Title: MODIFIED NUCLEOSIDE, NUCLEOTIDE, AND NUCLEIC ACID COMPOSITIONS

Abstract:
The present disclosure provides, inter alia, formulation compositions comprising modified nucleic acid molecules which may encode a protein, a protein precursor, or a partially or fully processed form of the protein or a protein precursor. The formulation composition may further include a modified nucleic acid molecule and a delivery agent. The present invention further provides nucleic acids useful for encoding polypeptides capable of modulating a cell's function and/or activity.
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[Continued on next page]

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NOTE POUR LE TOME / VOLUME NOTE:
MODIFIED NUCLEOSIDE, NUCLEOTIDE, AND NUCLEIC ACID COMPOSITIONS

REFERENCE TO SEQUENCE LISTING
[0001] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing file, entitled M11PCTSQLST.txt, was created on December 14, 2012 and is 25,579 bytes in size. The information in electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

CROSS REFERENCE TO RELATED APPLICATIONS

BACKGROUND OF THE INVENTION
[0003] In general, exogenous unmodified nucleic acid molecules, particularly viral nucleic acids, introduced into the cell induce an innate immune response which results in cytokine and interferon (IFN) production and ultimately cell death. It is of great interest for therapeutics, diagnostics, reagents and for biological assays to be able to deliver a nucleic acid, e.g., a ribonucleic acid (RNA), into a cell, such as to cause intracellular translation of the nucleic acid and production of the encoded
protein instead of generating an innate immune response. Thus, there is a need to develop formulation compositions comprising a delivery agent that can effectively facilitate the in vivo delivery of nucleic acids to targeted cells without generating an innate immune response.

SUMMARY OF THE INVENTION

[0004] The present disclosure provides, inter alia, formulation compositions comprising modified nucleic acid molecules which may encode a protein, a protein precursor, or a partially or fully processed form of the protein or a protein precursor. The formulation compositions may further include a modified nucleic acid molecule and a delivery agent. The present invention further provides nucleic acids useful for encoding polypeptides capable of modulating a cell’s function and/or activity.

[0005] In one aspect a method of producing a polypeptide of interest in a mammalian cell or tissue is described. The method comprises contacting the mammalian cell or tissue with a formulation comprising a modified mRNA encoding a polypeptide of interest. The formulation may be, but is not limited to, nanoparticles, poly(lactic-co-glycolic acid)(PLGA) microspheres, lipidoids, lipoplex, liposome, polymers, carbohydrates (including simple sugars), cationic lipids, fibrin gel, fibrin hydrogel, fibrin glue, fibrin sealant, fibrinogen, thrombin, rapidly eliminated lipid nanoparticles (reLNPs) and combinations thereof. The modified mRNA may comprise a purified IVT transcript.

[0006] In one embodiment, the formulation comprising the modified mRNA is a nanoparticle which may comprise at least one lipid. The lipid may be selected from, but is not limited to, DLinDMA, DLin-K-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, PLGA, PEG, PEG-DMG and PEGylated lipids. In another aspect, the lipid may be a cationic lipid such as, but not limited to, DLin-DMA, DLin-D-DMA, DLin-MC3-DMA, DLin-KC2-DMA and DODMA.

[0007] The lipid to modified mRNA ration in the formulation may be between 10:1 and 30:1. The mean size of the nanoparticle formulation may comprise the modified mRNA between 60 and 225 nm. The PDI of the nanoparticle formulation comprising the modified mRNA is between 0.03 and 0.15. The zeta potential of the lipid may be from -10 to +10 at a pH of 7.4

[0008] The formulations of modified mRNA may comprise a fusogenic lipid, cholesterol and a PEG lipid. The formulation may have a molar ratio 50:10:38.5:1.5-3.0 (cationic lipid:fusogenic lipid: cholesterol: PEG lipid). The PEG lipid may be selected from, but is not limited to PEG-c-DOMG, PEG-DMG. The fusogenic lipid may be DSPC.
[0009] The mammalian cell or tissue may be contacted using a device such as, but not limited to, a syringe pump, internal osmotic pump and external osmotic pump.

[0010] The formulation of modified mRNA may be a PLGA microsphere which may be between 4 and 20 μm in size. The modified mRNA may be released from the formulation at less than 50% in a 48 hour time period. The PLGA microsphere formulation may be stable in serum. Stability may be determined relative to unformulated modified mRNA in 90%.

[0011] The loading weight percent of the modified mRNA PLGA microsphere may be at least 0.05%, at least 0.1%, at least 0.2%, at least 0.3%, at least 0.4% or at least 0.5%. The encapsulation efficiency of the modified mRNA in the PLGA microsphere may be at least 50%, at least 70%, at least 90% or at least 97%.

[0012] A lipid nanoparticle of the present invention may be formulated in a sealant such as, but not limited to, a fibrin sealant.

[0013] The mammalian cells or tissues may be contacted by a route of administration such as, but not limited to, intravenous, intramuscular, intravitreal, intrathecal, intratumoral, pulmonary and subcutaneous. The mammalian cells or tissues may be contacted using a split dosing schedule.

The mammalian cell or tissue may be contacted by injection. The injection may be made to tissue selected from the group consisting of intradermal space, epidermis, subcutaneous tissue and muscle. The polypeptide of interest may be produced in the cell or tissue in a location systemic from the location of contacting.

[0014] The polypeptide of interest may be detectable in serum for up to 72 hours after contacting. The level of the polypeptide of interest can be higher than the levels prior to dosing. The level of the polypeptide of interest may be greater in the serum of female subjects than in the serum of male subjects.

[0015] The formulation of modified mRNA may comprise more than one modified mRNA. The formulation may have two or three modified mRNA.

[0016] The formulation comprising the modified mRNA may comprise a rapidly eliminated lipid nanoparticle (reLNP) which may comprise a reLNP lipid, fusogenic lipid, cholesterol and a PEG lipid at a molar ratio of 50: 10: 38.5: 1.5 (reLNP lipid:fusogenic lipid: cholesterol: PEG lipid). The fusogenic lipid may be DSPC and the PEG lipid may be PEG-c-DOMG. The reLNP lipid may be DLin-DMA with an internal or terminal ester or DLin-MC3-DMA with an internal or terminal ester. The total lipid to modified mRNA weight ratio may be between 10:1 and 30:1.
[0017] The formulation comprising modified mRNA may comprise a fibrin sealant.

[0018] The formulation comprising modified mRNA may comprise a lipidoid where the lipid is selected from the group consisting of C12-200 and 98N12-5.

[0019] The formulation comprising modified mRNA may include a polymer. The polymer may be coated, covered, surrounded, enclosed or comprise a layer of a hydrogel or surgical sealant. The polymer may be selected from the group consisting of PLGA, ethylene vinyl acetate, poloxamer and GELSITE®.

[0020] A polypeptide of interest may be produced in a mammalian cell or tissue by contacting the mammalian cell or tissue with a buffer formulation comprising a modified mRNA encoding the polypeptide of interest. The buffer formulation may be selected from, but is not limited to, saline, phosphate buffered saline and Ringer’s lactate. The buffer formulation may comprise a calcium concentration of between 1 to 10 mM. The modified mRNA in the buffer formulation may comprise a purified IVT transcript.

[0021] A pharmacologic effect in a primate may be produced by contacting the primate with a composition comprising a formulated modified mRNA encoding a polypeptide of interest. The modified mRNA may comprise a purified IVT transcript and/or may be formulated in nanoparticles, poly(lactic-co-glycolic acid)(PLGA) microspheres, lipidoids, lipoplex, liposome, polymers, carbohydrates (including simple sugars), cationic lipids, fibrin gel, fibrin hydrogel, fibrin glue, fibrin sealant, fibrinogen, thrombin, rapidly eliminated lipid nanoparticles (reLNPs) and combinations thereof. The pharmacological effect may be greater than the pharmacologic effect associated with a therapeutic agent and/or composition known to produce said pharmacologic effect. The composition may comprise a formulated or unformulated modified mRNA. The pharmacologic effect may result in a therapeutically effective outcome of a disease, disorder, condition or infection. Such therapeutically effective outcome may include, but is not limited to, treatment, improvement of one or more symptoms, diagnosis, prevention, and delay of onset. The pharmacologic effect may include, but is not limited to, change in cell count, alteration in serum chemistry, alteration of enzyme activity, increase in hemoglobin, and increase in hematocrit.

[0022] In one embodiment, the present disclosure provides a formulation composition which comprises a modified nucleic acid molecule and a delivery agent. The modified nucleic acid molecule may be selected from the group consisting of DNA, complimentary DNA (cDNA), RNA, messenger RNA (mRNA), RNAi-inducing agents, RNAi agents, siRNA, shRNA, miRNA, antisense
RNA, ribozymes, catalytic DNA, RNA that induce triple helix formation, aptamers, vectors and combinations thereof. If the modified nucleic acid molecule is mRNA the mRNA may be derived from cDNA.

[0023] In one embodiment, the modified nucleic acid molecule may comprise at least one modification and a translatable region. In some instances, the modified nucleic acid comprises at least two modifications and a translatable region. The modification may be located on the backbone and/or a nucleoside of the nucleic acid molecule. The modification may be located on both a nucleoside and a backbone linkage.

[0024] In one embodiment, a modification may be located on the backbone linkage of the modified nucleic acid molecule. The backbone linkage may be modified by replacing of one or more oxygen atoms. The modification of the backbone linkage may comprise replacing at least one phosphodiester linkage with a phosphorothioate linkage.

[0025] In one embodiment, a modification may be located on a nucleoside of the modified nucleic acid molecule. The modification on the nucleoside may be located on the sugar of said nucleoside. The modification of the nucleoside may occur at the 2’ position on the nucleoside.

[0026] The nucleoside modification may include a compound selected from the group consisting of pyridin-4-one ribonucleoside, 5-aza-uridine, 2-thio-5-aza-uridine, 2-thiouridine, 4-thio-pseudouridine, 2-thio-pseudouridin, 5-hydroxyuridine, 3-methyluridine, 5-carboxymethyl-uridine, 1-carboxymethyl-pseudouridine, 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-taurinomethyluridine, 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uridine, 1-taurinomethyl-4-thio-uridine, 5-methyl-uridine, 1-methyl-pseudouridine, 4-thio-1-methyl-pseudouridine, 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouridine, dihydrospseudouridine, 2-thio-dihydrouridine, 2-thio-dihydrosseudouridine, 2-methoxyuridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, 5-aza-cytidine, pseudoisocytidine, 3-methyl-cytidine, N4-acetylcytidine, 5-formylecytidine, N4-methylcytidine, 5-hydroxymethylcytidine, 1-methyl-pseudoisocytidine, pyrrolo-cytidine, pyrrolo-pseudoisocytidine, 2-thio-cytidine, 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, zebaraline, 5-aza-zebaraline, methyl-zebaraline, 5-aza-2-thio-zebaraline, 2-thio-zebaraline, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, 2-aminopurine, 2,
6-diaminopurine, 7-deaza-adenine, 7-deaza-8-aza-adenine, 7-deaza-2-aminopurine, 7-deaza-8-aza-2-aminopurine, 7-deaza-2,6-diaminopurine, 7-deaza-8-aza-2,6-diaminopurine, 1-methyladenosine, N6-methyladenosine, N6-isopentenyladenosine, N6-(cis-hydroxyisopentenyl)adenosine, 2-methylthio-N6-(cis-hydroxyisopentenyl) adenosine, N6-glycinylcarnamoyladenosine, N6-threonylcarnamoyladenosine, 2-methylthio-N6-threonyl carbamoyladenosine, N6,N6-dimethyladenosine, 7-methyladenine, 2-methylthio-adenine, 2-methoxy-adenine, inosine, 1-methylinosine, wyosine, wybutosine, 7-deaza-guanosine, 7-deaza-8-aza-guanosine, 6-thio-guanosine, 6-thio-7-deaza-guanosine, 6-thio-7-deaza-8-aza-guanosine, 7-methyl-guanosine, 6-thio-7-methyl-guanosine, 7-methylinosine, 6-methoxy-guanosine, 1-methylguanosine, N2-methylguanosine, N2,N2-dimethylguanosine, 8-oxo-guanosine, 7-methyl-8-oxo-guanosine, 1-methyl-6-thio-guanosine, N2-methyl-6-thio-guanosine, and N2,N2-dimethyl-6-thio-guanosine. In another embodiment, the modifications are independently selected from the group consisting of 5-methylcytosine, pseudouridine and 1-methylpseudouridine.

[0027] In one embodiment, a modification may be located on a nucleobase of the modified nucleic acid molecule. The modification on the nucleobase may be selected from the group consisting of cytosine, guanine, adenine, thymine and uracil. The modification on the nucleobase may be selected from the group consisting of deaza-adenosine and deaza-guanosine, and the linker may be attached at a C-7 or C-8 position of said deaza-adenosine or deaza-guanosine. The modified nucleobase may be selected from the group consisting of cytosine and uracil, and the linker may be attached to the modified nucleobase at an N-3 or C-5 position. The linker attached to the nucleobase may be selected from the group consisting of diethylene glycol, dipropylene glycol, triethylene glycol, tripropylene glycol, tetrathylene glycol, tetaethylene glycol, divalent alkyl, alkenyl, alkynyl moiety, ester, amide, and ether moiety.

[0028] In one embodiment, two modifications of the nucleic acid molecule may be located on nucleosides of the modified nucleic acid molecule. The modified nucleosides may be selected from 5-methylcytosine and pseudouridine.

[0029] In one embodiment, two modifications of the modified nucleic acid molecule may be located on a nucleotide or a nucleoside. In one embodiment, the present disclosure provides a formulation comprising a nucleic acid molecule such as, but not limited to, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9 and SEQ ID NO: 10 and a delivery agent. The nucleic acid molecule may comprise a polyA tail about 160 nucleotides in length. Further, the nucleic acid molecule may
comprise at least one 5’ terminal cap such as, but not limited to, Cap0, Cap1, ARCA, inosine, N1-methyl-guanosine, 2’ fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

[0030] In one embodiment, the present disclosure provides a nucleic acid of SEQ ID NO: 6, a 5’ terminal cap which is Cap1, a poly A tail of approximately 160 nucleotides in length and a delivery agent.

[0031] In one embodiment, the present disclosure provides a nucleic acid of SEQ ID NO: 7, a 5’ terminal cap which is Cap1, a poly A tail of approximately 160 nucleotides in length and a delivery agent.

[0032] In one embodiment, the present disclosure provides a nucleic acid of SEQ ID NO: 9, a 5’ terminal cap which is Cap1, a poly A tail of approximately 160 nucleotides in length and a delivery agent.

[0033] In one embodiment, the present disclosure provides a nucleic acid of SEQ ID NO: 10, a 5’ terminal cap which is Cap1, a poly A tail of approximately 160 nucleotides in length and a delivery agent.

[0034] In one embodiment, the delivery agent comprises at least one method to improve delivery selected from the group consisting of lipidoids, liposomes, lipid nanoparticles, rapidly eliminated lipid nanoparticles (reLNs), polymers, lipoplexes, peptides, proteins, hydrogels, sealants, chemical modifications, conjugation, cells and enhancers. The lipidoid, lipid nanoparticle and rapidly eliminated lipid nanoparticles which may be used as a delivery agent may include a lipid which may be selected from the group consisting of C12-200, MD1, 98N12-5, DLin-DMA, DLin-K-DMA, DLin-KC2-DMA, DLin-MC3-DMA, PLGA, PEG, PEG-DMG, PEGylated lipids and analogs thereof. The rapidly eliminated lipid nanoparticle may have an ester linkage at the terminal end of the lipid chain, or an ester linkage may be an internal linkage located to the right or left of a saturated carbon in the lipid chain. The rapidly eliminated lipid nanoparticle which may be used as a delivery agent may be, but is not limited to, DLin-MC3-DMA and DLin-DMA.

[0035] In one embodiment, the lipid nanoparticle may comprise PEG and at least one component such as, but not limited to, cholesterol, cationic lipid and fusogenic lipid.

[0036] In one embodiment, the lipid nanoparticle may comprise at least one of a PEG, cholesterol, cationic lipid and fusogenic lipid.
In one embodiment, the fusogenic lipid is disteyrylphosphatidylcholine (DSPC). In another embodiment, the PEG lipid is PEG-DMG. In yet another embodiment, the cationic lipid may be, but not limited to, DLin-DMA, DLin-MC3-DMA, C12-200, 98N12-5 and DLin-KC2-DMA.

In one embodiment, the lipid nanoparticle composition may comprise 50 mol% cationic lipid, 10 mol% DSPC, 1.5-3.0 mol% PEG and 37-38.5 mol% cholesterol.

In one embodiment, a modified nucleic acid may be formulated with PLGA to form a sustained release formulation. In another embodiment, a modified nucleic acid may be formulated with PLGA and other active and/or inactive components to form a sustained release formulation. In one embodiment, the modified nucleic acid molecule may include, but is not limited to, SEQ ID NO: 9 and SEQ ID NO: 10.

In one embodiment, a sustained release formulation may comprise a sustained release microsphere. The sustained release microsphere may be about 10 to about 50 μm in diameter. In another embodiment, the sustained release microsphere may contain about 0.001 to about 1.0 weight percent of at least one modified nucleic acid molecule.

In one embodiment, the modified nucleic acids of the present invention may include at least one stop codon before the 3’ untranslated region (UTR). The stop codon may be selected from TGA, TAA and TAG. In one embodiment, the modified nucleic acids of the present invention include the stop codon TGA and one additional stop codon. In a further embodiment the addition stop codon may be TAA. In another embodiment, the modified nucleic acid of the present invention includes three stop codons.

In one embodiment, the present disclosure provides a controlled release formulation comprising a modified nucleic acid which may encode a polypeptide of interest. The modified nucleic acid may be encapsulated or substantially encapsulated in a delivery agent. The delivery agent may be coated, covered, surrounded, enclosed or comprise a layer of polymer, hydrogel and/or surgical sealant. In a further embodiment, the controlled release formulation may comprise a second layer of polymer, hydrogel and/or surgical sealant.

In one embodiment, the delivery agent of the controlled release formulation may include, but is not limited to, lipidoids, liposomes, lipid nanoparticles, rapidly eliminated lipid nanoparticles, lipoplexes and self-assembled lipid nanoparticles.
[0045] The polymer which may be used in the controlled release formulation may include, but is not limited to, PLGA, ethylene vinyl acetate, poloxamer and GELSITE®. The surgical sealant which may be used in the controlled release formulation may include, but is not limited to, fibrinogen polymers, TISSEELL®, PEG-based sealants and COSEAL®.

[0046] In one embodiment, the delivery agent of the controlled release formulation comprises a lipid nanoparticle or a rapidly eliminated lipid nanoparticle delivery agent. In one aspect, the lipid nanoparticle or rapidly eliminated lipid nanoparticle may be coated, substantially coated, covered, substantially covered, surrounded, substantially surrounded, enclosed, substantially enclosed or comprises a layer of polymer, hydrogel and/or surgical sealant. In another aspect, the delivery agent may be a lipid nanoparticle which may be coated, substantially coated, covered, substantially covered, surrounded, substantially surrounded, enclosed, substantially enclosed or comprises a layer of PLGA.

BRIEF DESCRIPTION OF THE DRAWINGS

[0047] The foregoing and other objects, features and advantages will be apparent from the following description of particular embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of various embodiments of the invention.

[0048] FIG. 1 illustrates lipid structures in the prior art useful in the present invention. Shown are the structures for 98N12-5 (TETA5-LAP), DLin-DMA, DLin-K-DMA (2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane), DLin-KC2-DMA, DLin-MC3-DMA and C12-200.

[0049] FIG. 2 is a representative plasmid useful in the IVT reactions taught herein. The plasmid contains Insert 64818, designed by the instant inventors.

[0050] FIG. 3 is a gel profile of modified mRNA encapsulated in PLGA microspheres.

DETAILED DESCRIPTION

[0051] The delivery of nucleic acids into cells has many undesired complications including the integration of the nucleic acid into the target cell genome which may result in imprecise expression levels, the deleterious transfer of the nucleic acid to progeny and neighbor cells and a substantial risk of causing mutations. The modified nucleic acid molecules of the present disclosure are capable of
reducing the innate immune activity of a population of cells into which they are introduced, thus increasing the efficiency of protein production in that cell population. Further, one or more additional advantageous activities and/or properties of the nucleic acids and proteins of the present disclosure are described herein.

[0052] In addition, provided herein are methods of treating a subject having or being suspected of having a disease, disorder and/or condition the methods comprising administering to a subject in need of such treatment a composition described herein in an amount sufficient to treat the disease, disorder and/or condition.

[0053] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of methods featured in the invention, suitable methods and materials are described below.

**Modified Nucleic Acid Molecules**

[0054] The present disclosure provides nucleic acids, including RNA such as mRNA, which contain one or more modified nucleosides or nucleotides (termed “modified nucleic acid molecules,” “modified mRNA” or “modified mRNA molecules”) as described herein. The modification of the nucleic acid molecules of the present invention may have useful properties including, but not limited to, a significant decrease in or a lack of a substantial induction of the innate immune response of a cell into which the modified mRNA is introduced. The modified nucleic acid molecules may also exhibit enhanced efficiency of protein production, intracellular retention of nucleic acids, and viability of contacted cells, as well as having reduced immunogenicity as compared to unmodified nucleic acid molecules.

[0055] Provided are modified nucleic acid molecules containing a translatable region and one, two, or more than two different nucleoside modifications. Exemplary nucleic acids for use in this disclosure include ribonucleic acids (RNA), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), locked nucleic acids (LNAs) or a hybrid thereof. In preferred embodiments, the modified nucleic acid molecules include messenger RNA (mRNA). As described herein, the modified nucleic acid molecules of the present disclosure may not substantially induce an innate immune response of a cell into which the modified mRNA is introduced. In another embodiment, the modified nucleic acid molecule may exhibit reduced degradation, as compared to a
nucleic acid that has not been modified, in a cell where the modified nucleic acid molecule is introduced.

[0056] The term “nucleic acid” includes any compound and/or substance that is or can be incorporated into an oligonucleotide chain. Exemplary nucleic acids for use in accordance with the present disclosure include, but are not limited to, one or more of DNA, cDNA, RNA including messenger RNA (mRNA), hybrids thereof, RNAi-inducing agents, RNAi agents, siRNA, shRNA, miRNA, antisense RNA, ribozymes, catalytic DNA, RNA that induce triple helix formation, aptamers, vectors and the like.

[0057] In certain embodiments, it is desirable to intracellularly degrade a modified nucleic acid molecule introduced into the cell. For example it would be desirable to degrade a modified nucleic acid molecule if precise timing of protein production was desired. Thus, the present disclosure provides a modified nucleic acid molecule containing a degradation domain, which is capable of being acted on in a directed manner within a cell.

[0058] In some embodiments, the modified nucleic acid molecules may be chemically modified on the sugar, nucleobase (e.g., in the 5’ position of the nucleobase), or phosphate backbone (e.g., replacing the phosphate with another moiety such as a thio phosphate). In some embodiments, the modification may result in a disruption of a major groove binding partner interaction, which may contribute to an innate immune response. In some embodiments, the formulation composition, when administered to a subject, can result in improved bioavailability, therapeutic window, or volume of distribution of the modified nucleic acid molecule relative to administration of the modified nucleic acid molecule without the incorporation of the delivery agent. In some embodiments, the modified nucleosides and nucleotides of the modified nucleic acid molecules of the present invention may be synthesized using the O-protected compounds described in International Pub. No. WO2012138530, the contents of which is herein incorporated by reference in its entirety.

[0059] In certain embodiments, the modified nucleic acid molecule may comprise mRNA. In particular embodiments, the modified mRNA (mmRNA) may be derived from cDNA. In certain embodiments, mmRNA may comprise at least two nucleoside modifications. In one embodiment, the nucleoside modifications may be selected from 5-methylcytosine and pseudouridine. In another embodiment, at least one of the nucleoside modifications is not 5-methylcytosine and/or pseudouridine. In certain embodiments the delivery agent may comprise formulations allowing for localized and systemic delivery of mmRNA. The formulations of the modified nucleic acids
molecules and/or mmRNA may be selected from, but are not limited to, lipoidoids, liposomes and lipid nanoparticles, rapidly eliminated lipid nanoparticles, polymers, lipoplexes, peptides and proteins, at least one chemical modification and conjugation, enhancers, and/or cells.

[0060] In one embodiment, the modified nucleic acid molecules of the present invention may include at least two stop codons before the 3’ untranslated region (UTR). The stop codon may be selected from TGA, TAA and TAG. In one embodiment, the nucleic acids of the present invention include the stop codon TGA and one additional stop codon. In a further embodiment the addition stop codon may be TAA. In another embodiment, the modified nucleic acid molecules may comprise three stop codons.

[0061] Other components of a nucleic acid are optional in a modified nucleic acid molecule but these components may be beneficial in some embodiments.

Untranslated Regions (UTRs)

[0062] Untranslated regions (UTRs) of a gene are transcribed but not translated. 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 a 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 modified mRNA molecules of the present invention to 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.

5’ UTR and Translation Initiation

[0063] Natural 5’ UTRs bear features which play roles in for 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: 1), 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.

[0064] By engineering the features typically found in abundantly expressed genes of specific target organs, one can enhance the stability and protein production of the modified mRNA molecules of the invention. For example, introduction of 5’ UTR of liver-expressed mRNA, such as albumin, serum amyloid A, Apolipoprotein A/B/E, transferrin, alpha fetoprotein, erythropoietin, or Factor
VIII, could be used to enhance expression of a modified nucleic acid molecule, such as a mmRNA, in hepatic cell lines or liver. Likewise, use of 5' UTR from other tissue-specific mRNA to improve expression in that tissue is possible for muscle (MyoD, Myosin, Myoglobin, Myogenin, Herculun), for endothelial cells (Tie-1, CD36), for myeloid cells (C/EBP, AML1, G-CSF, GM-CSF, CD11b, MSR, Fr-1, i-NOS), for leukocytes (CD45, CD18), for adipose tissue (CD36, GLUT4, ACRP30, adiponectin) and for lung epithelial cells (SP-A/B/C/D).

[0065] Other non-UTR sequences may be incorporated into the 5' (or 3' UTR) UTRs of the modified nucleic acid molecules of the present invention. For example, introns or portions of introns sequences may be incorporated into the flanking regions of the modified mRNA of the invention. Incorporation of intronic sequences may increase protein production as well as mRNA levels.

3' UTR and the AU Rich Elements

[0066] 3' UTRs are known to have stretches of Adenoses and Uriddines 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) (SEQ ID NO: 2) nonamers. Molecules containing this type of AREs include GM-CSF and TNF-a. 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.

[0067] Introduction, removal or modification of 3' UTR AU rich elements (AREs) can be used to modulate the stability of modified mRNA of the invention. When engineering specific modified mRNA, one or more copies of an ARE can be introduced to make modified mRNA of the invention less stable and thereby curtail translation and decrease production of the resultant protein.

[0068] 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 modified mRNA of the invention 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 hours, 12 hours, 24 hours, 48 hours, and 7 days post-transfection.

_Incorporating microRNA Binding Sites_

**[0069]** microRNAs (or miRNA) are 19-25 nucleotide long noncoding RNAs that bind to the 3’ UTR of nucleic acid molecules and down-regulate gene expression either by reducing nucleic acid molecule stability or by inhibiting translation. The modified mRNA of the invention may comprise one or more microRNA target sequences, microRNA sequences, or microRNA seeds. Such sequences may correspond to any known microRNA such as those taught in US Publication US2005/0261218 and US Publication US2005/0059005, the contents of which are incorporated herein by reference in their entirety.

**[0070]** A microRNA sequence comprises a “seed” region, i.e., a sequence in the region of positions 2-8 of the mature microRNA, which sequence has perfect Watson-Crick complementarity to the miRNA target sequence. A microRNA seed may comprise positions 2-8 or 2-7 of the mature microRNA. In some embodiments, a microRNA seed may comprise 7 nucleotides (e.g., nucleotides 2-8 of the mature microRNA), wherein the seed-complementary site in the corresponding miRNA target is flanked by an adenine (A) opposed to microRNA position 1. In some embodiments, a microRNA seed may comprise 6 nucleotides (e.g., nucleotides 2-7 of the mature microRNA), wherein the seed-complementary site in the corresponding miRNA target is flanked by an adenine (A) opposed to microRNA position 1. See for example, Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP; Mol Cell. 2007 Jul 6;27(1):91-105; each of which is herein incorporated by reference in their entirety. The bases of the microRNA seed have complete complementarity with the target sequence. By engineering microRNA target sequences into the 3’UTR of modified mRNA of the invention one can target the molecule for degradation or reduced translation, provided the microRNA in question is available. This process will reduce the hazard of off target effects upon nucleic acid molecule delivery. Identification of microRNA, microRNA target regions, and their expression patterns and role in biology have been reported (Bonauer et al., Curr Drug Targets 2010 11:943-949; Anand and Cheresh 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; each of which is herein incorporated by reference in its entirety).
[0071] For example, if the modified nucleic acid molecule is a modified mRNA and is not intended to be delivered to the liver but ends up there, then miR-122, a microRNA abundant in liver, can inhibit the expression of the gene of interest if one or multiple target sites of miR-122 are engineered into the 3’ UTR of the modified mRNA. Introduction of one or multiple binding sites for different microRNA can be engineered to further decrease the longevity, stability, and protein translation of a modified nucleic acid molecule and/or modified mRNA.

[0072] As used herein, the term “microRNA site” refers to a microRNA target site or a microRNA recognition site, or any nucleotide sequence to which a microRNA binds or associates. It should be understood that “binding” may follow traditional Watson-Crick hybridization rules or may reflect any stable association of the microRNA with the target sequence at or adjacent to the microRNA site.

[0073] Conversely, for the purposes of the modified mRNA of the present invention, microRNA binding sites can be engineered out of (i.e. removed from) sequences in which they naturally occur in order to increase protein expression in specific tissues. For example, miR-122 binding sites may be removed to improve protein expression in the liver. Regulation of expression in multiple tissues can be accomplished through introduction or removal or one or several microRNA binding sites.

[0074] Examples of tissues where microRNA 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). MicroRNA can also regulate complex biological processes such as angiogenesis (miR-132) (Anand and Cheresh Curr Opin Hematol 2011 18:171-176; herein incorporated by reference in its entirety). In the modified mRNA of the present invention, binding sites for microRNAs that are involved in such processes may be removed or introduced, in order to tailor the expression of the modified mRNA expression to biologically relevant cell types or to the context of relevant biological processes.

[0075] Lastly, through an understanding of the expression patterns of microRNA in different cell types, modified mRNA can be engineered for more targeted expression in specific cell types or only under specific biological conditions. Through introduction of tissue-specific microRNA binding sites, modified mRNA could be designed that would be optimal for protein expression in a tissue or in the context of a biological condition.
Transfection experiments can be conducted in relevant cell lines, using engineered modified mRNA and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different microRNA binding site-engineering modified mRNA and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hour, 12 hour, 24 hour, 48 hour, 72 hour and 7 days post-transfection. In vivo experiments can also be conducted using microRNA-binding site-engineered molecules to examine changes in tissue-specific expression of formulated modified mRNA.

5’ Capping

The 5’ cap structure of an 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 removal during mRNA splicing.

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

Modifications to the modified mRNA of the present invention may generate a non-hydrolyzable cap structure preventing decapping and thus increasing mRNA half-life. Because cap structure hydrolysis requires cleavage of 5’-ppp-5’ phosphorodiester linkages, modified nucleotides may be used during the capping reaction. For example, a Vaccinia Capping Enzyme from New England Biolabs (Ipswich, MA) may 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 may 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 mRNA (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 an mRNA molecule.

[0081] Cap analogs, which herein are also referred to as synthetic cap analogs, chemical caps, chemical cap analogs, or structural or functional cap analogs, differ from natural (i.e. endogenous, wild-type or physiological) 5'-caps in their chemical structure, while retaining cap function. Cap analogs may be chemically (i.e. non-enzymatically) or enzymatically synthesized and/or linked to a nucleic acid molecule.

[0082] 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 (m^7G-3'mppp-G; which may 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 nucleic acid molecule (e.g. an mRNA or mmRNA). The N7- and 3'-O-methylated guanine provides the terminal moiety of the capped nucleic acid molecule (e.g. mRNA or mmRNA).

[0083] 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, m^7Gm-ppp-G).

[0084] While cap analogs allow for the concomitant capping of a nucleic acid molecule 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, may lead to reduced translational competency and reduced cellular stability.

[0085] Modified mRNA of the present invention may also be capped post-transcriptionally, using enzymes, in order to generate more authentic 5'-cap structures. As used herein, the phrase “more authentic” refers to a feature that closely mirrors or mimics, either structurally or functionally, an endogenous or wild type feature. That is, a “more authentic” feature is better representative of an endogenous, wild-type, natural or physiological cellular function and/or structure as 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 invention are those which, among other things, have enhanced binding of cap binding proteins, increased half life, reduced susceptibility to 5' endonucleases and/or reduced 5'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 an mRNA 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’)NlmpNp (cap 1), and 7mG(5’)ppp(5’)NlmpN2mp (cap 2).

[0086] Because the modified mRNA may be capped post-transcriptionally, and because this process is more efficient, nearly 100% of the modified mRNA may be capped. This is in contrast to ~80% when a cap analog is linked to an mRNA in the course of an in vitro transcription reaction.

[0087] According to the present invention, 5’ terminal caps may include endogenous caps or cap analogs. According to the present invention, a 5’ terminal cap may 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.

Viral Sequences

[0088] Additional viral sequences such as, but not limited to, the translation enhancer sequence of the barley yellow dwarf virus (BYDV-PAV) can be engineered and inserted in the 3’ UTR of the modified mRNA of the invention and can stimulate the translation of the mRNA in vitro and in vivo. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12 hour, 24 hour, 48 hour, 72 hour and day 7 post-transfection.

IRES Sequences

[0089] Further, provided are modified mRNA which may contain an internal ribosome entry site (IRES). First identified as a feature Picorna virus RNA, IRES plays an important role in initiating protein synthesis in absence of the 5’ cap structure. An IRES may act as the sole ribosome binding site, or may serve as one of multiple ribosome binding sites of an mRNA. Modified mRNA containing more than one functional ribosome binding site may encode several peptides or polypeptides that are translated independently by the ribosomes (“multicistronic nucleic acid molecules”). When modified mRNA are provided with an IRES, further optionally provided is a
second translatable region. Examples of IRES sequences that can be used according to the invention include without limitation, those from picornaviruses (e.g. FMDV), pest viruses (CFFV), polioviruses (PV), encephalomyocarditis viruses (ECMV), foot-and-mouth disease viruses (FMDV), hepatitis C viruses (HCV), classical swine fever viruses (CSFV), murine leukemia virus (MLV), simian immune deficiency viruses (SIV) or cricket paralysis viruses (CrPV).

Poly-A tails

[0090] During RNA processing, a long chain of adenine nucleotides (poly-A tail) may be added to a modified nucleic acid molecule such as a modified mRNA molecules in order to increase stability. Immediately after transcription, the 3’ end of the transcript may 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 100 and 250 residues long.

[0091] It has been discovered that unique poly-A tail lengths provide certain advantages to the modified mRNA of the present invention.

[0092] Generally, the length of a poly-A tail of the present invention 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 modified mRNA 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 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).

[0093] In one embodiment, the poly-A tail is designed relative to the length of the overall modified mRNA. This design may be based on the length of the coding region, the length of a
particular feature or region (such as the flanking regions), or based on the length of the ultimate product expressed from the modified mRNA.

[0094] In this context the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% greater in length than the modified mRNA, region or feature thereof. The poly-A tail may also be designed as a fraction of modified mRNA to which it belongs. In this context, the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, or 90% or more of the total length of the molecule or the total length of the molecule minus the poly-A tail. Further, engineered binding sites and conjugation of modified mRNA for Poly-A binding protein may enhance expression.

[0095] Additionally, multiple distinct modified mRNA may be linked together to 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 12 hour, 24 hour, 48 hour, 72 hour and day 7 post-transfection.

[0096] In one embodiment, the modified mRNA of the present invention are designed to include a polyA-G Quartet. 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 mmRNA molecule 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 equivalent to at least 75% of that seen using a poly-A tail of 120 nucleotides alone.

Modifications

[0097] The modified nucleic acids and modified mRNA (mmRNA) of the invention may contain one, two, or more different modifications. In some embodiments, modified nucleic acids and mmRNA may contain one, two, or more different nucleoside or nucleotide modifications. In some embodiments, a modified nucleic acid or mmRNA (e.g., having one or more mmRNA molecules) introduced to a cell may exhibit reduced degradation in the cell, as compared to an unmodified nucleic acid or mmRNA.

[0098] The modified nucleic acids and mmRNA can include any useful modification, such as to the sugar, the nucleobase (e.g., one or more modifications of a nucleobase, such as by replacing or substituting an atom of a pyrimidine nucleobase with optionally substituted amino, optionally substituted thiol, optionally substituted alkyl (e.g., methyl or ethyl), or halo (e.g., chloro or fluoro), or the internucleoside linkage (e.g., one or more modification to the phosphodiester backbone).
certain embodiments, modifications are present in both the sugar and the internucleoside linkage (e.g., one or modifications, such as those present in ribonucleic acids (RNA), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs) or hybrids thereof). Additional modifications are described herein.

[0099] As described herein, the modified nucleic acids and mmRNA of the invention do not substantially induce an innate immune response of a cell into which the mRNA is introduced. In certain embodiments, it may desirable to intracellularly degrade a modified nucleic acid molecule or modified nucleic acid molecule introduced into the cell. For example, degradation of a modified nucleic acid molecule or modified mRNA may be preferable if precise timing of protein production is desired. Thus, in some embodiments, the invention provides a modified nucleic acid molecule containing a degradation domain, which is capable of being acted on in a directed manner within a cell. In another aspect, the present disclosure provides nucleic acids comprising a nucleoside or nucleotide that can disrupt the binding of a major groove interacting, e.g. binding, partner with the nucleic acid (e.g., where the modified nucleotide has decreased binding affinity to major groove interacting partner, as compared to an unmodified nucleotide).

[0100] The modified nucleic acid and mmRNA can optionally include other agents (e.g., RNAi-inducing agents, RNAi agents, siRNA, shRNA, miRNA, antisense RNA, ribozymes, catalytic DNA, tRNA, RNA that induce triple helix formation, aptamers, vectors, etc.). In some embodiments, the modified nucleic acids or mmRNA may include one or more messenger RNA (mRNA) and one or more modified nucleoside or nucleotides (e.g., mmRNA molecules). Details for these modified nucleic acids and mmRNA follow.

Modified Nucleic Acids

[0101] The modified nucleic acids or mmRNA of the invention may include a first region of linked nucleosides encoding a polypeptide of interest, a first flanking region located at the 5’ terminus of the first region, and a second flanking region located at the 3’ terminus of the first region.

[0102] In some embodiments, the modified nucleic acids or mmRNA includes n number of linked nucleosides having Formula (Ia) or Formula (Ia-1):
acceptable salt or stereoisomer thereof, wherein

[U00103] U is O, S, N(R^μ)^nu, or C(R^μ)^nu, wherein nu is an integer from 0 to 2 and each R^μ is, independently, H, halo, or optionally substituted alkyl;

[U00104] --- is a single bond or absent;

[U00105] each of R^1', R^2', R^1'', R^2'', R^1, R^2, R^3, R^4, and R^5 is, if present, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alknyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or absent; wherein the combination of R^3 with one or more of R^1', R^1'', R^2', R^2'', or R^5 (e.g., the combination of R^1' and R^3, the combination of R^1'' and R^3, the combination of R^2' and R^3, the combination of R^2'' and R^3, or the combination of R^5 and R^3) can join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl), wherein the combination of R^3 with one or more of R^1', R^1'', R^2', or R^2'' (e.g., the combination of R^1' and R^5, the combination of R^1'' and R^5, the combination of R^2' and R^5, or the combination of R^2'' and R^5) can join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl); and wherein the combination of R^3 and one or more of R^1', R^1'', R^2', R^2'', R^3, or R^5 can join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl);
[00106] each of \( m^1 \) and \( m^2 \) is, independently, an integer from 0 to 3 (e.g., from 0 to 2, from 0 to 1, from 1 to 3, or from 1 to 2);

[00107] each of \( Y^1 \), \( Y^2 \), and \( Y^3 \), is, independently, O, S, Se, -NR\(^{N1}\), optionally substituted alkylene, or optionally substituted heteroalkylene, wherein \( R^{N1} \) is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, or absent;

[00108] each \( Y^4 \) is, independently, H, hydroxy, thiol, boranyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

[00109] each \( Y^5 \) is, independently, O, S, Se, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene;

[00110] \( n \) is an integer from 1 to 100,000; and

[00111] B is a nucleobase (e.g., a purine, a pyrimidine, or derivatives thereof), wherein the combination of \( B \) and \( R^1 \), the combination of \( B \) and \( R^2 \), the combination of \( B \) and \( R^1^\prime \), or the combination of \( B \) and \( R^2^\prime \) can, taken together with the carbons to which they are attached, optionally form a bicyclic group (e.g., a bicyclic heterocyclcyl) or wherein the combination of \( B \), \( R^1^\prime \), and \( R^3 \) or the combination of \( B \), \( R^2^\prime \), and \( R^3 \) can optionally form a tricyclic or tetracyclic group (e.g., a tricyclic or tetracyclic heterocyclcyl, such as in Formula (IIo)-(IIp) herein). In some embodiments, the modified nucleic acid or mmRNA includes a modified ribose.

[00112] In some embodiments, the modified nucleic acid or mmRNA includes \( n \) number of linked nucleosides having Formula (Ia-2)-(Ia-5) or a pharmaceutically acceptable salt or stereoisomer thereof.
[00113] In some embodiments, the modified nucleic acid or mRNA includes n number of linked nucleosides having Formula (Ib) or Formula (Ib-1):

or a pharmaceutically acceptable salt or stereoisomer thereof, wherein

[00114] U is O, S, N(R\text{I}_{\text{nu}}), or C(R\text{I}_{\text{nu}}), wherein nu is an integer from 0 to 2 and each R\text{I} is, independently, H, halo, or optionally substituted alkyl;

[00115] \(\text{---}\) is a single bond or absent;

[00116] each of \(R^1, R^2, R^3,\) and \(R^4\) is, independently, H, halo, hydroxy, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynloxy, optionally substituted aminooalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted aminoalkoxy, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or absent; and wherein the combination of \(R^1\) and \(R^3\) or the combination of \(R^1\) and
R³" can be taken together to form optionally substituted alkylene or optionally substituted heteroalkylene (e.g., to produce a locked nucleic acid);

[00117] each R⁵ is, independently, H, halo, hydroxy, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, or absent;

[00118] each of Y¹, Y², and Y³ is, independently, O, S, Se, -NR¹¹¹, - optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R¹¹¹ is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl;
each Y⁴ is, independently, H, hydroxy, thiol, boranyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

[00119] n is an integer from 1 to 100,000; and

[00120] B is a nucleobase.

[00121] In some embodiments, the modified nucleic acid or mRNA includes n number of linked nucleosides having Formula (Ic):

![Diagram](image)

(Ic), or a pharmaceutically acceptable salt or stereoisomer thereof,

wherein

[00122] U is O, S, N(R⁴)₉, or C(R⁴)₉, wherein nu is an integer from 0 to 2 and each R⁴ is, independently, H, halo, or optionally substituted alkyl;

[00123] --- is a single bond or absent;

[00124] each of B¹, B², and B³ is, independently, a nucleobase (e.g., a purine, a pyrimidine, or derivatives thereof, as described herein), H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally
substituted aminoalkyl, optionally substituted aminoalkeny1, or optionally substituted aminoalkynyl, wherein one and only one of B₁, B₂, and B₃ is a nucleobase;

[00125] each of R₁, R₂, R₃, and R₄ is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkenyloxy, optionally substituted alkynyl, optionally substituted aminoalkoxy, optionally substituted alkoxylalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminooalkeny1 or optionally substituted aminoalkynyl;

[00126] each of Y₁, Y₂, and Y₃ is, independently, O, S, Se, -NR⁻¹⁻, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R⁻¹ is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl;

[00127] each Y₄ is, independently, H, hydroxy, thiol, boranyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyl, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

[00128] each Y₅ is, independently, O, S, Se, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene;

[00129] n is an integer from 1 to 100,000; and

[00130] wherein the ring including U can include one or more double bonds.

[00131] In particular embodiments, the ring including U does not have a double bond between U-CB⁻³⁻R⁻³⁻ or between CB⁻³⁻R⁻³⁻-C⁻²⁻⁻R⁻²⁻⁻.

[00132] In some embodiments, the modified nucleic acid or mmRNA includes n number of linked nucleosides having Formula (Id):
[00134] each R³ is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl;

[00135] each of Y¹, Y², and Y³, is, independently, O, S, Se, -NR⁴⁻¹, optionally substituted alkyylene, or optionally substituted heteroalkylene, wherein R⁴ is H, optionally substituted alkyln, optionally substituted alkenyl, or optionally substituted aryl;

[00136] each Y⁴ is, independently, H, hydroxy, thiol, borany, optionally substituted alkyln, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

[00137] each Y⁵ is, independently, O, S, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene;

[00138] n is an integer from 1 to 100,000; and

[00139] B is a nucleobase (e.g., a purine, a pyrimidine, or derivatives thereof).

[00140] In some embodiments, the modified nucleic acid molecules or modified mRNA includes n number of linked nucleosides having Formula (Ie):

(ie), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein

[00141] each of U' and U'' is, independently, O, S, N(R⁴)µ, or C(R⁴)µ, wherein µ is an integer from 0 to 2 and each R⁴ is, independently, H, halo, or optionally substituted alkyl;

each R⁵ is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl;

[00142] each Y⁶ is, independently, O, S, optionally substituted alkylene (e.g., methylene or ethylene), or optionally substituted heteroalkylene;
[00143] n is an integer from 1 to 100,000; and

[00144] B is a nucleobase (e.g., a purine, a pyrimidine, or derivatives thereof).

[00145] In some embodiments, the modified nucleic acid or mRNA includes n number of linked nucleosides having Formula (I) or (I-1):

\[
\begin{align*}
\text{(I),} \\
\text{(I-1), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein}\end{align*}
\]

[00146] each of U’ and U” is, independently, O, S, N, N(R\(^\text{I}\))_{nu}, or C(R\(^\text{I}\))_{nu}, wherein nu is an integer from 0 to 2 and each R\(^\text{U}\) is, independently, H, halo, or optionally substituted alkyl (e.g., U’ is O and U” is N);

[00147] --- is a single bond or absent;

[00148] each of R\(^{1^\text{I}}\), R\(^{2^\text{I}}\), R\(^{1^\text{II}}\), R\(^{2^\text{II}}\), R\(^3\) and R\(^4\) is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyl, optionally substituted aminoalkoxy, optionally substituted alkoxalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or absent; and wherein the combination of R\(^{1^\text{I}}\) and R\(^3\), the combination of R\(^{1^\text{II}}\) and R\(^3\), the combination of R\(^{2^\text{I}}\) and R\(^3\), or the combination of R\(^{2^\text{II}}\) and R\(^3\) can be taken together to form optionally substituted alkyne or optionally substituted heteroalkylene (e.g., to produce a locked nucleic acid); each of m’ and m” is, independently, an integer from 0 to 3 (e.g., from 0 to 2, from 0 to 1, from 1 to 3, or from 1 to 2);

[00149] each of Y\(^1\), Y\(^2\), and Y\(^3\) is, independently, O, S, Se, -NR\(^N\text{I}\)-, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R\(^N\text{I}\) is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, or absent;

[00150] each Y\(^4\) is, independently, H, hydroxy, thiol, boranyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy,
optionally substituted alkenyloxy, optionally substituted alknyloxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

[00151] each Y is, independently, O, S, Se, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene;

[00152] n is an integer from 1 to 100,000; and

[00153] B is a nucleobase (e.g., a purine, a pyrimidine, or derivatives thereof).

[00154] In some embodiments of the modified nucleic acid or mmRNA (e.g., (Ia)-(Ia-5), (Ib)-(If-1), (Ia)-(I Ip), (Ib-1), (I lb-2), (illac)-illac-2), (Inl-1), (Inl-2), (IVa)-(IVI), and (IXa)-(IXr)), the ring including U has one or two double bonds.

[00155] In some embodiments of the modified nucleic acid or mmRNA (e.g., Formulas (Ia)-Ia-5), (Ib)-(If-1), (Ia)-(I Ip), (Ib-1), (I lb-2), (illac)-illac-2), (Inl-1), (Inl-2), (IVa)-(IVI), and (IXa)-(IXr)), each of R, R’ and R” is, if present, H. In further embodiments, each of R, R’ and R” is, if present, independently, H, halo (e.g., fluoro), hydroxy, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy. In particular embodiments, alkoxyalkoxy is -(CH₂)ₙ₂(OCH₂CH₂)ₘ₁(CH₂)ₙ₃OR’, wherein n₁ is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of n₂ and n₃, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R’ is H or C₁₋₂₀ alkyl. In some embodiments, n₂ is 0, n₁ is 1 or 2, n₃ is 0 or 1, and R’ is C₁₋₆ alkyl.

[00156] In some embodiments of the modified nucleic acid or mmRNA (e.g., Formulas (Ia)-Ia-5), (Ib)-(If-1), (Ia)-(I Ip), (Ib-1), (I lb-2), (illac)-illac-2), (Inl-1), (Inl-2), (IVa)-(IVI), and (IXa)-(IXr)), each of R, R’ and R” is, if present, H. In further embodiments, each of R, R’ and R” is, if present, independently, H, halo (e.g., fluoro), hydroxy, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy. In particular embodiments, alkoxyalkoxy is -(CH₂)ₙ₁(OCH₂CH₂)ₘ₁(CH₂)ₙ₃OR’, wherein n₁ is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of n₂ and n₃, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R’ is H or C₁₋₂₀ alkyl. In some embodiments, n₂ is 0, n₁ is 1 or 2, n₃ is 0 or 1, and R’ is C₁₋₆ alkyl.

[00157] In some embodiments of the modified nucleic acids or mmRNA (e.g., Formulas (Ia)-Ia-5), (Ib)-(If-1), (Ia)-(I Ip), (Ib-1), (I lb-2), (illac)-illac-2), (Inl-1), (Inl-2), (IVa)-(IVI), and (IXa)-(IXr)), each of R, R’ and R” is, independently, H, halo (e.g., fluoro), hydroxy, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy. In
particular embodiments, $R^3$ is H, $R^4$ is H, $R^5$ is H, or $R^3$, $R^4$, and $R^5$ are all H. In particular embodiments, $R^3$ is C$_{1-6}$ alkyl, $R^4$ is C$_{1-6}$ alkyl, $R^5$ is C$_{1-6}$ alkyl, or $R^3$, $R^4$, and $R^5$ are all C$_{1-6}$ alkyl. In particular embodiments, $R^3$ and $R^4$ are both H, and $R^5$ is C$_{1-6}$ alkyl.

[00158] In some embodiments of the modified nucleic acids or mRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(Ib-1), (IIa)-(IIa), (IIb-1), (IIc-2), (IIc-2-1), (IIc-2-2), (IIc-2-3), (IVa)-(IVa), and (IXa)-(IXa)), $R^3$ and $R^5$ join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl, such as trans-3',4' analogs, wherein $R^3$ and $R^5$ join together to form heterocyclyl (e.g., -(CH$_2$)$_b$(O(CH$_2$)$_{b_2}$O(CH$_2$)$_{b_3}$, wherein each of $b_1$, $b_2$, and $b_3$ are, independently, an integer from 0 to 3).

[00159] In some embodiments of the modified nucleic acids or mRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(Ib-1), (IIa)-(IIa), (IIb-1), (IIc-2), (IIc-2-1), (IIc-2-2), (IIc-2-3), (IVa)-(IVa), and (IXa)-(IXa)), $R^3$ and one or more of $R^1$, $R^1'$, $R^2$, $R^2'$, or $R^5$ join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl, $R^3$ and one or more of $R^1$, $R^1'$, $R^2$, $R^2'$, or $R^5$ join together to form heterocyclyl (e.g., -(CH$_2$)$_b$(O(CH$_2$)$_{b_2}$O(CH$_2$)$_{b_3}$, wherein each of $b_1$, $b_2$, and $b_3$ are, independently, an integer from 0 to 3).

[00160] In some embodiments of the modified nucleic acids or mRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(Ib-1), (IIa)-(IIa), (IIb-1), (IIc-2), (IIc-2-1), (IIc-2-2), (IIc-2-3), (IVa)-(IVa), and (IXa)-(IXa)), $R^5$ and one or more of $R^1$, $R^1'$, $R^2$, or $R^2'$ join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl, $R^5$ and one or more of $R^1$, $R^1'$, $R^2$, or $R^2'$ join together to form heterocyclyl (e.g., -(CH$_2$)$_b$(O(CH$_2$)$_{b_2}$O(CH$_2$)$_{b_3}$, wherein each of $b_1$, $b_2$, and $b_3$ are, independently, an integer from 0 to 3).

[00161] In some embodiments of the modified nucleic acids or mRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(Ib-1), (IIa)-(IIa), (IIb-1), (IIc-2), (IIc-2-1), (IIc-2-2), (IIc-2-3), (IVa)-(IVa), and (IXa)-(IXa)), each $Y^2$ is, independently, O, S, or -NR$^1$-, wherein $R^1$ is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl. In particular
embodiments, Y² is NR⁴⁻¹, wherein R⁴⁻¹ is H or optionally substituted alkyl (e.g., C₁₋₆ alkyl, such as methyl, ethyl, isopropyl, or n-propyl).

[00162] In some embodiments of the modified nucleic acids or mRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (III-1), (III-2), (IVA)-(IVl), and (IXa)-(IXr)), each Y³ is, independently, O or S.

[00163] In some embodiments of the modified nucleic acids or mRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (III-1), (III-2), (IVA)-(IVl), and (IXa)-(IXr)), R¹ is H; each R² is, independently, H, halo (e.g., fluoro), hydroxy, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy (e.g., -(CH₂)ₛ₂(OCH₂CH₂)ₛ₁(CH₂)ₓ₃OR’, wherein s₁ is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s₂ and s₃, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R’ is H or C₁₋₂₀ alkyl, such as wherein s₂ is 0, s₁ is 1 or 2, s₃ is 0 or 1, and R’ is C₁₋₆ alkyl); each Y² is, independently, O or -NR⁴⁻¹, wherein R⁴⁻¹ is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl (e.g., wherein R⁴⁻¹ is H or optionally substituted alkyl (e.g., C₁₋₆ alkyl, such as methyl, ethyl, isopropyl, or n-propyl)); and each Y³ is, independently, O or S (e.g., S). In further embodiments, R³ is H, halo (e.g., fluoro), hydroxy, optionally substituted alkyl, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy. In yet further embodiments, each Y¹ is, independently, O or -NR⁴⁻¹, wherein R⁴⁻¹ is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl (e.g., wherein R⁴⁻¹ is H or optionally substituted alkyl (e.g., C₁₋₆ alkyl, such as methyl, ethyl, isopropyl, or n-propyl)); and each Y² is, independently, H, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino.

[00164] In some embodiments of the modified nucleic acids or mRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (III-1), (III-2), (IVA)-(IVl), and (IXa)-(IXr)), each R¹ is, independently, H, halo (e.g., fluoro), hydroxy, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy (e.g., -(CH₂)ₛ₂(OCH₂CH₂)ₛ₁(CH₂)ₓ₃OR’, wherein s₁ is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s₂ and s₃, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R’ is H or C₁₋₂₀ alkyl, such as wherein s₂ is 0, s₁ is 1 or 2, s₃ is 0 or 1, and R’ is
C_{1-6} alkyl); R^2 is H; each Y^2 is, independently, O or -NR^{N1}-, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl (e.g., wherein R^{N1} is H or optionally substituted alkyl (e.g., C_{1-6} alkyl, such as methyl, ethyl, isopropyl, or n-propyl)); and each Y^1 is, independently, O or S (e.g., S). In further embodiments, R^3 is H, halo (e.g., fluoro), hydroxy, optionally substituted alkyl, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy. In yet further embodiments, each Y^1 is, independently, O or -NR^{N1}-, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl (e.g., wherein R^{N1} is H or optionally substituted alkyl (e.g., C_{1-6} alkyl, such as methyl, ethyl, isopropyl, or n-propyl)); and each Y^4 is, independently, H, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino.

[00165] In some embodiments of the modified nucleic acids or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(Ib-1), (I1a)-(I1p), (I1b-1), (I1b-2), (I1c-1)-(I1c-2), (I1n-1), (I1n-2), (I1v)-I(V1), and (IXa)-(IXr)), the ring including U is in the β-D (e.g., β-D-ribo) configuration.

[00166] In some embodiments of the modified nucleic acids or mmRNA (e.g Formulas (Ia)-(Ia-5), (Ib)-(Ib-1), (I1a)-(I1p), (I1b-1), (I1b-2), (I1c-1)-(I1c-2), (I1n-1), (I1n-2), (I1v)-I(V1), and (IXa)-(IXr)), the ring including U is in the α-L (e.g., α-L-ribo) configuration.

[00167] In some embodiments of the modified nucleic acids or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(Ib-1), (I1a)-(I1p), (I1b-1), (I1b-2), (I1c-1)-(I1c-2), (I1n-1), (I1n-2), (I1v)-I(V1), and (IXa)-(IXr)), one or more B is not pseudouridine (ψ) or 5-methyl-cytidine (m{5}C). In some embodiments, about 10% to about 100% of B nucleobases is not ψ or m{5}C (e.g., from 10% to 20%, from 10% to 35%, from 10% to 50%, from 10% to 60%, from 10% to 75%, from 10% to 90%, from 10% to 95%, from 10% to 98%, from 10% to 99%, from 20% to 35%, from 20% to 50%, from 20% to 60%, from 20% to 75%, from 20% to 90%, from 20% to 95%, from 20% to 98%, from 20% to 99%, from 20% to 95%, from 20% to 98%, from 20% to 99%, from 20% to 95%, from 20% to 98%, from 20% to 99%, from 20% to 98%, from 20% to 99%, from 20% to 95%, from 20% to 98%, from 20% to 99%, from 20% to 95%, from 20% to 98%, from 20% to 99%, from 20% to 95%, from 20% to 98%, from 20% to 99%, and from 75% to 100% of n number of B is not ψ or m{5}C). In some embodiments, B is not ψ or m{5}C.

[00168] In some embodiments of the modified nucleic acids or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(Ib-1), (I1a)-(I1p), (I1b-1), (I1b-2), (I1c-1)-(I1c-2), (I1n-1), (I1n-2), (I1v)-I(V1), and (IXa)-(IXr)),
when B is an unmodified nucleobase selected from cytosine, guanine, uracil and adenine, then at least one of \( Y^1 \), \( Y^2 \), or \( Y^3 \) is not O.

[00169] In some embodiments, the modified nucleic acids or mRNA includes a modified ribose. In some embodiments, modified nucleic acids or mRNA includes n number of linked nucleosides having Formula (IIa)-(IIc):

\[
\begin{align*}
&\text{(IIa),} \\
&(\text{IIb), or} \\
&(\text{IIc), or}
\end{align*}
\]

a pharmaceutically acceptable salt or stereoisomer thereof. In particular embodiments, U is O or C(R^U)_{nu}, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl (e.g., U is –CH_2– or –CH–). In other embodiments, each of R^1, R^2, R^3, R^4, and R^5 is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or absent (e.g., each R^1 and R^2 is, independently, H, halo, hydroxy, optionally substituted alkyl, or optionally substituted alkoxy; each R^3 and R^4 is, independently, H or optionally substituted alkyl; and R^5 is H or hydroxy), and __ is a single bond or double bond.

[00170] In particular embodiments, the modified nucleic acid or mRNA includes n number of linked nucleosides having Formula (IIb-1)-(IIb-2):

\[
\begin{align*}
&(\text{IIb-1) or} \\
&(\text{IIb-2) or a pharmaceutically acceptable salt or stereoisomer thereof. In some embodiments, U is O or C(R^U)_{nu}, wherein nu is an integer from 0}
\end{align*}
\]
to 2 and each \( R^U \) is, independently, H, halo, or optionally substituted alkyl (e.g., \( U = -\text{CH}_2- \) or \(-\text{CH}-\)). In other embodiments, each of \( R^1 \) and \( R^2 \) is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or absent (e.g., each \( R^1 \) and \( R^2 \) is, independently, H, halo, hydroxy, optionally substituted alkyl, or optionally substituted alkoxy, e.g., H, halo, hydroxy, alkyl, or alkoxy). In particular embodiments, \( R^2 \) is hydroxy or optionally substituted alkoxy (e.g., methoxy, ethoxy, or any described herein).

[00171] In particular embodiments, the modified nucleic acid or mRNA includes \( n \) number of linked nucleosides having Formula (IIc-1)-(IIc-4):

(Iic-1),

(Iic-2),

(Iic-3),

(Iic-4), or a pharmaceutically acceptable salt or stereoisomer thereof. In some embodiments, \( U = O \) or \( C(R^U)_{nu} \), wherein \( nu \) is an integer from 0 to 2 and each \( R^U \) is, independently, H, halo, or optionally substituted alkyl (e.g., \( U = -\text{CH}_2- \) or \(-\text{CH}-\)). In some embodiments, each of \( R^1, R^2, \) and \( R^3 \) is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted
aminoalkynyl, or absent (e.g., each R¹ and R² is, independently, H, halo, hydroxy, optionally substituted alkyl, or optionally substituted alkoxy, e.g., H, halo, hydroxy, alkyl, or alkoxy; and each R³ is, independently, H or optionally substituted alkyl)). In particular embodiments, R² is optionally substituted alkoxy (e.g., methoxy or ethoxy, or any described herein). In particular embodiments, R¹ is optionally substituted alkyl, and R² is hydroxy. In other embodiments, R¹ is hydroxy, and R² is optionally substituted alkyl. In further embodiments, R³ is optionally substituted alkyl.

[00172] In some embodiments, the modified nucleic acids or mmRNA includes an acyclic modified ribose. In some embodiments, the modified nucleic acids or mmRNA includes n number of linked nucleosides having Formula (IIa)-(IIf):

(IIa), (IIb), (IIc), (IId), (IIe), or (IIf), or a pharmaceutically acceptable salt or stereoisomer thereof.

[00173] In some embodiments, the modified nucleic acids or mmRNA includes an acyclic modified hexitol. In some embodiments, the modified nucleic acids or mmRNA includes n number of linked nucleosides having Formula (IIg)-(IIj):
acceptable salt or stereoisomer thereof.

[00174] In some embodiments, the modified nucleic acids or mRNA includes a sugar moiety having a contracted or an expanded ribose ring. In some embodiments, the modified nucleic acids or mRNA includes n number of linked nucleosides having Formula (IIk)-(IIm):

wherein each of R', R'', R', and R'' is, independently, H, halo, hydroxy, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted...
alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, or absent; and wherein the combination of \( R^2 \) and \( R^3 \) or the combination of \( R^2' \) and \( R^3' \) can be taken together to form optionally substituted alkylene or optionally substituted heteroalkylene.

[00175] In some embodiments, the modified nucleic acids or mRNA includes a locked modified ribose. In some embodiments, the modified nucleic acids or mRNA includes \( n \) number of linked nucleosides having Formula (IIa):

![Diagram of nucleoside structure](image)

(IIa), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein \( R^3 \) is O, S, or \(-NR^N_1\), wherein \( R^N_1 \) is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl and \( R^3'' \) is optionally substituted alkylene (e.g., \(-CH_2\), \(-CH_2CH_2\), or \(-CH_2CH_2CH_2\)) or optionally substituted heteroalkylene (e.g., \(-CH_2NH\), \(-CH_2CH_2NH\), \(-CH_2OCH\), or \(-CH_2CH_2OCH\))(e.g., \( R^3' \) is O and \( R^3'' \) is optionally substituted alkylene (e.g., \(-CH_2\), \(-CH_2CH_2\), or \(-CH_2CH_2CH_2\))).

[00176] In some embodiments, the modified nucleic acid or mRNA includes \( n \) number of linked nucleosides having Formula (II-1)-(II-n2):

![Diagram of nucleoside structure with variations](image)

(II-1) or (II-2), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein \( R^3' \) is O, S, or \(-NR^N_1\), wherein \( R^N_1 \) is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl and \( R^3'' \) is optionally substituted alkylene (e.g., \(-CH_2\), \(-CH_2CH_2\), or \(-CH_2CH_2CH_2\)) or optionally substituted heteroalkylene (e.g., \(-CH_2NH\), \(-CH_2CH_2NH\), \(-CH_2OCH\), or \(-CH_2CH_2OCH\))(e.g., \( R^3' \) is O and \( R^3'' \) is optionally substituted alkylene (e.g., \(-CH_2\), \(-CH_2CH_2\), or \(-CH_2CH_2CH_2\))).
In some embodiments, the modified nucleic acids or mmRNA includes a locked modified ribose that forms a tetracyclic heterocyclyl. In some embodiments, the modified nucleic acids or mmRNA includes n number of linked nucleosides having Formula (Ilo):

![Chemical Structure Image]

(Ilo) or (IIp), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein R$^{12a}$, R$^{12c}$, T$^{1'}$, T$^{1''}$, T$^{2'}$, T$^{2''}$, V$^1$, and V$^3$ are as described herein.

Any of the formulas for the modified nucleic acids or mmRNA can include one or more nucleobases described herein (e.g., Formulas (b1)-(b43)).

In one embodiment, the present invention provides methods of preparing a modified nucleic acids or mmRNA comprising at least one nucleotide (e.g., mmRNA molecule), wherein the modified nucleic acid comprises n number of nucleosides having Formula (Ia), as defined herein:

![Chemical Structure Image]

(Ia), the method comprising reacting a compound of Formula (IIIa), as defined herein:
with an RNA polymerase, and a cDNA template.

[00180] In a further embodiment, the present invention provides methods of amplifying a modified nucleic acids or mRNA comprising at least one nucleotide (e.g., mRNA molecule), the method comprising: reacting a compound of Formula (IIIa), as defined herein, with a primer, a cDNA template, and an RNA polymerase.

[00181] In one embodiment, the present invention provides methods of preparing a modified nucleic acids or mRNA comprising at least one nucleotide (e.g., mRNA molecule), wherein the modified nucleic acid comprises n number of nucleosides having Formula (Ia-1), as defined herein:

\[
\begin{align*}
Y^1 & \quad U \quad B \quad R^4 \\
Y^2 & \quad R^1' \quad R^1'' \quad R^2 \quad R^2' \quad m' \\
Y^3 = P & \quad Y^7 \\
Y^4 & \quad q
\end{align*}
\]  

(Ia-1),

the method comprising reacting a compound of Formula (IIIa-1), as defined herein:
(IIIa-1), with an RNA polymerase, and a cDNA template.

[00182] In a further embodiment, the present invention provides methods of amplifying a modified nucleic acids or mmRNA comprising at least one nucleotide (e.g., mmRNA molecule), the method comprising reacting a compound of Formula (IIIa-1), as defined herein, with a primer, a cDNA template, and an RNA polymerase.

[00183] In one embodiment, the present invention provides methods of preparing a modified mRNA comprising at least one nucleotide (e.g., mmRNA molecule), wherein the polynucleotide comprises n number of nucleosides having Formula (Ia-2), as defined herein:

the method comprising reacting a compound of Formula (IIIa-2), as defined herein:

(IIIa-2), with an RNA polymerase, and a cDNA template.
[00184] In a further embodiment, the present invention provides methods of amplifying a modified mRNA comprising at least one nucleotide (e.g., mmRNA molecule), the method comprising:

[00185] reacting a compound of Formula (IIIa-2), as defined herein, with a primer, a cDNA template, and an RNA polymerase.

[00186] In some embodiments, the reaction may be repeated from 1 to about 7,000 times. In any of the embodiments herein, B may be a nucleobase of Formula (b1)-(b43).

[00187] The modified nucleic acids and mmRNA can optionally include 5’ and/or 3’ flanking regions, which are described herein.

**Modified RNA (e.g. mmRNA) Molecules**

[00188] The present invention also includes building blocks, e.g., modified ribonucleosides, modified ribonucleotides, of modified RNA (mmRNA) molecules. For example, these mmRNA can be useful for preparing the modified nucleic acids or mmRNA of the invention.

[00189] In some embodiments, the building block molecule has Formula (IIIa) or (IIIa-1):

![Chemical Structures]

pharmaceutically acceptable salt or stereoisomer thereof, wherein the substituents are as described herein (e.g., for Formula (Ia) and (Ia-1)), and wherein when B is an unmodified nucleobase selected from cytosine, guanine, uracil and adenine, then at least one of Y¹, Y², or Y³ is not O.

[00190] In some embodiments, the building block molecule, which may be incorporated into a modified nucleic acid or mmRNA, has Formula (IVa)-(IVb):

![Chemical Structures]

(IVa) or (IVb), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein B is as described herein (e.g., any one of (b1)-(b43)). In particular
embodiments, Formula (IVa) or (IVb) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, Formula (IVa) or (IVb) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, Formula (IVa) or (IVb) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, Formula (IVa) or (IVb) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

[00191] In some embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mRNA, has Formula (IVc)-(IVk):

- Formula (IVc)
- Formula (IVd)
- Formula (IVe)
- Formula (IVf)
- Formula (IVg)
- Formula (IVh)
- Formula (IVi)
- Formula (IVj)
- Formula (IVk)
wherein B is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, one of Formulas (IVc)-(IVk) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, one of Formulas (IVc)-(IVk) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, one of Formulas (IVc)-(IVk) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, one of Formulas (IVc)-(IVk) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

[00192] In other embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mRNA, has Formula (Va) or (Vb):

![Diagram](image)

pharmacologically acceptable salt or stereoisomer thereof, wherein B is as described herein (e.g., any one of (b1)-(b43)).

[00193] In other embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mRNA, has Formula (IXa)-(IXd):

![Diagram](image)
or HO 1 (IXd), or a pharmaceutically acceptable salt or stereoisomer thereof,
wherein B is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, one of Formulas (IXa)-(IXd) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, one of Formulas (IXa)-(IXd) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, one of Formulas (IXa)-(IXd) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, one of Formulas (IXa)-(IXd) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

[00194] In other embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mRNA, has Formula (IXe)-(IXg):

\[
\begin{align*}
\text{HO} & \quad \text{R}^2 \quad \text{(IXe)}, \\
\text{HO} & \quad \text{R}^2 \quad \text{(IXf)}, \quad \text{or} \\
\text{HO} & \quad \text{R}^2 \quad \text{(IXg)}, \quad \text{or}
\end{align*}
\]

pharmaceutically acceptable salt or stereoisomer thereof, wherein B is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, one of Formulas (IXe)-(IXg) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, one of Formulas (IXe)-(IXg) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, one of Formulas (IXe)-(IXg) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In
particular embodiments, one of Formulas (IXe)-(IXg) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

[00195] In other embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mmRNA, has Formula (IXh)-(IXk):

\[
\text{(IXh), (IXi), (IXj), (IXk)}
\]

or \(\text{OH(IXk)}\), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein B is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, one of Formulas (IXh)-(IXk) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, one of Formulas (IXh)-(IXk) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, one of Formulas (IXh)-(IXk) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, one of Formulas (IXh)-(IXk) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

[00196] In other embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mmRNA, has Formula (IXl)-(IXr):
salt or stereoisomer thereof, wherein each \( r_1 \) and \( r_2 \) is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5) and \( B \) is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, one of Formulas (IX1)-(IXr) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, one of Formulas (IX1)-(IXr) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, one of Formulas (IX1)-(IXr) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, one of Formulas (IX1)-(IXr) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

[00197] In some embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecules or mmRNA, can be selected from the group consisting of:
or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each \( r \) is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5).

In some embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mRNA, can be selected from the group consisting of:
thereof, wherein each \( r \) is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5) and \( s_1 \) is as described herein.

[00199] In some embodiments, the building block molecule, which may be incorporated into a nucleic acid (e.g., RNA, mRNA, or mmRNA), is a modified uridine (e.g., selected from the group consisting of:

\[ \text{(BB-18), (BB-19), and (BB-20), or a pharmaceutically acceptable salt or stereoisomer thereof.} \]
(BB- 24),

(BB- 25),

(BB- 26),

(BB- 27),

(BB- 28),

(BB- 29),

(BB- 30),

(BB- 31),

(BB- 32),

(BB- 33),

- 50 -
(BB- 66),

(BB- 67),

(BB- 68),

(BB- 69),
(BB-119),

(BB-120),

(BB-121),

(BB-122),

(BB-123),

(BB-124), and

(BB-125), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein $Y^1$, $Y^3$, $Y^4$, $Y^6$, and $r$ are as described herein (e.g., each $r$ is, independently, an integer from 0 to 5, such as from 0 to 3, from 1 to 3, or from 1 to 5)).
[00200] In some embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mRNA, is a modified cytidine (e.g., selected from the group consisting of:

(BB-126),

(BB-127),

(BB-128),

(BB-129),

(BB-130),

(BB-131),
stereoisomer thereof, wherein \( Y^1, Y^2, Y^4, Y^6 \), and \( r \) are as described herein (e.g., each \( r \) is, independently, an integer from 0 to 5, such as from 0 to 3, from 1 to 3, or from 1 to 5). For example, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mRNA, can be:

(BB-159), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each \( r \) is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5).
In some embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mRNA, is a modified adenosine (e.g., selected from the group consisting of:

- (BB-162),
- (BB-163),
- (BB-164),
- (BB-165),
- (BB-166),
- (BB-167),
- (BB-168),
- (BB-169),
and (BB- 200) or a pharmaceutically acceptable salt or stereoisomer thereof, wherein \( Y^1, Y^3, Y^4, Y^6 \), and \( r \) are as described herein (e.g., each \( r \) is, independently, an integer from 0 to 5, such as from 0 to 3, from 1 to 3, or from 1 to 5)).

[00202] In some embodiments, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mRNA, is a modified guanosine (e.g., selected from the group consisting of:

- (BB- 201),
- (BB- 202),
- (BB- 203),
- (BB- 204),
thereof, wherein \( Y^1, Y^3, Y^4, Y^6 \), and \( r \) are as described herein (e.g., each \( r \) is, independently, an integer from 0 to 5, such as from 0 to 3, from 1 to 3, or from 1 to 5).

[00203] In some embodiments, the chemical modification can include replacement of C group at C-5 of the ring (e.g., for a pyrimidine nucleoside, such as cytosine or uracil) with N (e.g., replacement of the \( \text{CH} \) group at C-5 with \( \text{NR}^N \) group, wherein \( R^N \) is H or optionally substituted alkyl). For example, the mmRNA molecule, which may be incorporated into a modified nucleic acid molecule or mmRNA, can be:

(BB-235), (BB-236), and (BB-237), or a pharmaceutically acceptable salt or stereoisomer thereof.
pharmaceutically acceptable salt or stereoisomer thereof, wherein each r is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5).

[00204] In another embodiment, the chemical modification can include replacement of the hydrogen at C-5 of cytosine with halo (e.g., Br, Cl, F, or I) or optionally substituted alkyl (e.g., methyl). For example, the mmRNA molecule, which may be incorporated into a modified nucleic acid or mmRNA, can be:

![Chemical structures](image1)

(BB-242) or

![Chemical structures](image2)

(BB-243) or

![Chemical structures](image3)

(BB-244) or

![Chemical structures](image4)

(BB-245), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each r is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5).

[00205] In yet a further embodiment, the chemical modification can include a fused ring that is formed by the NH$_2$ at the C-4 position and the carbon atom at the C-5 position. For example, the building block molecule, which may be incorporated into a modified nucleic acid molecule or mmRNA, can be:
(BB- 246), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each r is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5).

**Modifications on the Sugar**

[00206] The modified nucleosides and nucleotides (e.g., building block molecules), which may be incorporated into a modified nucleic acid or mmRNA (e.g., RNA or mRNA, as described herein), can be modified on the sugar of the ribonucleic acid. For example, the 2’ hydroxyl group (OH) can be modified or replaced with a number of different substituents. Exemplary substitutions at the 2’-position include, but are not limited to, H, halo, optionally substituted C1-6 alkyl; optionally substituted C1-6 alkoxy; optionally substituted C6-10 aryloxy; optionally substituted C3-8 cycloalkyl; optionally substituted C3-8 cycloalkoxy; optionally substituted C6-10 aryloxy; optionally substituted C6-10 aryl-C1-6 alkoxy, optionally substituted C1-12 (heterocyclyl)oxy; a sugar (e.g., ribose, pentose, or any described herein); a polyethyleneglycol (PEG), -O(CH2CH2O)nCH2CH2OR, where R is H or optionally substituted alkyl, and n is an integer from 0 to 20 (e.g., from 0 to 4, from 0 to 8, from 0 to 10, from 0 to 16, from 1 to 4, from 1 to 8, from 1 to 10, from 1 to 16, from 1 to 20, from 2 to 4, from 2 to 8, from 2 to 10, from 2 to 16, from 2 to 20, from 4 to 8, from 4 to 10, from 4 to 16, and from 4 to 20); “locked” nucleic acids (LNA) in which the 2’-hydroxyl is connected by a C1,6 alkylene or C1,6 heteroalkylene bridge to the 4’-carbon of the same ribose sugar, where exemplary bridges included methylene, propylene, ether, or amino bridges; aminoalkyl, as defined herein; aminoalkoxy, as defined herein; amino as defined herein; and amino acid, as defined herein.

[00207] Generally, RNA includes the sugar group ribose, which is a 5-membered ring having an oxygen. Exemplary, non-limiting modified nucleotides include replacement of the oxygen in ribose (e.g., with S, Se, or alkylene, such as methylene or ethylene); addition of a double bond (e.g., to replace ribose with cyclopentenyl or cyclohexenyl); ring contraction of ribose (e.g., to form a 4-membered ring of cyclobutane or oxetane); ring expansion of ribose (e.g., to form a 6- or 7-membered ring having an additional carbon or heteroatom, such as for anhydrohexitol, altitol,
mannitol, cyclohexanyl, cyclohexenyl, and morpholino that also has a phosphoramidate backbone; multicyclic forms (e.g., tricyclo; and “unlocked” forms, such as glycol nucleic acid (GNA) (e.g., R-GNA or S-GNA, where ribose is replaced by glycol units attached to phosphodiester bonds), threose nucleic acid (TNA, where ribose is replace with α-L-threofuranosyl-(3’→2’)), and peptide nucleic acid (PNA, where 2-amino-ethyl-glycine linkages replace the ribose and phosphodiester backbone). The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a modified nucleic acid molecule or mRNA can include nucleotides containing, e.g., arabinose, as the sugar.

**Modifications on the Nucleobase**

[00208] The present disclosure provides for modified nucleosides and nucleotides. As described herein “nucleoside” is defined as 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. As described herein, “nucleotide” is defined as a nucleoside including a phosphate group. The modified nucleotides (e.g., modified mRNA) may by synthesized by any useful method, as described herein (e.g., chemically, enzymatically, or recombinantly to include one or more modified or non-natural nucleosides).

[00209] The 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.

[00210] The modified nucleosides and nucleotides can include a modified nucleobase. Examples of nucleobases found in RNA include, but are not limited to, adenine, guanine, cytosine, and uracil. Examples of nucleobase found in DNA include, but are not limited to, adenine, guanine, cytosine, and thymine. These nucleobases can be modified or wholly replaced to provide modified nucleic acids or mRNA molecules having enhanced properties, e.g., resistance to nucleases through disruption of the binding of a major groove binding partner. Table 1 below identifies the chemical faces of each canonical nucleotide. Circles identify the atoms comprising the respective chemical regions.
Table 1

Pyrimidines

<table>
<thead>
<tr>
<th>Major Groove Face</th>
<th>Minor Groove Face</th>
<th>Watson-Crick Base-pairing Face</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytidine:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uridine:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Purines

<table>
<thead>
<tr>
<th>Major Groove Face</th>
<th>Minor Groove Face</th>
<th>Watson-Crick Base-pairing Face</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanosine:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[00211] In some embodiments, B is a modified uracil. Exemplary modified uracils include those having Formula (b1)-(b5):

![Chemical Structures]

(b1), (b2), (b3), (b4), or (b5), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein

[00212] is a single or double bond;
[00213] each of T', T", T", and T" is, independently, H, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy, or the combination of T' or T" or the combination of T' and T" join together (e.g., as in T²) to form O (oxo), S (thio), or Se (seleno);

[00214] each of V¹ and V² is, independently, O, S, N(R²)ₙ, or C(R²)ₙ, wherein n is an integer from 0 to 2 and each R² is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted haloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynloxy, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl), optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted acylaminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl), optionally substituted alkoxy carbonylalkyl, optionally substituted alkoxy carbonylalkenyl, optionally substituted alkoxy carbonylalkynyl, or optionally substituted alkoxy carbonylalkoxy (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl);

[00215] R¹ is H, halo, optionally substituted amino acid, hydroxy, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aminoalkyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted alkoxy carbonylalkyl, optionally substituted alkoxy carbonylalkenyl, optionally substituted alkoxy carbonylalkynyl, optionally substituted alkoxy carbonylalkoxy, optionally substituted carboxyalkoxy, optionally substituted carboxyalkyl, or optionally substituted carbamoylalkyl;

[00216] R¹ is H or optionally substituted alkyl;

[00217] R² is H, optionally substituted alkyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl, optionally substituted carboxyalkyl (e.g., optionally substituted with hydroxy), optionally substituted carboxyalkoxy, optionally substituted carboxyaminoalkyl, or optionally substituted carbamoylalkyl; and
[00218] R\textsuperscript{12c} is H, halo, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted thioalkoxy, optionally substituted amino, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl.

[00219] Other exemplary modified uracils include those having Formula (b6)-(b9):

![Diagram](image)

\(\sim\) is a single or double bond;

[00220] each of T\textsuperscript{1}, T\textsuperscript{1'}, T\textsuperscript{2}, and T\textsuperscript{2'\prime}\ is, independently, H, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy, or the combination of T\textsuperscript{1} and T\textsuperscript{1'} join together (e.g., as in T\textsuperscript{1} \(\sim\) T\textsuperscript{1'}), or the combination of T\textsuperscript{2} and T\textsuperscript{2'} join together (e.g., as in T\textsuperscript{2} \(\sim\) T\textsuperscript{2'}), or form O (oxo), S (thio), or Se (seleno); each T\textsuperscript{1} and T\textsuperscript{2} is, independently, O (oxo), S (thio), or Se (seleno);

[00221] each of W\textsuperscript{1} and W\textsuperscript{2} is, independently, N(R\textsuperscript{WA}\textsubscript{nw})\textsubscript{nw} or C(R\textsuperscript{WA}\textsubscript{nw}), wherein nw is an integer from 0 to 2 and each R\textsuperscript{WA} is, independently, H, optionally substituted alkyl, or optionally substituted alkoxy;

[00222] each V\textsuperscript{3} is, independently, O, S, N(R\textsuperscript{VA}\textsubscript{nv})\textsubscript{nv} or C(R\textsuperscript{VA}\textsubscript{nv}), wherein nv is an integer from 0 to 2 and each R\textsuperscript{VA} is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted alkynyl, optionally substituted heterocycl, optionally substituted alk heterocycl, optionally substituted alkoxy, optionally substituted alkenylox, or optionally substituted alkynyl, or optionally substituted alkoxy, optionally substituted aminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl), optionally substituted aminoaalkenyl, optionally substituted aminoaalkynyl, optionally substituted acylaminooalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl), optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkenyl, optionally substituted alkoxycarbonylalkynyl, optionally substituted alkoxycarbonylalkynyl, optionally substituted alkoxyalkyl (e.g., optionally substituted with hydroxy and/or an O-protecting group),
optionally substituted carboxyalkoxy, optionally substituted carboxyaminoalkyl, or optionally substituted carbamoylalkyl (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl), and wherein $\text{R}^{\text{Va}}$ and $\text{R}^{\text{Vc}}$ taken together with the carbon atoms to which they are attached can form optionally substituted cycloalkyl, optionally substituted aryl, or optionally substituted heterocyclyl (e.g., a 5- or 6-membered ring);

[00224] $\text{R}^{\text{La}}$ is H, optionally substituted alkyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted carboxyalkyl (e.g., optionally substituted with hydroxy and/or an $O$-protecting group), optionally substituted carboxyalkoxy, optionally substituted carboxyaminoalkyl, optionally substituted carbamoylalkyl, or absent;

[00225] $\text{R}^{\text{Lb}}$ is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted alkaryl, optionally substituted heterocyclyl, optionally substituted alkhetercyclyl, optionally substituted amino acid, optionally substituted alkoxy carbonylacetyl, optionally substituted alkoxy carbonylalkoxy, optionally substituted alkoxy carbonylalkyl, optionally substituted alkoxy carbonylalkenyl, optionally substituted alkoxy carbonylalkynyl, optionally substituted alkoxy carbonylalkoxy, optionally substituted carboxyalkyl (e.g., optionally substituted with hydroxy and/or an $O$-protecting group), optionally substituted carboxyalkoxy, optionally substituted carboxyaminoalkyl, or optionally substituted carbamoylalkyl,

[00226] wherein the combination of $\text{R}^{\text{Lb}}$ and $\text{T}^{\text{L}}$ or the combination of $\text{R}^{\text{Lb}}$ and $\text{R}^{\text{Lc}}$ can join together to form optionally substituted heterocyclyl; and

[00227] $\text{R}^{\text{Lc}}$ is H, halo, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted thioalkoxy, optionally substituted amino, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl.

[00228] Further exemplary modified uracils include those having Formula (b28)-(b31):
[00229] each of $T^1$ and $T^2$ is, independently, O (oxo), S (thio), or Se (seleno);

[00230] each $R^{Vb'}$ and $R^{Vb''}$ is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted haloalkyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynloxy, optionally substituted aminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl), optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted acylaminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl), optionally substituted alkoxy carbonylalkyl, optionally substituted alkoxy carbonylalkenyl, optionally substituted alkoxy carbonylalkynyl, optionally substituted alkoxy carbonylacyl, optionally substituted alkoxy carbonylalkoxy, optionally substituted carboxyalkyl (e.g., optionally substituted with hydroxy and/or an O-protecting group), optionally substituted carboxyalkoxy, optionally substituted carboxyaminoalkyl, or optionally substituted carbamoylalkyl (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl) (e.g., $R^{Vb'}$ is optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted aminoalkyl, e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl);

[00231] $R^{12a}$ is H, optionally substituted alkyl, optionally substituted carboxyaminoalkyl, optionally substituted aminoalkyl (e.g., e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl), optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl; and
[00232] R^{12b} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl),

[00233] optionally substituted alkoxy carbonyl acyl, optionally substituted alkoxy carbonyl alkoxy, optionally substituted alkoxy carbonyl alkyl, optionally substituted alkoxy carbonyl alkenyl, optionally substituted alkoxy carbonyl alkyne, optionally substituted alkoxy carbonyl alkynyl, optionally substituted alkoxy carbonyl alkoxy, optionally substituted carboxyalkoxy, optionally substituted carboxyalkyl, or optionally substituted carbamoylalkyl.

[00234] In particular embodiments, T^1 is O (oxo), and T^2 is S (thio) or Se (seleno). In other embodiments, T^1 is S (thio), and T^2 is O (oxo) or Se (seleno). In some embodiments, R^{Vb} is H, optionally substituted alkyl, or optionally substituted alkoxy.

[00235] In other embodiments, each R^{12a} and R^{12b} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted hydroxyalkyl. In particular embodiments, R^{12a} is H. In other embodiments, both R^{12a} and R^{12b} are H.

[00236] In some embodiments, each R^{Vb} of R^{12b} is, independently, optionally substituted aminoalkyl (e.g., substituted with a N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl), optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or optionally substituted acylaminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl). In some embodiments, the amino and/or alkyl of the optionally substituted aminoalkyl is substituted with one or more of optionally substituted alkyl, optionally substituted alkenyl, optionally substituted sulfoalkyl, optionally substituted carboxy (e.g., substituted with an O-protecting group), optionally substituted hydroxy (e.g., substituted with an O-protecting group), optionally substituted carboxyalkyl (e.g., substituted with an O-protecting group), optionally substituted alkoxy carbonyl alkyl (e.g., substituted with an O-protecting group), or N-protecting group. In some embodiments, optionally substituted aminoalkyl is substituted with an optionally substituted sulfoalkyl or optionally substituted alkenyl. In particular embodiments, R^{12a} and R^{Vb} are both H. In particular embodiments, T^1 is O (oxo), and T^2 is S (thio) or Se (seleno).

[00237] In some embodiments, R^{Vb} is optionally substituted alkoxy carbonyl alkyl or optionally substituted carbamoyl alkyl.
[00238] In particular embodiments, the optional substituent for R_{12a}, R_{12b}, R_{12c}, or R_{Va} is a polyethylene glycol group (e.g., -(CH₂)_s(OCH₂CH₂)_s(CH₂)_sOR’, wherein s is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s₂ and s₃, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R’ is H or C₁₋₅ alkyl; or an amino-polyethylene glycol group (e.g., -NR{N₁}(CH₂)ₘ(CH₂CH₂O)ₙ₋ₙ(CH₂)ₙNR{N₁}, wherein s is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s₂ and s₃, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R{N₁} is, independently, hydrogen or optionally substituted C₁₋₅ alkyl).

[00239] In some embodiments, B is a modified cytosine. Exemplary modified cytosines include compounds (b10)-(b14):

(b10), (b11), (b12), (b13), or (b14), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein

[00240] each of T^3 and T^5 is, independently, H, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy, or the combination of T^3 and T^5 join together (e.g., as in T^5) to form O (oxo), S (thio), or Se (seleno);

[00241] each V^4 is, independently, O, S, N(R^Vc)ₙ₋ₙ, or C(R^Vc)ₙ₋ₙ, wherein n is an integer from 0 to 2 and each R^Vc is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, or optionally substituted alkynyloxy (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl), wherein the combination of R_{13b} and R^Vc can be taken together to form optionally substituted heterocyclyl;

[00242] each V^5 is, independently, N(R^Vd)ₙ₋ₙ, or C(R^Vd)ₙ₋ₙ, wherein n is an integer from 0 to 2 and each R^Vd is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted heterocyclyl, optionally substituted...
alkheterocycl, or optionally substituted alkynylloxy (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl) (e.g., V is –CH or N);

[00243] each of \( R^{13a} \) and \( R^{13b} \) is, independently, H, optionally substituted acyl, optionally substituted acyloxyalkyl, optionally substituted alkyl, or optionally substituted alkoxy, wherein the combination of \( R^{13b} \) and \( R^{14} \) can be taken together to form optionally substituted heterocycl,

[00244] each \( R^{14} \) is, independently, H, halo, hydroxy, thiol, optionally substituted acyl, optionally substituted amino acid, optionally substituted alkyl, optionally substituted haloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl (e.g., substituted with an \( O \)-protecting group), optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkoxyalkyl, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted acyloxyalkyl, optionally substituted amino (e.g., \(-\text{NHR}, \text{ wherein R is H, alkyl, aryl, or phosphoryl}, \text{ azido, optionally substituted aryl, optionally substituted heterocycl, optionally substituted alkylheterocyclyl, optionally substituted aminoalkyl, optionally substituted aminoalkynyl, or optionally substituted aminoalkyl; and}

[00245] each of \( R^{15} \) and \( R^{16} \) is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl.

[00246] Further exemplary modified cytosines include those having Formula (b32)-(b35):

![Chemical Structures](image)

(b32), (b33), (b34), or (b35),

or a pharmaceutically acceptable salt or stereoisomer thereof, wherein

[00247] each of \( T^1 \) and \( T^3 \) is, independently, O (oxo), S (thio), or Se (seleno);

[00248] each of \( R^{13a} \) and \( R^{13b} \) is, independently, H, optionally substituted acyl, optionally substituted acyloxyalkyl, optionally substituted alkyl, or optionally substituted alkoxy, wherein the combination of \( R^{13b} \) and \( R^{14} \) can be taken together to form optionally substituted heterocycl,

[00249] each \( R^{14} \) is, independently, H, halo, hydroxy, thiol, optionally substituted acyl, optionally substituted amino acid, optionally substituted alkyl, optionally substituted haloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl (e.g., substituted with an \( O \)-protecting group), optionally substituted hydroxyalkenyl, optionally
substituted hydroxyalkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynylxyloxy, optionally substituted aminooalkoxy, optionally substituted alkoxyalkoxy, optionally substituted acyloxyalkyl, optionally substituted amino (e.g., -NHR, wherein R is H, alkyl, aryl, or phosphoryl), azido, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted alkhetereocyclyl, optionally substituted aminoalkyl (e.g., hydroxyalkyl, alkyl, alkenyl, or alkynyl), optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl; and

[00250] each of R\textsuperscript{15} and R\textsuperscript{16} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl (e.g., R\textsuperscript{15} is H, and R\textsuperscript{16} is H or optionally substituted alkyl).

[00251] In some embodiments, R\textsuperscript{15} is H, and R\textsuperscript{16} is H or optionally substituted alkyl. In particular embodiments, R\textsuperscript{14} is H, acyl, or hydroxyalkyl. In some embodiments, R\textsuperscript{14} is halo. In some embodiments, both R\textsuperscript{14} and R\textsuperscript{15} are H. In some embodiments, both R\textsuperscript{15} and R\textsuperscript{16} are H. In some embodiments, each of R\textsuperscript{14} and R\textsuperscript{15} and R\textsuperscript{16} is H. In further embodiments, each of R\textsuperscript{13a} and R\textsuperscript{13b} is independently, H or optionally substituted alkyl.

[00252] Further non-limiting examples of modified cytosines include compounds of Formula (b36):

(b36) or a pharmaceutically acceptable salt or stereoisomer thereof, wherein

[00253] each R\textsuperscript{13b} is, independently, H, optionally substituted acyl, optionally substituted acyloxyalkyl, optionally substituted alkyl, or optionally substituted alkoxy, wherein the combination of R\textsuperscript{13b} and R\textsuperscript{14b} can be taken together to form optionally substituted heterocyclyl;

[00254] each R\textsuperscript{14a} and R\textsuperscript{14b} is, independently, H, halo, hydroxy, thiol, optionally substituted acyl, optionally substituted amino acid, optionally substituted alkyl, optionally substituted haloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl (e.g., substituted with an O-protecting group), optionally substituted hydroxyalkenyl, optionally substituted alkoy, optionally substituted alkenyloxy, optionally substituted alkynlyloxy, optionally substituted aminooalkoxy, optionally substituted alkoxyalkoxy, optionally substituted acyloxyalkyl, optionally substituted amino (e.g., -NHR, wherein R is H, alkyl, aryl, phosphoryl, optionally substituted aminoalkyl, or optionally substituted carboxyminoalkyl), azido, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted alkhetereocyclyl, optionally
substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl; and

[00255] each of $R^{15}$ is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl.

[00256] In particular embodiments, $R^{14b}$ is an optionally substituted amino acid (e.g., optionally substituted lysine). In some embodiments, $R^{14a}$ is H.

[00257] In some embodiments, B is a modified guanine. Exemplary modified guanines include compounds of Formula (b15)-(b17):

or a pharmaceutically acceptable salt or stereoisomer thereof, wherein

[00258] each of $T^{4}$, $T^{4'}$, $T^{5'}$, $T^{5}$, and $T^{6'}$ is, independently, H, optionally substituted alkyl, or optionally substituted alkoxy, and wherein the combination of $T^{4'}$ and $T^{4}$ (e.g., as in $T^{4}$) or the combination of $T^{5'}$ and $T^{5}$ (e.g., as in $T^{5}$) or the combination of $T^{6'}$ and $T^{6}$ join together (e.g., as in $T^{6}$) form O (oxo), S (thio), or Se (seleno);

[00259] each of $V^{5}$ and $V^{6}$ is, independently, O, S, N($R_{vd}^{vd}$)$_{nv}$, or C($R_{vd}^{vd}$)$_{nv}$, wherein nv is an integer from 0 to 2 and each $R_{vd}^{vd}$ is, independently, H, halo, thiol, optionally substituted amino acid, cyano, amidine, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynylloxy (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl), optionally substituted thioalkoxy, or optionally substituted amino; and

[00260] each of $R^{17}$, $R^{18}$, $R^{19a}$, $R^{19b}$, $R^{21}$, $R^{22}$, $R^{23}$, and $R^{24}$ is, independently, H, halo, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted thioalkoxy, optionally substituted amino, or optionally substituted amino acid.

[00261] Exemplary modified guanosines include compounds of Formula (b37)-(b40):
wherein

[00262] each of T⁴ is, independently, H, optionally substituted alkyl, or optionally substituted alkoxy, and each T¹ is, independently, O (oxo), S (thio), or Se (seleno);

[00263] each of R¹⁸, R¹⁹a, R¹⁹b, and R²¹ is, independently, H, halo, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted thioalkoxy, optionally substituted amino, or optionally substituted amino acid.

[00264] In some embodiments, R¹⁸ is H or optionally substituted alkyl. In further embodiments, T⁴ is oxo. In some embodiments, each of R¹⁹a and R¹⁹b is, independently, H or optionally substituted alkyl.

[00265] In some embodiments, B is a modified adenine. Exemplary modified adenines include compounds of Formula (b18)-(b20):

or a pharmaceutically acceptable salt or stereoisomer thereof, wherein

[00266] each V⁷ is, independently, O, S, N(R³⁶)ₙ⁷, or C(R³⁶)ₙ⁷, wherein n is an integer from 0 to 2 and each R³⁶ is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, or optionally substituted alkynyloxy (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl);
each R^{25} is, independently, H, halo, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted thioalkoxy, or optionally substituted amino;

each of R^{26a} and R^{26b} is, independently, H, optionally substituted acyl, optionally substituted amino acid, optionally substituted carbamoylalkyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxalkenyl, optionally substituted hydroxalkynyl, optionally substituted alkoxy, or polyethylene glycol group (e.g., -(CH_{2})_{s2}(OCH_{2}CH_{2})_{s3}(CH_{2})_{s4}OR’), wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R’ is H or C_{1-20} alkyl; or an amino-polyethylene glycol group (e.g., -NR^{N1}(CH_{2})_{s2}(CH_{2}CH_{2}O)_{s3}(CH_{2})_{s4}NR^{N1}), wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; 

each R^{27} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted thioalkoxy or optionally substituted amino;

each R^{28} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl; and

each R^{29} is, independently, H, optionally substituted acyl, optionally substituted amino acid, optionally substituted carbamoylalkyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxalkenyl, optionally substituted alkoxy, or optionally substituted amino.

Exemplary modified adenines include compounds of Formula (b41)-(b43):

pharmaceutically acceptable salt or stereoisomer thereof, wherein
[00273] each R\textsuperscript{25} is, independently, H, halo, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted thioalkoxy, or optionally substituted amino;

[00274] each of R\textsuperscript{26a} and R\textsuperscript{26b} is, independently, H, optionally substituted acyl, optionally substituted amino acid, optionally substituted carbamoylalkyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted alkoxy, or polyethylene glycol group (e.g., -(CH\textsubscript{2})\textsubscript{s1}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{s2}(CH\textsubscript{2})\textsubscript{s3}OR', wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C\textsubscript{1-20} alkyl); or an amino-polyethylene glycol group (e.g., -NR\textsuperscript{N1}(CH\textsubscript{2})\textsubscript{s1}(CH\textsubscript{2}CH\textsubscript{2}O)\textsubscript{s2}(CH\textsubscript{2})\textsubscript{s3}NR\textsuperscript{N1}, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R\textsuperscript{N1} is, independently, hydrogen or optionally substituted C\textsubscript{1-6} alkyl); and

[00275] each R\textsuperscript{27} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted thioalkoxy, or optionally substituted amino.

[00276] In some embodiments, R\textsuperscript{26a} is H, and R\textsuperscript{26b} is optionally substituted alkyl. In some embodiments, each of R\textsuperscript{26a} and R\textsuperscript{26b} is, independently, optionally substituted alkyl. In particular embodiments, R\textsuperscript{27} is optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy. In other embodiments, R\textsuperscript{25} is optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkoxy, or optionally substituted thioalkoxy.

[00277] In particular embodiments, the optional substituent for R\textsuperscript{26a}, R\textsuperscript{26b}, or R\textsuperscript{29} is a polyethylene glycol group (e.g., -(CH\textsubscript{2})\textsubscript{s1}(OCH\textsubscript{2}CH\textsubscript{2})\textsubscript{s2}(CH\textsubscript{2})\textsubscript{s3}OR', wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C\textsubscript{1-20} alkyl); or an amino-polyethylene glycol group (e.g., -NR\textsuperscript{N1}(CH\textsubscript{2})\textsubscript{s1}(CH\textsubscript{2}CH\textsubscript{2}O)\textsubscript{s2}(CH\textsubscript{2})\textsubscript{s3}NR\textsuperscript{N1}, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R\textsuperscript{N1} is, independently, hydrogen or optionally substituted C\textsubscript{1-6} alkyl).

[00278] In some embodiments, B may have Formula (b21):
(b21), wherein X^{12} is, independently, O, S, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene, xa is an integer from 0 to 3, and R^{12a} and T^{2} are as described herein.

[00279] In some embodiments, B may have Formula (b22):

(b22), wherein R^{10} is, independently, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted alkoxy, optionally substituted alkoxyethylalkyl, optionally substituted alkoxyethylalkenyl, optionally substituted alkoxyethylalkynyl, optionally substituted alkoxyethylalkoxy, optionally substituted alkoxyethylalkoxy, optionally substituted carboxyalkyl, or optionally substituted carbamoylalkyl, and R^{11}, R^{12a}, T^{1}, and T^{2} are as described herein.

[00280] In some embodiments, B may have Formula (b23):

(b23), wherein R^{10} is optionally substituted heterocyclyl (e.g., optionally substituted furyl, optionally substituted thienyl, or optionally substituted pyrrolyl), optionally substituted aryl (e.g., optionally substituted phenyl or optionally substituted naphthyl), or any substituent described herein (e.g., for R^{10}); and wherein R^{11} (e.g., H or any substituent described herein), R^{12a} (e.g., H or any substituent described herein), T^{1} (e.g., oxo or any substituent described herein), and T^{2} (e.g., oxo or any substituent described herein) are as described herein.

[00281] In some embodiments, B may have Formula (b24):
(b24), wherein R\textsuperscript{14} is, independently, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted alkaryl, optionally substituted alkhetercyclyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted alkoxy, optionally substituted alkoxyarylalkyl, optionally substituted alkoxyarylalkenyl, optionally substituted alkoxyarylalkynyl, optionally substituted alkoxyarylalkoxy, optionally substituted carboxyalkoxy, optionally substituted carboxyalkyl, or optionally substituted carboxyarylalkyl, and R\textsuperscript{13a}, R\textsuperscript{13b}, R\textsuperscript{15}, and T\textsuperscript{3} are as described herein.

[00282] In some embodiments, B may have Formula (b25):

(b25), wherein R\textsuperscript{14} is optionally substituted heterocyclyl (e.g., optionally substituted furyl, optionally substituted thiophenyl, or optionally substituted pyrrolidinyl), optionally substituted aryl (e.g., optionally substituted phenyl or optionally substituted naphthyl), or any substituent described herein (e.g., for R\textsuperscript{14} or R\textsuperscript{14}); and wherein R\textsuperscript{13a} (e.g., H or any substituent described herein), R\textsuperscript{13b} (e.g., H or any substituent described herein), R\textsuperscript{15} (e.g., H or any substituent described herein), and T\textsuperscript{3} (e.g., oxo or any substituent described herein) are as described herein.

[00283] In some embodiments, B is a nucleobase selected from the group consisting of cytosine, guanine, adenine, and uracil. In some embodiments, B may be:

(b26) or (b27).

[00284] 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 (\(\text{s}^2\text{U}\)), 4-thio-
uridine (sU), 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxy-uridine (hoU), 5-aminopropyl-
uridine, 5-halo-uridine (e.g., 5-iodo-uridine or 5-bromo-uridine), 3-methyl-uridine (mU), 5-
methoxy-uridine (moU), uridine 5-oxyacetic acid (cmoU), uridine 5-oxyacetic acid methyl ester
(mcmoU), 5-carboxymethyl-uridine (cmU), 1-carboxymethyl-pseudouridine, 5-
carboxyhydroxymethyl-uridine (chmU), 5-carboxyhydroxymethyl-uridine methyl ester (mehmU),
5-methoxycarbonyl-uridine (mcmU), 5-methoxycarbonylmethyl-2-thio-uridine (mcmU),
5-aminomethyl-2-thio-uridine (nmU), 5-methylaminomethyl-uridine (mmU), 5-
methylaminomethyl-2-thio-uridine (mmU), 5-methylaminomethyl-2-seleno-uridine (msU),
5-carbamoylmethyl-uridine (ncmU), 5-carboxymethylaminomethyl-uridine (ccmU), 5-
carboxyethylaminomethyl-2-thio-uridine (ccmU), 5-propynyl-uridine, 1-propynyl-
pseudouridine, 2-thio-1-propynyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-
pseudouridine, dihydrouridine (D), dihydropseudouridine, 5,6-dihydrouridine, 5-methyl-
dihydrouridine (mD), 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, 3-(3-amino-3-carboxypropyl)uridine (acpU), 1-methyl-3-(3-amino-3-
carboxypropyl)pseudouridine (acpU), 5-(isopentenylaminomethyl)uridine (ImmU), 5-
(isopentenylaminomethyl)-2-thio-uridine (ImmU), α-thio-uridine, 2′-O-methyl-uridine (Um), 5,2′-
O-dimethyl-uridine (mUm), 2′-O-methyl-pseudouridine (pUm), 2-thio-2′-O-methyl-uridine (sUm),
5-methoxycarbonylmethyl-2′-O-methyl-uridine (mcmUm), 5-carbamoylmethyl-2′-O-methyl-
uridine (nmmUm), 5-carboxymethylaminomethyl-2′-O-methyl-urate (mmmmUm), 3,2′-O-
dimethyl-uridine (mUm), 5-(isopentenylaminomethyl)-2′-O-methyl-uridine (immU), 1-thio-
uridine, deoxythymidine, 2′-F-arabinoside, 2′-F-uridine, 2′-OH-arabinoside, 5-(2-carboxyethoxy vinyl)
uridine, and 5-[3-(1-E-propenylamino)uridine].

[00285] In some embodiments, the modified nucleobase is a modified cytosine. Exemplary
nucleobases and nucleosides having a modified cytosine include 5-aza-9-cytidine, 6-aza-cytidine,
pseudoisocytidine, 3-methyl-cytidine (mC), N4-acetyl-cytidine (acC), 5-formyl-cytidine (fC), N4-
methyl-cytidine (mC), 5-methyl-cytidine (mC), 5-halo-cytidine (e.g., 5-iodo-cytidine), 5-
hydroxymethyl-cytidine (hm³C), 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, zebaruline, 5-aza-zebaruline, 5-methyl-zebaruline, 5-aza-2-thio-zebaruline, 2-
thio-zebaruline, 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 (m²Cm), 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 (m³₂Cm),
1-thio-cytidine, 2'-F-ara-cytidine, 2'-F-cytidine, and 2'-OH-ara-cytidine.

[00286] 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 (t⁶A), 2-methylthio-N6-
isopentenyl-adenosine (ms²t⁶A), N6-(cis-hydroxyisopentenyl)adenosine (io⁶A), 2-methylthio-N6-
(cis-hydroxyisopentenyl)adenosine (ms²io⁶A), N6-glycinylcarbamoyl-adenosine (g⁶A), N6-
threonylcarbamoyl-adenosine (t⁶A), N6-methyl-N6-threonylcarbamoyl-adenosine (m⁶t⁶A), 2-
methylthio-N6-threonylcarbamoyl-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 (m³₂Am), 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-pentaoxanoaddeyl)-adenosine.

[00287] 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), epoxycuesuosine (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-thioguanosine, 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 (m²²⁻⁷G), 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.

[00288] The nucleobase of the nucleotide can be independently selected from a purine, a pyrimidine, a purine or pyrimidine analog. For example, the nucleobase can each be independently selected from adenine, cytosine, guanine, uracil, or hypoxanthine. In another embodiment, the nucleobase can also include, for example, naturally-occurring and synthetic derivatives of a base, including pyrazolo[3,4-d]pyrimidines, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo (e.g., 8-bromo), 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adeneses and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, deazaguanine, 7-deazaguanine, 3-deazaguanine, deazaadenine, 7-deazaadenine, 3-deazaadenine, pyrazolo[3,4-d]pyrimidine, imidazo[1,5-a]1,3,5 triazinones, 9-deazapurines, imidazo[4,5-d]pyrazines, thiazolo[4,5-d]pyrimidines, pyrazin-2-ones, 1,2,4-triazine, pyridazine; and 1,3,5 triazine. When the nucleotides are depicted using the shorthand A, G, C, T or U, each letter refers to the representative base and/or derivatives thereof, e.g., A includes adenine or adenine analogs, e.g., 7-deaza adenine).
Modifications on the Internucleoside Linkage

[00289] The modified nucleosides and nucleotides, which may be incorporated into a modified nucleic acid or mmRNA molecule, can be modified on the internucleoside linkage (e.g., phosphate backbone). The phosphate groups of the backbone can be modified by replacing one or more of the oxygen atoms with a different substituent. Further, the modified nucleosides and nucleotides can include the wholesale replacement of an unmodified phosphate moiety with a modified phosphate as described herein. Examples of modified phosphate groups include, but are not limited to, phosphorothioate, phosphoroselenates, boranophosphates, boranophosphate esters, hydrogen phosphonates, phosphoramidates, phosphorodiamidates, alkyl or aryl phosphonates, and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. The phosphate linker can also be modified by the replacement of a linking oxygen with nitrogen (bridged phosphoramidates), sulfur (bridged phosphorothioates), and carbon (bridged methylene-phosphonates).

[00290] The α-thio substituted phosphate moiety is provided to confer stability to RNA and DNA polymers through the unnatural phosphorothioate backbone linkages. Phosphorothioate DNA and RNA have increased nuclease resistance and subsequently a longer half-life in a cellular environment. Phosphorothioate linked modified nucleic acids or mmRNA molecules are expected to also reduce the innate immune response through weaker binding/activation of cellular innate immune molecules.

[00291] In specific embodiments, a modified nucleoside includes an alpha-thio-nucleoside (e.g., 5'-O-(1-thiophosphate)-adenosine, 5'-O-(1-thiophosphate)-cytidine (α-thio-cytidine), 5'-O-(1-thiophosphate)-guanosine, 5'-O-(1-thiophosphate)-uridine, or 5'-O-(1-thiophosphate)- pseudouridine).

Combinations of Modified Sugars, Nucleobases, and Internucleoside Linkages

[00292] The modified nucleic acids and mmRNA of the invention can include a combination of modifications to the sugar, the nucleobase, and/or the internucleoside linkage. These combinations can include any one or more modifications described herein. For examples, any of the nucleotides described herein in Formulas (Ia), (Ia-1)-(Ia-3), (Ib)-(Ilf), (Iia)-(IIp), (Iib-1), (Iib-2), (Iic-1)-(Iic-2), (Iin-1), (Iin-2), (IVa)-(IVl), and (IXa)-(IXr) can be combined with any of the nucleobases described herein (e.g., in Formulas (b1)-(b43) or any other described herein).

Synthesis of Modified Nucleic Acids and mmRNA Molecules
The modified nucleic acid and mmRNA molecules for use in accordance with the invention may be prepared according to any useful technique, as described herein. The modified nucleosides and nucleotides used in the synthesis of modified nucleic acid and mmRNA molecules disclosed herein can be prepared from readily available starting materials using the following general methods and procedures. Where typical or preferred process conditions (e.g., reaction temperatures, times, mole ratios of reactants, solvents, pressures, etc.) are provided, a skilled artisan would be able to optimize and develop additional process conditions. Optimum reaction conditions may vary with the particular reactants or solvent used, but such conditions can be determined by one skilled in the art by routine optimization procedures.

The processes described herein can be monitored according to any suitable method known in the art. For example, product formation can be monitored by spectroscopic means, such as nuclear magnetic resonance spectroscopy (e.g., $^1$H or $^{13}$C) infrared spectroscopy, spectrophotometry (e.g., UV-visible), or mass spectrometry, or by chromatography such as high performance liquid chromatography (HPLC) or thin layer chromatography.

Preparation of modified nucleic acid and mmRNA molecules of the present invention can involve the protection and deprotection of various chemical groups. The need for protection and deprotection, and the selection of appropriate protecting groups can be readily determined by one skilled in the art. The chemistry of protecting groups can be found, for example, in Greene, et al., Protective Groups in Organic Synthesis, 2d. Ed., Wiley & Sons, 1991, which is incorporated herein by reference in its entirety.

The reactions of the processes described herein can be carried out in suitable solvents, which can be readily selected by one of skill in the art of organic synthesis. Suitable solvents can be substantially nonreactive with the starting materials (reactants), the intermediates, or products at the temperatures at which the reactions are carried out, i.e., temperatures which can range from the solvent’s freezing temperature to the solvent’s boiling temperature. A given reaction can be carried out in one solvent or a mixture of more than one solvent. Depending on the particular reaction step, suitable solvents for a particular reaction step can be selected.

Resolution of racemic mixtures of modified nucleosides and nucleotides can be carried out by any of numerous methods known in the art. An example method includes fractional recrystallization using a “chiral resolving acid” which is an optically active, salt-forming organic acid. Suitable resolving agents for fractional recrystallization methods are, for example, optically
active acids, such as the D and L forms of tartaric acid, diacetyltartaric acid, dibenzoyltartaric acid, mandelic acid, malic acid, lactic acid or the various optically active camphorsulfonic acids. Resolution of racemic mixtures can also be carried out by elution on a column packed with an optically active resolving agent (e.g., dinitrobenzoylephosphorylglycine). Suitable elution solvent composition can be determined by one skilled in the art.

[00298] Modified nucleosides and nucleotides (e.g., binding block molecules) can be prepared according to the synthetic methods described in Ogata et al., J. Org. Chem. 74:2585-2588 (2009); Purmal et al., Nucl. Acids Res. 22(1): 72-78, (1994); Fukuwara et al., Biochemistry, 1(4): 563-568 (1962); and Xu et al., Tetrahedron, 48(9): 1729-1740 (1992), each of which are incorporated by reference in their entirety.

[00299] The modified nucleic acid and mmRNA of the invention need not be uniformly modified along the entire length of the molecule. For example, one or more or all types of nucleotide (e.g., purine or pyrimidine, or any one or more or all of A, G, U, C) may or may not be uniformly modified in a polynucleotide of the invention, or in a given predetermined sequence region thereof. In some embodiments, all nucleotides X in a polynucleotide of the invention (or in a given sequence region thereof) are modified, 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

[00300] Different sugar modifications, nucleotide modifications, and/or internucleoside linkages (e.g., backbone structures) may exist at various positions in the modified nucleic acid or mmRNA. One of ordinary skill in the art will appreciate that the nucleotide analogs or other modification(s) may be located at any position(s) of a modified nucleic acid or mmRNA such that the function of the modified nucleic acid or mmRNA is not substantially decreased. A modification may also be a 5’ or 3’ terminal modification. The modified nucleic acid or mmRNA may contain from about 1% to about 100% modified nucleotides, 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 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%).

[00301] In some embodiments, the modified nucleic acid or mmRNA includes a modified pyrimidine (e.g., a modified uracil/uridine or modified cytosine/cytidine). In some embodiments, the uracil or uridine in the modified nucleic acid or mmRNA molecule may be replaced with from about 1% to about 100% of a modified uracil or modified uridine (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 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% of a modified uracil or modified uridine). The modified uracil or uridine can be replaced by a compound having a single unique structure or by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures, as described herein). In some embodiments, the cytosine or cytidine in the modified nucleic acid or mmRNA molecule may be replaced with from about 1% to about 100% of a modified cytosine or modified cytidine (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% of a modified cytosine or modified cytidine). The modified cytosine or cytidine can be replaced by a compound having a single unique structure or by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures, as described herein).
[00302] In some embodiments, the present disclosure provides methods of synthesizing a modified nucleic acid or mmRNA including n number of linked nucleosides having Formula (Ia-1):

\[
\begin{array}{c}
Y^1 - Y^5 \\
Y^3 - Y^2 \\
Y^4 \\
B \\
R^1 \\
R^2 \\
R^3 \\
R^5 \\
(\text{Ia-1}), \text{ comprising:}
\end{array}
\]

[00303] a) reacting a nucleotide of Formula (IV-1):

\[
\begin{array}{c}
Y^1 - Y^5 \\
Y^3 - Y^2 \\
Y^4 \\
B \\
R^1 \\
R^2 \\
R^3 \\
R^5 \\
(\text{IV-1}),
\end{array}
\]

[00304] with a phosphoramidite compound of Formula (V-1):

\[
\begin{array}{c}
p^1 - Y^1 - Y^5 \\
Y^3 - Y^2 \\
Y^4 \\
B \\
R^1 \\
R^2 \\
R^3 \\
R^5 \\
(\text{V-1}),
\end{array}
\]

[00305] wherein \(Y^3\) is H, hydroxy, phosphoryl, pyrophosphate, sulfate, amino, thiol, optionally substituted amino acid, or a peptide (e.g., including from 2 to 12 amino acids); and each \(p^1\), \(p^2\), and \(p^3\) is, independently, a suitable protecting group; and \(\bigcirc\) denotes a solid support;

[00306] to provide a modified nucleic acid or mmRNA of Formula (VI-1):
b) oxidizing or sulfurizing the modified nucleic acid or mmRNA of Formula (V) to yield a modified nucleic acid or mmRNA of Formula (VII-1):

\[ \text{(VI-1), and} \]

\[ \text{(VII-1), and} \]

[00307]  c) removing the protecting groups to yield the modified nucleic acid or mmRNA of Formula (Ia).

[00308]  In some embodiments, steps a) and b) are repeated from 1 to about 10,000 times. In some embodiments, the methods further comprise a nucleotide (e.g., building block molecule) selected from the group consisting of adenosine, cytosine, guanosine, and uracil. In some embodiments, the nucleobase may be a pyrimidine or derivative thereof. In some embodiments, the modified nucleic acid or mmRNA is translatable.

[00309]  Other components of modified nucleic acids and mmRNA are optional, and are beneficial in some embodiments. For example, a 5′ untranslated region (UTR) and/or a 3′UTR are provided, wherein either or both may independently contain one or more different nucleoside modifications. In such embodiments, nucleoside modifications may also be present in the translatable region. Also provided are modified nucleic acids and mmRNA containing a Kozak sequence.
Exemplary syntheses of modified nucleotides, which are incorporated into a modified nucleic acid or mRNA, e.g., RNA or mRNA, are provided below in Scheme 1 through Scheme 11. Scheme 1 provides a general method for phosphorylation of nucleosides, including modified nucleosides.

Scheme 1

Various protecting groups may be used to control the reaction. For example, Scheme 2 provides the use of multiple protecting and deprotecting steps to promote phosphorylation at the 5' position of the sugar, rather than the 2' and 3' hydroxyl groups.
Modified nucleotides can be synthesized in any useful manner. Schemes 3, 4, and 7 provide exemplary methods for synthesizing modified nucleotides having a modified purine nucleobase; and Schemes 5 and 6 provide exemplary methods for synthesizing modified nucleotides having a modified pseudouridine or pseudoisocytidine, respectively.
Scheme 6

\[
\text{RBr/Heat}
\]

R = alkyl, alkenyl, allyl, and benzyl

1) POCl₃
2) Pyrophosphate
[00314] Schemes 8 and 9 provide exemplary syntheses of modified nucleotides. Scheme 10 provides a non-limiting biocatalytic method for producing nucleotides.
Scheme 8
Scheme 9

Scheme 10

[00315] Scheme 11 provides an exemplary synthesis of a modified uracil, where the N1 position is modified with R\textsuperscript{12b}, as provided elsewhere, and the 5′-position of ribose is phosphorylated. T\textsuperscript{1}, T\textsuperscript{2}, R\textsuperscript{12a}, R\textsuperscript{12b}, and r are as provided herein. This synthesis, as well as optimized versions thereof, can be used to modify other pyrimidine nucleobases and purine nucleobases (see e.g., Formulas (b1)-(b43)) and/or to install one or more phosphate groups (e.g., at the 5′ position of the sugar). This alkylating reaction can also be used to include one or more optionally substituted alkyl group at any reactive group (e.g., amino group) in any nucleobase described herein (e.g., the amino groups in the Watson-Crick base-pairing face for cytosine, uracil, adenine, and guanine).

Scheme 11
Combinations of Nucleotides in mmRNA

[00316] Further examples of modified nucleotides and modified nucleotide combinations are provided below in Table 2. These combinations of modified nucleotides can be used to form the modified nucleic acids or mmRNA of the invention. Unless otherwise noted, the modified nucleotides may be completely substituted for the natural nucleotides of the modified nucleic acids or mmRNA of the invention. As a non-limiting example, the natural nucleotide uridine may be substituted with a modified nucleoside described herein. In another non-limiting example, the natural nucleotide uridine may be partially substituted (e.g., about 0.1%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99.9%) with at least one of the modified nucleoside disclosed herein.

Table 2

<table>
<thead>
<tr>
<th>Modified Nucleotide</th>
<th>Modified Nucleotide Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-thio-cytidine</td>
<td>α-thio-cytidine/5-ido-uridine</td>
</tr>
<tr>
<td></td>
<td>α-thio-cytidine/N1-methyl-pseudouridine</td>
</tr>
<tr>
<td></td>
<td>α-thio-cytidine/α-thio-uridine</td>
</tr>
<tr>
<td></td>
<td>α-thio-cytidine/5-methyl-uridine</td>
</tr>
<tr>
<td></td>
<td>α-thio-cytidine/pseudouridine</td>
</tr>
<tr>
<td></td>
<td>about 50% of the cytosines are α-thio-cytidine</td>
</tr>
<tr>
<td>pseudoisocytidine</td>
<td>pseudoisocytidine/5-ido-uridine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/N1-methyl-pseudouridine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/α-thio-uridine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/5-methyl-uridine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/pseudouridine</td>
</tr>
<tr>
<td></td>
<td>about 25% of cytosines are pseudoisocytidine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/about 50% of uridines are N1-methyl-pseudouridine and about 50% of uridines are pseudouridine</td>
</tr>
<tr>
<td></td>
<td>pseudoisocytidine/about 25% of uridines are N1-methyl-pseudouridine and about 25% of uridines are pseudouridine</td>
</tr>
<tr>
<td>pyrrolo-cytidine</td>
<td>pyrrolo-cytidine/5-ido-uridine</td>
</tr>
<tr>
<td></td>
<td>pyrrolo-cytidine/N1-methyl-pseudouridine</td>
</tr>
<tr>
<td></td>
<td>pyrrolo-cytidine/α-thio-uridine</td>
</tr>
<tr>
<td></td>
<td>pyrrolo-cytidine/5-methyl-uridine</td>
</tr>
</tbody>
</table>
Further examples of modified nucleotide combinations are provided below in Table 3.
These combinations of modified nucleotides can be used to form the modified nucleic acid molecules or mRNA of the invention.

<table>
<thead>
<tr>
<th>Modified Nucleotide</th>
<th>Modified Nucleotide Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>modified cytidine having one or more nucleobases of Formula (b10)</td>
<td>modified cytidine with (b10)/pseudouridine</td>
</tr>
<tr>
<td></td>
<td>modified cytidine with (b10)/N1-methyl-pseudouridine</td>
</tr>
<tr>
<td></td>
<td>modified cytidine with (b10)/5-methoxy-uridine</td>
</tr>
<tr>
<td></td>
<td>modified cytidine with (b10)/5-methyl-uridine</td>
</tr>
<tr>
<td></td>
<td>modified cytidine with (b10)/5-bromo-uridine</td>
</tr>
<tr>
<td></td>
<td>modified cytidine with (b10)/2-thio-uridine</td>
</tr>
<tr>
<td></td>
<td>about 50% of cytidine substituted with modified cytidine (b10)/ about 50% of uridines are 2-thio-uridine</td>
</tr>
<tr>
<td>modified cytidine having one or more nucleobases of Formula (b32)</td>
<td>modified cytidine with (b32)/pseudouridine</td>
</tr>
<tr>
<td></td>
<td>modified cytidine with (b32)/N1-methyl-pseudouridine</td>
</tr>
<tr>
<td></td>
<td>modified cytidine with (b32)/5-methoxy-uridine</td>
</tr>
<tr>
<td></td>
<td>modified cytidine with (b32)/5-methyl-uridine</td>
</tr>
<tr>
<td></td>
<td>modified cytidine with (b32)/5-bromo-uridine</td>
</tr>
<tr>
<td>modified uridine having one or more nucleobases of Formula (b1)</td>
<td>modified uridine with (b1)/ N4-acetyl-cytidine</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>modified uridine having one or more nucleobases of Formula (b8)</td>
<td>modified uridine with (b8)/ N4-acetyl-cytidine</td>
</tr>
<tr>
<td>modified uridine having one or more nucleobases of Formula (b28)</td>
<td>modified uridine with (b28)/ N4-acetyl-cytidine</td>
</tr>
<tr>
<td>modified uridine having one or more nucleobases of Formula (b29)</td>
<td>modified uridine with (b29)/ N4-acetyl-cytidine</td>
</tr>
<tr>
<td>modified uridine having one or more nucleobases of Formula (b30)</td>
<td>modified uridine with (b30)/ N4-acetyl-cytidine</td>
</tr>
</tbody>
</table>

**[00318]** In some embodiments, at least 25% of the cytosines are replaced by a compound of Formula (b10)-(b14) (e.g., at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%).

**[00319]** In some embodiments, at least 25% of the uracils are replaced by a compound of Formula (b1)-(b9) (e.g., at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%).

**[00320]** In some embodiments, at least 25% of the cytosines are replaced by a compound of Formula (b10)-(b14), and at least 25% of the uracils are replaced by a compound of Formula (b1)-(b9) (e.g., at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%).
Synthesis of Modified Nucleic Acid Molecules

[00321] Modified nucleic acid molecules for use in accordance with the present disclosure may be prepared according to any available technique including, but not limited to, in vitro transcription such as chemical synthesis and enzymatic synthesis, or enzymatic and chemical cleavage of a longer precursor, etc. Methods of synthesizing RNA are known in the art (see, e.g., Gait, M.J. (ed.) Oligonucleotide synthesis: a practical approach, Oxford [Oxfordshire], Washington, DC: IRL Press, 1984; and Herdewijn, P. (ed.) Oligonucleotide synthesis: methods and applications, Methods in Molecular Biology, v. 288 (Clifton, N.J.) Totowa, N.J.: Humana Press, 2005; both of which are incorporated herein by reference).

[00322] The modified nucleic acid molecules disclosed herein can be prepared from readily available starting materials using the following general methods and procedures. It is understood that where typical or preferred process conditions (i.e., reaction temperatures, times, mole ratios of reactants, solvents, pressures, etc.) are given other process conditions can also be used unless otherwise stated. Optimum reaction conditions may vary with the particular reactants or solvent used, but such conditions can be determined by one skilled in the art by routine optimization procedures.

[00323] The processes described herein can be monitored according to any suitable method known in the art. For example, product formation can be monitored by spectroscopic means, such as nuclear magnetic resonance spectroscopy (e.g., $^1$H or $^{13}$C) infrared spectroscopy, spectrophotometry (e.g., UV-visible), mass spectrometry, or by chromatography such as high performance liquid chromatography (HPLC) or thin layer chromatography.

[00324] Preparation of modified nucleic acid molecules can involve the protection and deprotection of various chemical groups. The need for protection and deprotection, and the selection of appropriate protecting groups can be readily determined by one skilled in the art. The chemistry of protecting groups can be found, for example, in Greene, et al., Protective Groups in Organic Synthesis, 2d. Ed., Wiley & Sons, 1991, which is incorporated herein by reference in its entirety.

[00325] The reactions of the processes described herein can be carried out in suitable solvents, which can be readily selected by one of skill in the art of organic synthesis. Suitable solvents can be substantially nonreactive with the starting materials (reactants), the intermediates, or products at the temperatures at which the reactions are carried out, i.e., temperatures which can range from the solvent’s freezing temperature to the solvent’s boiling temperature. A given reaction can be carried
out in one solvent or a mixture of more than one solvent. Depending on the particular reaction step, suitable solvents for a particular reaction step can be selected.

[00326] Resolution of racemic mixtures of modified nucleic acid molecules can be carried out by any of numerous methods known in the art. An example method includes, but is not limited to, fractional recrystallization using a “chiral resolving acid” which is an optically active, salt-forming organic acid. Suitable resolving agents for fractional recrystallization methods are, for example, optically active acids, such as the D and L forms of tartaric acid, diacetyltartaric acid, dibenzoyltartaric acid, mandelic acid, malic acid, lactic acid or the various optically active camphorsulfonic acids. Resolution of racemic mixtures can also be carried out by elution on a column packed with an optically active resolving agent (e.g., dinitrobenzoylphenylglycine). Suitable elution solvent composition can be determined by one skilled in the art.

[00327] Modified nucleic acid molecules need not be uniformly modified along the entire length of the molecule. Different nucleic acid modifications and/or backbone structures may exist at various positions in the nucleic acid. One of ordinary skill in the art will appreciate that the nucleotide analogs or other modification(s) may be located at any position(s) of a nucleic acid such that the function of the nucleic acid is not substantially decreased. A modification may also be a 5’ or 3’ terminal modification. The nucleic acids may contain at a minimum one modified nucleotide 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 nucleic acids may contain a modified pyrimidine such as 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 may be replaced with a modified 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 nucleic acid may be replaced with a modified 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).

[00328] Generally, the shortest length of a modified mRNA, herein “mmRNA,” of the present disclosure can be the length of an mRNA sequence that may be sufficient to encode for a dipeptide.
In another embodiment, the length of the mRNA sequence may be sufficient to encode for a tripeptide. In another embodiment, the length of an mRNA sequence may be sufficient to encode for a tetrapeptide. In another embodiment, the length of an mRNA sequence may be sufficient to encode for a pentapeptide. In another embodiment, the length of an mRNA sequence may be sufficient to encode for a hexapeptide. In another embodiment, the length of an mRNA sequence may be sufficient to encode for a heptapeptide. In another embodiment, the length of an mRNA sequence may be sufficient to encode for an octapeptide. In another embodiment, the length of an mRNA sequence may be sufficient to encode for a nonapeptide. In another embodiment, the length of an mRNA sequence may be sufficient to encode for a decapeptide.

[00329] Examples of dipeptides that the modified nucleic acid molecule sequences can encode for include, but are not limited to, carnosine and anserine.

[00330] In a further embodiment, the mRNA may be greater than 30 nucleotides in length. In another embodiment, the RNA molecule may be 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, 3,000, 4,000 and 5,000 nucleotides).

**Exemplary Properties of Modified Nucleic Acid Molecules**

**Major Groove Interacting Partners**

[00331] The modified nucleic acid molecules, e.g., modified mRNA (mmRNA), described herein can disrupt interactions with recognition receptors that detect and respond to RNA ligands through interactions, e.g. binding, with the major groove face of a nucleotide or nucleic acid. As such, RNA ligands comprising modified nucleotides or modified nucleic acid molecules, as described herein, decrease interactions with major groove binding partners, and therefore decrease an innate immune response, or expression and secretion of pro-inflammatory cytokines, or both.

[00332] Example major groove interacting, e.g. binding, partners include, but are not limited to, the following nucleases and helicases. Within membranes, TLRs (Toll-like Receptors) 3, 7, and 8 can respond to single- and double-stranded RNA. Within the cytoplasm, members of the superfamily 2 class of DEX(D/H) helicases and ATPases can sense RNA to initiate antiviral responses. These helicases include the RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation-associated gene 5). Other examples include laboratory of genetics and physiology 2 (LGP2), HIN-200 domain containing proteins, or Helicase-domain containing proteins.
Prevention or Reduction of Innate Cellular Immune Response Activation Using Modified Nucleic Acid Molecules

[00333] The modified nucleic acid molecules, e.g., mmRNA, described herein, decrease the innate immune response in a cell. The term “innate immune response” includes a cellular response to exogenous nucleic acids, including, but not limited to, single stranded nucleic acids, generally of viral or bacterial origin, which involve the induction of cytokine expression and release, particularly the interferons, and cell death. Protein synthesis may also be reduced during the innate cellular immune response. While it is advantageous to eliminate the innate immune response in a cell, the present disclosure provides modified mRNA that substantially reduce the immune response, including interferon signaling, without entirely eliminating such a response. In some embodiments, the immune response may be reduced by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, 99.9%, or greater than 99.9% as compared to the immune response induced by a corresponding unmodified nucleic acid molecule. Such a reduction can be measured by the expression or activity level of Type 1 interferons or the expression of interferon-regulated genes such as the toll-like receptors (e.g., TLR7 and TLR8). Reduction of the innate immune response can also be measured by decreased cell death following one or more administrations of modified RNA to a cell population; e.g., cell death is 10%, 25%, 50%, 75%, 85%, 90%, 95%, or over 95% less than the cell death frequency observed with a corresponding unmodified nucleic acid molecule. Moreover, cell death may affect fewer than 50%, 40%, 30%, 20%, 10%, 5%, 1%, 0.1%, 0.01% or fewer than 0.01% of cells contacted with the modified nucleic acid molecules.

[00334] The present disclosure provides for the repeated introduction (e.g., transfection) of modified nucleic acid molecules into a target cell population, e.g., in vitro, ex vivo, or in vivo. The step of contacting the cell population may be repeated one or more times (such as two, three, four, five or more than five times). In some embodiments, the step of contacting the cell population with the modified nucleic acid molecules may be repeated a number of times sufficient such that a predetermined efficiency of protein translation in the cell population is achieved. Given the reduced cytotoxicity of the target cell population by the nucleic acid modifications, such repeated transfections are achievable in a variety of cell types.

[00335] The modified nucleic acids of the invention, including the combination of modifications taught herein may have superior properties making them more suitable as therapeutic modalities.
It has been determined that the “all or none” model in the art is sorely insufficient to describe the biological phenomena associated with the therapeutic utility of modified mRNA. The present inventors have determined that to improve protein production, one may consider the nature of the modification, or combination of modifications, the percent modification and survey more than one cytokine or metric to determine the efficacy and risk profile of a particular modified mRNA.

In one aspect of the invention, methods of determining the effectiveness of a modified mRNA as compared to unmodified involves the measure and analysis of one or more cytokines whose expression is triggered by the administration of the exogenous nucleic acid of the invention. These values are compared to administration of an unmodified nucleic acid or to a standard metric such as cytokine response, PolyIC, R-848 or other standard known in the art.

One example of a standard metric developed herein is the measure of the ratio of the level or amount of encoded polypeptide (protein) produced in the cell, tissue or organism to the level or amount of one or more (or a panel) of cytokines whose expression is triggered in the cell, tissue or organism as a result of administration or contact with the modified nucleic acid. Such ratios are referred to herein as the Protein:Cytokine Ratio or “PC” Ratio. The higher the PC ratio, the more efficacious the modified nucleic acid (polynucleotide encoding the protein measured). Preferred PC Ratios, by cytokine, of the present invention may be greater than 1, greater than 10, greater than 100, greater than 1000, greater than 10,000 or more. Modified nucleic acids having higher PC Ratios than a modified nucleic acid of a different or unmodified construct are preferred.

The PC ratio may be further qualified by the percent modification present in the polynucleotide. For example, normalized to a 100% modified nucleic acid, the protein production as a function of cytokine (or risk) or cytokine profile can be determined.

In one embodiment, the present invention provides a method for determining, across chemistries, cytokines or percent modification, the relative efficacy of any particular modified polynucleotide by comparing the PC Ratio of the modified nucleic acid (polynucleotide).

Activation of the immune response: Vaccines

In one embodiment of the present invention, mRNA molecules may be used to elicit or provoke an immune response in an organism. The mRNA molecules to be delivered may encode an immunogenic peptide or polypeptide and may encode more than one such peptide or polypeptide.

Additionally, certain modified nucleosides, or combinations thereof, when introduced into the modified nucleic acid molecules or mRNA of the invention will activate the innate immune
response. Such activating molecules are useful as adjuvants when combined with polypeptides and/or other vaccines. In certain embodiments, the activating molecules contain a translatable region which encodes for a polypeptide sequence useful as a vaccine, thus providing the ability to be a self-adjuvant.

[00343] In one embodiment, the modified nucleic acid molecules and/or mmRNA of the invention may encode an immunogen. The delivery of modified nucleic acid molecules and/or mmRNA encoding an immunogen may activate the immune response. As a non-limiting example, the modified nucleic acid molecules and/or mmRNA encoding an immunogen may be delivered to cells to trigger multiple innate response pathways (see International Pub. No. WO2012006377; herein incorporated by reference in its entirety). As another non-limiting example, the modified nucleic acid molecules and mmRNA of the present invention encoding an immunogen may be delivered to a vertebrate in a dose amount large enough to be immunogenic to the vertebrate (see International Pub. No. WO2012006372 and WO2012006369; each of which is herein incorporated by reference in their entirety).

[00344] The modified nucleic acid molecules or mmRNA of invention may encode a polypeptide sequence for a vaccine and may further comprise an inhibitor. The inhibitor may impair antigen presentation and/or inhibit various pathways known in the art. As a non-limiting example, the modified nucleic acid molecules or mmRNA of the invention may be used for a vaccine in combination with an inhibitor which can impair antigen presentation (see International Pub. No. WO2012089225 and WO2012089338; each of which is herein incorporated by reference in their entirety).

[00345] In one embodiment, the modified nucleic acid molecules or mmRNA of the invention may be self-replicating RNA. Self-replicating RNA molecules can enhance efficiency of RNA delivery and expression of the enclosed gene product. In one embodiment, the modified nucleic acid molecules or mmRNA may comprise at least one modification described herein and/or known in the art. In one embodiment, the self-replicating RNA can be designed so that the self-replicating RNA does not induce production of infectious viral particles. As a non-limiting example the self-replicating RNA may be designed by the methods described in US Pub. No. US20110300205 and International Pub. No. WO2011005799, each of which is herein incorporated by reference in their entirety.
In one embodiment, the self-replicating modified nucleic acid molecules or mmRNA of the invention may encode a protein which may raise the immune response. As a non-limiting example, the modified nucleic acid molecules and/or mmRNA may be self-replicating mRNA may encode at least one antigen (see US Pub. No. US20110300205 and International Pub. No. WO2011005799; each of which is herein incorporated by reference in their entirety).

In one embodiment, the self-replicating modified nucleic acids or mmRNA of the invention may be formulated using methods described herein or known in the art. As a non-limiting example, the self-replicating RNA may be formulated for delivery by the methods described in Geall et al (Nonviral delivery of self-amplifying RNA vaccines, PNAS 2012; PMID: 22908294; herein incorporated by reference in its entirety).

In one embodiment, the modified nucleic acid molecules or mmRNA of the present invention may encode amphipathic and/or immunogenic amphipathic peptides.

In one embodiment, a formulation of the modified nucleic acid molecules or mmRNA of the present invention may further comprise an amphipathic and/or immunogenic amphipathic peptide. As a non-limiting example, the modified nucleic acid molecule or mmRNA comprising an amphipathic and/or immunogenic amphipathic peptide may be formulated as described in US. Pub. No. US20110250237 and International Pub. Nos. WO2010009277 and WO2010009065; each of which is herein incorporated by reference in their entirety.

In one embodiment, the modified nucleic acid molecules and mmRNA of the present invention may be immunostimulatory. As a non-limiting example, the modified nucleic acid molecules and mmRNA may encode all or a part of a positive-sense or a negative-sense stranded RNA virus genome (see International Pub No. WO2012092569 and US Pub No. US20120177701, each of which is herein incorporated by reference in their entirety). In another non-limiting example, the immunostimulatory modified nucleic acid molecules or mmRNA of the present invention may be formulated with an excipient for administration as described herein and/or known in the art (see International Pub No. WO2012068295 and US Pub No. US20120213812, each of which is herein incorporated by reference in their entirety).

In one embodiment, the response of the vaccine formulated by the methods described herein may be enhanced by the addition of various compounds to induce the therapeutic effect. As a non-limiting example, the vaccine formulation may include a MHC II binding peptide or a peptide having a similar sequence to a MHC II binding peptide (see International Pub Nos. WO2012027365,
WO2011031298 and US Pub No. US20120070493, US20110110965, each of which is herein incorporated by reference in their entirety). As another example, the vaccine formulations may comprise modified nicotinic compounds which may generate an antibody response to nicotine residue in a subject (see International Pub No. WO2012061717 and US Pub No. US20120114677, each of which is herein incorporated by reference in their entirety).

Polypeptide variants

[00352] The modified nucleic acid molecules encode polypeptides, e.g., a variant polypeptides, which have a certain identity to a reference polypeptide sequence. The term “identity,” as known in the art, refers to a relationship between the sequences of two or more peptides, determined by comparing the sequences. In the art, "identity" also refers to the degree of sequence relatedness between peptides, as determined by the number of matches between strings of two or more amino 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 (i.e., “algorithms”). Identity of related peptides can be readily calculated by known methods. Such methods include, but are not limited to, 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; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math. 48, 1073 (1988); all of which are herein incorporated by reference in their entirety.

[00353] In some embodiments, the polypeptide variant may have the same or a similar activity as the reference polypeptide. Alternatively, the variant may have an altered activity (e.g., increased or decreased) relative to a reference polypeptide. Generally, variants of a particular polynucleotide or polypeptide of the present disclosure will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more 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.

[00354] 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 this present disclosure. For
example, provided herein is any protein fragment of a reference protein (meaning a polypeptide sequence which is at least one amino acid residue shorter than a reference polypeptide sequence but otherwise identical) 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 about 20, about 30, about 40, about 50, or about 100 amino acids which are about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or about 100% identical to any of the sequences described herein can be utilized in accordance with the present disclosure. In certain embodiments, a protein sequence to be utilized in accordance with the present disclosure includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations as shown in any of the sequences provided or referenced herein.

**Polypeptide-nucleic acid complexes**

[00355] Proper protein translation involves the physical aggregation of a number of polypeptides and nucleic acids associated with the mRNA. Provided by the present disclosure are protein-nucleic acid complexes, containing a translatable mRNA having one or more nucleoside modifications (e.g., at least two different nucleoside modifications) and one or more polypeptides bound to the mRNA. Generally, the proteins are provided in an amount effective to prevent or to reduce an innate immune response of a cell into which the complex is introduced.

**Untranslatable Modified Nucleic Acid Molecules**

[00356] As described herein, provided are mRNA having sequences that are substantially not translatable. Such mRNA may be effective as a vaccine when administered to a subject. It is further provided that the subject administered the vaccine may be a mammal, more preferably a human and most preferably a patient.

[00357] Also provided are modified nucleic acid molecules that contain one or more noncoding regions. Such modified nucleic acid molecules are generally not translated, but are capable of binding to and sequestering one or more translational machinery component such as a ribosomal protein or a transfer RNA (tRNA), thereby effectively reducing the protein expression in the cell. The modified nucleic acid molecule may contain a small nucleolar RNA (sno-RNA), micro RNA (miRNA), small interfering RNA (siRNA) or Piwi-interacting RNA (piRNA).

**Pharmaceutical Compositions**

**Formulation, Administration, Delivery and Dosing**

[00358] The present invention provides modified nucleic acids and mmRNA compositions and complexes in combination with one or more pharmaceutically acceptable excipients. Pharmaceutical compositions may optionally comprise one or more additional active substances, e.g. therapeutically
and/or prophylactically active substances. General considerations in the formulation and/or manufacture of pharmaceutical agents 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).

[00359] In some embodiments, compositions are administered to humans, human patients or subjects. For the purposes of the present disclosure, the phrase “active ingredient” generally refers to modified nucleic acids and mRNA to be delivered as described herein.

[00360] Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, e.g., to non-human animals, e.g. non-human mammals. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, humans and/or other primates; mammals, including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, dogs, mice, and/or rats; and/or birds, including commercially relevant birds such as poultry, chickens, ducks, geese, and/or turkeys.

[00361] Formulations of the pharmaceutical 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 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.

[00362] A pharmaceutical composition in accordance with the invention may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a “unit dose” is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.
[00363] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the invention 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 .5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

**Formulations**

[00364] The modified nucleic acid, and mmRNA of the invention 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 of the modified nucleic acid, or mmRNA); (4) alter the biodistribution (e.g., target the modified nucleic acid, or mmRNA 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 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 of the present invention can include, without limitation, lipidoids, liposomes, lipid nanoparticles, polymers, lipoplexes, core-shell nanoparticles, peptides, proteins, cells transfected with modified nucleic acid, or mmRNA (e.g., for transplantation into a subject), hyaluronidase, nanoparticle mimics and combinations thereof. Accordingly, the formulations of the invention can include one or more excipients, each in an amount that together increases the stability of the modified nucleic acid, or mmRNA, increases cell transfection by the modified nucleic acid, or mmRNA, increases the expression of modified nucleic acid, or mmRNA encoded protein, and/or alters the release profile of modified nucleic acid, or mmRNA encoded proteins. Further, the modified nucleic acids and mmRNA of the present invention may be formulated using self-assembled nucleic acid nanoparticles.

[00365] Formulations of the pharmaceutical 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 associating the active ingredient with an excipient and/or one or more other accessory ingredients.

[00366] A pharmaceutical composition in accordance with the present disclosure may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a “unit dose” refers to a discrete amount of the pharmaceutical composition comprising
a predetermined amount of the active ingredient. The amount of the active ingredient may generally be equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage including, but not limited to, one-half or one-third of such a dosage.

[00367] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the present disclosure may vary, depending upon the identity, size, and/or condition of the subject being treated and further depending upon the route by which the composition is to be administered. For example, the composition may comprise between 0.1% and 99% (w/w) of the active ingredient.

[00368] In some embodiments, the modified mRNA formulations described herein may contain at least one modified mRNA. The formulations may contain 1, 2, 3, 4 or 5 modified mRNA. In one embodiment, the formulation contains at least three modified mRNA encoding proteins. In one embodiment, the formulation contains at least five modified mRNA encoding proteins.

[00369] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but is not limited to, 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, and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see Remington: The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium may be incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

[00370] In some embodiments, the particle size of the lipid nanoparticle may be increased and/or decreased. The change in particle size may be able to help counter biological reaction such as, but not limited to, inflammation or may increase the biological effect of the modified mRNA delivered to mammals.

[00371] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, surface active agents and/or emulsifiers,
preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in the pharmaceutical formulations of the invention.

Lipidoids

[00372] The synthesis of lipidoids has been extensively described and formulations containing these compounds are particularly suited for delivery of modified nucleic acid molecules or mRNA (see Mahon et al., Bioconj Chem. 2010 21:1448-1454; Schroeder et al., J Intern Med. 2010 267:9-21; Akinc et al., Nat Biotechnol. 2008 26:561-569; Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869; Siegwart et al., Proc Natl Acad Sci U S A. 2011 108:12996-3001; all of which are incorporated herein in their entireties).

[00373] While these lipidoids have been used to effectively deliver double stranded small interfering RNA molecules in rodents and non-human primates (see Akinc et al., Nat Biotechnol. 2008 26:561-569; Frank-Kamenetsky et al., Proc Natl Acad Sci U S A. 2008 105:11915-11920; Akinc et al., Mol Ther. 2009 17:872-879; Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869; Leuschner et al., Nat Biotechnol. 2011 29:1005-1010; all of which is incorporated herein in their entirety), the present disclosure describes their formulation and use in delivering single stranded modified nucleic acid molecules or mRNA. Complexes, micelles, liposomes or particles can be prepared containing these lipidoids and therefore, can result in an effective delivery of the modified nucleic acid molecules or mRNA, as judged by the production of an encoded protein, following the injection of a lipidoid formulation via localized and/or systemic routes of administration. Lipidoid complexes of modified nucleic acid molecules or mRNA can be administered by various means including, but not limited to, intravenous, intramuscular, or subcutaneous routes.

[00374] In vivo delivery of nucleic acids may be affected by many parameters, including, but not limited to, the formulation composition, nature of particle PEGylation, degree of loading, oligonucleotide to lipid ratio, and biophysical parameters such as, but not limited to, particle size (Akinc et al., Mol Ther. 2009 17:872-879; herein incorporated by reference in its entirety). As an example, small changes in the anchor chain length of poly(ethylene glycol) (PEG) lipids may result in significant effects on in vivo efficacy. Formulations with the different lipidoids, including, but not limited to penta[3-(1-laurylaminopropionyl)]-triethylenetetramine hydrochloride (TETA-5LAP; aka 98N12-5, see Murugaiah et al., Analytical Biochemistry, 401:61 (2010); herein incorporated by
reference in its entirety), C12-200 (including derivatives and variants), and MD1, can be tested for in vivo activity.

[00375] The lipidoid referred to herein as “98N12-5” is disclosed by Akinc et al., Mol Ther. 2009 17:872-879 and is incorporated by reference in its entirety (See Figure 1).

[00376] The lipidoid referred to herein as “C12-200” is disclosed by Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869 and Liu and Huang, Molecular Therapy. 2010 669-670 (see Figure 1); both of which are herein incorporated by reference in their entirety. The lipidoid formulations can include particles comprising either 3 or 4 or more components in addition to modified nucleic acid molecules or mmRNA. As an example, formulations with certain lipidoids, include, but are not limited to, 98N12-5 and may contain 42% lipidoid, 48% cholesterol and 10% PEG (C14 alkyl chain length). As another example, formulations with certain lipidoids, include, but are not limited to, C12-200 and may contain 50% lipidoid, 10% disterylphosphatidyl choline, 38.5% cholesterol, and 1.5% PEG-DMG.

[00377] In one embodiment, a modified nucleic acid molecule or mRNA formulated with a lipidoid for systemic intravenous administration can target the liver. For example, a final optimized intravenous formulation using modified nucleic acid molecule or mRNA, and comprising a lipid molar composition of 42% 98N12-5, 48% cholesterol, and 10% PEG-lipid with a final weight ratio of about 7.5 to 1 total lipid to modified nucleic acid, or mRNA, and a C14 alkyl chain length on the PEG lipid, with a mean particle size of roughly 50–60 nm, can result in the distribution of the formulation to be greater than 90% to the liver.(see, Akinc et al., Mol Ther. 2009 17:872-879; herein incorporated by reference in its entirety). In another example, an intravenous formulation using a C12-200 (see US provisional application 61/175,770 and published international application WO2010129709, each of which is herein incorporated by reference in their entirety) lipidoid may have a molar ratio of 50/10/38.5/1.5 of C12-200/disterylphosphatidyl choline/cholesterol/PEG-DMG, with a weight ratio of 7 to 1 total lipid to modified nucleic acid molecule or mRNA, and a mean particle size of 80 nm may be effective to deliver modified nucleic acid molecule or mRNA to hepatocytes (see, Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869 herein incorporated by reference in its entirety). In another embodiment, an MD1 lipidoid-containing formulation may be used to effectively deliver modified nucleic acid molecule or mRNA to hepatocytes in vivo. The characteristics of optimized lipidoid formulations for intramuscular or subcutaneous routes may vary significantly depending on the target cell type and the ability of formulations to diffuse through the
extracellular matrix into the blood stream. While a particle size of less than 150 nm may be desired for effective hepatocyte delivery due to the size of the endothelial fenestrae (see, Akinc et al., Mol Ther. 2009 17:872-879 herein incorporated by reference in its entirety), use of a lipidoid-formulated modified nucleic acid molecules or mmRNA to deliver the formulation to other cells types including, but not limited to, endothelial cells, myeloid cells, and muscle cells may not be similarly size-limited. Use of lipidoid formulations to deliver siRNA in vivo to other non-hepatocyte cells such as myeloid cells and endothelium has been reported (see Akinc et al., Nat Biotechnol. 2008 26:561-569; Leuschner et al., Nat Biotechnol. 2011 29:1005-1010; Cho et al. Adv. Funct. Mater. 2009 19:3112-3118; 8th International Judah Folkman Conference, Cambridge, MA October 8-9, 2010; each of which is herein incorporated by reference in its entirety). Effective delivery to myeloid cells, such as monocytes, lipidoid formulations may have a similar component molar ratio. Different ratios of lipidoids and other components including, but not limited to, disteroylphosphatidyl choline, cholesterol and PEG-DMG, may be used to optimize the formulation of the modified nucleic acid, or mmRNA for delivery to different cell types including, but not limited to, hepatocytes, myeloid cells, muscle cells, etc. For example, the component molar ratio may include, but is not limited to, 50% C12-200, 10% disteroylphosphatidyl choline, 38.5% cholesterol, and %1.5 PEG-DMG (see Leuschner et al., Nat Biotechnol 2011 29:1005-1010; herein incorporated by reference in its entirety). The use of lipidoid formulations for the localized delivery of nucleic acids to cells (such as, but not limited to, adipose cells and muscle cells) via either subcutaneous or intramuscular delivery, may not require all of the formulation components desired for systemic delivery, and as such may comprise only the lipidoid and the modified nucleic acid molecule or mmRNA.

[00378] Combinations of different lipidoids may be used to improve the efficacy of modified nucleic acid molecule or mmRNA directed protein production as the lipidoids may be able to increase cell transfection by the modified nucleic acid molecule or mmRNA; and/or increase the translation of encoded protein (see Whitehead et al., Mol. Ther. 2011, 19:1688-1694, herein incorporated by reference in its entirety).

*Liposomes, Lipoplexes, and Lipid Nanoparticles*

[00379] The modified nucleic acid molecules and mmRNA of the invention can be formulated using one or more liposomes, lipoplexes, or lipid nanoparticles. In one embodiment, pharmaceutical compositions of modified nucleic acid molecule or mmRNA include liposomes. Liposomes are artificially-prepared vesicles which may primarily be composed of a lipid bilayer and may be used as
a delivery vehicle for the administration of nutrients and pharmaceutical formulations. Liposomes can be of different sizes such as, but not limited to, a multilamellar vesicle (MLV) which may be hundreds of nanometers in diameter and may contain a series of concentric bilayers separated by narrow aqueous compartments, a small unicellular vesicle (SUV) which may be smaller than 50 nm in diameter, and a large unilamellar vesicle (LUV) which may be between 50 and 500 nm in diameter. Liposome design may include, but is not limited to, opsonins or ligands in order to improve the attachment of liposomes to unhealthy tissue or to activate events such as, but not limited to, endocytosis. Liposomes may contain a low or a high pH in order to improve the delivery of the pharmaceutical formulations.

[00380] The formation of liposomes may depend on the physicochemical characteristics such as, but not limited to, the pharmaceutical formulation entrapped and the liposomal ingredients, the nature of the medium in which the lipid vesicles are dispersed, the effective concentration of the entrapped substance and its potential toxicity, any additional processes involved during the application and/or delivery of the vesicles, the optimization size, polydispersity and the shelf-life of the vesicles for the intended application, and the batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.

[00381] In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA) liposomes, DiLa2 liposomes from Marina Biotech (Bothell, WA), 1,2-dilinoleyloxy-3-dimethylaminopropane (DLin-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), and MC3 (US20100324120; herein incorporated by reference in its entirety) and liposomes which may deliver small molecule drugs such as, but not limited to, DOXIL® from Janssen Biotech, Inc. (Horsham, PA). In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from the synthesis of stabilized plasmid-lipid particles (SPLP) or stabilized nucleic acid lipid particle (SNALP) that have been previously described and shown to be suitable for oligonucleotide delivery in vitro and in vivo (see Wheeler et al. Gene Therapy. 1999 6:271-281; Zhang et al. Gene Therapy. 1999 6:1438-1447; Jeffs et al. Pharm Res. 2005 22:362-372; Morrissey et al., Nat Biotechnol. 2005 2:1002-1007; Zimmermann et al., Nature. 2006 441:111-114; Heyes et al. J Contr Rel. 2005 107:276-287; Semple et al. Nature Biotech. 2010 28:172-176; Judge et al. J Clin Invest. 2009 119:661-673; deFougerolles and others). The
original manufacture method by Wheeler et al. was a detergent dialysis method, which was later improved by Jeffs et al. and is referred to as the spontaneous vesicle formation method. The liposome formulations are composed of 3 to 4 lipid components in addition to the modified nucleic acid molecule or mmRNA. As an example a liposome can contain, but is not limited to, 55% cholesterol, 20% disteroylphosphatidyl choline (DSPC), 10% PEG-S-DSG, and 15% 1,2-dioleloxy-N,N-dimethylaminopropane (DODMA), as described by Jeffs et al. As another example, certain liposome formulations may contain, but are not limited to, 48% cholesterol, 20% DSPC, 2% PEG-cDMA, and 30% cationic lipid, where the cationic lipid can be 1,2-distearloxy-N,N-dimethylaminopropane (DSDMA), DODMA, DLin-DMA, or 1,2-dilinolenyloxy-3-dimethylaminopropane (DLenDMA), as described by Heyes et al.

In one embodiment, pharmaceutical compositions may include liposomes which may be formed to deliver mmRNA which may encode at least one immunogen. The mmRNA may be encapsulated by the liposome and/or it may be contained in an aqueous core which may then be encapsulated by the liposome (see International Pub. Nos. WO2012031046, WO2012031043, WO2012030901 and WO2012006378; each of which is herein incorporated by reference in their entirety). In another embodiment, the mmRNA which may encode an immunogen may be formulated in a cationic oil-in-water emulsion where the emulsion particle comprises an oil core and a cationic lipid which can interact with the mmRNA anchoring the molecule to the emulsion particle (see International Pub. No. WO2012006380; herein incorporated by reference in its entirety). In yet another embodiment, the lipid formulation may include at least cationic lipid, a lipid which may enhance transfection and a least one lipid which contains a hydrophilic head group linked to a lipid moiety (International Pub. No. WO2011076807 and U.S. Pub. No. 20110200582; each of which is herein incorporated by reference in their entirety). In another embodiment, the modified mRNA encoding an immunogen may be formulated in a lipid vesicle which may have crosslinks between functionalized lipid bilayers (see U.S. Pub. No. 20120177724, herein incorporated by reference in its entirety).

In one embodiment, the modified mRNA may be formulated in a lipid vesicle which may have crosslinks between functionalized lipid bilayers.

In one embodiment, the modified mRNA may be formulated in a lipid-polycation complex. The formation of the lipid-polycation complex may be accomplished by methods known in the art and/or as described in U.S. Pub. No. 20120178702, herein incorporated by reference in its entirety.
As a non-limiting example, the polycation may include a cationic peptide or a polypeptide such as, but not limited to, polylysine, polymornithine and/or polyarginine and the cationic peptides described in International Pub. No. WO2012013326; herein incorporated by reference in its entirety. In another embodiment, the modified mRNA may be formulated in a lipid-polycation complex which may further include a neutral lipid such as, but not limited to, cholesterol or dioleoyl phosphatidylethanolamine (DOPE).

[00385] The liposome formulation may be influenced by, but not limited to, the selection of the cationic lipid component, the degree of cationic lipid saturation, the nature of the PEGylation, ratio of all components and biophysical parameters such as size. In one example by Semple et al. (Semple et al. Nature Biotech. 2010 28: 172-176; herein incorporated by reference in its entirety), the liposome formulation was composed of 57.1 % cationic lipid, 7.1% dipalmitoylphosphatidylcholine, 34.3 % cholesterol, and 1.4% PEG-c-DMA. As another example, changing the composition of the cationic lipid could more effectively deliver siRNA to various antigen presenting cells (Basha et al. Mol Ther. 2011 19: 2186-2200; herein incorporated by reference in its entirety).

[00386] In some embodiments, the ratio of PEG in the lipid nanoparticle (LNP) formulations may be increased or decreased and/or the carbon chain length of the PEG lipid may be modified from C14 to C18 to alter the pharmacokinetics and/or biodistribution of the LNP formulations. As a non-limiting example, LNP formulations may contain 1-5% of the lipid molar ratio of PEG-c-DOMG as compared to the cationic lipid, DSPC and cholesterol. In another embodiment the PEG-c-DOMG may be replaced with a PEG lipid such as, but not limited to, PEG-DSG (1,2-Distearoyl-sn-glycerol, methoxypolyethylene glycol) or PEG-DSG (1,2-Dipalmitoyl-sn-glycerol, methoxypolyethylene glycol). The cationic lipid may be selected from any lipid known in the art such as, but not limited to, DLin-MC3-DMA, DLin-DMA, C12-200 and DLin-KC2-DMA.

[00387] In one embodiment, the cationic lipid may be selected from, but not limited to, a cationic lipid described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365, WO2012044638, WO2010080724, WO201021865 and WO2008103276, US Patent Nos. 7,893,302, 7,404,969 and 8,283,333 and US Patent Publication No. US20100036115 and US20120202871; each of which is herein incorporated by reference in their entirety. In another embodiment, the cationic lipid may be selected from, but not limited to, formula A described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733,

**[00388]** In one embodiment, the cationic lipid may be synthesized by methods known in the art and/or as described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365, WO2012044638, WO2010080724 and WO201021865; each of which is herein incorporated by reference in their entirety.
In one embodiment, the LNP formulation may contain PEG-c-DOMG at 3% lipid molar ratio. In another embodiment, the LNP formulation may contain PEG-c-DOMG at 1.5% lipid molar ratio.

In one embodiment, the LNP formulation may contain PEG-DMG 2000 (1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)]-2000). In one embodiment, the LNP formulation may contain PEG-DMG 2000, a cationic lipid known in the art and at least one other component. In another embodiment, the LNP formulation may contain PEG-DMG 2000, a cationic lipid known in the art, DSPC and cholesterol. As a non-limiting example, the LNP formulation may contain PEG-DMG 2000, DLin-DMA, DSPC and cholesterol. As another non-limiting example the LNP formulation may contain PEG-DMG 2000, DLin-DMA, DSPC and cholesterol in a molar ratio of 2:40:10:48 (see e.g. Geall et al., Nonviral delivery of self-amplifying RNA vaccines, PNAS 2012; PMID: 22908294; herein incorporated by reference in its entirety).

In one embodiment, the LNP formulation may be formulated by the methods described in International Publication Nos. WO2011127255 or WO2008103276, each of which is herein incorporated by reference in their entirety. As a non-limiting example, modified RNA described herein may be encapsulated in LNP formulations as described in WO2011127255 and/or WO2008103276; each of which is herein incorporated by reference in their entirety. As another non-limiting example, modified RNA described herein may be formulated in a nanoparticle to be delivered by a parenteral route as described in U.S. Pub. No. 20120207845; herein incorporated by reference in its entirety.

In one embodiment, LNP formulations described herein may comprise a polycationic composition. As a non-limiting example, the polycationic composition may be selected from formula 1-60 of US Patent Publication No. US20050222064; herein incorporated by reference in its entirety. In another embodiment, the LNP formulations comprising a polycationic composition may be used for the delivery of the modified RNA described herein in vivo and/or in vitro.

In one embodiment, the LNP formulations described herein may additionally comprise a permeability enhancer molecule. Non-limiting permeability enhancer molecules are described in US Patent Publication No. US20050222064; herein incorporated by reference in its entirety.

In one embodiment, the pharmaceutical compositions may be formulated in liposomes such as, but not limited to, DiLa2 liposomes (Marina Biotech, Bothell, WA), SMARTICLES® (Marina Biotech, Bothell, WA), neutral DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) based liposomes.
(e.g., siRNA delivery for ovarian cancer (Landen et al. Cancer Biology & Therapy 2006 5(12)1708-1713); herein incorporated by reference in its entirety) and hyaluronan-coated liposomes (Quiet Therapeutics, Israel).

[00395] The nanoparticle formulations may be a carbohydrate nanoparticle comprising a carbohydrate carrier and a modified nucleic acid molecule (e.g., mRNA). As a non-limiting example, the carbohydrate carrier may include, but is not limited to, an anhydride-modified phytoglycerogen or glycogen-type material, phytoglycerogen octenyl succinate, phytoglycerogen beta-dextrin, anhydride-modified phytoglycerogen beta-dextrin. (See e.g., International Publication No. WO2012109121; herein incorporated by reference in its entirety).

[00396] Lipid nanoparticle formulations may be improved by replacing the cationic lipid with a biodegradable cationic lipid which is known as a rapidly eliminated lipid nanoparticle (reLNP). Ionizable cationic lipids, such as, but not limited to, DLinDMA, DLin-KC2-DMA, and DLin-MC3-DMA, have been shown to accumulate in plasma and tissues over time and may be a potential source of toxicity. The rapid metabolism of the rapidly eliminated lipids can improve the tolerability and therapeutic index of the lipid nanoparticles by an order of magnitude from a 1 mg/kg dose to a 10 mg/kg dose in rat. Inclusion of an enzymatically degraded ester linkage can improve the degradation and metabolism profile of the cationic component, while still maintaining the activity of the reLNP formulation. The ester linkage can be internally located within the lipid chain or it may be terminally located at the terminal end of the lipid chain. The internal ester linkage may replace any carbon in the lipid chain.

[00397] In one embodiment, the internal ester linkage may be located on either side of the saturated carbon. Non-limiting examples of reLNP's include,

[00398] In one embodiment, an immune response may be elicited by delivering a lipid nanoparticle which may include a nanospecies, a polymer and an immunogen. (U.S. Publication No. 20120189700 and International Publication No. WO2012099805; each of which is herein incorporated by reference in their entirety). The polymer may encapsulate the nanospecies or
partially encapsulate the nanospecies. The immunogen may be a recombinant protein, a modified RNA described herein. In one embodiment, the lipid nanoparticle may be formulated for use in a vaccine such as, but not limited to, against a pathogen.

[00399] Lipid nanoparticles may be engineered to alter the surface properties of particles so the lipid nanoparticles may penetrate the mucosal barrier. Mucus is located on mucosal tissue such as, but not limited to, oral (e.g., the buccal and esophageal membranes and tonsil tissue), ophthalmic, gastrointestinal (e.g., stomach, small intestine, large intestine, colon, rectum), nasal, respiratory (e.g., nasal, pharyngeal, tracheal and bronchial membranes), genital (e.g., vaginal, cervical and urethral membranes). Nanoparticles larger than 10-200 nm which are preferred for higher drug encapsulation efficiency and the ability to provide the sustained delivery of a wide array of drugs have been thought to be too large to rapidly diffuse through mucosal barriers. Mucus is continuously secreted, shed, discarded or digested and recycled so most of the trapped particles may be removed from the mucosa tissue within seconds or within a few hours. Large polymeric nanoparticles (200nm -500nm in diameter) which have been coated densely with a low molecular weight polyethylene glycol (PEG) diffused through mucus only 4 to 6-fold lower than the same particles diffusing in water (Lai et al. PNAS 2007 104(5):1482-487; Lai et al. Adv Drug Deliv Rev. 2009 61(2): 158-171; each of which is herein incorporated by reference in their entirety). The transport of nanoparticles may be determined using rates of permeation and/or fluorescent microscopy techniques including, but not limited to, fluorescence recovery after photobleaching (FRAP) and high resolution multiple particle tracking (MPT). As a non-limiting example, compositions which can penetrate a mucosal barrier may be made as described in U.S. Pat. No. 8,241,670, herein incorporated by reference in its entirety.

[00400] The lipid nanoparticle engineered to penetrate mucus may comprise a polymeric material (i.e. a polymeric core) and/or a polymer-vitamin conjugate and/or a tri-block co-polymer. The polymeric material may include, but is not limited to, polyamines, polyethers, polyamides, polyesters, polycarbamates, polyureas, polycarbonates, poly(styrenes), polyimides, polysulfones, polyurethanes, polyacetylenes, polyethylene, polyethyleneimines, polysiscyanates, polyacrylates, polymethacrylates, polyacrylonitriles, and polyarylates. The polymeric material may be biodegradable and/or biocompatible. The polymeric material may additionally be irradiated. As a non-limiting example, the polymeric material may be gamma irradiated (See e.g., International App. No. WO201282165, herein incorporated by reference in its entirety). Non-limiting examples of
specific polymers include poly(caprolactone) (PCL), ethylene vinyl acetate polymer (EVA),
poly(lactic acid) (PLA), poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(lactic acid-co-
glycolic acid) (PLGA), poly(L-lactic acid-co-glycolic acid) ( PLLGA), poly(D,L-lactide) (PDLA),
poly(L-lactide) ( PLLA), poly(D,L-lactide-co-caprolactone), poly(D,L-lactide-co-caprolactone-co-
glycolide), poly(D,L-lactide-co-PEO-co-D,L-lactide), poly(D,L-lactide-co-PPO-co-D,L-lactide),
polyalkyl cyanoacrylate, polyurethane, poly-L-lysine (PLL), hydroxypropyl methacrylate (HPMA),
polyethylene glycol, poly-L-glutamic acid, poly(hydroxy acids), polyanhydrides, polyorthoesters,
poly(ester amides), polyamides, poly(ester ethers), polycarbonates, polyalkylenes such as
polyethylene and polypropylene, polyalkylene glycols such as poly(ethylene glycol) (PEG),
polyalkylene oxides (PEO), polyalkylene terephthalates such as poly(ethylene terephthalate),
polyvinyl alcohols (PVA), polyvinyl ethers, polyvinyl esters such as poly(vinyl acetate), polyvinyl
halides such as poly(vinyl chloride) (PVC), polyvinylpyrrolidone, polysiloxanes, polystyrene (PS),
polyurethanes, derivatized celluloses such as alkyl celluloses, hydroxyalkyl celluloses, cellulose
ethers, cellulose esters, nitro celluloses, hydroxypropylcellulose, carboxymethylcellulose, polymers
of acrylic acids, such as poly(methyl(meth)acrylate) (PMMA), poly(ethyl(meth)acrylate),
poly(butyl(meth)acrylate), poly(isobutyl(meth)acrylate), poly(hexyl(meth)acrylate),
poly(isodecyl(meth)acrylate), poly(lauryl(meth)acrylate), poly(phenyl(meth)acrylate), poly(methyl
acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) and copolymers
and mixtures thereof, polydioxyanone and its copolymers, polyhydroxyalkanoates, polypropylene
fumarate, polyoxymethylene, poloxamers, poly(ortho)esters, poly(butyric acid), poly(valeric acid),
poly(lactide-co-caprolactone), and trimethylene carbonate, polyvinylpyrrolidone. The lipid
nanoparticle may be coated or associated with a co-polymer such as, but not limited to, a block co-
polymer, and (poly(ethylene glycol))-(poly(propylene oxide))-(poly(ethylene glycol)) triblock
copolymer (see e.g., US Publication 20120121718 and US Publication 20100033337 and U.S. Pat.
No. 8,263,665; each of which is herein incorporated by reference in their entirety). The co-polymer
may be a polymer that is generally regarded as safe (GRAS) and the formation of the lipid
nanoparticle may be in such a way that no new chemical entities are created. For example, the lipid
nanoparticle may comprise poloxamers coating PLGA nanoparticles without forming new chemical
entities which are still able to rapidly penetrate human mucus (Yang et al. Angew. Chem. Int. Ed.
2011 50:2597-2600; herein incorporated by reference in its entirety).
[00401] The vitamin of the polymer-vitamin conjugate may be vitamin E. The vitamin portion of the conjugate may be substituted with other suitable components such as, but not limited to, vitamin A, vitamin E, other vitamins, cholesterol, a hydrophobic moiety, or a hydrophobic component of other surfactants (e.g., sterol chains, fatty acids, hydrocarbon chains and alkylene oxide chains).

[00402] The lipid nanoparticle engineered to penetrate mucus may include surface altering agents such as, but not limited to, mmRNA, anionic proteins (e.g., bovine serum albumin), surfactants (e.g., cationic surfactants such as for example dimethyldioctadecyl-ammonium bromide), sugars or sugar derivatives (e.g., cyclodextrin), nucleic acids, polymers (e.g., heparin, polyethylene glycol and poloxamer), mucolytic agents (e.g., N-acetylcysteine, mugwort, bromelain, papain, clerodendrum, acetylcysteine, bromhexine, carbocisteine, eprazinone, mesna, ambroxol, sobrerol, domiodol, letostene, stepronin, tiopronin, gelsolin, thymosin β4 dornase alfa, neltenerexine, erdostecine) and various DNases including rhDNase.. The surface altering agent may be embedded or enmeshed in the particle’s surface or disposed (e.g., by coating, adsorption, covalent linkage, or other process) on the surface of the lipid nanoparticle. (see e.g., US Publication 20100215580 and US Publication 20080166414; each of which is herein incorporated by reference in their entirety).

[00403] The mucus penetrating lipid nanoparticles may comprise at least one mmRNA described herein. The mmRNA may be encapsulated in the lipid nanoparticle and/or disposed on the surface of the particle. The mmRNA may be covalently coupled to the lipid nanoparticle. Formulations of mucus penetrating lipid nanoparticles may comprise a plurality of nanoparticles. Further, the formulations may contain particles which may interact with the mucus and alter the structural and/or adhesive properties of the surrounding mucus to decrease mucoadhesion which may increase the delivery of the mucus penetrating lipid nanoparticles to the mucosal tissue.

[00404] In one embodiment, the modified nucleic acid molecule or mmRNA is formulated as a lipoplex, such as, without limitation, the ATUPLEX™ system, the DACC system, the DBTC system and other siRNA-lipoplex technology from Silence Therapeutics (London, United Kingdom), STEMFECT™ from STEMGENT® (Cambridge, MA), and polyethyleneimine (PEI) or protamine-based targeted and non-targeted delivery of nucleic acids acids (Aleku et al. Cancer Res. 2008 68:9788-9798; Strumberg et al. Int J Clin Pharmacol Ther 2012 50:76-78; Santel et al., Gene Ther 2006 13:1222-1234; Santel et al., Gene Ther 2006 13:1360-1370; Gutbier et al., Pulm Pharmacol. Ther. 2010 23:334-344; Kaufmann et al. Microvasc Res 2010 80:286-293Weide et al. J Immunother. 2009 32:498-507; Weide et al. J Immunother. 2008 31:180-188; Pascolo Expert Opin. Biol. Ther.


[00406] In one embodiment, the modified nucleic acid molecules or mRNA are formulated as a solid lipid nanoparticle. A solid lipid nanoparticle (SLN) may be spherical with an average diameter between 10 to 1000 nm. SLN possess a solid lipid core matrix that can solubilize lipophilic
molecules and may be stabilized with surfactants and/or emulsifiers. In a further embodiment, the lipid nanoparticle may be a self-assembly lipid-polymer nanoparticle (see Zhang et al., ACS Nano, 2008, 2 (8), pp 1696–1702; herein incorporated by reference in its entirety).

[00407] Liposomes, lipoplexes, or lipid nanoparticles may be used to improve the efficacy of modified nucleic acid molecules or mmRNA directed protein production as these formulations may be able to increase cell transfection by the modified nucleic acid molecule or mmRNA; and/or increase the translation of encoded protein. One such example involves the use of lipid encapsulation to enable the effective systemic delivery of polyplex plasmid DNA (Heyes et al., Mol Ther. 2007 15:713-720; herein incorporated by reference in its entirety). The liposomes, lipoplexes, or lipid nanoparticles may also be used to increase the stability of the modified nucleic acid molecules or mmRNA.

[00408] In one embodiment, the modified nucleic acid molecules and/or the mmRNA of the present invention can be formulated for controlled release and/or targeted delivery. As used herein, “controlled release” refers to a pharmaceutical composition or compound release profile that conforms to a particular pattern of release to effect a therapeutic outcome. In one embodiment, the modified nucleic acids molecules or the mmRNA may be encapsulated into a delivery agent described herein and/or known in the art for controlled release and/or targeted delivery. As used herein, the term “encapsulate” means to enclose, surround or enlace. As it relates to the formulation of the compounds of the invention, encapsulation may be substantial, complete or partial. The term “substantially encapsulated” means that at least greater than 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.9 or greater than 99.99% of the pharmaceutical composition or compound of the invention may be enclosed, surrounded or encased within the delivery agent. “Partially encapsulation” means that less than 10, 10, 20, 30, 40 50 or less of the pharmaceutical composition or compound of the invention may be enclosed, surrounded or encased within the delivery agent. Advantageously, encapsulation may be determined by measuring the escape or the activity of the pharmaceutical composition or compound of the invention using fluorescence and/or electron micrograph. For example, at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the pharmaceutical composition or compound of the invention are encapsulated in the delivery agent.

[00409] In one embodiment, the controlled release formulation may include, but is not limited to, tri-block co-polymers. As a non-limiting example, the formulation may include two different types
of tri-block co-polymers (International Pub. No. WO2012131104 and WO2012131106; each of which is herein incorporated by reference in its entirety).

[00410] In another embodiment, the modified nucleic acid molecules or the mRNA may be encapsulated into a lipid nanoparticle or a rapidly eliminated lipid nanoparticle and the lipid nanoparticles or a rapidly eliminated lipid nanoparticle may then be encapsulated into a polymer, hydrogel and/or surgical sealant described herein and/or known in the art. As a non-limiting example, the polymer, hydrogel or surgical sealant may be PLGA, ethylene vinyl acetate (EVAc), poloxamer, GELSITE® (Nanotherapeutics, Inc. Alachua, FL), HYLENEX® (Halozyme Therapeutics, San Diego CA), surgical sealants such as fibrinogen polymers (Ethicon Inc. Cornelia, GA), TISSELL® (Baxter International, Inc Deerfield, IL), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, IL).

[00411] In another embodiment, the lipid nanoparticle may be encapsulated into any polymer known in the art which may form a gel when injected into a subject. As a non-limiting example, the lipid nanoparticle may be encapsulated into a polymer matrix which may be biodegradable.

[00412] In one embodiment, the modified nucleic acid molecules or mRNA formulation for controlled release and/or targeted delivery may also include at least one controlled release coating. Controlled release coatings include, but are not limited to, OPADRY®, polyvinylpyrrolidone/vinyl acetate copolymer, polyvinylpyrrolidone, hydroxypropyl methylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, EUDRAGIT RL®, EUDRAGIT RS® and cellulose derivatives such as ethylcellulose aqueous dispersions (AQUACOAT® and SURELEASE®).

[00413] In one embodiment, the controlled release and/or targeted delivery formulation may comprise at least one degradable polyester which may contain polyacrylic side chains. Degradable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

8,206,747, 8,293,276 8,318,208 and 8,318,211; each of which is herein incorporated by reference in their entirety. In another embodiment, therapeutic polymer nanoparticles may be identified by the methods described in US Pub No. US20120140790, herein incorporated by reference in its entirety.

[00415] In one embodiment, the therapeutic nanoparticle may be formulated for sustained release. As used herein, “sustained release” refers to a pharmaceutical composition or compound that conforms to a release rate over a specific period of time. The period of time may include, but is not limited to, hours, days, weeks, months and years. As a non-limiting example, the sustained release nanoparticle may comprise a polymer and a therapeutic agent such as, but not limited to, the modified nucleic acid molecules and mmRNA of the present invention (see International Pub No. 2010075072 and US Pub No. US20100216804, US20110217377 and US20120201859, each of which is herein incorporated by reference in their entirety).

[00416] In one embodiment, the therapeutic nanoparticles may be formulated to be target specific. As a non-limiting example, the therapeutic nanoparticles may include a corticosteroid (see International Pub. No. WO2011084518 herein incorporated by reference in its entirety). In one embodiment, the therapeutic nanoparticles of the present invention may be formulated to be cancer specific. As a non-limiting example, the therapeutic nanoparticles may be formulated in nanoparticles described in International Pub No. WO2008121949, WO2010005726, WO2010005725, WO2011084521 and US Pub No. US20100069426, US20120004293 and US20120104655, each of which is herein incorporated by reference in their entirety.

[00417] In one embodiment, the nanoparticles of the present invention may comprise a polymeric matrix. As a non-limiting example, the nanoparticle may comprise two or more polymers such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polyurethanes, polyamines, polylsine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester) or combinations thereof.

[00418] In one embodiment, the therapeutic nanoparticle comprises a diblock copolymer. In one embodiment, the diblock copolymer may include PEG in combination with a polymer such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters,
poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester) or combinations thereof.

[00419] As a non-limiting example the therapeutic nanoparticle comprises a PLGA-PEG block copolymer (see US Pub. No. US20120004293 and US Pat No. 8,236,330, each of which is herein incorporated by reference in their entirety). In another non-limiting example, the therapeutic nanoparticle is a stealth nanoparticle comprising a diblock copolymer of PEG and PLA or PEG and PLGA (see US Pat No 8,246,968, herein incorporated by reference in its entirety).

[00420] In one embodiment, the therapeutic nanoparticle may comprise a multiblock copolymer (See e.g., U.S. Pat. No. 8,263,665 and 8,287,910; each of which is herein incorporated by reference in its entirety).

[00421] In one embodiment, the block copolymers described herein may be included in a polyion complex comprising a non-polymeric micelle and the block copolymer. (See e.g., U.S. Pub. No. 20120076836; herein incorporated by reference in its entirety).

[00422] In one embodiment, the therapeutic nanoparticle may comprise at least one acrylic polymer. Acrylic polymers include but are not limited to, acrylic acid, methacrylic acid, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), polycyanoacrylates and combinations thereof.

[00423] In one embodiment, the therapeutic nanoparticles may comprise at least one cationic polymer described herein and/or known in the art.

[00424] In one embodiment, the therapeutic nanoparticles may comprise at least one amine-containing polymer such as, but not limited to polylysine, polyethylene imine, poly(amidoamine) dendrimers, poly(beta-amino esters) (See e.g., U.S. Pat. No. 8,287,849; herein incorporated by reference in its entirety) and combinations thereof.

[00425] In one embodiment, the therapeutic nanoparticles may comprise at least one degradable polyester which may contain polycationic side chains. Degradable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.
[00426] In another embodiment, the therapeutic nanoparticle may include a conjugation of at least one targeting ligand. The targeting ligand may be any ligand known in the art such as, but not limited to, a monoclonal antibody. (Kirpotin et al, Cancer Res. 2006 66:6732-6740; herein incorporated by reference in its entirety).

[00427] In one embodiment, the therapeutic nanoparticle may be formulated in an aqueous solution which may be used to target cancer (see International Pub No. WO2011084513 and US Pub No. US20110294717, each of which is herein incorporated by reference in their entirety).

[00428] In one embodiment, the modified nucleic acid molecules or mmRNA may be encapsulated in, linked to and/or associated with synthetic nanocarriers. Synthetic nanocarriers include, but are not limited to, those described in International Pub. Nos. WO2010005740, WO2010030763, WO201213501, WO2012149252, WO2012149255, WO2012149259, WO2012149265, WO2012149268, WO2012149282, WO2012149301, WO2012149393, WO2012149405, WO2012149411 and WO2012149454 and US Pub. Nos. US20110262491, US20100104645, US20100087337 and US20120244222, each of which is herein incorporated by reference in their entirety. The synthetic nanocarriers may be formulated using methods known in the art and/or described herein. As a non-limiting example, the synthetic nanocarriers may be formulated by the methods described in International Pub Nos. WO2010005740, WO2010030763 and WO201213501 and US Pub. Nos. US20110262491, US20100104645, US20100087337 and US20120244222, each of which is herein incorporated by reference in their entirety. In another embodiment, the synthetic nanocarrier formulations may be lyophilized by methods described in International Pub. No. WO2011072218 and US Pat No. 8,211,473; each of which is herein incorporated by reference in their entirety.

[00429] In one embodiment, the synthetic nanocarriers may contain reactive groups to release the modified nucleic acid molecules and/or mmRNA described herein (see International Pub. No. WO20120952552 and US Pub No. US20120171229, each of which is herein incorporated by reference in their entirety).

[00430] In one embodiment, the synthetic nanocarriers may contain an immunostimulatory agent to enhance the immune response from delivery of the synthetic nanocarrier. As a non-limiting example, the synthetic nanocarrier may comprise a Th1 immunostimulatory agent which may enhance a Th1-based response of the immune system (see International Pub No. WO2010123569 and US Pub. No. US20110223201, each of which is herein incorporated by reference in its entirety).
In one embodiment, the synthetic nanocarriers may be formulated for targeted release. In one embodiment, the synthetic nanocarrier is formulated to release the modified nucleic acid molecules and/or mmRNA at a specified pH and/or after a desired time interval. As a non-limiting example, the synthetic nanoparticle may be formulated to release the modified mRNA molecules and/or mmRNA after 24 hours and/or at a pH of 4.5 (see International Pub. Nos. WO2010138193 and WO2010138194 and US Pub Nos. US20110020388 and US20110027217, each of which is herein incorporated by reference in their entirety).

In one embodiment, the synthetic nanocarriers may be formulated for controlled and/or sustained release of the modified nucleic acid molecules and/or mmRNA described herein. As a non-limiting example, the synthetic nanocarriers for sustained release may be formulated by methods known in the art, described herein and/or as described in International Pub No. WO2010138192 and US Pub No. 20100303850, each of which is herein incorporated by reference in their entirety.

In one embodiment, the synthetic nanocarrier may be formulated for use as a vaccine. In one embodiment, the synthetic nanocarrier may encapsulate at least one modified nucleic acid molecule and/or mmRNA which encodes at least one antigen. As a non-limiting example, the synthetic nanocarrier may include at least one antigen and an excipient for a vaccine dosage form (see International Pub No. WO2011150264 and US Pub No. US20110293723, each of which is herein incorporated by reference in their entirety). As another non-limiting example, a vaccine dosage form may include at least two synthetic nanocarriers with the same or different antigens and an excipient (see International Pub No. WO2011150249 and US Pub No. US20110293701, each of which is herein incorporated by reference in their entirety). The vaccine dosage form may be selected by methods described herein, known in the art and/or described in International Pub No. WO2011150258 and US Pub No. US20120027806, each of which is herein incorporated by reference in their entirety.

In one embodiment, the synthetic nanocarrier may comprise at least one modified nucleic acid molecule and/or mmRNA which encodes at least one adjuvant. In another embodiment, the synthetic nanocarrier may comprise at least one modified nucleic molecule acid and/or mmRNA and an adjuvant. As a non-limiting example, the synthetic nanocarrier comprising and adjuvant may be formulated by the methods described in International Pub No. WO2011150240 and US Pub No. US20110293700, each of which is herein incorporated by reference in its entirety.
[00435] In one embodiment, the synthetic nanocarrier may encapsulate at least one modified nucleic acid molecule and/or mRNA which encodes a peptide, fragment or region from a virus. As a non-limiting example, the synthetic nanocarrier may include, but is not limited to, the nanocarriers described in International Pub No. WO2012024621, WO201202629, WO2012024632 and US Pub No. US20120064110, US20120058153 and US20120058154, each of which is herein incorporated by reference in their entirety.

[00436] In one embodiment, the nanoparticle may be optimized for oral administration. The nanoparticle may comprise at least one cationic biopolymer such as, but not limited to, chitosan or a derivative thereof. As a non-limiting example, the nanoparticle may be formulated by the methods described in U.S. Pub. No. 20120282343; herein incorporated by reference in its entirety.

*Polymers, Biodegradable Nanoparticles, and Core-Shell Nanoparticles*

[00437] The modified nucleic acid molecules and mRNA of the invention can be formulated using natural and/or synthetic polymers. Non-limiting examples of polymers which may be used for delivery include, but are not limited to, DYNAMIC POLYCONJUGATE® (Arrowhead Research Corp., Pasadena, CA) formulations from MIRUS® Bio (Madison, WI) and Roche Madison (Madison, WI), PHASERX™ polymer formulations such as, without limitation, SMARTT POLYMER TECHNOLOGY™ (Seattle, WA), DMRI/DOPE, poloxamer, VAXFECTIN® adjuvant from Vical (San Diego, CA), chitosan, cyclodextrin from Calando Pharmaceuticals (Pasadena, CA), dendrimers and poly(lactic-co-glycolic acid) (PLGA) polymers, RONDEL™ (RNAi/Oligonucleotide Nanoparticle Delivery) polymers (Arrowhead Research Corporation, Pasadena, CA) and pH responsive co-block polymers such as, but not limited to, PHASERX™ (Seattle, WA).

[00438] A non-limiting example of chitosan formulation includes a core of positively charged chitosan and an outer portion of negatively charged substrate (U.S. Pub. No. 20120258176; herein incorporated by reference in its entirety). Chitosan includes, but is not limited to N-trimethyl chitosan, mono-N-carboxymethyl chitosan (MCC), N-palmitoyl chitosan (NPCS), EDTA-chitosan, low molecular weight chitosan, chitosan derivatives, or combinations thereof.

[00439] In one embodiment, the polymers used in the present invention have undergone processing to reduce and/or inhibit the attachment of unwanted substances such as, but not limited to, bacteria, to the surface of the polymer. The polymer may be processed by methods known and/or described
in the art and/or described in International Pub. No. WO2012150467, herein incorporated by reference in its entirety.

[00440] A non-limiting example of PLGA formulations include, but are not limited to, PLGA injectable depots (e.g., ELIGARD® which is formed by dissolving PLGA in 66% N-methyl-2-pyrrolidone (NMP) and the remainder being aqueous solvent and leuprolide. Once injected, the PLGA and leuprolide peptide precipitates into the subcutaneous space).

[00441] Many of these polymer approaches have demonstrated efficacy in delivering oligonucleotides in vivo into the cell cytoplasm (reviewed in deFougerrolles Hum Gene Ther. 2008 19:125-132; herein incorporated by reference in its entirety). Two polymer approaches that have yielded robust in vivo delivery of nucleic acids, in this case with small interfering RNA (siRNA), are dynamic polyconjugates and cyclodextrin-based nanoparticles. The first of these delivery approaches uses dynamic polyconjugates and has been shown in vivo in mice to effectively deliver siRNA and silence endogenous target mRNA in hepatocytes (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; herein incorporated by reference in its entirety). This particular approach is a multicomponent polymer system whose key features include a membrane-active polymer to which nucleic acid, in this case siRNA, is covalently coupled via a disulfide bond and where both PEG (for charge masking) and N-acetylgalactosamine (for hepatocyte targeting) groups are linked via pH-sensitive bonds (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; herein incorporated by reference in its entirety). On binding to the hepatocyte and entry into the endosome, the polymer complex disassembles in the low-pH environment, with the polymer exposing its positive charge, leading to endosomal escape and cytoplasmic release of the siRNA from the polymer. Through replacement of the N-acetylgalactosamine group with a mannose group, it was shown one could alter targeting from asialoglycoprotein receptor-expressing hepatocytes to sinusoidal endothelium and Kupffer cells. Another polymer approach involves using transferrin-targeted cyclodextrin-containing polycation nanoparticles. These nanoparticles have demonstrated targeted silencing of the EWS-FLI1 gene product in transferrin receptor-expressing Ewing’s sarcoma tumor cells (Hu-Lieskovskyt al., Cancer Res.2005 65: 8984-8982; herein incorporated by reference in its entirety) and siRNA formulated in these nanoparticles was well tolerated in non-human primates (Heidel et al., Proc Natl Acad Sci USA 2007 104:5715-21; herein incorporated by reference in its entirety). Both of these delivery strategies incorporate rational approaches using both targeted delivery and endosomal escape mechanisms.
The polymer formulation can permit the sustained or delayed release of modified nucleic acid molecules or mRNA (e.g., following intramuscular or subcutaneous injection). The altered release profile for the modified nucleic acid molecule or mRNA can result in, for example, translation of an encoded protein over an extended period of time. The polymer formulation may also be used to increase the stability of the modified nucleic acid molecule or mRNA. Biodegradable polymers have been previously used to protect nucleic acids other than mRNA from degradation and been shown to result in sustained release of payloads in vivo (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; Sullivan et al., Expert Opin Drug Deliv. 2010 7:1433-1446; Convertine et al., Biomacromolecules. 2010 Oct 1; Chu et al., Acc Chem Res. 2012 Jan 13; Manganiello et al., Biomaterials. 2012 33:2301-2309; Benoit et al., Biomacromolecules. 2011 12:2708-2714; Singha et al., Nucleic Acid Ther. 2011 2:133-147; de Fougerolles Hum Gene Ther. 2008 19:125-132; Schaffert and Wagner, Gene Ther. 2008 16:1131-1138; Chaturvedi et al., Expert Opin Drug Deliv. 2011 8:1455-1468; Davis, Mol Pharm. 2009 6:659-668; Davis, Nature 2010 464:1067-1070; each of which is herein incorporated by reference in its entirety).

In one embodiment, the pharmaceutical compositions may be sustained release formulations. In a further embodiment, the sustained release formulations may be for subcutaneous delivery. Sustained release formulations may include, but are not limited to, PLGA microspheres, ethylene vinyl acetate (EVAe), poloxamer, GELSITE® (NanoTherapeutics, Inc. Alachua, FL), HYLENEX® (Halozyme Therapeutics, San Diego CA), surgical sealants such as fibrinogen polymers (Ethicon Inc. Cornelia, GA), TISSELL® (Baxter International, Inc Deerfield, IL), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, IL).

As a non-limiting example modified mRNA may be formulated in PLGA microspheres by preparing the PLGA microspheres with tunable release rates (e.g., days and weeks) and encapsulating the modified mRNA in the PLGA microspheres while maintaining the integrity of the modified mRNA during the encapsulation process. EVAe are non-biodegradable, biocompatible polymers which are used extensively in pre-clinical sustained release implant applications (e.g., extended release products Ocusert a pilocarpine ophthalmic insert for glaucoma or progestasert a sustained release progesterone intrauterine device; transdermal delivery systems Testoderm, Duragesic and Selegiline; catheters). Poloxamer F-407 NF is a hydrophilic, non-ionic surfactant triblock copolymer of polyoxyethylene-polyoxypropylene-polyoxyethylene having a low viscosity at temperatures less than 5°C and forms a solid gel at temperatures greater than 15°C. PEG-based
surgical sealants comprise two synthetic PEG components mixed in a delivery device which can be prepared in one minute, seals in 3 minutes and is reabsorbed within 30 days. GELSITE® and natural polymers are capable of in-situ gelation at the site of administration. They have been shown to interact with protein and peptide therapeutic candidates through ionic ineraction to provide a stabilizing effect.

Polymer formulations can also be selectively targeted through expression of different ligands as exemplified by, but not limited by, folate, transferrin, and N-acetylgalactosamine (GalNAc) (Benoit et al., Biomacromolecules. 2011 12:2708-2714; Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; Davis, Mol Pharm. 2009 6:659-668; Davis, Nature 2010 464:1067-1070; each of which is herein incorporated by reference in its entirety).

The modified nucleic acid molecules and mmRNA of the invention may be formulated with or in a polymeric compound. The polymer may include at least one polymer such as, but not limited to, polyethenes, polyethylene glycol (PEG), poly(l-lysine)(PLL), PEG grafted to PLL, cationic lipopolymer, biodegradable cationic lipopolymer, polyethyleneimine (PEI), cross-linked branched poly(alkylene imines), a polyamine derivative, a modified poloxamer, a biodegradable polymer, elastic biodegradable polymer, biodegradable block copolymer, biodegradable random copolymer, biodegradable polyester copolymer, biodegradable polyester block copolymer, biodegradable polyester block random copolymer, multiblock copolymers, linear biodegradable copolymer, poly[α-(4-aminobutyl)-l-glycolic acid] (PAPA), biodegradable cross-linked cationic multi-block copolymers, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polyacrolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanooacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanooacrylates, polylureas, polystyrenes, polymamines, polyllysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), acrylic polymers, amine-containing polymers, dextran polymers, dextran polymer derivatives or combinations thereof.

As a non-limiting example, the modified nucleic acid molecules or mmRNA of the invention may be formulated with the polymeric compound of PEG grafted with PLL as described in U.S. Pat. No. 6,177,274; herein incorporated by reference in its entirety. The formulation may be used for transfecting cells in vitro or for in vivo delivery of the modified nucleic acid molecules and mmRNA. In another example, the modified nucleic acid molecules and mmRNA may be suspended
in a solution or medium with a cationic polymer, in a dry pharmaceutical composition or in a solution that is capable of being dried as described in U.S. Pub. Nos. 20090042829 and 20090042825; each of which are herein incorporated by reference in their entireties.

[00448] As another non-limiting example the modified nucleic acid molecules or mmRNA of the invention may be formulated with a PLGA-PEG block copolymer (see US Pub. No. US20120004293 and US Pat No. 8,236,330, each of which are herein incorporated by reference in their entireties) or PLGA-PEG-PLGA block copolymers (See U.S. Pat. No. 6,004,573, herein incorporated by reference in its entirety). As a non-limiting example, the modified nucleic acid molecules or mmRNA of the invention may be formulated with a diblock copolymer of PEG and PLA or PEG and PLGA (see US Pat No 8,246,968, herein incorporated by reference in its entirety).

[00449] A polyamine derivative may be used to deliver nucleic acid molecules and/or mmRNA or to treat and/or prevent a disease or to be included in an implantable or injectable device (U.S. Pub. No. 20100260817 herein incorporated by reference in its entirety). As a non-limiting example, a pharmaceutical composition may include the modified nucleic acid molecules and mmRNA and the polyamine derivative described in U.S. Pub. No. 20100260817 (the contents of which are incorporated herein by reference in its entirety). As a non-limiting example the modified nucleic acids or mmRNA of the present invention may be delivered using a polyamidine polymer such as, but not limited to, a polymer comprising a 1,3-dipolar addition polymer prepared by combining a carbohydrate diazide monomer with a dilkyne unite comprising oligoamines (U.S. Pat. No. 8,236,280; herein incorporated by reference in its entirety).

[00450] The modified nucleic acid molecules and/or mmRNA of the invention may be formulated with at least one acrylic polymer. Acrylic polymers include but are not limited to, acrylic acid, methacrylic acid, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), polycyanoacrylates and combinations thereof.

[00451] In one embodiment, the modified nucleic acid molecules and/or mmRNA of the present invention may be formulated with at least one polymer and/or derivatives thereof described in International Publication Nos. WO2011115862, WO2012082574 and WO2012068187 and U.S. Pub. No. 20120283427, each of which are herein incorporated by reference in their entireties. In another embodiment, the modified nucleic acid molecules or mmRNA of the present invention may be formulated with a polymer of formula Z as described in WO2011115862, herein incorporated by
reference in its entirety. In yet another embodiment, the modified nucleic acid molecules or mmRNA may be formulated with a polymer of formula Z, Z’ or Z” as described in International Pub. Nos. WO2012082574 or WO2012068187, each of which are herein incorporated by reference in their entireties. The polymers formulated with the modified nucleic acids and/or modified mRNA of the present invention may be synthesized by the methods described in International Pub. Nos. WO2012082574 or WO2012068187, each of which are herein incorporated by reference in their entireties.

[00452] Formulations of modified nucleic acid molecules and/or mmRNA of the invention may include at least one amine-containing polymer such as, but not limited to polylysine, polyethylene imine, poly(amidoamine) dendrimers or combinations thereof.

[00453] For example, the modified nucleic acid molecules and/or mmRNA of the invention may be formulated in a pharmaceutical compound including a poly(alkylene imine), a biodegradable cationic lipopolymer, a biodegradable block copolymer, a biodegradable polymer, or a biodegradable random copolymer, a biodegradable polyester block copolymer, a biodegradable polyester polymer, a biodegradable polyester random copolymer, a linear biodegradable copolymer, PAGA, a biodegradable cross-linked cationic multi-block copolymer or combinations thereof. The biodegradable cationic lipopolymer may be made by methods known in the art and/or described in U.S. Pat. No. 6,696,038, U.S. App. Nos. 20030073619 and 20040142474 each of which is herein incorporated by reference in their entireties. The poly(alkylene imine) may be made using methods known in the art and/or as described in U.S. Pub. No. 20100004315, herein incorporated by reference in its entirety. The biodegradable polymer, biodegradable block copolymer, the biodegradable random copolymer, biodegradable polyester block copolymer, biodegradable polyester polymer, or biodegradable polyester random copolymer may be made using methods known in the art and/or as described in U.S. Pat. Nos. 6,517,869 and 6,267,987, the contents of which are each incorporated herein by reference in their entirety. The linear biodegradable copolymer may be made using methods known in the art and/or as described in U.S. Pat. No. 6,652,886. The PAGA polymer may be made using methods known in the art and/or as described in U.S. Pat. No. 6,217,912 herein incorporated by reference in its entirety. The PAGA polymer may be copolymerized to form a copolymer or block copolymer with polymers such as but not limited to, poly-L-lysine, polyargin, polyornithine, histones, avidin, protamines, polylactides and poly(lactide-co-glycolides). The biodegradable cross-linked cationic multi-block copolymers may be made my
methods known in the art and/or as described in U.S. Pat. No. 8,057,821 or U.S. Pub. No. 2012009145 each of which are herein incorporated by reference in their entireties. For example, the multi-block copolymers may be synthesized using linear polyethyleneimine (LPEI) blocks which have distinct patterns as compared to branched polyethyleneimines. Further, the composition or pharmaceutical composition may be made by the methods known in the art, described herein, or as described in U.S. Pub. No. 20100004315 or U.S. Pat. Nos. 6,267,987 and 6,217,912 each of which are herein incorporated by reference in their entireties.

[00454] The modified nucleic acid molecules and mmRNA of the invention may be formulated with at least one degradable polyester which may contain polycationic side chains. Degradable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

[00455] The modified nucleic acid molecules and mmRNA of the invention may be formulated with at least one crosslinkable polyester. Crosslinkable polyesters include those known in the art and described in US Pub. No. 20120269761, herein incorporated by reference in its entirety.

[00456] In one embodiment, the polymers described herein may be conjugated to a lipid-terminating PEG. As a non-limiting example, PLGA may be conjugated to a lipid-terminating PEG forming PLGA-DSPE-PEG. As another non-limiting example, PEG conjugates for use with the present invention are described in International Publication No. WO2008103276, herein incorporated by reference in its entirety. The polymers may be conjugated using a ligand conjugate such as, but not limited to, the conjugates described in U.S. Pat. No. 8,273,363, herein incorporated by reference in its entirety.

[00457] In one embodiment, the modified nucleic acid molecules and/or mmRNA described herein may be conjugated with another compound. Non-limiting examples of conjugates are described in US Patent Nos. 7,964,578 and 7,833,992, each of which are herein incorporated by reference in their entireties. In another embodiment, modified RNA of the present invention may be conjugated with conjugates of formula 1-122 as described in US Patent Nos. 7,964,578 and 7,833,992, each of which are herein incorporated by reference in their entireties. The modified RNA described herein may be conjugated with a metal such as, but not limited to, gold. (See e.g., Giljohann et al. Journ. Amer. Chem. Soc. 2009 131(6): 2072-2073; herein incorporated by reference in its entirety). In another embodiment, the modified nucleic acid molecules and/or mmRNA described herein may be
conjugated and/or encapsulated in gold-nanoparticles. (Interantional Pub. No. WO201216269 and U.S. Pub. No. 20120302940; each of which is herein incorporated by reference in its entirety).

[00458] As described in U.S. Pub. No. 20100004313, herein incorporated by reference in its entirety, a gene delivery composition may include a nucleotide sequence and a poloxamer. For example, the modified nucleic acid and mmRNA of the present invenion may be used in a gene delivery composition with the poloxamer described in U.S. Pub. No. 20100004313.

[00459] In one embodiment, the polymer formulation of the present invention may be stabilized by contacting the polymer formulation, which may include a cationic carrier, with a cationic lipopolymer which may be covalently linked to cholesterol and polyethylene glycol groups. The polymer formulation may be contacted with a cationic lipopolymer using the methods described in U.S. Pub. No. 20090042829 herein incorporated by reference in its entirety. The cationic carrier may include, but is not limited to, polyethyleneimine, poly(trimethylenimine), poly(tetramethylenimine), poly(propylelenimine), aminoglycoside-polymamine, dideoxy-diamino-b-cyclodextrin, spermine, spermidine, poly(2-dimethylamino)ethyl methacrylate, poly(lysine), poly(histidine), poly(arginine), cationized gelatin, dendrimers, chitosan, 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1-[2-(oleoxyloxy)ethyl]-2-oleyl-(2-hydroxyethyl)imidazolinium chloride (DOTIM), 2,3-dioleoxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 3B-[N—(N’N’-Dimethylaminoethane)carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HCl) diheptadecylamidoglycyl spermidine (DOGS), N,N-distearyl-N,N-dimethalammonium bromide (DDAB), N-(1,2-dimyristoylprop-3-yl)-N,N-dimethyl-N-hydroxyethyl amonium bromide (DMRIE), N,N-dioleyl-N,N-dimethalammonium chloride:DODAC) and combinations thereof.

[00460] The modified nucleic acid molecules and/or mmRNA of the invention may be formulated in a polyplex of one or more polymers (U.S. Pub. No. 20120237565 and 20120270927; each of which is herein incorporated by reference in its entirety). In one embodiment, the polyplex comprises two or more cationic polymers. The cationic polymer may comprise a poly(ethylene imine) (PEI) such as linear PEI.

[00461] The modified nucleic acid molecules and mmRNA of the invention can also be formulated as a nanoparticle using a combination of polymers, lipids, and/or other biodegradable agents, such as, but not limited to, calcium phosphate. Components may be combined in a core-shell, hybrid,
and/or layer-by-layer architecture, to allow for fine-tuning of the nanoparticle so to delivery of the modified nucleic acid molecule and mmRNA may be enhanced (Wang et al., Nat Mater. 2006 5:791-796; Fuller et al., Biomaterials. 2008 29:1526-1532; DeKoker et al., Adv Drug Deliv Rev. 2011 63:748-761; Endres et al., Biomaterials. 2011 32:7721-7731; Su et al., Mol Pharm. 2011 Jun 6;8(3):774-87; each of which is herein incorporated by reference in its entirety). As a non-limiting example, the nanoparticle may comprise a plurality of polymers such as, but not limited to hydrophilic-hydrophobic polymers (e.g., PEG-PLGA), hydrophobic polymers (e.g., PEG) and/or hydrophilic polymers (International Pub. No. WO20120225129; herein incorporated by reference in its entirety).

[00462] Biodegradable calcium phosphate nanoparticles in combination with lipids and/or polymers have been shown to deliver modified nucleic acid molecules and mmRNA in vivo. In one embodiment, a lipid coated calcium phosphate nanoparticle, which may also contain a targeting ligand such as anisamide, may be used to deliver the modified nucleic acid molecule and mmRNA of the present invention. For example, to effectively deliver siRNA in a mouse metastatic lung model a lipid coated calcium phosphate nanoparticle was used (Li et al., J Contr Rel. 2010 142: 416-421; Li et al., J Contr Rel. 2012 158:108-114; Yang et al., Mol Ther. 2012 20:609-615; herein incorporated by reference in its entirety). This delivery system combines both a targeted nanoparticle and a component to enhance the endosomal escape, calcium phosphate, in order to improve delivery of the siRNA.

[00463] In one embodiment, calcium phosphate with a PEG-polyanion block copolymer may be used to deliver modified nucleic acid molecules and mmRNA (Kazikawa et al., J Contr Rel. 2004 97:345-356; Kazikawa et al., J Contr Rel. 2006 111:368-370; herein incorporated by reference in its entirety).

[00464] In one embodiment, a PEG-charge-conversional polymer (Pitella et al., Biomaterials. 2011 32:3106-3114) may be used to form a nanoparticle to deliver the modified nucleic acid molecules and mmRNA of the present invention. The PEG-charge-conversional polymer may improve upon the PEG-polyanion block copolymers by being cleaved into a polycation at acidic pH, thus enhancing endosomal escape.

[00465] The use of core-shell nanoparticles has additionally focused on a high-throughput approach to synthesize cationic cross-linked nanogel cores and various shells (Siegwart et al., Proc Natl Acad Sci U S A. 2011 108:12996-13001). The complexation, delivery, and internalization of the
polymeric nanoparticles can be precisely controlled by altering the chemical composition in both the core and shell components of the nanoparticle. For example, the core-shell nanoparticles may efficiently deliver siRNA to mouse hepatocytes after they covalently attach cholesterol to the nanoparticle.

In one embodiment, a hollow lipid core comprising a middle PLGA layer and an outer neutral lipid layer containing PEG may be used to delivery of the modified nucleic acid molecules and mRNA of the present invention. As a non-limiting example, in mice bearing a luciferase-expressing tumor, it was determined that the lipid-polymer-lipid hybrid nanoparticle significantly suppressed luciferase expression, as compared to a conventional lipoplex (Shi et al, Angew Chem Int Ed. 2011 50:7027-7031; herein incorporated by reference in its entirety).

In one embodiment, the lipid nanoparticles may comprise a core of the modified nucleic acid molecules disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the modified nucleic acids in the core.

Core–shell nanoparticles for use with the modified nucleic acid molecules of the present invention are described and may be formed by the methods described in U.S. Pat. No. 8,313,777 herein incorporated by reference in its entirety.

In one embodiment, the core-shell nanoparticles may comprise a core of the modified nucleic acid molecules disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the modified nucleic acid molecules in the core.

**Peptides and Proteins**

The modified nucleic acid molecules and mRNA of the invention can be formulated with peptides and/or proteins in order to increase transfection of cells by the modified nucleic acid molecules or mRNA. In one embodiment, peptides such as, but not limited to, cell penetrating peptides and proteins and peptides that enable intracellular delivery may be used to deliver pharmaceutical formulations. A non-limiting example of a cell penetrating peptide which may be used with the pharmaceutical formulations of the present invention include a cell-penetrating peptide sequence attached to polyethylene that facilitates delivery to the intracellular space, e.g., HIV-derived TAT peptide, penetratin, transportan, or hCT derived cell-penetrating peptides (see, e.g., Caron et al., Mol. Ther. 3(3):310-8 (2001); Langel, Cell-Penetrating Peptides: Processes and
Applications (CRC Press, Boca Raton FL, 2002); El-Andaloussy et al., Curr. Pharm. Des. 11(28):3597-611 (2003); and Deshayes et al., Cell. Mol. Life Sci. 62(16):1839-49 (2005), all of which are incorporated herein by reference). The compositions can also be formulated to include a cell penetrating agent, e.g., liposomes, which enhance delivery of the compositions to the intracellular space. Modified nucleic acid molecules and mmRNA of the invention may be complexed to peptides and/or proteins such as, but not limited to, peptides and/or proteins from Aileron Therapeutics (Cambridge, MA) and Permeon Biologics (Cambridge, MA) in order to enable intracellular delivery (Cronican et al., ACS Chem. Biol. 2010 5:747-752; McNaughton et al., Proc. Natl. Acad. Sci. USA 2009 106:6111-6116; Sawyer, Chem Biol Drug Des. 2009 73:3-6; Verdin and Hilinski, Methods Enzymol. 2012;503:3-33; all of which are herein incorporated by reference in its entirety).

In one embodiment, the cell-penetrating polypeptide may comprise a first domain and a second domain. The first domain may comprise a supercharged polypeptide. The second domain may comprise a protein-binding partner. As used herein, “protein-binding partner” includes, but are not limited to, antibodies and functional fragments thereof, scaffold proteins, or peptides. The cell-penetrating polypeptide may further comprise an intracellular binding partner for the protein-binding partner. The cell-penetrating polypeptide may be capable of being secreted from a cell where the modified nucleic acid molecules or mmRNA may be introduced.

Formulations of the including peptides or proteins may be used to increase cell transfection by the modified nucleic acid molecule or mmRNA, alter the biodistribution of the modified nucleic acid molecule or mmRNA (e.g., by targeting specific tissues or cell types), and/or increase the translation of encoded protein. (See e.g., International Pub. No. WO2012110636; herein incorporated by reference in its entirety).

Cells

The modified nucleic acid molecule and mmRNA of the invention can be transfected ex vivo into cells, which are subsequently transplanted into a subject. As non-limiting examples, the pharmaceutical compositions may include red blood cells to deliver modified RNA to liver and myeloid cells, virosomes to deliver modified nucleic acid molecules and mmRNA in virus-like particles (VLPs), and electroporated cells such as, but not limited to, from MAXCYTE® (Gaithersburg, MD) and from ERYTECH® (Lyon, France) to deliver modified RNA. Examples of use of red blood cells, viral particles and electroporated cells to deliver payloads other than mmRNA
have been documented (Godfrin et al., Expert Opin Biol Ther. 2012 12:127-133; Fang et al., Expert Opin Biol Ther. 2012 12:385-389; Hu et al., Proc Natl Acad Sci U S A. 2011 108:10980-10985; Lund et al., Pharm Res. 2010 27:400-420; Huckriede et al., J Liposome Res. 2007;17:39-47; Cusi, Hum Vaccin. 2006 2:1-7; de Jonge et al., Gene Ther. 2006 13:400-411; all of which are herein incorporated by reference in its entirety). The modified nucleic acid molecules and mmRNA may be delivered in synthetic VLPs synthesized by the methods described in International Pub No. WO2011085231 and US Pub No. 20110171248, each of which are herein incorporated by reference in their entireties.

[00474] Cell-based formulations of the modified nucleic acid molecules and mmRNA of the invention may be used to ensure cell transfection (e.g., in the cellular carrier), alter the biodistribution of the modified nucleic acid molecule or mmRNA (e.g., by targeting the cell carrier to specific tissues or cell types), and/or increase the translation of encoded protein.

Introduction into cells

[00475] A variety of methods are known in the art and suitable for introduction of nucleic acid into a cell, including viral and non-viral mediated techniques. Examples of typical non-viral mediated techniques include, but are not limited to, electroporation, calcium phosphate mediated transfer, nucleofection, sonoporation, heat shock, magnetofection, liposome mediated transfer, microinjection, microprojectile mediated transfer (nanoparticles), cationic polymer mediated transfer (DEAE-dextran, polyethyleneimine, polyethylene glycol (PEG) and the like) or cell fusion.

[00476] The technique of sonoporation, or cellular sonication, is the use of sound (e.g., ultrasonic frequencies) for modifying the permeability of the cell plasma membrane. Sonoporation methods are known to those in the art and are taught for example as it relates to bacteria in US Patent Publication 20100196983 and as it relates to other cell types in, for example, US Patent Publication 20100009424, each of which are incorporated herein by reference in their entirety.

[00477] Electroporation techniques are also well known in the art. In one embodiment, modified nucleic acid molecules or mmRNA may be delivered by electroporation as described in Example 8.

Hyaluronidase

[00478] The intramuscular or subcutaneous localized injection of modified nucleic acid molecules or mmRNA of the invention can include hyaluronidase, which catalyzes the hydrolysis of hyaluronan. By catalyzing the hydrolysis of hyaluronan, a constituent of the interstitial barrier, hyaluronidase lowers the viscosity of hyaluronan, thereby increasing tissue permeability (Frost,
Expert Opin. Drug Deliv. (2007) 4:427-440; herein incorporated by reference in its entirety). It is useful to speed their dispersion and systemic distribution of encoded proteins produced by transfected cells. Alternatively, the hyaluronidase can be used to increase the number of cells exposed to a modified nucleic acid molecule or mRNA of the invention administered intramuscularly or subcutaneously.

*Nanoparticle Mimics*

[00479] The modified nucleic acid molecules and mRNA of the invention may be encapsulated within and/or absorbed to a nanoparticle mimic. A nanoparticle mimic can mimic the delivery function organisms or particles such as, but not limited to, pathogens, viruses, bacteria, fungus, parasites, prions and cells. As a non-limiting example the modified mRNA of the invention may be encapsulated in a non-viron particle which can mimic the delivery function of a virus (see International Pub. No. WO2012006376 herein incorporated by reference in its entirety).

*Nanotubes*

[00480] The modified nucleic acid molecules or mRNA of the invention can be attached or otherwise bound to at least one nanotube such as, but not limited to, rosette nanotubes, rosette nanotubes having twin bases with a linker, carbon nanotubes and/or single-walled carbon nanotubes. The modified nucleic acid molecules or mRNA may be bound to the nanotubes through forces such as, but not limited to, steric, ionic, covalent and/or other forces.

[00481] In one embodiment, the nanotube can release one or more modified nucleic acid molecule or mRNA into cells. The size and/or the surface structure of at least one nanotube may be altered so as to govern the interaction of the nanotubes within the body and/or to attach or bind to the modified nucleic acid molecule or mRNA disclosed herein. In one embodiment, the building block and/or the functional groups attached to the building block of the at least one nanotube may be altered to adjust the dimensions and/or properties of the nanotube. As a non-limiting example, the length of the nanotubes may be altered to hinder the nanotubes from passing through the holes in the walls of normal blood vessels but still small enough to pass through the larger holes in the blood vessels of tumor tissue.

[00482] In one embodiment, at least one nanotube may also be coated with delivery enhancing compounds including polymers, such as, but not limited to, polyethylene glycol. In another embodiment, at least one nanotube and/or the modified mRNA may be mixed with pharmaceutically acceptable excipients and/or delivery vehicles.
In one embodiment, the modified mRNA are attached and/or otherwise bound to at least one rosette nanotube. The rosette nanotubes may be formed by a process known in the art and/or by the process described in International Publication No. WO2012094304, herein incorporated by reference in its entirety. At least one modified mRNA may be attached and/or otherwise bound to at least one rosette nanotube by a process as described in International Publication No. WO2012094304, herein incorporated by reference in its entirety, where rosette nanotubes or modules forming rosette nanotubes are mixed in aqueous media with at least one modified mRNA under conditions which may cause at least one modified mRNA to attach or otherwise bind to the rosette nanotubes.

In one embodiment, the modified nucleic acid molecule or mmRNA may be attached to and/or otherwise bound to at least one carbon nanotube. As a non-limiting example, the modified nucleic acid molecule or mmRNA may be bound to a linking agent and the linked agent may be bound to the carbon nanotube (See e.g., U.S. Pat No. 8,246,995; herein incorporated by reference in its entirety). The carbon nanotube may be a single-walled nanotube (See e.g., U.S. Pat No. 8,246,995; herein incorporated by reference in its entirety).

Conjugates

The modified nucleic acids molecules and mmRNA of the invention include conjugates, such as a modified nucleic acid molecule or mmRNA covalently linked to a carrier or targeting group, or including two encoding regions that together produce a fusion protein (e.g., bearing a targeting group and therapeutic protein or peptide).

The conjugates of the invention include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin); an carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid, an oligonucleotide (e.g. an aptamer). Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HPMA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazene. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine,
peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

[00487] Representative U.S. patents that teach the preparation of polynucleotide conjugates, particularly to RNA, include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,73; 5,317,098; 5,371,245; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941; 6,294,664; 6,320,017; 6,576,752; 6,783,931; 6,900,297; 7,037,646; each of which is herein incorporated by reference in their entirety.

[00488] In one embodiment, the conjugate of the present invention may function as a carrier for the modified nucleic acid molecules and mRNA of the present invention. The conjugate may comprise a cationic polymer such as, but not limited to, polyamine, polylysine, polyalkylenimine, and polyethyleneimine which may be grafted to with poly(ethylene glycol). As a non-limiting example, the conjugate may be similar to the polymeric conjugate and the method of synthesizing the polymeric conjugate described in U.S. Pat. No. 6,586,524 herein incorporated by reference in its entirety.

[00489] The conjugates can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimic or an aptamer.

[00490] Targeting groups can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Targeting groups may also include hormones and
hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, or aptamers. The ligand can be, for example, a lipopolysaccharide, or an activator of p38 MAP kinase.

[00491] The targeting group can be any ligand that is capable of targeting a specific receptor. Examples include, without limitation, folate, GalNAc, galactose, mannose, mannose-6P, apatamers, integrin receptor ligands, chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL, and HDL ligands. In particular embodiments, the targeting group is an aptamer. The aptamer can be unmodified or have any combination of modifications disclosed herein.

[00492] In one embodiment, pharmaceutical compositions of the present invention may include chemical modifications such as, but not limited to, modifications similar to locked nucleic acids.

[00493] Representative U.S. Patents that teach the preparation of locked nucleic acid (LNA) such as those from Santarisi, include, but are not limited to, the following: U.S. Pat. Nos. 6,268,490; 6,670,461; 6,794,499; 6,998,484; 7,053,207; 7,084,125; and 7,399,845, each of which is herein incorporated by reference in its entirety.

[00494] Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found, for example, in Nielsen et al., Science, 1991, 254, 1497-1500.

[00495] Some embodiments featured in the invention include modified nucleic acids or mRNA with phosphorothioate backbones and oligonucleosides with other modified backbones, and in particular --CH₂--NH--CH₂--, --CH₂--N(CH₃)₂--O--CH₂--[known as a methylene (methylimino) or MMI backbone], --CH₂--O--N(CH₃)₂--CH₂--, --CH₂--N(CH₃)₂--N(CH₃)₂--CH₂-- and --N(CH₃)₂--CH₂--CH₂--[wherein the native phosphodiester backbone is represented as --O—P(O)₂--O--CH₂--] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240. In some embodiments, the polynucleotides featured herein have morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

[00496] Modifications at the 2' position may also aid in delivery. Preferably, modifications at the 2' position are not located in a polypeptide-coding sequence, i.e., not in a translatable region. Modifications at the 2' position may be located in a 5' UTR, a 3' UTR and/or a tailing region.
Modifications at the 2' position can include one of the following at the 2' position: H (i.e., 2'-deoxy); F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C1 to C10 alkyl or C2 to C10 alkenyl and alkynyl. Exemplary suitable modifications include O[(CH2)nO]mCH3, O(CH2)nOCH3, O(CH2)nNH2, O(CH2)nCH3, O(CH2)nONH2, and O(CH2)nON[(CH2)nCH3], where n and m are from 1 to about 10. In other embodiments, the modified nucleic acids or mRNA include one of the following at the 2' position: C1 to C10 lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCHR, OCN, Cl, Br, CN, CF3, OCF3, SOCH3, SO2CH3, ONO2, NO2, N3, NH2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties, or a group for improving the pharmacodynamic properties, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O--CH2CH2OCH3, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., Helv. Chim. Acta, 1995, 78:486-504) i.e., an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminoxyethoxy, i.e., a O(CH2)2ON(CH3)2 group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O--CH2--O--CH2--N(CH3)2, also described in examples herein below. Other modifications include 2'-methoxy (2'-OCH3), 2'-aminopropoxy (2'-OCH2CH2CH2NH2) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. Polynucleotides of the invention may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920 and each of which is herein incorporated by reference.

[00497] In still other embodiments, the modified nucleic acid molecule or mRNA is covalently conjugated to a cell-penetrating polypeptide. The cell-penetrating peptide may also include a signal sequence. The conjugates of the invention can be designed to have increased stability; increased cell transfection; and/or altered the biodistribution (e.g., targeted to specific tissues or cell types).
Self-Assembled Nanoparticles

Nucleic Acid Self-Assembled Nanoparticles

[00498] Self-assembled nanoparticles have a well-defined size which may be precisely controlled as the nucleic acid strands may be easily reprogrammable. For example, the optimal particle size for a cancer-targeting nanodelivery carrier is 20-100 nm as a diameter greater than 20 nm avoids renal clearance and enhances delivery to certain tumors through enhanced permeability and retention effect. Using self-assembled nucleic acid nanoparticles a single uniform population in size and shape having a precisely controlled spatial orientation and density of cancer-targeting ligands for enhanced delivery. As a non-limiting example, oligonucleotide nanoparticles were prepared using programmable self-assembly of short DNA fragments and therapeutic siRNAs. These nanoparticles are molecularly identical with controllable particle size and target ligand location and density. The DNA fragments and siRNAs self-assembled into a one-step reaction to generate DNA/siRNA tetrahedral nanoparticles for targeted in vivo delivery. (Lee et al., Nature Nanotechnology 2012 7:389-393; herein incorporated by reference in its entirety).

[00499] In one embodiment, the modified nucleic acid molecules and mmRNA disclosed herein may be formulated as self-assembled nanoparticles. As a non-limiting example, nucleic acids may be used to make nanoparticles which may be used in a delivery system for the modified nucleic acid molecules and/or mmRNA of the present invention (See e.g., International Pub. No. WO2012125987; herein incorporated by reference in its entirety).

[00500] In one embodiment, the nucleic acid self-assembled nanoparticles may comprise a core of the modified nucleic acid molecules or mmRNA disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the modified nucleic acid molecules and mmRNA in the core.

Polymer-Based Self-Assembled Nanoparticles

[00501] Polymers may be used to form sheets which self-assembled into nanoparticles. These nanoparticles may be used to deliver the modified nucleic acids and mmRNA of the present invention. In one embodiment, these self-assembled nanoparticles may be microsponges formed of long polymers of RNA hairpins which form into crystalline ‘pleated’ sheets before self-assembling into microsponges. These microsponges are densely-packed sponge like microparticles which may function as an efficient carrier and may be able to deliver cargo to a cell. The microsponges may be
from 1 um to 300 nm in diameter. The microsponges may be complexed with other agents known in the art to form larger microsponges. As a non-limiting example, the microsponge may be complexed with an agent to form an outer layer to promote cellular uptake such as polycation polyethyleneimine (PEI). This complex can form a 250-nm diameter particle that can remain stable at high temperatures (150°C) (Grabow and Jaegar, Nature Materials 2012, 11:269-269; herein incorporated by reference in its entirety). Additionally these microsponges may be able to exhibit an extraordinary degree of protection from degradation by ribonucleases.

[00502] In another embodiment, the polymer-based self-assembled nanoparticles such as, but not limited to, microsponges, may be fully programmable nanoparticles. The geometry, size and stoichiometry of the nanoparticle may be precisely controlled to create the optimal nanoparticle for delivery of cargo such as, but not limited to, modified nucleic acid molecules and mmRNA.

[00503] In one embodiment, the polymer based nanoparticles may comprise a core of the modified nucleic acid molecules and mmRNA disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the modified nucleic acid molecules and mmRNA in the core.

Inorganic Nanoparticles

[00504] The modified nucleic acid molecules or mmRNAs of the present invention may be formulated in inorganic nanoparticles (U.S. Pat. No. 8,257,745, herein incorporated by reference in its entirety). The inorganic nanoparticles may include, but are not limited to, clay substances that are water swellable. As a non-limiting example, the inorganic nanoparticle may include synthetic smectite clays which are made from simple silicates (See e.g., U.S. Pat. No. 5,585,108 and 8,257,745 each of which are herein incorporated by reference in their entirety).

[00505] In one embodiment, the inorganic nanoparticles may comprise a core of the modified nucleic acids disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the modified nucleic acids in the core.

Semi-conductive and Metallic Nanoparticles

[00506] The modified nucleic acid molecules or mmRNAs of the present invention may be formulated in water-dispersible nanoparticle comprising a semiconductive or metallic material (U.S. Pub. No. 20120228565; herein incorporated by reference in its entirety) or formed in a magnetic nanoparticle (U.S. Pub. No. 20120265001 and 20120283503; each of which is herein incorporated
by reference in its entirety). The water-dispersible nanoparticles may be hydrophobic nanoparticles or hydrophilic nanoparticles.

[00507] In one embodiment, the semi-conductive and/or metallic nanoparticles may comprise a core of the modified nucleic acids disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the modified nucleic acids in the core.

Gels and Hydrogels

[00508] In one embodiment, the modified mRNA disclosed herein may be encapsulated into any hydrogel known in the art which may form a gel when injected into a subject. Hydrogels are a network of polymer chains that are hydrophilic, and are sometimes found as a colloidal gel in which water is the dispersion medium. Hydrogels are highly absorbent (they can contain over 99% water) natural or synthetic polymers. Hydrogels also possess a degree of flexibility very similar to natural tissue, due to their significant water content. The hydrogel described herein may used to encapsulate lipid nanoparticles which are biocompatible, biodegradable and/or porous.

[00509] As a non-limiting example, the hydrogel may be an aptamer-functionalized hydrogel. The aptamer-functionalized hydrogel may be programmed to release one or more modified nucleic acid molecules and/or mmRNA using nucleic acid hybridization. (Battig et al., J. Am. Chem. Society. 2012 134:12410-12413; herein incorporated by reference in its entirety).

[00510] As another non-limiting example, the hydrogel may be a shaped as an inverted opal. The opal hydrogels exhibit higher swelling ratios and the swelling kinetics is an order of magnitude faster as well. Methods of producing opal hydrogels and description of opal hydrogels are described in International Pub. No. WO2012148684, herein incorporated by reference in its entirety.

[00511] In yet another non-limiting example, the hydrogel may be an antibacterial hydrogel. The antibacterial hydrogel may comprise a pharmaceutical acceptable salt or organic material such as, but not limited to pharmaceutical grade and/or medical grade silver salt and aloe vera gel or extract. (International Pub. No. WO2012151438, herein incorporated by reference in its entirety).

[00512] In one embodiment, the modified mRNA may be encapsulated in a lipid nanoparticle and then the lipid nanoparticle may be encapsulated into a hyrdogel.

[00513] In one embodiment, the modified mRNA disclosed herein may be encapsulated into any gel known in the art. As a non-limiting example the gel may be a fluorouracil injectable gel or a fluorouracil injectable gel containing a chemical compound and/or drug known in the art. As
another example, the modified mRNA may be encapsulated in a fluorouracil gel containing epinephrine (See e.g., Smith et al. Cancer Chemotherapy and Pharmacology, 1999 44(4):267-274; herein incorporated by reference in its entirety).

[00514] In one embodiment, the modified nucleic acid molecules and/or mRNA disclosed herein may be encapsulated into a fibrin gel, fibrin hydrogel or fibrin glue. In another embodiment, the modified nucleic acid molecules and/or mRNA may be formulated in a lipid nanoparticle or a rapidly eliminated lipid nanoparticle prior to being encapsulated into a fibrin gel, fibrin hydrogel or a fibrin glue. In yet another embodiment, the modified nucleic acid molecules and/or mRNA may be formulated as a lipoplex prior to being encapsulated into a fibrin gel, hydrogel or a fibrin glue. Fibrin gels, hydrogels and glues comprise two components, a fibrinogen solution and a thrombin solution which is rich in calcium (See e.g., Spicer and Mikos, Journal of Controlled Release 2010. 148: 49-55; Kidd et al. Journal of Controlled Release 2012. 157:80-85; each of which is herein incorporated by reference in its entirety). The concentration of the components of the fibrin gel, hydrogel and/or glue can be altered to change the characteristics, the network mesh size, and/or the degradation characteristics of the gel, hydrogel and/or glue such as, but not limited to changing the release characteristics of the fibrin gel, hydrogel and/or glue. (See e.g., Spicer and Mikos, Journal of Controlled Release 2010. 148: 49-55; Kidd et al. Journal of Controlled Release 2012. 157:80-85; Catelas et al. Tissue Engineering 2008. 14:119-128; each of which is herein incorporated by reference in its entirety). This feature may be advantageous when used to deliver the modified mRNA disclosed herein. (See e.g., Kidd et al. Journal of Controlled Release 2012. 157:80-85; Catelas et al. Tissue Engineering 2008. 14:119-128; each of which is herein incorporated by reference in its entirety).

Cations and Anions

[00515] Formulations of modified nucleic acid molecules disclosed herein may include cations or anions. In one embodiment, the formulations include metal cations such as, but not limited to, Zn2+, Ca2+, Cu2+, Mg+ and combinations thereof. As a non-limiting example, formulations may include polymers and a modified mRNA complexed with a metal cation (See e.g., U.S. Pat. Nos. 6,265,389 and 6,555,525, each of which is herein incorporated by reference in its entirety).

Molded Nanoparticles and Microparticles

[00516] The modified nucleic acid molecules and/or mRNA disclosed herein may be formulated in nanoparticles and/or microparticles. These nanoparticles and/or microparticles may be molded
into any size shape and chemistry. As an example, the nanoparticles and/or microparticles may be made using the PRINT® technology by LIQUIDA TECHNOLOGIES® (Morrisville, NC) (See e.g., International Pub. No. WO2007024323; herein incorporated by reference in its entirety).

[00517] In one embodiment, the molded nanoparticles may comprise a core of the modified nucleic acid molecules and/or mmRNA disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the modified nucleic acid molecules and/or mmRNA in the core.

NanoJackets and NanoLiposomes

[00518] The modified nucleic acid molecules and/or mmRNA disclosed herein may be formulated in NanoJackets and NanoLiposomes by Keystone Nano (State College, PA). NanoJackets are made of compounds that are naturally found in the body including calcium, phosphate and may also include a small amount of silicates. Nanojackets may range in size from 5 to 50 nm and may be used to deliver hydrophilic and hydrophobic compounds such as, but not limited to, modified nucleic acid molecules and/or mmRNA.

[00519] NanoLiposomes are made of lipids such as, but not limited to, lipids which naturally occur in the body. NanoLiposomes may range in size from 60-80 nm and may be used to deliver hydrophilic and hydrophobic compounds such as, but not limited to, modified nucleic acid molecules and/or mmRNA. In one aspect, the modified nucleic acids disclosed herein are formulated in a NanoLiposome such as, but not limited to, Ceramide NanoLiposomes.

Excipients

[00520] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but are not limited to, 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, solid binders, lubricants and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see Remington: The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium may be incompatible with a
substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

[00521] In some embodiments, a pharmaceutically acceptable excipient may be at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient may be approved for use for humans and for veterinary use. In some embodiments, an excipient may be approved by United States Food and Drug Administration. In some embodiments, an excipient may be of pharmaceutical grade. In some embodiments, an excipient may meet the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[00522] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in pharmaceutical formulations. The composition may also include excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents.

[00523] Exemplary diluents include, but are not limited to, calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, etc., and/or combinations thereof.

[00524] Exemplary granulating and/or dispersing agents include, but are not limited to, potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic acid, guar gum, citrus pulp, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, calcium carbonate, silicates, sodium carbonate, cross-linked poly(vinyl-pyrrolidone) (crosipovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose (crosacarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate (VEEGUM®), sodium lauryl sulfate, quaternary ammonium compounds, etc., and/or combinations thereof.

[00525] Exemplary surface active agents and/or emulsifiers include, but are not limited to, natural emulsifiers (e.g. acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays
(e.g. bentonite [aluminum silicate] and VEEGUM® [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (e.g. stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan monolaurate [TWEEN® 20], polyoxyethylene sorbitan [TWEEN® 60], polyoxyethylene sorbitan monooleate [TWEEN® 80], sorbitan monopalmitate [SPAN® 40], sorbitan monostearate [SPAN® 60], sorbitan tristearate [SPAN® 65], glyceryl monooleate, sorbitan monoooleate [SPAN® 80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [MYRJ® 45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxyethylene stearate, and SOLUTOL®), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. CREMOPHOR®), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [BRIJ® 30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, PLUORINC® F 68, POLOXAMER® 188, cetrionium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, etc. and/or combinations thereof.

[00526] Exemplary binding agents include, but are not limited to, starch (e.g. cornstarch and starch paste); gelatin; sugars (e.g. sucrose, glucose, dextrose, dextrin, molasses, lactose, lactitol, mannitol); natural and synthetic gums (e.g. acacia, sodium alginate, extract of Irish moss, panwar gum, ghatti gum, mucilage of isapol husks, carboxymethylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, cellulose acetate, poly(vinyl-pyrrolidone), magnesium aluminum silicate (VEEGUM®), and larch arabogalactan); alginates; polyethylene oxide; polyethylene glycol; inorganic calcium salts; silicic acid; polymethacrylates; waxes; water; alcohol; etc.; and combinations thereof.

[00527] Exemplary preservatives may include, but are not limited to, antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and/or other preservatives. Exemplary antioxidants include, but are not limited to, alpha tocopherol, ascorbic acid, acorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium
bisulfite, sodium metabisulfite, and/or sodium sulfite. Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, disodium edetate, dipotassium edetate, edetic acid, fumaric acid, malic acid, phosphoric acid, sodium edetate, tartaric acid, and/or trisodium edetate. Exemplary antimicrobial preservatives include, but are not limited to, benzalkonium chloride, benzethonium chloride, benzyl alcohol, bronopol, cetrimide, cetlypyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxylenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and/or thimerosal. Exemplary antifungal preservatives include, but are not limited to, butyl paraben, methyl paraben, ethyl paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and/or sorbic acid. Exemplary alcohol preservatives include, but are not limited to, ethanol, polyethylene glycol, phenol, phenolic compounds, bisphenol, chlorobutanol, hydroxybenzoate, and/or phenylethyl alcohol. Exemplary acidic preservatives include, but are not limited to, vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroacetic acid, ascorbic acid, sorbic acid, and/or phytic acid. Other preservatives include, but are not limited to, tocopherol, tocopherol acetate, dextrose mesylate, cetrimide, butylated hydroxyanisol (BHA), butylated hydroxytoluened (BHT), ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium sulfite, potassium metabisulfite, GLYDANT PLUS®, PHENONIP®, methylparaben, GERMALL®115, GERMABEN®II, NEOLONE™, KATHON™, and/or EUXYL®.

[00528] Exemplary buffering agents include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium gluconate, calcium gluceptate, calcium gluconate, d-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer’s solution, ethyl alcohol, etc., and/or combinations thereof.
Exemplary lubricating agents include, but are not limited to, magnesium stearate, calcium stearate, stearic acid, silica, talc, malt, glyceryl behenate, hydrogenated vegetable oils, polyethylene glycol, sodium benzoate, sodium acetate, sodium chloride, leucine, magnesium lauryl sulfate, sodium lauryl sulfate, etc., and combinations thereof.

Exemplary oils include, but are not limited to, almond, apricot kernel, avocado, babassu, bergamot, black current seed, borage, cade, camomile, canola, caraway, carnauba, castor, cinnamon, cocoa butter, coconut, cod liver, coffee, corn, cotton seed, emu, eucalyptus, evening primrose, fish, flaxseed, geraniol, gourd, grape seed, hazel nut, hyssop, isopropyl myristate, jojoba, kukui nut, lavandin, lavender, lemon, litsea cubeba, macadamia nut, mallow, mango seed, meadowfoam seed, mink, nutmeg, olive, orange, orange roughy, palm, palm kernel, peach kernel, peanut, poppy seed, pumpkin seed, rapeseed, rice bran, rosemary, safflower, sandalwood, sasquana, savoury, sea buckthorn, sesame, shea butter, silicone, soybean, sunflower, tea tree, thistle, tsubaki, vetiver, walnut, and wheat germ oils. Exemplary oils include, but are not limited to, butyl stearate, caprylic triglyceride, capric triglyceride, cyclomethicone, diethyl sebacate, dimethicone 360, isopropyl myristate, mineral oil, octyldecaneol, oleyl alcohol, silicone oil, and/or combinations thereof.

Excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents can be present in the composition, according to the judgment of the formulator.

**Delivery**

The present disclosure encompasses the delivery of modified nucleic acid molecules or mmRNA for any of therapeutic, pharmaceutical, diagnostic or imaging by any appropriate route taking into consideration likely advances in the sciences of drug delivery. Delivery may be naked or formulated.

**Naked Delivery**

The modified nucleic acid molecules or mmRNA of the present invention may be delivered to a cell naked. As used herein in, “naked” refers to delivering modified nucleic acid molecules or mmRNA free from agents which promote transfection. For example, the modified nucleic acid molecules or mmRNA delivered to the cell may contain no modifications. The naked modified nucleic acid molecules or mmRNA may be delivered to the cell using routes of administration known in the art and described herein.

**Formulated Delivery**
[00534] The modified nucleic acid molecules or mRNA of the present invention may be formulated, using the methods described herein. The formulations may contain modified nucleic acid molecules or mRNA which may be modified and/or unmodified. The formulations may further include, but are not limited to, cell penetration agents, a pharmaceutically acceptable carrier, a delivery agent, a bioerodible or biocompatible polymer, a solvent, and a sustained-release delivery depot. The formulated modified nucleic acid molecules or mRNA may be delivered to the cell using routes of administration known in the art and described herein.

[00535] The compositions may also be formulated for direct delivery to an organ or tissue in any of several ways in the art including, but not limited to, direct soaking or bathing, via a catheter, by gels, powder, ointments, creams, gels, lotions, and/or drops, by using substrates such as fabric or biodegradable materials coated or impregnated with the compositions, and the like.

Administration

[00536] The modified nucleic acid molecules or mRNA of the present invention may be administered by any route which results in a therapeutically effective outcome. These include, but are not limited to enteral, gastroenteral, epidural, oral, transdermal, epidural (peridural), intracerebral (into the cerebrum), intracerebroventricular (into the cerebral ventricles), epicutaneous (application onto the skin), intradermal, (into the skin itself), subcutaneous (under the skin), nasal administration (through the nose), intravenous (into a vein), intraarterial (into an artery), intramuscular (into a muscle), intracardiac (into the heart), intraosseous infusion (into the bone marrow), intrathecal (into the spinal canal), intraperitoneal, (infusion or injection into the peritoneum), intravesical infusion, intravitreal, (through the eye), intracavernous injection, (into the base of the penis), intravaginal administration, intrauterine, extra-amniotic administration, transdermal (diffusion through the intact skin for systemic distribution), transmucosal (diffusion through a mucous membrane), insufflation (snorting), sublingual, sublabial, enema, eye drops (onto the conjunctiva), or in ear drops. In specific embodiments, compositions may be administered in a way which allows them cross the blood-brain barrier, vascular barrier, or other epithelial barrier. Non-limiting routes of administration for the modified nucleic acids or mRNA of the present invention are described below.

Parenteral and Injectable Administration

[00537] Liquid dosage forms for parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or
elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylen glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

[00538] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[00539] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[00540] In order to prolong the effect of an active ingredient, it is often desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be
controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

Rectal and Vaginal Administration

[00541] Compositions for rectal or vaginal administration are typically suppositories which can be prepared by mixing compositions with suitable non-irritating excipients such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active ingredient.

Oral Administration

[00542] Liquid dosage forms for oral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

[00543] Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, an active ingredient is mixed with at least one inert, pharmaceutically acceptable excipient such as sodium citrate or dicalcium phosphate and/or fillers or extenders (e.g. starches, lactose, sucrose, glucose, mannitol, and silicic acid), binders (e.g. carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, and acacia), humectants (e.g. glycerol), disintegrating agents (e.g. agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate), solution retarding agents (e.g. paraffin), absorption accelerators (e.g. quaternary ammonium compounds), wetting agents (e.g. cetyl alcohol and glycerol monostearate), absorbents (e.g. kaolin and bentonite clay), and lubricants (e.g. talc, calcium stearate,
magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate), and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may comprise buffering agents.

Topical or Transdermal Administration

[00544] As described herein, compositions containing the modified nucleic acid molecules or mRNA of the invention may be formulated for administration topically. The skin may be an ideal target site for delivery as it is readily accessible. Gene expression may be restricted not only to the skin, potentially avoiding nonspecific toxicity, but also to specific layers and cell types within the skin.

[00545] The site of cutaneous expression of the delivered compositions will depend on the route of nucleic acid delivery. Three routes are commonly considered to deliver modified nucleic acid molecules or mRNA to the skin: (i) topical application (e.g. for local/regional treatment); (ii) intradermal injection (e.g. for local/regional treatment); and (iii) systemic delivery (e.g. for treatment of dermatologic diseases that affect both cutaneous and extracutaneous regions). Modified nucleic acid molecules or mRNA can be delivered to the skin by several different approaches known in the art. Most topical delivery approaches have been shown to work for delivery of DNA, such as but not limited to, topical application of non-cationic liposome–DNA complex, cationic liposome–DNA complex, particle-mediated (gene gun), puncture-mediated gene transfections, and viral delivery approaches. After delivery of the nucleic acid, gene products have been detected in a number of different skin cell types, including, but not limited to, basal keratinocytes, sebaceous gland cells, dermal fibroblasts and dermal macrophages.

[00546] In one embodiment, the invention provides for a variety of dressings (e.g., wound dressings) or bandages (e.g., adhesive bandages) for conveniently and/or effectively carrying out methods of the present invention. Typically dressing or bandages may comprise sufficient amounts of pharmaceutical compositions and/or modified nucleic acid molecules or mRNA described herein to allow a user to perform multiple treatments of a subject(s).

[00547] In one embodiment, the invention provides for the modified nucleic acid molecules or mRNA compositions to be delivered in more than one injection.

[00548] In one embodiment, before topical and/or transdermal administration at least one area of tissue, such as skin, may be subjected to a device and/or solution which may increase permeability. In one embodiment, the tissue may be subjected to an abrasion device to increase the permeability of the skin (see U.S. Patent Publication No. 20080275468, herein incorporated by reference in its
entirety). In another embodiment, the tissue may be subjected to an ultrasound enhancement device. An ultrasound enhancement device may include, but is not limited to, the devices described in U.S. Publication No. 20040236268 and U.S. Patent Nos. 6,491,657 and 6,234,990; each of which are herein incorporated by reference in their entireties. Methods of enhancing the permeability of tissue are described in U.S. Publication Nos. 20040171980 and 20040236268 and U.S. Pat. No. 6,190,315; each of which are herein incorporated by reference in their entireties.

[00549] In one embodiment, a device may be used to increase permeability of tissue before delivering formulations of modified mRNA described herein. The permeability of skin may be measured by methods known in the art and/or described in U.S. Patent No. 6,190,315, herein incorporated by reference in its entirety. As a non-limiting example, a modified mRNA formulation may be delivered by the drug delivery methods described in U.S. Patent No. 6,190,315, herein incorporated by reference in its entirety.

[00550] In another non-limiting example tissue may be treated with a eutectic mixture of local anesthetics (EMLA) cream before, during and/or after the tissue may be subjected to a device which may increase permeability. Katz et al. (Anesth Analg (2004); 98:371-76; herein incorporated by reference in its entirety) showed that using the EMLA cream in combination with a low energy, an onset of superficial cutaneous analgesia was seen as fast as 5 minutes after a pretreatment with a low energy ultrasound.

[00551] In one embodiment, enhancers may be applied to the tissue before, during, and/or after the tissue has been treated to increase permeability. Enhancers include, but are not limited to, transport enhancers, physical enhancers, and cavitation enhancers. Non-limiting examples of enhancers are described in U.S. Patent No. 6,190,315, herein incorporated by reference in its entirety.

[00552] In one embodiment, a device may be used to increase permeability of tissue before delivering formulations of modified mRNA described herein, which may further contain a substance that invokes an immune response. In another non-limiting example, a formulation containing a substance to invoke an immune response may be delivered by the methods described in U.S. Publication Nos. 20040171980 and 20040236268; each of which are herein incorporated by reference in their entireties.

[00553] Dosage forms for topical and/or transdermal administration of a composition may include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants and/or patches.
Generally, an active ingredient is admixed under sterile conditions with a pharmaceutically acceptable excipient and/or any needed preservatives and/or buffers as may be required.

[00554] Additionally, the present invention contemplates the use of transdermal patches, which often have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms may be prepared, for example, by dissolving and/or dispensing the compound in the proper medium. Alternatively or additionally, rate may be controlled by either providing a rate controlling membrane and/or by dispersing the compound in a polymer matrix and/or gel.

[00555] Formulations suitable for topical administration include, but are not limited to, liquid and/or semi liquid preparations such as liniments, lotions, oil in water and/or water in oil emulsions such as creams, ointments and/or pastes, and/or solutions and/or suspensions. Topically-administrable formulations may, for example, comprise from about 0.1% to about 10% (w/w) active ingredient, although the concentration of active ingredient may be as high as the solubility limit of the active ingredient in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

Depot Administration

[00556] As described herein, in some embodiments, the composition is formulated in depots for extended release. Generally, a specific organ or tissue (a “target tissue”) is targeted for administration.

[00557] In some aspects of the invention, the modified nucleic acid molecules or mRNA are spatially retained within or proximal to a target tissue. Provided are method of providing a composition to a target tissue of a mammalian subject by contacting the target tissue (which contains one or more target cells) with the composition under conditions such that the composition, in particular the nucleic acid component(s) of the composition, is substantially retained in the target tissue, meaning that at least 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the composition is retained in the target tissue. Advantageously, retention is determined by measuring the amount of the nucleic acid present in the composition that enters one or more target cells. For example, at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the nucleic acids administered to the subject are present intracellularly at a period of time following administration. For example, intramuscular injection to a mammalian subject is performed using an aqueous composition containing a ribonucleic acid and a
transfection reagent, and retention of the composition is determined by measuring the amount of the ribonucleic acid present in the muscle cells.

[00558] Aspects of the invention are directed to methods of providing a composition to a target tissue of a mammalian subject, by contacting the target tissue (containing one or more target cells) with the composition under conditions such that the composition is substantially retained in the target tissue. The composition contains an effective amount of a nucleic acid molecules or mmRNA such that the polypeptide of interest is produced in at least one target cell. The compositions generally contain a cell penetration agent, although “naked” nucleic acid (such as nucleic acids without a cell penetration agent or other agent) is also contemplated, and a pharmaceutically acceptable carrier.

[00559] In some circumstances, the amount of a protein produced by cells in a tissue is desirably increased. Preferably, this increase in protein production is spatially restricted to cells within the target tissue. Thus, provided are methods of increasing production of a protein of interest in a tissue of a mammalian subject. A composition is provided that contains modified nucleic acid molecule or mmRNA characterized in that a unit quantity of composition has been determined to produce the polypeptide of interest in a substantial percentage of cells contained within a predetermined volume of the target tissue.

[00560] In some embodiments, the composition includes a plurality of different modified nucleic acid molecules or mmRNA, where one or more than one of the modified nucleic acid molecules or mmRNA encodes a polypeptide of interest. Optionally, the composition also contains a cell penetration agent to assist in the intracellular delivery of the composition. A determination is made of the dose of the composition required to produce the polypeptide of interest in a substantial percentage of cells contained within the predetermined volume of the target tissue (generally, without inducing significant production of the polypeptide of interest in tissue adjacent to the predetermined volume, or distally to the target tissue). Subsequent to this determination, the determined dose is introduced directly into the tissue of the mammalian subject.

[00561] In one embodiment, the invention provides for the modified nucleic acid molecules or mmRNA to be delivered in more than one injection or by split dose injections.

[00562] In one embodiment, the invention may be retained near target tissue using a small disposable drug reservoir, patch pump or osmotic pump. Non-limiting examples of patch pumps include those manufactured and/or sold by BD®, (Franklin Lakes, NJ), Insulet Corporation
(Bedford, MA), SteadyMed Therapeutics (San Francisco, CA), Medtronic (Minneapolis, MN) (e.g., MiniMed), UniLife (York, PA), Valeritas (Bridgewater, NJ), and SpringLeaf Therapeutics (Boston, MA). A non-limiting example of an osmotic pump include those manufactured by DURECT® (Cupertino, CA) (e.g., DUROS® and ALZET®).

**Pulmonary Administration**

[00563] A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 nm to about 7 nm or from about 1 nm to about 6 nm. Such compositions are suitably in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder and/or using a self-propelling solvent/powder dispensing container such as a device comprising the active ingredient dissolved and/or suspended in a low-boiling propellant in a sealed container. Such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nm and at least 95% of the particles by number have a diameter less than 7 nm. Alternatively, at least 95% of the particles by weight have a diameter greater than 1 nm and at least 90% of the particles by number have a diameter less than 6 nm. Dry powder compositions may include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

[00564] Low boiling propellants generally include liquid propellants having a boiling point of below 65 °F at atmospheric pressure. Generally the propellant may constitute 50% to 99.9% (w/w) of the composition, and active ingredient may constitute 0.1% to 20% (w/w) of the composition. A propellant may further comprise additional ingredients such as a liquid non-ionic and/or solid anionic surfactant and/or a solid diluent (which may have a particle size of the same order as particles comprising the active ingredient).

[00565] As a non-limiting example, the modified nucleic acid molecules or mRNA described herein may be formulated for pulmonary delivery by the methods described in U.S. Pat. No. 8,257,685; herein incorporated by reference in its entirety.

[00566] Pharmaceutical compositions formulated for pulmonary delivery may provide an active ingredient in the form of droplets of a solution and/or suspension. Such formulations may be prepared, packaged, and/or sold as aqueous and/or dilute alcoholic solutions and/or suspensions, optionally sterile, comprising active ingredient, and may conveniently be administered using any
nebulization and/or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, and/or a preservative such as methylhydroxybenzoate. Droplets provided by this route of administration may have an average diameter in the range from about 0.1 nm to about 200 nm.

_Intranasal, nasal and buccal Administration_

**[00567]** Formulations described herein as being useful for pulmonary delivery are useful for intranasal delivery of a pharmaceutical composition. Another formulation suitable for intranasal administration is a coarse powder comprising the active ingredient and having an average particle from about 0.2 μm to 500 μm. Such a formulation is administered in the manner in which snuff is taken, _i.e._ by rapid inhalation through the nasal passage from a container of the powder held close to the nose.

**[00568]** Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of active ingredient, and may comprise one or more of the additional ingredients described herein. A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets and/or lozenges made using conventional methods, and may, for example, 0.1% to 20% (w/w) active ingredient, the balance comprising an orally dissolvable and/or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise a powder and/or an aerosolized and/or atomized solution and/or suspension comprising active ingredient. Such powdered, aerosolized, and/or aerosolized formulations, when dispersed, may have an average particle and/or droplet size in the range from about 0.1 nm to about 200 nm, and may further comprise one or more of any additional ingredients described herein.

_Ophthalmic Administration_

**[00569]** A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for ophthalmic administration. Such formulations may, for example, be in the form of eye drops including, for example, a 0.1/1.0% (w/w) solution and/or suspension of the active ingredient in an aqueous or oily liquid excipient. Such drops may further comprise buffering agents, salts, and/or one or more other of any additional ingredients described herein. Other ophthalmically-administrable formulations which are useful include those which comprise the active ingredient in
microcrystalline form and/or in a liposomal preparation. Ear drops and/or eye drops are contemplated as being within the scope of this invention. A multilayer thin film device may be prepared to contain a pharmaceutical composition for delivery to the eye and/or surrounding tissue.

Payload Administration: Detectable Agents and Therapeutic Agents

[00570] The modified nucleic acid molecules or mmRNA described herein can be used in a number of different scenarios in which delivery of a substance (the “payload”) to a biological target is desired, for example delivery of detectable substances for detection of the target, or delivery of a therapeutic agent. Detection methods can include, but are not limited to, both imaging in vitro and in vivo imaging methods, e.g., immunohistochemistry, bioluminescence imaging (BLI), Magnetic Resonance Imaging (MRI), positron emission tomography (PET), electron microscopy, X-ray computed tomography, Raman imaging, optical coherence tomography, absorption imaging, thermal imaging, fluorescence reflectance imaging, fluorescence microscopy, fluorescence molecular tomographic imaging, nuclear magnetic resonance imaging, X-ray imaging, ultrasound imaging, photoacoustic imaging, lab assays, or in any situation where tagging/staining/imaging is required.

[00571] The modified nucleic acid molecules or mmRNA can be designed to include both a linker and a payload in any useful orientation. For example, a linker having two ends is used to attach one end to the payload and the other end to the nucleobase, such as at the C-7 or C-8 positions of the deaza-adenosine or deaza-guanosine or to the N-3 or C-5 positions of cytosine or uracil. The polynucleotide of the invention can include more than one payload (e.g., a label and a transcription inhibitor), as well as a cleavable linker.

[00572] In one embodiment, the modified nucleotide is a modified 7-deaza-adenosine triphosphate, where one end of a cleavable linker is attached to the C7 position of 7-deaza-adenine, the other end of the linker is attached to an inhibitor (e.g., to the C5 position of the nucleobase on a cytidine), and a label (e.g., Cy5) is attached to the center of the linker (see, e.g., compound 1 of A*pCp C5 Parg Capless in Fig. 5 and columns 9 and 10 of U.S. Pat. No. 7,994,304, incorporated herein by reference). Upon incorporation of the modified 7-deaza-adenosine triphosphate to an encoding region, the resulting polynucleotide having a cleavable linker attached to a label and an inhibitor (e.g., a polymerase inhibitor). Upon cleavage of the linker (e.g., with reductive conditions to reduce a linker having a cleavable disulfide moiety), the label and inhibitor are released. Additional linkers and payloads (e.g., therapeutic agents, detectable labels, and cell penetrating payloads) are described herein.
Scheme 12 below depicts an exemplary modified nucleotide wherein the nucleobase, adenine, is attached to a linker at the C-7 carbon of 7-deaza adenine. In addition, Scheme 12 depicts the modified nucleotide with the linker and payload, e.g., a detectable agent, incorporated onto the 3’ end of the mRNA. Disulfide cleavage and 1,2-addition of the thiol group onto the propargyl ester releases the detectable agent. The remaining structure (depicted, for example, as pApC5Parg in Scheme 12) is the inhibitor. The rationale for the structure of the modified nucleotides is that the tethered inhibitor sterically interferes with the ability of the polymerase to incorporate a second base. Thus, it is critical that the tether be long enough to affect this function and that the inhibitor be in a stereochemical orientation that inhibits or prohibits second and follow on nucleotides into the growing polynucleotide strand.
[00574] For example, the modified nucleic acid molecules or mmRNA described herein can be used in reprogramming induced pluripotent stem cells (iPS cells), which can directly track cells that are transfected compared to total cells in the cluster. In another example, a drug that may be attached to the modified nucleic acid molecules or mmRNA via a linker and may be fluorescently labeled can be used to track the drug in vivo, e.g. intracellularly. Other examples include, but are not limited to, the use of modified nucleic acid molecules or mmRNA in reversible drug delivery into cells.

[00575] The modified nucleic acid molecules or mmRNA described herein can be used in intracellular targeting of a payload, e.g., detectable or therapeutic agent, to specific organelle. Exemplary intracellular targets can include, but are not limited to, the nuclear localization for advanced mRNA processing, or a nuclear localization sequence (NLS) linked to the mRNA containing an inhibitor.
[00576] In addition, the modified nucleic acid molecules or mmRNA described herein can be used to deliver therapeutic agents to cells or tissues, e.g., in living animals. For example, the modified nucleic acids or mmRNA described herein can be used to deliver highly polar chemotherapeutics agents to kill cancer cells. The modified nucleic acid molecules or mmRNA attached to the therapeutic agent through a linker can facilitate member permeation allowing the therapeutic agent to travel into a cell to reach an intracellular target.

[00577] In one example, the linker is attached at the 2'-position of the ribose ring and/or at the 3' and/or 5' position of the modified nucleic acid molecule or mmRNA (See e.g., International Pub. No. WO2012030683, herein incorporated by reference in its entirety). The linker may be any linker disclosed herein, known in the art and/or disclosed in International Pub. No. WO2012030683, herein incorporated by reference in its entirety.

[00578] In another example, the modified nucleic acid molecules or mmRNA can be attached to the modified nucleic acid molecules or mmRNA a viral inhibitory peptide (VIP) through a cleavable linker. The cleavable linker can release the VIP and dye into the cell. In another example, the modified nucleic acid molecules or mmRNA can be attached through the linker to an ADP-ribosylate, which is responsible for the actions of some bacterial toxins, such as cholera toxin, diphtheria toxin, and pertussis toxin. These toxin proteins are ADP-ribosyltransferases that modify target proteins in human cells. For example, cholera toxin ADP-ribosylates G proteins modifies human cells by causing massive fluid secretion from the lining of the small intestine, which results in life-threatening diarrhea.

[00579] In some embodiments, the payload may be a therapeutic agent such as a cytotoxin, radioactive ion, chemotherapeutic, or other therapeutic agent. A cytotoxin or cytotoxic agent includes any agent that may be detrimental to cells. Examples include, but are not limited to, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicine, doxorubicin, daunorubicin, dihydroxyanthracenedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansinoids, e.g., maytansinol (see U.S. Pat. No. 5,208,020 incorporated herein in its entirety), rachemycin (CC-1065, see U.S. Pat. Nos. 5,475,092, 5,585,499, and 5,846,545, all of which are incorporated herein by reference), and analogs or homologs thereof. Radioactive ions include, but are not limited to iodine (e.g., iodine 125 or iodine 131), strontium 89, phosphorous, palladium, cesium, iridium, phosphate, cobalt, yttrium 90,
samarium 153, and praseodymium. Other therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thiopeta chlorambucil, rachelmycin (CC-1065), melphalan, carmustine (BSNU), lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine, vinblastine, taxol and maytansinoids).

[00580] In some embodiments, the payload may be a detectable agent, such as various organic small molecules, inorganic compounds, nanoparticles, enzymes or enzyme substrates, fluorescent materials, luminescent materials (e.g., luminol), bioluminescent materials (e.g., luciferase, luciferin, and aerugin), chemiluminescent materials, radioactive materials (e.g., $^{18}$F, $^{67}$Ga, $^{81}$mKr, $^{88}$Rb, $^{111}$In, $^{123}$I, $^{133}$Xe, $^{201}$Tl, $^{125}$I, $^{35}$S $^{14}$C, $^{3}$H, or $^{99m}$Tc (e.g., as pertechnetate (technetate(VII), TeO$_4^-$)), and contrast agents (e.g., gold (e.g., gold nanoparticles), gadolinium (e.g., chelated Gd), iron oxides (e.g., superparamagnetic iron oxide (SPIO), monocrystalline iron oxide nanoparticles (MIONs), and ultrasmall superparamagnetic iron oxide (USPIO)), manganese chelates (e.g., Mn-DPDP), barium sulfate, iodinated contrast media (iohexol), microbubbles, or perfluorocarbons. Such optically-detectable labels include for example, without limitation, 4-acetamido-4′-isothiocyanatostilbene-2,2′disulfonic acid; acridine and derivatives (e.g., acridine and acridine isothiocyanate); 5-(2′-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS); 4-amino-N-[3-vinylsulfonyl]phenyl)naphthalimide-3,5 disulfonate; N-(4-anilino-l-naphthyl)maleimide; anthranilamide; BODIPY; Brilliant Yellow; coumarin and derivatives (e.g., coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), and 7-amino-4-trifluoromethylcoumarin (Coumarin 151)); cyanine dyes; cyanosine; 4′,6-diaminidino-2-phenylindole (DAPI); 5′ 5″-dibromopyrogallol-sulfonaphthalein (Bromopyrogallol Red); 7-diethylamino-3-(4′-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4′-diisothiocyanatodihydro-stilbene-2,2′-disulfonic acid; 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; 5-[dimethylamino]-naphthalene-1-sulfonyl chloride (DNS, dansylchloride); 4-dimethylaminophenylazophenyl-4′-isothiocyanate (DABITC); eosin and derivatives (e.g., eosin and eosin isothiocyanate); erythrosin and derivatives (e.g., erythrosin B and erythrosin isothiocyanate); ethidium; fluorescein and derivatives (e.g., 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotiazin-2-yl)aminofluorescein (DTAF), 2′,7′-dimethoxy-
4′,5′-dichloro-6-carboxyfluorescein, fluorescein, fluorescein isothiocyanate, X-rhodamine-5-(and-6)-isothiocyanate (QFITC or XRITC), and fluorescamine; 2-[2-[3-[[1,3-dihydro-1,1-dimethyl-3-(3-sulphopropyl)-2H-benz[e]indol-2-ylidene]ethylidene]-2-[4-(ethoxycarbonyl)-1-piperazinyl]-1-cyclopenten-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulphopropyl)-1H-benz[e]indolium hydroxide, inner salt, compound with n,n-diethylethanamine (1:1) (IR144); 5-chloro-2-[2-[3-[(5-chloro-3-ethyl-2(3H)-benzothiazol-ylidene)ethylidene]-2-(diphenylamino)-1-cyclopenten-1-yl]ethenyl]-3-ethyl benzothiazolium perchlorate (IR140); Malachite Green isothiocyanate; 4-methylumbelliferone orthocresolsolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives (e.g., pyrene, pyrene butyrate, and succinimidyl 1-pyrene); butyrate quantum dots; Reactive Red 4 (CibaconTM Brilliant Red 3B-A); rhodamine and derivatives (e.g., 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulfhorhodamine B, sulfhorhodamine 101, sulfonyl chloride derivative of sulfhorhodamine 101 (Texas Red), N,N,N′,N′-tetramethyl-6-carboxyrhodamine (TAMRA) tetramethyl rhodamine, and tetramethyl rhodamine isothiocyanate (TRITC)); riboflavin; rosolic acid; terbium chelate derivatives; Cyanine-3 (Cy3); Cyanine-5 (Cy5); cyanine-5.5 (Cy5.5), Cyanine-7 (Cy7); IRD 700; IRD 800; Alexa 647; La Jolta Blue; phthalo cyanine; and naphthalo cyanine.

[00581] In some embodiments, the detectable agent may be a non-detectable pre-cursor that becomes detectable upon activation (e.g., fluorogenic tetrazine-fluorophore constructs (e.g., tetrazine-BODIPY FL, tetrazine-Oregon Green 488, or tetrazine-BODIPY TMR-X) or enzyme activatable fluorogenic agents (e.g., PROSENSE® (VisEn Medical)). In vitro assays in which the enzyme labeled compositions can be used include, but are not limited to, enzyme linked immunosorbent assays (ELISAs), immunoprecipitation assays, immunofluorescence, enzyme immunoassays (EIA), radioimmunoassays (RIA), and Western blot analysis. Combinations

[00582] The nucleic acid molecules or mRNA may be used in combination with one or more other therapeutic, prophylactic, diagnostic, or imaging agents. By “in combination with,” it is not intended to imply that the agents must be administered at the same time and/or formulated for delivery together, although these methods of delivery are within the scope of the present disclosure. Compositions can be administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics or medical procedures. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent. In some embodiments, the present disclosure
encompasses the delivery of pharmaceutical, prophylactic, diagnostic, or imaging compositions in combination with agents that may improve their bioavailability, reduce and/or modify their metabolism, inhibit their excretion, and/or modify their distribution within the body. As a non-limiting example, the nucleic acid molecules or mRNA may be used in combination with a pharmaceutical agent for the treatment of cancer or to control hyperproliferative cells. In U.S. Pat. No. 7,964,571, herein incorporated by reference in its entirety, a combination therapy for the treatment of solid primary or metastasized tumor is described using a pharmaceutical composition including a DNA plasmid encoding for interleukin-12 with a lipopolymer and also administering at least one anticancer agent or chemotherapeutic. Further, the nucleic acid molecules and mRNA of the present invention that encodes anti-proliferative molecules may be in a pharmaceutical composition with a lipopolymer (see e.g., U.S. Pub. No. 20110218231, herein incorporated by reference in its entirety, claiming a pharmaceutical composition comprising a DNA plasmid encoding an anti-proliferative molecule and a lipopolymer) which may be administered with at least one chemotherapeutic or anticancer agent.

Cell Penetrating Payloads

[00583] In some embodiments, the modified nucleotides and modified nucleic acid molecules, which are incorporated into a nucleic acid, e.g., RNA or mRNA, can also include a payload that can be a cell penetrating moiety or agent that enhances intracellular delivery of the compositions. For example, the compositions can include, but are not limited to, a cell-penetrating peptide sequence that facilitates delivery to the intracellular space, e.g., HIV-derived TAT peptide, penetratin, transportan, or hCT derived cell-penetrating peptides, see, e.g., Caron et al., (2001) Mol Ther. 3(3):310-8; Langel, Cell-Penetrating Peptides: Processes and Applications (CRC Press, Boca Raton FL 2002); El-Andaloussi et al., (2005) Curr Pharm Des. 11(28):3597-611; and Deshayes et al., (2005) Cell Mol Life Sci. 62(16):1839-49; all of which are incorporated herein by reference. The compositions can also be formulated to include a cell penetrating agent, e.g., liposomes, which enhance delivery of the compositions to the intracellular space.

Biological Targets

[00584] The modified nucleotides and modified nucleic acid molecules described herein, which are incorporated into a nucleic acid, e.g., RNA or mRNA, can be used to deliver a payload to any biological target for which a specific ligand exists or can be generated. The ligand can bind to the biological target either covalently or non-covalently.
[00585] Examples of biological targets include, but are not limited to, biopolymers, e.g., antibodies, nucleic acids such as RNA and DNA, proteins, enzymes; examples of proteins include, but are not limited to, enzymes, receptors, and ion channels. In some embodiments the target may be a tissue- or a cell-type specific marker, e.g., a protein that is expressed specifically on a selected tissue or cell type. In some embodiments, the target may be a receptor, such as, but not limited to, plasma membrane receptors and nuclear receptors; more specific examples include, but are not limited to, G-protein-coupled receptors, cell pore proteins, transporter proteins, surface-expressed antibodies, HLA proteins, MHC proteins and growth factor receptors.

Dosing

[00586] The present invention provides methods comprising administering modified mRNAs and their encoded proteins or complexes in accordance with the invention to a subject in need thereof. Nucleic acids, proteins or complexes, or pharmaceutical, imaging, diagnostic, or prophylactic compositions thereof, may be administered to a subject using any amount and any route of administration effective for preventing, treating, diagnosing, or imaging a disease, disorder, and/or condition (e.g., a disease, disorder, and/or condition relating to working memory deficits). 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. Compositions in accordance with the invention 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 the compositions of the present invention 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.

[00587] In certain embodiments, compositions in accordance with the present invention may be administered at dosage levels sufficient to deliver from about 0.0001 mg/kg to about 100 mg/kg, from about 0.001 mg/kg to about 0.05 mg/kg, from about 0.005 mg/kg to about 0.05 mg/kg, from
about 0.001 mg/kg to about 0.005 mg/kg, from about 0.05 mg/kg to about 0.5 mg/kg, from about 0.01 mg/kg to about 50 mg/kg, from about 0.1 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 25 mg/kg, of subject body weight per day, one or more times a day, to obtain the desired therapeutic, diagnostic, prophylactic, or imaging effect. 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, or every four weeks. 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).

[00588] According to the present invention, it has been discovered that administration of mRNA in split-dose regimens produces higher levels of proteins in mammalian subjects. As used herein, a “split dose” is the division of a single unit dose or total daily dose into two or more doses, e.g., two or more administrations of the single unit dose. As used herein, a “single unit dose” is a dose of any therapeutic administered in one dose at one time/single route/single point of contact, i.e., single administration event. As used herein, a “total daily dose” is an amount given or prescribed in 24 hr period. It may be administered as a single unit dose. In one embodiment, the mRNA of the present invention are administered to a subject in split doses. The mRNA may be formulated in buffer only or in a formulation described herein.

Dosage Forms

[00589] A pharmaceutical composition described herein can be formulated into a dosage form described herein, such as a topical, intranasal, intratracheal, or injectable (e.g., intravenous, intraocular, intravitreal, intramuscular, intracardiac, intraperitoneal, subcutaneous).

Liquid dosage forms

[00590] Liquid dosage forms for parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art including, but not limited to, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. In certain
embodiments for parenteral administration, compositions may be mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

Injectable

[00591] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art and may include suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed include, but are not limited to, water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[00592] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[00593] In order to prolong the effect of an active ingredient, it may be desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of modified mRNA then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered modified mRNA may be accomplished by dissolving or suspending the modified mRNA in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the modified mRNA in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of modified mRNA to polymer and the nature of the particular polymer employed, the rate of modified mRNA release can be controlled. Examples of other biodegradable polymers include, but are not limited to, poly(orthoesters) and poly(anhydrides). Depot injectable formulations may be prepared by entrapping the modified mRNA in liposomes or microemulsions which are compatible with body tissues.
Pulmonary

[00594] Formulations described herein as being useful for pulmonary delivery may also be used for intranasal delivery of a pharmaceutical composition. Another formulation suitable for intranasal administration may be a coarse powder comprising the active ingredient and having an average particle from about 0.2 μm to 500 μm. Such a formulation may be administered in the manner in which snuff is taken, i.e. by rapid inhalation through the nasal passage from a container of the powder held close to the nose.

[00595] Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of active ingredient, and may comprise one or more of the additional ingredients described herein. A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets and/or lozenges made using conventional methods, and may, for example, contain about 0.1% to 20% (w/w) active ingredient, where the balance may comprise an orally dissolvable and/or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise a powder and/or an aerosolized and/or atomized solution and/or suspension comprising active ingredient. Such powdered, aerosolized, and/or aerosolized formulations, when dispersed, may have an average particle and/or droplet size in the range from about 0.1 nm to about 200 nm, and may further comprise one or more of any additional ingredients described herein.

[00596] General considerations in the formulation and/or manufacture of pharmaceutical agents 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).

Coatings or Shells

[00597] Solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally comprise opacifying agents and can be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.
Properties of the Pharmaceutical Compositions

[00598] The pharmaceutical compositions described herein can be characterized by one or more of the following properties:

Bioavailability

[00599] The modified nucleic acid molecules and mmRNA, when formulated into a composition with a delivery agent as described herein, can exhibit an increase in bioavailability as compared to a composition lacking a delivery agent as described herein. As used herein, the term “bioavailability” refers to the systemic availability of a given amount of a modified nucleic acid molecule administered to a mammal. Bioavailability can be assessed by measuring the area under the curve (AUC) or the maximum serum or plasma concentration (C_{max}) of the unchanged form of a compound following administration of the compound to a mammal. AUC is a determination of the area under the curve plotting the serum or plasma concentration of a compound along the ordinate (Y-axis) against time along the abscissa (X-axis). Generally, the AUC for a particular compound can be calculated using methods known to those of ordinary skill in the art and as described in G. S. Banker, Modern Pharmaceutics, Drugs and the Pharmaceutical Sciences, v. 72, Marcel Dekker, New York, Inc., 1996, herein incorporated by reference in its entirety.

[00600] The C_{max} value is the maximum concentration of the compound achieved in the serum or plasma of a mammal following administration of the compound to the mammal. The C_{max} value of a particular compound can be measured using methods known to those of ordinary skill in the art. The phrases “increasing bioavailability” or “improving the pharmacokinetics,” as used herein mean that the systemic availability of a first modified nucleic acid molecule, measured as AUC, C_{max}, or C_{min} in a mammal is greater, when co-administered with a delivery agent as described herein, than when such co-administration does not take place. In some embodiments, the bioavailability of the modified nucleic acid molecule can increase by at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%.

Therapeutic Window

[00601] The modified nucleic acid molecules and mmRNA when formulated into a composition with a delivery agent as described herein, can exhibit an increase in the therapeutic window of the
administered modified nucleic acid molecule composition as compared to the therapeutic window of the administrated modified nucleic acid molecule composition lacking a delivery agent as described herein. As used herein “therapeutic window” refers to the range of plasma concentrations, or the range of levels of therapeutically active substance at the site of action, with a high probability of eliciting a therapeutic effect. In some embodiments, the therapeutic window of the modified nucleic acid molecule when co-administered with a delivery agent as described herein can increase by at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%.

Volume of Distribution

[00602] The modified nucleic acid molecules, when formulated into a composition with a delivery agent as described herein, can exhibit an improved volume of distribution ($V_{\text{dist}}$), e.g., reduced or targeted, relative to a modified nucleic acid molecule composition lacking a delivery agent as described herein. The volume of distribution ($V_{\text{dist}}$) relates the amount of the drug in the body to the concentration of the drug in the blood or plasma. As used herein, the term “volume of distribution” refers to the fluid volume that would be required to contain the total amount of the drug in the body at the same concentration as in the blood or plasma: $V_{\text{dist}}$ equals the amount of drug in the body/concentration of drug in blood or plasma. For example, for a 10 mg dose and a plasma concentration of 10 mg/L, the volume of distribution would be 1 liter. The volume of distribution reflects the extent to which the drug is present in the extravascular tissue. A large volume of distribution reflects the tendency of a compound to bind to the tissue components compared with plasma protein binding. In a clinical setting, $V_{\text{dist}}$ can be used to determine a loading dose to achieve a steady state concentration. In some embodiments, the volume of distribution of the modified nucleic acid molecule when co-administered with a delivery agent as described herein can decrease at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%.

Biological Effect
In one embodiment, the biological effect of the modified mRNA delivered to the animals may be categorized by analyzing the protein expression in the animals. The protein expression may be determined from analyzing a biological sample collected from a mammal administered the modified mRNA of the present invention. In one embodiment, the expression protein encoded by the modified mRNA administered to the mammal of at least 50 pg/ml may be preferred. For example, a protein expression of 50-200 pg/ml for the protein encoded by the modified mRNA delivered to the mammal may be seen as a therapeutically effective amount of protein in the mammal.

Detection of Modified Nucleic Acids by Mass Spectrometry

Mass spectrometry (MS) is an analytical technique that can provide structural and molecular mass/concentration information on molecules after their conversion to ions. The molecules are first ionized to acquire positive or negative charges and then they travel through the mass analyzer to arrive at different areas of the detector according to their mass/charge (m/z) ratio.

Mass spectrometry is performed using a mass spectrometer which includes an ion source for ionizing the fractionated sample and creating charged molecules for further analysis. For example ionization of the sample may be performed by electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), photoionization, electron ionization, fast atom bombardment (FAB)/liquid secondary ionization (LSIMS), matrix assisted laser desorption/ionization (MALDI), field ionization, field desorption, thermospray/plasma spray ionization, and particle beam ionization. The skilled artisan will understand that the choice of ionization method can be determined based on the analyte to be measured, type of sample, the type of detector, the choice of positive versus negative mode, etc.

After the sample has been ionized, the positively charged or negatively charged ions thereby created may be analyzed to determine a mass-to-charge ratio (i.e., m/z). Suitable analyzers for determining mass-to-charge ratios include quadrupole analyzers, ion traps analyzers, and time-of-flight analyzers. The ions may be detected using several detection modes. For example, selected ions may be detected (i.e., using a selective ion monitoring mode (SIM)), or alternatively, ions may be detected using a scanning mode, e.g., multiple reaction monitoring (MRM) or selected reaction monitoring (SRM).

Liquid chromatography-multiple reaction monitoring (LC-MS/MRM) coupled with stable isotope labeled dilution of peptide standards has been shown to be an effective method for protein
verification (e.g., Keshishian et al., Mol Cell Proteomics 2009 8: 2339-2349; Kuhn et al., Clin Chem 2009 55:1108-1117; Lopez et al., Clin Chem 2010 56:281-290; each of which are herein incorporated by reference in its entirety). Unlike untargeted mass spectrometry frequently used in biomarker discovery studies, targeted MS methods are peptide sequence–based modes of MS that focus the full analytical capacity of the instrument on tens to hundreds of selected peptides in a complex mixture. By restricting detection and fragmentation to only those peptides derived from proteins of interest, sensitivity and reproducibility are improved dramatically compared to discovery-mode MS methods. This method of mass spectrometry-based multiple reaction monitoring (MRM) quantitation of proteins can dramatically impact the discovery and quantitation of biomarkers via rapid, targeted, multiplexed protein expression profiling of clinical samples.

[00608] In one embodiment, a biological sample which may contain at least one protein encoded by at least one modified mRNA of the present invention may be analyzed by the method of MRM-MS. The quantification of the biological sample may further include, but is not limited to, isotopically labeled peptides or proteins as internal standards.

[00609] According to the present invention, the biological sample, once obtained from the subject, may be subjected to enzyme digestion. As used herein, the term “digest” means to break apart into shorter peptides. As used herein, the phrase “treating a sample to digest proteins” means manipulating a sample in such a way as to break down proteins in a sample. These enzymes include, but are not limited to, trypsin, endoproteinase Glu-C and chymotrypsin. In one embodiment, a biological sample which may contain at least one protein encoded by at least one modified mRNA of the present invention may be digested using enzymes.

[00610] In one embodiment, a biological sample which may contain protein encoded by modified mRNA of the present invention may be analyzed for protein using electrospray ionization. Electrospray ionization (ESI) mass spectrometry (ESIMS) uses electrical energy to aid in the transfer of ions from the solution to the gaseous phase before they are analyzed by mass spectrometry. Samples may be analyzed using methods known in the art (e.g., Ho et al., Clin Biochem Rev. 2003 24(1):3-12; herein incorporated by reference in its entirety). The ionic species contained in solution may be transferred into the gas phase by dispersing a fine spray of charge droplets, evaporating the solvent and ejecting the ions from the charged droplets to generate a mist of highly charged droplets. The mist of highly charged droplets may be analyzed using at least 1, at least 2, at least 3 or at least 4 mass analyzers such as, but not limited to, a quadropole mass analyzer. Further, the mass
spectrometry method may include a purification step. As a non-limiting example, the first quadrupole may be set to select a single m/z ratio so it may filter out other molecular ions having a different m/z ratio which may eliminate complicated and time-consuming sample purification procedures prior to MS analysis.

[00611] In one embodiment, a biological sample which may contain protein encoded by modified mRNA of the present invention may be analyzed for protein in a tandem ESIMS system (e.g., MS/MS). As non-limiting examples, the droplets may be analyzed using a product scan (or daughter scan) a precursor scan (parent scan) a neutral loss or a multiple reaction monitoring.

[00612] In one embodiment, a biological sample which may contain protein encoded by modified mRNA of the present invention may be analyzed using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MALDIMS). MALDI provides for the nondestructive vaporization and ionization of both large and small molecules, such as proteins. In MALDI analysis, the analyte is first co-crystallized with a large molar excess of a matrix compound, which may also include, but is not limited to, an ultraviolet absorbing weak organic acid. Non-limiting examples of matrices used in MALDI are α-cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid. Laser radiation of the analyte-matrix mixture may result in the vaporization of the matrix and the analyte. The laser induced desorption provides high ion yields of the intact analyte and allows for measurement of compounds with high accuracy. Samples may be analyzed using methods known in the art (e.g., Lewis, Wei and Siuzdak, Encyclopedia of Analytical Chemistry 2000:5880-5894; herein incorporated by reference in its entirety). As non-limiting examples, mass analyzers used in the MALDI analysis may include a linear time-of-flight (TOF), a TOF reflectron or a Fourier transform mass analyzer.

[00613] In one embodiment, the analyte-matrix mixture may be formed using the dried-droplet method. A biologic sample is mixed with a matrix to create a saturated matrix solution where the matrix-to-sample ratio is approximately 5000:1. An aliquot (approximately 0.5-2.0 µL) of the saturated matrix solution is then allowed to dry to form the analyte-matrix mixture.

[00614] In one embodiment, the analyte-matrix mixture may be formed using the thin-layer method. A matrix homogeneous film is first formed and then the sample is then applied and may be absorbed by the matrix to form the analyte-matrix mixture.

[00615] In one embodiment, the analyte-matrix mixture may be formed using the thick-layer method. A matrix homogeneous film is formed with a nitro-cellulose matrix additive. Once the
uniform nitro-cellulose matrix layer is obtained the sample is applied and absorbed into the matrix to form the analyte-matrix mixture.

**[00616]** In one embodiment, the analyte-matrix mixture may be formed using the sandwich method. A thin layer of matrix crystals is prepared as in the thin-layer method followed by the addition of droplets of aqueous trifluoroacetic acid, the sample and matrix. The sample is then absorbed into the matrix to form the analyte-matrix mixture.

**Uses of Modified Nucleic Acid Molecules**

**Therapeutic Agents**

**[00617]** The modified nucleic acid molecules and the proteins translated from the modified nucleic acid molecules described herein can be used as therapeutic agents. For example, a modified nucleic acid molecule described herein can be administered to a subject, wherein the modified nucleic acid molecule is translated *in vivo* to produce a therapeutic peptide in the subject. Accordingly, provided herein are compositions, methods, kits, and reagents for treatment or prevention of disease or conditions in humans and other mammals. The active therapeutic agents of the present disclosure include, but are not limited to, modified nucleic acid molecules, cells containing modified nucleic acid molecules or polypeptides translated from the modified nucleic acid molecules, polypeptides translated from modified nucleic acid molecules, and cells contacted with cells containing modified nucleic acid molecules or polypeptides translated from the modified nucleic acid molecules.

**[00618]** In certain embodiments, combination therapeutics are provided which may containing one or more modified nucleic acid molecules containing translatable regions along with a protein that induces antibody-dependent cellular toxicity. As used herein “translatable regions” encode for a protein or proteins that may boost a subject’s immunity. For example, provided herein are therapeutics containing one or more nucleic acids that encode trastuzumab and granulocyte-colony stimulating factor (G-CSF). In particular, such combination therapeutics may be useful in Her2+ breast cancer patients who develop induced resistance to trastuzumab. (See, e.g., Albrecht, Immunotherapy. 2(6):795-8 (2010); herein incorporated by reference in its entirety).

**[00619]** Methods of inducing translation of a recombinant polypeptide in a cell population using the modified nucleic acid molecules described herein are also provided. Such translation can be *in vivo*, *ex vivo*, *in culture*, or *in vitro*. The cell population may be contacted with an effective amount of a composition containing a nucleic acid that has at least one nucleoside modification, and a translatable region encoding the recombinant polypeptide. The population may be contacted under
conditions such that the nucleic acid may be localized into one or more cells of the cell population and the recombinant polypeptide may be translated in the cell from the nucleic acid.

[00620] An effective amount of the composition may be provided based, at least in part, on the target tissue, target cell type, means of administration, physical characteristics of the nucleic acid (e.g., size, and extent of modified nucleosides), and other determinants. In general, an effective amount of the composition provides efficient protein production in the cell, preferably more efficient than a composition containing a corresponding unmodified nucleic acid molecule. Increased efficiency may be demonstrated by increased cell transfection (i.e., the percentage of cells transfected with the nucleic acid), increased protein translation from the nucleic acid, decreased nucleic acid degradation (as demonstrated, e.g., by increased duration of protein translation from a modified nucleic acid molecule), or reduced innate immune response of the host cell.

[00621] Aspects of the present disclosure are directed to methods of inducing in vivo translation of a recombinant polypeptide in a mammalian subject in need thereof. Therein, an effective amount of a composition containing a nucleic acid that has at least one nucleoside modification and a translatable region encoding the recombinant polypeptide may be administered to the subject using the delivery methods described herein. The nucleic acid may be provided in an amount and under other conditions such that the nucleic acid is localized into a cell of the subject and the recombinant polypeptide may be translated in the cell from the nucleic acid. The cell in which the nucleic acid is localized, or the tissue in which the cell is present, may be targeted with one or more than one rounds of nucleic acid administration.

[00622] Other aspects of the present disclosure relate to transplantation of cells containing modified nucleic acid molecules to a mammalian subject. Administration of cells to mammalian subjects is known to those of ordinary skill in the art, and include, but is not limited to, local implantation (e.g., topical or subcutaneous administration), organ delivery or systemic injection (e.g., intravenous injection or inhalation), and the formulation of cells in pharmaceutically acceptable carrier. Compositions containing modified nucleic acid molecules are formulated for administration intramuscularly, transarterially, intraperitoneally, intravenously, intransally, subcutaneously, endoscopically, transdermally, or intrathecally. In some embodiments, the composition may be formulated for extended release.

[00623] The subject to whom the therapeutic agent may be administered suffers from or may be at risk of developing a disease, disorder, or deleterious condition. Provided are methods of identifying,
diagnosing, and classifying subjects on these bases, which may include clinical diagnosis, biomarker levels, genome-wide association studies (GWAS), and other methods known in the art.

[00624] In certain embodiments, the administered modified nucleic acid molecule directs production of one or more recombinant polypeptides that provide a functional activity which may be substantially absent in the cell in which the recombinant polypeptide may be translated. For example, the missing functional activity may be enzymatic, structural, or gene regulatory in nature.

[00625] In other embodiments, the administration of a modified nucleic acid molecule directs production of one or more recombinant polypeptides that replace a polypeptide (or multiple polypeptides) that may be substantially absent in the cell in which the recombinant polypeptide may be translated. Such absence may be due to a genetic mutation of the encoding gene or a regulatory pathway thereof. Alternatively, the recombinant polypeptide functions to antagonize the activity of an endogenous protein present in, on the surface of, or secreted from the cell. Usually, the activity of the endogenous protein may be deleterious to the subject, for example, due to the mutation of the endogenous protein resulting in altered activity or localization. Additionally, the recombinant polypeptide antagonizes, directly or indirectly, the activity of a biological moiety present in, on the surface of, or secreted from the cell. Examples of antagonized biological moieties include, but are not limited to, lipids (e.g., cholesterol), a lipoprotein (e.g., low density lipoprotein), a nucleic acid, a carbohydrate, or a small molecule toxin.

[00626] The recombinant proteins described herein may be engineered for localization within the cell, potentially within a specific compartment such as the nucleus, or are engineered for secretion from the cell or translocation to the plasma membrane of the cell.

[00627] As described herein, a useful feature of the modified nucleic acid molecules of the present disclosure is the capacity to reduce the innate immune response of a cell to an exogenous nucleic acid. Provided are methods for performing the titration, reduction or elimination of the immune response in a cell or a population of cells. In some embodiments, the cell may be contacted with a first composition that contains a first dose of a first exogenous nucleic acid including a translatable region and at least one nucleoside modification, and the level of the innate immune response of the cell to the first exogenous nucleic acid may be determined. Subsequently, the cell may be contacted with a second composition, which includes a second dose of the first exogenous nucleic acid, the second dose containing a lesser amount of the first exogenous nucleic acid as compared to the first dose. Alternatively, the cell may be contacted with a first dose of a second exogenous nucleic acid.
The second exogenous nucleic acid may contain one or more modified nucleosides, which may be the same or different from the first exogenous nucleic acid or, alternatively, the second exogenous nucleic acid may not contain modified nucleosides. The steps of contacting the cell with the first composition and/or the second composition may be repeated one or more times. Additionally, efficiency of protein production (e.g., protein translation) in the cell may be optionally determined, and the cell may be re-transfected with the first and/or second composition repeatedly until a target protein production efficiency is achieved.

*Therapeutics for diseases and conditions*

**[00628]** Provided herein are methods for treating or preventing a symptom of diseases, characterized by missing or aberrant protein activity, by supplying the missing protein activity or overcoming the aberrant protein activity. Because of the rapid initiation of protein production following introduction of modified mRNA, as compared to viral DNA vectors, the compounds of the present disclosure are particularly advantageous in treating acute diseases such as sepsis, stroke, and myocardial infarction. Moreover, an accurate titration of protein may be achievable using the modified mRNA of the present disclosure as the modified mRNA may be able to alter transcription rates and thus cause changes in gene expression.

**[00629]** Diseases characterized by dysfunctional or aberrant protein activity include, but are not limited to, cancer and proliferative diseases, genetic diseases (e.g., cystic fibrosis), autoimmune diseases, diabetes, neurodegenerative diseases, cardiovascular diseases, and metabolic diseases. The present disclosure provides a method for treating such conditions or diseases in a subject by introducing nucleic acid or cell-based therapeutics containing the modified nucleic acid molecules provided herein, wherein the modified nucleic acid molecules encode for a protein that antagonizes or otherwise overcomes the aberrant protein activity present in the cell of the subject. Specific examples of a dysfunctional protein include, but are not limited to, the missense mutation variants of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which produce a dysfunctional protein variant of CFTR protein, which causes cystic fibrosis.

**[00630]** Multiple diseases may be characterized by missing (or substantially diminished such that proper protein function does not occur) protein activity. Such proteins may not be present, or they may be essentially non-functional.

**[00631]** Thus, provided are methods of treating cystic fibrosis in a mammalian subject by contacting a cell of the subject with a modified nucleic acid molecule having a translatable region
that encodes a functional CFTR polypeptide, under conditions such that an effective amount of the CFTR polypeptide is present in the cell. Preferred target cells are epithelial cells, such as the lung, and methods of administration are determined in view of the target tissue; i.e., for lung delivery, the RNA molecules are formulated for administration by inhalation.

[00632] In another embodiment, the present disclosure provides a method for treating hyperlipidemia in a subject, by introducing into a cell population of the subject with a modified mRNA molecule encoding Sortilin, a protein recently characterized by genomic studies, thereby ameliorating the hyperlipidemia in a subject. The SORTI gene encodes a trans-Golgi network (TGN) transmembrane protein called Sortilin. Genetic studies have shown that one of five individuals has a single nucleotide polymorphism, rs12740374, in the 1p13 locus of the SORT1 gene that predisposes them to having low levels of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL). Each copy of the minor allele, present in about 30% of people, alters LDL cholesterol by 8 mg/dL, while two copies of the minor allele, present in about 5% of the population, lowers LDL cholesterol 16 mg/dL. Carriers of the minor allele have also been shown to have a 40% decreased risk of myocardial infarction. Functional in vivo studies in mice describes that overexpression of SORT1 in mouse liver tissue led to significantly lower LDL-cholesterol levels, as much as 80% lower, and that silencing SORT1 increased LDL cholesterol approximately 200% (Musunuru K et al. From noncoding variant to phenotype via SORT1 at the 1p13 cholesterol locus. Nature 2010; 466: 714-721; herein incorporated by reference in its entirety).

Methods of cellular nucleic acid delivery

[00633] Methods of the present disclosure enhance nucleic acid delivery into a cell population, in vivo, ex vivo, or in culture. For example, a cell culture containing a plurality of host cells (e.g., eukaryotic cells such as yeast or mammalian cells) may be contacted with a composition that contains an modified nucleic acid molecule having at least one nucleoside modification and, optionally, a translatable region. The composition may also generally contain a transfection reagent or other compound that may increases the efficiency of modified nucleic acid molecule uptake into the host cells. The modified nucleic acid molecule may exhibit enhanced retention in the cell population, relative to a corresponding unmodified nucleic acid molecule. The retention of the modified nucleic acid molecule may greater than the retention of the unmodified nucleic acid molecule. In some embodiments, it is at least about 50%, 75%, 90%, 95%, 100%, 150%, 200% or more than 200% greater than the retention of the unmodified nucleic acid molecule. Such retention
advantage may be achieved by one round of transfection with the modified nucleic acid molecule, or
may be obtained following repeated rounds of transfection.

[00634] In some embodiments, the modified nucleic acid molecule may be delivered to a target cell
population with one or more additional nucleic acids. Such delivery may be at the same time, or the
modified nucleic acid molecule is delivered prior to delivery of the one or more additional nucleic
acids. The additional one or more nucleic acids may be modified nucleic acid molecules or
unmodified nucleic acid molecules. It is understood that the initial presence of the modified nucleic
acid molecules may not substantially induce an innate immune response of the cell population and,
moreover, that the innate immune response may not be activated by the later presence of the
unmodified nucleic acid molecules. In this regard, the enhanced nucleic acid may not itself contain a
translatable region, if the protein desired to be present in the target cell population is translated from
the unmodified nucleic acid molecules.

Targeting Moieties

[00635] In some embodiments, modified nucleic acid molecules are provided to express a protein-
binding partner or a receptor on the surface of the cell, which may function to target the cell to a
specific tissue space or to interact with a specific moiety, either in vivo or in vitro. Suitable protein-
binding partners include, but are not limited to, antibodies and functional fragments thereof, scaffold
proteins, or peptides. Additionally, modified nucleic acid molecules may be employed to direct the
synthesis and extracellular localization of lipids, carbohydrates, or other biological moieties.

Permanent Gene Expression Silencing

[00636] A method for epigenetically silencing gene expression in a mammalian subject, comprising
a nucleic acid where the translatable region encodes a polypeptide or polypeptides capable of
directing sequence-specific histone H3 methylation to initiate heterochromatin formation and reduce
gene transcription around specific genes for the purpose of silencing the gene. For example, a gain-
of-function mutation in the Janus Kinase 2 gene is responsible for the family of Myeloproliferative
Diseases.

Expression of Ligand or Receptor on Cell Surface

[00637] In some aspects and embodiments of the aspects described herein, the modified RNA can
be used to express a ligand or ligand receptor on the surface of a cell (e.g., a homing moiety). A
ligand or ligand receptor moiety attached to a cell surface can permit the cell to have a desired
biological interaction with a tissue or an agent in vivo. A ligand can be an antibody, an antibody
fragment, an aptamer, a peptide, a vitamin, a carbohydrate, a protein or polypeptide, a receptor, e.g., cell-surface receptor, an adhesion molecule, a glycoprotein, a sugar residue, a therapeutic agent, a drug, a glycosaminoglycan, or any combination thereof. For example, a ligand can be an antibody that recognizes a cancer-cell specific antigen, rendering the cell capable of preferentially interacting with tumor cells to permit tumor-specific localization of a modified cell. A ligand can confer the ability of a cell composition to accumulate in a tissue to be treated, since a preferred ligand may be capable of interacting with a target molecule on the external face of a tissue to be treated. Ligands having limited cross-reactivity to other tissues are generally preferred.

In some cases, a ligand can act as a homing moiety which permits the cell to target to a specific tissue or interact with a specific ligand. Such homing moieties can include, but are not limited to, any member of a specific binding pair, antibodies, monoclonal antibodies, or derivatives or analogs thereof, including without limitation: Fv fragments, single chain Fv (scFv) fragments, Fab' fragments, F(ab')2 fragments, single domain antibodies, camelized antibodies and antibody fragments, humanized antibodies and antibody fragments, and multivalent versions of the foregoing; multivalent binding reagents including without limitation: monospecific or bispecific antibodies, such as disulfide stabilized Fv fragments, scFv tandems ((SCFV)2 fragments), diabodies, tribodies or tetrabodies, which typically are covalently linked or otherwise stabilized (i.e., leucine zipper or helix stabilized) scFv fragments; and other homing moieties include for example, aptamers, receptors, and fusion proteins.

In some embodiments, the homing moiety may be a surface-bound antibody, which can permit tuning of cell targeting specificity. This is especially useful since highly specific antibodies can be raised against an epitope of interest for the desired targeting site. In one embodiment, multiple antibodies are expressed on the surface of a cell, and each antibody can have a different specificity for a desired target. Such approaches can increase the avidity and specificity of homing interactions.

A skilled artisan can select any homing moiety based on the desired localization or function of the cell, for example an estrogen receptor ligand, such as tamoxifen, can target cells to estrogen-dependent breast cancer cells that have an increased number of estrogen receptors on the cell surface. Other non-limiting examples of ligand/receptor interactions include CCRI (e.g., for treatment of inflamed joint tissues or brain in rheumatoid arthritis, and/or multiple sclerosis), CCR7, CCR8 (e.g., targeting to lymph node tissue), CCR6, CCR9, CCR10 (e.g., to target to intestinal
tissue), CCR4, CCR10 (e.g., for targeting to skin), CXCR4 (e.g., for general enhanced transmigration), HCELL (e.g., for treatment of inflammation and inflammatory disorders, bone marrow), Alpha4beta7 (e.g., for intestinal mucosa targeting), VLA-4/VCAM-1 (e.g., targeting to endothelium). In general, any receptor involved in targeting (e.g., cancer metastasis) can be harnessed for use in the methods and compositions described herein.

*Meditators of Cell Death*

[00641] In one embodiment, a modified nucleic acid molecule composition can be used to induce apoptosis in a cell (e.g., a cancer cell) by increasing the expression of a death receptor, a death receptor ligand or a combination thereof. This method can be used to induce cell death in any desired cell and has particular usefulness in the treatment of cancer where cells escape natural apoptotic signals.

[00642] Apoptosis can be induced by multiple independent signaling pathways that converge upon a final effector mechanism consisting of multiple interactions between several “death receptors” and their ligands, which belong to the tumor necrosis factor (TNF) receptor/ligand superfamily. The best-characterized death receptors are CD95 (“Fas”), TNFRI (p55), death receptor 3 (DR3 or Apo3/TRAMO), DR4 and DR5 (apo2-TRAIL-R2). The final effector mechanism of apoptosis may be the activation of a series of proteinases designated as caspases. The activation of these caspases results in the cleavage of a series of vital cellular proteins and cell death. The molecular mechanism of death receptors/ligands-induced apoptosis is well known in the art. For example, Fas/FasL-mediated apoptosis is induced by binding of three FasL molecules which induces trimerization of Fas receptor via C-terminus death domains (DDs), which in turn recruits an adapter protein FADD (Fas-associated protein with death domain) and Caspase-8. The oligomerization of this trimeric complex, Fas/FAIDD/caspase-8, results in proteolytic cleavage of proenzyme caspase-8 into active caspase-8 that, in turn, initiates the apoptosis process by activating other downstream caspases through proteolysis, including caspase-3. Death ligands in general are apoptotic when formed into trimers or higher order of structures. As monomers, they may serve as antiapoptotic agents by competing with the trimers for binding to the death receptors.

[00643] In one embodiment, the modified nucleic acid molecule composition encodes for a death receptor (e.g., Fas, TRAIL, TRAMO, TNFR, TLR etc). Cells made to express a death receptor by transfection of modified RNA become susceptible to death induced by the ligand that activates that receptor. Similarly, cells made to express a death ligand, e.g., on their surface, will induce death of
cells with the receptor when the transfected cell contacts the target cell. In another embodiment, the modified RNA composition encodes for a death receptor ligand (e.g., FasL, TNF, etc). In another embodiment, the modified RNA composition encodes a caspase (e.g., caspase 3, caspase 8, caspase 9 etc). Where cancer cells often exhibit a failure to properly differentiate to a non-proliferative or controlled proliferative form, in another embodiment, the synthetic, modified RNA composition encodes for both a death receptor and its appropriate activating ligand. In another embodiment, the synthetic, modified RNA composition encodes for a differentiation factor that when expressed in the cancer cell, such as a cancer stem cell, will induce the cell to differentiate to a non-pathogenic or nonself-renewing phenotype (e.g., reduced cell growth rate, reduced cell division etc) or to induce the cell to enter a dormant cell phase (e.g., G0 resting phase).

[00644] One of skill in the art will appreciate that the use of apoptosis-inducing techniques may require that the modified nucleic acid molecules are appropriately targeted to e.g., tumor cells to prevent unwanted wide-spread cell death. Thus, one can use a delivery mechanism (e.g., attached ligand or antibody, targeted liposome etc) that recognizes a cancer antigen such that the modified nucleic acid molecules are expressed only in cancer cells.

**Kits and Devices**

**Kits**

[00645] The invention provides a variety of kits for conveniently and/or effectively carrying out methods of the present invention. Typically kits will comprise sufficient amounts and/or numbers of components to allow a user to perform multiple treatments of a subject(s) and/or to perform multiple experiments.

[00646] In one aspect, the present invention provides kits for protein production, comprising a first modified nucleic acid molecule or mmRNA comprising a translatable region. The kit may further comprise packaging and instructions and/or a delivery agent to form a formulation composition. The delivery agent may comprise a saline, a buffered solution, a lipidoid or any delivery agent disclosed herein.

[00647] In one embodiment, the buffer solution may include sodium chloride, calcium chloride, phosphate and/or EDTA. In another embodiment, the buffer solution may include, but is not limited to, saline, saline with 2mM calcium, 5% sucrose, 5% sucrose with 2mM calcium, 5% Mannitol, 5% Mannitol with 2mM calcium, Ringer’s lactate, sodium chloride, sodium chloride with 2mM calcium and mannose (See e.g., U.S. Pub. No. 20120258046; herein incorporated by reference in its entirety).
In a further embodiment, the buffer solutions may be precipitated or it may be lyophilized. The amount of each component may be varied to enable consistent, reproducible higher concentration saline or simple buffer formulations. The components may also be varied in order to increase the stability of modified nucleic acid molecules and mmRNA in the buffer solution over a period of time and/or under a variety of conditions.

[00648] In one aspect, the present invention provides kits for protein production, comprising a modified nucleic acid molecule or mmRNA comprising a translatable region, provided in an amount effective to produce a desired amount of a protein encoded by the translatable region when introduced into a target cell; a second modified nucleic acid molecule or mmRNA comprising an inhibitory nucleic acid, provided in an amount effective to substantially inhibit the innate immune response of the cell; and packaging and instructions.

[00649] In one aspect, the present invention provides kits for protein production, comprising a modified nucleic acid molecule or mmRNA comprising a translatable region, wherein the nucleic acid exhibits reduced degradation by a cellular nuclease, and packaging and instructions.

[00650] In one aspect, the present invention provides kits for protein production, comprising a modified nucleic acid molecule or mmRNA comprising a translatable region, wherein the nucleic acid exhibits reduced degradation by a cellular nuclease, and a mammalian cell suitable for translation of the translatable region of the first nucleic acid.

Devices

[00651] The present invention provides for devices which may incorporate modified nucleic acid molecules or mmRNA that encode polypeptides of interest. These devices contain in a stable formulation the reagents to synthesize a nucleic acid in a formulation available to be immediately delivered to a subject in need thereof, such as a human patient. Non-limiting examples of such a polypeptide of interest include a growth factor and/or angiogenesis stimulator for wound healing, a peptide antibiotic to facilitate infection control, and an antigen to rapidly stimulate an immune response to a newly identified virus.

[00652] In some embodiments the device is self-contained, and is optionally capable of wireless remote access to obtain instructions for synthesis and/or analysis of the generated modified nucleic acid molecule or mmRNA. The device is capable of mobile synthesis of at least one modified nucleic acid molecule or mmRNA and preferably an unlimited number of different modified nucleic acid molecules or mmRNA. In certain embodiments, the device is capable of being transported by
one or a small number of individuals. In other embodiments, the device is scaled to fit on a benchtop or desk. In other embodiments, the device is scaled to fit into a suitcase, backpack or similarly sized object.

[00653] In another embodiment, the device may be a point of care or handheld device. In further embodiments, the device is scaled to fit into a vehicle, such as a car, truck or ambulance, or a military vehicle such as a tank or personnel carrier. The information necessary to generate a modified mRNA encoding polypeptide of interest is present within a computer readable medium present in the device.

[00654] In one embodiment, a device may be used to assess levels of a protein which has been administered in the form of a modified nucleic acid or mRNA. The device may comprise a blood, urine or other biofluidic test.

[00655] In some embodiments, the device is capable of communication (e.g., wireless communication) with a database of nucleic acid and polypeptide sequences. The device contains at least one sample block for insertion of one or more sample vessels. Such sample vessels are capable of accepting in liquid or other form any number of materials such as template DNA, nucleotides, enzymes, buffers, and other reagents. The sample vessels are also capable of being heated and cooled by contact with the sample block. The sample block is generally in communication with a device base with one or more electronic control units for the at least one sample block. The sample block preferably contains a heating module, such heating molecule capable of heating and/or cooling the sample vessels and contents thereof to temperatures between about -20°C and above +100°C. The device base is in communication with a voltage supply such as a battery or external voltage supply. The device also contains means for storing and distributing the materials for RNA synthesis.

[00656] Optionally, the sample block contains a module for separating the synthesized nucleic acids. Alternatively, the device contains a separation module operably linked to the sample block. Preferably the device contains a means for analysis of the synthesized nucleic acid. Such analysis includes sequence identity (demonstrated such as by hybridization), absence of non-desired sequences, measurement of integrity of synthesized mRNA (such has by microfluidic viscometry combined with spectrophotometry), and concentration and/or potency of modified RNA (such as by spectrophotometry).
In certain embodiments, the device is combined with a means for detection of pathogens present in a biological material obtained from a subject, e.g., the IBIS PLEX-ID system (Abbott, Abbott Park, IL) for microbial identification.

Suitable devices for use in delivering intradermal pharmaceutical compositions described herein include short needle devices such as those described in U.S. Patents 4,886,499; 5,190,521; 5,328,483; 5,527,288; 4,270,537; 5,015,235; 5,141,496; and 5,417,662; each of which is herein incorporated by reference in their entirety. Intradermal compositions may be administered by devices which limit the effective penetration length of a needle into the skin, such as those described in PCT publication WO 99/34850 (herein incorporated by reference in its entirety) and functional equivalents thereof. Jet injection devices which deliver liquid compositions to the dermis via a liquid jet injector and/or via a needle which pierces the stratum corneum and produces a jet which reaches the dermis are suitable. Jet injection devices are described, for example, in U.S. Patents 5,480,381; 5,599,302; 5,334,144; 5,993,412; 5,649,912; 5,569,189; 5,704,911; 5,383,851; 5,893,397; 5,466,220; 5,339,163; 5,312,335; 5,503,627; 5,064,413; 5,520,639; 4,596,556; 4,790,824; 4,941,880; 4,940,460; and PCT publications WO 97/37705 and WO 97/13537; each of which are herein incorporated by reference in their entirety. Ballistic powder/particle delivery devices which use compressed gas to accelerate vaccine in powder form through the outer layers of the skin to the dermis are suitable. Alternatively or additionally, conventional syringes may be used in the classical mantoux method of intradermal administration.

In some embodiments, the device may be a pump or comprise a catheter for administration of compounds or compositions of the invention across the blood brain barrier. Such devices include but are not limited to a pressurized olfactory delivery device, iontophoresis devices, multi-layered microfluidic devices, and the like. Such devices may be portable or stationary. They may be implantable or externally tethered to the body or combinations thereof.

Devices for administration may be employed to deliver the modified nucleic acid molecules or mRNA of the present invention according to single, multi- or split-dosing regimens taught herein. Such devices are described below.

Method and devices known in the art for multi-administration to cells, organs and tissues are contemplated for use in conjunction with the methods and compositions disclosed herein as embodiments of the present invention. These include, for example, those methods and devices...
having multiple needles, hybrid devices employing for example lumens or catheters as well as devices utilizing heat, electric current or radiation driven mechanisms.

[00662] According to the present invention, these multi-administration devices may be utilized to deliver the single, multi- or split doses contemplated herein.

[00663] A method for delivering therapeutic agents to a solid tissue has been described by Bahrami et al. and is taught for example in US Patent Publication 20110230839, the contents of which are incorporated herein by reference in their entirety. According to Bahrami, an array of needles is incorporated into a device which delivers a substantially equal amount of fluid at any location in said solid tissue along each needle’s length.

[00664] A device for delivery of biological material across the biological tissue has been described by Kodgule et al. and is taught for example in US Patent Publication 20110172610, the contents of which are incorporated herein by reference in their entirety. According to Kodgule, multiple hollow micro-needles made of one or more metals and having outer diameters from about 200 microns to about 350 microns and lengths of at least 100 microns are incorporated into the device which delivers peptides, proteins, carbohydrates, nucleic acid molecules, lipids and other pharmaceutically active ingredients or combinations thereof.

[00665] A delivery probe for delivering a therapeutic agent to a tissue has been described by Gunday et al. and is taught for example in US Patent Publication 20110270184, the contents of each of which are incorporated herein by reference in their entirety. According to Gunday, multiple needles are incorporated into the device which moves the attached capsules between an activated position and an inactivated position to force the agent out of the capsules through the needles.

[00666] A multiple-injection medical apparatus has been described by Assaf and is taught for example in US Patent Publication 20110218497, the contents of which are incorporated herein by reference in their entirety. According to Assaf, multiple needles are incorporated into the device which has a chamber connected to one or more of said needles and a means for continuously refilling the chamber with the medical fluid after each injection.

[00667] In one embodiment, the modified nucleic acid molecule or mRNA is administered subcutaneously or intramuscularly via at least 3 needles to three different, optionally adjacent, sites simultaneously, or within a 60 minutes period (e.g., administration to 4, 5, 6, 7, 8, 9, or 10 sites simultaneously or within a 60 minute period). The split doses can be administered simultaneously to
adjacent tissue using the devices described in U.S. Patent Publication Nos. 20110230839 and 20110218497, each of which is incorporated herein by reference in their entirety.

[00668] An at least partially implantable system for injecting a substance into a patient’s body, in particular a penis erection stimulation system has been described by Forsell and is taught for example in US Patent Publication 20110196198, the contents of which are incorporated herein by reference in their entirety. According to Forsell, multiple needles are incorporated into the device which is implanted along with one or more housings adjacent the patient's left and right corpora cavernosa. A reservoir and a pump are also implanted to supply drugs through the needles.

[00669] A method for the transdermal delivery of a therapeutic effective amount of iron has been described by Berenson and is taught for example in US Patent Publication 20100130910, the contents of which are incorporated herein by reference in their entirety. According to Berenson, multiple needles may be used to create multiple micro channels in stratum corneum to enhance transdermal delivery of the ionic iron on an iontophoretic patch.

[00670] A method for delivery of biological material across the biological tissue has been described by Kodgule et al and is taught for example in US Patent Publication 20110196308, the contents of which are incorporated herein by reference in their entirety. According to Kodgule, multiple biodegradable microneedles containing a therapeutic active ingredient are incorporated in a device which delivers proteins, carbohydrates, nucleic acid molecules, lipids and other pharmaceutically active ingredients or combinations thereof.

[00671] A transdermal patch comprising a botulinum toxin composition has been described by Donovan and is taught for example in US Patent Publication 20080220020, the contents of which are incorporated herein by reference in their entirety. According to Donovan, multiple needles are incorporated into the patch which delivers botulinum toxin under stratum corneum through said needles which project through the stratum corneum of the skin without rupturing a blood vessel.

[00672] A small, disposable drug reservoir, or patch pump, which can hold approximately 0.2 to 15 mL of liquid formulations can be placed on the skin and deliver the formulation continuously subcutaneously using a small bore needle (e.g., 26 to 34 gauge). As non-limiting examples, the patch pump may be 50 mm by 76 mm by 20 mm spring loaded having a 30 to 34 gauge needle (BD™ Microinfuser, Franklin Lakes NJ), 41 mm by 62 mm by 17 mm with a 2 mL reservoir used for drug delivery such as insulin (OMNIPOD®, Insulet Corporation Bedford, MA), or 43-60 mm
diameter, 10 mm thick with a 0.5 to 10 mL reservoir (PATCHPUMP®, SteadyMed Therapeutics, San Francisco, CA). Further, the patch pump may be battery powered and/or rechargeable.

[00673] A cryoprobe for administration of an active agent to a location of cryogenic treatment has been described by Toubia and is taught for example in US Patent Publication 20080140061, the contents of which are incorporated herein by reference in their entirety. According to Toubia, multiple needles are incorporated into the probe which receives the active agent into a chamber and administers the agent to the tissue.

[00674] A method for treating or preventing inflammation or promoting healthy joints has been described by Stock et al and is taught for example in US Patent Publication 20090155186, the contents of which are incorporated herein by reference in their entirety. According to Stock, multiple needles are incorporated in a device which administers compositions containing signal transduction modulator compounds.

[00675] A multi-site injection system has been described by Kimmell et al. and is taught for example in US Patent Publication 20100256594, the contents of which are incorporated herein by reference in their entirety. According to Kimmell, multiple needles are incorporated into a device which delivers a medication into a stratum corneum through the needles.

[00676] A method for delivering interferons to the intradermal compartment has been described by Dekker et al. and is taught for example in US Patent Publication 20050181033, the contents of which are incorporated herein by reference in their entirety. According to Dekker, multiple needles having an outlet with an exposed height between 0 and 1 mm are incorporated into a device which improves pharmacokinetics and bioavailability by delivering the substance at a depth between 0.3 mm and 2 mm.

[00677] A method for delivering genes, enzymes and biological agents to tissue cells has described by Desai and is taught for example in US Patent Publication 20030073908, the contents of which are incorporated herein by reference in their entirety. According to Desai, multiple needles are incorporated into a device which is inserted into a body and delivers a medication fluid through said needles.

[00678] A method for treating cardiac arrhythmias with fibroblast cells has been described by Lee et al and is taught for example in US Patent Publication 20040005295, the contents of which are incorporated herein by reference in their entirety. According to Lee, multiple needles are incorporated into the device which delivers fibroblast cells into the local region of the tissue.
A method using a magnetically controlled pump for treating a brain tumor has been described by Shachar et al. and is taught for example in US Patent 7,799,012 (method) and 7,799,016 (device), the contents of which are incorporated herein by reference in their entirety. According Shachar, multiple needles were incorporated into the pump which pushes a medicating agent through the needles at a controlled rate.

Methods of treating functional disorders of the bladder in mammalian females have been described by Versi et al. and are taught for example in US Patent 8,029,496, the contents of which are incorporated herein by reference in their entirety. According to Versi, an array of micro-needles is incorporated into a device which delivers a therapeutic agent through the needles directly into the trigone of the bladder.

A micro-needle transdermal transport device has been described by Angel et al and is taught for example in US Patent 7,364,568, the contents of which are incorporated herein by reference in their entirety. According to Angel, multiple needles are incorporated into the device which transports a substance into a body surface through the needles which are inserted into the surface from different directions. The micro-needle transdermal transport device may be a solid micro-needle system or a hollow micro-needle system. As a non-limiting example, the solid micro-needle system may have up to a 0.5 mg capacity, with 300-1500 solid micro-needles per cm² about 150-700 µm tall coated with a drug. The micro-needles penetrate the stratum corneum and remain in the skin for short duration (e.g., 20 seconds to 15 minutes). In another example, the hollow micro-needle system has up to a 3 mL capacity to deliver liquid formulations using 15-20 microneedles per cm² being approximately 950 µm tall. The micro-needles penetrate the skin to allow the liquid formulations to flow from the device into the skin. The hollow micro-needle system may be worn from 1 to 30 minutes depending on the formulation volume and viscosity.

A device for subcutaneous infusion has been described by Dalton et al and is taught for example in US Patent 7,150,726, the contents of which are incorporated herein by reference in their entirety. According to Dalton, multiple needles are incorporated into the device which delivers fluid through the needles into a subcutaneous tissue.

A device and a method for intradermal delivery of vaccines and gene therapeutic agents through microcannula have been described by Mikszta et al. and are taught for example in US Patent 7,473,247, the contents of which are incorporated herein by reference in their entirety. According to
Mitszta, at least one hollow micro-needle is incorporated into the device which delivers the vaccines to the subject’s skin to a depth of between 0.025 mm and 2 mm. [00684] A method of delivering insulin has been described by Pettis et al and is taught for example in US Patent 7,722,595, the contents of which are incorporated herein by reference in their entirety. According to Pettis, two needles are incorporated into a device wherein both needles insert essentially simultaneously into the skin with the first at a depth of less than 2.5 mm to deliver insulin to intradermal compartment and the second at a depth of greater than 2.5 mm and less than 5.0 mm to deliver insulin to subcutaneous compartment.

[00685] Cutaneous injection delivery under suction has been described by Kochamba et al. and is taught for example in US Patent 6,896,666, the contents of which are incorporated herein by reference in their entirety. According to Kochamba, multiple needles in relative adjacency with each other are incorporated into a device which injects a fluid below the cutaneous layer.

[00686] A device for withdrawing or delivering a substance through the skin has been described by Down et al and is taught for example in US Patent 6,607,513, the contents of which are incorporated herein by reference in their entirety. According to Down, multiple skin penetrating members which are incorporated into the device have lengths of about 100 microns to about 2000 microns and are about 30 to 50 gauge.

[00687] A device for delivering a substance to the skin has been described by Palmer et al and is taught for example in US Patent 6,537,242, the contents of which are incorporated herein by reference in their entirety. According to Palmer, an array of micro-needles is incorporated into the device which uses a stretching assembly to enhance the contact of the needles with the skin and provides a more uniform delivery of the substance.

[00688] A perfusion device for localized drug delivery has been described by Zamoyski and is taught for example in US Patent 6,468,247, the contents of which are incorporated herein by reference in their entirety. According to Zamoyski, multiple hypodermic needles are incorporated into the device which injects the contents of the hypodermics into a tissue as said hypodermics are being retracted.

[00689] A method for enhanced transport of drugs and biological molecules across tissue by improving the interaction between micro-needles and human skin has been described by Prausnitz et al. and is taught for example in US Patent 6,743,211, the contents of which are incorporated herein by reference in their entirety. According to Prausnitz, multiple micro-needles are incorporated into a
device which is able to present a more rigid and less deformable surface to which the micro-needles are applied.

[00690] A device for intraorgan administration of medicinal agents has been described by Ting et al and is taught for example in US Patent 6,077,251, the contents of which are incorporated herein by reference in their entirety. According to Ting, multiple needles having side openings for enhanced administration are incorporated into a device which by extending and retracting said needles from and into the needle chamber forces a medicinal agent from a reservoir into said needles and injects said medicinal agent into a target organ.

[00691] A multiple needle holder and a subcutaneous multiple channel infusion port has been described by Brown and is taught for example in US Patent 4,695,273, the contents of which are incorporated herein by reference in their entirety. According to Brown, multiple needles on the needle holder are inserted through the septum of the infusion port and communicate with isolated chambers in said infusion port.

[00692] A dual hypodermic syringe has been described by Horn and is taught for example in US Patent 3,552,394, the contents of which are incorporated herein by reference in their entirety. According to Horn, two needles incorporated into the device are spaced apart less than 68 mm and may be of different styles and lengths, thus enabling injections to be made to different depths.

[00693] A syringe with multiple needles and multiple fluid compartments has been described by Hershberg and is taught for example in US Patent 3,572,336, the contents of which are incorporated herein by reference in their entirety. According to Hershberg, multiple needles are incorporated into the syringe which has multiple fluid compartments and is capable of simultaneously administering incompatible drugs which are not able to be mixed for one injection.

[00694] A surgical instrument for intradermal injection of fluids has been described by Eliscu et al. and is taught for example in US Patent 2,588,623, the contents of which are incorporated herein by reference in their entirety. According to Eliscu, multiple needles are incorporated into the instrument which injects fluids intradermally with a wider disperse.

[00695] An apparatus for simultaneous delivery of a substance to multiple breast milk ducts has been described by Hung and is taught for example in EP 1818017, the contents of which are incorporated herein by reference in their entirety. According to Hung, multiple lumens are incorporated into the device which inserts though the orifices of the ductal networks and delivers a fluid to the ductal networks.
[00696] A catheter for introduction of medications to the tissue of a heart or other organs has been described by Tkebuchava and is taught for example in WO2006138109, the contents of which are incorporated herein by reference in their entirety. According to Tkebuchava, two curved needles are incorporated which enter the organ wall in a flattened trajectory.

[00697] Devices for delivering medical agents have been described by Mckay et al. and are taught for example in WO2006118804, the content of which are incorporated herein by reference in their entirety. According to Mckay, multiple needles with multiple orifices on each needle are incorporated into the devices to facilitate regional delivery to a tissue, such as the interior disc space of a spinal disc.

[00698] A method for directly delivering an immunomodulatory substance into an intradermal space within a mammalian skin has been described by Pettis and is taught for example in WO2004020014, the contents of which are incorporated herein by reference in their entirety. According to Pettis, multiple needles are incorporated into a device which delivers the substance through the needles to a depth between 0.3 mm and 2 mm.

[00699] Methods and devices for administration of substances into at least two compartments in skin for systemic absorption and improved pharmacokinetics have been described by Pettis et al. and are taught for example in WO2003094995, the contents of which are incorporated herein by reference in their entirety. According to Pettis, multiple needles having lengths between about 300 µm and about 5 mm are incorporated into a device which delivers to intradermal and subcutaneous tissue compartments simultaneously.

[00700] A drug delivery device with needles and a roller has been described by Zimmerman et al. and is taught for example in WO2012006259, the contents of which are incorporated herein by reference in their entirety. According to Zimmerman, multiple hollow needles positioned in a roller are incorporated into the device which delivers the content in a reservoir through the needles as the roller rotates.

[00701] A drug delivery device such as a stent is known in the art and is taught for example in U.S. Pub. Nos. US20060020329, US20040172127 and US20100161032; the contents of which are herein incorporated by reference in their entirety. Formulations of the modified nucleic acid molecules and mRNA described herein may be delivered using stents. Additionally, stents used herein may be able to deliver multiple modified nucleic acid molecules and/or formulations at the same or varied rates of delivery. Non-limiting examples of manufacturers of stents include CORDIS® (Miami, FL)
Methods and Devices utilizing catheters and/or lumens

[00702] Methods and devices using catheters and lumens may be employed to administer the mRNA of the present invention on a single, multi- or split dosing schedule. Such methods and devices are described below.

[00703] A catheter-based delivery of skeletal myoblasts to the myocardium of damaged hearts has been described by Jacoby et al and is taught for example in US Patent Publication 20060263338, the contents of which are incorporated herein by reference in their entirety. According to Jacoby, multiple needles are incorporated into the device at least part of which is inserted into a blood vessel and delivers the cell composition through the needles into the localized region of the subject’s heart.

[00704] An apparatus for treating asthma using neurotoxin has been described by Deem et al and is taught for example in US Patent Publication 20060225742, the contents of which are incorporated herein by reference in their entirety. According to Deem, multiple needles are incorporated into the device which delivers neurotoxin through the needles into the bronchial tissue.

[00705] A method for administering multiple-component therapies has been described by Nayak and is taught for example in US Patent 7,699,803, the contents of which are incorporated herein by reference in their entirety. According to Nayak, multiple injection cannulas may be incorporated into a device wherein depth slots may be included for controlling the depth at which the therapeutic substance is delivered within the tissue.

[00706] A surgical device for ablating a channel and delivering at least one therapeutic agent into a desired region of the tissue has been described by McIntyre et al and is taught for example in US Patent 8,012,096, the contents of which are incorporated herein by reference in their entirety. According to McIntyre, multiple needles are incorporated into the device which dispenses a therapeutic agent into a region of tissue surrounding the channel and is particularly well suited for transmyocardial revascularization operations.

[00707] Methods of treating functional disorders of the bladder in mammalian females have been described by Versi et al and are taught for example in US Patent 8,029,496, the contents of which are incorporated herein by reference in their entirety. According to Versi, an array of micro-needles is incorporated into a device which delivers a therapeutic agent through the needles directly into the trigone of the bladder.
[00708] A device and a method for delivering fluid into a flexible biological barrier have been described by Yeshurun et al. and are taught for example in US Patent 7,998,119 (device) and 8,007,466 (method), the contents of which are incorporated herein by reference in their entirety. According to Yeshurun, the micro-needles on the device penetrate and extend into the flexible biological barrier and fluid is injected through the bore of the hollow micro-needles.

[00709] A method for epicardially injecting a substance into an area of tissue of a heart having an epicardial surface and disposed within a torso has been described by Bonner et al and is taught for example in US Patent 7,628,780, the contents of which are incorporated herein by reference in their entirety. According to Bonner, the devices have elongate shafts and distal injection heads for driving needles into tissue and injecting medical agents into the tissue through the needles.

[00710] A device for sealing a puncture has been described by Nielsen et al and is taught for example in US Patent 7,972,358, the contents of which are incorporated herein by reference in their entirety. According to Nielsen, multiple needles are incorporated into the device which delivers a closure agent into the tissue surrounding the puncture tract.

[00711] A method for myogenesis and angiogenesis has been described by Chiu et al. and is taught for example in US Patent 6,551,338, the contents of which are incorporated herein by reference in their entirety. According to Chiu, 5 to 15 needles having a maximum diameter of at least 1.25 mm and a length effective to provide a puncture depth of 6 to 20 mm are incorporated into a device which inserts into proximity with a myocardium and supplies an exogeneous angiogenic or myogenic factor to said myocardium through the conduits which are in at least some of said needles.

[00712] A method for the treatment of prostate tissue has been described by Bolmsjø et al. and is taught for example in US Patent 6,524,270, the contents of which are incorporated herein by reference in their entirety. According to Bolmsjø, a device comprising a catheter which is inserted through the urethra has at least one hollow tip extendible into the surrounding prostate tissue. An astringent and analgesic medicine is administered through said tip into said prostate tissue.

[00713] A method for infusing fluids to an intraosseous site has been described by Findlay et al. and is taught for example in US Patent 6,761,726, the contents of which are incorporated herein by reference in their entirety. According to Findlay, multiple needles are incorporated into a device which is capable of penetrating a hard shell of material covered by a layer of soft material and delivers a fluid at a predetermined distance below said hard shell of material.
A device for injecting medications into a vessel wall has been described by Vigil et al. and is taught for example in US Patent 5,713,863, the contents of which are incorporated herein by reference in their entirety. According to Vigil, multiple injectors are mounted on each of the flexible tubes in the device which introduces a medication fluid through a multi-lumen catheter, into said flexible tubes and out of said injectors for infusion into the vessel wall.

A catheter for delivering therapeutic and/or diagnostic agents to the tissue surrounding a bodily passageway has been described by Faxon et al. and is taught for example in US Patent 5,464,395, the contents of which are incorporated herein by reference in their entirety. According to Faxon, at least one needle cannula is incorporated into the catheter which delivers the desired agents to the tissue through said needles which project outboard of the catheter.

Balloon catheters for delivering therapeutic agents have been described by Orr and are taught for example in WO2010024871, the contents of which are incorporated herein by reference in their entirety. According to Orr, multiple needles are incorporated into the devices which deliver the therapeutic agents to different depths within the tissue. In another aspect, drug-eluting balloons may be used to deliver the formulations described herein. The drug-eluting balloons may be used in target lesion applications such as, but are not limited to, in-stent restenosis, treating lesion in tortuous vessels, bifurcation lesions, femoral/popliteal lesions and below the knee lesions.

A device for delivering therapeutic agents (e.g., modified nucleic acid molecules or mmRNA) to tissue disposed about a lumen has been described by Perry et al. and is taught for example in U.S. Pat. Pub. US20100125239, the contents of which are herein incorporated by reference in their entirety. According to Perry, the catheter has a balloon which may be coated with a therapeutic agent by methods known in the art and described in Perry. When the balloon expands, the therapeutic agent will contact the surrounding tissue. The device may additionally have a heat source to change the temperature of the coating on the balloon to release the therapeutic agent to the tissue.

Methods and Devices utilizing electrical current

Methods and devices utilizing electric current may be employed to deliver the mmRNA of the present invention according to the single, multi- or split dosing regimens taught herein. Such methods and devices are described below.

An electro collagen induction therapy device has been described by Marquez and is taught for example in US Patent Publication 20090137945, the contents of which are incorporated herein
by reference in their entirety. According to Marquez, multiple needles are incorporated into the device which repeatedly pierce the skin and draw in the skin a portion of the substance which is applied to the skin first.

An electrokinetic system has been described by Etheredge et al. and is taught for example in US Patent Publication 20070185432, the contents of which are incorporated herein by reference in their entirety. According to Etheredge, micro-needles are incorporated into a device which drives by an electrical current the medication through the needles into the targeted treatment site.

An iontophoresis device has been described by Matsumura et al. and is taught for example in US Patent 7,437,189, the contents of which are incorporated herein by reference in their entirety. According to Matsumura, multiple needles are incorporated into the device which is capable of delivering ionizable drug into a living body at higher speed or with higher efficiency.

Intradermal delivery of biologically active agents by needle-free injection and electroporation has been described by Hoffmann et al and is taught for example in US Patent 7,171,264, the contents of which are incorporated herein by reference in their entirety. According to Hoffmann, one or more needle-free injectors are incorporated into an electroporation device and the combination of needle-free injection and electroporation is sufficient to introduce the agent into cells in skin, muscle or mucosa.

A method for electroporation-mediated intracellular delivery has been described by Lundkvist et al. and is taught for example in US Patent 6,625,486, the contents of which are incorporated herein by reference in their entirety. According to Lundkvist, a pair of needle electrodes is incorporated into a catheter. Said catheter is positioned into a body lumen followed by extending said needle electrodes to penetrate into the tissue surrounding said lumen. Then the device introduces an agent through at least one of said needle electrodes and applies electric field by said pair of needle electrodes to allow said agent pass through the cell membranes into the cells at the treatment site.

A delivery system for transdermal immunization has been described by Levin et al. and is taught for example in WO2006003659, the contents of which are incorporated herein by reference in their entirety. According to Levin, multiple electrodes are incorporated into the device which applies electrical energy between the electrodes to generate micro channels in the skin to facilitate transdermal delivery.
[00725] A method for delivering RF energy into skin has been described by Schomacker and is taught for example in WO2011163264, the contents of which are incorporated herein by reference in their entirety. According to Schomacker, multiple needles are incorporated into a device which applies vacuum to draw skin into contact with a plate so that needles insert into skin through the holes on the plate and deliver RF energy.

Definitions

[00726] At various places in the present specification, substituents of compounds of the present disclosure are disclosed in groups or in ranges. It is specifically intended that the present disclosure include each and every individual subcombination of the members of such groups and ranges. For example, the term “C<sub>1-6</sub> alkyl” is specifically intended to individually disclose methyl, ethyl, C<sub>3</sub> alkyl, C<sub>4</sub> alkyl, C<sub>5</sub> alkyl, and C<sub>6</sub> alkyl.

[00727] About: As used herein, the term “about” means +/- 10% of the recited value.

[00728] Administered in combination: As used herein, the term “administered in combination” or “combined administration” means that two or more agents (e.g., a modified nucleic acid or mmRNA encoding an anti-microbial polypeptide (e.g., an anti-bacterial polypeptide), e.g., an anti-microbial polypeptide described herein and an anti-microbial agent (e.g., an anti-microbial polypeptide or a small molecule anti-microbial compound described herein)) are administered to a subject at the same time or within an interval such that there may be an overlap of an effect of each agent on the patient. In some embodiments, they are administered within about 60, 30, 15, 10, 5, or 1 minute of one another. In some embodiments, the administrations of the agents are spaced sufficiently close together such that a combinatorial (e.g., a synergistic) effect is achieved.

[00729] Animal: As used herein, the term “animal” refers to any member of the animal kingdom. In some embodiments, “animal” refers to humans at any stage of development. In some embodiments, “animal” refers to non-human animals at any stage of development. In certain embodiments, the non-human animal is a mammal (e.g., a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, and worms. In some embodiments, the animal is a transgenic animal, genetically-engineered animal, or a clone.

[00730] Antigens of interest or desired antigens: As used herein, the terms “antigens of interest” or “desired antigens” include those proteins and other biomolecules provided herein that are immunospecifically bound by the antibodies and fragments, mutants, variants, and alterations thereof.
described herein. Examples of antigens of interest include, but are not limited to, insulin, insulin-like growth factor, hGH, tPA, cytokines, such as interleukins (IL), e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon (IFN) alpha, IFN beta, IFN gamma, IFN omega or IFN tau, tumor necrosis factor (TNF), such as TNF alpha and TNF beta, TNF gamma, TRAIL; G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF.

[00731] **Approximately:** As used herein, the term “approximately” or “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term “approximately” or “about” refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[00732] **Associated with:** As used herein, the terms “associated with,” “conjugated,” “linked,” “attached,” and “tethered,” when used with respect to two or more moieties, means that the moieties are physically associated or connected with one another, either directly or via one or more additional moieties that serves as a linking agent, to form a structure that is sufficiently stable so that the moieties remain physically associated under the conditions in which the structure is used, e.g., physiological conditions. An “association” need not be strictly through direct covalent chemical bonding. It may also suggest ionic or hydrogen bonding or a hybridization based connectivity sufficiently stable such that the “associated” entities remain physically associated.

[00733] **Bifunctional:** As used herein, the term “bifunctional” refers to any substance, molecule or moiety which is capable of or maintains at least two functions. The functions may effect the same outcome or a different outcome. The structure that produces the function may be the same or different. For example, bifunctional modified RNA of the present invention may encode a cytotoxic peptide (a first function) while those nucleosides which comprise the encoding RNA are, in and of themselves, cytotoxic (second function). In this example, delivery of the bifunctional modified RNA to a cancer cell would produce not only a peptide or protein molecule which may ameliorate or treat the cancer but would also deliver a cytotoxic payload of nucleosides to the cell should degradation, instead of translation of the modified RNA, occur.
[00734] **Biocompatible**: As used herein, the term “biocompatible” means compatible with living cells, tissues, organs or systems posing little to no risk of injury, toxicity or rejection by the immune system.

[00735] **Biodegradable**: As used herein, the term “biodegradable” means capable of being broken down into innocuous products by the action of living things.

[00736] **Biologically active**: As used herein, the phrase “biologically active” refers to a characteristic of any substance that has activity in a biological system and/or organism. For instance, a substance that, when administered to an organism, has a biological affect on that organism, is considered to be biologically active. In particular embodiments, the modified nucleic acid or mRNA of the present invention may be considered biologically active if even a portion of the modified nucleic acid or mRNA is biologically active or mimics an activity considered biologically relevant.

[00737] **Chemical terms**: The following provides the definition of various chemical terms from “acyl” to “thiol.”

[00738] The term “acyl,” as used herein, represents a hydrogen or an alkyl group (e.g., a haloalkyl group), as defined herein, that is attached to the parent molecular group through a carbonyl group, as defined herein, and is exemplified by formyl (i.e., a carboxylaldehyde group), acetyl, propionyl, butanoyl and the like. Exemplary unsubstituted acyl groups include from 1 to 7, from 1 to 11, or from 1 to 21 carbons. In some embodiments, the alkyl group is further substituted with 1, 2, 3, or 4 substituents as described herein.

[00739] The term “acylamino,” as used herein, represents an acyl group, as defined herein, attached to the parent molecular group though an amino group, as defined herein (i.e., –N(R^N1)-C(O)-R, where R is H or an optionally substituted C_{1-6}, C_{1-10}, or C_{1-20} alkyl group and R^N1 is as defined herein). Exemplary unsubstituted acylamino groups include from 1 to 41 carbons (e.g., from 1 to 7, from 1 to 13, from 1 to 21, from 2 to 7, from 2 to 13, from 2 to 21, or from 2 to 41 carbons). In some embodiments, the alkyl group is further substituted with 1, 2, 3, or 4 substituents as described herein, and/or the amino group is –NH_2 or –NHR^N1, wherein R^N1 is, independently, OH, NO_2, NH_2, NR^N2, SO_2OR^N2, SO_2R^N2, SOR^N2, alkyl, or aryl, and each R^N2 can be H, alkyl, or aryl.

[00740] The term “acyloxy,” as used herein, represents an acyl group, as defined herein, attached to the parent molecular group though an oxygen atom (i.e., –O-C(O)-R, where R is H or an optionally substituted C_{1-6}, C_{1-10}, or C_{1-20} alkyl group). Exemplary unsubstituted acyloxy groups include from 1
to 21 carbons (e.g., from 1 to 7 or from 1 to 11 carbons). In some embodiments, the alkyl group is further substituted with 1, 2, 3, or 4 substituents as described herein, and/or the amino group is \(-\text{NH}_2\) or \(-\text{NHR}^{\text{N1}}\), wherein \(\text{R}^{\text{N1}}\) is, independently, \(\text{OH}, \text{NO}_2, \text{NH}_2, \text{NR}^{\text{N2}}, \text{SO}_2\text{OR}^{\text{N2}}, \text{SO}_2\text{R}^{\text{N2}}, \text{SOR}^{\text{N2}}, \text{alkyl}, \) or ary1, and each \(\text{R}^{\text{N2}}\) can be \(\text{H}, \text{alkyl}, \text{or aryl.}\)

[00741] The term “alkaryl,” as used herein, represents an aryl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein. Exemplary unsubstituted alkaryl groups are from 7 to 30 carbons (e.g., from 7 to 16 or from 7 to 20 carbons, such as \(\text{C}_{\text{1-6}}\) alk-C_{6-10} aryl, \(\text{C}_{\text{1-10}}\) alk-C_{6-10} aryl, or \(\text{C}_{\text{1-20}}\) alk-C_{6-10} aryl). In some embodiments, the alkylene and the aryl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective groups. Other groups preceded by the prefix “alk-“ are defined in the same manner, where “alk” refers to a \(\text{C}_{\text{1-6}}\) alkylene, unless otherwise noted, and the attached chemical structure is as defined herein.

[00742] The term “alkycycloalkyl” represents a cycloalkyl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein (e.g., an alkylene group of from 1 to 4, from 1 to 6, from 1 to 10, or form 1 to 20 carbons). In some embodiments, the alkylene and the cycloalkyl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group.

[00743] The term “alkenyl,” as used herein, represents monovalent straight or branched chain groups of, unless otherwise specified, from 2 to 20 carbons (e.g., from 2 to 6 or from 2 to 10 carbons) containing one or more carbon-carbon double bonds and is exemplified by ethenyl, 1-propenyl, 2-propenyl, 2-methyl-1-propenyl, 1-butenyl, 2-butenyl, and the like. Alkenyls include both cis and trans isomers. Alkenyl groups may be optionally substituted with 1, 2, 3, or 4 substituent groups that are selected, independently, from amino, aryl, cycloalkyl, or heterocyclyl (e.g., heteroaryl), as defined herein, or any of the exemplary alkyl substituent groups described herein.

[00744] The term “alkenyloxy” represents a chemical substituent of formula \(-\text{OR}\), where \(\text{R}\) is a \(\text{C}_{\text{2-20}}\) alkenyl group (e.g., \(\text{C}_{\text{2-6}}\) or \(\text{C}_{\text{2-10}}\) alkenyl), unless otherwise specified. Exemplary alkenyloxy groups include ethenyloxy, propenyloxy, and the like. In some embodiments, the alkenyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein (e.g., a hydroxy group).

[00745] The term “alkheteroaryl” refers to a heteroaryl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein. Exemplary unsubstituted
alkheteroaryl groups are from 2 to 32 carbons (e.g., from 2 to 22, from 2 to 18, from 2 to 17, from 2 to 16, from 3 to 15, from 2 to 14, from 2 to 13, or from 2 to 12 carbons, such as C_{1-6} alk-C_{1-12} heteroaryl, C_{1-10} alk-C_{1-12} heteroaryl, or C_{1-20} alk-C_{1-12} heteroaryl). In some embodiments, the alkylene and the heteroaryl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group. Alkheteroaryl groups are a subset of alkheterocyclyl groups.

[00746] The term “alkheterocyclyl” represents a heterocyclyl group, as defined herein, attached to the parent molecular group through an alkylene group, as defined herein. Exemplary unsubstituted alkheterocyclyl groups are from 2 to 32 carbons (e.g., from 2 to 22, from 2 to 18, from 2 to 17, from 2 to 16, from 3 to 15, from 2 to 14, from 2 to 13, or from 2 to 12 carbons, such as C_{1-6} alk-C_{1-12} heterocyclyl, C_{1-10} alk-C_{1-12} heterocyclyl, or C_{1-20} alk-C_{1-12} heterocyclyl). In some embodiments, the alkylene and the heterocyclyl each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group.

[00747] The term “alkoxy” represents a chemical substituent of formula 

$$\text{OR}$$

where R is a C_{1-20} alkyl group (e.g., C_{1-6} or C_{1-10} alkyl), unless otherwise specified. Exemplary alkoxy groups include methoxy, ethoxy, propoxy (e.g., n-propoxy and isopropoxy), t-butoxy, and the like. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein (e.g., hydroxy or alkoxy).

[00748] The term “alkoxyalkoxy” represents an alkoxy group that is substituted with an alkoxy group. Exemplary unsubstituted alkoxyalkoxy groups include between 2 to 40 carbons (e.g., from 2 to 12 or from 2 to 20 carbons, such as C_{1-6} alkoxy-C_{1-6} alkoxy, C_{1-10} alkoxy-C_{1-10} alkoxy, or C_{1-20} alkoxy-C_{1-20} alkoxy). In some embodiments, the alkoxy group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[00749] The term “alkoxyalkyl” represents an alkyl group that is substituted with an alkoxy group. Exemplary unsubstituted alkoxyalkyl groups include between 2 to 40 carbons (e.g., from 2 to 12 or from 2 to 20 carbons, such as C_{1-6} alkoxy-C_{1-6} alkyl, C_{1-10} alkoxy-C_{1-10} alkyl, or C_{1-20} alkoxy-C_{1-20} alkyl). In some embodiments, the alkyl and the alkoxy each can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for the respective group.

[00750] The term “alkoxycarbonyl,” as used herein, represents an alkoxy, as defined herein, attached to the parent molecular group through a carbonyl atom (e.g., -C(O)-OR, where R is H or an optionally substituted C_{1-6}, C_{1-10}, or C_{1-20} alkyl group). Exemplary unsubstituted alkoxy carbonyl
include from 1 to 21 carbons (e.g., from 1 to 11 or from 1 to 7 carbons). In some embodiments, the alkoxy group is further substituted with 1, 2, 3, or 4 substituents as described herein.

[00751] The term “alkoxycarbonylalkoxy,” as used herein, represents an alkoxy group, as defined herein, that is substituted with an alkoxy carbonyl group, as defined herein (e.g., -O-alkyl-C(O)-OR, where R is an optionally substituted C1-6, C1-10, or C1-20 alkyl group). Exemplary unsubstituted alkoxy carbonylalkoxy include from 3 to 41 carbons (e.g., from 3 to 10, from 3 to 13, from 3 to 17, from 3 to 21, or from 3 to 31 carbons, such as C1-6 alkoxy carbonyl-C1-6 alkoxy, C1-10 alkoxy carbonyl-C1-10 alkoxy, or C1-20 alkoxy carbonyl-C1-20 alkoxy). In some embodiments, each alkoxy group is further independently substituted with 1, 2, 3, or 4 substituents, as described herein (e.g., a hydroxy group).

[00752] The term “alkoxycarbonylalkyl,” as used herein, represents an alkyl group, as defined herein, that is substituted with an alkoxy carbonyl group, as defined herein (e.g., -alkyl-C(O)-OR, where R is an optionally substituted C1-20, C1-10, or C1-6 alkyl group). Exemplary unsubstituted alkoxy carbonylalkyl include from 3 to 41 carbons (e.g., from 3 to 10, from 3 to 13, from 3 to 17, from 3 to 21, or from 3 to 31 carbons, such as C1-6 alkoxy carbonyl-C1-6 alkyl, C1-10 alkoxy carbonyl-C1-10 alkyl, or C1-20 alkoxy carbonyl-C1-20 alkyl). In some embodiments, each alkyl and alkoxy group is further independently substituted with 1, 2, 3, or 4 substituents as described herein (e.g., a hydroxy group).

[00753] The term “alkyl,” as used herein, is inclusive of both straight chain and branched chain saturated groups from 1 to 20 carbons (e.g., from 1 to 10 or from 1 to 6), unless otherwise specified. Alkyl groups are exemplified by methyl, ethyl, n- and iso-propyl, n-, sec-, iso- and tert-butyl, neopentyl, and the like, and may be optionally substituted with one, two, three, or, in the case of alkyl groups of two carbons or more, four substituents independently selected from the group consisting of: (1) C1-6 alkoxy; (2) C1-6 alkylsulfinyl; (3) amino, as defined herein (e.g., unsubstituted amino (i.e., -NH2) or a substituted amino (i.e., -N(RN)2, where RN is as defined for amino); (4) C6-10 aryl-C1-6 alkoxy; (5) azido; (6) halo; (7) C2-9 heterocyclyl oxy; (8) hydroxy; (9) nitro; (10) oxo (e.g., carboxyaldehyde or acyl); (11) C1-7 spirocyclyl; (12) thioalkoxy; (13) thiol; (14) CO2R′, where R′ is selected from the group consisting of (a) C1-20 alkyl (e.g., C1-6 alkyl), (b) C2-20 alkenyl (e.g., C2-6 alkenyl), (c) C6-10 aryl, (d) hydrogen, (e) C1-6 alk-C6-10 aryl, (f) amino-C1-20 alkyl, (g) polyethylene glycol of -(CH2)s(OCH2CH2)s(CH2)sOR′, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0
to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of -NR^N(CH_2)_a(CH_2CH_2O)_b(CH_2)_cNR^N, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^N is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (15) -C(O)NR^B'RC, where each of R^{B'} and R^C is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (16) -SO_2R^{D'}, where R^{D'} is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) C_{1-6} alk-C_{6-10} aryl, and (d) hydroxy; (17) -SO_2NR^E'R^F', where each of R^E' and R^F' is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl and (d) C_{1-6} alk-C_{6-10} aryl; (18) -C(O)R^{G'}, where R^{G'} is selected from the group consisting of (a) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c) C_{6-10} aryl, (d) hydrogen, (e) C_{1-6} alk-C_{6-10} aryl, (f) amino-C_{1-20} alkyl, (g) polyethylene glycol of -(CH_2)_a(OCH_2CH_2)_b(H_2)_cOR', wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of -NR^N(CH_2)_a(CH_2CH_2O)_b(CH_2)_cNR^N, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^N is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (19) -NR^W'C(O)R^F', wherein R^W' is selected from the group consisting of (a1) hydrogen and (b1) C_{1-6} alkyl, and R^F' is selected from the group consisting of (a2) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b2) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c2) C_{6-10} aryl, and (d2) hydrogen, (e2) C_{1-6} alk-C_{6-10} aryl, (f2) amino-C_{1-20} alkyl, (g2) polyethylene glycol of -(CH_2)_a(OCH_2CH_2)_b(H_2)_cOR', wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of -NR^N(CH_2)_a(CH_2CH_2O)_b(CH_2)_cNR^N, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^N is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (20) -NR^W'C(O)OR^{K'}, wherein R^{K'} is selected from the group consisting of (a) hydrogen and (b) C_{1-6} alkyl, and R^{K'} is selected from the group consisting of (a) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c) C_{6-10} aryl, (d) hydrogen, (e) C_{1-6} alk-C_{6-10} aryl, (f) amino-C_{1-20}
alkyl, (g2) polyethylene glycol of \(-(\text{CH}_2)_2(\text{OCH}_2\text{CH}_2)_s(\text{CH}_2)_a\)OR', wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, and (h2) amino-polyethylene glycol of -NR^N_{1}(\text{CH}_2)_2(\text{CH}_2\text{CH}_2\text{O})_s(\text{CH}_2)_a\)NR^N_{1}, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^N_{1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; and (21) amidine. In some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a C_{1-alkaryl} can be further substituted with an oxo group to afford the respective aryloyl substituent.

[00754] The term “alkylene” and the prefix “alk-“, as used herein, represent a saturated divalent hydrocarbon group derived from a straight or branched chain saturated hydrocarbon by the removal of two hydrogen atoms, and is exemplified by methylene, ethylene, isopropylene, and the like. The term “C_{x-y} alkylene” and the prefix “C_{x-y} alk-“ represent alkylene groups having between x and y carbons. Exemplary values for x are 1, 2, 3, 4, 5, and 6, and exemplary values for y are 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, or 20 (e.g., C_{1-6}, C_{1-10}, C_{2-20}, C_{2-6}, C_{2-10}, or C_{2-20} alkylene). In some embodiments, the alkylene can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein for an alkyl group.

[00755] The term “alkylsulfenyl,” as used herein, represents an alkyl group attached to the parent molecular group through an -S(O)- group. Exemplary unsubstituted alkylsulfenyl groups are from 1 to 6, from 1 to 10, or from 1 to 20 carbons. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[00756] The term “alkylsulfenylalkyl,” as used herein, represents an alkyl group, as defined herein, substituted by an alkylsulfenyl group. Exemplary unsubstituted alkylsulfenylalkyl groups are from 2 to 12, from 2 to 20, or from 2 to 40 carbons. In some embodiments, each alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[00757] The term “alkynyl,” as used herein, represents monovalent straight or branched chain groups from 2 to 20 carbon atoms (e.g., from 2 to 4, from 2 to 6, or from 2 to 10 carbons) containing a carbon-carbon triple bond and is exemplified by ethynyl, 1-propynyl, and the like. Alkynyl groups may be optionally substituted with 1, 2, 3, or 4 substituent groups that are selected, independently,
from aryl, cycloalkyl, or heterocycyl (e.g., heteroaryl), as defined herein, or any of the exemplary alkyl substituent groups described herein.

[00758] The term “alkynyloxy” represents a chemical substituent of formula −OR, where R is a C_{2-20} alkynyl group (e.g., C_{2-6} or C_{2-10} alkynyl), unless otherwise specified. Exemplary alkynyloxy groups include ethynyloxy, propynyloxy, and the like. In some embodiments, the alkynyl group can be further substituted with 1, 2, 3, or 4 substituent groups as defined herein (e.g., a hydroxy group).

[00759] The term “amidine,” as used herein, represents a −C(=NH)NH_{2} group.

[00760] The term “amino,” as used herein, represents −N(R^{N1})_{2}, wherein each R^{N1} is, independently, H, OH, NO_{2}, N(R^{N2})_{2}, SO_{2}OR^{N2}, SO_{2}R^{N2}, SOR^{N2}, an N-protecting group, alkyl, alkenyl, alkynyl, alkoxy, aryl, alkaryl, cycloalkyl, alky cycloalkyl, carboxyalkyl, sulfoalkyl, heterocycyl (e.g., heteroaryl), or alk heterocycyl (e.g., alk heteroaryl), wherein each of these recited R^{N1} groups can be optionally substituted, as defined herein for each group; or two R^{N1} combine to form a heterocycyl or an N-protecting group, and wherein each R^{N2} is, independently, H, alkyl, or aryl. The amino groups of the invention can be an unsubstituted amino (i.e., −NH_{2}) or a substituted amino (i.e., −N(R^{N1})_{2}). In a preferred embodiment, amino is −NH_{2} or −NHR^{N1}, wherein R^{N1} is, independently, OH, NO_{2}, NH_{2}, NR^{N2}, SO_{2}OR^{N2}, SO_{2}R^{N2}, SOR^{N2}, alkyl, carboxyalkyl, sulfoalkyl, or aryl, and each R^{N2} can be H, C_{1-20} alkyl (e.g., C_{1-6} alkyl), or C_{6-10} aryl.

[00761] The term “amino acid,” as described herein, refers to a molecule having a side chain, an amino group, and an acid group (e.g., a carboxy group of −CO_{2}H or a sulf group of −SO_{2}H), wherein the amino acid is attached to the parent molecular group by the side chain, amino group, or acid group (e.g., the side chain). In some embodiments, the amino acid is attached to the parent molecular group by a carbonyl group, where the side chain or amino group is attached to the carbonyl group. Exemplary side chains include an optionally substituted alkyl, aryl, heterocycyl, alkaryl, alkheterocycyl, aminoalkyl, carbamoylalkyl, and carboxyalkyl. Exemplary amino acids include alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, hydroxyornithine, isoleucine, leucine, lysine, methionine, norvaline, ornithine, phenylalanine, proline, pyrroline, selenocysteine, serine, taurine, threonine, tryptophan, tyrosine, and valine. Amino acid groups may be optionally substituted with one, two, three, or, in the case of amino acid groups of two carbons or more, four substituents independently selected from the group consisting of: (1) C_{1-6} alkoxy; (2) C_{1-6} alkylsulfinyl; (3) amino, as defined herein (e.g., unsubstituted amino (i.e., −NH_{2}) or a substituted amino (i.e., −N(R^{N1})_{2}, where R^{N1} is as defined for amino); (4) C_{6-10}
aryl-C_{1-6} alkoxy; (5) azido; (6) halo; (7) (C_{2-9} heterocyclyl)oxy; (8) hydroxy; (9) nitro; (10) oxo (e.g., carboxyaldehyde or acyl); (11) C_{1-7} spirocycl; (12) thioalkoxy; (13) thiol; (14) -CO_{2}R^{A}, where R^{A} is selected from the group consisting of (a) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c) C_{6-10} aryl, (d) hydrogen, (e) C_{1-6} alk-C_{6-10} aryl, (f) amino-C_{1-20} alkyl, (g) polyethylene glycol of -(CH_{2})_{2e}(OCH_{2}CH_{2})_{s1}(CH_{2})_{s3}OR^{*}, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R^{*} is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of -NR^{N1}(CH_{2})_{2e}(CH_{2}CH_{2}O)_{s1}(CH_{2})_{s3}NR^{N1}, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (15) -C(O)NR^{B}R^{C}, where each of R^{B} and R^{C} is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (16) -SO_{2}R^{D}, where R^{D} is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) C_{1-6} alk-C_{6-10} aryl, and (d) hydroxy; (17) -SO_{2}NR^{E}R^{F}, where each of R^{E} and R^{F} is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl and (d) C_{1-6} alk-C_{6-10} aryl; (18) -C(O)R^{G}, where R^{G} is selected from the group consisting of (a) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c) C_{6-10} aryl, (d) hydrogen, (e) C_{1-6} alk-C_{6-10} aryl, (f) amino-C_{1-20} alkyl, (g) polyethylene glycol of -(CH_{2})_{2e}(OCH_{2}CH_{2})_{s1}(CH_{2})_{s3}OR^{*}, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R^{*} is H or C_{1-20} alkyl, and (h) amino-polyethylene glycol of -NR^{N1}(CH_{2})_{2e}(CH_{2}CH_{2}O)_{s1}(CH_{2})_{s3}NR^{N1}, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (19) -NR^{N1}C(O)R^{F}, where R^{F} is selected from the group consisting of (a1) hydrogen and (b1) C_{1-6} alkyl, and R^{F} is selected from the group consisting of (a2) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b2) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c2) C_{6-10} aryl, (d2) hydrogen, (e2) C_{1-6} alk-C_{6-10} aryl, (f2) amino-C_{1-20} alkyl, (g2) polyethylene glycol of -(CH_{2})_{2e}(OCH_{2}CH_{2})_{s1}(CH_{2})_{s3}OR^{*}, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R^{*} is H or C_{1-20} alkyl, and (h2) amino-polyethylene glycol of -NR^{N1}(CH_{2})_{2e}(CH_{2}CH_{2}O)_{s1}(CH_{2})_{s3}NR^{N1},
wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; (20) -NR^{J}C(=O)OR^{K}, wherein R^{J} is selected from the group consisting of (a1) hydrogen and (b1) C_{1-6} alkyl, and R^{K} is selected from the group consisting of (a2) C_{1-20} alkyl (e.g., C_{1-6} alkyl), (b2) C_{2-20} alkenyl (e.g., C_{2-6} alkenyl), (c2) C_{6-10} aryl, (d2) hydrogen, (e2) C_{1-6} alk-C_{6-10} aryl, (f2) amino-C_{1-20} alkyl, (g2) polyethylene glycol of -(CH_{2})_{x}(OCH_{2}CH_{2})_{y}(CH_{2})_{z}OR^{K}, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R^{J} is H or C_{1-20} alkyl, and (h2) amino-polyethylene glycol of -NR^{N1}(CH_{2})_{x}(CH_{2}CH_{2}O)_{y}(CH_{2})_{z}NR^{N1}, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl; and (21) amidine. In some embodiments, each of these groups can be further substituted as described herein.

[00762] The term “aminoalkoxy,” as used herein, represents an alkoxy group, as defined herein, substituted by an amino group, as defined herein. The alkyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., CO_{2}R^{A'}, where R^{A'} is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl, e.g., carboxy).

[00763] The term “aminoalkyl,” as used herein, represents an alkyl group, as defined herein, substituted by an amino group, as defined herein. The alkyl and amino each can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the respective group (e.g., CO_{2}R^{A'}, where R^{A'} is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl, e.g., carboxy).

[00764] The term “aryl,” as used herein, represents a mono-, bicyclic, or multicyclic carbocyclic ring system having one or two aromatic rings and is exemplified by phenyl, naphthyl, 1,2-dihydronaphthyl, 1,2,3,4-tetrahydronaphthyl, anthracenyl, phenanthrenyl, fluorenlyl, indanlyl, indenyl, and the like, and may be optionally substituted with 1, 2, 3, 4, or 5 substituents independently selected from the group consisting of: (1) C_{1-7} acyl (e.g., carboxyaldehyde); (2) C_{1-20} alkyl (e.g., C_{1-6} alkyl, C_{1-6} alkoxy-C_{1-6} alkyl, C_{1-6} alkylsulfanyl-C_{1-6} alkyl, amino-C_{1-6} alkyl, azido-C_{1-6} alkyl, (carboxyaldehyde)-C_{1-6} alkyl, halo-C_{1-6} alkyl (e.g., perfluoroalkyl), hydroxy-C_{1-6} alkyl, nitro-
C_{1-6} alkyl, or C_{1-6} thioalkoxy-C_{1-6} alkyl; (3) C_{1-20} alkoxy (e.g., C_{1-6} alkoxy, such as perfluoroalkoxy); (4) C_{1-6} alkylsulfanyl; (5) C_{6-10} aryl; (6) amino; (7) C_{1-6} alk-C_{6-10} aryl; (8) azido; (9) C_{3-8} cycloalkyl; (10) C_{1-6} alk-C_{3-8} cycloalkyl; (11) halo; (12) C_{1-12} heterocyclyl (e.g., C_{1-12} heteroaryl); (13) (C_{1-12} heterocyclyl)oxy; (14) hydroxy; (15) nitro; (16) C_{1-20} thioalkoxy (e.g., C_{1-6} thioalkoxy); (17) -(CH_2)_qCO_2R^A, where q is an integer from zero to four, and R^A is selected from the group consisting of (a) C_{1-6} alkyl, (b) C_{6-10} aryl, (c) hydrogen, and (d) C_{1-6} alk-C_{6-10} aryl; (18) -(CH_2)_qCONR^B^CR^C, where q is an integer from zero to four and where R^B and R^C are independently selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (19) -(CH_2)_qSO_2R^D, where q is an integer from zero to four and where R^D is selected from the group consisting of (a) alkyl, (b) C_{6-10} aryl, and (c) alk-C_{6-10} aryl; (20) -(CH_2)_qSO_2NR^E^FR^F, where q is an integer from zero to four and where each of R^E and R^F is, independently, selected from the group consisting of (a) hydrogen, (b) C_{1-6} alkyl, (c) C_{6-10} aryl, and (d) C_{1-6} alk-C_{6-10} aryl; (21) thiol; (22) C_{6-10} arloxy; (23) C_{3-8} cycloalkoxy; (24) C_{6-10} aryl-C_{1-6} alkoxy; (25) C_{1-6} alk-C_{1-12} heterocyclyl (e.g., C_{1-6} alk-C_{1-12} heteroaryl); (26) C_{2-20} alkenyl; and (27) C_{2-20} alkynyl. In some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a C_{1}-alkaryl or a C_{1}-alkheterocyclyl can be further substituted with an oxo group to afford the respective arylloyl and (heterocyclyl)oyl substituent group.

The term “arylalkoxy,” as used herein, represents an alkaryl group, as defined herein, attached to the parent molecular group through an oxygen atom. Exemplary unsubstituted alkoxyalkyl groups include from 7 to 30 carbons (e.g., from 7 to 16 or from 7 to 20 carbons, such as C_{6-10} aryl-C_{1-6} alkoxy, C_{6-10} aryl-C_{1-10} alkoxy, or C_{6-10} aryl-C_{1-20} alkoxy). In some embodiments, the arylalkoxy group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

The term “aryloxy” represents a chemical substituent of formula –OR’, where R’ is an aryl group of 6 to 18 carbons, unless otherwise specified. In some embodiments, the aryl group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

The term “aryloyl,” as used herein, represents an aryl group, as defined herein, that is attached to the parent molecular group through a carbonyl group. Exemplary unsubstituted aryloyl groups are of 7 to 11 carbons. In some embodiments, the aryl group can be substituted with 1, 2, 3, or 4 substituents as defined herein.

The term “azido” represents an –N_3 group, which can also be represented as –N=N=N.
[00769] The term “bicyclic,” as used herein, refer to a structure having two rings, which may be aromatic or non-aromatic. Bicyclic structures include spirocyclyl groups, as defined herein, and two rings that share one or more bridges, where such bridges can include one atom or a chain including two, three, or more atoms. Exemplary bicyclic groups include a bicyclic carbocyclyl group, where the first and second rings are carbocyclyl groups, as defined herein; a bicyclic aryl groups, where the first and second rings are aryl groups, as defined herein; bicyclic heterocyclyl groups, where the first ring is a heterocyclyl group and the second ring is a carbocyclyl (e.g., aryl) or heterocyclyl (e.g., heteroaryl) group; and bicyclic heteroaaryl groups, where the first ring is a heteroaryl group and the second ring is a carbocyclyl (e.g., aryl) or heterocyclyl (e.g., heteroaryl) group. In some embodiments, the bicyclic group can be substituted with 1, 2, 3, or 4 substituents as defined herein for cycloalkyl, heterocyclyl, and aryl groups.

[00770] The terms “carbocyclic” and “carbocyclyl,” as used herein, refer to an optionally substituted $C_{3-12}$ monocyclic, bicyclic, or tricyclic structure in which the rings, which may be aromatic or non-aromatic, are formed by carbon atoms. Carbocyclic structures include cycloalkyl, cycloalkenyl, and aryl groups.

[00771] The term “carbamoyl,” as used herein, represents $\text{C}($O$)\text{-N}(\text{R}^{N1})_2$, where the meaning of each $\text{R}^{N1}$ is found in the definition of “amino” provided herein.

[00772] The term “carbamoylalkyl,” as used herein, represents an alkyl group, as defined herein, substituted by a carbamoyl group, as defined herein. The alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[00773] The term “carbamyl,” as used herein, refers to a carbamate group having the structure $\text{-N}(\text{R}^{N1})\text{C(=O)}\text{OR}$ or $\text{-OC(=O)}\text{N}(\text{R}^{N1})_2$, where the meaning of each $\text{R}^{N1}$ is found in the definition of “amino” provided herein, and $\text{R}$ is alkyl, cycloalkyl, alkyloalkyl, aryl, alkaryl, heterocyclyl (e.g., heteroaryyl), or alkhetocyclyl (e.g., alkhetoaryl), as defined herein.

[00774] The term “carbonyl,” as used herein, represents a $\text{C}$(O) group, which can also be represented as $\text{C}=$O.

[00775] The term “carboxyaldehyde” represents an acyl group having the structure $\text{C}$(O)H.

[00776] The term “carbonyl,” as used herein, means $\text{C}$(O)H.

[00777] The term “carboxyalkoxy,” as used herein, represents an alkoxy group, as defined herein, substituted by a carboxy group, as defined herein. The alkoxy group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for the alkyl group.
The term “carboxyalkyl,” as used herein, represents an alkyl group, as defined herein, substituted by a carboxy group, as defined herein. The alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

The term “cyano,” as used herein, represents an –CN group.

The term “cycloalkoxy” represents a chemical substituent of formula –OR, where R is a C₃-8 cycloalkyl group, as defined herein, unless otherwise specified. The cycloalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein. Exemplary unsubstituted cycloalkoxy groups are from 3 to 8 carbons.

The term “cycloalkyl,” as used herein represents a monovalent saturated or unsaturated non-aromatic cyclic hydrocarbon group from three to eight carbons, unless otherwise specified, and is exemplified by cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, bicyclo[2.2.1.]heptyl, and the like. When the cycloalkyl group includes one carbon-carbon double bond, the cycloalkyl group can be referred to as a “cycloalkenyl” group. Exemplary cycloalkenyl groups include cyclopentenyl, cyclohexenyl, and the like. The cycloalkyl groups of this invention can be optionally substituted with: (1) C₁₋₇ acyl (e.g., carboxyaldehyde); (2) C₁₋₂₀ alkyl (e.g., C₁₋₆ alkyl, C₁₋₆ alkoxy-C₁₋₆ alkyl, C₁₋₆ alkylsulfanyl-C₁₋₆ alkyl, amino-C₁₋₆ alkyl, azido-C₁₋₆ alkyl, (carboxyaldehyde)-C₁₋₆ alkyl, halo-C₁₋₆ alkyl (e.g., perfluoroalkyl), hydroxy-C₁₋₆ alkyl, nitro-C₁₋₆ alkyl, or C₁₋₆ thioalkoxy-C₁₋₆ alkyl); (3) C₁₋₂₀ alkoxy (e.g., C₁₋₆ alkoxy, such as perfluoroalkoxy); (4) C₁₋₆ alkylsulfanyl; (5) C₆₋₁₀ aryl; (6) amino; (7) C₁₋₆ alk-C₆₋₁₀ aryl; (8) azido; (9) C₃₋₈ cycloalkyl; (10) C₁₋₆ alk-C₃₋₈ cycloalkyl; (11) halo; (12) C₁₋₁₂ heterocyclyl (e.g., C₁₋₁₂ heteroaryl); (13) (C₁₋₁₂ heterocyclyl)oxy; (14) hydroxy; (15) nitro; (16) C₁₋₂₀ thioalkoxy (e.g., C₁₋₆ thioalkoxy); (17) –(CH₂)₉CO₂R⁺, where q is an integer from zero to four, and R⁺ is selected from the group consisting of (a) C₁₋₆ alkyl, (b) C₆₋₁₀ aryl, (c) hydrogen, and (d) C₁₋₆ alk-C₆₋₁₀ aryl; (18) –(CH₂)₉CONR⁺R⁻, where q is an integer from zero to four and where R⁺ and R⁻ are independently selected from the group consisting of (a) hydrogen, (b) C₆₋₁₀ alkyl, (c) C₆₋₁₀ aryl, and (d) C₁₋₆ alk-C₆₋₁₀ aryl; (19) –(CH₂)₉SO₂R⁻, where q is an integer from zero to four and where R⁻ is selected from the group consisting of (a) C₆₋₁₀ alkyl, (b) C₆₋₁₀ aryl, and (c) C₁₋₆ alk-C₆₋₁₀ aryl; (20) –(CH₂)₉SO₂NR⁺R⁻, where q is an integer from zero to four and where each of R⁺ and R⁻ is, independently, selected from the group consisting of (a) hydrogen, (b) C₆₋₁₀ alkyl, (c) C₆₋₁₀ aryl, and (d) C₁₋₆ alk-C₆₋₁₀ aryl; (21) thiol; (22) C₆₋₁₀ aryloxy; (23) C₃₋₈ cycloalkoxy; (24) C₆₋₁₀ aryloxy-C₁₋₆ aryl; (25) C₁₋₆ alk-C₁₋₁₂ heterocyclyl (e.g., C₁₋₆ alk-C₁₋₁₂ heteroaryl); (26) oxo; (27) C₂₋₂₀ alkenyl; and (28) C₂₋₂₀ alkynyl. In
some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a C₁-alkaryl or a C₁-alkheterocyclyl can be further substituted with an oxo group to afford the respective aryloxy and (heterocyclyloxy) substituent group.

[00782] The term “diasteromer” means stereoisomers that are not mirror images of one another and are non-superimposable.

[00783] The term “effective amount” of an agent, as used herein, is that amount sufficient to effect beneficial or desired results, for example, clinical results, and, as such, an “effective amount” depends upon the context in which it is being applied. For example, in the context of administering an agent that treats cancer, an effective amount of an agent is, for example, an amount sufficient to achieve treatment, as defined herein, of cancer, as compared to the response obtained without administration of the agent.

[00784] The term “enantiomer,” as used herein, means each individual optically active form of a compound of the invention, having an optical purity or enantiomeric excess (as determined by methods standard in the art) of at least 80% (i.e., at least 90% of one enantiomer and at most 10% of the other enantiomer), preferably at least 90% and more preferably at least 98%.

[00785] The term “halo,” as used herein, represents a halogen selected from bromine, chlorine, iodine, or fluorine.

[00786] The term “haloalkoxy,” as used herein, represents an alkoxy group, as defined herein, substituted by a halogen group (i.e., F, Cl, Br, or I). A haloalkoxy may be substituted with one, two, three, or, in the case of alkyl groups of two carbons or more, four halogens. Haloalkoxy groups include perfluoroalkoxys (e.g., -OCF₃, -OCHF₂, -OCH₂F, -OCCl₃, -OCH₂CH₂Br, -OCH₂CH(CH₂CH₂Br)CH₃, and -OCHICH₃. In some embodiments, the haloalkoxy group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkyl groups.

[00787] The term “haloalkyl,” as used herein, represents an alkyl group, as defined herein, substituted by a halogen group (i.e., F, Cl, Br, or I). A haloalkyl may be substituted with one, two, three, or, in the case of alkyl groups of two carbons or more, four halogens. Haloalkyl groups include perfluoroalkyls (e.g., -CF₃, -CHF₂, -CH₂F, -CCl₃, -CH₂CH₂Br, -CH₂CH(CH₂CH₂Br)CH₃, and -CHICH₃. In some embodiments, the haloalkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkyl groups.

[00788] The term “heteroalkylene,” as used herein, refers to an alkylene group, as defined herein, in which one or two of the constituent carbon atoms have each been replaced by nitrogen, oxygen, or
sulfur. In some embodiments, the heteroalkylene group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein for alkylene groups.

[00789] The term “heteroaryl,” as used herein, represents that subset of heterocyclyls, as defined herein, which are aromatic: i.e., they contain 4\(n\)+2 pi electrons within the mono- or multicyclic ring system. Exemplary unsubstituted heteroaryl groups are of 1 to 12 (e.g., 1 to 11, 1 to 10, 1 to 9, 2 to 12, 2 to 11, 2 to 10, or 2 to 9) carbons. In some embodiment, the heteroaryl is substituted with 1, 2, 3, or 4 substituents groups as defined for a heterocyclyl group.

[00790] The term “heterocyclyl,” as used herein represents a 5-, 6- or 7-membered ring, unless otherwise specified, containing one, two, three, or four heteroatoms independently selected from the group consisting of nitrogen, oxygen, and sulfur. The 5-membered ring has zero to two double bonds, and the 6- and 7-membered rings have zero to three double bonds. Exemplary unsubstituted heterocyclyl groups are of 1 to 12 (e.g., 1 to 11, 1 to 10, 1 to 9, 2 to 12, 2 to 11, 2 to 10, or 2 to 9) carbons. The term “heterocyclyl” also represents a heterocyclic compound having a bridged multicyclic structure in which one or more carbons and/or heteroatoms bridges two non-adjacent members of a monocyclic ring, e.g., a quinuclidinyl group. The term “heterocyclyl” includes bicyclic, tricyclic, and tetracyclic groups in which any of the above heterocyclic rings is fused to one, two, or three carbocyclic rings, e.g., an aryl ring, a cyclohexane ring, a cyclohexene ring, a cyclopentane ring, a cyclopentene ring, or another monocyclic heterocyclic ring, such as indolyl, quinolyl, isoquinolyl, tetrahydroquinolyl, benzofuranyl, benzothienyl and the like. Examples of fused heterocyclyls include tropanes and 1,2,3,5,8a-hexahydroindolizine. Heterocycles include pyrrolyl, pyrrolinyl, pyrrolidinyl, pyrazolyl, pyrazolinyl, pyrazolidinyl, imidazolyl, imidazolinyl, imidazolidinyl, pyridyl, piperidinyl, homopiperidinyl, pyrazinyl, pyrazinyl, pyrimidinyl, pyridazinyl, oxazolyl, oxazolidinyl, isoxazolyl, isoxazolidinyl, morpholinyl, thiomorpholinyl, thiazolyl, thiazolidinyl, isothiazolyl, isothiazolidinyl, indolyl, indazolyl, quinolyl, isoquinolyl, quinoxalinyl, dihydroquinoxalinyl, quinazolinyl, cinnolinyl, phthalazinyl, benzimidazolyl, benzothiazolyl, benzoxazolyl, benzothiazolyl, furyl, thiényl, thiazolidinyl, isothiazolyl, triazolyl, tetrazolyl, oxadiazolyl (e.g., 1,2,3-oxadiazolyl), purinyl, thiadiazolyl (e.g., 1,2,3-thiadiazolyl), tetrahydrofuranyl, dihydrofuranyl, tetrahydrothienyl, dihydrothienyl, dihydroindolyl, dihydroquinolyl, tetrahydroquinolyl, tetrahydroisoquinolyl, dihydroisoquinolyl, pyranyl, dihydropyranyl, dithiazolyl, benzofuranyl, isobenzofuranyl, benzothienyl, and the like, including dihydro and tetrahydro forms thereof, where one or more double bonds are reduced and replaced.
with hydrogens. Still other exemplary heterocycls include: 2,3,4,5-tetrahydro-2-oxo-oxazolyl; 2,3-dihydro-2-oxo-1H-imidazolyl; 2,3,4,5-tetrahydro-5-oxo-1H-pyrazolyl (e.g., 2,3,4,5-tetrahydro-2-phenyl-5-oxo-1H-pyrazolyl); 2,3,4,5-tetrahydro-2,4-dioxo-1H-imidazolyl (e.g., 2,3,4,5-tetrahydro-2,4-dioxo-5-methyl-5-phenyl-1H-imidazolyl); 2,3-dihydro-2-thioxo-1,3,4-oxadiazoazol (e.g., 2,3-dihydro-2-thioxo-5-phenyl-1,3,4-oxadiazoazol); 4,5-dihydro-5-oxo-1H-triazolyl (e.g., 4,5-dihydro-3-methyl-4-amino 5-oxo-1H-triazolyl); 1,2,3,4-tetrahydro-2,4-dioxopyridinyl (e.g., 1,2,3,4-tetrahydro-2,4-dioxo-3,3-diethylpyridinyl); 2,6-dioxo-piperidinyl (e.g., 2,6-dioxo-3-ethyl-3-phenylpiperidinyl); 1,6-dihydro-6-oxopyridinyl; 1,6-dihydro-4-oxopyrimidinyl (e.g., 2-(methylthio)-1,6-dihydro-4-oxo-5-methylpyrimidin-1-yl); 1,2,3,4-tetrahydro-2,4-dioxopyrimidinyl (e.g., 1,2,3,4-tetrahydro-2,4-dioxo-3-ethylpyrimidinyl); 1,6-dihydro-6-oxo-pyridazinyl (e.g., 1,6-dihydro-6-oxo-3-ethylpyridazinyl); 1,6-dihydro-6-oxo-1,2,4-triazinyl (e.g., 1,6-dihydro-5-isopropyl-6-oxo-1,2,4-triazinyl); 2,3-dihydro-2-oxo-1H-indolyl (e.g., 3,3-dimethyl-2,3-dihydro-2-oxo-1H-indolyl and 2,3-dihydro-2-oxo-3,3'-spiropropane-1H-indol-1-y1); 1,3-dihydro-1-oxo-2H-isouindolyl; 1,3-dihydro-1,3-dioxo-2H-iso-indolyl; 1H-benzopyrazolyl (e.g., 1-(ethoxycarbonyl)-1H-benzopyrazolyl); 2,3-dihydro-2-oxo-1H-benzimidazolyl (e.g., 3-ethyl-2,3-dihydro-2-oxo-1H-benzimidazolyl); 2,3-dihydro-2-oxo-benzoazolyl (e.g., 5-chloro-2,3-dihydro-2-oxo-benzoazolyl); 2,3-dihydro-2-oxo-benzoazolyl; 2-oxo-2H-benzyopyranyl; 1,4-benzodioxanyl; 1,3-benzodioxanly; 2,3-dihydro-3-oxo-4H-1,3-benzothiazinyl; 3,4-dihydro-4-oxo-3H-quinazolinyl (e.g., 2-methyl-3,4-dihydro-4-oxo-3H-quiazolinyl); 1,2,3,4-tetrahydro-2,4-dioxo-3H-quiazolyl (e.g., 1-ethyl-1,2,3,4-tetrahydro-2,4-dioxo-3H-quiazolyl); 1,2,3,6-tetrahydro-2,6-dioxo-7H-purinyl (e.g., 1,2,3,6-tetrahydro-1,3-dimethyl-2,6-dioxo-7 H-purinyl); 1,2,3,6-tetrahydro-2,6-dioxo-1 H-purinyl (e.g., 1,2,3,6-tetrahydro-3,7-dimethyl-2,6-dioxo-1 H-purinyl); 2-oxobenz[c,d]indolyl; 1,1-dioxo-2H-naphth[1,8-c,d]isothiazolyl; and 1,8-naphthylenedicarboxamido. Additional heterocyclics include 3,3a,4,5,6,6a-hexahydro-pyrrolo[3,4-b]pyrrol-(2H)-yl, and 2,5-diazabicyclo[2.2.1]heptan-2-yl, homopiperazinyl (or diazepanyl), tetrahydropranyln, dithiazolyl, benzo furanyln, benzo thiencn, oxeapanyln, thiepa nyl, azocanyl, oxecanyln, and thiocanyl. Heterocyclic groups also include groups of the formula.

E’ is selected from the group consisting of -N- and -CH-; F’ is selected from the group consisting of -N=CH-, -NH-CH2-, -NH-C(O)-, -NH-, -CH=N-, -CH2-NH-, -(O)-NH-, -CH=CH-, -CH2-, -CH2CH2-, -CH3O-, -OCH2-, -O-, and -S-; and G’ is selected from the group consisting of -CH- and
N-. Any of the heterocyclol groups mentioned herein may be optionally substituted with one, two, three, four or five substituents independently selected from the group consisting of: (1) C\textsubscript{1-7} acyl (e.g., carboxyaldehyde); (2) C\textsubscript{1-20} alkyl (e.g., C\textsubscript{1-6} alkyl, C\textsubscript{1-6} alkoxy-C\textsubscript{1-6} alkyl, C\textsubscript{1-6} alkylsulfinyl-C\textsubscript{1-6} alkyl, amino-C\textsubscript{1-6} alkyl, azido-C\textsubscript{1-6} alkyl, (carboxyaldehyde)-C\textsubscript{1-6} alkyl, halo-C\textsubscript{1-6} alkyl (e.g., perfluoroalkyl), hydroxy-C\textsubscript{1-6} alkyl, nitro-C\textsubscript{1-6} alkyl, or C\textsubscript{1-6} thioalkoxy-C\textsubscript{1-6} alkyl); (3) C\textsubscript{1-20} alkoxy (e.g., C\textsubscript{1-6} alkoxy, such as perfluoroalkoxy); (4) C\textsubscript{1-6} alkylsulfinyl; (5) C\textsubscript{6-10} aryl; (6) amino; (7) C\textsubscript{1-6} alk-C\textsubscript{6-10} aryl; (8) azido; (9) C\textsubscript{3-8} cycloalkyl; (10) C\textsubscript{1-6} alk-C\textsubscript{3-8} cycloalkyl; (11) halo; (12) C\textsubscript{1-12} heterocyclol (e.g., C\textsubscript{2-12} heteroaryl); (13) (C\textsubscript{1-12} heterocyclo)oxy; (14) hydroxy; (15) nitro; (16) C\textsubscript{1-20} thioalkoxy (e.g., C\textsubscript{1-6} thioalkoxy); (17) -(CH\textsubscript{2})\textsubscript{q}CO\textsubscript{2}R\textsuperscript{A}, where q is an integer from zero to four, and R\textsuperscript{A} is selected from the group consisting of (a) C\textsubscript{1-6} alkyl, (b) C\textsubscript{6-10} aryl, (c) hydrogen, and (d) C\textsubscript{1-6} alk-C\textsubscript{6-10} aryl; (18) -(CH\textsubscript{2})\textsubscript{q}CONR\textsuperscript{B}R\textsuperscript{C}, where q is an integer from zero to four and where R\textsuperscript{B} and R\textsuperscript{C} are independently selected from the group consisting of (a) hydrogen, (b) C\textsubscript{1-6} alkyl, (c) C\textsubscript{6-10} aryl, and (d) C\textsubscript{1-6} alk-C\textsubscript{6-10} aryl; (19) -(CH\textsubscript{2})\textsubscript{q}SO\textsubscript{2}R\textsuperscript{D}, where q is an integer from zero to four and where R\textsuperscript{D} is selected from the group consisting of (a) C\textsubscript{1-6} alkyl, (b) C\textsubscript{6-10} aryl, and (c) C\textsubscript{1-6} alk-C\textsubscript{6-10} aryl; (20) -(CH\textsubscript{2})\textsubscript{q}SO\textsubscript{2}NR\textsuperscript{E}R\textsuperscript{F}, where q is an integer from zero to four and where each of R\textsuperscript{E} and R\textsuperscript{F} is, independently, selected from the group consisting of (a) hydrogen, (b) C\textsubscript{1-6} alkyl, (c) C\textsubscript{6-10} aryl, and (d) C\textsubscript{1-6} alk-C\textsubscript{6-10} aryl; (21) thiol; (22) C\textsubscript{6-10} aryloxy; (23) C\textsubscript{3-8} cycloalkoxy; (24) aryalkoxy; (25) C\textsubscript{1-6} alk-C\textsubscript{1-12} heterocyclol (e.g., C\textsubscript{1-6} alk-C\textsubscript{1-12} heteroaryl); (26) oxo; (27) (C\textsubscript{1-12} heterocyclo)jimo; (28) C\textsubscript{2-20} alkenyl; and (29) C\textsubscript{2-20} alkynyl. In some embodiments, each of these groups can be further substituted as described herein. For example, the alkylene group of a C\textsubscript{1}-alkaryl or a C\textsubscript{1}-alkheterocyclol can be further substituted with an oxo group to afford the respective aryloyl and (heterocyclo)jyoil substituent group.

[00791] The term “(heterocyclo)jimo,” as used herein, represents a heterocyclol group, as defined herein, attached to the parent molecular group through an imino group. In some embodiments, the heterocyclol group can be substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[00792] The term “(heterocyclo)oxy,” as used herein, represents a heterocyclol group, as defined herein, attached to the parent molecular group through an oxygen atom. In some embodiments, the heterocyclol group can be substituted with 1, 2, 3, or 4 substituent groups as defined herein.

[00793] The term “(heterocyclo)oyl,” as used herein, represents a heterocyclol group, as defined herein, attached to the parent molecular group through a carbonyl group. In some embodiments, the heterocyclol group can be substituted with 1, 2, 3, or 4 substituent groups as defined herein.
The term “hydrocarbon,” as used herein, represents a group consisting only of carbon and hydrogen atoms.

The term “hydroxy,” as used herein, represents an –OH group.

The term “hydroxyalkenyl,” as used herein, represents an alkenyl group, as defined herein, substituted by one to three hydroxy groups, with the proviso that no more than one hydroxy group may be attached to a single carbon atom of the alkyl group, and is exemplified by dihydroxypropenyl, hydroxyisopentenyl, and the like.

The term “hydroxyalkyl,” as used herein, represents an alkyl group, as defined herein, substituted by one to three hydroxy groups, with the proviso that no more than one hydroxy group may be attached to a single carbon atom of the alkyl group, and is exemplified by hydroxymethyl, dihydroxypropyl, and the like.

The term “isomer,” as used herein, means any tautomer, stereoisomer, enantiomer, or diastereomer of any compound of the invention. It is recognized that the compounds of the invention can have one or more chiral centers and/or double bonds and, therefore, exist as stereoisomers, such as double-bond isomers (i.e., geometric E/Z isomers) or diastereomers (e.g., enantiomers (i.e., (+) or (-)) or cis/trans isomers). According to the invention, the chemical structures depicted herein, and therefore the compounds of the invention, encompass all of the corresponding stereoisomers, that is, both the stereomerically pure form (e.g., geometrically pure, enantiomerically pure, or diastereomerically pure) and enantiomeric and stereoisomeric mixtures, e.g., racemates. Enantiomeric and stereoisomeric mixtures of compounds of the invention can typically be resolved into their component enantiomers or stereoisomers by well-known methods, such as chiral-phase gas chromatography, chiral-phase high performance liquid chromatography, crystallizing the compound as a chiral salt complex, or crystallizing the compound in a chiral solvent. Enantiomers and stereoisomers can also be obtained from stereomerically or enantiomerically pure intermediates, reagents, and catalysts by well-known asymmetric synthetic methods.

The term “N-protected amino,” as used herein, refers to an amino group, as defined herein, to which is attached one or two N-protecting groups, as defined herein.

The term “N-protecting group,” as used herein, represents those groups intended to protect an amino group against undesirable reactions during synthetic procedures. Commonly used N-protecting groups are disclosed in Greene, “Protective Groups in Organic Synthesis,” 3rd Edition
(John Wiley & Sons, New York, 1999), which is incorporated herein by reference. N-protecting groups include acyl, aryloyl, or carbamyl groups such as formyl, acetyl, propionyl, pivaloyl, t-butyrlactyl, 2-chloroacetyl, 2-bromoacetyl, trifluoroacetyl, trichloroacetyl, phthalyl, o-nitrophenoxycetyl, α-chlorobutryl, benzoyl, 4-chlorobenzoyl, 4-bromobenzoyl, 4-nitrobenzoyl, and chiral auxiliaries such as protected or unprotected D, L or D, L-amino acids such as alanine, leucine, phenylalanine, and the like; sulfonyl-containing groups such as benzenesulfonyl, p-toluenesulfonyl, and the like; carbamate forming groups such as benzoyloxycarbonyl, p-chlorobenzoyloxycarbonyl, p-methoxybenzoyloxycarbonyl, p-nitrobenzoyloxycarbonyl, 2-nitrobenzylcarbonyl, 3,4-dimethoxybenzoyloxycarbonyl, 3,5-dimethoxybenzyloxycarbonyl, 2,4-dimethoxybenzyloxycarbonyl, 4-methoxybenzyloxycarbonyl, 2-nitro-4,5-dimethoxybenzyloxycarbonyl, 3,4,5-trimethoxybenzyloxycarbonyl, 1-(p-biphenylyl)-1-methylethoxycarbonyl, α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl, benzhydryloxy carbonyl, t-butyloxycarbonyl, diisopropylmethoxycarbonyl, isopropylcarbonyl, ethoxycarbonyl, methoxycarbonyl, allyloxy carbonyl, 2,2,2-trichloroethoxycarbonyl, phenoxycarbonyl, 4-nitrophenoxycarbonyl, fluorenyl-9-methoxy carbonyl, cyclopentylcarbonyl, adamantylcarbonyl, cyclohexyloxycarbonyl, phenylthiocarbonyl, and the like, alkaryl groups such as benzyl, triphenylmethyl, benzoxymethyl, and the like and silyl groups, such as trimethylsilyl, and the like. Preferred N-protecting groups are formyl, acetyl, benzoyl, pivaloyl, t-butyrlactyl, alanyl, phenylsulfonetyl, benzyl, t-butyloxycarbonyl (Boc), and benzoxycarbonyl (Cbz).

[00801] The term “nitro,” as used herein, represents an −NO₂ group.

[00802] The term “oxo” as used herein, represents =O.

[00803] The term “perfluoroalkyl,” as used herein, represents an alkyl group, as defined herein, where each hydrogen radical bound to the alkyl group has been replaced by a fluoride radical. Perfluoroalkyl groups are exemplified by trifluoromethyl, pentafluoroethyl, and the like.

[00804] The term “perfluoroalkoxy,” as used herein, represents an alkoxy group, as defined herein, where each hydrogen radical bound to the alkoxy group has been replaced by a fluoride radical. Perfluoroalkoxy groups are exemplified by trifluoromethoxy, pentafluoroethoxy, and the like.

[00805] The term “spirocyclyl,” as used herein, represents a C₂₋₇ alkylene diradical, both ends of which are bonded to the same carbon atom of the parent group to form a spirocyclic group, and also a C₁₋₆ heteroalkylene diradical, both ends of which are bonded to the same atom. The heteroalkylene radical forming the spirocyclyl group can containing one, two, three, or four heteroatoms
independently selected from the group consisting of nitrogen, oxygen, and sulfur. In some embodiments, the spirocycyl group includes one to seven carbons, excluding the carbon atom to which the diradical is attached. The spirocycyl groups of the invention may be optionally substituted with 1, 2, 3, or 4 substituents provided herein as optional substituents for cycloalkyl and/or heterocycycl groups.

[00806] The term “stereoisomer,” as used herein, refers to all possible different isomeric as well as conformational forms which a compound may possess (e.g., a compound of any formula described herein), in particular all possible stereochemically and conformationally isomeric forms, all diastereomers, enantiomers and/or conformers of the basic molecular structure. Some compounds of the present invention may exist in different tautomeric forms, all of the latter being included within the scope of the present invention.

[00807] The term “sulfoalkyl,” as used herein, represents an alkyl group, as defined herein, substituted by a sulfo group of –SO₃H. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[00808] The term “sulfonyl,” as used herein, represents an –SO₂- group.

[00809] The term “thioalkaryl,” as used herein, represents a chemical substituent of formula –SR, where R is an alkaryl group. In some embodiments, the alkaryl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[00810] The term “thioalkheterocycyl,” as used herein, represents a chemical substituent of formula –SR, where R is an alkheterocycyl group. In some embodiments, the alkylheterocycyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[00811] The term “thioalkoxy,” as used herein, represents a chemical substituent of formula –SR, where R is an alkyl group, as defined herein. In some embodiments, the alkyl group can be further substituted with 1, 2, 3, or 4 substituent groups as described herein.

[00812] The term “thiol” represents an –SH group.

[00813] Compound: As used herein, the term “compound,” is meant to include all stereoisomers, geometric isomers, tautomers, and isotopes of the structures depicted.

[00814] The compounds described herein can be asymmetric (e.g., having one or more stereocenters). All stereoisomers, such as enantiomers and diastereomers, are intended unless otherwise indicated. Compounds of the present disclosure that contain asymmetrically substituted carbon atoms can be isolated in optically active or racemic forms. Methods on how to prepare
optically active forms from optically active starting materials are known in the art, such as by resolution of racemic mixtures or by stereoselective synthesis. Many geometric isomers of olefins, C=N double bonds, and the like can also be present in the compounds described herein, and all such stable isomers are contemplated in the present disclosure. Cis and trans geometric isomers of the compounds of the present disclosure are described and may be isolated as a mixture of isomers or as separated isomeric forms.

[00815] Compounds of the present disclosure also include tautomeric forms. Tautomeric forms result from the swapping of a single bond with an adjacent double bond and the concomitant migration of a proton. Tautomeric forms include prototropic tautomers which are isomeric protonation states having the same empirical formula and total charge. Examples prototropic tautomers include ketone – enol pairs, amide – imidic acid pairs, lactam – lactim pairs, amide – imidic acid pairs, enamine – imine pairs, and annular forms where a proton can occupy two or more positions of a heterocyclic system, such as, 1H- and 3H-imidazole, 1H-, 2H- and 4H- 1,2,4-triazole, 1H- and 2H- isoindole, and 1H- and 2H-pyrazole. Tautomeric forms can be in equilibrium or sterically locked into one form by appropriate substitution.

[00816] Compounds of the present disclosure also include all of the isotopes of the atoms occurring in the intermediate or final compounds. “Isotopes” refers to atoms having the same atomic number but different mass numbers resulting from a different number of neutrons in the nuclei. For example, isotopes of hydrogen include tritium and deuterium.

[00817] The compounds and salts of the present disclosure can be prepared in combination with solvent or water molecules to form solvates and hydrates by routine methods.

[00818] Conserved: As used herein, the term “conserved” refers to nucleotides or amino acid residues of a polynucleotide sequence or polypeptide sequence, respectively, that are those that occur unaltered in the same position of two or more sequences being compared. Nucleotides or amino acids that are relatively conserved are those that are conserved amongst more related sequences than nucleotides or amino acids appearing elsewhere in the sequences.

[00819] In some embodiments, two or more sequences are said to be “completely conserved” if they are 100% identical to one another. In some embodiments, two or more sequences are said to be “highly conserved” if they are at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some embodiments, two or more sequences are said to be “highly conserved” if they are about 70% identical, about 80% identical, about 90% identical, about
95%, about 98%, or about 99% identical to one another. In some embodiments, two or more sequences are said to be “conserved” if they are at least 30% identical, at least 40% identical, at least 50% identical, at least 60% identical, at least 70% identical, at least 80% identical, at least 90% identical, or at least 95% identical to one another. In some embodiments, two or more sequences are said to be “conserved” if they are about 30% identical, about 40% identical, about 50% identical, about 60% identical, about 70% identical, about 80% identical, about 90% identical, about 95% identical, about 98% identical, or about 99% identical to one another. Conservation of sequence may apply to the entire length of an oligonucleotide or polypeptide or may apply to a portion, region or feature thereof.

[00820] Controlled Release: As used herein, the term “controlled release” refers to a pharmaceutical composition or compound release profile that conforms to a particular pattern of release to effect a therapeutic outcome.

[00821] Cyclic or Cyclized: As used herein, the term “cyclic” refers to the presence of a continuous loop. Cyclic molecules need not be circular, only joined to form an unbroken chain of subunits. Cyclic molecules such as the engineered RNA or mRNA of the present invention may be single units or multimers or comprise one or more components of a complex or higher order structure.

[00822] Cytostatic: As used herein, “cytostatic” refers to inhibiting, reducing, suppressing the growth, division, or multiplication of a cell (e.g., a mammalian cell (e.g., a human cell)), bacterium, virus, fungus, protozoan, parasite, prion, or a combination thereof.

[00823] Cytotoxic: As used herein, “cytotoxic” refers to killing or causing injurious, toxic, or deadly effect on a cell (e.g., a mammalian cell (e.g., a human cell)), bacterium, virus, fungus, protozoan, parasite, prion, or a combination thereof.

[00824] Delivery: As used herein, “delivery” refers to the act or manner of delivering a compound, substance, entity, moiety, cargo or payload.

[00825] Delivery Agent: As used herein, “delivery agent” refers to any substance which facilitates, at least in part, the in vivo delivery of a modified nucleic acid or mRNA to targeted cells.

[00826] Destabilized: As used herein, the term “destable,” “destabilize,” or “destabilizing region” means a region or molecule that is less stable than a starting, wild-type or native form of the same region or molecule.

[00827] Detectable label: As used herein, “detectable label” refers to one or more markers, signals, or moieties which are attached, incorporated or associated with another entity that is readily detected
by methods known in the art including radiography, fluorescence, chemiluminescence, enzymatic activity, absorbance and the like. Detectable labels include radioisotopes, fluorophores, chromophores, enzymes, dyes, metal ions, ligands such as biotin, avidin, streptavidin and haptens, quantum dots, and the like. Detectable labels may be located at any position in the peptides or proteins disclosed herein. They may be within the amino acids, the peptides, or proteins, or located at the N- or C- termini.

[00828] *Digest:* As used herein, the term “digest” means to break apart into smaller pieces or components. When referring to polypeptides or proteins, digestion results in the production of peptides.

[00829] *Distal:* As used herein, the term “distal” means situated away from the center or away from a point or region of interest.

[00830] *Dose splitting factor (DSF)-ratio of PUD of dose split treatment divided by PUD of total daily dose or single unit dose. The value is derived from comparison of dosing regimens groups.*

[00831] *Encapsulate:* As used herein, the term “encapsulate” means to enclose, surround or encase.

[00832] *Engineered:* As used herein, embodiments of the invention are “engineered” when they are designed to have a feature or property, whether structural or chemical, that varies from a starting point, wild type or native molecule.

[00833] *Exosome:* As used herein, “exosome” is a vesicle secreted by mammalian cells.

[00834] *Expression:* As used herein, “expression” of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (*e.g.*, by transcription); (2) processing of an RNA transcript (*e.g.*, by splicing, editing, 5’ cap formation, and/or 3’ end processing); (3) translation of an RNA into a polypeptide or protein; and (4) post-translational modification of a polypeptide or protein.

[00835] *Feature:* As used herein, a “feature” refers to a characteristic, a property, or a distinctive element.

[00836] *Formulation:* As used herein, a “formulation” includes at least a modified nucleic acid or mmRNA and a delivery agent.

[00837] *Fragment:* A “fragment,” as used herein, refers to a portion. For example, fragments of proteins may comprise polypeptides obtained by digesting full-length protein isolated from cultured cells.
[00838] **Functional:** As used herein, a “functional” biological molecule is a biological molecule in a form in which it exhibits a property and/or activity by which it is characterized.

[00839] **Homology:** 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. 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). In accordance with the invention, two polynucleotide sequences are considered to be homologous if the polypeptides they encode are at least about 50%, 60%, 70%, 80%, 90%, 95%, or even 99% for at least one stretch of at least about 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. In accordance with the invention, two protein sequences are considered to be homologous if the proteins are at least about 50%, 60%, 70%, 80%, or 90% identical for at least one stretch of at least about 20 amino acids.

[00840] **Identity:** As used herein, the term “identity” refers to the overall relatedness between polymeric molecules, e.g., between oligonucleotide molecules (e.g. DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of the percent identity of two polynucleotide 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 nucleotide 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 nucleotide 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 nucleotide 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)).

[00841] Inhibit expression of a gene: As used herein, the phrase “inhibit expression of a gene” means to cause a reduction in the amount of an expression product of the gene. The expression product can be an RNA transcribed from the gene (e.g., an mRNA) or a polypeptide translated from an mRNA transcribed from the gene. Typically a reduction in the level of an mRNA results in a reduction in the level of a polypeptide translated therefrom. The level of expression may be determined using standard techniques for measuring mRNA or protein.

[00842] In vitro: As used herein, the term “in vitro” refers to events that occur in an artificial environment, e.g., in a test tube or reaction vessel, in cell culture, in a Petri dish, etc., rather than within an organism (e.g., animal, plant, or microbe).

[00843] In vivo: As used herein, the term “in vivo” refers to events that occur within an organism (e.g., animal, plant, or microbe or cell or tissue thereof).
[00844] **Isolated:** As used herein, the term “isolated” refers to a substance or entity that has been separated from at least some of the components with which it was associated (whether in nature or in an experimental setting). Isolated substances may have varying levels of purity in reference to the substances from which they have been associated. Isolated substances and/or entities may be separated from at least about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or more of the other components with which they were initially associated. In some embodiments, isolated agents are more than about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is “pure” if it is substantially free of other components. **Substantially isolated:** By “substantially isolated” is meant that the compound is substantially separated from the environment in which it was formed or detected. Partial separation can include, for example, a composition enriched in the compound of the present disclosure. Substantial separation can include compositions containing at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, at least about 97%, or at least about 99% by weight of the compound of the present disclosure, or salt thereof. Methods for isolating compounds and their salts are routine in the art.

[00845] **Linker:** As used herein, a linker refers to a group of atoms, e.g., 10-1,000 atoms, and can be comprised of the atoms or groups such as, but not limited to, carbon, amino, alkylamino, oxygen, sulfur, sulfoxide, sulfonyl, carbonyl, and imine. The linker can be attached to a modified nucleoside or nucleotide on the nucleobase or sugar moiety at a first end, and to a payload, e.g., a detectable or therapeutic agent, at a second end. The linker may be of sufficient length as to not interfere with incorporation into a nucleic acid sequence. The linker can be used for any useful purpose, such as to form mRNA multimers (e.g., through linkage of two or more modified nucleic acid molecules or mRNA molecules) or mRNA conjugates, as well as to administer a payload, as described herein. Examples of chemical groups that can be incorporated into the linker include, but are not limited to, alkyl, alkenyl, alkynyl, amido, amino, ether, thioether, ester, alkylene, heteroalkylene, aryl, or heterocyclyl, each of which can be optionally substituted, as described herein. Examples of linkers include, but are not limited to, unsaturated alkanes, polyethylene glycols (e.g., ethylene or propylene glycol monomeric units, e.g., diethylene glycol, dipropylene glycol, triethylene glycol, tripropylene glycol, tetracyethylene glycol, or tetracyethylene glycol), and dextran polymers and derivatives thereof. Other examples include, but are not limited to, cleavable moieties within the linker, such as, for
example, a disulfide bond (-S-S-) or an azo bond (-N=N-), which can be cleaved using a reducing agent or photolysis. Non-limiting examples of a selectively cleavable bond include an amido bond can be cleaved for example by the use of tris(2-carboxyethyl)phosphine (TCEP), or other reducing agents, and/or photolysis, as well as an ester bond can be cleaved for example by acidic or basic hydrolysis.

[00846] MicroRNA (miRNA) binding site: As used herein, a microRNA (miRNA) binding site represents a nucleotide location or region of a nucleic acid transcript to which at least the “seed” region of a miRNA binds.

[00847] Modified: As used herein “modified” refers to a changed state or structure of a molecule of the invention. Molecules may be modified in many ways including chemically, structurally, and functionally. In one embodiment, the mRNA molecules of the present invention are modified by the introduction of non-natural nucleosides and/or nucleotides.

[00848] Mucus: As used herein, “mucus” refers to a natural substance that is viscous and comprises mucin glycoproteins.

[00849] Naturally occurring: As used herein, “naturally occurring” means existing in nature without artificial aid.

[00850] Non-human vertebrate: As used herein, a “non human vertebrate” includes all vertebrates except Homo sapiens, including wild and domesticated species. Examples of non-human vertebrates include, but are not limited to, mammals, such as alpaca, banteng, bison, camel, cat, cattle, deer, dog, donkey, gayal, goat, guinea pig, horse, llama, mule, pig, rabbit, reindeer, sheep water buffalo, and yak.

[00851] Off-target: As used herein, “off target” refers to any unintended effect on any one or more target, gene, or cellular transcript.

[00852] Open reading frame: As used herein, “open reading frame” or “ORF” refers to a sequence which does not contain a stop codon in a given reading frame.

[00853] Operably linked: As used herein, the phrase “operably linked” refers to a functional connection between two or more molecules, constructs, transcripts, entities, moieties or the like.

[00854] Paratope: As used herein, a “paratope” refers to the antigen-binding site of an antibody.

[00855] Patient: As used herein, “patient” refers to a subject who may seek or be in need of treatment, requires treatment, is receiving treatment, will receive treatment, or a subject who is under care by a trained professional for a particular disease or condition.
[00856] **Peptide:** As used herein, “peptide” is less than or equal to 50 amino acids long, e.g., about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

[00857] **Pharmaceutically acceptable:** The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00858] **Pharmaceutically acceptable excipients:** The phrase “pharmaceutically acceptable excipient,” as used herein, refers any ingredient other than the compounds described herein (for example, a vehicle capable of suspending or dissolving the active compound) and having the properties of being substantially nontoxic and non-inflammatory in a patient. Excipients may include, for example: antiadherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes (colors), emollients, emulsifiers, fillers (diluents), film formers or coatings, flavors, fragrances, glidants (flow enhancers), lubricants, preservatives, printing inks, sorbents, suspending or dispersing agents, sweeteners, and waters of hydration. Exemplary excipients include, but are not limited to: butylated hydroxytoluene (BHT), calcium carbonate, calcium phosphate (dibasic), calcium stearate, croscarmellose, crosslinked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactose, magnesium stearate, maltitol, mannitol, methionine, methylcellulose, methyl paraben, microcrystalline cellulose, polyethylene glycol, polyvinyl pyrrolidone, povidone, pregelatinized starch, propyl paraben, retinyl palmitate, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium citrate, sodium starch glycolate, sorbitol, starch (corn), stearic acid, sucrose, talc, titanium dioxide, vitamin A, vitamin E, vitamin C, and xylitol.

[00859] **Pharmaceutically acceptable salts:** The present disclosure also includes pharmaceutically acceptable salts of the compounds described herein. As used herein, “pharmaceutically acceptable salts” refers to derivatives of the disclosed compounds wherein the parent compound is modified by converting an existing acid or base moiety to its salt form (e.g., by reacting the free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate,
camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, maleate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. The pharmaceutically acceptable salts of the present disclosure include the conventional non-toxic salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. The pharmaceutically acceptable salts of the present disclosure can be synthesized from the parent compound which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, Pharmaceutical Salts: Properties, Selection, and Use, P.H. Stahl and C.G. Wermuth (eds.), Wiley-VCH, 2008, and Berge et al., Journal of Pharmaceutical Science, 66, 1-19 (1977), each of which is incorporated herein by reference in its entirety.

[00860] Pharmaceutically acceptable solvate: The term “pharmaceutically acceptable solvate,” as used herein, means a compound of the invention wherein molecules of a suitable solvant are incorporated in the crystal lattice. A suitable solvent is physiologically tolerable at the dosage administered. For example, solvates may be prepared by crystallization, recrystallization, or precipitation from a solution that includes organic solvents, water, or a mixture thereof. Examples of suitable solvents are ethanol, water (for example, mono-, di-, and tri-hydrates), N-methylpyrrolidinone (NMP), dimethyl sulfoxide (DMSO), N,N'-dimethylformamide (DMF), N,N'-dimethylacetamide (DMAC), 1,3-dimethyl-2-imidazolidinone (DMEU), 1,3-dimethyl-3,4,5,6-tetrahydro-2-(1H)-pyrimidinone (DMPU), acetonitrile (ACN), propylene glycol, ethyl acetate,
benzyl alcohol, 2-pyrrolidone, benzyl benzoate, and the like. When water is the solvent, the solvate is referred to as a “hydrate.”

[00861] **Pharmacokinetic:** As used herein, “pharmacokinetic” refers to any one or more properties of a molecule or compound as it relates to the determination of the fate of substances administered to a living organism. Pharmacokinetics is divided into several areas including the extent and rate of absorption, distribution, metabolism and excretion. This is commonly referred to as ADME where: (A) Absorption is the process of a substance entering the blood circulation; (D) Distribution is the dispersion or dissemination of substances throughout the fluids and tissues of the body; (M) Metabolism (or Biotransformation) is the irreversible transformation of parent compounds into daughter metabolites; and (E) Excretion (or Elimination) refers to the elimination of the substances from the body. In rare cases, some drugs irreversibly accumulate in body tissue.

[00862] **Pharmacologic effect:** As used herein, a “pharmacologic effect” is a measurable biologic phenomenon in an organism or system which occurs after the organism or system has been contacted with or exposed to an exogenous agent. Pharmacologic effects may result in therapeutically effective outcomes such as the treatment, improvement of one or more symptoms, diagnosis, prevention, and delay of onset of disease, disorder, condition or infection. Measurement of such biologic phenomena may be quantitative, qualitative or relative to another biologic phenomenon. Quantitative measurements may be statistically significant. Qualitative measurements may be by degree or kind and may be at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more different. They may be observable as present or absent, better or worse, greater or less. Exogenous agents, when referring to pharmacologic effects are those agents which are, in whole or in part, foreign to the organism or system. For example, modifications to a wild type biomolecule, whether structural or chemical, would produce an exogenous agent. Likewise, incorporation or combination of a wild type molecule into or with a compound, molecule or substance not found naturally in the organism or system would also produce an exogenous agent. The modified mRNA of the present invention, comprise exogenous agents. Examples of pharmacologic effects include, but are not limited to, alteration in cell count such as an increase or decrease in neutrophils, reticulocytes, granulocytes, erythrocytes (red blood cells), megakaryocytes, platelets, monocytes, connective tissue macrophages, epidermal langerhans cells, osteoclasts, dendritic cells, microglial cells, neutrophils, eosinophils, basophils, mast cells, helper T cells, suppressor T cells, cytotoxic T cells, natural killer T cells, B cells, natural killer cells, or reticulocytes. Pharmacologic effects also include
alterations in blood chemistry, pH, hemoglobin, hematocrit, changes in levels of enzymes such as, but not limited to, liver enzymes AST and ALT, changes in lipid profiles, electrolytes, metabolic markers, hormones or other marker or profile known to those of skill in the art.

[00863] Physicochemical: As used herein, “physicochemical” means of or relating to a physical and/or chemical property.

[00864] Preventing: As used herein, the term “preventing” refers to partially or completely delaying onset of an infection, disease, disorder and/or condition; partially or completely delaying onset of one or more symptoms, features, or clinical manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying onset of one or more symptoms, features, or manifestations of a particular infection, disease, disorder, and/or condition; partially or completely delaying progression from an infection, a particular disease, disorder and/or condition; and/or decreasing the risk of developing pathology associated with the infection, the disease, disorder, and/or condition.

[00865] Prodrug: The present disclosure also includes prodrugs of the compounds described herein. As used herein, “prodrugs” refer to any substance, molecule or entity which is in a form predicate for that substance, molecule or entity to act as a therapeutic upon chemical or physical alteration. Prodrugs may by covalently bonded or sequestered in some way and which release or are converted into the active drug moiety prior to, upon or after administered to a mammalian subject. Prodrugs can be prepared by modifying functional groups present in the compounds in such a way that the modifications are cleaved, either in routine manipulation or in vivo, to the parent compounds. Prodrugs include compounds wherein hydroxyl, amino, sulphydryl, or carboxyl groups are bonded to any group that, when administered to a mammalian subject, cleaves to form a free hydroxyl, amino, sulphydryl, or carboxyl group respectively. Preparation and use of prodrugs is discussed in T. Higuchi and V. Stella, “Pro-drugs as Novel Delivery Systems,” Vol. 14 of the A.C.S. Symposium Series, and in Bioreversible Carriers in Drug Design, ed. Edward B. Roche, American Pharmaceutical Association and Pergamon Press, 1987, both of which are hereby incorporated by reference in their entirety.

[00866] Proliferate: As used herein, the term “proliferate” means to grow, expand or increase or cause to grow, expand or increase rapidly. “Proliferative” means having the ability to proliferate. “Anti-proliferative” means having properties counter to or inapposite to proliferative properties.
[00867] **Protein of interest:** As used herein, the terms “proteins of interest” or “desired proteins” include those provided herein and fragments, mutants, variants, and alterations thereof.

[00868] **Proximal:** As used herein, the term “proximal” means situated nearer to the center or to a point or region of interest.

[00869] **Pseudouridine:** As used herein, pseudouridine refers to the C-glycoside isomer of the nucleoside uridine. A “pseudouridine analog” is any modification, variant, isoform or derivative of pseudouridine. For example, pseudouridine analogs include but are not limited to 1-carboxymethyl-pseudouridine, 1-propynyl-pseudouridine, 1-taurinomethyl-pseudouridine, 1-taurinomethyl-4-thio-pseudouridine, 1-methyl-pseudouridine (m1Ψ), 1-methyl-4-thio-pseudouridine (m1s4Ψ), 4-thio-1-methyl-pseudouridine, 3-methyl-pseudouridine (m3Ψ), 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydropseudouridine, 2-thio-dihydropseudouridine, 2-methoxyuridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine, 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine (acp3Ψ), and 2'-O-methyl-pseudouridine (Ψm).

[00870] **Purified:** As used herein, “purify,” “purified,” “purification” means to make substantially pure or clear from unwanted components, material defilement, admixture or imperfection.

[00871] **Sample:** As used herein, the term “sample” or “biological sample” refers to a subset of its tissues, cells or component parts (e.g. body fluids, including but not limited to blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, amniotic cord blood, urine, vaginal fluid and semen). A sample further may include a homogenate, lysate or extract prepared from a whole organism or a subset of its tissues, cells or component parts, or a fraction or portion thereof, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, tumors, organs. A sample further refers to a medium, such as a nutrient broth or gel, which may contain cellular components, such as proteins or nucleic acid molecule.

[00872] **Signal Sequences:** As used herein, the phrase “signal sequences” refers to a sequence which can direct the transport or localization of a protein.

[00873] **Single unit dose:** As used herein, a “single unit dose” is a dose of any therapeutic administered in one dose/at one time/single route/single point of contact, i.e., single administration event.
[00874] Similarity: As used herein, the term “similarity” refers to the overall relatedness between polymeric molecules, e.g. between polynucleotide molecules (e.g. DNA molecules and/or RNA molecules) and/or between polypeptide molecules. Calculation of percent similarity of polymeric molecules to one another can be performed in the same manner as a calculation of percent identity, except that calculation of percent similarity takes into account conservative substitutions as is understood in the art.

[00875] Split dose: As used herein, a “split dose” is the division of single unit dose or total daily dose into two or more doses.

[00876] Stable: As used herein “stable” refers to a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and preferably capable of formulation into an efficacious therapeutic agent.

[00877] Stabilized: As used herein, the term “stabilize”, “stabilized,” “stabilized region” means to make or become stable.

[00878] Subject: As used herein, the term “subject” or “patient” refers to any organism to which a composition in accordance with the invention may be administered, e.g., for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (e.g., mammals such as mice, rats, rabbits, non-human primates, and humans) and/or plants.

[00879] Substantially: As used herein, the term “substantially” refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term “substantially” is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[00880] Substantially equal: As used herein as it relates to time differences between doses, the term means plus/minus 2%.

[00881] Substantially simultaneously: As used herein and as it relates to plurality of doses, the term means within 2 seconds.

[00882] Suffering from: An individual who is “suffering from” a disease, disorder, and/or condition has been diagnosed with or displays one or more symptoms of a disease, disorder, and/or condition.

[00883] Susceptible to: An individual who is “susceptible to” a disease, disorder, and/or condition has not been diagnosed with and/or may not exhibit symptoms of the disease, disorder, and/or
condition but harbors a propensity to develop a disease or its symptoms. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition (for example, cancer) may be characterized by one or more of the following: (1) a genetic mutation associated with development of the disease, disorder, and/or condition; (2) a genetic polymorphism associated with development of the disease, disorder, and/or condition; (3) increased and/or decreased expression and/or activity of a protein and/or nucleic acid associated with the disease, disorder, and/or condition; (4) habits and/or lifestyles associated with development of the disease, disorder, and/or condition; (5) a family history of the disease, disorder, and/or condition; and (6) exposure to and/or infection with a microbe associated with development of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will develop the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will not develop the disease, disorder, and/or condition.

[00884] Sustained release: As used herein, the term “sustained release” refers to a pharmaceutical composition or compound release profile that conforms to a release rate over a specific period of time.

[00885] Synthetic: The term “synthetic” means produced, prepared, and/or manufactured by the hand of man. Synthesis of polynucleotides or polypeptides or other molecules of the present invention may be chemical or enzymatic.

[00886] Targeted Cells: As used herein, “targeted cells” refers to any one or more cells of interest. The cells may be found in vitro, in vivo, in situ or in the tissue or organ of an organism. The organism may be an animal, preferably a mammal, more preferably a human and most preferably a patient.

[00887] Therapeutic Agent: The term “therapeutic agent” refers to any agent that, when administered to a subject, has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

[00888] Therapeutically effective amount: As used herein, the term “therapeutically effective amount” means an amount of an agent to be delivered (e.g., nucleic acid, drug, therapeutic agent, diagnostic agent, prophylactic agent, etc.) that is sufficient, when administered to a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.
Therapeutically effective outcome: As used herein, the term “therapeutically effective outcome” means an outcome that is sufficient in a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition.

Total daily dose: As used herein, a “total daily dose” is an amount given or prescribed in 24 hr period. It may be administered as a single unit dose.

Transcription factor: As used herein, the term “transcription factor” refers to a DNA-binding protein that regulates transcription of DNA into RNA, for example, by activation or repression of transcription. Some transcription factors effect regulation of transcription alone, while others act in concert with other proteins. Some transcription factor can both activate and repress transcription under certain conditions. In general, transcription factors bind a specific target sequence or sequences highly similar to a specific consensus sequence in a regulatory region of a target gene. Transcription factors may regulate transcription of a target gene alone or in a complex with other molecules.

Treating: As used herein, the term “treating” refers to partially or completely alleviating, ameliorating, improving, relieving, delaying onset of, inhibiting progression of, reducing severity of, and/or reducing incidence of one or more symptoms or features of a particular infection, disease, disorder, and/or condition. For example, “treating” cancer may refer to inhibiting survival, growth, and/or spread of a tumor. Treatment may be administered to a subject who does not exhibit signs of a disease, disorder, and/or condition and/or to a subject who exhibits only early signs of a disease, disorder, and/or condition for the purpose of decreasing the risk of developing pathology associated with the disease, disorder, and/or condition.

Unmodified: As used herein, “unmodified” refers to any substance, compound or molecule prior to being changed in any way. Unmodified may, but does not always, refer to the wild type or native form of a biomolecule. Molecules may undergo a series of modifications whereby each modified molecule may serve as the “unmodified” starting molecule for a subsequent modification.

Equivalents and Scope

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the appended claims.
In the claims, articles such as “a,” “an,” and “the” may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

It is also noted that the term “comprising” is intended to be open and permits the inclusion of additional elements or steps.

Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

In addition, it is to be understood that any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the invention (e.g., any nucleic acid or protein encoded thereby; any method of production; any method of use; etc.) can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

All cited sources, for example, references, publications, databases, database entries, and art cited herein, are incorporated into this application by reference, even if not expressly stated in the citation. In case of conflicting statements of a cited source and the instant application, the statement in the instant application shall control.

Section and table headings are not intended to be limiting.

**EXAMPLES**

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.
Example 1. Modified mRNA Production

[00902] Modified mRNAs (mmRNA) according to the invention may be made using standard laboratory methods and materials. The open reading frame (ORF) of the gene of interest may be flanked by a 5′ untranslated region (UTR) which may contain a strong Kozak translational initiation signal and/or an alpha-globin 3′ UTR which may include an oligo(dT) sequence for templated addition of a poly-A tail. The modified mRNAs may be modified to reduce the cellular innate immune response. The modifications to reduce the cellular response may include pseudouridine (ψ) and 5-methyl-cytidine (5meC or m⁵C). (see, Kariko K et al. Immunity 23:165-75 (2005), Kariko K et al. Mol Ther 16:1833-40 (2008), Anderson BR et al. NAR (2010); each of which are herein incorporated by reference in their entirety).

[00903] The ORF may also include various upstream or downstream additions (such as, but not limited to, β-globin, tags, etc.) may be ordered from an optimization service such as, but limited to, DNA2.0 (Menlo Park, CA) and may contain multiple cloning sites which may have XbaI recognition. Upon receipt of the plasmid DNA, it may be reconstituted and transformed into chemically competent E. coli.

[00904] For the present invention, NEB DH5-alpha Competent E. coli are used. Transformations are performed according to NEB instructions using 100 ng of plasmid. The protocol is as follows:

1. Thaw a tube of NEB 5-alpha Competent E. coli cells on ice for 10 minutes.
2. Add 1-5 μl containing 1 pg-100 ng of plasmid DNA to the cell mixture. Carefully flick the tube 4-5 times to mix cells and DNA. Do not vortex.
3. Place the mixture on ice for 30 minutes. Do not mix.
5. Place on ice for 5 minutes. Do not mix.
6. Pipette 950 μl of room temperature SOC into the mixture.
7. Place at 37°C for 60 minutes. Shake vigorously (250 rpm) or rotate.
8. Warm selection plates to 37°C.
9. Mix the cells thoroughly by flicking the tube and inverting.

[00905] Spread 50-100 μl of each dilution onto a selection plate and incubate overnight at 37°C. Alternatively, incubate at 30°C for 24-36 hours or 25°C for 48 hours.

[00906] A single colony is then used to inoculate 5 ml of LB growth media using the appropriate antibiotic and then allowed to grow (250 RPM, 37°C) for 5 hours. This is then used to inoculate a 200 ml culture medium and allowed to grow overnight under the same conditions.
[00907] To isolate the plasmid (up to 850 µg), a maxi prep is performed using the Invitrogen PURELINK™ HiPure Maxiprep Kit (Carlsbad, CA), following the manufacturer’s instructions.

[00908] In order to generate cDNA for In Vitro Transcription (IVT), the plasmid (an Example of which is shown in Figure 2) is first linearized using a restriction enzyme such as XbaI. A typical restriction digest with XbaI will comprise the following: Plasmid 1.0 µg; 10x Buffer 1.0 µl; XbaI 1.5 µl; dH₂O up to 10 µl; incubated at 37° C for 1 hr. If performing at lab scale (< 5µg), the reaction is cleaned up using Invitrogen’s PURELINK™ PCR Micro Kit (Carlsbad, CA) per manufacturer’s instructions. Larger scale purifications may need to be done with a product that has a larger load capacity such as Invitrogen’s standard PURELINK™ PCR Kit (Carlsbad, CA). Following the cleanup, the linearized vector is quantified using the NanoDrop and analyzed to confirm linearization using agarose gel electrophoresis.

[00909] The methods described herein to make modified mRNA may be used to produce molecules of all sizes including long molecules. Modified mRNA using the described methods has been made for different sized molecules including glucosidase, alpha; acid (GAA) (3.2 kb), cystic fibrosis transmembrane conductance regulator (CFTR) (4.7 kb), Factor VII (7.3 kb), lysosomal acid lipase (45.4kDa), glucocerebrosidase (59.7 kDa) and iduronate 2-sulfatase (76 kDa).

[00910] As a non-limiting example, G-CSF may represent the polypeptide of interest. Sequences used in the steps outlined in Examples 1-5 are shown in Table 4. It should be noted that the start codon (ATG) has been underlined in each sequence of Table 4.

<table>
<thead>
<tr>
<th>SEQ ID NO</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>cDNA sequence: ATGGCTGGACCTGCCACCCAGAGCCCATGAAGCTGATGCGCCTGCA</td>
</tr>
</tbody>
</table>
4 cDNA having T7 polymerase site, AfeI and Xba restriction site:
TAATACGACTCATA
GGGAAATAAGAGAGAAAAAGAAGTAGAAGAAGAAATATAAGAGCCACC
ATGCTGGACCTGCACCCCCATGAAGCTGTGAGCCTGGAGG
CTGCTGCTGTGCGCAGTGCACCTGAGCAAGCTGGACATGGCCCGCAG
CTGGTGGCACCCTAAGTGGCACCACCCAGGAGTAGGGGCTGCCACTGCTCT
GAGACACTCTCTGGGCATCCCTCTGGCTCTCTCTGAGCACTGGCCACGCC
AGCCCCATGACAGTGGCTGCTGTGACTTCAAGCCGCTTTTT
CTCTACCAAGGGCTCTGACGGGCTCCTGAGAAGGATCTCCCCCCAGTT
GGTGCCCACTTGGGACGAGCTGAGCAGTCTGGACGGCTGGCATCTTTGAC
CACCACCTGTGCAAGATGGAAAGACTGGAAATGGCCACCTGGAGG
GCCACACAGGGTGCAATGCGCCCTCTGACCTGTCTTTCACGCCAGCAG
GACAGGGGATGGTCTGGCTCCATGCTAGCTTCGTCTCTCGAGGCTGA
TCGTACCCTGGCTACTACGCCACCTTGGCCAGCCCTAG
AGCCTGGTCCTCTGGGCTGCGCTTTCCTGGCCATGGGCTCTTCCTCCTCC
CTTGACACCTGTACCTCTGGCTCTTCGGAATAAAGCCTGAGTAAGGAAGCG
GCCGGCTGAGCAGCATGCTAG

5 Optimized sequence; containing T7 polymerase site, AfeI and Xba restriction site
TAATACGACTCATA
GGGAAATAAGAGAGAAAAAGAAGTAGAAGAAGAAATATAAGAGCCACC
ATGCTGGACCTGCACCCCCATGAAGCTGTGAGCCTGGAGG
CTGCTGCTGTGCGCAGTGCACCTGAGCAAGCTGGACATGGCCCGCAG
CTGGTGGCACCCTAAGTGGCACCACCCAGGAGTAGGGGCTGCCACTGCTCT
GAGACACTCTCTGGGCATCCCTCTGGCTCTCTCTGAGCACTGGCCACGCC
AGCCCCATGACAGTGGCTGCTGTGACTTCAAGCCGCTTTTT
CTCTACCAAGGGCTCTGACGGGCTCCTGAGAAGGATCTCCCCCCAGTT
GGTGCCCACTTGGGACGAGCTGAGCAGTCTGGACGGCTGGCATCTTTGAC
CACCACCTGTGCAAGATGGAAAGACTGGAAATGGCCACCTGGAGG
GCCACACAGGGTGCAATGCGCCCTCTGACCTGTCTTTCACGCCAGCAG
GACAGGGGATGGTCTGGCTCCATGCTAGCTTCGTCTCTCGAGGCTGA
TCGTACCCTGGCTACTACGCCACCTTGGCCAGCCCTAG
AGCCTGGTCCTCTGGGCTGCGCTTTCCTGGCCATGGGCTCTTCCTCCTCC
CTTGACACCTGTACCTCTGGCTCTTCGGAATAAAGCCTGAGTAAGGAAGCG
GCCGGCTGAGCAGCATGCTAG

6 mRNA sequence (transcribed)
GGGAAATAAGAGAGAAAAAGAAGTAGAAGAAGAAATATAAGAGCCACC
AUGGCGGCGGCGGACCCCAAGAGCCCAUGGAAACUUUAGGGCCCGA

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| GUUGCUGCUUUUGGACUCUGCCUCUCUUGGACAGUCCAAGAGCGACUCUCUCCGAGGCGCCUCUACUGUUGCCGCAGUCUAUCUCCUUUUGAGUGUCUGGAGCCAGAGGCAAGAGGCAAGGCGACAGUCCUUCUCUCUCGCUGUG UCCGUCCAGGCUUUUCAGUGGCAGGGCAGGCUUUCCAGCCACUCCACCUCCGGUUCUUCUUGGUAACAGGGACUGCUGCAAGCCCUUGAGGGAUCCGCCACAAUUGGGCGCCAGCGACGUUGG CGGAUUCGCAACAACCAUCUGGAGCAGAUGGAGAAGCUGGGGAUGGCCACCGCGCGGCUUCAGCGGCGCUUUCGGUG GCGGCGGCGGCUUCAGCGGCGGCGGCAUUGCCGGCGCCUUUUCCCU CCUGCUUUCAGCGGCGGCGGCGGCAUUGCCGGCGCCUUUUCCCU UCCUUGCAACCUGUACCUCUUGGUUUUGAUAAGCGCGGAGUAGGA AG |

**Example 2: PCR for cDNA Production**

[00911] PCR procedures for the preparation of cDNA are performed using 2x KAPA HIFI™ HotStart ReadyMix by Kapa Biosystems (Woburn, MA). This system includes 2x KAPA ReadyMix12.5 µl; Forward Primer (10 uM) 0.75 µl; Reverse Primer (10 uM) 0.75 µl; Template cDNA 100 ng; and dH2O diluted to 25.0 µl. The reaction conditions are at 95°C for 5 min. and 25 cycles of 98°C for 20 sec, then 58°C for 15 sec, then 72°C for 45 sec, then 72°C for 5 min. then 4°C C to termination.

[00912] The reverse primer of the instant invention incorporates a poly-T₁₂₀ for a poly-A₁₂₀ in the mRNA. Other reverse primers with longer or shorter poly(T) tracts can be used to adjust the length of the poly(A) tail in the mRNA.

[00913] The reaction is cleaned up using Invitrogen’s PURELINK™ PCR Micro Kit (Carlsbad, CA) per manufacturer’s instructions (up to 5 µg). Larger reactions will require a cleanup using a product with a larger capacity. Following the cleanup, the cDNA is quantified using the NANODROP™ and analyzed by agarose gel electrophoresis to confirm the cDNA is the expected size. The cDNA is then submitted for sequencing analysis before proceeding to the in vitro transcription reaction.

**Example 3. In vitro Transcription**
[00914] The *in vitro* transcription reaction generates mRNA containing modified nucleotides or modified RNA. The input nucleotide triphosphate (NTP) mix is made in-house using natural and unnatural NTPs.

[00915] A typical *in vitro* transcription reaction includes the following:

1. Template cDNA 1.0 µg
2. 10x transcription buffer (400 mM Tris-HCl pH 8.0, 190 mM MgCl₂, 50 mM DTT, 10 mM Spermidine) 2.0 µl
3. Custom NTPs (25mM each) 7.2 µl
4. RNase Inhibitor 20 U
5. T7 RNA polymerase 3000 U
6. dH₂O Up to 20.0 µl and
7. Incubation at 37°C for 3 hr-5 hrs.

[00916] The crude IVT mix may be stored at 4°C overnight for cleanup the next day. 1 U of RNase-free DNase is then used to digest the original template. After 15 minutes of incubation at 37°C, the mRNA is purified using Ambion’s MEGACLEAR™ Kit (Austin, TX) following the manufacturer’s instructions. This kit can purify up to 500 µg of RNA. Following the cleanup, the RNA is quantified using the NanoDrop and analyzed by agarose gel electrophoresis to confirm the RNA is the proper size and that no degradation of the RNA has occurred.

**Example 4. Enzymatic Capping of mRNA**

[00917] Capping of the mRNA is performed as follows where the mixture includes: IVT RNA 60 µg-180µg and dH₂O up to 72 µl. The mixture is incubated at 65°C for 5 minutes to denature RNA, and then is transferred immediately to ice.

[00918] The protocol then involves the mixing of 10x Capping Buffer (0.5 M Tris-HCl (pH 8.0), 60 mM KCl, 12.5 mM MgCl₂) (10.0 µl); 20 mM GTP (5.0 µl); 20 mM S-Adenosyl Methionine (2.5 µl); RNase Inhibitor (100 U); 2'-O-Methyltransferase (400U); Vaccinia capping enzyme (Guanylyl transferase) (40 U); dH₂O (Up to 28 µl); and incubation at 37°C for 30 minutes for 60 µg RNA or up to 2 hours for 180 µg of RNA.

[00919] The mRNA is then purified using Ambion’s MEGACLEAR™ Kit (Austin, TX) following the manufacturer’s instructions. Following the cleanup, the RNA is quantified using the NANODROP™ (ThermoFisher, Waltham, MA) and analyzed by agarose gel electrophoresis to confirm the RNA is the proper size and that no degradation of the RNA has occurred. The RNA
product may also be sequenced by running a reverse-transcription-PCR to generate the cDNA for sequencing.

**Example 5. PolyA Tailing Reaction**

[00920] Without a poly-T in the cDNA, a poly-A tailing reaction must be performed before cleaning the final product. This is done by mixing Capped IVT RNA (100 µl); RNase Inhibitor (20 U); 10x Tailing Buffer (0.5 M Tris-HCl (pH 8.0), 2.5 M NaCl, 100 mM MgCl₂(12.0 µl); 20 mM ATP (6.0 µl); Poly-A Polymerase (20 U); dH₂O up to 123.5 µl and incubation at 37°C for 30 min. If the poly-A tail is already in the transcript, then the tailing reaction may be skipped and proceed directly to cleanup with Ambion’s MEGACLEAR™ kit (Austin, TX) (up to 500 µg). Poly-A Polymerase is preferably a recombinant enzyme expressed in yeast.

[00921] For studies performed and described herein, the poly-A tail is encoded in the IVT template to comprise 160 nucleotides in length. However, it should be understood that the processivity or integrity of the polyA tailing reaction may not always result in exactly 160 nucleotides. Hence polyA tails of approximately 160 nucleotides, e.g., about 150-165, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164 or 165 are within the scope of the invention.

**Example 6. Natural 5’ Caps and 5’ Cap Analogues**

[00922] 5’-capping of modified RNA may be completed concomitantly during the *in vitro*-transcription reaction using the following chemical RNA cap analogs to generate the 5’-guanosine cap structure according to manufacturer protocols: 3’-O-Me-m7G(5’)ppp(5’) G [the ARCA cap]; G(5’)ppp(5’)A; G(5’)ppp(5’)G; m7G(5’)ppp(5’)A; m7G(5’)ppp(5’)G (New England BioLabs, Ipswich, MA). 5’-capping of modified RNA may be completed post-transcriptionally using a Vaccinia Virus Capping Enzyme to generate the “Cap 0” structure: m7G(5’)ppp(5’)G (New England BioLabs, Ipswich, MA). Cap 1 structure may be generated using both Vaccinia Virus Capping Enzyme and a 2’-O methyl-transferase to generate: m7G(5’)ppp(5’)G-2’-O-methyl. Cap 2 structure may be generated from the Cap 1 structure followed by the 2’-O-methylation of the 5’-antepenultimate nucleotide using a 2’-O methyl-transferase. Cap 3 structure may be generated from the Cap 2 structure followed by the 2’-O-methylation of the 5’-preantepenultimate nucleotide using a 2’-O methyl-transferase. Enzymes are preferably derived from a recombinant source.

[00923] When transfected into mammalian cells, the modified mRNAs have a stability of between 12-18 hours or more than 18 hours, e.g., 24, 36, 48, 60, 72 or greater than 72 hours.

**Example 7. Capping**

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A. Protein Expression Assay

[00924] Synthetic mRNAs encoding human G-CSF (cDNA shown in SEQ ID NO: 5; mRNA sequence fully modified with 5-methylcytosine at each cytosine and pseudouridine replacement at each uridine site shown in SEQ ID NO: 6 with a polyA tail approximately 160 nucleotides in length not shown in sequence) containing the ARCA (3’ O-Me-m7G(5’ppp(5’))G) cap analog or the Cap1 structure can be transfected into human primary keratinocytes at equal concentrations. 6, 12, 24 and 36 hours post-transfection the amount of G-CSF secreted into the culture medium can be assayed by ELISA. Synthetic mRNAs that secrete higher levels of G-CSF into the medium would correspond to a synthetic mRNA with a higher translationally-competent Cap structure.

B. Purity Analysis Synthesis

[00925] Synthetic mRNAs encoding human G-CSF (cDNA shown in SEQ ID NO: 5; mRNA sequence fully modified with 5-methylcytosine at each cytosine and pseudouridine replacement at each uridine site shown in SEQ ID NO: 6 with a polyA tail approximately 160 nucleotides in length not shown in sequence) containing the ARCA cap analog or the Cap1 structure crude synthesis products can be compared for purity using denaturing Agarose-Urea gel electrophoresis or HPLC analysis. Synthetic mRNAs with a single, consolidated band by electrophoresis correspond to the higher purity product compared to a synthetic mRNA with multiple bands or streaking bands. Synthetic mRNAs with a single HPLC peak would also correspond to a higher purity product. The capping reaction with a higher efficiency would provide a more pure mRNA population.

C. Cytokine Analysis

[00926] Synthetic mRNAs encoding human G-CSF (cDNA shown in SEQ ID NO: 5; mRNA sequence fully modified with 5-methylcytosine at each cytosine and pseudouridine replacement at each uridine site shown in SEQ ID NO: 6 with a polyA tail approximately 160 nucleotides in length not shown in sequence) containing the ARCA cap analog or the Cap1 structure can be transfected into human primary keratinocytes at multiple concentrations. 6, 12, 24 and 36 hours post-transfection the amount of pro-inflammatory cytokines such as TNF-alpha and IFN-beta secreted into the culture medium can be assayed by ELISA. Synthetic mRNAs that secrete higher levels of pro-inflammatory cytokines into the medium would correspond to a synthetic mRNA containing an immune-activating cap structure.

D. Capping Reaction Efficiency
Synthetic mRNAs encoding human G-CSF (cDNA shown in SEQ ID NO: 5; mRNA sequence fully modified with 5-methylcytosine at each cytosine and pseudouridine replacement at each uridine site shown in SEQ ID NO: 6 with a polyA tail approximately 160 nucleotides in length not shown in sequence) containing the ARCA cap analog or the Cap1 structure can be analyzed for capping reaction efficiency by LC-MS after capped mRNA nuclease treatment. Nuclease treatment of capped mRNAs would yield a mixture of free nucleotides and the capped 5′-5′-triphosphate cap structure detectable by LC-MS. The amount of capped product on the LC-MS spectra can be expressed as a percent of total mRNA from the reaction and would correspond to capping reaction efficiency. The cap structure with higher capping reaction efficiency would have a higher amount of capped product by LC-MS.

**Example 8. Agarose Gel Electrophoresis of Modified RNA or RT PCR Products**

Individual modified RNAs (200-400 ng in a 20 μl volume) or reverse transcribed PCR products (200-400 ng) are loaded into a well on a non-denaturing 1.2% Agarose E-Gel (Invitrogen, Carlsbad, CA) and run for 12-15 minutes according to the manufacturer protocol.

**Example 9. Formulation of Modified mRNA Using Lipidoids**

Modified mRNAs (mmRNA) are formulated for *in vitro* experiments by mixing the mmRNA with the lipidoid at a set ratio prior to addition to cells. *In vivo* formulation may require the addition of extra ingredients to facilitate circulation throughout the body. To test the ability of these lipidoids to form particles suitable for *in vivo* work, a standard formulation process used for siRNA-lipidoid formulations was used as a starting point. Initial mmRNA-lipidoid formulations may consist of particles composed of 42% lipidoid, 48% cholesterol and 10% PEG, with further optimization of ratios possible. After formation of the particle, mmRNA is added and allowed to integrate with the complex. The encapsulation efficiency is determined using a standard dye exclusion assays.

**Materials and Methods for Examples 10-14**

A. **Lipid Synthesis**

Six lipids, DLin-DMA, DLin-K-DMA, DLin-KC2-DMA, 98N12-5, C12-200 and DLin-MC3-DMA, were synthesized by methods outlined in the art in order to be formulated with modified RNA. DLin-DMA and precursors were synthesized as described in Heyes et. al, J. Control Release, 2005, 107, 276-287. DLin-K-DMA and DLin-KC2-DMA and precursors were synthesized as
described in Semple et. al., Nature Biotechnology, 2010, 28, 172-176. 98N12-5 and precursor were synthesized as described in Akinc et. al., Nature Biotechnology, 2008, 26, 561-569.

[00931] C12-200 and precursors were synthesized according to the method outlined in Love et. al, PNAS, 2010, 107, 1864-1869. 2-epoxydodecane (5.10 g, 27.7 mmol, 8.2 eq) was added to a vial containing Amine 200 (0.723 g, 3.36 mmol, 1 eq) and a stirring bar. The vial was sealed and warmed to 80°C. The reaction was stirred for 4 days at 80°C. Then the mixture was purified by silica gel chromatography using a gradient from pure dichloromethane (DCM) to DCM:MeOH 98:2. The target compound was further purified by RP-HPLC to afford the desired compound.

[00932] DLin-MC3-DMA and precursors were synthesized according to procedures described in WO 2010054401 herein incorporated by reference in its entirety. A mixture of dilinoleyl methanol (1.5 g, 2.8 mmol, 1 eq), N,N-dimethylaminobutyric acid (1.5 g, 2.8 mmol, 1eq), DIPEA (0.73 mL, 4.2 mmol, 1.5 eq) and TBTU(1.35 g, 4.2 mmol, 1.5 eq) in 10 mL of DMF was stirred for 10 h at room temperature. Then the reaction mixture was diluted in ether and washed with water. The organic layer was dried over anhydrous sodium sulfate, filtrated and concentrated under reduced pressure. The crude product was purified by silica gel chromatography using a gradient DCM to DCM:MeOH 98:2. Subsequently the target compound was subjected to an additional RP-HPLC purification which was done using a YMC – Pack C4 column to afford the target compound.

B. Formulation of Modified RNA Nanoparticles

[00933] Solutions of synthesized lipid, 1,2-distearoyl-3-phosphatidylcholine (DSPC) (Avanti Polar Lipids, Alabaster, AL), cholesterol (Sigma-Aldrich, Taufkirchen, Germany), and α-[3‘-(1,2-dimyristoyl-3-propanoxy)-carboxamide-propyl]-ω-methoxy-polyoxyethylene (PEG-c-DOMG) (NOF, Bouwelven, Belgium) were prepared at concentrations of 50 mM in ethanol and stored at -20°C. The lipids were combined to yield molar ratio of 50:10:38.5:1.5 (Lipid: DSPC: Cholesterol: PEG-c-DOMG) and diluted with ethanol to a final lipid concentration of 25 mM. Solutions of modified mRNA at a concentration of 1-2 mg/mL in water were diluted in 50 mM sodium citrate buffer at a pH of 3 to form a stock modified mRNA solution. Formulations of the lipid and modified mRNA were prepared by combining the synthesized lipid solution with the modified mRNA solution at total lipid to modified mRNA weight ratio of 10:1, 15:1, 20:1 and 30:1. The lipid ethanolic solution was rapidly injected into aqueous modified mRNA solution to afford a suspension containing 33% ethanol. The solutions were injected either manually (MI) or by the aid of a syringe pump (SP) (Harvard Pump 33 Dual Syringe Pump Harvard Apparatus Holliston, MA).
To remove the ethanol and to achieve the buffer exchange, the formulations were dialyzed twice against phosphate buffered saline (PBS), pH 7.4 at volumes 200-times of the primary product using a Slide-A-Lyzer cassettes (Thermo Fisher Scientific Inc. Rockford, IL) with a molecular weight cutoff (MWCO) of 10 kDa. The first dialysis was carried at room temperature for 3 hours and then the formulations were dialyzed overnight at 4°C. The resulting nanoparticle suspension was filtered through 0.2 µm sterile filter (Sarstedt, Nümbrecht, Germany) into glass vials and sealed with a crimp closure.

C. Characterization of formulations

A Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK) was used to determine the particle size, the polydispersity index (PDI) and the zeta potential of the modified mRNA nanoparticles in 1X PBS in determining particle size and 15 mM PBS in determining zeta potential.

Ultraviolet–visible spectroscopy was used to determine the concentration of modified mRNA nanoparticle formulation. 100 µL of the diluted formulation in 1X PBS was added to 900 µL of a 4:1 (v/v) mixture of methanol and chloroform. After mixing, the absorbance spectrum of the solution was recorded between 230 nm and 330 nm on a DU 800 spectrophotometer (Beckman Coulter, Beckman Coulter, Inc., Brea, CA). The modified RNA concentration in the nanoparticle formulation was calculated based on the extinction coefficient of the modified RNA used in the formulation and on the difference between the absorbance at a wavelength of 260 nm and the baseline value at a wavelength of 330 nm.

QUANT-IT™ RIBOGREEN® RNA assay (Invitrogen Corporation Carlsbad, CA) was used to evaluate the encapsulation of modified RNA by the nanoparticle. The samples were diluted to a concentration of approximately 5 µg/mL in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5). 50 µL of the diluted samples were transferred to a polystyrene 96 well plate, then either 50 µL of TE buffer or 50 µL of a 2% Triton X-100 solution was added. The plate was incubated at a temperature of 37°C for 15 minutes. The RIBOGREEN® reagent was diluted 1:100 in TE buffer, 100 µL of this solution was added to each well. The fluorescence intensity was measured using a fluorescence plate reader (Wallac Victor 1420 Multilabel Counter; Perkin Elmer, Waltham, MA) at an excitation wavelength of ~480 nm and an emission wavelength of ~520 nm. The fluorescence values of the reagent blank were subtracted from that of each of the samples and the percentage of free modified RNA was determined by dividing the fluorescence intensity of the intact sample (without addition of
Triton X-100) by the fluorescence value of the disrupted sample (caused by the addition of Triton X-100).

D. **In Vitro Incubation**

[00938] Human embryonic kidney epithelial (HEK293) and hepatocellular carcinoma epithelial (HepG2) cells (LGC standards GmbH, Wesel, Germany) were seeded on 96-well plates (Greiner Bio-one GmbH, Frickenhausen, Germany) and plates for HEK293 cells were precoated with collagen type1. HEK293 were seeded at a density of 30,000 and HepG2 were seeded at a density of 35,000 cells per well in 100 µl cell culture medium. For HEK293 the cell culture medium was DMEM, 10% FCS, adding 2mM L-Glutamine, 1 mM Sodiumpyruvate and 1x non-essential amino acids (Biochrom AG, Berlin, Germany) and 1.2 mg/ml Sodiumbicarbonate (Sigma-Aldrich, Munich, Germany) and for HepG2 the culture medium was MEM (Gibco Life Technologies, Darmstadt, Germany), 10% FCS adding 2mM L-Glutamine, 1 mM Sodiumpyruvate and 1x non-essential amino acids (Biochrom AG, Berlin, Germany). Formulations containing mCherry mRNA (mRNA sequence shown in SEQ ID NO: 7; polyA tail of approximately 160 nucleotides not shown in sequence; 5’ cap, Cap1); were added in quadruplicates directly after seeding the cells and incubated. The mCherry cDNA with the T7 promoter, 5’ untranslated region (UTR) and 3’ UTR used in **in vitro** transcription (IVT) is given in SEQ ID NO: 8. The mCherry mRNA was modified with 5meC at each cytosine and pseudouridine replacement at each uridine site.

[00939] Cells were harvested by transferring the culture media supernatants to a 96-well Pro-Bind U-bottom plate (Beckton Dickinson GmbH, Heidelberg, Germany). Cells were trypsinized with ½ volume Trypsin/EDTA (Biochrom AG, Berlin, Germany), pooled with respective supernatants and fixed by adding one volume PBS/2%FCS (both Biochrom AG, Berlin, Germany)/0.5% formaldehyde (Merek, Darmstadt, Germany). Samples then were submitted to a flow cytometer measurement with a 532nm excitation laser and the 610/20 filter for PE-Texas Red in a LSRII cytometer (Beckton Dickinson GmbH, Heidelberg, Germany). The mean fluorescence intensity (MFI) of all events and the standard deviation of four independent wells are presented in for samples analyzed.

**Example 10. Purification of Nanoparticle Formulations**

[00940] Nanoparticle formulations of DLin-KC2-DMA and 98N12-5 in HEK293 and HepG2 were tested to determine if the mean fluorescent intensity (MFI) was dependent on the lipid to modified RNA ratio and/or purification. Three formulations of DLin-KC2-DMA and two formulations of
98N12-5 were produced using a syringe pump to the specifications described in Table 5. Purified samples were purified by SEPHADEX™ G-25 DNA grade (GE Healthcare, Sweden). Each formulation before and after purification (aP) was tested at concentration of 250 ng modified RNA per well in a 24 well plate. The percentage of cells that are positive for the marker for FL4 channel (%FL4-positive) when analyzed by the flow cytometer for each formulation and the background sample and the MFI of the marker for the FL4 channel for each formulation and the background sample are shown in Table 6. The formulations which had been purified had a slightly higher MFI than those formulations tested before purification.

Table 5. Formulations

<table>
<thead>
<tr>
<th>Formulation #</th>
<th>Lipid</th>
<th>Lipid/RNA wt/wt</th>
<th>Mean size (nm)</th>
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<tbody>
<tr>
<td>NPA-001-1</td>
<td>DLin-KC2-DMA</td>
<td>10</td>
<td>155 nm PDI: 0.08</td>
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<tr>
<td>NPA-001-1 aP</td>
<td>DLin-KC2-DMA</td>
<td>10</td>
<td>141 nm PDI: 0.14</td>
</tr>
<tr>
<td>NPA-002-1</td>
<td>DLin-KC2-DMA</td>
<td>15</td>
<td>140 nm PDI: 0.11</td>
</tr>
<tr>
<td>NPA-002-1 aP</td>
<td>DLin-KC2-DMA</td>
<td>15</td>
<td>125 nm PDI: 0.12</td>
</tr>
<tr>
<td>NPA-003-1</td>
<td>DLin-KC2-DMA</td>
<td>20</td>
<td>114 nm PDI: 0.08</td>
</tr>
<tr>
<td>NPA-003-1 aP</td>
<td>DLin-KC2-DMA</td>
<td>20</td>
<td>104 nm PDI: 0.06</td>
</tr>
<tr>
<td>NPA-005-1</td>
<td>98N12-5</td>
<td>15</td>
<td>127 nm PDI: 0.12</td>
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<tr>
<td>NPA-005-1 aP</td>
<td>98N12-5</td>
<td>15</td>
<td>134 nm PDI: 0.17</td>
</tr>
<tr>
<td>NPA-006-1</td>
<td>98N12</td>
<td>20</td>
<td>126 nm PDI: 0.08</td>
</tr>
<tr>
<td>NPA-006-1 aP</td>
<td>98N12</td>
<td>20</td>
<td>118 nm PDI: 0.13</td>
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</tbody>
</table>

Table 6. HEK293 and HepG2, 24-well, 250 ng Modified RNA/well

<table>
<thead>
<tr>
<th>Formulation</th>
<th>%FL4-positive</th>
<th>FL4 MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td>HepG2</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>0.33</td>
<td>0.40</td>
</tr>
<tr>
<td>NPA-001-1</td>
<td>62.42</td>
<td>5.68</td>
</tr>
<tr>
<td>NPA-001-ap</td>
<td>87.32</td>
<td>9.02</td>
</tr>
<tr>
<td>NPA-002-1</td>
<td>91.28</td>
<td>9.90</td>
</tr>
<tr>
<td>NPA-002-ap</td>
<td>92.68</td>
<td>14.02</td>
</tr>
<tr>
<td>NPA-003-1</td>
<td>87.70</td>
<td>11.76</td>
</tr>
<tr>
<td>NPA-003-ap</td>
<td>88.88</td>
<td>15.46</td>
</tr>
<tr>
<td>NPA-005-1</td>
<td>50.60</td>
<td>4.75</td>
</tr>
<tr>
<td>NPA-005-ap</td>
<td>38.64</td>
<td>5.16</td>
</tr>
<tr>
<td>NPA-006-1</td>
<td>54.19</td>
<td>13.16</td>
</tr>
<tr>
<td>NPA-006-ap</td>
<td>49.97</td>
<td>13.74</td>
</tr>
</tbody>
</table>
Example 11. Concentration Response Curve

NanoGraft formulations of 98N12-5 (NPA-005) and DLin-KC2-DMA (NPA-003) were tested at varying concentrations to determine the MFI of FL4 or mCherry (mRNA sequence shown in SEQ ID NO: 7; polyA tail of approximately 160 nucleotides not shown in sequence; 5’ cap, Capi; fully modified with 5-methylcytosine and pseudouridine) over a range of doses. The formulations tested are outlined in Table 7. To determine the optimal concentration of nanoparticle formulations of 98N12-5, varying concentrations of formulated modified RNA (100 ng, 10 ng, 1.0 ng, 0.1 ng and 0.01 ng per well) were tested in a 24-well plate of HEK293, and the results of the FL4 MFI of each dose are shown in Table 8. Likewise, to determine the optimal concentration of nanoparticle formulations of DLin-KC2-DMA, varying concentrations of formulated modified RNA (250 ng 100 ng, 10 ng, 1.0 ng, 0.1 ng and 0.01 ng per well) were tested in a 24-well plate of HEK293, and the results of the FL4 MFI of each dose are shown in Table 9. Nanoparticle formulations of DLin-KC2-DMA were also tested at varying concentrations of formulated modified RNA (250 ng, 100 ng and 30 ng per well) in a 24 well plate of HEK293, and the results of the FL4 MFI of each dose are shown in Table 10. A dose of 1 ng/well for 98N12-5 and a dose of 10 ng/well for DLin-KC2-DMA were found to resemble the FL4 MFI of the background.

<table>
<thead>
<tr>
<th>Table 7. Formulations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formulation #</td>
</tr>
<tr>
<td>Lipid</td>
</tr>
<tr>
<td>Lipid/RNA wt/wt</td>
</tr>
<tr>
<td>Mean size</td>
</tr>
<tr>
<td>PDI: 0.08</td>
</tr>
</tbody>
</table>

Table 8. HEK293, NPA-005, 24-well, n=4

<table>
<thead>
<tr>
<th>Formulation</th>
<th>FL4 MFI</th>
</tr>
</thead>
</table>

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Table 9. HEK293, NPA-003, 24-well, n=4

<table>
<thead>
<tr>
<th>Formulation</th>
<th>FL4 MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>0.3225</td>
</tr>
<tr>
<td>NPA-003 250 ng</td>
<td>2.9575</td>
</tr>
<tr>
<td>NPA-003 100 ng</td>
<td>1.255</td>
</tr>
<tr>
<td>NPA-003 10 ng</td>
<td>0.40025</td>
</tr>
<tr>
<td>NPA-003 1 ng</td>
<td>0.33025</td>
</tr>
<tr>
<td>NPA-003 0.1 ng</td>
<td>0.34625</td>
</tr>
<tr>
<td>NPA-003 0.01 ng</td>
<td>0.3475</td>
</tr>
</tbody>
</table>

Table 10. HEK293, NPA-003, 24-well, n=4

<table>
<thead>
<tr>
<th>Formulation</th>
<th>FL4 MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>0.27425</td>
</tr>
<tr>
<td>NPA-003 250 ng</td>
<td>5.6075</td>
</tr>
<tr>
<td>NPA-003 100 ng</td>
<td>3.7825</td>
</tr>
<tr>
<td>NPA-003 30 ng</td>
<td>1.5525</td>
</tr>
</tbody>
</table>

Table 11. Concentration and MFI

<table>
<thead>
<tr>
<th>Formulation</th>
<th>NPA-003</th>
<th>NPA-005</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 ng/well</td>
<td>11963.25</td>
<td>12256.75</td>
</tr>
<tr>
<td>0.25 ng/well</td>
<td>1349.75</td>
<td>2572.75</td>
</tr>
<tr>
<td>0.025 ng/well</td>
<td>459.50</td>
<td>534.75</td>
</tr>
<tr>
<td>0.0025 ng/well</td>
<td>310.75</td>
<td>471.75</td>
</tr>
</tbody>
</table>

Example 12. Manual Injection and Syringe Pump Formulations

[00943] Two formulations of DLin-KC2-DMA and 98N12-5 were prepared by manual injection (MI) and syringe pump injection (SP) and analyzed along with a background sample to compare the MFI of mCherry (mRNA sequence shown in SEQ ID NO: 7; polyA tail of approximately 160 nucleotides not shown in sequence; 5’ cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) of the different formulations. Table 12 shows that the syringe pump formulations had a higher MFI as compared to the manual injection formulations of the same lipid and lipid/RNA ratio.

Table 12. Formulations and MFI
<table>
<thead>
<tr>
<th>Formulation #</th>
<th>Lipid</th>
<th>Lipid/RNA wt/wt</th>
<th>Mean size (nm)</th>
<th>Method of formulation</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Control</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>674.67</td>
</tr>
<tr>
<td>NPA-002</td>
<td>DLin-KC2-DMA</td>
<td>15</td>
<td>140 nm PDI: 0.11</td>
<td>MI</td>
<td>10318.25</td>
</tr>
<tr>
<td>NPA-002-2</td>
<td>DLin-KC2-DMA</td>
<td>15</td>
<td>105 nm PDI: 0.04</td>
<td>SP</td>
<td>37054.75</td>
</tr>
<tr>
<td>NPA-003</td>
<td>DLin-KC2-DMA</td>
<td>20</td>
<td>114 nm PDI: 0.08</td>
<td>MI</td>
<td>22037.5</td>
</tr>
<tr>
<td>NPA-003-2</td>
<td>DLin-KC2-DMA</td>
<td>20</td>
<td>95 nm PDI: 0.02</td>
<td>SP</td>
<td>37868.75</td>
</tr>
<tr>
<td>NPA-005</td>
<td>98N12-5</td>
<td>15</td>
<td>127 nm PDI: 0.12</td>
<td>MI</td>
<td>11504.75</td>
</tr>
<tr>
<td>NPA-005-2</td>
<td>98N12-5</td>
<td>15</td>
<td>106 nm PDI: 0.07</td>
<td>SP</td>
<td>9343.75</td>
</tr>
<tr>
<td>NPA-006</td>
<td>98N12-5</td>
<td>20</td>
<td>126 nm PDI: 0.08</td>
<td>MI</td>
<td>11182.25</td>
</tr>
<tr>
<td>NPA-006-2</td>
<td>98N12-5</td>
<td>20</td>
<td>93 nm PDI: 0.08</td>
<td>SP</td>
<td>5167</td>
</tr>
</tbody>
</table>

**Example 13. LNP Formulations**

[00944] Formulations of DLin-DMA, DLin-K-DMA, DLin-KC2-DMA, 98N12-5, C12-200 and DLin-MC3-DMA were incubated at a concentration of 60 ng/well or 62.5 ng/well in a plate of HEK293 and 62.5 ng/well in a plate of HepG2 cells for 24 hours to determine the MFI of mCherry (mRNA sequence shown in SEQ ID NO: 7; polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) for each formulation. The formulations tested are outlined in Table 13 below. As shown in Table 14 for the 60 ng/well and Tables 15, 16, 17 and 18 for the 62.5 ng/well, the formulation of NPA-003 and NPA-018 have the highest mCherry MFI and the formulations of NPA-008, NPA-010 and NPA-013 are most similar to the background sample mCherry MFI value.

**Table 13. Formulations**

<table>
<thead>
<tr>
<th>Formulation #</th>
<th>Lipid</th>
<th>Lipid/RNA wt/wt</th>
<th>Mean size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPA-001</td>
<td>DLin-KC2-DMA</td>
<td>10</td>
<td>155 nm PDI: 0.08</td>
</tr>
<tr>
<td>NPA-002</td>
<td>DLin-KC2-DMA</td>
<td>15</td>
<td>140 nm PDI: 0.11</td>
</tr>
<tr>
<td>NPA-002-2</td>
<td>DLin-KC2-DMA</td>
<td>15</td>
<td>105 nm PDI: 0.04</td>
</tr>
<tr>
<td>NPA-003</td>
<td>DLin-KC2-DMA</td>
<td>20</td>
<td>114 nm PDI: 0.08</td>
</tr>
<tr>
<td>NPA-003-2</td>
<td>DLin-KC2-DMA</td>
<td>20</td>
<td>95 nm PDI: 0.02</td>
</tr>
<tr>
<td>Formulation</td>
<td>MFI mCherry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>871.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-001</td>
<td>6407.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-002</td>
<td>14995</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-003</td>
<td>29499.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-005</td>
<td>3762</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-006</td>
<td>2676</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-007</td>
<td>9905.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-008</td>
<td>1648.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-009</td>
<td>2348.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-010</td>
<td>4426.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-012</td>
<td>11466</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-013</td>
<td>2098.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-014</td>
<td>3194.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPA-015</td>
<td>14524</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 16. HEK293, 62.5 ng /well

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MFI mCherry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>295</td>
</tr>
<tr>
<td>NPA-007</td>
<td>3504</td>
</tr>
<tr>
<td>NPA-012</td>
<td>8286</td>
</tr>
<tr>
<td>NPA-017</td>
<td>6128</td>
</tr>
<tr>
<td>NPA-003-2</td>
<td>17528</td>
</tr>
<tr>
<td>NPA-018</td>
<td>34142</td>
</tr>
<tr>
<td>NPA-010</td>
<td>1095</td>
</tr>
<tr>
<td>NPA-015</td>
<td>5859</td>
</tr>
<tr>
<td>NPA-019</td>
<td>3229</td>
</tr>
</tbody>
</table>

### Table 17. HepG2, 62.5 ng /well

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MFI mCherry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>649.94</td>
</tr>
<tr>
<td>NPA-001</td>
<td>6006.25</td>
</tr>
<tr>
<td>NPA-002</td>
<td>8705</td>
</tr>
<tr>
<td>NPA-002-2</td>
<td>15860.25</td>
</tr>
<tr>
<td>NPA-003</td>
<td>15059.25</td>
</tr>
<tr>
<td>NPA-003-2</td>
<td>28881</td>
</tr>
<tr>
<td>NPA-005</td>
<td>1676</td>
</tr>
<tr>
<td>NPA-006</td>
<td>1473</td>
</tr>
<tr>
<td>NPA-007</td>
<td>15678</td>
</tr>
<tr>
<td>NPA-008</td>
<td>2976.25</td>
</tr>
<tr>
<td>NPA-009</td>
<td>961.75</td>
</tr>
<tr>
<td>NPA-010</td>
<td>3301.75</td>
</tr>
<tr>
<td>NPA-012</td>
<td>18333.25</td>
</tr>
<tr>
<td>NPA-013</td>
<td>5853</td>
</tr>
<tr>
<td>NPA-014</td>
<td>2257</td>
</tr>
<tr>
<td>NPA-015</td>
<td>16225.75</td>
</tr>
</tbody>
</table>

### Table 18. HepG2, 62.5 ng /well

<table>
<thead>
<tr>
<th>Formulation</th>
<th>MFI mCherry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>656</td>
</tr>
<tr>
<td>NPA-007</td>
<td>16798</td>
</tr>
<tr>
<td>NPA-012</td>
<td>21993</td>
</tr>
<tr>
<td>NPA-017</td>
<td>20377</td>
</tr>
<tr>
<td>NPA-003-2</td>
<td>35651</td>
</tr>
<tr>
<td>NPA-018</td>
<td>40154</td>
</tr>
<tr>
<td>NPA-010</td>
<td>2496</td>
</tr>
<tr>
<td>NPA-015</td>
<td>19741</td>
</tr>
<tr>
<td>NPA-019</td>
<td>16373</td>
</tr>
</tbody>
</table>
Example 14. *In vivo* formulation studies

**[00945]** Rodents (n=5) are administered intravenously, subcutaneously or intramuscularly a single dose of a formulation containing at least one modified mRNA and a lipid. The modified mRNA administered to the rodents is selected from G-CSF (mRNA sequence shown in SEQ ID NO: 6; polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap1), erythropoietin (EPO) (mRNA sequence shown in SEQ ID NO: 9; polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap1), Factor IX (mRNA shown in SEQ ID NO: 10; polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap1) or mCherry (mRNA sequence shown in SEQ ID NO: 7; polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap1). The erythropoietin cDNA with the T7 promoter, 5’untranslated region (UTR) and 3’ UTR used in *in vitro* transcription (IVT) is given in SEQ ID NO: 11 and SEQ ID NO: 12.

**[00946]** Each formulation also contains a lipid which is selected from one of DLin-DMA, DLin-KDMA, DLin-KC2-DMA, 98N12-5, C12-200, DLin-MC3-DMA, reLNP, ATUPLEX®, DACC, and DBTC. The rodents are injected with 100µg, 10 µg or 1 µg of the formulated modified mRNA and samples are collected at specified time intervals.

**[00947]** Serum from the rodents administered formulations containing human G-CSF modified mRNA are measured by specific G-CSF ELISA and serum from mice administered human factor IX modified RNA is analyzed by specific factor IX ELISA or chromogenic assay. The liver and spleen from the mice administered with mCherry modified mRNA are analyzed by immunohistochemistry (IHC) or fluorescence-activated cell sorting (FACS). As a control, a group of mice are not injected with any formulation and their serum and tissue are collected analyzed by ELISA, FACS and/or IHC.

A. Time Course

**[00948]** The rodents are administered formulations containing at least one modified mRNA to study the time course of protein expression for the administered formulation. The rodents are bled at specified time intervals prior to and after administration of the modified mRNA formulations to determine protein expression and complete blood count. Samples are also collected from the site of administration of rodents administered modified mRNA formulations subcutaneously and intramuscularly to determine the protein expression in the tissue.

B. Dose Response
[00949] The rodents are administered formulations containing at least one modified mRNA to determine dose response of each formulation. The rodents are bled at specified time intervals prior to and after administration of the modified mRNA formulations to determine protein expression and complete blood count. The rodents are also sacrificed to analyze the effect of the modified mRNA formulation on the internal tissue. Samples are also collected from the site of administration of rodents administered modified mRNA formulations subcutaneously and intramuscularly to determine the protein expression in the tissue.

C. Toxicity

[00950] The rodents are administered formulations containing at least one modified mRNA to study toxicity of each formulation. The rodents are bled at specified time intervals prior to and after administration of the modified mRNA formulations to determine protein expression and complete blood count. The rodents are also sacrificed to analyze the effect of the modified mRNA formulation on the internal tissue. Samples are also collected from the site of administration of rodents administered modified mRNA formulations subcutaneously and intramuscularly to determine the protein expression in the tissue.

**Example 15. PLGA Microsphere Formulations**

[00951] Optimization of parameters used in the formulation of PLGA microspheres may allow for tunable release rates and high encapsulation efficiencies while maintaining the integrity of the modified RNA encapsulated in the microspheres. Parameters such as, but not limited to, particle size, recovery rates and encapsulation efficiency may be optimized to achieve the optimal formulation.

A. Synthesis of PLGA microspheres

[00952] Polylacticglycolic acid (PLGA) microspheres were synthesized using the water/oil/water double emulsification methods known in the art using PLGA (Lactel, Cat# B6010-2, inherent viscosity 0.55-0.75, 50:50 LA:GA), polyvinylalcohol (PVA) (Sigma, Cat# 348406-25G, MW 13-23k) dichloromethane and water. Briefly, 0.1 ml of water (W1) was added to 2 ml of PLGA dissolved in dichloromethane (DCM) (O1) at concentrations ranging from 50 – 200 mg/ml of PLGA. The W1/O1 emulsion was homogenized (IKA Ultra-Turrax Homogenizer, T18) for 30 seconds at speed 4 (~15,000 rpm). The W1/O1 emulsion was then added to 100 to 200 ml of 0.3 to 1% PVA (W2) and homogenized for 1 minute at varied speeds. Formulations were left to stir for 3 hours and then washed by centrifugation (20-25 min, 4,000 rpm, 4°C). The supernatant was discarded and the
PLGA pellets were resuspended in 5-10 ml of water, which was repeated 2x. Average particle size (represents 20–30 particles) for each formulation was determined by microscopy after washing. Table 19 shows that an increase in the PLGA concentration led to larger sized microspheres. A PLGA concentration of 200 mg/mL gave an average particle size of 14.8 μm, 100 mg/mL was 8.7 μm, and 50 mg/mL of PLGA gave an average particle size of 4.0 μm.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>O1 Volume (mL)</th>
<th>PLGA Concentration (mg/mL)</th>
<th>W2 Volume (mL)</th>
<th>PVA Concentration (%)</th>
<th>Speed</th>
<th>Average Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>200</td>
<td>100</td>
<td>0.3</td>
<td>5</td>
<td>14.8</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>100</td>
<td>100</td>
<td>0.3</td>
<td>5</td>
<td>8.7</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>50</td>
<td>100</td>
<td>0.3</td>
<td>5</td>
<td>4.0</td>
</tr>
</tbody>
</table>

[00953] Table 20 shows that decreasing the homogenization speed from 5 (~20,000 rpm) to speed 4 (~15,000 rpm) led to an increase in particle size from 14.8 μm to 29.7 μm.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>O1 Volume (mL)</th>
<th>PLGA Concentration (mg/mL)</th>
<th>W2 Volume (mL)</th>
<th>PVA Concentration (%)</th>
<th>Speed</th>
<th>Average Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>200</td>
<td>100</td>
<td>0.3</td>
<td>5</td>
<td>14.8</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>200</td>
<td>100</td>
<td>0.3</td>
<td>4</td>
<td>29.7</td>
</tr>
</tbody>
</table>

[00954] Table 21 shows that increasing the W2 volume (i.e. increasing the ratio of W2:O1 from 50:1 to 100:1), decreased average particle size slightly. Altering the PVA concentration from 0.3 to 1 wt% had little impact on PLGA microsphere size.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>O1 Volume (mL)</th>
<th>PLGA Concentration (mg/mL)</th>
<th>W2 Volume (mL)</th>
<th>PVA Concentration (%)</th>
<th>Speed</th>
<th>Average Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>200</td>
<td>100</td>
<td>0.3</td>
<td>5</td>
<td>14.8</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>200</td>
<td>200</td>
<td>0.3</td>
<td>5</td>
<td>11.7</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>200</td>
<td>190</td>
<td>0.3</td>
<td>5</td>
<td>11.4</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>200</td>
<td>190</td>
<td>1.0</td>
<td>5</td>
<td>12.3</td>
</tr>
</tbody>
</table>

B. Encapsulation of modified mRNA

[00955] Modified G-CSF mRNA (SEQ ID NO: 6; polyA tail of approximately 160 nucleotides not shown in sequence; 5’ cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) was
dissolved in water at a concentration of 2 mg/ml (W3). Three batches of PLGA microsphere formulations were made as described above with the following parameters: 0.1 ml of W3 at 2 mg/ml, 1.6 ml of O1 at 200 mg/ml, 160 ml of W2 at 1%, and homogenized at a speed of 4 for the first emulsion (W3/O1) and homogenized at a speed of 5 for the second emulsion (W3/O1/W2). After washing by centrifugation, the formulations were frozen in liquid nitrogen and then lyophilized for 3 days. To test the encapsulation efficiency of the formulations, the lyophilized material was deformulated in DCM for 6 hours followed by an overnight extraction in water. The modified RNA concentration in the samples was then determined by OD260. Encapsulation efficiency was calculated by taking the actual amount of modified RNA and dividing by the starting amount of modified RNA. In the three batches tested, there was an encapsulation efficiency of 59.2, 49.8 and 61.3.

C. Integrity of modified mRNA encapsulated in PLGA microspheres

Modified Factor IX mRNA (SEQ ID NO: 10; polyA tail of approximately 160 nucleotides not shown in sequence; 5'cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) was dissolved in water at varied concentrations (W4) to vary the weight percent loading in the formulation (mg modified RNA/mg PLGA * 100) and to determine encapsulation efficiency. The parameters in Table 22 were used to make four different batches of PLGA microsphere formulations with a homogenization speed of 4 for the first emulsion (W4/O1) and a homogenization speed of 5 for the second emulsion (W4/O1/W2).

<table>
<thead>
<tr>
<th>Table 22. Factor IX PLGA Microsphere Formulation Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ID</strong></td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
</tbody>
</table>

After lyophilization, PLGA microspheres were weighed out in 2 ml eppendorf tubes to correspond to ~ 10 ug of modified RNA. Lyophilization was found to not destroy the overall structure of the PLGA microspheres. To increase weight percent loading (wt%) for the PLGA microspheres, increasing amounts of modified RNA were added to the samples. PLGA microspheres were deformulated by adding 1.0 ml of DCM to each tube and then shaking the samples for 6 hours.
For modified RNA extraction, 0.5 ml of water was added to each sample and the samples were shaken overnight before the concentration of modified RNA in the samples was determined by OD260. To determine the recovery of the extraction process, unformulated Factor IX modified RNA (SEQ ID NO: 10; polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) (deformation control) was spiked into DCM and was subjected to the deformation process. Table 23 shows the loading and encapsulation efficiency for the samples. All encapsulation efficiency samples were normalized to the deformation control.

Table 23. Weight Percent Loading and Encapsulation Efficiency

<table>
<thead>
<tr>
<th>ID</th>
<th>Theoretical modified RNA loading (wt%)</th>
<th>Actual modified RNA loading (wt%)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.05</td>
<td>0.06</td>
<td>97.1</td>
</tr>
<tr>
<td>B</td>
<td>0.10</td>
<td>0.10</td>
<td>85.7</td>
</tr>
<tr>
<td>C</td>
<td>0.20</td>
<td>0.18</td>
<td>77.6</td>
</tr>
<tr>
<td>D</td>
<td>0.40</td>
<td>0.31</td>
<td>68.1</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>100.0</td>
</tr>
</tbody>
</table>

D. Release study of modified mRNA encapsulated in PLGA microspheres

[00958] PLGA microspheres formulated with Factor IX modified RNA (SEQ ID NO: 10) were deformulated as described above and the integrity of the extracted modified RNA was determined by automated electrophoresis (Bio-Rad Experion). The extracted modified mRNA was compared against unformulated modified mRNA and the deformation control in order to test the integrity of the encapsulated modified mRNA. As shown in Figure 3, the majority of modRNA was intact for batch ID A, B, C and D, for the deformulated control (Deform control) and the unformulated control (Uniform control).

E. Protein Expression of modified mRNA encapsulated in PLGA microspheres

[00959] PLGA microspheres formulated with Factor IX modified RNA (SEQ ID NO: 10; polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) were deformulated as described above and the protein expression of the extracted modified RNA was determined by an in vitro transfection assay. HEK293 cells were reverse transfected with 250 ng of Factor IX modified RNA complexed with RNAiMAX (Invitrogen) in triplicate.
Factor IX modified RNA was diluted in nuclease-free water to a concentration of 25 ng/μl and RNAiMAX was diluted 13.3x in serum-free EMEM. Equal volumes of diluted modified RNA and diluted RNAiMAX were mixed together and were allowed to stand for 20 to 30 minutes at room temperature. Subsequently, 20 μl of the transfection mix containing 250 ng of Factor IX modified RNA was added to 80 μl of a cell suspension containing 30,000 cells. Cells were then incubated for 16h in a humidified 37°C/5% CO2 cell culture incubator before harvesting the cell culture supernatant. Factor IX protein expression in the cell supernatant was analyzed by an ELISA kit specific for Factor IX (Molecular Innovations, Cat # HFIXKT-TOT) and the protein expression is shown in Table 24. In all PLGA microsphere batches tested, Factor IX modified RNA remained active and expressed Factor IX protein after formulation in PLGA microspheres and subsequent deformation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Factor IX Protein Expression (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch A</td>
<td>0.83</td>
</tr>
<tr>
<td>Batch B</td>
<td>1.83</td>
</tr>
<tr>
<td>Batch C</td>
<td>1.54</td>
</tr>
<tr>
<td>Batch D</td>
<td>2.52</td>
</tr>
<tr>
<td>Deformulated Control</td>
<td>4.34</td>
</tr>
<tr>
<td>Unformulated Control</td>
<td>3.35</td>
</tr>
</tbody>
</table>

Table 24. Protein Expression

F. Release study of modified mRNA encapsulated in PLGA microspheres

PLGA microspheres formulated with Factor IX modified RNA (SEQ ID NO: 10; polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) were resuspended in water to a PLGA microsphere concentration of 24 mg/ml. After resuspension, 150 μl of the PLGA microsphere suspension was aliquoted into eppendorf tubes. Samples were kept incubating and shaking at 37°C during the course of the study. Triplicate samples were pulled at 0.2, 1, 2, 8, 14, and 21 days. To determine the amount of modified RNA released from the PLGA microspheres, samples were centrifuged, the supernatant was removed, and the modified RNA concentration in the supernatant was determined by OD 260. The percent release, shown in Table 25, was calculated based on the total amount of modified RNA in each sample. After 31 days, 96% of the Factor IX modified RNA was released from the PLGA microsphere formulations.

Table 25. Percent Release
<table>
<thead>
<tr>
<th>Time (days)</th>
<th>% Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.2</td>
<td>27.0</td>
</tr>
<tr>
<td>1</td>
<td>37.7</td>
</tr>
<tr>
<td>2</td>
<td>45.3</td>
</tr>
<tr>
<td>4</td>
<td>50.9</td>
</tr>
<tr>
<td>8</td>
<td>57.0</td>
</tr>
<tr>
<td>14</td>
<td>61.8</td>
</tr>
<tr>
<td>21</td>
<td>75.5</td>
</tr>
<tr>
<td>31</td>
<td>96.4</td>
</tr>
</tbody>
</table>

G. Particle size reproducibility of PLGA microspheres

[00962] Three batches of Factor IX modified RNA (SEQ ID NO: 10 polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) PLGA microspheres were made using the same conditions described for Batch D, shown in Table 22, (0.4 ml of W4 at 4 mg/ml, 2.0 ml of O1 at 200 mg/ml, 200 ml of W2 at 1%, and homogenized at a speed of 5 for the W4/O1/W2 emulsion). To improve the homogeneity of the PLGA microsphere suspension, filtration was incorporated prior to centrifugation. After stirring for 3 hours and before centrifuging, all formulated material was passed through a 100 µm nylon mesh strainer (Fisherbrand Cell Strainer, Cat # 22-363-549) to remove larger aggregates. After washing and resuspension with water, 100-200 µl of a PLGA microspheres sample was used to measure particle size of the formulations by laser diffraction (Malvern Mastersizer2000). The particle size of the samples is shown in Table 26.

**Table 26. Particle Size Summary**

<table>
<thead>
<tr>
<th>ID</th>
<th>D10 (µm)</th>
<th>D50 (µm)</th>
<th>D90 (µm)</th>
<th>Volume Weighted Mean (µm)</th>
<th>Filtration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.2</td>
<td>62.5</td>
<td>722.4</td>
<td>223.1</td>
<td>No</td>
</tr>
<tr>
<td>A</td>
<td>9.8</td>
<td>31.6</td>
<td>65.5</td>
<td>35.2</td>
<td>Yes</td>
</tr>
<tr>
<td>B</td>
<td>10.5</td>
<td>32.3</td>
<td>66.9</td>
<td>36.1</td>
<td>Yes</td>
</tr>
<tr>
<td>C</td>
<td>10.8</td>
<td>35.7</td>
<td>79.8</td>
<td>41.4</td>
<td>Yes</td>
</tr>
</tbody>
</table>

[00963] Results of the 3 PLGA microsphere batches using filtration were compared to a PLGA microsphere batch made under the same conditions without filtration. The inclusion of a filtration
step before washing reduced the mean particle size and demonstrated a consistent particle size
distribution between 3 PLGA microsphere batches.

H. Serum Stability of Factor IX PLGA Microspheres

[00964] Factor IX mRNA RNA (SEQ ID NO: 10 polyA tail of approximately 160 nucleotides not
shown in sequence; 5’cap, Cap1; fully modified with 5-methylcytosine and pseudouridine) in buffer
(TE) or 90% serum (Se), or Factor IX mRNA in PLGA in buffer, 90% serum or 1% serum was
incubated in buffer, 90% serum or 1% serum at an mRNA concentration of 50 ng/ul in a total
volume of 70 ul. The samples were removed at 0, 30, 60 or 120 minutes. RNases were inactivated
with proteinase K digestion for 20 minutes at 55°C by adding 25 ul of 4x proteinase K buffer (0.4 ml
1M TRIS-HCl pH 7.5, 0.1 ml 0.5M EDTA, 0.12 ml 5M NaCl, and 0.4ml 10% SDS) and 8 ul of
proteinase K at 20 mg/ml. The Factor IX mRNA was precipitated (add 250 ul 95% ethanol for 1
hour, centrifuge for 10 min at 13 k rpm and remove supernatant, add 200 ul 70% ethanol to the
pellet, centrifuge again for 5 min at 13 k rpm and remove supernatant and resuspend the pellet in 70
ul water) or extracted from PLGA microspheres (centrifuge 5 min at 13k rpm and remove
supernatant, wash pellet with 1 ml water, centrifuge 5 min at 13k rpm and remove supernatant, add
280 ul dichloromethane to the pellet and shake for 15 minutes, add 70 ul water and then shake for 2
hours and remove the aqueous phase) before being analyzed by bioanalyzer. PLGA microspheres
protect Factor IX modified mRNA from degradation in 90% and 1% serum over 2 hours. Factor IX
modified mRNA completely degrades in 90% serum at the initial time point.

Example 16. Lipid nanoparticle in vivo studies

[00965] G-CSF (cDNA with the T7 promoter, 5’ Untranslated region (UTR) and 3’UTR used in in
vitro transcription is given in SEQ ID NO: 5. mRNA sequence shown in SEQ ID NO: 6; polyA tail
of approximately 160 nucleotides not shown in sequence; 5’cap, Cap 1; fully modified with 5-
methylcytosine and pseudouridine) and Factor IX (cDNA with the T7 promoter, 5’ UTR and 3’UTR
used in in vitro transcription is given in SEQ ID NO: 13. mRNA sequence shown in SEQ ID NO:10;
polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap 1; fully modified
with 5-methylcytosine and pseudouridine) modified mRNA were formulated as lipid nanoparticles
(LNPs) using the syringe pump method. The LNPs were formulated at a 20:1 weight ratio of total
lipid to modified mRNA with a final lipid molar ratio of 50:10:38.5:1.5 (DLin-KC2-DMA: DSPC:
Cholesterol: PEG-c-DOMG). Formulations, listed in Table 27, were characterized by particle size,
zeta potential, and encapsulation.
Table 27. Formulations

<table>
<thead>
<tr>
<th>Formulation #</th>
<th>NPA-029-1</th>
<th>NPA-030-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified mRNA</td>
<td>Factor IX</td>
<td>G-CSF</td>
</tr>
<tr>
<td>Mean size</td>
<td>91 nm</td>
<td>106 nm</td>
</tr>
<tr>
<td></td>
<td>PDI: 0.04</td>
<td>PDI: 0.06</td>
</tr>
<tr>
<td>Zeta at pH 7.4</td>
<td>1.8 mV</td>
<td>0.9 mV</td>
</tr>
<tr>
<td>Encaps. (RiboGr)</td>
<td>92%</td>
<td>100%</td>
</tr>
</tbody>
</table>

[00966] LNP formulations were administered to mice (n=5) intravenously at a modified mRNA dose of 100, 10, or 1 ug. Mice were sacrificed at 8 hrs after dosing. Serum was collected by cardiac puncture from mice that were administered with G-CSF or Factor IX modified mRNA formulations. Protein expression was determined by ELISA.

[00967] There was no significant body weight loss (<5%) in the G-CSF or Factor IX dose groups. Protein expression for G-CSF or Factor IX dose groups was determined by ELISA from a standard curve. Serum samples were diluted (about 20-2500x for G-CSF and about 10-250x for Factor IX) to ensure samples were within the linear range of the standard curve. As shown in Table 28, G-CSF protein expression determined by ELISA was approximately 17, 1200, and 4700 ng/ml for the 1, 10, and 100 ug dose groups, respectively. As shown in Table 29, Factor IX protein expression determined by ELISA was approximately 36, 380, and 3000-11000 ng/ml for the 1, 10, and 100 ug dose groups, respectively.

Table 28. G-CSF Protein Expression

<table>
<thead>
<tr>
<th>Dose (ug)</th>
<th>Conc (ng/ml)</th>
<th>Dilution Factor</th>
<th>Sample Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.73</td>
<td>20x</td>
<td>5 ul</td>
</tr>
<tr>
<td>10</td>
<td>1204.82</td>
<td>2500x</td>
<td>0.04 ul</td>
</tr>
<tr>
<td>100</td>
<td>4722.20</td>
<td>2500x</td>
<td>0.04 ul</td>
</tr>
</tbody>
</table>

Table 29. Factor IX Protein Expression

<table>
<thead>
<tr>
<th>Dose (ug)</th>
<th>Conc (ng/ml)</th>
<th>Dilution Factor</th>
<th>Sample Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36.05</td>
<td>10x</td>
<td>5 ul</td>
</tr>
<tr>
<td>10</td>
<td>383.04</td>
<td>10x</td>
<td>5 ul</td>
</tr>
<tr>
<td>100*</td>
<td>3247.75</td>
<td>50x</td>
<td>1 ul</td>
</tr>
</tbody>
</table>
As shown in Table 30, the LNP formulations described above have about a 10,000-100,000-fold increase in protein production compared to an administration of an intravenous (IV)-lipoplex formulation for the same dosage of modified mRNA and intramuscular (IM) or subcutaneous (SC) administration of the same dose of modified mRNA in saline. As used in Table 30, the symbol “~” means about.

### Table 30. Protein Production

<table>
<thead>
<tr>
<th></th>
<th>G-CSF</th>
<th>Serum Concentration (pg/ml) 8-12 hours after administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (µg)</td>
<td></td>
</tr>
<tr>
<td>IM</td>
<td>100</td>
<td>~20-80</td>
</tr>
<tr>
<td>SC</td>
<td>100</td>
<td>~10-40</td>
</tr>
<tr>
<td>IV (Lipoplex)</td>
<td>100</td>
<td>~50</td>
</tr>
<tr>
<td>IV (LNP)</td>
<td>100</td>
<td>~5,000,000</td>
</tr>
<tr>
<td>IV (LNP)</td>
<td>10</td>
<td>~1,000,000</td>
</tr>
<tr>
<td>IV (LNP)</td>
<td>1</td>
<td>~20,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Factor IX</th>
<th>Serum Concentration (ng/ml) 8-12 hours after administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (µg)</td>
<td></td>
</tr>
<tr>
<td>IM</td>
<td>2 x 100</td>
<td>~1.6 ng/ml</td>
</tr>
<tr>
<td>IV (LNP)</td>
<td>100</td>
<td>~3,000-10,000 ng/ml</td>
</tr>
<tr>
<td>IV (LNP)</td>
<td>10</td>
<td>~400 ng/ml</td>
</tr>
<tr>
<td>IV (LNP)</td>
<td>1</td>
<td>~40 ng/ml</td>
</tr>
</tbody>
</table>

### Materials and Methods for Examples 17-22

G-CSF (mRNA sequence shown in SEQ ID NO: 6; polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap 1; fully modified with 5-methylcytosine and pseudouridine) and EPO (mRNA sequence shown in SEQ ID NO: 9; polyA tail of approximately 160 nucleotides not shown in sequence; 5’cap, Cap 1; fully modified with 5-methylcytosine and pseudouridine) modified mRNA were formulated as lipid nanoparticles (LNPs) using the syringe pump method. The LNPs were formulated at a 20:1 weight ratio of total lipid to modified mRNA with a final lipid molar ratio of 50:10:38.5:1.5 (DLin-KC2-DMA: DSPC: Cholesterol: PEG-ε-DOMG). Formulations, listed in Table 31, were characterized by particle size, zeta potential, and encapsulation.

### Table 31. Formulations

<table>
<thead>
<tr>
<th>Formulation #</th>
<th>NPA-030-2</th>
<th>NPA-060-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified mRNA</td>
<td>G-CSF</td>
<td>EPO</td>
</tr>
<tr>
<td>Mean size</td>
<td>84 nm</td>
<td>85 nm</td>
</tr>
</tbody>
</table>
Example 17. Lipid nanoparticle in vivo studies with modified mRNA

[00970] LNP formulations, shown in Table 31 (above), were administered to rats (n=5) intravenously (IV), intramuscularly (IM) or subcutaneously (SC) at a single modified mRNA dose of 0.05 mg/kg. A control group of rats (n=4) was untreated. The rats were bled at 2 hours, 8 hours, 24 hours, 48 hours and 96 hours and after they were administered with G-CSF or EPO modified mRNA formulations to determine protein expression using ELISA. The rats administered EPO modified mRNA intravenously were also bled at 7 days.

[00971] As shown in Table 32, EPO protein expression in the rats intravenously administered modified EPO mRNA was detectable out to 5 days. G-CSF in the rats intravenously administered modified G-CSF mRNA was detectable to 7 days. Subcutaneous and intramuscular administration of EPO modified mRNA was detectable to at least 24 hours and G-CSF modified mRNA was detectable to at least 8 hours. In Table 32, “OSC” refers to values that were outside the standard curve and “NT” means not tested.

Table 32. G-CSF and EPO Protein Expression

<table>
<thead>
<tr>
<th>Route</th>
<th>Time</th>
<th>EPO Serum Concentration (pg/ml)</th>
<th>G-CSF Serum Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>2 hours</td>
<td>36,981.0</td>
<td>31,331.9</td>
</tr>
<tr>
<td>IV</td>
<td>8 hours</td>
<td>62,053.3</td>
<td>70,532.4</td>
</tr>
<tr>
<td>IV</td>
<td>24 hours</td>
<td>42,077.0</td>
<td>5,738.6</td>
</tr>
<tr>
<td>IV</td>
<td>48 hours</td>
<td>5,561.5</td>
<td>233.8</td>
</tr>
<tr>
<td>IV</td>
<td>5 days</td>
<td>0.0</td>
<td>60.4</td>
</tr>
<tr>
<td>IV</td>
<td>7 days</td>
<td>0.0</td>
<td>NT</td>
</tr>
<tr>
<td>IM</td>
<td>2 hours</td>
<td>1395.4</td>
<td>1620.4</td>
</tr>
<tr>
<td>IM</td>
<td>8 hours</td>
<td>8974.6</td>
<td>7910.4</td>
</tr>
<tr>
<td>IM</td>
<td>24 hours</td>
<td>4678.3</td>
<td>893.3</td>
</tr>
<tr>
<td>IM</td>
<td>48 hours</td>
<td>NT</td>
<td>OSC</td>
</tr>
<tr>
<td>IM</td>
<td>5 days</td>
<td>NT</td>
<td>OSC</td>
</tr>
<tr>
<td>SC</td>
<td>2 hours</td>
<td>386.2</td>
<td>80.3</td>
</tr>
<tr>
<td>SC</td>
<td>8 hours</td>
<td>985.6</td>
<td>164.2</td>
</tr>
<tr>
<td>SC</td>
<td>24 hours</td>
<td>544.2</td>
<td>OSC</td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>---------</td>
<td>-----</td>
</tr>
<tr>
<td>SC</td>
<td>48 hours</td>
<td>NT</td>
<td>OSC</td>
</tr>
<tr>
<td>SC</td>
<td>5 days</td>
<td>NT</td>
<td>OSC</td>
</tr>
<tr>
<td>Untreated</td>
<td>All bleeds</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Example 18. Time course in vivo study**

[00972] LNP formulations, shown in Table 31 (above), were administered to mice (n=5) intravenously (IV) at a single modified mRNA dose of 0.5, 0.05 or 0.005 mg/kg. The mice were bled at 8 hours, 24 hours, 72 hours and 6 days after they were administered with G-CSF or EPO modified mRNA formulations to determine protein expression using ELISA.

[00973] As shown in Table 33, EPO and G-CSF protein expression in the mice administered with the modified mRNA intravenously was detectable out to 72 hours for the mice dosed with 0.005 mg/kg and 0.05 mg/kg of modified mRNA and out to 6 days for the mice administered the EPO modified mRNA. In Table 33, “>” means greater than and “ND” means not detected.

**Table 33. Protein Expression**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Time</th>
<th>EPO Serum Concentration (pg/ml)</th>
<th>G-CSF Serum Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>8 hours</td>
<td>12,508.3</td>
<td>11,550.6</td>
</tr>
<tr>
<td>0.005</td>
<td>24 hours</td>
<td>6,803.0</td>
<td>5,068.9</td>
</tr>
<tr>
<td>0.005</td>
<td>72 hours</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.005</td>
<td>6 days</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.05</td>
<td>8 hours</td>
<td>92,139.9</td>
<td>462,312.5</td>
</tr>
<tr>
<td>0.05</td>
<td>24 hours</td>
<td>54,389.4</td>
<td>80,903.8</td>
</tr>
<tr>
<td>0.05</td>
<td>72 hours</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.05</td>
<td>6 days</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>0.5</td>
<td>8 hours</td>
<td>498,515.3</td>
<td>&gt;1,250,000</td>
</tr>
<tr>
<td>0.5</td>
<td>24 hours</td>
<td>160,566.3</td>
<td>495,812.5</td>
</tr>
<tr>
<td>0.5</td>
<td>72 hours</td>
<td>3,492.5</td>
<td>1,325.6</td>
</tr>
<tr>
<td>0.5</td>
<td>6 days</td>
<td>21.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Example 19. LNP formulations in vivo study in rodents**

A. LNP Formulations in Mice

[00974] LNP formulations, shown in Table 31 (above), were administered to mice (n=4) intravenously (IV) at a single modified mRNA dose 0.05 mg/kg or 0.005 mg/kg. There was also 3 control groups of mice (n=4) that were untreated. The mice were bled at 2 hours, 8 hours, 24 hours, 48 hours and 72 hours after they were administered with G-CSF or EPO modified mRNA.
formulations to determine the protein expression. Protein expression of G-CSF and EPO were determined using ELISA.

[00975] As shown in Table 34, EPO and G-CSF protein expression in the mice was detectable at least out to 48 hours for the mice that received a dose of 0.005 mg/kg modified RNA and 72 hours for the mice that received a dose of 0.05 mg/kg modified RNA. In Table 34, “OSC” refers to values that were outside the standard curve and “NT” means not tested.

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Time</th>
<th>EPO Serum Concentration (pg/ml)</th>
<th>G-CSF Serum Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>2 hours</td>
<td>OSC</td>
<td>3,447.8</td>
</tr>
<tr>
<td>0.005</td>
<td>8 hours</td>
<td>1,632.8</td>
<td>11,454.0</td>
</tr>
<tr>
<td>0.005</td>
<td>24 hours</td>
<td>1,141.0</td>
<td>4,960.2</td>
</tr>
<tr>
<td>0.005</td>
<td>48 hours</td>
<td>137.4</td>
<td>686.4</td>
</tr>
<tr>
<td>0.005</td>
<td>72 hours</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>0.05</td>
<td>2 hours</td>
<td>10,027.3</td>
<td>20,951.4</td>
</tr>
<tr>
<td>0.05</td>
<td>8 hours</td>
<td>56,547.2</td>
<td>70,012.8</td>
</tr>
<tr>
<td>0.05</td>
<td>24 hours</td>
<td>25,027.3</td>
<td>19,356.2</td>
</tr>
<tr>
<td>0.05</td>
<td>48 hours</td>
<td>1,432.3</td>
<td>1,963.0</td>
</tr>
<tr>
<td>0.05</td>
<td>72 hours</td>
<td>82.2</td>
<td>47.3</td>
</tr>
</tbody>
</table>

B. LNP Formulations in Rats

[00976] LNP formulations, shown in Table 31 (above), are administered to rats (n=4) intravenously (IV) at a single modified mRNA dose 0.05 mg/kg. There is also a control group of rats (n=4) that are untreated. The rats are bled at 2 hours, 8 hours, 24 hours, 48 hours, 72 hours, 7 days and 14 days after they were administered with G-CSF or EPO modified mRNA formulations to determine the protein expression. Protein expression of G-CSF and EPO are determined using ELISA.

Example 20. Early Time Course Study of LNPs

[00977] LNP formulations, shown in Table 31 (above), are administered to mammals intravenously (IV), intramuscularly (IM) or subcutaneously (SC) at a single modified mRNA dose of 0.5 mg/kg, 0.05 mg/kg or 0.005 mg/kg. A control group of mammals are not treated. The mammals are bled at 5 minutes, 10 minutes, 20 minutes, 30 minutes, 45 minutes, 1 hour, 1.5 hours and/or 2 hours after they are administered with the modified mRNA LNP formulations to determine protein expression using ELISA. The mammals are also bled to determine the complete blood count such as the granulocyte levels and red blood cell count.

Example 21. Non-human primate in vivo study
LNP formulations, shown in Table 31 (above), were administered to non-human primates (NHP) (cynomolgus monkey) (n=2) as a bolus intravenous injection (IV) over approximately 30 seconds using a hypodermic needle, which may be attached to a syringe/abbocath or butterfly if needed. The NHP were administered a single modified mRNA IV dose of 0.05mg/kg of EPO or G-CSF or 0.005 mg/kg of EPO in a dose volume of 0.5 mL/kg. The NHPs were bled 5-6 days before dosing with the modified mRNA LNP formulations to determine protein expression in the serum and a baseline complete blood count. After administration with the modified mRNA formulation the NHP were bled at 8, 24, 48 and 72 hours to determined protein expression. At 24 and 72 hours after administration the complete blood count of the NHP was also determined. Protein expression of G-CSF and EPO was determined by ELISA. Urine from the NHPs was collected over the course of the entire experiment and analyzed to evaluate clinical safety. Samples were collected from the NHPs after they were administered with G-CSF or EPO modified mRNA formulations to determine protein expression using ELISA. Clinical chemistry, hematology, urinalysis and cytokines of the non-human primates were also analyzed.

As shown in Table 35, EPO protein expression in the NHPs administered 0.05 mg/kg is detectable out to 72 hours and the 0.005 mg/kg dosing of the EPO formulation is detectable out to 48 hours. In Table 35, the “<” means less than a given value. G-CSF protein expression was seen out to 24 hours after administration with the modified mRNA formulation. Preliminary, there was an increase in granulocytes and reticulocytes levels seen in the NHP after administration with the modified mRNA formulations.

Table 35. Protein Expression in Non-Human Primates

<table>
<thead>
<tr>
<th>Modified mRNA</th>
<th>Dose (mg/kg)</th>
<th>Time</th>
<th>Female NHP Serum Concentration (pg/ml)</th>
<th>Male NHP Serum Concentration (pg/ml)</th>
<th>Average Serum Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>0.05</td>
<td>Pre-bleed</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 hours</td>
<td>3289</td>
<td>1722</td>
<td>2.506</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
<td>722</td>
<td>307</td>
<td>515</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48 hours</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 hours</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EPO</td>
<td>0.05</td>
<td>Pre-bleed</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Time</td>
<td>8 hours</td>
<td>24 hours</td>
<td>48 hours</td>
<td>72 hours</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
<td>----------</td>
<td>----------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>19,858</td>
<td>18,178</td>
<td>5,291</td>
<td>744</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7,072</td>
<td>4,913</td>
<td>498</td>
<td>60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13,465</td>
<td>11,546</td>
<td>2,895</td>
<td>402</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Example 22. Non-human primate in vivo study for G-CSF and EPO**

[00980] LNP formulations, shown in Table 31 (above), were administered to non-human primates (NHP) (cynomolgus monkey) (n=2) as intravenous injection (IV). The NHP were administered a single modified mRNA IV dose of 0.5 mg/kg, 0.05mg/kg or 0.005 mg/kg of G-CSF or EPO in a dose volume of 0.5 mL/kg. The NHPs were bled before dosing with the modified mRNA LNP formulations to determine protein expression in the serum and a baseline complete blood count. After administration with the G-CSF modified mRNA formulation the NHP were bled at 8, 24, 48 and 72 hours to determined protein expression. After administration with the EPO modified mRNA formulation the NHP were bled at 8, 24, 48, 72 hours and 7 days to determined protein expression.

[00981] Samples collected from the NHPs after they were administered with G-CSF or EPO modified mRNA formulations were analyzed by ELISA to determine protein expression. Neutrophil and reticulocyte count was also determined pre-dose, 24 hours, 3 days, 7 days, 14 days and 18 days after administration of the modified G-CSF or EPO formulation.

[00982] As shown in Table 36, G-CSF protein expression was not detected beyond 72 hours. In Table 36, “<39” refers to a value below the lower limit of detection of 39 pg/ml.

### Table 36. G-CSF Protein Expression

<table>
<thead>
<tr>
<th>Modified mRNA</th>
<th>Dose (mg/kg)</th>
<th>Time</th>
<th>Female NHP Serum G-CSF Concentration (pg/ml)</th>
<th>Male NHP Serum G-CSF Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>0.5</td>
<td>Pre-bleed</td>
<td>&lt;39</td>
<td>&lt;39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 hours</td>
<td>43,525</td>
<td>43,594</td>
</tr>
<tr>
<td>G-CSF</td>
<td>0.05</td>
<td>24 hours</td>
<td>11,374</td>
<td>3,628</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>----------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48 hours</td>
<td>1,100</td>
<td>833</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 hours</td>
<td>&lt;39</td>
<td>306</td>
</tr>
<tr>
<td></td>
<td>Pre-bleed</td>
<td>&lt;39</td>
<td>&lt;39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 hours</td>
<td>3,289</td>
<td>1,722</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>722</td>
<td>307</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td>&lt;39</td>
<td>&lt;39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 hours</td>
<td>&lt;39</td>
<td>&lt;39</td>
<td></td>
</tr>
<tr>
<td>G-CSF</td>
<td>0.005</td>
<td>Pre-bleed</td>
<td>&lt;39</td>
<td>&lt;39</td>
</tr>
<tr>
<td></td>
<td>8 hours</td>
<td>559</td>
<td>700</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>155</td>
<td>&lt;39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48 hours</td>
<td>&lt;39</td>
<td>&lt;39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72 hours</td>
<td>&lt;39</td>
<td>&lt;39</td>
<td></td>
</tr>
</tbody>
</table>

[00983] As shown in Table 37, EPO protein expression was not detected beyond 7 days. In Table 37, “<7.8” refers to a value below the lower limit of detection of 7.8 pg/ml.

**Table 37. EPO Protein Expression**

<table>
<thead>
<tr>
<th>Modified mRNA</th>
<th>Dose (mg/kg)</th>
<th>Time</th>
<th>Female NHP Serum EPO Concentration (pg/ml)</th>
<th>Male NHP Serum EPO Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPO</td>
<td>0.5</td>
<td>Pre-bleed</td>
<td>&lt;7.8</td>
<td>&lt;7.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 hours</td>
<td>158,771</td>
<td>119,086</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
<td>133,978</td>
<td>85,825</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48 hours</td>
<td>45,250</td>
<td>64,793</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 hours</td>
<td>15,097</td>
<td>20,407</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 days</td>
<td>&lt;7.8</td>
<td>&lt;7.8</td>
</tr>
<tr>
<td>EPO</td>
<td>0.05</td>
<td>Pre-bleed</td>
<td>&lt;7.8</td>
<td>&lt;7.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 hours</td>
<td>19,858</td>
<td>7,072</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 hours</td>
<td>18,187</td>
<td>4,913</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48 hours</td>
<td>5,291</td>
<td>498</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72 hours</td>
<td>744</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 days</td>
<td>&lt;7.8</td>
<td>&lt;7.8</td>
</tr>
</tbody>
</table>
As shown in Table 38, there was an increase in neutrophils in all G-CSF groups relative to pre-dose levels.

### Table 38. Pharmacologic Effect of G-CSF mRNA in NHP

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Time</th>
<th>Male NHP (G-CSF) Neutrophils (10^9/L)</th>
<th>Female NHP (G-CSF) Neutrophils (10^9/L)</th>
<th>Male NHP (EPO) Neutrophils (10^9/L)</th>
<th>Female NHP (EPO) Neutrophils (10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>Pre-dose</td>
<td>1.53</td>
<td>1.27</td>
<td>9.72</td>
<td>1.82</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>14.92</td>
<td>13.96</td>
<td>7.5</td>
<td>11.85</td>
</tr>
<tr>
<td></td>
<td>3 days</td>
<td>9.76</td>
<td>13.7</td>
<td>11.07</td>
<td>5.22</td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td>2.74</td>
<td>3.81</td>
<td>11.8</td>
<td>2.85</td>
</tr>
<tr>
<td></td>
<td>14/18 days</td>
<td>2.58</td>
<td>1.98</td>
<td>7.16</td>
<td>2.36</td>
</tr>
<tr>
<td>0.05</td>
<td>Pre-dose</td>
<td>13.74</td>
<td>3.05</td>
<td>0.97</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>19.92</td>
<td>29.91</td>
<td>2.51</td>
<td>2.63</td>
</tr>
<tr>
<td></td>
<td>3 days</td>
<td>7.49</td>
<td>10.77</td>
<td>1.73</td>
<td>4.08</td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td>4.13</td>
<td>3.8</td>
<td>1.23</td>
<td>2.77</td>
</tr>
<tr>
<td></td>
<td>14/18 days</td>
<td>3.59</td>
<td>1.82</td>
<td>1.53</td>
<td>1.27</td>
</tr>
<tr>
<td>0.005</td>
<td>Pre-dose</td>
<td>1.52</td>
<td>2.54</td>
<td>5.46</td>
<td>5.96</td>
</tr>
<tr>
<td></td>
<td>24 hours</td>
<td>16.44</td>
<td>8.6</td>
<td>5.37</td>
<td>2.59</td>
</tr>
<tr>
<td></td>
<td>3 days</td>
<td>3.74</td>
<td>1.78</td>
<td>6.08</td>
<td>2.83</td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td>7.28</td>
<td>2.27</td>
<td>3.51</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td>14/18 days</td>
<td>4.31</td>
<td>2.28</td>
<td>1.52</td>
<td>2.54</td>
</tr>
</tbody>
</table>

As shown in Table 39, there was an increase in reticulocytes in all EPO groups 3 days to 14/18 days after dosing relative to reticulocyte levels 24 hours after dosing.
DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 293

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 293

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:
WHAT IS CLAIMED IS:

1. A method of producing a polypeptide of interest in a mammalian cell or tissue, the method comprising, contacting said mammalian cell or tissue with a formulation comprising a modified mRNA encoding the polypeptide of interest, wherein the formulation is selected from the group consisting of nanoparticles, poly(lactic-co-glycolic acid) (PLGA) microspheres, lipidoid, lipoplex, liposome, polymers, carbohydrates (including simple sugars), cationic lipids, fibrin gel, fibrin hydrogel, fibrin glue, fibrin sealant, fibrinogen, thrombin, rapidly eliminated lipid nanoparticles (reLNPs) and combinations thereof.

2. The method of claim 1, wherein the modified mRNA comprises a purified IVT transcript.

3. The method of claim 1, wherein the formulation comprising the modified mRNA is a nanoparticle and wherein said nanoparticle comprises at least one lipid.

4. The method of claim 3, wherein the lipid is selected from the group consisting of DLin-DMA, DLin-K-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, PLGA, PEG, PEG-DMG and PEGylated lipids.

5. The method of claim 3, wherein the lipid is cationic lipid.

6. The method of claim 5, wherein the cationic lipid is selected from the group consisting of DLin-DMA, DLin-K-DMA, DLin-MC3-DMA, DLin-KC2-DMA and DODMA.

7. The method of claim 3, wherein the lipid to modified mRNA weight ratio is between 10:1, and 30:1.

8. The method of claim 7, wherein the mean size of the nanoparticle formulation comprising the modified mRNA is between 60-225 nm.

9. The method of claim 8, wherein the PDI of the nanoparticle formulation comprising the modified mRNA is between 0.03 and 0.15.

10. The method of claim 3, wherein the zeta potential of the lipid is from -10 to +10 at a pH of 7.4.
11. The method of claim 7, wherein the nanoparticle formulation comprising the modified mRNA further comprises a fusogenic lipid, cholesterol, and a PEG lipid.

12. The method of claim 11, wherein the nanoparticle formulation comprising the modified mRNA has a molar ratio of 50:10:38.5:1.5-3.0 (Cationic Lipid: fusogenic lipid: Cholesterol: PEG lipid).

13. The method of claim 12, wherein the PEG lipid is selected from PEG-c-DOMG and PEG-DMG and the fusogenic lipid is DSPC.

14. The method of claim 1, wherein contacting is through the use of a device selected from the group consisting of syringe pump, internal osmotic pump and external osmotic pump.

15. The method of claim 1, wherein the formulation comprising the modified mRNA is a poly(lactic-co-glycolic acid) (PLGA) microsphere.

16. The method of claim 15, wherein microspheres of the PLGA microsphere formulation comprising the modified mRNA are between 4 and 20 μm in size.

17. The method of claim 15, wherein the PLGA microsphere formulation comprising the modified mRNA release less than 50% of the modified mRNA in a 48 hour time period.

18. The method of claim 15, wherein the PLGA microsphere formulation comprising the modified mRNA is stable in serum.

19. The method of claim 18, wherein the stability is determined relative to unformulated modified mRNA in 90% serum.

20. The method of claim 15, wherein the loading weight percent is at least 0.05%, at least 0.1%, at least 0.2%, at least 0.3%, or at least 0.4%.

21. The method of claim 15, wherein the encapsulation efficiency of the modified mRNA in the PLGA microspheres is at least 50%.

22. The method of claim 15, wherein the encapsulation efficiency of the modified mRNA in the PLGA microspheres is at least 70%.
23. The method of claim 15, wherein the encapsulation efficiency of the modified mRNA in the PLGA microspheres is at least 90%.

24. The method of claim 15, wherein the encapsulation efficiency of the modified mRNA in the PLGA microspheres is at least 97%.

25. The method of claim 11, wherein contacting said mammalian cells or tissues occurs via a route of administration selected from the group consisting of intravenous, intramuscular, intravitreal, intrathecal, intratumoral, pulmonary, and subcutaneous.

26. The method of claim 25, wherein the polypeptide of interest is detectable in the serum for up to 72 hours after contacting at levels higher than the levels prior to contacting.

27. The method of claim 26, wherein the polypeptide of interest is detectable in the serum of female subjects at levels greater than in the serum of male subjects.

28. The method of claim 1, wherein the formulation further comprises a second modified mRNA.

29. The method of claim 28, wherein the formulation further comprises a third modified mRNA.

30. The method of claim 1, wherein the formulation comprising the modified mRNA comprises a rapidly eliminated lipid nanoparticle.

31. The method of claim 30, wherein the rapidly eliminated lipid nanoparticle comprises an reLNP lipid, fusogenic lipid, cholesterol and a PEG lipid at a molar ratio of 50:10:38.5:1.5(reLNP Lipid: Fusogenic lipid: Cholesterol: PEG lipid).

32. The method of claim 31, wherein the fusogenic lipid is DSPC and the PEG lipid is PEG-c-DOMG.

33. The method of claim 31 wherein the reLNP lipid is selected from the group consisting of DLin-DMA with an internal ester, DLin-DMA with a terminal ester, DLin-MC3-DMA-with an internal ester, and DLin-MC3-DMA with a terminal ester.
34. The method of claim 30, wherein in the total lipid to modified mRNA weight ratio is between 10:1 and 30:1.

35. The method of claim 1, wherein contacting occurs via injection using a split dosing schedule.

36. The method of claim 35, wherein the injection is made to the tissue selected from the group consisting of intradermal space, epidermis, subcutaneous tissue, and muscle.

37. The method of claim 1, wherein the formulation comprising the modified mRNA comprises a fibrin sealant.

38. The method of claim 1, wherein the formulation comprising the modified mRNA comprises a lipidoid and wherein the lipid is selected from the group consisting of C12-200 and 98N12-5.

39. The method of claim 1, wherein the formulation comprising the modified mRNA is a polymer and said polymer is coated, covered, surrounded, enclosed or comprises a layer of hydrogel or surgical sealant.

40. The method of claim 39, wherein the polymer is selected from the group consisting of PLGA, ethylene vinyl acetate, poloxamer and GELSITE®.

41. The method of claim 40, further comprising an additional layer of polymer, hydrogel or surgical sealant.

42. The method of claim 2, wherein the modified mRNA comprises at least one 5’ terminal cap selected from the group consisting of Cap0, Cap1, ARCA, inosine, N1-methyl-guanosine, 2’fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

43. The method of claim 42, wherein the 5’ terminal cap is Cap1.

44. The method of claim 43, wherein the modified mRNA comprises at least two modifications.

45. The method of claim 44, wherein the at least two modifications are independently selected from the group consisting of 5-methylcytidine, pseudouridine, and 1-methyl-pseudouridine.
46. A method of producing a polypeptide of interest in a mammalian cell or tissue, the method comprising, contacting said mammalian cell or tissue with a buffer formulation comprising a modified mRNA encoding the polypeptide of interest.

47. The method of claim 46, wherein the buffer formulation is selected from the group consisting of saline, phosphate buffered saline, and Ringer’s lactate.

48. The method of claim 46, wherein the buffer formulation comprises a calcium concentration of between 1-10 mM.

49. The method of claim 46, wherein the modified mRNA comprises a purified IVT transcript.

50. The method of claim 46, wherein contacting said mammalian cells or tissues occurs via a route of administration selected from the group consisting of intravenous, intramuscular, intravitreal, intrathecal, intratumoral, pulmonary, and subcutaneous.

51. The method of claim 25 or 50, wherein said polypeptide of interest is produced in said cell or tissue in a location systemic from the location of contacting.

52. The method of claim 51 wherein the route of administration is either via intramuscular or subcutaneous.

53. The method of claim 3, wherein the lipid nanoparticle formulation is further formulated in a sealant.

54. The method of claim 53, wherein said sealant is a fibrin sealant.

55. A method of producing a pharmacologic effect in a primate comprising contacting said primate with a composition comprising a formulated modified mRNA encoding a polypeptide of interest.

56. The method of claim 55, wherein the modified mRNA comprises a purified IVT transcript.

57. The method of claim 56, wherein the formulation is selected from the group consisting of nanoparticles, poly(lactic-co-glycolic acid) (PLGA) microspheres, lipidoid, lipoplex, liposome, polymers, carbohydrates (including simple sugars), cationic lipids, fibrin gel, fibrin
hydrogel, fibrin glue, fibrin sealant, fibrinogen, thrombin, rapidly eliminated lipid nanoparticles (reLNPs) and combinations thereof.

58. The method of claim 57, wherein the pharmacologic effect is greater than the pharmacologic effect associated with a therapeutic agent known to produce said pharmacologic effect.

59. The method of claim 57, wherein the pharmacologic effect is greater than the pharmacologic effect produced by a composition comprising an unformulated modified mRNA encoding the polypeptide of interest.

60. The method of claim 57, wherein the pharmacologic effect is greater than the pharmacologic effect produced by a composition comprising a formulated unmodified mRNA encoding the polypeptide of interest.

61. The method of claim 57, wherein the pharmacologic effect results in a therapeutically effective outcome of a disease, disorder, condition or infection.

62. The method of claim 61, wherein the pharmacologic effect is selected from the group consisting of a change in cell count, alteration in serum chemistry, alteration of enzyme activity, increase in hemoglobin, and increase in hematocrit.

63. The method of claim 62, wherein the therapeutically effective outcome is selected from the group consisting of treatment, improvement of one or more symptoms, diagnosis, prevention, and delay of onset.
98N12-5 (TETA5-LAP)

DLin-DMA

DLin-K-DMA (2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane)

DLin-KC2-DMA

DLin-MC3-DMA

C12-200

PRIOR ART
Only single cutters are shown in the map.
Figure 3