



(43) International Publication Date
22 November 2012 (22.11.2012)

(10) International Publication Number
WO 2012/158736 A1

- (51) International Patent Classification:
C12P 21/06 (2006.01)
- (21) International Application Number:
PCT/US2012/038028
- (22) International Filing Date:
16 May 2012 (16.05.2012)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
61/519,158 17 May 2011 (17.05.2011) US
- (71) Applicant (for all designated States except US): **MOD-ERNA THERAPEUTICS** [US/US]; 161 First Street, First Floor, Cambridge, Massachusetts 02142 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **AFEYAN, Noubar B.** [US/US]; 1 Memorial Drive, 7th Floor, Cambridge, Massachusetts 02142 (US). **SIECZKIEWICZ, Gregory J.** [US/US]; 1 Memorial Drive, 7th Floor, Cambridge, Massachusetts 02142 (US). **BANCEL, Stephane** [FR/US]; 68 Pinckney Road, Boston, Massachusetts 02114 (US).
- (74) Agent: **WARD, Donna T.**; DT Ward, P.C., 292 Main Street, Suite 2, Groton, Massachusetts 01450 (US).
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

Published:

- with international search report (Art. 21(3))

(54) Title: ENGINEERED NUCLEIC ACIDS AND METHODS OF USE THEREOF FOR NON-HUMAN VERTEBRATES

(57) Abstract: Provided are formulations, compositions, kits and methods for delivering biological moieties such as modified nucleic acids into cells to induce, reduce or modulate protein expression in non-human vertebrates.



WO 2012/158736 A1

**ENGINEERED NUCLEIC ACIDS AND METHODS OF USE THEREOF FOR NON-HUMAN
VERTEBRATES**

CROSS REFERENCE TO RELATED APPLICATIONS

5 This application claims priority to U.S.S.N. 61/519,158 filed on May 17, 2011, the contents of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

10 The invention relates to compositions, methods, processes, kits and devices for the design, preparation, manufacture and/or formulation of modified nucleic acid molecules and/or enhanced nucleic acid molecules for non-human vertebrates.

BACKGROUND OF THE INVENTION

15 Methods and devices for administering active agents such as therapeutic and/or bioactive substances to non-human vertebrates, particularly livestock, are known in the art, including tablets, solutions for oral administration and injection and topical administration by means including pour-on and spot-on formulations. For administering active agents to ruminant animals, formulations, such as capsules, have been adapted to be located and retained in the rumen. These formulations provide a gradual release of a therapeutic and/or bioactive substance
20 into the rumen over varying time periods. Such formulations are only appropriate for administering substances which are capable of being absorbed from the gastrointestinal tract. However, such formulations are not desirable when the therapeutic and/or bioactive agent may potentially be toxic to the animal(s), when the administration of an agent such as anti-parasitics or antibiotics, may cause the animal(s) to develop a resistance to the therapeutic and/or bioactive
25 agent or the therapeutic and/or bioactive agent may induce an altered physiological state in the animal(s) potentially harming the well-being of the animal(s).

 Currently, protein-based administered therapeutics such as growth factors, cytokines and antibodies in veterinary applications have raised concerns relating to immunogenicity, efficacy and cost. For example, introduced DNA can integrate into host cell genomic DNA at some
30 frequency, resulting in alterations and/or damage to the host cell genomic DNA. Alternatively, the heterologous deoxyribonucleic acid (DNA) introduced into a cell can be inherited by

daughter cells (whether or not the heterologous DNA has integrated into the chromosome) or by offspring.

In addition, assuming proper delivery and no damage or integration into the host genome, there are multiple steps which must occur before the encoded protein is made. Once inside the cell, DNA must be transported into the nucleus where it is transcribed into RNA. The RNA transcribed from DNA must then enter the cytoplasm where it is translated into protein. Not only do the multiple processing steps from administered DNA to protein create lag times before the generation of the functional protein, each step represents an opportunity for error and damage to the cell. Further, it is known to be difficult to obtain DNA expression in cells as DNA frequently enters a cell but is not expressed or not expressed at reasonable rates or concentrations. This can be a particular problem when DNA is introduced into primary cells or modified cell lines.

The present invention overcomes these concerns by providing nucleic acid based compounds or polynucleotides which encode a polypeptide of interest (e.g., modified mRNA or modified nucleic acids) and which have structural and/or chemical features that avoid one or more of the problems in the art, for example, features which are useful for optimizing formulation and delivery of nucleic acid-based therapeutics while retaining structural and functional integrity, overcoming the threshold of expression, improving expression rates, half life and/or protein concentrations, optimizing protein localization, and avoiding deleterious bio-responses such as the immune response and/or degradation pathways.

SUMMARY OF THE INVENTION

Described herein are compositions, methods, processes, kits and devices for the design, preparation, manufacture and/or formulation of modified nucleic acid or enhanced nucleic acid molecules.

The details of various embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and the drawings, and from the claims.

In one embodiment, the present invention provides a method of producing a polypeptide of interest in a cell, tissue or bodily fluid of a non-human vertebrate subject in need thereof by administering a pharmaceutical composition comprising a nucleic acid encoding the polypeptide

of interest. The pharmaceutical composition may be formulated such as, but not limited to, in saline and/or a lipid formulation. The formulation may be administered by a route such as, but not limited to, intravenous, intramuscular, subcutaneous and local. The formulation may be administered on a schedule selected from three times a day, twice a day, once a day, every other day, every third day, weekly, biweekly, every three weeks, every four weekly, and monthly. Further, the formulation may be administered by multiple administrations.

In one embodiment, the non-human vertebrate may be selected from alpaca, banteng, bison, camel, cat, cattle, deer, dog, donkey, elk, gayal, goat, guinea pig, horse, llama, mouse, mule, pig, rabbit, rat, reindeer, sheep water buffalo, yak, caiques, canary, cattle egret, chicken, cockatiel, cockatoo, conure, dove, duck, finch, geese, lovebird, macaw, parakeet, parrot, parrotlet, pigeon, pious, rosella, turkey, iguana, lizard, snake, turtle, tortoise, caecilian, frog, newt, salamander, and toad. In a further embodiment, the non-human vertebrate is a mouse which can be a transgenic, knock-in and/or a knock-out mouse.

In one embodiment, the polypeptide of interest may be provided in a bodily fluid such as, but not limited to, peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous humor, amniotic fluid, cerumen, breast milk, bronchoalveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, sweat, fecal matter, hair, tears, cyst fluid, pleural and peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, blastocyl cavity fluid, and umbilical cord blood.

In one embodiment, the polypeptide of interest may be provided in a tissue such as, but not limited to, liver, spleen, kidney, lung, heart, peri-renal adipose tissue, thymus and muscle.

The polypeptide of interest considered by the present invention may include, but is not limited to, insulin, feline interferon, erythropoietin, cyclosporine, Thymosin Beta-4, arginine vasopressin, bovine somatotropin, oxytocin, ghrelin, gonadorelin, pregnant mare serum gonadotrophin (PMSG), equine chorionic gonadotrophin (ECG), human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone analog (GRHa), pancreatic enzymes, Cre recombinase, an insulin-like growth factor, hGH, tPA, Interleukin (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, IFN tau, tumor necrosis factor (TNF) alpha, TNF beta, TNF

gamma, TRAIL, G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF.

In one embodiment, pharmaceutical composition includes a nucleic acid with one or more modifications. The modifications may include, but are not limited to, pyridin-4-one ribonucleoside, 5-aza-uridine, 2-thio-5-aza-uridine, 2-thiouridine, 4-thio-pseudouridine, 2-thio-pseudouridine, 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, dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 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-formylcytidine, 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, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-thio-zebularine, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, 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-glycinylicarbamoyladenosine, N6-threonylicarbamoyladenosine, 2-methylthio-N6-threonylicarbamoyladenosine, N6,N6-dimethyladenosine, 7-methyladenine, 2-methylthio-adenine, and 2-methoxy-adenine, inosine, 1-methyl-inosine, 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, and combinations thereof.

In one embodiment, the present invention provides a kit for producing a first polypeptide

of interest in a cell, tissue and/or bodily fluid of a non-human vertebrate in need thereof. In a further embodiment, the kit may comprise a second nucleic acid which may encode a second polypeptide of interest. The second polypeptide of interest may be the same or different than the first polypeptide of interest.

5

DETAILED DESCRIPTION OF THE INVENTION

It is of great interest for therapeutics, diagnostics, reagents and for biological assays to deliver a nucleic acid, e.g., a ribonucleic acid (RNA) inside a cell, either *in vivo* or *ex vivo*, such as to cause intracellular translation of the nucleic acid and production of the encoded

10 polypeptide. Of particular importance is the delivery and function of a non-integrative nucleic acid to a non-human vertebrate.

Provided herein are compositions (including pharmaceutical compositions) and methods for the design, preparation, manufacture and/or formulation of nucleic acids encoding polypeptides capable of functioning as biological moieties of interest in a non-human vertebrate

15 subject. As described herein, these nucleic acids 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.

Modified nucleic acids

The present invention provides nucleic acids, including RNAs such as mRNAs that

20 contain one or more modified nucleosides (termed "modified nucleic acids"), which have useful properties including the lack of a substantial induction of the innate immune response of a cell into which the mRNA is introduced. Because these modified nucleic acids enhance the efficiency of protein production, intracellular retention of nucleic acids, and viability of contacted cells, as well as possess reduced immunogenicity, these nucleic acids having these

25 properties are termed "enhanced nucleic acids" herein.

The term "nucleic acid," in its broadest sense, 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 invention include, but are not limited to, one or more of DNA, RNA including messenger mRNA (mRNA), hybrids thereof, RNAi-inducing agents, RNAi agents,

30 siRNAs, shRNAs, miRNAs, antisense RNAs, ribozymes, catalytic DNA, RNAs that induce triple helix formation, aptamers, vectors, *etc.*, described in detail herein.

Modifications to the Nucleic Acids

Provided are modified nucleic acids containing a translatable region and one, two, or more than two different nucleoside modifications. In some embodiments, the modified nucleic acid exhibits reduced degradation in a cell into which the nucleic acid is introduced, relative to a
 5 corresponding unmodified nucleic acid. Exemplary nucleic acids include ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), or a hybrid thereof. In preferred embodiments, the modified nucleic acid includes messenger RNAs (mRNAs). As described herein, the nucleic acids of the invention do not substantially induce an innate immune response of a cell into which the mRNA is introduced.

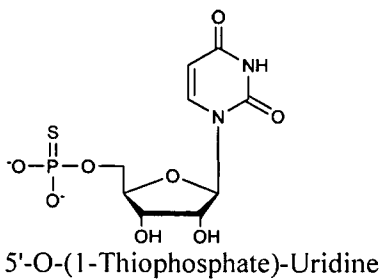
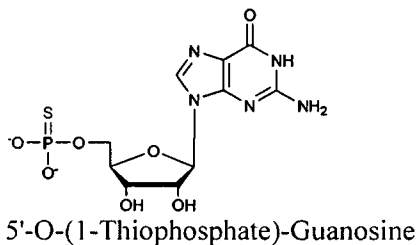
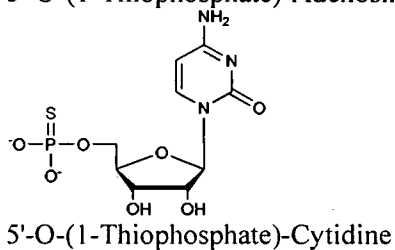
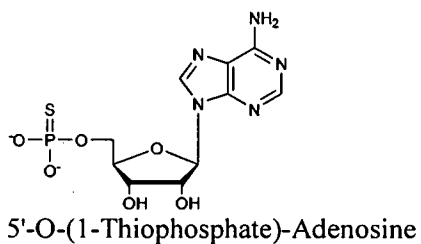
10 In some embodiments, modified nucleosides include pyridin-4-one ribonucleoside, 5-aza-uridine, 2-thio-5-aza-uridine, 2-thiouridine, 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxyuridine, 3-methyluridine, 5-carboxymethyl-uridine, 1-carboxymethyl-pseudouridine, 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-taurinomethyluridine, 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uridine, 1-taurinomethyl-4-thio-uridine, 5-methyl-uridine,
 15 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, dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 2-methoxyuridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, and 4-methoxy-2-thio-pseudouridine.

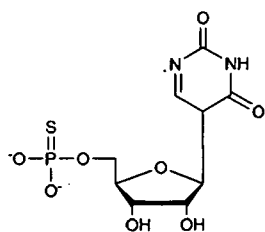
In some embodiments, modified nucleosides include 5-aza-cytidine, pseudoisocytidine,
 20 3-methyl-cytidine, N4-acetylcytidine, 5-formylcytidine, N4-methylcytidine, 5-hydroxymethylcytidine, 1-methyl-pseudoisocytidine, pyrrolo-cytidine, pyrrolo-pseudoisocytidine, 2-thio-cytidine, 2-thio-5-methyl-cytidine, 4-thio-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-1-deaza-pseudoisocytidine, 1-methyl-1-deaza-pseudoisocytidine, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-
 25 thio-zebularine, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-methoxy-pseudoisocytidine, and 4-methoxy-1-methyl-pseudoisocytidine.

In other embodiments, modified nucleosides include 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-
 30 methyladenosine, N6-isopentenyladenosine, N6-(cis-hydroxyisopentenyl)adenosine, 2-methylthio-N6-(cis-hydroxyisopentenyl) adenosine, N6-glycinylicarbamoyladenosine, N6-

threonylcarbamoyladenosine, 2-methylthio-N6-threonyl carbamoyladenosine, N6,N6-dimethyladenosine, 7-methyladenine, 2-methylthio-adenine, and 2-methoxy-adenine.

In specific embodiments, a modified nucleoside is 5'-O-(1-Thiophosphate)-Adenosine, 5'-O-(1-Thiophosphate)-Cytidine, 5'-O-(1-Thiophosphate)-Guanosine, 5'-O-(1-Thiophosphate)-Uridine or 5'-O-(1-Thiophosphate)-Pseudouridine.





5'-O-(1-Thiophosphate)-Pseudouridine

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 nucleic acids are expected to also reduce the innate immune response through weaker binding/activation of cellular innate immune molecules.

In certain embodiments it is desirable to intracellularly degrade a modified nucleic acid introduced into the cell, for example if precise timing of protein production is desired. Thus, the invention provides a modified nucleic acid containing a degradation domain, which is capable of being acted on in a directed manner within a cell.

In other embodiments, modified nucleosides include inosine, 1-methyl-inosine, 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.

Optional Components of the Modified Nucleic Acids

In further embodiments, the modified nucleic acids may include other optional components, which can be beneficial in some embodiments. These optional components include, but are not limited to, untranslated regions, kozak sequences, intronic nucleotide sequences, internal ribosome entry site (IRES), caps and polyA tails. For example, a 5' untranslated region (UTR) and/or a 3'UTR may be 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 nucleic acids containing a Kozak sequence.

Additionally, provided are nucleic acids containing one or more intronic nucleotide sequences capable of being excised from the nucleic acid.

Untranslated Regions (UTRs)

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 the stop codon and continues until the transcriptional termination signal. There is growing body of evidence about the regulatory roles played by the UTRs in terms of stability of the nucleic acid molecule and translation. The regulatory features of a UTR can be incorporated into the modified nucleic acids of the present invention to increase 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' 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'-triphosphate 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-methyl-guanylate residue. The ribose sugars of the terminal and/or anteterminal 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.

IRES Sequences

Further, provided are nucleic acids containing an internal ribosome entry site (IRES). An IRES may act as the sole ribosome binding site, or may serve as one of multiple ribosome binding sites of an mRNA. An mRNA containing more than one functional ribosome binding site may encode several peptides or polypeptides that are translated independently by the ribosomes ("multicistronic mRNA"). When nucleic acids 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), polio viruses (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

During RNA processing, a long chain of adenine nucleotides (poly-A tail) may be added to a polynucleotide such as an 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 100 and 250 residues long.

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 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 nucleic acid. The poly-A tail may also be designed as a fraction of modified nucleic acids 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 modified nucleic acid or the total length of the modified nucleic acid minus the poly-A tail.

Polypeptides of interest

The invention provides modified nucleic acids and enhanced nucleic acids encoding polypeptides of interest or fragments thereof for therapeutic uses for non-human vertebrates. A polypeptide of interest may include, but is not limited to, whole polypeptides, a plurality of polypeptides or fragments of polypeptides, which independently may be encoded by one or more nucleic acids, a plurality of nucleic acids, fragments of nucleic acids or variants of any of the aforementioned. As used herein, the term "polypeptides of interest" refer to any polypeptide which is selected to be encoded by the modified nucleic acids and enhanced nucleic acids of the present invention. As used herein, "polypeptide" means a polymer of amino acid residues

(natural or unnatural) linked together most often by peptide bonds. The term, as used herein, refers to proteins, polypeptides, and peptides of any size, structure, or function. In some instances the polypeptide encoded is smaller than about 50 amino acids and the polypeptide is then termed a peptide. If the polypeptide is a peptide, it will be at least about 2, 3, 4, or at least 5 amino acid residues long. Thus, polypeptides include gene products, naturally occurring polypeptides, synthetic polypeptides, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing. A polypeptide may be a single molecule or may be a multi-molecular complex such as a dimer, trimer or tetramer. They may also comprise single chain or multi-chain polypeptides such as antibodies or insulin and may be associated or linked. Most commonly disulfide linkages are found in multichain polypeptides. The term polypeptide may also apply to amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid.

The term "polypeptide variant" refers to molecules which differ in their amino acid sequence from a native or reference sequence. The amino acid sequence variants may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence, as compared to a native or reference sequence. Ordinarily, variants will possess at least about 50% identity (homology) to a native or reference sequence, and preferably, they will be at least about 80%, more preferably at least about 90% identical (homologous) to a native or reference sequence.

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

"Homology" as it applies to amino acid sequences is defined as the percentage of residues in the candidate amino acid sequence that are identical with the residues in the amino acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. It is understood that homology depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation.

By “homologs” as it applies to polypeptide sequences means the corresponding sequence of other species having substantial identity to a second sequence of a second species.

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

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

As such, modified nucleic acids and enhanced nucleic acids encoding polypeptide containing substitutions, insertions and/or additions, deletions and covalent modifications with respect to reference sequences, in particular the polypeptide sequences disclosed herein, are included within the scope of this invention. For example, sequence tags or amino acids, such as one or more lysines, can be added to the peptide sequences of the invention (e.g., at the N-terminal or C-terminal ends). Sequence tags can be used for peptide purification or localization. Lysines can be used to increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g., C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support.

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

As used herein the term “conservative amino acid substitution” refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine and leucine for another non-polar residue.

Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine, methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

“Insertional variants” when referring to polypeptides are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native or starting sequence. “Immediately adjacent” to an amino acid means connected to either the alpha-carboxy or alpha-amino functional group of the amino acid.

“Deletional variants” when referring to polypeptides are those with one or more amino acids in the native or starting amino acid sequence removed. Ordinarily, deletional variants will have one or more amino acids deleted in a particular region of the molecule.

“Covalent derivatives” when referring to polypeptides include modifications of a native or starting protein with an organic proteinaceous or non-proteinaceous derivatizing agent, and/or post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues of the protein with an organic derivatizing agent that is capable of reacting with selected side-chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays, or for the preparation of anti-protein antibodies for immunoaffinity purification of the recombinant glycoprotein. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues may be present in the polypeptides produced in accordance with the present invention.

Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)).

5 Once any of the features have been identified or defined as a desired component of a polypeptide to be encoded by the modified nucleic acids and enhanced nucleic acid of the invention, any of several manipulations and/or modifications of these features may be performed by moving, swapping, inverting, deleting, randomizing or duplicating. Furthermore, it is understood that manipulation of features may result in the same outcome as a modification to the
10 molecules of the invention. For example, a manipulation which involved deleting a domain would result in the alteration of the length of a molecule just as modification of a nucleic acid to encode less than a full length molecule would.

Modifications and manipulations can be accomplished by methods known in the art such as, but not limited to, site directed mutagenesis. The resulting modified molecules may then be
15 tested for activity using *in vitro* or *in vivo* assays such as those described herein or any other suitable screening assay known in the art.

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

In one embodiment, the polypeptide of interest may encode, bind, associate and/or interact with an antibody, small molecule, agonist, antagonist, intracellular, intercellular and/or
30 extracellular proteins. Non-limiting examples include receptors, enzymes, channels, pores, scaffolding proteins, cytoskeletal proteins, transcription factors, histones, lipids including

phospholipids, glycolipids, fatty acids, steroids, cholesterol and cholesterol-derived hormones, antibodies, vesicles, endosomes, exosomes, synaptic vesicles, signaling molecules including diacylglycerol, phosphatidyl inositol phosphate, prostaglandins, leukotrienes, lipoxins, growth factors, cytokines and neurotransmitters, DNA, RNA, mRNA, miRNA, tRNA, rRNA,
 5 ribonucleotides, deoxyribonucleotides, nitrogenous bases, sugars, glycans, proteoglycans, glycosaminoglycans, polysaccharides, lipopolysaccharide, integrins, cadherins and metabolites.

Encoded Polypeptides

The present invention provides modified nucleic acids and enhanced nucleic acids which may encode polypeptides of interest. The polypeptides of interest have various uses, as
 10 described herein, such as, but not limited to, the use as a therapeutic agents for non-human vertebrates in the treatment and/or prevention of various diseases and disorders. The encoded polypeptide of interest may be located in a cell, tissue and/or bodily fluid of a non-human vertebrate. The bodily fluid may include, but is not limited to, peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous
 15 humor, amniotic fluid, cerumen, breast milk, bronchoalveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, sweat, fecal matter, hair, tears, cyst fluid, pleural and peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, blastocyl cavity fluid, and umbilical cord
 20 blood. The encoded polypeptide of interest may be observed in a tissue such as, but not limited to, liver, spleen, kidney, lung, heart, peri-renal adipose tissue, thymus and muscle.

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the present invention may encode for a variety of polypeptides, variants and/or functional fragments thereof. Non-limiting examples of encoded polypeptides considered by the present invention
 25 include insulin, feline interferon, erythropoietin, cyclosporine, Thymosin Beta-4, arginine vasopressin, bovine somatotropin, oxytocin, ghrelin, gonadorelin, pregnant mare serum gonadotrophin (PMSG), equine chorionic gonadotrophin (ECG), human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone analog (GRHa), pancreatic enzymes, Cre recombinase, an insulin-like growth factor, hGH, tPA, Interleukin (IL)-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-
 30 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, IFN tau, tumor necrosis factor (TNF) alpha, TNF beta, TNF gamma, TRAIL, G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF.

Insulin

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for an insulin polypeptide, variant or functional fragment thereof. The insulin-encoding nucleic acids may be useful in the treatment and/or prevention of diabetes. Species to which the insulin-encoding nucleic acids may be administered include, but are not limited to, dogs and cats.

Feline Interferon

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for a feline interferon polypeptide, variant or functional fragment thereof. The feline interferon-encoding nucleic acids may be useful in the treatment and/or prevention of canine parvovirus, a contagious virus mainly affecting dogs. The virus may be spread by dog to dog contact or through contact with contaminated feces. The modified nucleic acid or enhanced nucleic acid may also be useful in the treatment of feline infectious peritonitis which may be transmitted by contact with contaminated feces, water bowls, food bowls and/or clothing. Species to which the feline interferon-encoding nucleic acids may be administered include, but are not limited to, dogs and cats. Currently, protein-based feline interferon therapeutics include Vibragen Omega (Virbac).

Erythropoietin

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for a human erythropoietin polypeptide, variant or functional fragment thereof. The erythropoietin-encoding nucleic acids may be useful in the treatment and/or prevention of chronic renal failure or kidney disease. This disease is common among older cats and is often a progressive disorder with may have a wide range of variation in the rate of progression. Species to which the erythropoietin-encoding nucleic acids may be administered include, but are not limited to, cats.

Cyclosporin

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for a cyclosporine polypeptide, variant or functional fragment thereof. Cyclosporin is an 11 amino acid cyclic protein that may be synthesized using a nonribosomal

enzyme, cyclosporine synthase. The cyclosporin-encoding nucleic acids may be useful in the treatment and/or prevention of atopic dermatitis, which is an allergic skin disease in dogs. Species to which the cyclosporin-encoding nucleic acids may be administered include, but are not limited to dogs. Currently, protein-based cyclosporine therapeutics include Atopica
5 (Novartis).

Thymosin Beta-4

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for an equine Thymosin Beta-4 polypeptide, variant or functional fragment thereof. The Thymosin Beta-4-encoding nucleic acids may be useful in the treatment
10 and/or prevention of weakness in non-human vertebrates as Thymosin Beta-4 may promote increased muscle mass and increased red blood cells. Species to which the Thymosin Beta-4-encoding nucleic acids may be administered include, but are not limited to, horses. Therapeutics containing Thymosin Beta-4 include TB-500 (DB Genetics).

Arginine Vasopressin

15 In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for an arginine vasopressin polypeptide, variant or functional fragment thereof. A proline-rich c-terminal portion of bovine arginine vasopressin may be used to treat and/or prevent cattle leukosis (also known as bovine leukosis and bovine leukemia).

Bovine Somatotropin

20 In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may for a bovine somatotropin polypeptide, variant or functional fragment thereof. The bovine somatotropin-encoding nucleic acids may be useful in increasing milk production in dairy cows. Species to which the bovine somatotropin-encoding nucleic acids may be administered include, but are not limited to, cows. Therapeutics containing bovine somatotropin include
25 Posilac (Elanco Animal Health).

Oxytocin

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may for an oxytocin polypeptide, variant or functional fragment thereof. The oxytocin-encoding nucleic acids may be useful in increasing milk production in dairy cows and as an aid
30 to precipitate labor. Species to which the oxytocin-encoding nucleic acids may be administered

include, but are not limited to, cows. Purified solutions of oxytocin are commercially available (VetTek).

Ghrelin

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for a chicken ghrelin polypeptide, variant or functional fragment thereof. The ghrelin-encoding nucleic acids may be useful in chickens to increase plasma levels of growth hormone and corticosterone levels. Species to which the oxytocin-encoding nucleic acids may be administered include, but are not limited to, chickens.

Gonadorelin

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for a gonadorelin polypeptide, variant or functional fragment thereof. The gonadorelin-encoding nucleic acids may be useful in the treatment and/or prevention of ovarian follicular cysts, and ovulation and fertility disorders. Species to which the gonadorelin-encoding nucleic acids may be administered include, but are not limited to, cattle and rabbits. Currently, protein-based gonadorelin therapeutics include Cystorelin (Merial), Fertagyl (Intervet - Schering-Plough), Factrel (Pfizer).

Pregnant Mare Serum Gonadotrophin (PMSG) and Equine Chorionic Gonadotrophin (ECG)

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for a PMSG or ECG polypeptide, variant or functional fragment thereof, or a combination thereof. The PMSG or ECG -encoding nucleic acids may be useful in the treatment and/or prevention of reproductive disorders and management of reproduction and the fertile estrous cycle. Species to which the PMSG or ECG-encoding nucleic acids may be administered include, but are not limited to, a variety of domesticated animals including cattle, horses, and pigs. Currently, protein-based PMSG or ECG therapeutics include Folligon / Chrono-Gest PMSG (Intervet - Schering-Plough).

Human Chorionic Gonadotrophin (hCG)

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for an hCG polypeptide, variant or functional fragment thereof. The hCG-encoding nucleic acids may be useful in the treatment and/or prevention of reproductive and/or fertility disorders. Species to which the hCG-encoding nucleic acids may be administered

include, but are not limited to, a variety of domesticated animals including cattle, horses, and pigs. Currently, protein-based hCG therapeutics include Chorulon (Intervet - Schering-Plough).
Gonadotrophin-Releasing Hormone analog (GRHa)

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for a GRHa polypeptide, variant or functional fragment thereof. The GRHa-encoding nucleic acids may be useful in the treatment and/or prevention of reduced fertility by ovarian dysfunction, and the induction of ovulation and improvement of conception rate. Species to which the GRHa-encoding nucleic acids may be administered include, but are not limited to, horses, cows and rabbits. Currently, protein-based GRHa therapeutics include Receptal (Intervet - Schering-Plough).

Pancreatic Enzymes.

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for one or a plurality of pancreatic polypeptide enzymes, as well as, variants or functional fragments thereof. The pancreatic enzymes include, but are not limited to, lipases, proteases, and amylases. Pancreatic enzyme-encoding nucleic acids may be useful in the treatment and/or prevention of deficiencies of pancreatic enzymes. Species to which the pancreatic enzyme-encoding nucleic acids may be administered include, but are not limited to, cats, dogs and livestock. Currently, protein-based pancreatic enzyme therapeutics include Viokase (Pfizer).

Cre recombinase

In one embodiment, the modified nucleic acids and/or enhanced nucleic acids of the invention may encode for a Cre recombinase polypeptide, as well as, variants or functional fragments thereof. Cre recombinase-encoding nucleic acids may be useful in transgenic mouse models used in research and pharmaceutical development. Expression of Cre recombinase in a cell containing DNA regions flanked by loxP sequences leads to the deletion of the flanked DNA region. Species to which the Cre recombinase-encoding nucleic acids may be administered include, but are not limited to, monkeys, dogs, cats, rabbits, rats, mice, xenopus and chickens. Currently, cross breeding with a Cre-expressing animal strain or viral delivery of the Cre gene is required.

Polypeptide libraries

In one embodiment, the modified nucleic acids and enhanced nucleic acids may be used to produce polynucleotide libraries containing nucleoside modifications. The polynucleotides may individually contain a first nucleic acid sequence encoding a polypeptide, such as an antibody, protein-binding partner, scaffold protein, and other polypeptides known in the art.

- 5 Preferably, the polynucleotides are mRNA in a form suitable for direct introduction into a target cell host, which in turn synthesizes the encoded polypeptide.

- In certain embodiments, multiple variants of a protein or antibody or functional fragment thereof, each with different amino acid modification(s), are produced and tested to determine the best variant in terms of antigen affinity, yield in a producing cell line, pharmacokinetics, stability, biocompatibility, and/or biological activity, or a biophysical property such as expression level. Such a library may contain 10 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , or over 10^9 possible variants (including substitutions, deletions of one or more residues, and insertion of one or more residues).

Polypeptide-nucleic acid complexes

- 15 Proper protein translation involves the physical aggregation of a number of polypeptides and nucleic acids associated with the mRNA. Provided by the invention 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 reduce an innate immune response of a cell into which the complex is introduced.

Untranslatable modified nucleic acids

As described herein, provided are mRNAs having sequences that are substantially not translatable. Such mRNA may be effective as a vaccine when administered to a mammalian subject.

- 25 Also provided are modified nucleic acids that contain one or more noncoding regions. Such modified nucleic acids 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 protein expression in the cell. The modified nucleic acid may contain a small nucleolar RNA (sno-RNA), micro RNA (miRNA), small interfering RNA (siRNA) or Piwi-interacting RNA (piRNA).

Modified nucleic acid synthesis

Nucleic acids for use in accordance with the invention may be prepared according to any available technique including, but not limited to chemical synthesis, enzymatic synthesis, which is generally termed *in vitro* transcription, enzymatic or chemical cleavage of a longer precursor, etc. Methods of synthesizing RNAs are known in the art (see, e.g., Gait, M.J. (ed.)

- 5 *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).

- 10 Modified nucleic acids need not be uniformly modified along the entire length of the molecule. Different nucleotide 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 and at maximum 100%
15 modified nucleotides, or any intervening percentage, such as at least 50% modified nucleotides, at least 80% modified nucleotides, or at least 90% modified nucleotides.

- Generally, the length of a modified mRNA of the present invention is greater than 30 nucleotides in length. In another embodiment, the RNA molecule is greater than 35 nucleotides in length. In another embodiment, the length is at least 40 nucleotides. In another embodiment,
20 the length is at least 45 nucleotides. In another embodiment, the length is at least 55 nucleotides. In another embodiment, the length is at least 60 nucleotides. In another embodiment, the length is at least 60 nucleotides. In another embodiment, the length is at least 80 nucleotides. In another embodiment, the length is at least 90 nucleotides. In another embodiment, the length is at least 100 nucleotides. In another embodiment, the length is at least 120 nucleotides. In another
25 embodiment, the length is at least 140 nucleotides. In another embodiment, the length is at least 160 nucleotides. In another embodiment, the length is at least 180 nucleotides. In another embodiment, the length is at least 200 nucleotides. In another embodiment, the length is at least 250 nucleotides. In another embodiment, the length is at least 300 nucleotides. In another embodiment, the length is at least 350 nucleotides. In another embodiment, the length is at least
30 400 nucleotides. In another embodiment, the length is at least 450 nucleotides. In another embodiment, the length is at least 500 nucleotides. In another embodiment, the length is at least

600 nucleotides. In another embodiment, the length is at least 700 nucleotides. In another embodiment, the length is at least 800 nucleotides. In another embodiment, the length is at least 900 nucleotides. In another embodiment, the length is at least 1000 nucleotides. In another embodiment, the length is at least 1100 nucleotides. In another embodiment, the length is at least 1200 nucleotides. In another embodiment, the length is at least 1300 nucleotides. In another embodiment, the length is at least 1400 nucleotides. In another embodiment, the length is at least 1500 nucleotides. In another embodiment, the length is at least 1600 nucleotides. In another embodiment, the length is at least 1800 nucleotides. In another embodiment, the length is at least 2000 nucleotides. In another embodiment, the length is at least 2500 nucleotides. In another embodiment, the length is at least 3000 nucleotides. In another embodiment, the length is at least 4000 nucleotides. In another embodiment, the length is at least 5000 nucleotides, or greater than 5000 nucleotides.

Pharmaceutical Compositions

The present invention provides modified nucleic acids or enhanced nucleic acids 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).

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.

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.

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 of modified nucleic acids

Provided are formulations containing an effective amount of a ribonucleic acid (e.g., an mRNA or a nucleic acid containing an mRNA) which may have been engineered to avoid an innate immune response of a cell into which the ribonucleic acid enters. The ribonucleic acid generally includes a nucleotide sequence encoding a polypeptide of interest.

The modified nucleic acids and enhanced nucleic acids 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 enhanced nucleic acid); (4) alter the biodistribution (e.g., target the modified nucleic acid or enhanced nucleic acid 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, rapidly eliminated lipid nanoparticles, polymers, lipoplexes, core-shell nanoparticles, peptides, proteins, cells transfected with modified nucleic acids or enhanced nucleic acids (e.g., for transplantation into a subject), hyaluronidase, 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 enhanced nucleic acid, increases cell transfection by the modified nucleic acid or enhanced nucleic acid, increases the expression of modified nucleic acid or enhanced nucleic acid encoded protein, and/or alters the release profile of the modified nucleic acid or enhanced nucleic acid encoded proteins.

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.

5 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
10 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). 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
15 by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

 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
20 optionally be included in the pharmaceutical formulations of the invention.

Lipidoids

 The synthesis of lipidoids has been extensively described and formulations containing these compounds are particularly suited for delivery of modified nucleic acids or enhanced nucleic acids (see Mahon et al., Bioconj Chem. 2010 21:1448-1454; Schroeder et al., J Intern
25 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).

 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.
30 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 acids or enhanced nucleic acids. Complexes, micelles, liposomes or particles can be prepared containing these lipidoids and therefore, can result in an effective
5 delivery of the modified nucleic acid or enhanced nucleic acid, 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 or enhanced nucleic acid can be administered by various means including, but not limited to, intravenous, intramuscular, or subcutaneous routes.

10 *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 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
15 effects on *in vivo* efficacy. Formulations with the different lipidoids, including, but not limited to penta[3-(1-laurylamino propionyl)]-triethylenetetramine hydrochloride (TETA-5LAP; aka 98N12-5, see Murugaiah et al., Analytical Biochemistry, 401:61 (2010)), C12-200 (including derivatives and variants), and MD1, can be tested for *in vivo* activity.

The lipidoid referred to herein as "98N12-5" is disclosed by Akinc et al., Mol Ther. 2009
20 17:872-879 and is incorporated by reference in its entirety.

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; 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
25 acid or enhanced nucleic acid. 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% distearylphosphatidyl choline, 38.5% cholesterol, and 1.5% PEG-DMG.

30 In one embodiment, a modified nucleic acid or enhanced nucleic acid formulated with a lipidoid for systemic intravenous administration can target the liver. For example, a final

optimized intravenous formulation using modified nucleic acid or enhanced nucleic acid, 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 enhanced nucleic acid, 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 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, herein incorporated by reference in their entirety) lipidoid may have a molar ratio of 50/10/38.5/1.5 of C12-200/disteroylphosphatidyl choline/cholesterol/PEG-DMG, with a weight ratio of 7 to 1 total lipid to modified nucleic acid or enhanced nucleic acid, and a mean particle size of 80 nm may be effective to deliver modified nucleic acid or enhanced nucleic acid to hepatocytes (see, Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869 herein incorporated by reference). In another embodiment, an MD1 lipidoid-containing formulation may be used to effectively deliver modified nucleic acids or enhanced nucleic acids 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), use of a lipidoid-formulated modified nucleic acids or enhanced nucleic acids 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 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 enhanced nucleic acid 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% distearylphosphatidyl 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 or enhanced nucleic acid.

Combinations of different lipidoids may be used to improve the efficacy of modified nucleic acid or enhanced nucleic acid directed protein production as the lipidoids may be able to increase cell transfection by the modified nucleic acid or enhanced nucleic acid; 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

The modified nucleic acid and enhanced nucleic acid of the invention can be formulated using one or more liposomes, lipoplexes, or lipid nanoparticles. In one embodiment, pharmaceutical compositions of modified nucleic acid or enhanced nucleic acid 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 unilamellar 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.

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.

In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from 1,2-dioleoyloxy-*N,N*-dimethylaminopropane (DODMA) liposomes, DiLa2 liposomes from Marina Biotech (Bothell, WA), 1,2-dilinoleoyloxy-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 Hum Gene Ther. 2008 19:125-132; all of which are incorporated herein in their entireties.) 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 or enhanced nucleic acid. As an example a liposome can contain, but is not limited to, 55% cholesterol, 20% distearylphosphatidyl choline (DSPC), 10% PEG-S-DSG, and 15% 1,2-dioleoyloxy-*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-c-DMA, 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.

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), 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 one embodiment, the modified nucleic acid or enhanced nucleic acid may be formulated as a lipoplex, such as, without limitation, the ATUPLEXTM system, the DACC system, the DBTC system and other siRNA-lipoplex technology from Silence Therapeutics (London, United Kingdom), STEMFECTTM from STEMGENT® (Cambridge, MA), and polyethylenimine (PEI) or protamine-based targeted and non-targeted delivery of nucleic 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 80:286-293; Weide et al. *J Immunother.* 2009 32:498-507; Weide et al. *J Immunother.* 2008 31:180-188; Pascolo *Expert Opin. Biol. Ther.* 4:1285-1294; Fotin-Mleczek et al., 2011 *J. Immunother.* 34:1-15; Song et al., *Nature Biotechnol.* 2005, 23:709-717; Peer et al., *Proc Natl Acad Sci U S A.* 2007 6;104:4095-4100; deFougerolles *Hum Gene Ther.* 2008 19:125-132; all of which are incorporated herein by reference in its entirety).

In one embodiment such formulations may also be constructed or compositions altered such that they passively or actively are directed to different cell types *in vivo*, including but not limited to hepatocytes, immune cells, tumor cells, endothelial cells, antigen presenting cells, and leukocytes (Akinc et al. *Mol Ther.* 2010 18:1357-1364; Song et al., *Nat Biotechnol.* 2005 23:709-717; Judge et al., *J Clin Invest.* 2009 119:661-673; Kaufmann et al., *Microvasc Res* 2010 80:286-293; 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; Basha et al., *Mol. Ther.* 2011 19:2186-2200; Fenske and Cullis, *Expert Opin Drug Deliv.* 2008 5:25-44; Peer et al., *Science.* 2008 319:627-630; Peer and Lieberman, *Gene Ther.* 2011 18:1127-1133; all of which are incorporated herein by reference in its entirety). One example of passive targeting of formulations to liver cells includes the DLin-DMA, DLin-KC2-DMA and MC3-based lipid nanoparticle formulations which have been shown to bind to apolipoprotein E and promote

binding and uptake of these formulations into hepatocytes in vivo (Akinc et al. Mol Ther. 2010 18:1357-1364; herein incorporated by reference in its entirety): Formulations can also be selectively targeted through expression of different ligands on their surface as exemplified by, but not limited by, folate, transferrin, N-acetylgalactosamine (GalNAc), and antibody targeted approaches (Kolhatkar et al., Curr Drug Discov Technol. 2011 8:197-206; Musacchio and Torchilin, *Front Biosci.* 2011 16:1388-1412; Yu et al., Mol Membr Biol. 2010 27:286-298; Patil et al., Crit Rev Ther Drug Carrier Syst. 2008 25:1-61; Benoit et al., Biomacromolecules. 2011 12:2708-2714; Zhao et al., Expert Opin Drug Deliv. 2008 5:309-319; Akinc et al., Mol Ther. 2010 18:1357-1364; Srinivasan et al., Methods Mol Biol. 2012 820:105-116; Ben-Arie et al., Methods Mol Biol. 2012 757:497-507; Peer 2010 J Control Release. 20:63-68; Peer et al., Proc Natl Acad Sci U S A. 2007 104:4095-4100; Kim et al., Methods Mol Biol. 2011 721:339-353; Subramanya et al., Mol Ther. 2010 18:2028-2037; Song et al., Nat Biotechnol. 2005 23:709-717; Peer et al., Science. 2008 319:627-630; Peer and Lieberman, Gene Ther. 2011 18:1127-1133; all of which are incorporated herein by reference in its entirety).

In one embodiment, the modified nucleic acid or enhanced nucleic acid may be 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).

Liposomes, lipoplexes, or lipid nanoparticles may be used to improve the efficacy of modified nucleic acid or enhanced nucleic acid directed protein production as these formulations may be able to increase cell transfection by the modified nucleic acid or enhanced nucleic acid; 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 or enhanced nucleic acid.

Polymers, Biodegradable Nanoparticles, and Core-Shell Nanoparticles

The modified nucleic acid and enhanced nucleic acid 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™ formulations from
5 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.

10 Many of these polymer approaches have demonstrated efficacy in delivering oligonucleotides *in vivo* into the cell cytoplasm (reviewed in deFougerolles *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
15 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). 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
20 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). 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
25 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-FLII* gene product in transferrin receptor-expressing Ewing's
30 sarcoma tumor cells (Hu-Lieskovan *et al.*, Cancer Res.2005 65: 8984-8982) 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). 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 acids or enhanced nucleic acids (e.g., following intramuscular or subcutaneous injection). The altered release profile for the modified nucleic acid or enhanced nucleic acid 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 or enhanced nucleic acid. Biodegradable polymers have been previously used to protect nucleic acids other than modified nucleic acids or enhanced nucleic acids 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 Fougères et al., 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; herein incorporated by reference in its entirety).

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; herein incorporated by reference in its entirety).

The modified nucleic acid and enhanced nucleic acid 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 and enhanced nucleic acid 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; herein incorporated by reference in its entirety).

Biodegradable calcium phosphate nanoparticles in combination with lipids and/or polymers have been shown to deliver modified nucleic acid and enhanced nucleic acid *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 and enhanced nucleic acid 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). 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.

In one embodiment, calcium phosphate with a PEG-polyanion block copolymer may be used to deliver modified nucleic acid and enhanced nucleic acid (Kazikawa et al., J Contr Rel. 2004 97:345-356; Kazikawa et al., J Contr Rel. 2006 111:368-370).

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 and enhanced nucleic acid 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.

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 or enhanced nucleic acid 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).

Peptides and Proteins

The modified nucleic acid and enhanced nucleic acid of the invention can be formulated with peptides and/or proteins in order to increase transfection of cells by the modified nucleic acid or enhanced nucleic acid. 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 includes a cell-penetrating peptide sequence attached to polycations that facilitates delivery to the intracellular space, e.g., HIV-derived TAT peptide, penetratins, transportans, 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-Andaloussi 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., transfection agents, and liposomes, which enhance delivery of the compositions to the intracellular space.

In one specific embodiment, a modified nucleic acid and enhanced nucleic acid can be mixed or admixed with a transfection agent (or mixture thereof) and the resulting mixture is employed to transfect cells. Preferred transfection agents include, but are not limited to, cationic lipid compositions, particularly monovalent and polyvalent cationic lipid compositions, more particularly LIPOFECTIN®, LIPOFECTACE®, LIPOFECTAMINE™, CELLFECTIN®, DMRIE-C, DMRIE, DOTAP, DOSPA, and DOSPER, and dendrimer compositions, particularly G5-G10 dendrimers, including dense star dendrimers, PAMAM dendrimers, grafted dendrimers, and dendrimers known as dendrigrafts and SUPERFECT®. In a second specific embodiment, a mixture of one or more transfection-enhancing peptides, proteins, or protein fragments, including fusagenic peptides or proteins, transport or trafficking peptides or proteins, receptor-ligand peptides or proteins, or nuclear localization peptides or proteins and/or their modified analogs (e.g., spermine modified peptides or proteins) or combinations thereof are mixed with and complexed with a modified nucleic acid and enhanced nucleic acid to be introduced into a cell, optionally being admixed with transfection agent and the resulting mixture is employed to transfect cells. Further, a component of a transfection agent (e.g., lipids, cationic lipids or dendrimers) may be covalently conjugated to selected peptides, proteins, or protein fragments

directly or via a linking or spacer group. Of particular interest in this embodiment are peptides or proteins that are fusogenic, membrane-permeabilizing, transport or trafficking, or which function for cell-targeting. The peptide- or protein-transfection agent complex is combined with a modified nucleic acid and enhanced nucleic acid and employed for transfection.

5 Modified nucleic acid and enhanced nucleic acid 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; 10 Verdine 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 15 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 or enhanced nucleic acid may be introduced.

Formulations of the including peptides or proteins may be used to increase cell 20 transfection by the modified nucleic acid or enhanced nucleic acid, alter the biodistribution of the modified nucleic acid or enhanced nucleic acid (e.g., by targeting specific tissues or cell types), and/or increase the translation of encoded protein.

Cells

The modified nucleic acid and enhanced nucleic acid of the invention can be transfected 25 *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 RNA 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 30 particles and electroporated cells to deliver payloads other than modified nucleic acids 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).

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

A variety of methods are known in the art and suitable for introduction of nucleic acid
10 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, polyethylenimine, polyethylene glycol (PEG) and the like) or cell
15 fusion.

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 used to deliver nucleic acids *in vivo* (Yoon and Park, Expert Opin Drug Deliv. 2010 7:321-330; Postema and Gilja, Curr Pharm Biotechnol.
20 2007 8:355-361; Newman and Bettinger, Gene Ther. 2007 14:465-475; all herein incorporated by reference in their entirety). Sonoporation methods are known in the art and are also 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.

25 Electroporation techniques are also well known in the art and are used to deliver nucleic acids *in vivo* and clinically (Andre et al., Curr Gene Ther. 2010 10:267-280; Chiarella et al., Curr Gene Ther. 2010 10:281-286; Hojman, Curr Gene Ther. 2010 10:128-138; all herein incorporated by reference in their entirety). In one embodiment, the modified nucleic acid or enhanced nucleic acid may be delivered by electroporation.

30 Hyaluronidase

The intramuscular or subcutaneous localized injection of the modified nucleic acid and enhanced nucleic acid 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 or enhanced nucleic acid of the invention administered intramuscularly or subcutaneously.

10 *Conjugates*

The modified nucleic acid and enhanced nucleic acid of the invention include conjugates, such as a modified nucleic acid or enhanced nucleic acid 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).

15 In one embodiment, a modified nucleic acid or enhanced nucleic acid may be conjugated to a nucleic acid-binding group, such as, but not limited to, a polyamine and more particularly a spermine. The nucleic acid-binding group may then be introduced into the cell or admixed with a transfection agent (or mixture thereof) and the resulting mixture may then be employed to transfect cells.

20 The conjugates of the invention include, but are not limited to, 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, but are not limited to, polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolide) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacrylic acid), N-isopropylacrylamide polymers, or polyphosphazene. Examples of polyamines include: 25 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.

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; 5,292,873; 5,317,098; 5,371,241; 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.

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 mimetic or an aptamer.

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.

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, aptamers, integrin receptor ligands, chemokine receptor ligands, transferrin, biotin, serotonin

receptor ligands, PSMA, endothelin, GCP II, 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.

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.

Representative U.S. Patents that teach the preparation of locked nucleic acid (LNA) such as those from Santaris, 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.

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.

Some embodiments featured in the invention include modified nucleic acids or enhanced nucleic acids with phosphorothioate backbones and oligonucleosides with other modified backbones, and in particular --CH₂--NH--CH₂--, --CH₂--N(CH₃)--O--CH₂--[known as a methylene (methylimino) or MMI backbone], --CH₂--O--N(CH₃)--CH₂--, --CH₂--N(CH₃)--N(CH₃)--CH₂-- and --N(CH₃)--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.

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]₂, where n and m are from 1 to about 10. In other embodiments, the modified nucleic acids or

enhanced nucleic acids may 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, SCH₃, 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'-dimethylaminoethoxyethoxy, *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(CH₂)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.

In still other embodiments, the modified nucleic acid or enhanced nucleic acid may be 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).

Excipients

Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes 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. Remington's *The Science and Practice of Pharmacy*, 21st Edition, A. R. Gennaro (Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference) discloses various excipients used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional excipient medium is 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, its use is contemplated to be within the scope of this invention.

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

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 compositions.

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.

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) (crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl

cellulose, cross-linked sodium carboxymethyl cellulose (croscarmellose), 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.

- 5 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), carbomers (e.g. carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (e.g. carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, 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 monooleate [SPAN®80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [MYRJ®45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and SOLUTOL®), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. CREMOPHOR®), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [BRIJ®30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, PLUORINC®F 68, POLOXAMER®188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, *etc.* and/or combinations thereof.

- 25 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.

Exemplary preservatives may include, but are not limited to, antioxidants, chelating
 5 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
 10 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, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol,
 15 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
 20 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,
 25 deteroxime mesylate, cetrimide, butylated hydroxyanisole (BHA), butylated hydroxytoluene (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[®].

30 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 glubionate, 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, 5 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.

10 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, 15 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, macademia nut, mallow, mango seed, meadowfoam seed, mink, nutmeg, olive, orange, orange roughy, palm, palm kernel, peach 20 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, octyldodecanol, oleyl alcohol, silicone oil, 25 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.

Pharmaceutically Acceptable Carrier

30 In some embodiments, the formulations may include a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may cause the effective amount of modified

nucleic acid or enhanced nucleic acid to be substantially retained in a target tissue containing the cell.

Delivery of modified nucleic acids

The present disclosure encompasses the delivery of modified nucleic acids or enhanced nucleic acids 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 acids or enhanced nucleic acids of the present invention may be delivered to a cell naked. As used herein in, "naked" refers to delivering modified nucleic acids or enhanced nucleic acids free from agents which promote transfection. For example, the modified nucleic acids or enhanced nucleic acids delivered to the cell may contain no modifications. The naked modified nucleic acids or enhanced nucleic acids may be delivered to the cell using routes of administration known in the art and described herein.

Formulated Delivery

The modified nucleic acids or enhanced nucleic acids of the present invention may be formulated, using the methods described herein. The formulations may contain ribonucleic acids 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 acids or enhanced nucleic acids may be delivered to the cell using routes of administration known in the art and described herein.

In one embodiment, provided are compositions for generation of an *in vivo* depot containing an engineered ribonucleotide such as a modified nucleic acid or an enhanced nucleic acid. For example, the composition contains a bioerodible, biocompatible polymer, a solvent present in an amount effective to plasticize the polymer and form a gel therewith, and an engineered ribonucleic acid. In certain embodiments the composition also includes a cell penetration agent as described herein. In other embodiments, the composition also contains a thixotropic amount of a thixotropic agent mixable with the polymer so as to be effective to form a thixotropic composition. Further compositions include a stabilizing agent, a bulking agent, a chelating agent, or a buffering agent.

In one embodiment, provided are sustained-release delivery depots, such as for administration of an engineered ribonucleic acid such as a modified nucleic acid or an enhanced nucleic acid to an environment (meaning an organ or tissue site) in a patient. Such depots generally contain an engineered ribonucleic acid and a flexible chain polymer where both the engineered ribonucleic acid and the flexible chain polymer are entrapped within a porous matrix of a crosslinked matrix protein. Usually, the pore size is less than 1mm, such as 900 nm, 800 nm, 700 nm, 600 nm, 500 nm, 400 nm, 300 nm, 200 nm, 100 nm, or less than 100 nm. Usually the flexible chain polymer is hydrophilic. Usually the flexible chain polymer has a molecular weight of at least 50 kDa, such as 75 kDa, 100 kDa, 150 kDa, 200 kDa, 250 kDa, 300 kDa, 400 kDa, 500 kDa, or greater than 500 kDa. Usually the flexible chain polymer has a persistence length of less than 10%, such as 9, 8, 7, 6, 5, 4, 3, 2, 1 or less than 1% of the persistence length of the matrix protein. Usually the flexible chain polymer has a charge similar to that of the matrix protein. In some embodiments, the flexible chain polymer alters the effective pore size of a matrix of crosslinked matrix protein to a size capable of sustaining the diffusion of the engineered ribonucleic acid from the matrix into a surrounding tissue comprising a cell into which the engineered ribonucleic acid is capable of entering.

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.

Methods of cellular nucleic acid delivery

Methods of the present invention enhance nucleic acid delivery into a cell population, particularly *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) is contacted with a composition that contains a modified nucleic acid, or an enhanced nucleic acid having at least one nucleoside modification and, optionally, a translatable region. The composition also generally contains a transfection reagent or other compound that increases the efficiency of modified nucleic acid or enhanced nucleic acid uptake into the host cells. The modified nucleic acid or enhanced nucleic acid may exhibit enhanced retention in the cell population, relative to a corresponding unmodified nucleic acid. The retention of the modified nucleic acid or enhanced nucleic acid is greater than the retention of the unmodified nucleic acid. 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. Such retention advantage may be achieved by one round of transfection with the modified nucleic acid or enhanced nucleic acid, or may be obtained following repeated rounds of transfection.

5 In some embodiments, the enhanced nucleic acid 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 enhanced nucleic acid may be delivered prior to delivery of the one or more additional nucleic acids. The additional one or more nucleic acids may be modified nucleic acids or unmodified nucleic acids. It is understood that the initial presence of the enhanced nucleic acids
10 does not substantially induce an innate immune response of the cell population and, moreover, that the innate immune response will not be activated by the later presence of the unmodified nucleic acids. 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 acids.

15 **Administration of modified nucleic acids**

As described herein, compositions containing the nucleic acids of the invention are formulated for administration intramuscularly, transarterially, intraperitoneally, intravenously, intranasally, subcutaneously, endoscopically, transdermally, and/or intrathecally. As described herein, in some embodiments, the composition is formulated in depots for extended release.

20 Generally, a specific organ or tissue (a "target tissue") may be targeted for administration.

In some aspects of the invention, the nucleic acids (particularly ribonucleic acids encoding polypeptides) are spatially retained within or proximal to a target tissue.

Advantageously, retention may be 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,
25 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 may be 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
30 present in the muscle cells.

In one embodiment, provided is a 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 may be 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 may be performed using an aqueous composition containing a ribonucleic acid and a transfection reagent, and retention of the composition may be determined by measuring the amount of the ribonucleic acid present in the muscle cells.

The subject to whom the therapeutic agent is administered suffers from or is 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.

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

In other embodiments, the administered modified nucleic acid directs production of one or more recombinant polypeptides that replace a polypeptide (or multiple polypeptides) that is substantially absent in the cell in which the recombinant polypeptide is translated. Such absence may be due to genetic mutation of the encoding gene or 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 is deleterious to the subject, for example, do to 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 lipids (e.g., cholesterol), a lipoprotein (e.g., low density lipoprotein), a nucleic acid, a carbohydrate, or a small molecule toxin.

5 **Uses of modified nucleic acids**

Therapeutic Agents

Provided are compositions, methods, kits, and reagents for treatment or prevention of disease or conditions in non-human vertebrates, particularly mammals. The active therapeutic agents of the invention include modified nucleic acids, cells containing modified nucleic acids or polypeptides translated from the modified nucleic acids, polypeptides translated from modified
10 nucleic acids, and cells contacted with cells containing modified nucleic acids or polypeptides translated from the modified nucleic acids.

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

20 An effective amount of the composition is 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. Increased
25 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), or reduced innate immune response of the host cell.

The modified nucleic acids and enhanced nucleic acids of the present invention exhibit
30 enhanced retention in the cell population, relative to a corresponding unmodified nucleic acid. The retention of the modified nucleic acid or enhanced nucleic acid is greater than the retention

of the unmodified nucleic acid. 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. Such retention advantage may be achieved by one round of transfection with the modified nucleic acid or enhanced nucleic acid, or may be obtained following repeated rounds of transfection.

In some embodiments, an enhanced nucleic acid 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 enhanced nucleic acid is delivered prior to delivery of the one or more additional nucleic acids. The additional one or more nucleic acids may be modified nucleic acids or unmodified nucleic acids. It is understood that the initial presence of the enhanced nucleic acids does not substantially induce an innate immune response of the cell population and, moreover, that the innate immune response will not be activated by the later presence of the unmodified nucleic acids. 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 acids.

Avoidance of the immune response

As described herein, a useful feature of the modified nucleic acids of the invention may be the capacity to reduce prevent the innate immune response of a cell to an exogenous nucleic acid. Provided are methods for performing the titration, prevention, 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 is 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 is 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.

The term "innate immune response" includes a cellular response to exogenous single stranded nucleic acids, generally of viral or bacterial origin, which involves the induction of cytokine expression and release, particularly the interferons, and cell death. Protein synthesis is also reduced during the innate cellular immune response. While it is advantageous to eliminate the innate immune response in a cell, the invention provides modified mRNAs that substantially reduce the immune response, including interferon signaling, without entirely eliminating such a response. In some embodiments, the immune response is 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. Such a reduction can be measured by 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 innate immune response can also be measured by decreased cell death following one or more administrations of modified RNAs 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. 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 acids.

The invention provides for the repeated introduction (e.g., transfection) of modified nucleic acids 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 acids is 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 provided by the nucleic acid modifications, such repeated transfections are achievable in a diverse array of cell types.

Production of Antibodies

The invention provides antibodies produced by any one of the methods of the present invention and fragments of such antibodies. The antibodies may be of any of the different

subclasses or isotypes of immunoglobulin, eg IgA, IgG or IgM, or any of the other subclasses. Exemplary antibody molecules and fragments that may be prepared according to the invention include intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope (the antigen-binding site of an antibody). Such portion of antibodies that contain the paratope include those portions known in the art as Fab, Fab', F(ab')₂ and F(v).

Antibody polypeptide variants.

Provided are nucleic acids that encode variant antibody polypeptides, which have a certain identity with a reference polypeptide sequence or, alternatively, have similar or dissimilar binding characteristics. The term "identity" as known in the art, refers to a relationship between the sequences of two or more peptides, as determined by comparing the sequences. In the art, "identity" also means 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).

Antibodies obtained by the methods of the present invention can be chimeric antibodies comprising non-human antibody-derived variable region(s) sequences, derived from the immunized animals, and human antibody-derived constant region(s) sequences. In addition, they can also be humanized antibodies comprising complementarity determining regions (CDRs) of non-human antibodies derived from the immunized animals and the framework regions (FRs) and constant regions derived from human antibodies.

In some embodiments, the polypeptide variant has the same or a similar activity as the reference polypeptide. Alternatively, the variant has an altered activity (e.g., increased or

decreased) relative to a reference polypeptide. Generally, variants of a particular polynucleotide or polypeptide of the invention 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.

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 invention. For example, provided herein is any protein fragment of a reference protein (meaning a polypeptide sequence 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 invention. In certain embodiments, a protein sequence to be utilized in accordance with the invention includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations as shown in any of the sequences provided or referenced herein.

Methods of antibody production

The methods provided herein are useful for enhancing antibody protein product yield in a cell culture process. In a cell culture containing a plurality of host cells, introduction of the modified mRNAs described herein results in increased protein production efficiency relative to a corresponding unmodified nucleic acid. Such increased protein production efficiency can be demonstrated, e.g., by showing increased cell transfection, increased protein translation from the nucleic acid, decreased nucleic acid degradation, and/or reduced innate immune response of the host cell. Protein production can be measured by ELISA, and protein activity can be measured by various functional assays known in the art. The protein production may be generated in a continuous or a fed-batch process.

Cell culture and growth

In the methods of the invention, the cells are cultured. Cells may be cultured in suspension or as adherent cultures. Cells may be cultured in a variety of vessels including, for example, bioreactors, cell bags, wave bags, culture plates, flasks and other vessels well known to those of ordinary skill in the art. Cells may be cultured in IMDM (Invitrogen, Catalog number

12440-53) or any other suitable media including chemically defined media formulations.

Ambient conditions suitable for cell culture, such as temperature and atmospheric composition, are also well known to those skilled in the art. The methods of the invention may be used with any cell that is suitable for use in protein production. In one embodiment, the cells are selected from the group consisting of mammalian cells, bacterial cells, plant, microbial, algal and fungal cells. In some embodiments, the cells are mammalian cells, such human, mouse, rat, goat, horse, rabbit, hamster or cow cells. For instance, the cells may be from any established cell line, including but not limited to HeLa, NS0, SP2/0, HEK 293T, Vero, Caco, Caco-2, MDCK, COS-1, COS-7, K562, Jurkat, CHO-K1, DG44, CHOK1SV, CHO-S, Huvec, CV-1, HuH-7, NIH3T3, HEK293, 293, A549, HepG2, IMR-90, MCF-7, U-20S, Per.C6, SF9, SF21 or Chinese Hamster Ovary (CHO) cells. In certain embodiments, the cells are fungal cells, such as cells selected from the group consisting of: Chrysosporium cells, Aspergillus cells, Trichoderma cells, Dictyostelium cells, Candida cells, Saccharomyces cells, Schizosaccharomyces cells, and Penicillium cells. In certain other embodiments, the cells are bacterial cells, such as E. coli, B. subtilis, or BL21 cells. Primary and secondary cells to be transfected by the present method can be obtained from a variety of tissues and include all cell types which can be maintained in culture. For example, primary and secondary cells which can be transfected by the present method include fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells and precursors of these somatic cell types. Primary cells can be obtained from a donor of the same species or another species (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse).

Therapeutics for diseases and conditions

Provided are methods for treating or preventing a symptom of diseases characterized by missing or aberrant protein activity, by replacing the missing protein activity or overcoming the aberrant protein activity. Because of the rapid initiation of protein production following introduction of modified mRNAs, as compared to viral DNA vectors, the compounds of the present invention are particularly advantageous in treating acute diseases such as sepsis, stroke, and myocardial infarction. Moreover, the lack of transcriptional regulation of the modified mRNAs of the invention is advantageous in that accurate titration of protein production is achievable.

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. In one embodiment, the composition contains an effective amount of a
5 ribonucleic acid engineered to avoid an innate immune response of a cell into which the ribonucleic acid enters, where the ribonucleic acid contains a nucleotide sequence encoding a polypeptide of interest, under conditions such that the polypeptide of interest is produced in at least one target cell. Generally, the compositions may contain a cell penetration agent, although “naked” nucleic acid (such as nucleic acids without a cell penetration agent or other agent) are
10 also contemplated, and a pharmaceutically acceptable carrier.

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).

15 Subsequent to this determination, the determined dose is introduced directly into the tissue of the non-human vertebrate subject.

Provided are methods of altering the differentiative state of a cell or a population of cells present in a non-human vertebrate subject. Such methods include the steps of i) providing a composition containing a plurality of different ribonucleic acids, wherein each ribonucleic acid is
20 engineered to avoid an innate immune response of a cell into which the ribonucleic acid enters and encodes a polypeptide of interest (thereby producing a plurality of different polypeptides), along with a cell penetration agent, and a pharmaceutically acceptable carrier. A unit quantity of composition may be determined to produce the plurality of different polypeptides of interest in a substantial percentage of cells contained within a predetermined volume of the tissue. The
25 method further includes step ii) determining a dose of the composition required to produce the different polypeptides of interest in a substantial percentage of cells contained within the predetermined volume of the tissue. The different polypeptides of interest may be produced in an amount affective to alter the differentiative state of a significant amount (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 95 or greater than 95%) of those protein
30 producing cells without altering the differentiative state of a significant percentage (50, 40, 30, 20, 10, 5, 4, 3, 2, 1 or less than 1%) of cells in tissue adjacent to the predetermined volume. The

method further comprises step iii) introducing directly into the tissue of the non-human vertebrate subject the dose of the composition.

Aspects of the invention are directed to methods of inducing *in vivo* translation of a recombinant polypeptide in a non-human vertebrate 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 is administered to the subject using the delivery methods described herein. The nucleic acid is 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 is 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.

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.

Other aspects of the invention relate to transplantation of cells containing modified nucleic acids to a non-human vertebrate subject. Administration of cells to non-human vertebrate subjects is known to those of ordinary skill in the art, such as local implantation (e.g., topical or subcutaneous administration), organ delivery or systemic injection (e.g., intravenous injection or inhalation), as is the formulation of cells in pharmaceutically acceptable carrier.

Diagnostic Agents

Provided are compositions, methods, kits, and reagents for detection of disease or conditions in non-human animals. The diagnostic agents of the invention include modified nucleic acids, cells containing modified nucleic acids or polypeptides translated from the modified nucleic acids, polypeptides translated from modified nucleic acids, and cells contacted with cells containing modified nucleic acids or polypeptides translated from the modified nucleic acids.

Provided are methods of inducing translation of a recombinant polypeptide in a cell population using the modified nucleic acids described herein. Such translation can be *in vivo*, *ex vivo*, or preferably, *in culture* or *in vitro*. The cell population is 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 is contacted

under conditions such that the nucleic acid is localized into one or more cells of the cell population and the recombinant polypeptide is translated in the cell from the nucleic acid.

An effective amount of the composition is provided based, at least in part, on the 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. 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), or reduced innate immune response of the host cell.

As described herein, a useful feature of the modified nucleic acids of the invention 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 is 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 is determined. Subsequently, the cell is 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 is 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 is 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.

Protein production

Transiently transfected cells may be generated by methods of transfection, electroporation, cationic agents, polymers, or lipid-based delivery molecules well known to those

of ordinary skill in the art. The modified transient RNAs can be introduced into the cultured cells in either traditional batch like steps or continuous flow through steps if appropriate. The methods and compositions of the present invention may be used to produce cells with increased production of one or more protein of interest. Cells can be transfected or otherwise introduced with one or more RNA. The cells may be transfected with the two or more RNA constructs simultaneously or sequentially. In certain embodiments, multiple rounds of the methods described herein may be used to obtain cells with increased expression of one or more RNAs or proteins of interest. For example, cells may be transfected with one or more RNA constructs that encode an RNA or protein of interest and isolated according to the methods described herein.

The isolated cells may then be subjected to further rounds of transfection with one or more other RNA that encode an RNA or protein of interest and isolated once again. This method is useful, for example, for generating cells with increased expression of a complex of proteins, RNAs or proteins in the same or related biological pathway, RNAs or proteins that act upstream or downstream of each other, RNAs or proteins that have a modulating, activating or repressing function to each other, RNAs or proteins that are dependent on each other for function or activity, or RNAs or proteins that share homology (e.g., sequence, structural, or functional homology). For example, this method may be used to generate a cell line with increased expression of the heavy and light chains of an immunoglobulin protein (e.g., IgA, IgD, IgE, IgG, and IgM) or antigen-binding fragments thereof. The immunoglobulin proteins may be fully human, humanized, or chimeric immunoglobulin proteins. In a particular embodiment the RNA encodes an immunoglobulin protein or an antigen-binding fragment thereof, such as an immunoglobulin heavy chain, an immunoglobulin light chain, a single chain Fv, a fragment of an antibody, such as Fab, Fab', or (Fab')₂, or an antigen binding fragment of an immunoglobulin.

In some embodiments, the amount of a protein produced by cells in a tissue may be desirably increased. Preferably, this increase in protein production may be 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 may be provided that contain a ribonucleic acid that may be engineered to avoid an innate immune response of a cell into which the ribonucleic acid enters and encodes the polypeptide of interest and the composition may be 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. In some embodiments, the composition includes a plurality of different ribonucleic acids, where one or more than one of the ribonucleic acids is engineered to avoid an innate immune response of a cell into which the ribonucleic acid enters, and where one or more than one of the ribonucleic acids encodes a polypeptide of interest. Optionally, the composition also contains a cell penetration agent to assist in the intracellular delivery of the ribonucleic acid.

Isolation and/or purification of proteins

Those of ordinary skill in the art can easily make a determination of the proper manner to purify or isolate the protein of interest from the cultured cells. Generally, this is done through a capture method using affinity binding or non-affinity purification. If the protein of interest is not secreted by the cultured cells, then a lysis of the cultured cells would be performed prior to purification or isolation as described above. One can use unclarified cell culture fluid containing the protein of interest along with cell culture media components as well as cell culture additives, such as anti-foam compounds and other nutrients and supplements, cells, cellular debris, host cell proteins, DNA, viruses and the like in the present invention. Moreover, the process can be conducted, if desired, in the bioreactor itself. The fluid may either be preconditioned to a desired stimulus such as pH, temperature or other stimulus characteristic or the fluid can be conditioned upon addition of the polymer(s) or the polymer(s) can be added to a carrier liquid that is properly conditioned to the required parameter for the stimulus condition required for that polymer to be solubilized in the fluid. The polymer(s) is allowed to circulate thoroughly with the fluid and then the stimulus is applied (change in pH, temperature, salt concentration, etc) and the desired protein and polymer(s) precipitate out of solution. The polymer and desired protein(s) is separated from the rest of the fluid and optionally washed one or more times to remove any trapped or loosely bound contaminants. The desired protein is then recovered from the polymer(s) such as by elution and the like. Preferably, the elution is done under a set of conditions such that the polymer remains in its solid (precipitated) form and retains any impurities to it during the selective elution of the desired protein. Alternatively, the polymer and protein as well as any impurities can be solubilized in a new fluid such as water or a buffered solution and the protein be recovered by a means such as affinity, ion exchange, hydrophobic, or some other type of chromatography that has a preference and selectivity for the protein over that of the polymer or impurities. The eluted protein is then recovered and if desired subjected to

additional processing steps, either traditional batch like steps or continuous flow through steps if appropriate.

Additionally, it is useful to optimize the expression of a specific polypeptide in a cell line or collection of cell lines of potential interest, particularly an engineered protein such as a protein variant of a reference protein having a known activity. In one embodiment, provided is a method of optimizing expression of an engineered protein in a target cell, by providing a plurality of target cell types, and independently contacting with each of the plurality of target cell types a modified mRNA encoding an engineered polypeptide. Additionally, culture conditions may be altered to increase protein production efficiency. Subsequently, the presence and/or level of the engineered polypeptide in the plurality of target cell types is detected and/or quantitated, allowing for the optimization of an engineered polypeptide's expression by selection of an efficient target cell and cell culture conditions relating thereto. Such methods are particularly useful when the engineered polypeptide contains one or more post-translational modifications or has substantial tertiary structure, situations which often complicate efficient protein production.

The method according to the invention can also be advantageously used for production of antibodies or fragments thereof. Such fragments include e.g. Fab fragments (Fragment antigen-binding). Fab fragments consist of the variable regions of both chains which are held together by the adjacent constant region.

The protein of interest is preferably recovered from the culture medium as a secreted polypeptide, or it can be recovered from host cell lysates if expressed without a secretory signal. It is necessary to purify the protein of interest from other recombinant proteins and host cell proteins in a way that substantially homogenous preparations of the protein of interest are obtained. As a first step, cells and/or particulate cell debris are removed from the culture medium or lysate. The product of interest thereafter is purified from contaminant soluble proteins, polypeptides and nucleic acids, for example, by fractionation on immunoaffinity or ion-exchange columns, ethanol precipitation, reverse phase HPLC, Sephadex chromatography, chromatography on silica or on a cation exchange resin such as DEAE. In general, methods teaching a skilled person how to purify a protein heterologous expressed by host cells, are well known in the art. Such methods are for example described by (Harris and Angal, 1995) or (Robert Scopes, 1988).

Targeting Moieties

In embodiments of the invention, modified nucleic acids are provided to express a protein-binding partner or a receptor on the surface of the cell, which functions 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 antibodies and functional fragments thereof, scaffold proteins, or peptides. Additionally, modified nucleic acids can be employed to direct the synthesis and extracellular localization of lipids, carbohydrates, or other biological moieties.

Altering the Differentiative State of Cells

Provided are methods of altering the differentiative state of a cell or a population of cells present in a non-human vertebrate subject. Such methods include the steps of i) providing a composition containing a plurality of different ribonucleic acids, wherein each ribonucleic acid is engineered to avoid an innate immune response of a cell into which the ribonucleic acid enters and encodes a polypeptide of interest (thereby producing a plurality of different polypeptides), along with a cell penetration agent, and a pharmaceutically acceptable carrier. A unit quantity of composition may be determined to produce the plurality of different polypeptides of interest in a substantial percentage of cells contained within a predetermined volume of the tissue. The method further includes step ii) determining a dose of the composition required to produce the different polypeptides of interest in a substantial percentage of cells contained within the predetermined volume of the tissue. The different polypeptides of interest may be produced in an amount affective to alter the differentiative state of a significant amount (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 95 or greater than 95%) of those protein producing cells without altering the differentiative state of a significant percentage (50, 40, 30, 20, 10, 5, 4, 3, 2, 1 or less than 1%) of cells in tissue adjacent to the predetermined volume. The method further comprises step iii) introducing directly into the tissue of the mammalian subject the dose of the composition.

In Vitro Translation

Aspects of the invention 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 is administered to the subject using the delivery methods described herein. The nucleic acid is 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 is 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.

Localization

5 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.

Transplantation of Cells

10 Other aspects of the invention relate to transplantation of cells containing modified nucleic acids to a non-human vertebrate subject. Administration of cells to mammalian subjects is known to those of ordinary skill in the art, such as local implantation (e.g., topical or subcutaneous administration), organ delivery or systemic injection (e.g., intravenous injection or inhalation), as is the formulation of cells in pharmaceutically acceptable carrier.

Animal Models

15 In one embodiment, provided are compositions, methods, kits, and reagents for using modified nucleic acids and enhanced nucleic acids in or to create non-human vertebrate animal models. Non-limiting examples of non-human vertebrates used in animal models include primates, dogs, cats, rabbits, rats, mice, xenopus, fish and chickens. In one embodiment, non-human vertebrates may be treated with the modified nucleic acids and enhanced nucleic acids of
20 the present invention which may be useful in biomedical research. In another embodiment, non-human vertebrates may be treated with the modified nucleic acids and enhanced nucleic acids of the present invention to screen and/or test compounds which may be analyzed for pharmaceutical development.

 In some embodiments, the enhanced nucleic acid may be delivered to a transgenic,
25 knock-out, knock-in or otherwise genetically manipulated mouse. Such delivery may be useful for the expression of non-native proteins, the over-expression of native proteins, the reduction of protein expression and/or for other genetic manipulations.

Knock-in Models

30 In some embodiments, the enhanced nucleic acid may be delivered to create a transient knock-in animal such as, but not limited to, mice, rats, rabbits, dogs and the like. Traditionally, knock-in models involve the insertion of a polynucleotide, gene, multiple genes and/or a gene

fragment into a specific locus within the genome of the animal. Such targeted insertion can avoid the disruption of other genes at the insertion site. This may be accomplished by flanking the desired DNA insert with a nucleic acid sequence corresponding to a non-critical locus in the genome of the target species. Upon insertion into a fertilized embryo, the process of homologous recombination allows the foreign gene to be inserted at the site of the non-critical locus.

According to the present invention, the modified mRNA or enhanced nucleic acid molecules may be used to create transient knock-in animal models. In this embodiment, proteins of interest are delivered via the nucleic acid molecule encoding them. Hence, protein expression in an animal may be evaluated transiently, for as long as the encoded protein is translated. This embodiment allows for the controlled temporal study of the effects of one or more proteins in a living system.

In a further embodiment, the gene may be a fluorescent or chemical reporter helping to make knock-in cells easily identifiable. In another embodiment, the knock-in gene may code for nucleic acid that targets and knocks down transcripts from another gene.

Knock-out Models

In some embodiments, modified nucleic acids or enhanced nucleic acids may be delivered to knock-out animals such as, but not limited to, mice and rats. Knock-out mice and/or rats are mice in which a segment of DNA, a gene, multiple genes or a portion of a gene has been deleted from their genome. These animals are generated much like knock-in animals but the flanking regions of the DNA insert contain sequences homologous to the flanking regions of the gene that is being knocked out. The insert then replaces the genomic DNA and knocks out expression of the gene. In a further embodiment, the insert may contain a fluorescent or chemical reporter gene to easily identify cells wherein the target gene has been deleted.

Transgenic Models

In some embodiments, modified nucleic acids and enhanced nucleic acids may be delivered to transgenic mice and/or rats. Similar to knock-in and knock-out animals, a transgenic animal is an animal in which additional genetic material, or transgene, has been introduced. Unlike knock-in and knock-out models, the additional genetic material may be integrated at a random site, such that integration can sometimes disrupt another gene. The transgene can be used to express or over-express a native protein, express a foreign protein, express a given gene under

the control of a desired promoter, express an inhibitor or express an RNA to knock down the expression of another gene.

As a non-limiting example, the protein expressed by the delivery of a modified nucleic acid or an enhanced nucleotides may be Cre recombinase. The delivery of Cre recombinase can be tissue or cell specific and may be delivered to knock-in or transgenic animals whose manipulated genome contains at least one DNA region flanked by a loxP site. The expression of Cre recombinase in cells containing the DNA regions can facilitates the removal and thus the knockout of a particular DNA segment.

Vaccine Production

In some embodiments, the enhanced nucleic acid may be delivered to specialized pathogen-free chickens whose eggs may be used in vaccine production. Vaccine manufacturers use pathogen-free fertilized chicken eggs to produce human and veterinary vaccines. Delivery of the modified nucleic acids and/or enhanced nucleic acids of the present invention to specialized pathogen-free chickens may be useful for the expression of non-native proteins, the over-expression of native proteins, the reduction of protein expression or for other purposes to maintain the pathogen-free state of the chickens, improve their health and/or to enhance egg production.

Kits

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.

In one aspect, the present invention provides kits comprising the modified nucleic acids or the enhanced nucleic acids of the invention. In one embodiment, the kit comprises one or more functional antibodies or function fragments thereof.

Said kits can be for protein production, comprising a first modified nucleic acid or the enhanced nucleic acid 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.

In one aspect, the present invention provides kits for protein production, comprising: a modified nucleic acid or the enhanced nucleic acid 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 or the enhanced nucleic acid comprising an inhibitory nucleic acid, provided in an amount effective to substantially inhibit the innate immune response of the cell; and packaging and instructions.

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

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

Definitions

15 *About:* As used herein, the term "about" means +/- 10% of the recited value.

Administered in combination: As used herein, the term "administered in combination" or "combined administration" means that two or more agents 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 subject. In some embodiments, they are administered within about 60, 30, 15, 10, 5, or 1
20 minute of one another. In some embodiments, the administrations of the agents are spaced sufficiently closely together such that a combinatorial (e.g., a synergistic) effect is achieved.

Animal: As used herein, the term "animal" refers to any member of the animal kingdom, of which vertebrates are a preferred subphylum of the phylum Chordata. In particular
25 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, and fish. In some embodiments, the animal is a transgenic animal, genetically-engineered animal, or a clone.

Antigens of interest or desired antigens: As used herein, the terms "antigens of interest" or
30 "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. Especially, desired antigens or antigens of interest are for example, but not limited to insulin, feline interferon, erythropoietin, cyclosporine, Thymosin Beta-4, arginine vasopressin, bovine somatotropin, oxytocin, ghrelin, gonadorelin, pregnant mare serum gonadotrophin (PMSG), equine chorionic gonadotrophin (ECG), human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone analog (GRHa), pancreatic enzymes, Cre recombinase, 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.

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).

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.

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 effect on that organism, is considered to be biologically active. In particular embodiments, where a nucleic acid is biologically active, a portion of that nucleic acid that shares at least one biological activity of the whole nucleic acid is typically referred to as a “biologically active” portion.

Conserved: As used herein, the term “conserved” refers to nucleotides or amino acid residues of a polynucleotide sequence or amino acid sequence, respectively, that are those that occur

unaltered in the same position of two or more related 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.

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
10 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,
15 about 80% identical, about 90% identical, about 95% identical, about 98% identical, or about 99% identical to one another.

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 non-human vertebrate cell)), bacterium, virus, fungus, protozoan, parasite, prion, or a combination thereof.

20 *Delivery*: As used herein, “delivery” refers to the act or manner of delivering a compound, substance, entity, moiety, cargo or payload.

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 to targeted cells.

Detectable label: As used herein, “detectable label” refers to one or more markers, signals, or
25 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
30 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.

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.

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.

Formulation: As used herein, a “formulation” includes at least a modified nucleic acid and a delivery agent.

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.

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.

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%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical. In some embodiments, polymeric molecules are considered to be “homologous” to one another if their sequences are at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% similar. The term “homologous” necessarily refers to a comparison between at least two sequences (nucleotide sequences or amino acid sequences). In accordance with the invention, two nucleotide sequences are considered to be homologous if the polypeptides they encode are at least about 50% identical, at least about 60% identical, at least about 70% identical, at least about 80% identical, or at least about 90% identical for at least one stretch of at least about 20 amino acids. In some embodiments, homologous nucleotide sequences are characterized by the ability to encode a stretch of at least 4–5 uniquely specified amino acids. Both the identity and the

approximate spacing of these amino acids relative to one another must be considered for nucleotide sequences to be considered homologous. For nucleotide 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% identical, at least about 60% identical, at least about 70% identical, at least about 80% identical, or at least about 90% identical for at least one stretch of at least about 20 amino acids.

Identity: As used herein, the term “identity” 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. Calculation of the percent identity of two nucleic acid sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second nucleic acid sequences for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or 100% of the length of the reference sequence. The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two 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 Atschul, S. F. *et al.*, *J. Molec. Biol.*, 215, 403 (1990)).
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.

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).

In vivo: As used herein, the term "*in vivo*" refers to events that occur within an organism (*e.g.*, animal, plant, or microbe).

Isolated: As used herein, the term "isolated" refers to a substance or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature or in an experimental setting), and/or (2) produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from 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.

Livestock: As used herein, “livestock” relates to domesticated animals raised in an agricultural setting to produce materials such as food, labor, and derived products such as fiber and

5 chemicals. Generally, livestock includes all mammals, avians and fish having potential agricultural significance. For example, livestock may include, four-legged slaughter animals such as, but not limited to, steers, heifers, cows, calves, bulls, cattle, swine and sheep.

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

10 functionally. In one embodiment, the modified nucleic acid molecules of the present invention are modified by the introduction of non-natural nucleosides and/or nucleotides, e.g., as it relates to the natural ribonucleotides A, U, G, and C. Noncanonical nucleotides such as the cap structures are not considered “modified” although they differ from the chemical structure of the A, C, G, U ribonucleotides.

15 *Naturally occurring:* As used herein, “naturally occurring” means existing in nature without artificial aid.

Non-human vertebrate: As described 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, but not limited to, alpaca, banteng,

20 bison, camel, cat, cattle, deer, dog, donkey, elk, gayal, goat, guinea pig, horse, llama, mouse, mule, pig, rabbit, rat, reindeer, sheep water buffalo, and yak; birds such as, but not limited to, caiques, canary, cattle egret, chicken, cockatiel, cockatoo, conure, dove, duck, finch, geese, lovebird, macaw, parakeet, parrot, parrotlet, pigeon, pionus, rosella, and turkey; reptiles such as, but not limited to, iguana, lizard, snake, turtle, tortoise; amphibians such as, but not limited to,

25 caecilian, frog, newt, salamander, and toad.

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.

Paratope: As used herein, “paratope” refers to the antigen-binding site of an antibody.

Pharmaceutically acceptable: The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the

30 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.

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 (diluent), 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.

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, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate,

palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, 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.

Pharmaceutically acceptable solvate: The term "pharmaceutically acceptable solvate," as used herein, means a compound of the invention wherein molecules of a suitable solvent 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."

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
5 condition; and/or decreasing the risk of developing pathology associated with the infection, the disease, disorder, and/or condition.

Protein of Interest: "Proteins of interest" or "desired proteins" include those provided herein and fragments, mutants, variants, and alterations thereof. Especially, desired proteins/polypeptides or proteins of interest are for example, but 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,
10 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.

Sample: As used herein, the term "sample" refers to a subset of its tissues, cells or component
15 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
20 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.

Similarity: As used herein, the term "similarity" refers to the overall relatedness between polymeric molecules, e.g. between nucleic acid molecules (e.g. DNA molecules and/or RNA
25 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.

Subject: As used herein, the term "subject" or "patient" refers to any organism to which a
30 composition in accordance with the invention may be administered, e.g., for experimental,

diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include non-human animals (*e.g.*, mammals such as mice, rats, rabbits, non-human primates).

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.

Suffering from: A non-human individual or population “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.

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. 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.

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.

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”

5 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.

Treating: As used herein, the term “treating” refers to partially or completely alleviating, ameliorating, improving, relieving, delaying onset of, inhibiting progression of, reducing severity

10 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

15 developing pathology associated with the disease, disorder, and/or condition.

Unit Dose: 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-

20 third of such a dosage.

Unmodified: As used herein, “unmodified” refers to a nucleic acid prior to being modified.

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, described herein. The scope of

25 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

30 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. Furthermore, it is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, *etc.*, from one or more of the listed claims is introduced into another claim. For example, any claim that is dependent on another claim can be modified to include one or more limitations found in any other claim that is dependent on the same base claim. Furthermore, where the claims recite a composition, it is to be understood that methods of using the composition for any of the purposes disclosed herein are included, and methods of making the composition according to any of the methods of making disclosed herein or other methods known in the art are included, unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise.

Where elements are presented as lists, *e.g.*, in Markush group format, it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, *etc.*, certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, *etc.* For purposes of simplicity those embodiments have not been specifically set forth *in haec verba* herein. 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 protein; any nucleic acid; 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.

EXAMPLES

10 Example 1. Modified mRNA Production

Modified nucleic acids (modified mRNA) 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); herein incorporated by reference).

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 construct, it may be reconstituted and transformed into chemically competent *E. coli*.

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 μ l 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.

4. Heat shock at 42°C for exactly 30 seconds. 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.
- 5 8. Warm selection plates to 37°C.
9. Mix the cells thoroughly by flicking the tube and inverting.
10. 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.

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.

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.

In order to generate cDNA for *In Vitro* Transcription (IVT), the plasmid 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.

Example 2: PCR for cDNA Production

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 ReadyMix 12.5 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 to termination.

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.

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.

10 **Example 3. *In vitro* Transcription (IVT)**

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 un-natural NTPs.

A typical *in vitro* transcription reaction includes the following:

- | | | |
|----|--|--------------------|
| 15 | 1. Template cDNA | 1.0 µg |
| | 2. 10x transcription buffer (400 mM Tris-HCl pH 8.0, 190 mM MgCl ₂ , 50 mM DTT, 10 mM Spermidine) | 2.0 µl |
| | 3. Custom NTPs (25mM each) | 7.2 µl |
| | 4. RNase Inhibitor | 20 U |
| 20 | 5. T7 RNA polymerase | 3000 U |
| | 6. dH ₂ O | Up to 20.0 µl. and |
| | 7. Incubation at 37° C for 3 hr-5 hrs. | |

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

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.

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.

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.

15 **Example 5. PolyA Tailing Reaction**

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.

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 Poly-A tailing reaction may not always result in exactly 160 nucleotides. Hence Poly-A 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. Formulation of Modified mRNA Using Lipidoids

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
5 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
10 from a recombinant source.

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.

Claims

What is claimed is:

1. A method of producing a polypeptide of interest in a cell, tissue or bodily fluid of a non-human vertebrate subject in need thereof comprising administering to said subject a pharmaceutical composition comprising a nucleic acid encoding said polypeptide of interest.
2. The method of claim 1, wherein the pharmaceutical composition is formulated.
3. The method of claim 2, wherein the formulation is selected from saline or a lipid formulation.
4. The method of claim 2, wherein the formulation is administered by a route selected from the group consisting of intravenous, intramuscular, subcutaneous, and local.
5. The method of claim 1, wherein the pharmaceutical composition is administered on a schedule selected from the group consisting of three times a day, twice a day, once a day, every other day, every third day, weekly, biweekly, every three weeks, every four weeks, and monthly.
6. The method of claim 5, wherein the formulation is administered by multiple administrations.
7. The method of claim 1, wherein the non-human vertebrate is selected from the group consisting of alpaca, banteng, bison, camel, cat, cattle, deer, dog, donkey, elk, gayal, goat, guinea pig, horse, llama, mouse, mule, pig, rabbit, rat, reindeer, sheep water buffalo, yak, caiques, canary, cattle egret, chicken, cockatiel, cockatoo, conure, dove, duck, finch, geese, lovebird, macaw, parakeet, parrot, parrotlet, pigeon, pionus, rosella, turkey, iguana, lizard, snake, turtle, tortoise, caecilian, frog, newt, salamander, and toad.
8. The method of claim 8, wherein the non-human vertebrate is a mouse.
9. The method of claim 9, wherein the mouse is selected from the group consisting of a transgenic mouse, a knock-in mouse and a knock-out mouse.
10. The method of claim 1, wherein the bodily fluid is selected from the group consisting of peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous humor, amniotic fluid, cerumen, breast milk, bronchoalveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, sweat, fecal matter, hair, tears, cyst fluid, pleural and peritoneal fluid, pericardial

fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, blastocyl cavity fluid, and umbilical cord blood.

11. The method of claim 1, wherein the tissue is selected from the group consisting of liver, spleen, kidney, lung, heart, peri-renal adipose tissue, thymus and muscle.

12. The method of claim 1, wherein the polypeptide of interest is selected from the group consisting of insulin, feline interferon, erythropoietin, cyclosporine, Thymosin Beta-4, arginine vasopressin, bovine somatotropin, oxytocin, ghrelin, gonadorelin, pregnant mare serum gonadotrophin (PMSG), equine chorionic gonadotrophin (ECG), human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone analog (GRHa), pancreatic enzymes, Cre recombinase, an insulin-like growth factor, hGH, tPA, Interleukin (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, IFN tau, tumor necrosis factor (TNF) alpha, TNF beta, TNF gamma, TRAIL, G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF.

13. The method of claim 12, wherein the polypeptide of interest is selected from the group consisting of The method of claim 1, wherein the polypeptide of interest is selected from the group consisting of insulin, feline interferon, erythropoietin, cyclosporine, Thymosin Beta-4, arginine vasopressin, bovine somatotropin, oxytocin, ghrelin, gonadorelin, pregnant mare serum gonadotrophin (PMSG), equine chorionic gonadotrophin (ECG), human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone analog (GRHa), pancreatic enzymes and Cre recombinase.

14. The method of claim 1, wherein the nucleic acid comprises one or more modifications 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-pseudouridine, 5-hydroxyuridine, 3-methyluridine, 5-carboxymethyl-uridine, 1-carboxymethyl-pseudouridine, 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-aurinomethyluridine, 1-aurinomethyl-pseudouridine, 5-aurinomethyl-2-thio-uridine, 1-aurinomethyl-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, dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-

dihydropseudouridine, 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-formylcytidine, 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, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-
 thio-zebularine, 2-thio-zebularine, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-
 methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, 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-glycinylicarbamoyl-adenosine, N6-threonylicarbamoyl-adenosine, 2-
 methylthio-N6-threonyl carbamoyl-adenosine, N6,N6-dimethyladenosine, 7-
 methyladenine, 2-methylthio-adenine, and 2-methoxy-adenine, inosine, 1-methyl-inosine,
 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, and combinations thereof.

15. A kit for producing a first polypeptide of interest in a cell, tissue or bodily fluid of a non-
 human vertebrate subject in need thereof comprising a first nucleic acid encoding said
 first polypeptide of interest.

16. The kit of claim 15, further comprising a second nucleic acid comprising a second
 nucleic acid encoding a second polypeptide of interest.

17. The kit of claim 16 where the second polypeptide of interest is different than the first
 polypeptide of interest.

18. The kit of claim 15, wherein the non-human vertebrate is selected from the group
 consisting of alpaca, banteng, bison, camel, cat, cattle, deer, dog, donkey, elk, geyal,

goat, guinea pig, horse, llama, mouse, mule, pig, rabbit, rat, reindeer, sheep water buffalo, yak, caiques, canary, cattle egret, chicken, cockatiel, cockatoo, conure, dove, duck, finch, geese, lovebird, macaw, parakeet, parrot, parrotlet, pigeon, pionus, rosella, turkey, iguana, lizard, snake, turtle, tortoise, caecilian, frog, newt, salamander, and toad.

- 5 19. The kit of claim 15, wherein the polypeptide of interest is selected from the group consisting of insulin, feline interferon, erythropoietin, cyclosporine, Thymosin Beta-4, arginine vasopressin, bovine somatotropin, oxytocin, ghrelin, gonadorelin, pregnant mare serum gonadotrophin (PMSG), equine chorionic gonadotrophin (ECG), human chorionic gonadotrophin (hCG), gonadotrophin-releasing hormone analog (GRHa),
10 pancreatic enzymes, Cre recombinase, an insulin-like growth factor, hGH, tPA, Interleukin (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, IFN tau, tumor necrosis factor (TNF) alpha, TNF beta, TNF gamma, TRAIL, G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF.
- 15 20. The kit of claim 15, wherein the nucleic acid comprises one or more modifications 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-pseudouridine, 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, dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 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-formylcytidine, 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, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-thio-zebularine, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-
- 20
- 25
- 30

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-
5 (cis-hydroxyisopentenyl)adenosine, 2-methylthio-N6-(cis-hydroxyisopentenyl)adenosine, N6-glycinylicarbamoyladenosine, N6-threonylicarbamoyladenosine, 2-methylthio-N6-threonyl carbamoyladenosine, N6,N6-dimethyladenosine, 7-methyladenine, 2-methylthio-adenine, and 2-methoxy-adenine, inosine, 1-methyl-inosine, wyosine, wybutosine, 7-deaza-guanosine, 7-deaza-8-aza-guanosine, 6-thio-guanosine, 6-
10 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, and combinations thereof.

15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 12/38028

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12P 21/06 (2012.01)

USPC - 435/69.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC - 435/69.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 435/69.1, 69.8, 70.1, 91.1

(Text Search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWEST (PGPB, USPT, USOC, EPAB, JPAB) and Google Scholar.

Search Terms: administe\$, DNA, RNA, nucleic acid, polynucleotide, protein, polypeptide, insulin, pyridin-4-one ribonucleoside, transgenic, 5-aza-uridine, mouse, 5-methyl-uridine, 5-hydroxyuridine, gene therapy, erythropoietin, oxytocin, ghrelin, gonadorelin, PMSG,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US 2009/0286852 A1 (KARIKO et al.) 19 November 2009 (19.11.2009) para [0010]-[0011], [0068]-[0070], [0080], [0104], [0125], [0158], [0163]-[0166], [0171]-[0172], [0189], [0237], [0261]-[0263]	1-8 and 10-20 9
Y	US 2002/0111471 A1 (LO et al.) 15 August 2002 (15.08.2002) para [0243].	9

☐ Further documents are listed in the continuation of Box C.


* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

05 August 2012 (05.08.2012)

Date of mailing of the international search report

14 AUG 2012

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774