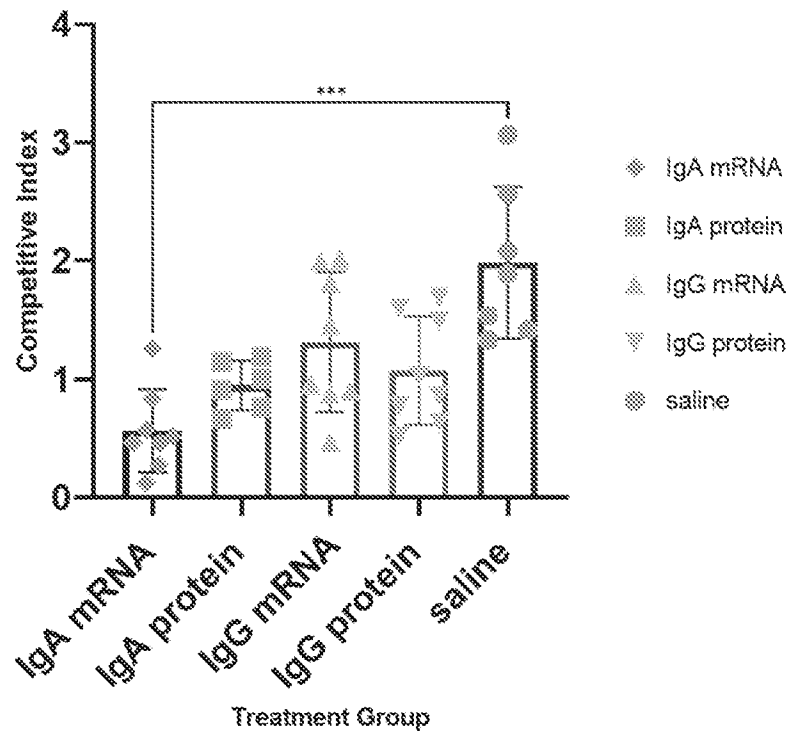


FIG. 28

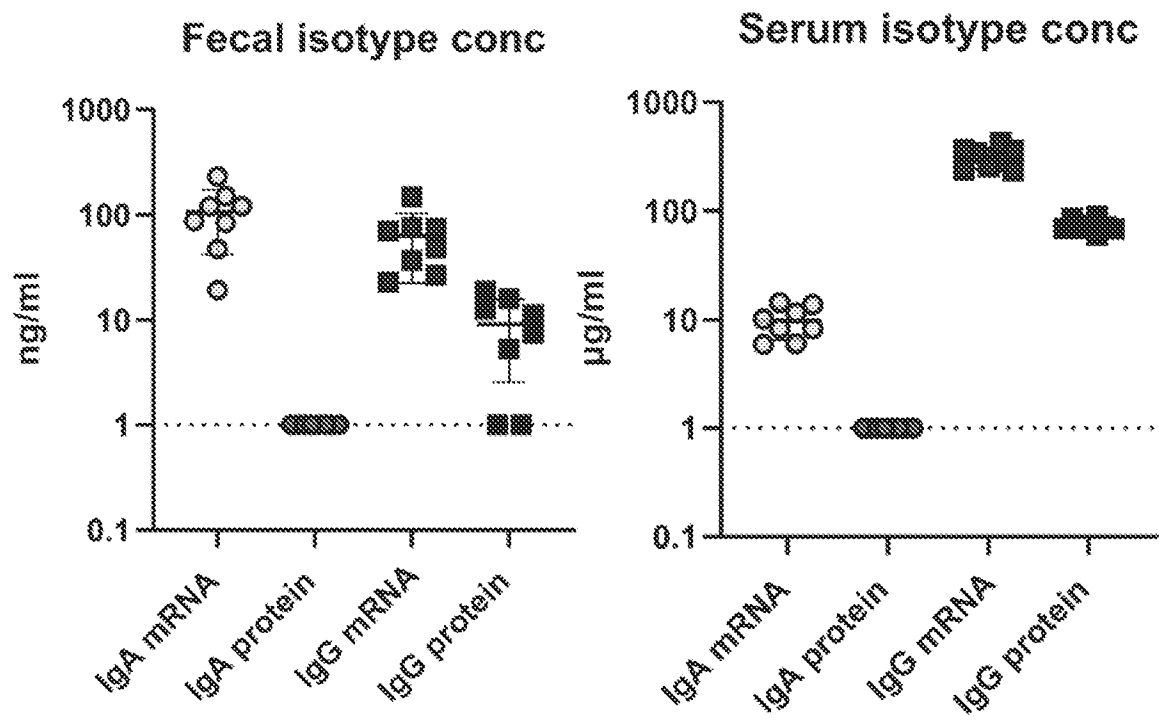


FIG. 29

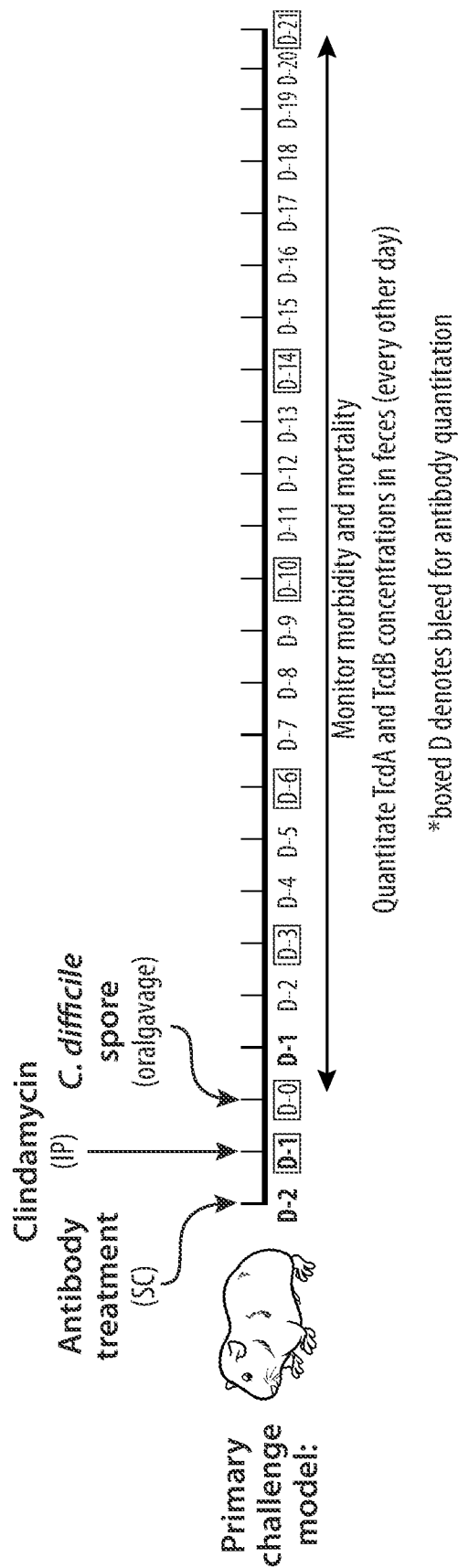


FIG. 30

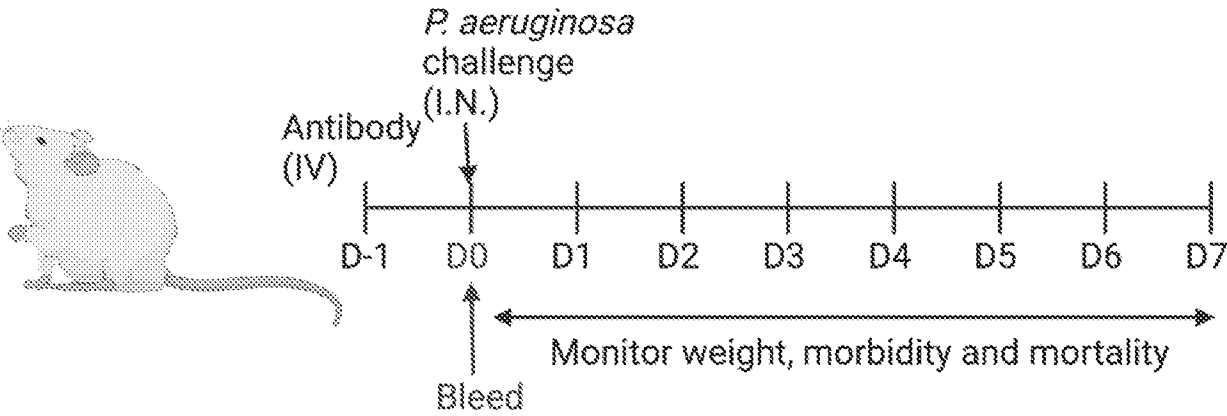


FIG. 31

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2022/021895

A. CLASSIFICATION OF SUBJECT MATTER

C07K 16/12 (2006.01) A61K 9/127 (2006.01) A61K 31/7105 (2006.01) A61K 39/40 (2006.01) A61K 39/42 (2006.01)
A61K 47/14 (2017.01) A61K 47/24 (2006.01) A61P 31/00 (2006.01) A61P 35/00 (2006.01) C07K 16/08 (2006.01)
C07K 16/30 (2006.01) C12N 15/88 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DATABASES: Epoque - MEDLINE, EPODOC, WPIAP, and STN - CAPLUS, EMBASE, BIOSIS, ClinicalTrials.gov with KEYWORDS: mRNA, IgA, IgM, antibody and like terms. GENOMEQUEST (all protein databases) search for SEQ ID NOS 10-1 at 90% homology. Applicant and/or Inventor searches of the patent and non-patent literature was performed in DOCDB and DWPI, PubMed, and in internal databases provided by IP Australia. MODERNATX; DEAL, C.; PLANTE, O.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

☒ Further documents are listed in the continuation of Box C☒ See patent family annex

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"D" document cited by the applicant in the international application	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
21 June 2022

Date of mailing of the international search report
21 June 2022

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
Email address: pct@ipaustalia.gov.au

Authorised officer

Johanna Lowery
AUSTRALIAN PATENT OFFICE
(ISO 9001 Quality Certified Service)
Telephone No. +61262832968

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/US2022/021895
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/148247 A2 (SHIRE HUMAN GENETIC THERAPIES, INC.) 01 October 2015 Claims, [0060]	1-65
X	WO 2018/232355 A1 (MODERNATX, INC.) 20 December 2018 Claims, page 50, page 165, compound 18.	1-65
X	WO 2021/055765 A2 (IGM BIOSCIENCES, INC.) 25 March 2021 Claims	1-65
X	TCHOUDAKOVA, A. et al "High level expression of functional human IgMs in human PER.C6® cells." MABS, 1:2, page 163-171. March/April 2009. Abstract	1-16, 21-29, 41-47 and 52-56
X	US 2011/0003336 A1 (VIDARSSON et al) 06 January 2011 claims	41-47 and 52-55
X	SEIJI SAITO et al "A stable engineered human IgG3 antibody with decreased aggregation during antibody expression and low pH stress". Protein Science, Vol. 28, page 900-909. Published online 22/03/2019. See abstract	41-47 and 52-55
X	WO 2017011773 A2 (MODERNATX, INC.) 19 January 2017 Claims, [0131]	1-65

Form PCT/ISA/210 (fifth sheet) (July 2019)

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
☒ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:
SEQ ID NO10 and SEQ ID NO 11 were used for search purposes

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/US2022/021895	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
WO 2015/148247 A2	01 October 2015	None	
WO 2018/232355 A1	20 December 2018	WO 2018232355 A1	20 Dec 2018
WO 2021/055765 A2	25 March 2021	WO 2021055765 A2	25 Mar 2021
		AU 2020351205 A1	14 Apr 2022
		CA 3151237 A1	25 Mar 2021
		CN 114616031 A	10 Jun 2022
US 2011/0003336 A1	06 January 2011	US 2011003336 A1	06 Jan 2011
		US 8426141 B2	23 Apr 2013
		AU 2008347438 A1	23 Jul 2009
		AU 2008347438 B2	29 Aug 2013
		CA 2710439 A1	23 Jul 2009
		EP 2229409 A1	22 Sep 2010
		EP 2229409 B1	26 Sep 2012
		JP 2011509951 A	31 Mar 2011
		JP 5406213 B2	05 Feb 2014
		WO 2009089846 A1	23 Jul 2009
WO 2017011773 A2	19 January 2017	WO 2017011773 A2	19 Jan 2017
End of Annex			
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2019)			