(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 May 2013 (30.05.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/078199 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/US2012/066055

(22) International Filing Date:
20 November 2012 (20.11.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/563,171 23 November 2011 (23.11.2011) US

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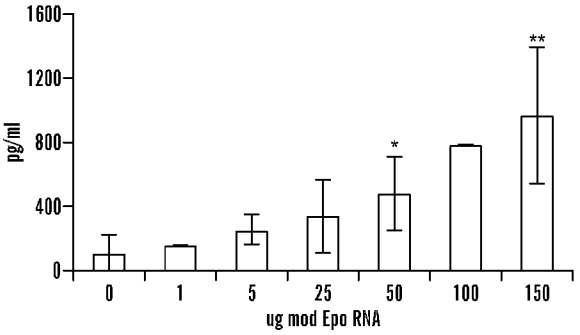
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, 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, PA, 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,

[Continued on next page]

(54) Title: METHODS FOR ENHANCED *IN VIVO* DELIVERY OF SYNTHETIC, MODIFIED RNAS

(57) Abstract: Described herein are unit dose compositions, kits, delivery devices, and methods for enhancing delivery of a synthetic, modified RNAs encoding a therapeutic agent to a subject. These unit dose compositions, kits, delivery devices, and methods for enhancing delivery can be used to express a desired therapeutic agent without the introduction of any exogenous DNA or viral vectors, and thus, do not cause permanent modification of the genome or have the potential for unintended mutagenic effects. The RNAs provided using the dosages as provided have been shown to provide a dose-dependent response both in terms of protein expression and function.



ug RNA	NUMBER OF MICE (n)	ttest
0	6	
1	1	n/a
5	3	0.118
25	4	0.083
50	4	0.015
100	1	n/a
150	4	0.003

NOTE: MALES AND FEMALES COMBINED; 0ug OUTLIER REMOVED

FIG. 1

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TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

METHODS FOR ENHANCED *IN VIVO* DELIVERY OF SYNTHETIC, MODIFIED RNAS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. §119(e) of U.S. Provisional Application Serial No.: 61/563,171 filed November 23, 2011, the contents of which are herein incorporated by reference in their entireties.

FIELD OF THE INVENTION

[0002] The field of the invention relates to unit dose compositions comprising synthetic, modified RNAs and methods of delivery thereof.

BACKGROUND OF THE INVENTION

[0003] Methodologies for deliverying pharmaceutical compositions comprising nucleic acids in order to achieve effective protein expression for therapeutics and bioprocessing applications can be problematic. For example, introduced DNA can integrate into host cell genomic DNA and/or can be inherited by daughter cells. Further, multiple steps occur after delivery but before the encoded protein is made, which can impact protein expression, including, for example, transportation into the nucleus for transcription into RNA, followed by transcribed RNA entering the cytoplasm where it is translated into protein. Furthermore, even if DNA is successfully introduced into a cell, expression levels and rates of expression can vary. In addition, effective delivery and achievement of thereapeutically relevant levels of proteins for a time sufficient to produce clinical results can be problematic.

SUMMARY

[0004] Accordingly, the present invention provides novel unit doses of synthetic, modified RNAs for optimizing protein expression from delivered pharmaceutical modalities.The present invention provides, in part, novel unit doses of synthetic, modified RNAs for intramuscular administration. The inventors have discovered particularly effective unit dosages for intramuscular administration of synthetic modified RNAs using a combination of a synthetic modified RNAs and a cationic lipid. The RNAs provided using the dosages as provided have been shown to provide a dose-dependent response both in terms of protein expression and function.

[0005] Accordingly, in some aspects, provided herein are unit dose compositions comprising at least one synthetic, modified RNA encoding a therapeutic agent, wherein the unit dose ranges from between 0.03 mg/kg of body weight and 10 mg/kg of body weight. In some embodiments of these aspects and all such aspects described herein, the synthetic, modified RNA comprises at least two modified nucleosides.

[0006] In some embodiments of these aspects and all such aspects described herein, the therapeutic agent is a polypeptide or a non-translated RNA molecule. In some such embodiments, the

polypeptide is a somatotrophic agent. In some embodiments, the somatotrophic agent is a growth hormone. In some such embodiments, the polypeptide is a cytokine. In some such embodiments, the polypeptide is a cellular growth factor.

[0007] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 0.03 mg per kg of body weight to about 0.0625 mg per kg of body weight.

[0008] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 0.1 mg per kg of body weight to about 0.4 mg per kg of body weight.

[0009] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 0.9 mg per kg of body weight to about 1.6 mg per kg of body weight.

[0010] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 1.8 mg per kg of body weight to about 3.2 mg per kg of body weight.

[0011] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 3.0 mg per kg of body weight to about 6.5 mg per kg of body weight.

[0012] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 5.5 mg per kg of body weight to about 10 mg per kg of body weight.

[0013] In some embodiments of these aspects and all such aspects described herein, the at least two modified nucleosides are selected from the group consisting of 5-methylcytidine (5mC), N6-methyladenosine (m6A), 3,2'-O-dimethyluridine (m4U), 2-thiouridine (s2U), 2' fluorouridine, pseudouridine, 2'-O-methyluridine (Um), 2'deoxy uridine (2' dU), 4-thiouridine (s4U), 5-methyluridine (m5U), 2'-O-methyladenosine (m6A), N6,2'-O-dimethyladenosine (m6Am), N6,N6,2'-O-trimethyladenosine (m62Am), 2'-O-methylcytidine (Cm), 7-methylguanosine (m7G), 2'-O-methylguanosine (Gm), N2,7-dimethylguanosine (m2,7G), N2, N2, 7-trimethylguanosine (m2,2,7G), and inosine (I).

[0014] In some embodiments of these aspects and all such aspects described herein, the at least two modified nucleosides are 5-methylcytidine (5mC) and pseudouridine.

[0015] In some embodiments of these aspects and all such aspects described herein, the unit dose compositions further comprise a cationic lipid.

[0016] Also provided herein, in some aspects, are pharmaceutical compositions for intramuscular delivery comprising a unit dose of a synthetic, modified RNA encoding a therapeutic agent and a pharmaceutically acceptable carrier, wherein the unit dose ranges from between 0.03 mg/kg of body weight and 10 mg/kg of body weight. In some embodiments of these aspects and all such aspects described herein, the synthetic, modified RNA comprises at least two modified nucleosides.

[0017] In some embodiments of these aspects and all such aspects described herein, the therapeutic agent is a polypeptide or a non-translated RNA molecule. In some such embodiments, the polypeptide is a somatotrophic agent. In some embodiments, the somatotrophic agent is a growth

hormone. In some such embodiments, the polypeptide is a cytokine. In some such embodiments, the polypeptide is a cellular growth factor.

[0018] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 0.03 mg per kg of body weight to about 0.0625 mg per kg of body weight.

[0019] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 0.1 mg per kg of body weight to about 0.4 mg per kg of body weight.

[0020] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 0.9 mg per kg of body weight to about 1.6 mg per kg of body weight.

[0021] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 1.8 mg per kg of body weight to about 3.2 mg per kg of body weight.

[0022] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 3.0 mg per kg of body weight to about 6.5 mg per kg of body weight.

[0023] In some embodiments of these aspects and all such aspects described herein, the unit dose ranges from about 5.5 mg per kg of body weight to about 10 mg per kg of body weight.

[0024] In some embodiments of these aspects and all such aspects described herein, the at least two modified nucleosides are selected from the group consisting of 5-methylcytidine (5mC), N6-methyladenosine (m6A), 3,2'-O-dimethyluridine (m4U), 2-thiouridine (s2U), 2' fluorouridine, pseudouridine, 2'-O-methyluridine (Um), 2'deoxy uridine (2' dU), 4-thiouridine (s4U), 5-methyluridine (m5U), 2'-O-methyladenosine (m6A), N6,2'-O-dimethyladenosine (m6Am), N6,N6,2'-O-trimethyladenosine (m62Am), 2'-O-methylcytidine (Cm), 7-methylguanosine (m7G), 2'-O-methylguanosine (Gm), N2,7-dimethylguanosine (m2,7G), N2, N2, 7-trimethylguanosine (m2,2,7G), and inosine (I).

[0025] In some embodiments of these aspects and all such aspects described herein, the at least two modified nucleosides are 5-methylcytidine (5mC) and pseudouridine.

[0026] In some embodiments of these aspects and all such aspects described herein, the pharmaceutical compositions further comprise a cationic lipid.

[0027] In some aspects, provided herein are kits comprising (a) a container or vial containing a unit dose of a synthetic, modified RNA encoding a therapeutic agent, wherein the unit dose ranges from between 0.03 mg/kg of body weight and 10 mg/kg of body weight, or a pharmaceutical composition for intramuscular delivery comprising a unit dose of a synthetic, modified RNA encoding a therapeutic agent and a pharmaceutically acceptable carrier, wherein the unit dose ranges from between 0.03 mg/kg of body weight and 10 mg/kg of body weight; and (b) packaging and instructions therefor. In some embodiments of these aspects and all such aspects described herein, the synthetic, modified RNA comprises at least two modified nucleosides.

[0028] In some embodiments of these kits, the unit dose is divided into at least two containers or vials.

[0029] In other aspects, provided herein are intramuscular delivery devices comprising a unit dose of a synthetic, modified RNA encoding a therapeutic agent, wherein the unit dose ranges from between 0.03 mg/kg of body weight and 10 mg/kg of body weight, or a pharmaceutical composition for intramuscular delivery comprising a unit dose of a synthetic, modified RNA encoding a therapeutic agent and a pharmaceutically acceptable carrier, wherein the unit dose ranges from between 0.03 mg/kg of body weight and 10 mg/kg of body weight.

[0030] In some embodiments of these intramuscular delivery devices, the intramuscular delivery device is a non-implantable delivery device or an implantable delivery device.

[0031] In some embodiments of these intramuscular delivery devices, the intramuscular delivery device is a syringe.

[0032] Also provided herein, in some aspects, are enhanced methods for delivering synthetic, modified RNA into a subject, such methods comprising administering intramuscularly to a subject at least one unit dose of a synthetic, modified RNA encoding a therapeutic agent, wherein the unit dose ranges from between 0.03 mg/kg of body weight and 10 mg/kg of body weight, or a pharmaceutical composition for intramuscular delivery comprising a unit dose of a synthetic, modified RNA encoding a therapeutic agent and a pharmaceutically acceptable carrier, wherein the unit dose ranges from between 0.03 mg/kg of body weight and 10 mg/kg of body weight.

[0033] In some embodiments of these methods and all such methods described herein, the unit dose is divided into at least two separate unit dosages and administered simultaneously into at least two muscular locations.

[0034] In some embodiments of these methods and all such methods described herein, the unit dose is divided into at least two separate unit dosages and administered sequentially into the same or a different muscular locations.

[0035] In some embodiments of these methods and all such methods described herein, the therapeutic agent is a polypeptide or a non-translated RNA molecule. In some such embodiments, the polypeptide is a somatotrophic agent. In some embodiments, the somatotrophic agent is a growth hormone. In some such embodiments, the polypeptide is a cytokine. In some such embodiments, the polypeptide is a cellular growth factor.

[0036] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 0.03 mg per kg of body weight to about 0.0625 mg per kg of body weight.

[0037] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 0.1 mg per kg of body weight to about 0.4 mg per kg of body weight.

[0038] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 0.9 mg per kg of body weight to about 1.6 mg per kg of body weight.

[0039] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 1.8 mg per kg of body weight to about 3.2 mg per kg of body weight.

[0040] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 3.0 mg per kg of body weight to about 6.5 mg per kg of body weight.

[0041] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 5.5 mg per kg of body weight to about 10 mg per kg of body weight.

[0042] In some embodiments of these methods and all such methods described herein, the at least two modified nucleosides are selected from the group consisting of 5-methylcytidine (5mC), N6-methyladenosine (m6A), 3,2'-O-dimethyluridine (m4U), 2-thiouridine (s2U), 2' fluorouridine, pseudouridine, 2'-O-methyluridine (Um), 2'deoxy uridine (2' dU), 4-thiouridine (s4U), 5-methyluridine (m5U), 2'-O-methyladenosine (m6A), N6,2'-O-dimethyladenosine (m6Am), N6,N6,2'-O-trimethyladenosine (m62Am), 2'-O-methylcytidine (Cm), 7-methylguanosine (m7G), 2'-O-methylguanosine (Gm), N2,7-dimethylguanosine (m2,7G), N2, N2, 7-trimethylguanosine (m2,2,7G), and inosine (I).

[0043] In some embodiments of these methods and all such methods described herein, the at least two modified nucleosides are 5-methylcytidine (5mC) and pseudouridine.

[0044] In some embodiments of these methods and all such methods described herein, the unit dose or pharmaceutical compositions further comprise a cationic lipid.

[0045] Also provided herein, in some aspects, are methods of stimulating erythropoiesis in a mammalian subject in need thereof, such methods comprising the step of administering intramuscularly a pharmaceutical composition for intramuscular delivery comprising a unit dose of a synthetic, modified RNA encoding a therapeutic agent and a pharmaceutically acceptable carrier, wherein the unit dose ranges from between 0.03 mg/kg of body weight and 10 mg/kg of body weight.

[0046] In some embodiments of these methods, the therapuetic agent is erythropoietin.

[0047] In some embodiments of these methods and all such methods described herein, the unit dose is divided into at least two separate unit dosages and administered simultaneously into at least two muscular locations.

[0048] In some embodiments of these methods and all such methods described herein, the unit dose is divided into at least two separate unit dosages and administered sequentially into the same or a different muscular locations.

[0049] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 0.03 mg per kg of body weight to about 0.0625 mg per kg of body weight.

[0050] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 0.1 mg per kg of body weight to about 0.4 mg per kg of body weight.

[0051] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 0.9 mg per kg of body weight to about 1.6 mg per kg of body weight.

[0052] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 1.8 mg per kg of body weight to about 3.2 mg per kg of body weight.

[0053] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 3.0 mg per kg of body weight to about 6.5 mg per kg of body weight.

[0054] In some embodiments of these methods and all such methods described herein, the unit dose ranges from about 5.5 mg per kg of body weight to about 10 mg per kg of body weight.

[0055] In some embodiments of these methods and all such methods described herein, the at least two modified nucleosides are selected from the group consisting of 5-methylcytidine (5mC), N6-methyladenosine (m6A), 3,2'-O-dimethyluridine (m4U), 2-thiouridine (s2U), 2' fluorouridine, pseudouridine, 2'-O-methyluridine (Um), 2'deoxy uridine (2' dU), 4-thiouridine (s4U), 5-methyluridine (m5U), 2'-O-methyladenosine (m6A), N6,2'-O-dimethyladenosine (m6Am), N6,N6,2'-O-trimethyladenosine (m62Am), 2'-O-methylcytidine (Cm), 7-methylguanosine (m7G), 2'-O-methylguanosine (Gm), N2,7-dimethylguanosine (m2,7G), N2, N2, 7-trimethylguanosine (m2,2,7G), and inosine (I).

[0056] In some embodiments of these methods and all such methods described herein, the at least two modified nucleosides are 5-methylcytidine (5mC) and pseudouridine.

[0057] In some embodiments of these methods and all such methods described herein, the unit dose or pharmaceutical compositions further comprise a cationic lipid.

BRIEF DESCRIPTION OF THE DRAWINGS

[0058] **FIG. 1** demonstrates dose-dependency of protein expression upon intramuscular injection of synthetic, modified RNAs. Various doses of a synthetic, modified RNA encoding Erythropoietin (EPO) were injected intramuscularly, and thirteen hours later plasma Erythropoietin levels were examined using ELISA. As shown, increasing the doses of the synthetic, modified RNA leads to a proportional increase in plasma levels of erythropoietin.

[0059] **FIG. 2** demonstrates that 1 week following injection of a synthetic, modified RNA encoding erythropoietin, functional parameters mediated by erythropoietin increase in a dose-dependent fashion. Synthetic, modified RNA encoding erythropoietin was injected with (+) or without (-) lipofectamine, with unmodified RNA with lipofectamine used as a control. The upper left, upper right, and lower left panels demonstrates that red blood cell (RBC) counts, hematocrit, and hemoglobin levels were increased in a dose-dependent fashion 1 week following injection of the synthetic, modified RNA encoding erythropoietin in the presence of lipfectamine. In the absence of liptofectamine or when unmodified RNAs were used, a dose-dependent effect was not observed for any of these functional parameters.

[0060] **FIG. 3** demonstrates that parameters and cells not responsive to erythropoietin are not impacted by the injection of the synthetic, modified RNA encoding erythropoietin, therefore demonstrating the specificity of the effects of the synthetic, modified RNA. Synthetic, modified RNA encoding erythropoietin was injected with (+) or without (-) lipofectamine, with unmodified RNA with lipofectamine used as a control. The upper left panel shows white blood cell (WBC) counts, the

upper right panel shows lymphocyte counts, the lower left panel shows neutrophil counts, and the lower right paner shows platelet numbers 1 week following injection. In contrast to the RBC data shown in FIG. 2, no dose-dependent effects are observed for any of the cells types.

DETAILED DESCRIPTION

[0061] Provided herein are unit dose compositions, kits, delivery devices, and methods for enhancing delivery of a synthetic, modified RNAs encoding a therapeutic agent to a subject. These unit dose compositions, kits, delivery devices, and methods for enhancing delivery can be used to express a desired therapeutic agent without the introduction of any exogenous DNA or viral vectors, and thus, do not cause permanent modification of the genome or have the potential for unintended mutagenic effects.

[0062] The inventors have demonstrated, using erythropoietin as an exemplary therapeutic agent, that intramuscular administration of a unit dose of a synthetic, modified RNA encoding a therapeutic agent, such as a growth factor, particularly when combined with a lipid carrier, permits dose-dependent protein expression that can be measured in the systemic circulation. As described herein, various doses of a synthetic, modified RNA encoding Erythropoietin (EPO) were injected intramuscularly, and thirteen hours later plasma erythropoietin levels were examined using ELISA. Increasing doses of the synthetic, modified RNA is demonstrated herein to result in a proportional increase in plasma levels of a therapeutic agent, such as erythropoietin.

[0063] The inventors further demonstrate the functional impact of intramuscular administration of unit dose compositions comprising synthetic, modified RNAs encoding a therapeutic agent. Again using erythropoietin as an exemplary therapeutic agent, the inventors show that there is a dose-dependent functional response to the increased levels of erythropoietin. More specifically, one week following the injection of a synthetic, modified RNA encoding erythropoietin, functional parameters mediated by erythropoietin were found to increase in a dose-dependent fashion. Synthetic, modified RNA encoding erythropoietin was injected with or without a cationic lipid agent, lipofectamine, with an unmodified RNA injected with lipofectamine used as a control. It was found that red blood cell (RBC) counts, hematocrit, and hemoglobin levels were increased in a dose-dependent fashion that was measurable one week following injection of the synthetic, modified RNA encoding erythropoietin in the presence of lipofectamine. In the absence of liptofectamine or when unmodified RNAs were used, a dose-dependent effect was not observed for any of these functional parameters. Thus, expression of a therapeutic agent encoded by a unit dose composition comprising synthetic, modified RNA is demonstrated herein to have functional implications that occur in a dose-dependent fashion.

[0064] The inventors also demonstrated that cells not responsive to a therapeutic agent, such as erythropoietin, are not impacted by the intramuscular injection of the synthetic, modified RNA

encoding the therapeutic agent, therefore demonstrating the specificity of the effects of the unit dose composition comprising synthetic, modified RNAs.

[0065] The unit dose compositions comprising synthetic, modified RNA encoding erythropoietin were injected with or without a cationic lipid agent, lipofectamine, and with unmodified RNA plus lipofectamine used as a control. The inventors demonstrated that the dose dependent response in protein expression as well as the functional effects of the protein expression was only seen when the intramuscular administration was performed with the modified RNA combined with the cationic lipid agent.

[0066] No effects of intramuscular injection of unit dose compositions comprising synthetic, modified RNAs encoding erythropoietin were found for other blood cell populations, as demonstrated by cell counts of white blood cells (WBC), lymphocytes, neutrophils, and platelets one week following injection. In contrast to the erythropoietin-responsive red blood cells, no dose-dependent effects are observed for any of the other blood cells types.

[0067] Accordingly, the inventors have shown a particularly effective composition for intramuscular delivery of RNA therapies comprising a unit dose of synthetic modified RNA encoding a protein of interest for the delivery of such synthetic modified RNAs to a subject. The unit dose compositions comprising synthetic, modified RNAs encoding therapeutic agents can be used to provide dose-dependent effects on specific cell types *in vivo*.

[0068] The specific dosage ranges have been shown to work for an exemplary RNA encoding EPO protein. However, these dosage ranges may be extrapolated to any other protein with the knowledge that the method allows a dose-dependent titration of the RNAs. Similarly, while the preliminary results were obtained in a mouse model, the results may be extrapolated into other animals, including humans based on the per kg amount calculated from the exemplary mouse models. Moreover, the discovery of a unit dose-dependent expression of the RNA allows one to more easily titrate effective dosages for any protein.

Unit Dose Compositions Comprising Synthetic, Modified RNAs and Administration Thereof

[0069] Provided herein, in some aspects, are unit dose compositions comprising synthetic, modified RNAs encoding therapeutic agents for enhancing delivery of such agents to a subject. Dosage and administration can vary with the condition to be treated and the therapeutic approach taken in a given instance, as described herein. The term "unit dose," as used herein, when used in reference to a therapeutic composition, refers to physically discrete units suitable as unitary dosage for the subject, each unit containing a predetermined quantity of active material, such as a synthetic, modified RNA calculated to produce the desired therapeutic effect in association with the required physiologically acceptable diluent, *i.e.*, carrier, or vehicle. As described herein, the unit dose compositions are administered intramuscularly in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered and timing depends on the

subject to be treated, capacity of the subject's system to utilize the active ingredient, and degree of therapeutic effect desired.

[0070] The success of treatment or therapy using the unit dose compositions comprising synthetic, modified RNAs encoding therapeutic agents described herein can be evaluated by the ordinarily skilled clinician by monitoring one or more symptoms or markers of the disease or disorder being treated by administration of the cells. Effective treatment includes any statistically significant improvement in one or more indicia of the disease or disorder. Where appropriate, a clinically accepted grade or scaling system for the given disease or disorder can be applied, with an improvement in the scale or grade being indicative of effective treatment.

[0071] Dosages of the unit dose compositions comprising synthetic, modified RNAs encoding therapeutic agents can vary depending upon the approach taken and the disease to be treated. For example, intramuscular administration without a targeting approach will generally require greater amounts of the unit dose compositions comprising synthetic, modified RNA than intramuscular administration that employs a targeting or homing approach.

[0072] As demonstrated herein, intramuscular injection of the unit dose compositions comprising synthetic, modified RNAs encoding therapeutic agents provide dose-dependent expression of the therapeutic agent. Depending upon the therapeutic agent and formulation of the unit dose compositions, effective dosages of unit dose compositions comprising synthetic, modified RNA can include, for example, 1 ng/kg of body weight up to a gram or more per kg of body weight and any amount in between. Preferred amounts, include, for example, unit dose ranges from about 0.03 mg per kg of body weight to about 0.0625 mg per kg of body weight; unit dose ranges from about 0.05 mg per kg of body weight to about 0.2 mg per kg of body weight; unit dose ranges from about 0.1 mg per kg of body weight to about 0.4 mg per kg of body weight; unit dose ranges from about 0.3 mg per kg of body weight to about 1.0 mg per kg of body weight; unit dose ranges from about 0.9 mg per kg of body weight to about 1.6 mg per kg of body weight; unit dose ranges from about 1.5 mg per kg of body weight to about 2.0 mg per kg of body weight; unit dose ranges from about 1.8 mg per kg of body weight to about 3.2 mg per kg of body weight; unit dose ranges from about 3.0 mg per kg of body weight to about 6.5 mg per kg of body weight; unit dose ranges from about 5.5 mg per kg of body weight to about 10 mg per kg of body weight; unit dose ranges from about 8.0 mg per kg of body weight to about 20 mg per kg of body weight or any amount in between.

[0073] Dosages in such ranges can be administered once, twice, three times, four times or more per day, or every two days, every three days, every four days, once a week, twice a month, once a month or less frequently over a duration of days, weeks or months, depending on the condition being treated - where the therapeutic approach treats or ameliorates but does not permanently cure the disease or disorder, *e.g.*, where the unit dose compositions comprising synthetic, modified RNAs effects treatment of a growth or metabolic disorder by expression of a protein that is deficient in the subject, administration of the unit dose compositions comprising synthetic, modified RNAs can be

repeated over time as needed. Where, instead, the expression of the therapeutic agent encoded by the unit dose compositions comprising synthetic, modified RNAs leads to the establishment of a cell compartment that maintains itself and treats the disease or disorder, readministration can become unnecessary. Sustained release formulations of unit dose compositions comprising synthetic, modified RNAs are specifically contemplated herein. Continuous, relatively low doses are contemplated after an initial higher therapeutic dose.

[0074] A unit dose composition comprising at least one synthetic, modified RNA as described herein can be delivered to or administered to a subject by an intramuscular delivery route or intramuscular injection. A unit dose composition comprising at least one synthetic, modified RNAs can be incorporated into pharmaceutical compositions suitable for intramuscular administration. For example, unit dose compositions can include one or more synthetic, modified RNAs and a pharmaceutically acceptable carrier. Supplementary active compounds, such as targeting moieties, can also be incorporated into the unit dose compositions, as described herein. Unit dose compositions for intramuscular administration of synthetic, modified RNAs can include sterile aqueous solutions that can also contain buffers, diluents and other suitable additives.

[0075] In some embodiments, the effective dose of a synthetic, modified RNA can be administered in a single dose or in two or more doses, as desired or considered appropriate under the specific circumstances.

[0076] In some embodiments, the effective dose of a synthetic, modified RNA can be administered as two or more separate unit dosages and administered simultaneously into at least two different muscular locations. In some embodiments, the effective dose of a synthetic, modified RNA can be administered as two or more separate unit dosages and administered sequentially into the same or different muscular locations.

[0077] If desired to facilitate repeated or frequent infusions, a non-implantable intramuscular delivery device, e.g., needle, syringe, pen device, or implantatable intramuscular delivery device, e.g., a pump, semi-permanent stent, or reservoir can be advisable. In some such embodiments, the delivery device can include a mechanism to dispense a unit dose of the pharmaceutical composition comprising a synthetic, modified RNA. In some embodiments, the device releases the pharmaceutical composition comprising a synthetic, modified RNAcontinuously, e.g., by diffusion. In some embodiments, the device can include a sensor that monitors a parameter within a subject. For example, the device can include pump, e.g., and, optionally, associated electronics.

[0078] A unit dose composition comprising at least one synthetic, modified RNA can be modified such that it is capable of traversing the blood brain barrier. For example, the synthetic, modified RNA can be conjugated to a molecule that enables the agent to traverse the barrier. Such conjugated synthetic, modified RNA can be administered by intramuscular injection.

[0079] A unit dose composition comprising a synthetic, modified RNA described herein can also be delivered through the use of implanted, indwelling catheters that provide a means for injecting

small volumes of fluid containing the synthetic, modified RNAs described herein directly into the muscle. The proximal end of these catheters can be connected to an implanted, access port surgically affixed to the patient's body.

[0080] Alternatively, implantable delivery devices, such as an implantable pump can be employed. The delivery of the unit dose compositions comprising at least one synthetic, modified RNA as described herein can be accomplished with a wide variety of devices, including but not limited to U.S. Pat. Nos. 5,735,814, 5,814,014, and 6,042,579, all of which are incorporated herein by reference. Using the teachings described herein, those of skill in the art will recognize that these and other devices and systems can be suitable for intramuscular delivery of unit dose compositions comprising the synthetic, modified RNAs described herein.

[0081] In some such embodiments, the delivery system further comprises implanting a pump outside the body, the pump coupled to a proximal end of the catheter, and operating the pump to deliver the predetermined dosage of a unit dose composition comprising a synthetic, modified RNA described herein through the discharge portion of the catheter. A further embodiment comprises periodically refreshing a supply of the unit dose composition comprising a synthetic, modified RNA to the pump outside the body.

[0082] Intramuscular administration of a unit dose composition comprising at least one synthetic, modified RNA can be provided by the subject or by another person, e.g., a another caregiver. A caregiver can be any entity involved with providing care to the human: for example, a hospital, hospice, doctor's office, outpatient clinic; a healthcare worker such as a doctor, nurse, or other practitioner; or a spouse or guardian, such as a parent.

[0083] Where cells expressing therapeutic agents, such as proteins, encoded by synthetic, modified RNA as described herein are administered to treat a malignancy or disease or disorder, the dose of cells administered will also vary with the therapeutic approach. For example, where a unit dose composition expresses a therapeutic agent for targeting a tumor cell, the dosage of cells administered will vary with, for example, the size of the tumor being treated - generally more cells or more frequent administration is warranted for larger tumors versus smaller ones. The unit dose composition amount administered will also vary with the level of expression of the polypeptide or polypeptides encoded by the synthetic, modified RNA - this is equally true of the administration of cells expressing proteins encoded by modified RNA for any purpose described herein. An important advantage of the unit dose compositions and methods described herein is that where, for example, more than one factor or polypeptide is expressed from a unit dose composition comprising at least one synthetic, modified RNA administered to a subject, the relative dosage of the expressed proteins can be tuned in a straightforward manner by adjusting the relative amounts of the unit dose composition administered to the subject. This is in contrast to the difficulty of tuning the expression of even a single gene product in a cell transduced with a viral or even a plasmid vector.

Pharmaceutical Compositions & Formulations

[0084] Also provided herein are pharmacuetical compositions for intramuscular delivery comprising the unit dose compositions comprising synthetic, modified RNAs dissolved or dispersed as an active ingredient and a physiologically tolerable carrier. In a preferred embodiment, the therapeutic composition is not immunogenic when administered to a mammal or human patient for therapeutic purposes, unless so desired. As used herein, the terms "pharmaceutically acceptable," "physiologically tolerable," and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable or unacceptable physiological effects such as toxicity, nausea, dizziness, gastric upset, immune reaction and the like. A pharmaceutically acceptable carrier will not promote the raising of an immune response to an agent with which it is admixed, unless so desired.

[0085] The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as injectable either as liquid solutions or suspensions, however, particularly where synthetic, modified RNA itself is administered, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified or presented as a liposome composition. The active ingredient, i.e., unit dose compositions comprising synthetic, modified RNAs, can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient. Physiologically tolerable carriers are well known in the art. Exemplary liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Saline-based carriers are most useful for the administration of cells or cell preparations. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes.

[0086] The unit dose compositions comprising synthetic, modified RNAs described herein can be formulated in conjunction with one or more penetration enhancers, surfactants and/or chelators for intramuscular delivery. Suitable surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Suitable bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Suitable fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid,

palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (*e.g.,* sodium). In some embodiments, combinations of penetration enhancers are used, for example, fatty acids/salts in combination with bile acids/salts. One exemplary combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether.

[0087] The unit dose compositions comprising synthetic, modified RNAs described herein can be formulated into any of many possible administration forms, including a sustained release form. In some embodiments of the aspects described herein, formulations comprising a plurality of different synthetic, modified RNAs are prepared by first mixing all members of a plurality of different synthetic, modified RNAs, and then complexing the mixture comprising the plurality of different synthetic, modified RNAs with a desired ligand or targeting moiety, such as a lipid. The unit dose compositions comprising synthetic, modified RNAs can be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions can further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension can also contain stabilizers.

[0088] The unit dose compositions comprising synthetic, modified RNAs described herein can be prepared and formulated as emulsions for the delivery of synthetic, modified RNAs. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1μm in diameter (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 301). Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions can be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions can contain further components in addition to the dispersed phases, and the active drug (*i.e.*, synthetic, modified RNA) which can be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants can also be present in emulsions as needed. Emulsions can also be

multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion. Emulsifiers can broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

[0089] Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

[0090] A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

[0091] Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

[0092] As noted above, liposomes can optionally be prepared to contain surface groups to facilitate delivery of liposomes and their contents to specific cell populations. For example, a liposome can comprise a surface groups such as antibodies or antibody fragments, small effector molecules for interacting with cell-surface receptors, antigens, and other like compounds.

[0093] Surface groups can be incorporated into the liposome by including in the liposomal lipids a lipid derivatized with the targeting molecule, or a lipid having a polar-head chemical group that can be derivatized with the targeting molecule in preformed liposomes. Alternatively, a targeting moiety can be inserted into preformed liposomes by incubating the preformed liposomes with a ligand-polymer-lipid conjugate.

[0094] A number of liposomes comprising nucleic acids are known in the art. WO 96/40062 (Thierry et al.) discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Pat. No. 5,264,221 (Tagawa et al.) discloses protein-bonded liposomes and asserts that the contents of such liposomes can include an RNA molecule. U.S. Pat. No. 5,665,710 (Rahman et al.) describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 (Love et al.) discloses liposomes comprising RNAi molecules targeted to the raf gene. In addition, methods for preparing a liposome composition comprising a nucleic acid can be found in *e.g.*, U.S. Patent Nos. 6,011,020; 6,074,667; 6,110,490; 6,147,204; 6, 271, 206; 6,312,956; 6,465,188; 6,506,564; 6,750,016; and 7,112,337. Each of these approaches can provide delivery of a synthetic, modified RNA as described herein to a cell.

[0095] In some embodiments of the aspects described herein, the unit dose compositions comprising synthetic, modified RNAs described herein can be encapsulated in a nanoparticle. Methods for nanoparticle packaging are well known in the art, and are described, for example, in Bose S, et al (Role of Nucleolin in Human Parainfluenza Virus Type 3 Infection of Human Lung Epithelial Cells. J. Virol. 78:8146. 2004); Dong Y et al. Poly(d,l-lactide-co-glycolide)/montmorillonite nanoparticles for oral delivery of anticancer drugs. Biomaterials 26:6068. 2005); Lobenberg R. et al (Improved body distribution of 14C-labelled AZT bound to nanoparticles in rats determined by radioluminography. J Drug Target 5:171.1998); Sakuma S R et al (Mucoadhesion of polystyrene nanoparticles having surface hydrophilic polymeric chains in the gastrointestinal tract. Int J Pharm 177:161. 1999); Virovic L et al. Novel delivery methods for treatment of viral hepatitis: an update. Expert Opin Drug Deliv 2:707.2005); and Zimmermann E et al, Electrolyte- and pH-stabilities of aqueous solid lipid nanoparticle (SLN) dispersions in artificial gastrointestinal media. Eur J Pharm Biopharm 52:203. 2001), the contents of which are herein incoporated in their entireties by reference.

[0096] In some embodiments, the unit dose compositions comprising synthetic, modified RNAs and methods thereof can comprise the use of other agents or measures to prevent or reduce any cytotoxicity caused by the administration procedure, the unit dose compositions, or a combination thereof. The cytotoxicity of synthetic, unmodified RNAs involves a cellular innate immune response designed to recognize a foreign pathogen (*e.g.*, virus) and to produce interferons, which in turn stimulates the activity of the protein kinase PKR, Toll-like receptors (TLRs) and RIG-1, among others, to mediate anti-viral actions. A significant part of an individual cell's innate immune response to foreign RNA is represented by the so-called "PKR response" triggered largely by double-stranded RNA.

[0097] It has been shown that the PKR response can be reduced by removing the 5'-triphosphate on an RNA molecule, and that RNAs having a 5'-monophosphate, -diphosphate or -7-methyl guanosine cap do not activate PKR. Thus, in some embodiments, the synthetic, modified RNA described herein comprises a 5'-monophosphate, a 5'-diphosphate, or a 5' 7-methyl guanosine cap to escape the immune response initiated by PKR. In other embodiments, the synthetic, modified RNA as described herein is treated to remove the 5'-triphosphate using an alkaline phosphatase, *e.g.*, calf intestinal phosphatase. Other modifications to prevent activation of the immune response mediators (*e.g.*, PKR, TLRs, and RIG-1) are discussed in detail in Nallagatla, SR, et al., (2008) *RNA Biol* 5(3):140-144, which is herein incorporated by reference in its entirety.

[0098] In some embodiments, RNA interference agents (*e.g.*, siRNA, shRNA, etc.) can be administered to a subject and used to inhibit expression of RIG-1, MYD88, VISA, PKR, TRIF, TRL7, or TLR8, which will result in a lower innate immune mediated response in the cells.

[0099] Another approach to reduce the innate immune mediated response is to inhibit the effect of secreted interferon on cellular receptors, for example, by scavenging secreted interferon using a soluble interferon receptor (*e.g.*, B18R) or a neutralizing antibody. Accordingly, in some embodiments, a unit dose composition comprising a synthetic, modified RNA encoding a therapeutic agent can further comprise or be administered to a subject with a modified RNA encoding an interferon scavenging agent (*e.g.*, a soluble interferon receptor) to further reduce the innate immune response of the cells.

[00100] Small molecules that inhibit the innate immune response in cells, such as chloroquine (a TLR signaling inhibitor) and 2-aminopurine (a PKR inhibitor), can also be administered with the unit dose compositions comprising synthetic, modified RNAs described herein. Some non-limiting examples of commercially available TLR-signaling inhibitors include BX795, chloroquine, CLI-095, OxPAPC, polymyxin B, and rapamycin (all available for purchase from INVIVOGEN™). In addition, inhibitors of pattern recognition receptors (PRR) (which are involved in innate immunity signaling) such as 2-aminopurine, BX795, chloroquine, and H-89, can also be used in the compositions and methods described herein.

[00101] In other embodiments, unit dose compositions comprising synthetic, modified RNAs encoding inhibitors of the innate immune system can be used to avoid the innate immune response generated in the cell.

Delivery Agents

[00102] Unit dose compositions comprising synthetic, modified RNAs can be intramuscularly administered or delivered into a subject using, for example, a drug delivery system such as a nanoparticle, a dendrimer, a polymer, a liposome, or a cationic delivery system. Positively charged cationic delivery systems facilitate binding of a synthetic, modified RNA (negatively charged polynucleotides) and also enhances interactions at the negatively charged cell membrane to permit efficient cellular uptake by the subject. Cationic lipids, dendrimers, or polymers can either be bound

to synthetic, modified RNAs, or induced to form a vesicle or micelle (see *e.g.*, Kim SH., et al (2008) Journal of Controlled Release 129(2):107-116) that encases the modified RNA. Methods for making and using cationic-modified RNA complexes are well within the abilities of those skilled in the art (see *e.g.*, Sorensen, DR., et al (2003) J. Mol. Biol 327:761-766; Verma, UN., et al (2003) Clin. Cancer Res. 9:1291-1300; Arnold, AS et al (2007) J. Hypertens. 25:197-205, which are incorporated herein by reference in their entirety).

[00103] It is also contemplated herein that unit dose compositions comprising a first and second synthetic, modified RNA respectively are administered in a separate and temporally distinct manner. Thus, each of a plurality of unit dose compositions comprising synthetic, modified RNAs can be administered at a separate time or at a different frequency interval to achieve the desired expression of a given set of therapeutic agents.

[00104] In some embodiments of the aspects described herein, the unit dose compositions comprising synthetic, modified RNAs further comprise a delivery reagent that facilitates uptake of a synthetic, modified RNA into a cell of a subject to which it is adminstered, such as an emulsion, a liposome, a cationic lipid, a non-cationic lipid, an anionic lipid, a charged lipid, a penetration enhancer, a modification to the synthetic, modified RNA to attach *e.g.*, a ligand, peptide, lipophillic group, or targeting moiety, or any combination thereof.

[00105] Suitable delivery agents that can be added to the unit dose compositions comprising synthetic, modified RNAs described herein include, for example, lIPOFECTIN, LIPOFECTAMINe, DIMRIE C™, SUPERFECT™, and EFFECTIN™ (QIAGEN™), UNIFECTIN™, MAXIFECTIN™, DOTMA, DOGS™ (Transfectam; dioctadecylamidoglycylspermine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), DOTAP (1,2-dioleoyl-3-trimethylammonium propane), DDAB (dimethyl dioctadecylammonium bromide), DHDEAB (N,N-di-n-hexadecyl-N,N-dihydroxyethyl ammonium bromide), HDEAB (N-n-hexadecyl-N,N-dihydroxyethylammonium bromide), polybrene, poly(ethylenimine) (PEI), and the like. (See, *e.g.*, Banerjee et al., Med. Chem. 42:4292-99 (1999); Godbey et al., Gene Ther. 6:1380-88 (1999); Kichler et al., Gene Ther. 5:855-60 (1998); Birchaa et al., J. Pharm. 183:195-207 (1999)).

[00106] In some embodiments, the unit dose compositions comprising synthetic, modified RNAs can further comprise cationic lipid carriers (*e.g.*, OLIGOFECTAMINE™) or non-cationic lipid-based carriers (*e.g.*, TRANSIT-TKOTM™, Mirus Bio LLC, Madison, WI) as delivery agents. Successful expression of the therapeutic agent encoded by the synthetic, modified RNA can be monitored using various known methods. For example, transient transfection can be signaled with a reporter, such as a fluorescent marker, such as Green Fluorescent Protein (GFP). Successful transfection of a modified RNA can also be determined by measuring the protein expression level of the therapeutic agent by *e.g.*, Western Blotting or immunocytochemistry, or ELISA, as described herein for the unit dose compositions encoding erythropoietin.

[00107] In some embodiments, the unit dose compositions comprising synthetic, modified RNAs can further comprise a transfection reagent as a delivery agent. Some exemplary transfection reagents contemplated for the administration of the unit dose compositions comprising synthetic, modified RNAs include, for example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731). Examples of commercially available transfection reagents for use in delivery include, but are not limited to, for example RNAiMAX (Invitrogen; Carlsbad, CA), LIPOFECTAMINE™ (Invitrogen; Carlsbad, CA), LIPOFECTAMINE 2000™ (Invitrogen; Carlsbad, CA), 293FECTIN™ (Invitrogen; Carlsbad, CA), CELLFECTIN™ (Invitrogen; Carlsbad, CA), DMRIE-C™ (Invitrogen; Carlsbad, CA), FREESTYLE™ MAX (Invitrogen; Carlsbad, CA), LIPOFECTAMINE™ 2000 CD (Invitrogen; Carlsbad, CA), LIPOFECTAMINE™ (Invitrogen; Carlsbad, CA), OLIGOFECTAMINE™ (Invitrogen; Carlsbad, CA), OPTIFECT™ (Invitrogen; Carlsbad, CA), X-TREMEGENE Q2 Transfection Reagent (Roche; Grenzacherstrasse, Switzerland), DOTAP Liposomal Transfection Reagent (Grenzacherstrasse, Switzerland), DOSPER Liposomal Transfection Reagent (Grenzacherstrasse, Switzerland), or FUGENE (Grenzacherstrasse, Switzerland), TRANSFECTAM® Reagent (Promega; Madison, WI), TRANSFAST™ Transfection Reagent (Promega; Madison, WI), TFX™-20 Reagent (Promega; Madison, WI), TFX™-50 Reagent (Promega; Madison, WI), DREAMFECT™ (OZ Biosciences; Marseille, France), ECOTRANSFECT (OZ Biosciences; Marseille, France), TRANSPASS[a] D1 Transfection Reagent (New England Biolabs; Ipswich, MA, USA), LYOVEC™/LIPOGEN™ (Invitrogen; San Diego, CA, USA), PERFECTIN Transfection Reagent (Genlantis; San Diego, CA, USA), NEUROPORTER Transfection Reagent (Genlantis; San Diego, CA, USA), GENEPORTER Transfection reagent (Genlantis; San Diego, CA, USA), GENEPORTER 2 Transfection reagent (Genlantis; San Diego, CA, USA), CYTOFECTIN Transfection Reagent (Genlantis; San Diego, CA, USA), BACULOPORTER Transfection Reagent (Genlantis; San Diego, CA, USA), TROGANPORTER™ transfection Reagent (Genlantis; San Diego, CA, USA), RIBOFECT (Bioline; Taunton, MA, USA), PlasFect (Bioline; Taunton, MA, USA), UNIFECTOR (B-Bridge International; Mountain View, CA, USA), SUREFECTOR (B-Bridge International; Mountain View, CA, USA), or HIFECT™ (B-Bridge International, Mountain View, CA, USA), among others.

[00108] In other embodiments, unit dose compositions comprising synthetic, modified RNAs can further comprise highly branched organic compounds, termed "dendrimers," that can bind the synthetic, modified RNAs.

[00109] Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols, such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes, such as limonene and menthone.

Synthetic, Modified RNAs

[00110] Described herein are unit dose compositions comprising synthetic, modified RNAs for expressing a therapeutic agent, such as a polypeptide or non-translated RNA. As used herein, the

term "synthetic, modified RNA" refers to a nucleic acid molecule encoding a factor, such as a polypeptide, to be expressed in a host cell, which comprises at least one modified nucleoside and has at least the following characteristics as the term is used herein: (i) it can be generated by *in vitro* transcription and is not isolated from a cell; (ii) it is translatable in a mammalian (and preferably human) cell; and (iii) it does not provoke or provokes a significantly reduced innate immune response or interferon response in a cell to which it is introduced or contacted relative to a synthetic, non-modified RNA of the same sequence .

[00111] A synthetic, modified RNA can be generated by *in vitro* transcription, using any method known to one of skill in the art, such as "splint-mediated ligation" technique described in International Publication WO 2011/130624 or using the IVT templates described in US 61/558,563. The transcribed, synthetic, modified RNA polymer can be modified further post-transcriptionally, *e.g.*, by adding a cap or other functional group.

[00112] To be suitable for *in vitro* transcription, the modified nucleoside(s) must be recognized as substrates by at least one RNA polymerase enzyme. Generally, RNA polymerase enzymes can tolerate a range of nucleoside base modifications, at least in part because the naturally occurring G, A, U, and C nucleoside bases differ from each other quite significantly. Thus, the structure of a modified nucleoside base for use in generating the synthetic, modified RNAs described herein can generally vary more than the sugar-phosphate moieties of the modified nucleoside. That said, ribose and phosphate-modified nucleosides or nucleoside analogs are known in the art that permit transcription by RNA polymerases. In some embodiments of the aspects described herein, the RNA polymerase is a phage RNA polymerase. The modified nucleotides pseudouridine, m5U, s2U, m6A, and m5C are known to be compatible with transcription using phage RNA polymerases, while N1-methylguanosine, N1-methyladenosine, N7-methylguanosine, 2'-)-methyluridine, and 2'-O-methylcytidine are not. Polymerases that accept modified nucleosides are known to those of skill in the art.

[00113] It is also contemplated that modified polymerases can be used to generate synthetic, modified RNAs, as described herein. Thus, for example, a polymerase that tolerates or accepts a particular modified nucleoside as a substrate can be used to generate a synthetic, modified RNA including that modified nucleoside.

[00114] Second, the synthetic, modified RNA must be translatable by the translation machinery of a eukaryotic, preferably mammalian, and more preferably, human cell. Translation generally requires at least a ribosome binding site, a methionine start codon, and an open reading frame encoding a polypeptide. Preferably, the synthetic, modified RNA also comprises a 5′ cap, a stop codon, a Kozak sequence, and a polyA tail. In addition, mRNAs in a eukaryotic cell are regulated by degradation, thus a synthetic, modified RNA as described herein can be further modified to extend its half-life in the cell by incorporating modifications to reduce the rate of RNA degradation (*e.g.*, by increasing serum stability of a synthetic, modified RNA).

[00115] Nucleoside modifications can interfere with translation. To the extent that a given modification interferes with translation, those modifications are not encompassed by the synthetic, modified RNA as described herein. One can test a synthetic, modified RNA for its ability to undergo translation and translation efficiency using an *in vitro* translation assay (*e.g.*, a rabbit reticulocyte lysate assay, a reporter activity assay, or measurement of a radioactive label in the translated protein) and detecting the amount of the polypeptide produced using SDS-PAGE, Western blot, or immunochemistry assays etc. The translation of a synthetic, modified RNA comprising a candidate modification is compared to the translation of an RNA lacking the candidate modification, such that if the translation of the synthetic, modified RNA having the candidate modification remains the same or is increased then the candidate modification is contemplated for use with the compositions and methods described herein. It is noted that fluoro-modified nucleosides are generally not translatable and can be used herein as a negative control for an *in vitro* translation assay.

[00116] Third, the synthetic, modified RNA provokes a reduced (or absent) innate immune response or interferon response by a transfected cell or population of cells thereof, or in the subject to whom the unit dose compositions are administered. mRNA produced in eukaryotic cells, *e.g.*, mammalian or human cells, is heavily modified, the modifications permitting the cell to detect RNA not produced by that cell. The cell responds by shutting down translation or otherwise initiating an innate immune or interferon response. Thus, to the extent that an exogenously added RNA can be modified to mimic the modifications occurring in the endogenous RNAs produced by a target cell, the exogenous RNA can avoid at least part of the target cell's defense against foreign nucleic acids. Thus, in some embodiments, synthetic, modified RNAs as described herein include *in vitro* transcribed RNAs including modifications as found in eukaryotic/mammalian/human RNA *in vivo*. Other modifications that mimic such naturally occurring modifications can also be helpful in producing a synthetic, modified RNA molecule that will be tolerated by a cell.

RNA Modifications

[00117] The unit dose compositions comprising synthetic, modified RNA molecules encoding therapeutic agents, comprise one or more nucleoside modifications, such that administering the synthetic, modified RNA molecules to a subject results in a reduced innate immune response relative to a subject contacted with synthetic RNA molecules encoding the therapeutic agents not comprising the one or more nucleoside modifications.

[00118] The unit dose compositions comprising synthetic, modified RNAs described herein include modifications to prevent rapid degradation by endo- and exo-nucleases and to avoid or reduce a subject's cellular innate immune or interferon response to the RNA. Modifications include, but are not limited to, for example, (a) end modifications, *e.g.*, 5' end modifications (phosphorylation dephosphorylation, conjugation, inverted linkages, etc.), 3' end modifications (conjugation, DNA nucleotides, inverted linkages, etc.), (b) base modifications, *e.g.*, replacement with modified bases, stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners,

or conjugated bases, (c) sugar modifications (*e.g.*, at the 2' position or 4' position) or replacement of the sugar, as well as (d) internucleoside linkage modifications, including modification or replacement of the phosphodiester linkages. To the extent that such modifications interfere with translation (*i.e.*, results in a reduction of 50% or more in translation relative to the lack of the modification - *e.g.*, in a rabbit reticulocyte *in vitro* translation assay), the modification is not suitable for the methods and compositions described herein. Specific examples of unit dose compositions comprising synthetic, modified RNA compositions described herein include, but are not limited to, RNA molecules containing modified or non-natural internucleoside linkages. Synthetic, modified RNAs having modified internucleoside linkages include, among others, those that do not have a phosphorus atom in the internucleoside linkage. In other embodiments, the synthetic, modified RNA has a phosphorus atom in its internucleoside linkage(s).

[00119] Non-limiting examples of modified internucleoside linkages include phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those) having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

[00120] Representative U.S. patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,195; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,316; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; 6,028,188; 6,124,445; 6,160,109; 6,169,170; 6,172,209; 6, 239,265; 6,277,603; 6,326,199; 6,346,614; 6,444,423; 6,531,590; 6,534,639; 6,608,035; 6,683,167; 6,858,715; 6,867,294; 6,878,805; 7,015,315; 7,041,816; 7,273,933; 7,321,029; and US Pat RE39464, each of which is herein incorporated by reference in its entirety.

[00121] Modified internucleoside linkages that do not include a phosphorus atom therein have internucleoside linkages that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatoms and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

[00122] Representative U.S. patents that teach the preparation of modified oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,64,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference in its entirety.

[00123] Some embodiments of the unit dose compositions comprising synthetic, modified RNAs described herein include nucleic acids with phosphorothioate internucleoside linkages and oligonucleosides with heteroatom internucleoside linkage, and in particular --CH2-NH-CH2-, -CH2-N(CH3)-O-CH2- [known as a methylene (methylimino) or MMI], -CH2-O-N(CH3)-CH2-, -CH2-N(CH3)-N(CH3)-CH2- and -N(CH3)-CH2-CH2- [wherein the native phosphodiester internucleoside linkage is represented as -O-P-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, both of which are herein incorporated by reference in their entirety. In some embodiments, the nucleic acid sequences featured herein have morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506, herein incorporated by reference in its entirety.

[00124] Unit dose compositions comprising synthetic, modified RNAs described herein can also contain one or more substituted sugar moieties. The nucleic acids featured herein can include one of the following at the 2' position: H (deoxyribose); OH (ribose); 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 can be substituted or unsubstituted C1 to C10 alkyl or C2 to C10 alkenyl and alkynyl. Exemplary 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 some embodiments, synthetic, modified RNAs 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, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an RNA, or a group for improving the pharmacodynamic properties of a synthetic, modified RNA, 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'-dimethylaminooxyethoxy, *i.e.*, a O(CH2)2ON(CH3)2 group, also known as 2'-DMAOE, 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.

[00125] Other modifications include 2'-methoxy (2'-OCH3), 2'-aminopropoxy (2'-OCH2CH2CH2NH2) and 2'-fluoro (2'-F). Similar modifications can also be made at other positions on the nucleic acid sequence, particularly the 3' position of the sugar on the 3' terminal nucleotide or

in 2'-5' linked nucleotides and the 5' position of 5' terminal nucleotide. A synthetic, modified RNA can 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, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

[00126] As non-limiting examples, unit dose compositions comprising synthetic, modified RNAs described herein can include at least one modified nucleoside including a 2'-O-methyl modified nucleoside, a nucleoside comprising a 5' phosphorothioate group, a 2'-amino-modified nucleoside, 2'-alkyl-modified nucleoside, morpholino nucleoside, a phosphoramidate or a non-natural base comprising nucleoside, or any combination thereof.

[00127] In some embodiments of this aspect and all other such aspects described herein, the at least one modified nucleoside is selected from the group consisting of 5-methylcytidine (5mC), N6-methyladenosine (m6A), 3,2'-O-dimethyluridine (m4U), 2-thiouridine (s2U), 2' fluorouridine, pseudouridine, 2'-O-methyluridine (Um), 2' deoxyuridine (2' dU), 4-thiouridine (s4U), 5-methyluridine (m5U), 2'-O-methyladenosine (m6A), N6,2'-O-dimethyladenosine (m6Am), N6,N6,2'-O-trimethyladenosine ($m6_2Am$), 2'-O-methylcytidine (Cm), 7-methylguanosine (m7G), 2'-O-methylguanosine (Gm), N2,7-dimethylguanosine (m2,7G), N2, N2, 7-trimethylguanosine (m2,2,7G), and inosine (I).

[00128] Alternatively, unit dose compositions comprising synthetic, modified RNA can comprise at least two modified nucleosides, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20 or more, up to the entire length of the oligonucleotide. At a minimum, a synthetic, modified RNA molecule comprising at least one modified nucleoside comprises a single nucleoside with a modification as described herein. It is not necessary for all positions in a given synthetic, modified RNA to be uniformly modified, and in fact more than one of the aforementioned modifications can be incorporated in a single synthetic, modified RNA or even at a single nucleoside within a synthetic, modified RNA. However, it is preferred, but not absolutely necessary, that each occurrence of a given nucleoside in a molecule is modified (*e.g.*, each cytosine is a modified cytosine *e.g.*, 5mC). However, it is also contemplated that different occurrences of the same nucleoside can be modified in a different way in a given synthetic, modified RNA molecule (*e.g.*, some cytosines modified as 5mC, others modified as 2'-O-methylcytidine or other cytosine analog). The modifications need not be the same for each of a plurality of modified nucleosides in a synthetic, modified RNA. Furthermore, in some embodiments of the aspects described herein, a unit dose composition comprising synthetic, modified RNA comprises at least two different modified nucleosides. In some such preferred embodiments of the aspects described herein, the at least two

different modified nucleosides are 5-methylcytidine and pseudouridine. A synthetic, modified RNA can also contain a mixture of both modified and unmodified nucleosides.

[00129] As used herein, "unmodified" or "natural" nucleosides or nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). In some embodiments, a synthetic, modified RNA comprises at least one nucleoside ("base") modification or substitution. Modified nucleosides include other synthetic and natural nucleobases such as inosine, xanthine, hypoxanthine, nubularine, isoguanisine, tubercidine, 2-(halo)adenine, 2-(alkyl)adenine, 2-(propyl)adenine, 2 (amino)adenine, 2-(aminoalkyll)adenine, 2 (aminopropyl)adenine, 2 (methylthio) N6 (isopentenyl)adenine, 6 (alkyl)adenine, 6 (methyl)adenine, 7 (deaza)adenine, 8 (alkenyl)adenine, 8-(alkyl)adenine, 8 (alkynyl)adenine, 8 (amino)adenine, 8-(halo)adenine, 8-(hydroxyl)adenine, 8 (thioalkyl)adenine, 8-(thiol)adenine, N6-(isopentyl)adenine, N6 (methyl)adenine, N6, N6 (dimethyl)adenine, 2-(alkyl)guanine,2 (propyl)guanine, 6-(alkyl)guanine, 6 (methyl)guanine, 7 (alkyl)guanine, 7 (methyl)guanine, 7 (deaza)guanine, 8 (alkyl)guanine, 8-(alkenyl)guanine, 8 (alkynyl)guanine, 8-(amino)guanine, 8 (halo)guanine, 8-(hydroxyl)guanine, 8 (thioalkyl)guanine, 8-(thiol)guanine, N (methyl)guanine, 2-(thio)cytosine, 3 (deaza) 5 (aza)cytosine, 3-(alkyl)cytosine, 3 (methyl)cytosine, 5-(alkyl)cytosine, 5-(alkynyl)cytosine, 5 (halo)cytosine, 5 (methyl)cytosine, 5 (propynyl)cytosine, 5 (propynyl)cytosine, 5 (trifluoromethyl)cytosine, 6-(azo)cytosine, N4 (acetyl)cytosine, 3 (3 amino-3 carboxypropyl)uracil, 2-(thio)uracil, 5 (methyl) 2 (thio)uracil, 5 (methylaminomethyl)-2 (thio)uracil, 4-(thio)uracil, 5 (methyl) 4 (thio)uracil, 5 (methylaminomethyl)-4 (thio)uracil, 5 (methyl) 2,4 (dithio)uracil, 5 (methylaminomethyl)-2,4 (dithio)uracil, 5 (2-aminopropyl)uracil, 5-(alkyl)uracil, 5-(alkynyl)uracil, 5-(allylamino)uracil, 5 (aminoallyl)uracil, 5 (aminoalkyl)uracil, 5 (guanidiniumalkyl)uracil, 5 (1,3-diazole-1-alkyl)uracil, 5-(cyanoalkyl)uracil, 5-(dialkylaminoalkyl)uracil, 5 (dimethylaminoalkyl)uracil, 5-(halo)uracil, 5-(methoxy)uracil, uracil-5 oxyacetic acid, 5 (methoxycarbonylmethyl)-2-(thio)uracil, 5 (methoxycarbonyl-methyl)uracil, 5 (propynyl)uracil, 5 (propynyl)uracil, 5 (trifluoromethyl)uracil, 6 (azo)uracil, dihydrouracil, N3 (methyl)uracil, 5-uracil (*i.e.*, pseudouracil), 2 (thio)pseudouracil,4 (thio)pseudouracil,2,4-(dithio)psuedouracil,5-(alkyl)pseudouracil, 5-(methyl)pseudouracil, 5-(alkyl)-2-(thio)pseudouracil, 5-(methyl)-2-(thio)pseudouracil, 5-(alkyl)-4 (thio)pseudouracil, 5-(methyl)-4 (thio)pseudouracil, 5-(alkyl)-2,4 (dithio)pseudouracil, 5-(methyl)-2,4 (dithio)pseudouracil, 1 substituted pseudouracil, 1 substituted 2(thio)-pseudouracil, 1 substituted 4 (thio)pseudouracil, 1 substituted 2,4-(dithio)pseudouracil, 1 (aminocarbonylethylenyl)-pseudouracil, 1 (aminocarbonylethylenyl)-2(thio)-pseudouracil, 1 (aminocarbonylethylenyl)-4 (thio)pseudouracil, 1 (aminocarbonylethylenyl)-2,4-(dithio)pseudouracil, 1 (aminoalkylaminocarbonylethylenyl)-pseudouracil, 1 (aminoalkylamino-carbonylethylenyl)-2(thio)-pseudouracil, 1 (aminoalkylaminocarbonylethylenyl)-4 (thio)pseudouracil, 1 (aminoalkylaminocarbonylethylenyl)-2,4-(dithio)pseudouracil, 1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-substituted 1,3-

(diaza)-2-(oxo)-phenoxazin-1-yl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-substituted 1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 7-substituted 1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-(aminoalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 7-(aminoalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 7-(guanidiniumalkylhydroxy)-1,3-(diaza)-2-(oxo)-phenoxazin-1-yl, 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenoxazin-1-yl, 7-(guanidiniumalkyl-hydroxy)-1,3-(diaza)-2-(oxo)-phenthiazin-1-yl, 7-(guanidiniumalkylhydroxy)-1-(aza)-2-(thio)-3-(aza)-phenthiazin-1-yl, 1,3,5-(triaza)-2,6-(dioxa)-naphthalene, inosine, xanthine, hypoxanthine, nubularine, tubercidine, isoguanisine, inosinyl, 2-aza-inosinyl, 7-deaza-inosinyl, nitroimidazolyl, nitropyrazolyl, nitrobenzimidazolyl, nitroindazolyl, aminoindolyl, pyrrolopyrimidinyl, 3-(methyl)isocarbostyrilyl, 5-(methyl)isocarbostyrilyl, 3-(methyl)-7-(propynyl)isocarbostyrilyl, 7-(aza)indolyl, 6-(methyl)-7-(aza)indolyl, imidizopyridinyl, 9-(methyl)-imidizopyridinyl, pyrrolopyrizinyl, isocarbostyrilyl, 7-(propynyl)isocarbostyrilyl, propynyl-7-(aza)indolyl, 2,4,5-(trimethyl)phenyl, 4-(methyl)indolyl, 4,6-(dimethyl)indolyl, phenyl, napthalenyl, anthracenyl, phenanthracenyl, pyrenyl, stilbenyl, tetracenyl, pentacenyl, difluorotolyl, 4-(fluoro)-6-(methyl)benzimidazole, 4-(methyl)benzimidazole, 6-(azo)thymine, 2-pyridinone, 5 nitroindole, 3 nitropyrrole, 6-(aza)pyrimidine, 2 (amino)purine, 2,6-(diamino)purine, 5 substituted pyrimidines, N2-substituted purines, N6-substituted purines, O6-substituted purines, substituted 1,2,4-triazoles, pyrrolo-pyrimidin-2-on-3-yl, 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, para-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, bis-ortho-substituted-6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, para-(aminoalkylhydroxy)- 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, ortho-(aminoalkylhydroxy)- 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, bis-ortho--(aminoalkylhydroxy)- 6-phenyl-pyrrolo-pyrimidin-2-on-3-yl, pyridopyrimidin-3-yl, 2-oxo-7-amino-pyridopyrimidin-3-yl, 2-oxo-pyridopyrimidine-3-yl, or any O-alkylated or N-alkylated derivatives thereof. Modified nucleosides also include natural bases that comprise conjugated moieties, *e.g.* a ligand. As discussed herein above, the RNA containing the modified nucleosides must be translatable in a subject's cell (*i.e.*, does not prevent translation of the polypeptide encoded by the modified RNA). For example, transcripts containing s2U and m6A are translated poorly in rabbit reticulocyte lysates, while pseudouridine, m5U, and m5C are compatible with efficient translation. In addition, it is known in the art that 2'-fluoro-modified bases useful for increasing nuclease resistance of a transcript, leads to very inefficient translation. Translation can be assayed by one of ordinary skill in the art using *e.g.*, a rabbit reticulocyte lysate translation assay.

[00130] Further modified nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in Modified Nucleosides in Biochemistry, Biotechnology and Medicine, Herdewijn, P. ed. Wiley-VCH, 2008; those disclosed in Int. Appl. No. PCT/US09/038425, filed March 26, 2009; those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859,

Kroschwitz, J. L, ed. John Wiley & Sons, 1990, and those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613.

[00131] Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,457,191; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,681,941; 6,015,886; 6,147,200; 6,166,197; 6,222,025; 6,235,887; 6,380,368; 6,528,640; 6,639,062; 6,617,438; 7,045,610; 7,427,672; and 7,495,088, each of which is herein incorporated by reference in its entirety, and U.S. Pat. No. 5,750,692, also herein incorporated by reference in its entirety.

[00132] Another modification for use with the unit dose compositions comprising synthetic, modified RNAs described herein involves chemically linking to the RNA one or more ligands, moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the RNA. Ligands can be particularly useful where, for example, a synthetic, modified RNA is directly administered *in vivo*, as described herein. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, Proc. Natl. Acid. Sci. USA, 1989, 86: 6553-6556, herein incorporated by reference in its entirety), cholic acid (Manoharan *et al.*, Biorg. Med. Chem. Let., 1994, 4:1053-1060, herein incorporated by reference in its entirety), a thioether, *e.g.*, beryl-S-tritylthiol (Manoharan *et al.*, Ann. N.Y. Acad. Sci., 1992, 660:306-309; Manoharan *et al.*, Biorg. Med. Chem. Let., 1993, 3:2765-2770, each of which is herein incorporated by reference in its entirety), a thiocholesterol (Oberhauser *et al.*, Nucl. Acids Res., 1992, 20:533-538, herein incorporated by reference in its entirety), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, EMBO J, 1991, 10:1111-1118; Kabanov *et al.*, FEBS Lett., 1990, 259:327-330; Svinarchuk *et al.*, Biochimie, 1993, 75:49-54, each of which is herein incorporated by reference in its entirety), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-phosphonate (Manoharan *et al.*, Tetrahedron Lett., 1995, 36:3651-3654; Shea *et al.*, Nucl. Acids Res., 1990, 18:3777-3783, each of which is herein incorporated by reference in its entirety), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, Nucleosides & Nucleotides, 1995, 14:969-973, herein incorporated by reference in its entirety), or adamantane acetic acid (Manoharan *et al.*, Tetrahedron Lett., 1995, 36:3651-3654, herein incorporated by reference in its entirety), a palmityl moiety (Mishra *et al.*, Biochim. Biophys. Acta, 1995, 1264:229-237, herein incorporated by reference in its entirety), or an octadecylamine or hexylamino-carbonyloxycholesterol moiety (Crooke *et al.*, J. Pharmacol. Exp. Ther., 1996, 277:923-937, herein incorporated by reference in its entirety).

[00133] The synthetic, modified RNAs described herein can further comprise a 5' cap. In some embodiments of the aspects described herein, the synthetic, modified RNAs comprise a 5' cap comprising a modified guanine nucleotide that is linked to the 5' end of an RNA molecule using a 5'-5'triphosphate linkage. As used herein, the term "5' cap" is also intended to encompass other 5' cap

analogs including, *e.g.*, 5' diguanosine cap, tetraphosphate cap analogs having a methylene-bis(phosphonate) moiety (see *e.g.*, Rydzik, AM et al., (2009) *Org Biomol Chem* 7(22):4763-76), dinucleotide cap analogs having a phosphorothioate modification (see *e.g.*, Kowalska, J. et al., (2008) *RNA* 14(6):1119-1131), cap analogs having a sulfur substitution for a non-bridging oxygen (see *e.g.*, Grudzien-Nogalska, E. et al., (2007) *RNA* 13(10): 1745-1755), N7-benzylated dinucleoside tetraphosphate analogs (see *e.g.*, Grudzien, E. et al., (2004) *RNA* 10(9):1479-1487), or anti-reverse cap analogs (see *e.g.*, Jemielity, J. et al., (2003) *RNA* 9(9): 1108-1122 and Stepinski, J. et al., (2001) *RNA* 7(10):1486-1495). In some such embodiments, the 5' cap analog is a 5' diguanosine cap. In some embodiments, the synthetic, modified RNA does not comprise a 5' triphosphate.

[00134] The 5' cap is important for recognition and attachment of an mRNA to a ribosome to initiate translation. The 5' cap also protects the synthetic, modified RNA from 5' exonuclease mediated degradation. It is not an absolute requirement that a synthetic, modified RNA comprise a 5' cap, and thus in other embodiments the synthetic, modified RNAs lack a 5' cap. However, due to the longer half-life of synthetic, modified RNAs comprising a 5' cap and the increased efficiency of translation, synthetic, modified RNAs comprising a 5' cap are preferred herein.

[00135] The synthetic, modified RNAs described herein can further comprise a 5' and/or 3' untranslated region (UTR). Untranslated regions are regions of the RNA before the start codon (5') and after the stop codon (3'), and are therefore not translated by the translation machinery. Modification of an RNA molecule with one or more untranslated regions can improve the stability of an mRNA, since the untranslated regions can interfere with ribonucleases and other proteins involved in RNA degradation. In addition, modification of an RNA with a 5' and/or 3' untranslated region can enhance translational efficiency by binding proteins that alter ribosome binding to an mRNA. Modification of an RNA with a 3' UTR can be used to maintain a cytoplasmic localization of the RNA, permitting translation to occur in the cytoplasm of the cell. In one embodiment, the synthetic, modified RNAs described herein do not comprise a 5' or 3' UTR. In another embodiment, the synthetic, modified RNAs comprise either a 5' or 3' UTR. In another embodiment, the synthetic, modified RNAs described herein comprise both a 5' and a 3' UTR. In one embodiment, the 5' and/or 3' UTR is selected from an mRNA known to have high stability in the cell (*e.g.*, a murine alpha-globin 3' UTR). In some embodiments, the 5' UTR, the 3' UTR, or both comprise one or more modified nucleosides.

[00136] In some embodiments, the synthetic, modified RNAs described herein further comprise a Kozak sequence. The "Kozak sequence" refers to a sequence on eukaryotic mRNA having the consensus (gcc)gccRccAUGG (SEQ ID NO: 1), where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. The Kozak consensus sequence is recognized by the ribosome to initiate translation of a polypeptide. Typically, initiation occurs at the first AUG codon encountered by the translation machinery that is proximal to the 5' end of the transcript. However, in some cases, this AUG codon can be bypassed in a process called leaky

scanning. The presence of a Kozak sequence near the AUG codon will strengthen that codon as the initiating site of translation, such that translation of the correct polypeptide occurs. Furthermore, addition of a Kozak sequence to a synthetic, modified RNA will promote more efficient translation, even if there is no ambiguity regarding the start codon. Thus, in some embodiments, the synthetic, modified RNAs described herein further comprise a Kozak consensus sequence at the desired site for initiation of translation to produce the correct length polypeptide. In some such embodiments, the Kozak sequence comprises one or more modified nucleosides.

[00137] In some embodiments, the synthetic, modified RNAs described herein further comprise a "poly (A) tail", which refers to a 3' homopolymeric tail of adenine nucleotides, which can vary in length (*e.g.*, at least 5 adenine nucleotides) and can be up to several hundred adenine nucleotides). The inclusion of a 3' poly(A) tail can protect the synthetic, modified RNA from degradation in the cell, and also facilitates extra-nuclear localization to enhance translation efficiency. In some embodiments, the poly(A) tail comprises between 1 and 500 adenine nucleotides; in other embodiments the poly(A) tail comprises at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 225, at least 250, at least 275, at least 300, at least 325, at least 350, at least 375, at least 400, at least 425, at least 450, at least 475, at least 500 adenine nucleotides or more. In one embodiment, the poly(A) tail comprises between 1 and 150 adenine nucleotides. In another embodiment, the poly(A) tail comprises between 90 and 120 adenine nucleotides. In some such embodiments, the poly(A) tail comprises one or more modified nucleosides.

[00138] It is contemplated that one or more modifications to the synthetic, modified RNAs described herein permit greater stability of the synthetic, modified RNA in a subject to which the unit dose compositions comprising one or more synthetic, modified RNAs is being administered. To the extent that such modifications permit translation and either reduce or do not exacerbate a cell's innate immune or interferon response to the synthetic, modified RNA with the modification, such modifications are specifically contemplated for use herein. Generally, the greater the stability of a synthetic, modified RNA, the more protein can be produced from that synthetic, modified RNA. Typically, the presence of AU-rich regions in mammalian mRNAs tend to destabilize transcripts, as cellular proteins are recruited to AU-rich regions to stimulate removal of the poly(A) tail of the transcript. Loss of a poly(A) tail of a synthetic, modified RNA can result in increased RNA degradation. Thus, in one embodiment, a synthetic, modified RNA as described herein does not comprise an AU-rich region. In particular, it is preferred that the 3' UTR substantially lacks AUUUA sequence elements.

Therapuetic Agents Encoded by the Synthetic, Modified RNAs

[00139] The unit dose compositions comprising synthetic, modified RNAs and methods of enhancing delivery described herein permit the long-term, safe, and efficient expression of therapeutic

agents, such as polypepeptides and non-translated RNAs, without the risk of permanent genomic alterations. Such unit dose compositions and methods are useful for a variety of applications, indications, and modalities, including, but not limited to, gene therapy, regenerative medicine, and cancer therapies.

[00140] Unit dose compositions comprising synthetic, modified RNAs as described herein can be made that direct the expression of essentially any gene product or open reading frame whose coding sequences can be cloned, such as those regulating cellular differentiation and growth, transcription factor, hormones, cytokines, as well as non-translated RNA products.

[00141] Accordingly, in some embodiments, the synthetic, modified RNA encodes a mRNA that undergoes translation into a peptide or polypeptide. In some embodiments, the synthetic, modified RNA encodes inhibitory RNAs, such as small interfering RNAs (siRNA) or micro RNAs (miRNA). For example, an interfering RNA that prevents expression of an mRNA, an RNA that is a pre-RNA, for example pre-miRNA, or a mature RNA, for example mature miRNA.

[00142] In those embodiments where the synthetic, modified RNA encodes for an mRNA, the mRNA can encode or be translated into essentially any polypeptide or peptide that is desired to be expressed. Such polypeptides include, but are not limited to, transcription factors, targeting moieties and other cell-surface polypeptides, cell-type specific polypeptides, differentiation factors, death receptors, death receptor ligands, structural proteins, enzymes, hormones, reprogramming factors, de-differentiation factors, cytokines, and any combination thereof. Further, such polypeptides or peptides to be expressed can include fusion proteins, truncated variants, protein domains, allelic variants and the like of any polypeptide. Additionally, the synthetic, modified RNA can encode essentially any non-translated RNA molecule that it is desired to synthesize, including, for example, shRNA molecules, siRNA molecules, dsRNA molecules, ribozymes, and any combinations thereof.

[00143] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for a cytokine, a hormone or a cellular growth factor, such as, for example, somatrophic hormone, insulin, glucagon, erythropoietin, a glucocorticoid, epidermal growth factor (EGF), insulin, transforming growth factors (TGF-α and TGF-β), heparin, hepatocyte growth factors (HGF), interleukins (IL-1 and IL-6), insulin-like growth factors (IGF-I and IGF-II), heparin-binding growth factors (HBGF-1), angiogenin, bone morphogenic protein-1, bone morphogenic protein-2, bone morphogenic protein-3, bone morphogenic protein-4, bone morphogenic protein-5, bone morphogenic protein-6, bone morphogenic protein-7, bone morphogenic protein-8, bone morphogenic protein-9, bone morphogenic protein-10, bone morphogenic protein-11, bone morphogenic protein-12, bone morphogenic protein-13, bone morphogenic protein-14, bone morphogenic protein-15, bone morphogenic protein receptor IA, bone morphogenic protein receptor IB, brain derived neurotrophic factor, ciliary neutrophic factor, ciliary neutrophic factor receptor-alpha, cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil, chemotactic factor 2-alpha, cytokine-induced neutrophil chemotactic factor 2-beta, beta-endothelial cell growth factor, endothelia 1, epidermal

growth factor, epithelial-derived neutrophil attractant, fibroblast growth factor 4, fibroblast growth factor 5, fibroblast growth factor 6 fibroblast growth factor 7, fibroblast growth factor 8, fibroblast growth factor b, fibroblast growth factor c, fibroblast growth factor 9, fibroblast growth factor 10, fibroblast growth factor acidic, fibroblast growth factor basic, glial cell line-derived neutrophil factor receptor-alpha-1, glial cell line-derived neutrophil factor receptor-alpha-2, granulocyte colony-stimulating factor (G-CSF), growth related protein, growth related protein-alpha, growth related protein-beta, growth related protein-gamma, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leptin, leukemia inhibitory factor, leukemia inhibitory factor receptor-alpha, nerve growth factor, nerve growth factor receptor, neurotrophin-3, neurotrophin-4, placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet derived growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor-alpha, platelet derived growth factor receptor-beta, pre-B cell growth stimulating factor, stem cell factor, stem cell factor receptor, thrombopoietin, transforming growth factor-alpha, transforming growth factor-beta, transforming growth factor-beta-1, transforming growth factor-beta-1-2, transforming growth factor-beta-2, transforming growth factor-beta-3, transforming growth factor-beta-5, latent transforming growth factor-beta-1, transforming growth factor-beta-binding protein I, transforming growth factor-beta-binding protein II, transforming growth factor-beta-binding protein III, tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, vascular endothelial growth factor, human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); epidermal growth factor; hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-alpha and -beta; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-alpha; platelet-growth factor; transforming growth factors (TGFs) such as TGF-alpha and TGF-beta; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-alpha, -beta and -gamma colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1alpha, 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-15, IL-16, IL-17, IL-18, IL-20, IL-21, IL-22, IL-23, IL-25, IL-27, IL-29, IL-33, etc.; a tumor necrosis factor such as TNF-alpha or TNF-beta; other polypeptide factors including LIF and kit ligand (KL), and chimeric proteins and biologically or immunologically active fragments thereof.

[00144] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for a mitogen or growth factor receptor. Mitogen receptors include those that bind ligands including, but not limited to: insulin, insulin-like growth factor (*e.g.*, IGF1, IGF2), platelet derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), fibroblast growth factor (FGF), bone morphogenic proteins (BMPs), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF), transforming growth factor (TGF)-alpha and -beta, among others.

[00145] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for a transcription factor. As used herein the term "transcription factor" refers to a protein that binds to specific DNA sequences and thereby controls the transfer (or transcription) of genetic information from DNA to mRNA. In one embodiment, the transcription factor encoded by the synthetic, modified RNA is a human transcription factor, such as those described in e.g., Messina DM, et al. (2004) Genome Res. 14(10B):2041-2047, which is herein incorporated by reference in its entirety. Some non-limiting examples of human transcription factors (and their mRNA IDs and sequence identifiers) for use in the aspects and embodiments described herein include those listed in Table 1 of International Patent Publication PCT/US2011/032679, the contents of which are herein incorporated by reference in their entireties.

[00146] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for a CD ("cluster of differentiation") molecules and/or other cell-surface/membrane bound molecule or receptor, such as transmembrane tyrosine kinase receptors, ABC transporters, and integrins. . Some non-limiting examples of CD molecules (and their mRNA IDs and sequence identifiers) for use in the aspects and embodiments described herein include those listed in Tables 2 and 3 of International Patent Publication PCT/US2011/032679, the contents of which are herein incorporated by reference in their entireties.

[00147] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for a ligand or ligand receptor on the surface of a cell (*e.g.*, a homing moiety). A ligand or ligand receptor moiety attached to a cell surface permits the cell to have a desired biological interaction with a tissue or an agent *in vivo*. A ligand can be an antibody, an antibody fragment, an aptamer, a peptide, a vitamin, a carbohydrate, a protein or polypeptide, a receptor, e.g., cell-surafce receptor, an adhesion molecule, a glycoprotein, a sugar residue, a therapeutic agent, a drug, a glycosaminoglycan, or any combination thereof. For example, a ligand can be an antibody that recognizes a cancer-cell specific antigen, rendering the cell capable of preferentially interacting with tumor cells to permit tumor-specific localization of a modified cell. A ligand can confer the ability of a cell composition to accumulate in a tissue to be treated, since a preferred ligand is capable of interacting with a target molecule on the external face of a tissue to be treated. Ligands having limited cross-reactivity to other tissues are generally preferred.

[00148] In some cases, a ligand can act as a homing moiety which permits the cell to target to a specific tissue or interact with a specific ligand. Such homing moieties can include, for example, any member of a specific binding pair, antibodies, monoclonal antibodies, or derivatives or analogs thereof, including without limitation: Fv fragments, single chain Fv (scFv) fragments, Fab' fragments, $F(ab')_2$ fragments, single domain antibodies, camelized antibodies and antibody fragments, humanized antibodies and antibody fragments, and multivalent versions of the foregoing; multivalent binding reagents including without limitation: monospecific or bispecific antibodies, such as disulfide stabilized Fv fragments, scFv tandems ($(scFv)_2$ fragments), diabodies, tribodies or tetrabodies, which typically are covalently linked or otherwise stabilized (*i.e.*, leucine zipper or helix stabilized) scFv fragments; and other homing moieties include for example, aptamers, receptors, and fusion proteins.

[00149] In some embodiments, the homing moiety is a surface-bound antibody, which can permit tuning of cell targeting specificity. This is especially useful since highly specific antibodies can be raised against an epitope of interest for the desired targeting site. In one embodiment, multiple antibodies are expressed on the surface of a cell, and each antibody can have a different specificity for a desired target. Such approaches can increase the avidity and specificity of homing interactions.

[00150] A skilled artisan can select any homing moiety based on the desired localization or function of the cell, for example an estrogen receptor ligand, such as tamoxifen, can target cells to estrogen-dependent breast cancer cells that have an increased number of estrogen receptors on the cell surface. Other non-limiting examples of ligand/receptor interactions include CCR1 (*e.g.*, for treatment of inflamed joint tissues or brain in rheumatoid arthritis, and/or multiple sclerosis), CCR7, CCR8 (*e.g.*, targeting to lymph node tissue), CCR6, CCR9,CCR10 (*e.g.*, to target to intestinal tissue), CCR4, CCR10 (*e.g.*, for targeting to skin), CXCR4 (*e.g.*, for general enhanced transmigration), HCELL (*e.g.*, for treatment of inflammation and inflammatory disorders, bone marrow), Alpha4beta7 (*e.g.*, for intestinal mucosa targeting), VLA-4 / VCAM-1 (*e.g.*, targeting to endothelium).

[00151] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for a reprogramming factor. The term a "reprogramming factor," as used herein, refers to a developmental potential altering factor, such as a protein, RNA, or small molecule, the expression of which contributes to the reprogramming of a cell, *e.g.* a somatic cell, to a less differentiated or undifferentiated state, *e.g.* to a cell of a pluripotent state or partially pluripotent state. A reprogramming factor can be, for example, transcription factors that can reprogram cells to a pluripotent state, such as SOX2, OCT3/4, KLF4, NANOG, LIN-28, c-MYC, and the like, including as any gene, protein, RNA or small molecule, that can substitute for one or more of these in a method of reprogramming cells *in vitro*. A reprogramming factor can also be termed a "de-differentiation factor," which refers to a developmental potential altering factor, such as a protein or RNA, that induces a cell to de-differentiate to a less differentiated phenotype, or, in other words, increases the developmental potential of a cell.

[00152] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for a differentiation factor. As used herein, the term "differentiation factor" refers to a developmental potential altering factor, such as a protein, RNA, or small molecule, that induces a cell to differentiate to a desired cell-type, *i.e.*, a differentiation factor reduces the developmental potential of a cell.

[00153] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for a cell-type specific polypeptide. As used herein, the term "cell-type specific polypeptide" refers to a polypeptide that is expressed in a cell having a particular phenotype (*e.g.*, a muscle cell) but is not generally expressed in other cell types with different phenotypes. For example, MyoD is expressed specifically in muscle cells but not in non-muscle cells, thus MyoD is a cell-type specific polypeptide. As another example, albumin is expressed in hepatocytes and is thus an hepatocyte-specific polypeptide. Such cell-specific polypeptides are well known in the art or can be identified using a gene array analysis and comparison of at least two different cell types. Methods for gene expressional array analysis is well known in the art.

[00154] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for a death receptor or death receptor ligand. By "death receptor" is meant a receptor that induces cellular apoptosis once bound by a ligand. Death receptors include, for example, tumor necrosis factor (TNF) receptor superfamily members having death domains (*e.g.*, TNFRI, Fas, DR3, 4, 5, 6) and TNF receptor superfamily members without death domains LTbetaR, CD40, CD27, HVEM. Death receptors and death receptor ligands are well known in the art. Some non-limiting examples of death receptors include FAS (CD95, Apo1), TNFR1 (p55, CD120a), DR3 (Apo3, WSL-1, TRAMP, LARD), DR4, DR5 (Apo2, TRAIL-R2, TRICK2, KILLER), CAR1, and the adaptor molecules FADD, TRADD, and DAXX. Some non-limiting examples of death receptor ligands include FASL (CD95L), TNF, lymphotoxin alpha, Apo3L (TWEAK), and TRAIL (Apo2L).

[00155] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for an RNA molecule found in a non-human species, including other mammalian RNAs, avian RNA, reptilian RNAs, bacterial RNA, and viral RNAs. Such sequences can encode for protein or peptides that have a desirable function, such as a reporter molecule, a secreted anti-microbial peptide, and the like.

[00156] In some embodiments, the unit dose composition comprises a synthetic, modified RNA that encodes for a tRNA (transfer RNA), an snRNA (small nuclear RNA), an rRNA (ribosomal RNA), an anti-sense RNA, a small interfering RNA (siRNA), a micro RNA (miRNA), or any other RNA sequence that does not encode a functional protein or peptide.

[00157] As used herein, an “antisense RNA” comprises one or more nucleotide sequences sufficient in identity, number and size to effect specific hybridization with a preselected nucleic acid sequence.

[00158] As used herein, "ribozymes" refer to RNA molecules having enzymatic activities usually associated with cleavage, splicing or ligation of nucleic acid sequences to which the ribozyme binds. Typical substrates for ribozymes include RNA molecules, although ribozymes can also catalyze reactions in which DNA molecules serve as substrates. Two distinct regions can be identified in a ribozyme: the binding region which gives the ribozyme its specificity through hybridization to a specific nucleic acid sequence, and a catalytic region which gives the ribozyme the activity of cleavage, ligation or splicing.

[00159] As used herein, "siRNA" refers to a 1-50 nucleotide double stranded RNA (dsRNA) molecule that has sequence-specific homology to its "target" nucleic acid sequences (Caplen, N. J., et al., Proc. Natl. Acad. Sci. USA 98:9742-9747 (2001)) and is derived from the processing of a larger dsRNA by an RNase known as Dicer (Bernstein, E., et al., Nature 409:363-366 (2001)).

[00160] As used herein, "shRNA" molecules are single stranded nucleic acid molecules that comprise two sequences complementary to each other, oriented such that one of the sequences is inverted relative to the other, which allows the two complementary sequences to base pair with each other, thereby forming a hairpin structure. The two copies of the inverted repeat need not be contiguous. There may be "n" additional nucleotides between the hairpin forming sequences, wherein "n" is any number of nucleotides.

[00161] As used herein, "microRNA" refers to molecules which are structurally similar to shRNA molecules, as described herein, but, typically, contain one or more mismatches or insertion/deletions in their regions of sequence complementary. The binding of miRNA of perfect complementarity to a target sequence results in mRNA degradation; single base mismatches can block translation.

Targeting Ligands & Moieties

[00162] In some embodiments, the unit dose compositions comprising synthetic, modified RNAs further comprise a ligand. A ligan can, for example, alter the cellular uptake, intracellular targeting or half-life of a synthetic, modified RNA into which it is incorporated. In some embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type, intracellular compartment, *e.g.*, mitochondria, cytoplasm, peroxisome, lysosome, as, *e.g.*, compared to a composition absent such a ligand. Preferred ligands do not interfere with expression of a polypeptide from the synthetic, modified RNA.

[00163] Ligands can include a naturally occurring substance, such as a protein (*e.g.*, human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (*e.g.*, a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand can also be a recombinant or synthetic molecule, such as a synthetic polymer, *e.g.*, a synthetic polyamino acid. Examples of polyamino acids include polylysine (PLL), poly L aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene

glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

[00164] Ligands can also include targeting groups, *e.g.*, a cell targeting agent, (*e.g.*, a lectin, glycoprotein, lipid or protein), or an antibody, that binds to a specified cell type such as a fibroblast cell. A targeting group can be, for example, a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-glucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, or an RGD peptide or RGD peptide mimetic, among others.

[00165] Other examples of ligands include dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), lipophilic molecules, *e.g.*, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid,O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine)and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, [MPEG]2, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), and transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid).

[00166] Ligands 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 fibroblast cell, or other cell useful in the production of polypeptides. Ligands can 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-glucosamine multivalent mannose, or multivalent fucose.

[00167] The ligand can be a substance, *e.g.*, a drug, which can increase the uptake of the unit dose compositions comprising synthetic, modified RNAs into a cell of a subject to which it is adminstered, for example, by disrupting the cell's cytoskeleton, *e.g.*, by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxol, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

[00168] One exemplary ligand is a lipid or lipid-based molecule. A lipid or lipid-based ligand can (a) increase resistance to degradation, and/or (b) increase targeting or transport into a target cell or

cell membrane. A lipid based ligand can be used to modulate, *e.g.*, binding of the modified RNA composition to a target cell.

[00169] In other embodiments, the ligand is a moiety, *e.g.*, a vitamin, which is taken up by a host cell. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include B vitamin, *e.g.*, folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up, for example, by cancer cells. Also included are HSA and low density lipoprotein (LDL).

[00170] In other embodiments, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

[00171] A "cell permeation peptide" is capable of permeating a cell, *e.g.*, a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α-helical linear peptide (*e.g.*, LL-37 or Ceropin P1), a disulfide bond-containing peptide (*e.g.*, α -defensin, β-defensin or bactenecin), or a peptide containing only one or two dominating amino acids (*e.g.*, PR-39 or indolicidin). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni et al., Nucl. Acids Res. 31:2717-2724, 2003).

[00172] As used herein, the term "targeting moiety" refers to an agent that directs a composition to a particular tissue, cell type, receptor, or other area of interest. As per this definition, a targeting moiety can be attached directly to a synthetic, modified RNA or indirectly to a composition used for delivering a synthetic, modified RNA (*e.g.*, a liposome, polymer etc) to direct expression in a particular cell etc. A targeting moiety can also be encoded or expressed by a synthetic, modified-NA as described herein, such that a cell expresses a targeting moiety on it surface, permitting a cell to be targeted to a desired tissue, organ etc. For the avoidance of confusion, targeting moieties expressed on a cell surface are referred to herein as "homing moieties."

[00173] Non-limiting examples of a targeting moiety or homing moiety include, but are not limited to, an oligonucleotide, an antigen, an antibody or functional fragment thereof, a ligand, a cell-surface receptor, a membrane-bound molecule, one member of a specific binding pair, a polyamide including a peptide having affinity for a biological receptor, an oligosaccharide, a polysaccharide, a steroid or steroid derivative, a hormone, *e.g.*, estradiol or histamine, a hormone-mimic, *e.g.*, morphine, or hormone-receptor, or other compound having binding specificity for a target. In the methods of the present invention, a targeting moiety promotes transport or preferential localization of a synthetic, modified RNA to a target cell, while a homing moiety permits the targeting of a cell modified using the synthetic, modified RNAs described herein to a particular tissue *in vivo*. It is contemplated herein

that the homing moiety can be also encoded in a cell by a synthetic, modified RNA as described herein.

[00174] A synthetic, modified RNA or unit dose composition thereof can be targeted by means of a targeting moiety, including, *e.g.*, an antibody or targeted liposome technology. In some embodiments, a synthetic, modified RNA or unit dose composition thereof is targeted to a specific tissue by using bispecific antibodies, for example produced by chemical linkage of an anti-ligand antibody (Ab) and an Ab directed toward a specific target. To avoid the limitations of chemical conjugates, molecular conjugates of antibodies can be used for production of recombinant, bispecific single-chain Abs directing ligands and/or chimeric inhibitors at cell surface molecules. The addition of an antibody to a synthetic, modified RNA composition permits the agent attached to accumulate additively at the desired target site. Antibody-based or non- antibody-based targeting moieties can be employed to deliver a ligand or the inhibitor to a target site. Preferably, a natural binding agent for an unregulated or disease associated antigen is used for this purpose.

Plurality of synthetic, modified RNAs

[00175] In some embodiments of the aspects described herein, a unit dose composition can comprise a plurality of different synthetic, modified RNA. In some embodiments, a unit dose composition can comprise two or more synthetic, modified RNAs, *e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more synthetic, modified RNAs. In some embodiments, the two or more synthetic, modified RNAs are capable of increasing expression of a desired therapeutic agent (*e.g.*, a transcription factor, a cell surface marker, a death receptor, etc.).

[00176] In some embodiments, when a plurality of different synthetic, modified RNAs are used to modulate expression of a desired set of therapeutic agents, the plurality of synthetic, modified RNAs can be administered to a subject simultaneously in a single unit dose composition, for example. In other embodiments, the plurality of synthetic, modified RNAs can be administered to a subject individually in separate unit dose compositions. In addition, each synthetic, modified RNA can be administered according to its own dosage regime. For example, in one embodiment, a unit dose composition can be prepared comprising a plurality of synthetic, modified RNAs, in differing relative amounts or in equal amounts, that is administered to a subject such that the plurality of synthetic, modified RNAs are administered simultaneously. Alternatively, one synthetic, modified RNA at a time can be administered to a subject. In this manner, the expression desired for each target therapeutic agentcan be easily tailored by altering the frequency of administration and/or the amount of a particular synthetic, modified RNA administered. By adminstereing unit dose composition comprising a unique synthetic, modified RNA separately, interactions between the synthetic, modified RNAs that can reduce efficiency of expression can be prevented.

[00177] The unit dose compositions and methods described herein permit the expression of one or more therapeutic agents to be tuned to a desired level by varying the amount of each synthetic, modified RNA adminstered. One of skill in the art can easily monitor the expression level of the

therapeutic agent encoded by a synthetic, modified RNA using *e.g.*, Western blotting techniques, immunocytochemistry techniques, or ELISA, as described herein for erythropoietin. A unit dose composition comprising one or more synthetic, modified RNAs can be administered at a frequency and dose that permit a desired level of expression of the therapeutic agent encoded by the synthetic, modified RNA. In addition, since the synthetic, modified RNAs administered toa subject are transient in nature (*i.e.*, are degraded over time) one of skill in the art can easily remove or stop expression of a synthetic, modified RNA by halting further administrations and permitting the subject to degrade the synthetic, modified RNA over time. The synthetic, modified RNAs will degrade in a manner similar to cellular mRNAs.

Synthesis of synthetic, modified RNAs

[00178] The synthetic, modified RNAs described herein can be synthesized and/or modified by methods well established in the art, such as those described in "Current Protocols in Nucleic Acid Chemistry," Beaucage, S.L. et al. (Edrs.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference in its entirety. Transcription methods are described further herein in the Examples.

[00179] In one embodiment of the aspects described herein, a template for a synthetic, modified RNA is synthesized using "splint-mediated ligation," which allows for the rapid synthesis of DNA constructs by controlled concatenation of long oligos and/or dsDNA PCR products and without the need to introduce restriction sites at the joining regions. It can be used to add generic untranslated regions (UTRs) to the coding sequences of genes during T7 template generation. Splint mediated ligation can also be used to add nuclear localization sequences to an open reading frame, and to make dominant-negative constructs with point mutations starting from a wild-type open reading frame. Briefly, single-stranded and/or denatured dsDNA components are annealed to splint oligos which bring the desired ends into conjunction, the ends are ligated by a thermostable DNA ligase and the desired constructs amplified by PCR. A synthetic, modified RNA is then synthesized from the template using an RNA polymerase *in vitro*. After synthesis of a synthetic, modified RNA is complete, the DNA template is removed from the transcription reaction prior to use with the methods described herein.

[00180] In some embodiments of these aspects, the synthetic, modified RNAs are further treated with an alkaline phosphatase.

[00181] In other embodiments, the synthetic modified RNAs can be synethesized using the IVT templates and constructs described in US Provisional Application 61/558,563, which permit easy and efficient generation of synethetic, modified RNAs.

Kits and Intramuscular Delivery Devices

[00182] Provided herein are kits and intramuscular delivery devices comprising unit dose compositions comprising synthetic, modified RNAs encoding therapeutic agents as described herein.

[00183] Accordingly, provided herein, in some aspects, are kits comprising (a) a container or vial containing a unit dose comprising between 0.03 mg/kg of body weight and 10 mg/kg of body weight of a synthetic, modified RNA encoding a therapeutic agent, wherein the synthetic, modified RNA comprises at least two modified nucleosides, or a pharmaceutical composition thereof and (b) packaging and instructions therefor.

[00184] In some embodiments of these kits, the therapeutic agent is a polypeptide or a non-translated RNA molecule. In some such embodiments, the polypeptide is a somatotrophic agent. In some such embodiments, the somatotrophic agent is a growth hormone. In some such embodiments, the polypeptide is a cytokine.

[00185] In some embodiments of these kits, the unit dose ranges from about 0.03 mg per kg of body weight to about 0.0625 mg per kg of body weight. In some embodiments of these kits, the unit dose ranges from about 0.1 mg per kg of body weight to about 0.4 mg per kg of body weight. In some embodiments of these kits, the unit dose ranges from about 0.9 mg per kg of body weight to about 1.6 mg per kg of body weight. In some embodiments of these kits, the unit dose ranges from about 1.8 mg per kg of body weight to about 3.2 mg per kg of body weight.In some embodiments of these kits, the unit dose ranges from about 3.0 mg per kg of body weight to about 6.5 mg per kg of body weight. In some embodiments of these kits, unit dose ranges from about 5.5 mg per kg of body weight to about 10 mg per kg of body weight.

[00186] In some embodiments of these kits, the at least two modified nucleosides are selected from the group consisting of 5-methylcytidine (5mC), N6-methyladenosine (m6A), 3,2'-O-dimethyluridine (m4U), 2-thiouridine (s2U), 2' fluorouridine, pseudouridine, 2'-O-methyluridine (Um), 2'deoxy uridine (2' dU), 4-thiouridine (s4U), 5-methyluridine (m5U), 2'-O-methyladenosine (m6A), N6,2'-O-dimethyladenosine (m6Am), N6,N6,2'-O-trimethyladenosine (m62Am), 2'-O-methylcytidine (Cm), 7-methylguanosine (m7G), 2'-O-methylguanosine (Gm), N2,7-dimethylguanosine (m2,7G), N2, N2, 7-trimethylguanosine (m2,2,7G), and inosine (I).

[00187] In some embodiments of these kits, the at least two modified nucleosides are 5-methylcytidine (5mC) and pseudouridine.

[00188] In some embodiments of these kits, the unit dose composition further comprises a cationic lipid or lipid carrier.

[00189] In some embodiments of these kits, the unit dose is divided into at least two containers or vials.

[00190] In other aspects, provided herein are intramuscular delivery devices comprising a unit dose comprising between 0.03 mg/kg of body weight and 10 mg/kg of body weight of a synthetic, modified RNA encoding a therapeutic agent, wherein the synthetic, modified RNA comprises at least two modified nucleosides, or a pharmaceutical composition thereof

[00191] In some embodiments of these intramuscular delivery devices, the intramuscular delivery device is a non-implantable delivery device or an implantable delivery device.

[00192] In some embodiments of these intramuscular delivery devices, the intramuscular delivery device is a syringe.

[00193] All kits and/or intramuscular delivery devices described herein can further comprise one or more buffers, cell culture mediums, transfection medium and/or a media supplement. In preferred embodiments, the buffers, cell culture mediums, transfection mediums, and/or media supplements are RNase-free. In some embodiments, the unit dose compositions comprising synthetic, modified RNAs provided in the kits can be in a non-solution form of specific quantity or mass, e.g., 20 μg, such as a lyophilized powder form, such that the end-user adds a suitable amount of buffer or medium to bring the synthetic, modified RNAs to a desired unit dose concentration, e.g., 100 ng/μl.

[00194] All kits and/or intramuscular delivery devices described herein can further comprise devices to facilitate single-adminstration or repeated or frequent infusions of a unit dose of a synthetic, modified RNA, such as a non-implantable delivery device, e.g., needle, syringe, pen device, or an implantatable delivery device, or reservoir. In some such embodiments, the delivery device can include a mechanism to dispense a unit dose composition comprising a synthetic, modified RNA. In some embodiments, the device releases the unit dose composition comprising a synthetic, modified RNA continuously, e.g., by diffusion. In some embodiments, the device can include a sensor that monitors a parameter within a subject. For example, the device can include pump, e.g., and, optionally, associated electronics.

[00195] Embodiments of the various aspects described herein can be illustrated by the following numbered paragraphs:

1. A unit dose comprising between 0.03 mg/kg of body weight and 10 mg/kg of body weight of a synthetic, modified RNA encoding a therapeutic agent, wherein the synthetic, modified RNA comprises at least two modified nucleosides.

2. The unit dose of paragraph 1, wherein the therapeutic agent is a polypeptide or a non-translated RNA molecule.

3. The unit dose of paragraph 2, wherein the polypeptide is a somatotrophic agent.

4. The unit dose of paragraph 3, wherein the somatotrophic agent is a growth hormone.

5. The unit dose of paragraph 2, wherein the polypeptide is a cytokine.

6. The unit dose of paragraph 2, wherein the polypeptide is a cellular growth factor.

7. The unit dose of any one of the preceding paragraphs, wherein the unit dose ranges from about 0.03 mg per kg of body weight to about 0.0625 mg per kg of body weight.

8. The unit dose of any one of paragraphs 1-6, wherein the unit dose ranges from about 0.1 mg per kg of body weight to about 0.4 mg per kg of body weight.

9. The unit dose of any one of paragraphs 1-6, wherein the unit dose ranges from about 0.9 mg per kg of body weight to about 1.6 mg per kg of body weight.

10. The unit dose of any one of paragraphs 1-6, wherein the unit dose ranges from about 1.8 mg per kg of body weight to about 3.2 mg per kg of body weight.

11. The unit dose of any one of paragraphs 1-6, wherein the unit dose ranges from about 3.0 mg per kg of body weight to about 6.5 mg per kg of body weight.

12. The unit dose of any one of paragraphs 1-6, wherein the unit dose ranges from about 5.5 mg per kg of body weight to about 10 mg per kg of body weight.

13. The unit dose of any one of the preceding paragraphs, wherein the at least two modified nucleosides are selected from the group consisting of 5-methylcytidine (5mC), N6-methyladenosine (m6A), 3,2'-O-dimethyluridine (m4U), 2-thiouridine (s2U), 2' fluorouridine, pseudouridine, 2'-O-methyluridine (Um), 2'deoxy uridine (2' dU), 4-thiouridine (s4U), 5-methyluridine (m5U), 2'-O-methyladenosine (m6A), N6,2'-O-dimethyladenosine (m6Am), N6,N6,2'-O-trimethyladenosine (m62Am), 2'-O-methylcytidine (Cm), 7-methylguanosine (m7G), 2'-O-methylguanosine (Gm), N2,7-dimethylguanosine (m2,7G), N2, N2, 7-trimethylguanosine (m2,2,7G), and inosine (I).

14. The unit dose of any one of the preceding paragraphs, wherein the at least two modified nucleosides are 5-methylcytidine (5mC) and pseudouridine.

15. The unit dose of any one of the preceding paragraphs, further comprising a cationic lipid.

16. A pharmaceutical composition for intramuscular delivery comprising the unit dose of a synthetic, modified RNA encoding a therapeutic agent of any one of paragraphs 1-15 and a pharmaceutically acceptable carrier.

17. A kit comprising (a) a container or vial containing the unit dose of a synthetic, modified RNA encoding a therapeutic agent of any one of paragraphs 1-15 or the pharmaceutical composition of paragraph 16, and (b) packaging and instructions therefor.

18. The kit of paragraph 17, wherein the unit dose is divided into at least two containers or vials.

19. An intramuscular delivery device comprising the unit dose of a synthetic, modified RNA encoding a therapeutic agent of any one of paragraphs 1-15 or the pharmaceutical composition of paragraph 16.

20. The intramuscular delivery device of paragraph 19, wherein the intramuscular delivery device is a non-implantable delivery device or an implantable delivery device.

21. The intramuscular delivery device of paragraph 19, wherein the intramuscular delivery device is a syringe.

22. An enhanced method for delivering synthetic, modified RNA into a subject comprising administering intramuscularly to a subject at least one unit dose of any one of paragraphs 1-15 or the pharmaceutical composition of paragraph 16.

23. The method of paragraph 22, wherein the unit dose is divided into at least two separate unit dosages and administered simultaneously into at least two muscular locations.

24. The method of paragraph 22, wherein the unit dose is divided into at least two separate unit dosages and administered sequentially into the same or a different muscular locations.

25. A method of stimulating erythropoiesis in a mammalian subject in need thereof, comprising the step of administering intramuscularly the pharmaceutical composition of paragraph 16 to the subject.

26. The method of paragraph 25, wherein the theapuetic agent is erythropoietin.

[00196] As used herein the term "comprising " or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not.

[00197] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[00198] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00199] As used in this specification and the appended claims, the singular forms “a,” "an," and "the" include plural references unless the context clearly dictates otherwise. Thus for example, references to "the method" includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth. In addition, the term ‘cell’ can be construed as a cell population, which can be either heterogeneous or homogeneous in nature, and can also refer to an aggregate of cells.

[00200] It is understood that the foregoing detailed description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments, which will be apparent to those of skill in the art, may be made without departing from the spirit and scope of the present invention. Further, all patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the dates or contents of these documents.

[00201] All references cited herein in the specification are incorporated by reference in their entirety.

EXAMPLES

[00202] Dose-dependency of protein expression upon intramuscular injection of synthetic, modified RNAs is shown in FIG. 1. Various doses of a synthetic, modified RNA encoding

Erythropoietin (EPO) were injected intramuscularly, and thirteen hours later plasma erythropoietin levels were examined using ELISA. As shown, increasing the doses of the synthetic, modified RNA leads to a proportional increase in plasma levels of erythropoietin.

[00203] As shown in FIG. 2, 1 week following injection of a synthetic, modified RNA encoding erythropoietin, functional parameters mediated by erythropoietin increase in a dose-dependent fashion. Synthetic, modified RNA encoding erythropoietin was injected with or without lipofectamine, with unmodified RNA plus lipofectamine used as a control. The upper left, upper right, and lower left panels of FIG. 2 demonstrate that red blood cell (RBC) counts, hematocrit, and hemoglobin levels were increased in a dose-dependent fashion 1 week following injection of the synthetic, modified RNA encoding erythropoietin in the presence of lipfectamine. In the absence of liptofectamine or when unmodified RNAs were used, a dose-dependent effect was not observed for any of these functional parameters.

[00204] As shown in FIG. 3, parameters and cell types not responsive to erythropoietin are not impacted by the injection of the synthetic, modified RNA encoding erythropoietin, therefore demonstrating the specificity of the effects of the synthetic, modified RNA. Synthetic, modified RNA encoding erythropoietin was injected with or without lipofectamine, with unmodified RNA with lipofectamine used as a control. The upper left panel of FIG. 3 shows white blood cell (WBC) counts, the upper right panel of FIG. 3 shows lymphocyte counts, the lower left panel of FIG. 3 shows neutrophil counts, and the lower right panel of FIG. 3 shows platelet numbers one week following injection. In contrast to the RBC data shown in FIG. 2, no dose-dependent effects are observed for any of the cells types.

CLAIMS

We claim:

1. A unit dose comprising between 0.03 mg/kg of body weight and 10 mg/kg of body weight of a synthetic, modified RNA encoding a therapeutic agent, wherein the synthetic, modified RNA comprises at least two modified nucleosides.

2. The unit dose of claim 1, wherein the therapeutic agent is a polypeptide or a non-translated RNA molecule.

3. The unit dose of claim 2, wherein the polypeptide is a somatotrophic agent.

4. The unit dose of claim 3, wherein the somatotrophic agent is a growth hormone.

5. The unit dose of claim 2, wherein the polypeptide is a cytokine.

6. The unit dose of claim 2, wherein the polypeptide is a cellular growth factor.

7. The unit dose of any one of preceding claims, wherein the unit dose ranges from about 0.03 mg per kg of body weight to about 0.0625 mg per kg of body weight.

8. The unit dose of any one of claims 1-6, wherein the unit dose ranges from about 0.1 mg per kg of body weight to about 0.4 mg per kg of body weight.

9. The unit dose of any one of claims 1-6, wherein the unit dose ranges from about 0.9 mg per kg of body weight to about 1.6 mg per kg of body weight.

10. The unit dose of any one of claims 1-6, wherein the unit dose ranges from about 1.8 mg per kg of body weight to about 3.2 mg per kg of body weight.

11. The unit dose of any one of claims 1-6, wherein the unit dose ranges from about 3.0 mg per kg of body weight to about 6.5 mg per kg of body weight.

12. The unit dose of any one of claims 1-6, wherein the unit dose ranges from about 5.5 mg per kg of body weight to about 10 mg per kg of body weight.

13. The unit dose of any one of the preceding claims, wherein the at least two modified nucleosides are selected from the group consisting of 5-methylcytidine (5mC), N6-methyladenosine (m6A), 3,2'-O-dimethyluridine (m4U), 2-thiouridine (s2U), 2' fluorouridine, pseudouridine, 2'-O-methyluridine (Um), 2'deoxy uridine (2' dU), 4-thiouridine (s4U), 5-methyluridine (m5U), 2'-O-methyladenosine (m6A), N6,2'-O-dimethyladenosine (m6Am), N6,N6,2'-O-trimethyladenosine (m62Am), 2'-O-methylcytidine (Cm), 7-methylguanosine (m7G), 2'-O-methylguanosine (Gm), N2,7-dimethylguanosine (m2,7G), N2, N2, 7-trimethylguanosine (m2,2,7G), and inosine (I).

14. The unit dose of any one of the preceding claims, wherein the at least two modified nucleosides are 5-methylcytidine (5mC) and pseudouridine.

15. The unit dose of any one of the preceding claims, further comprising a cationic lipid.

16. A pharmaceutical composition for intramuscular delivery comprising the unit dose of a synthetic, modified RNA encoding a therapeutic agent of any one of claims 1-15 and a pharmaceutically acceptable carrier.

17. A kit comprising (a) a container or vial containing the unit dose of a synthetic, modified RNA encoding a therapeutic agent of any one of claims 1-15 or the pharmaceutical composition of claim 16, and (b) packaging and instructions therefor.

18. The kit of claim 17, wherein the unit dose is divided into at least two containers or vials.

19. An intramuscular delivery device comprising the unit dose of a synthetic, modified RNA encoding a therapeutic agent of any one of claims 1-15 or the pharmaceutical composition of claim 16.

20. The intramuscular delivery device of claim 19, wherein the intramuscular delivery device is a non-implantable delivery device or an implantable delivery device.

21. The intramuscular delivery device of claim 19, wherein the intramuscular delivery device is a syringe.

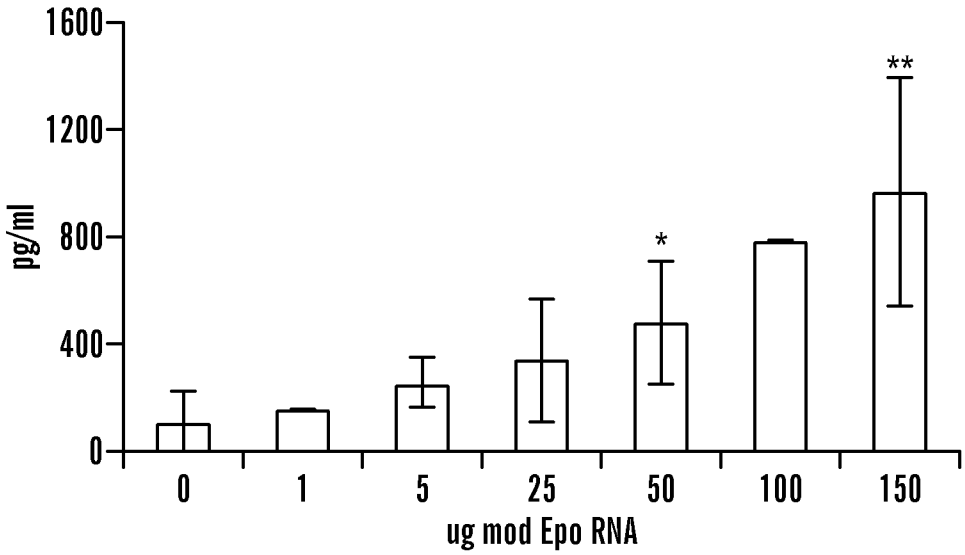
22. An enhanced method for delivering synthetic, modified RNA into a subject comprising administering intramuscularly to a subject at least one unit dose of any one of claims 1-15 or the pharmaceutical composition of claim 16.

23. The method of claim 22, wherein the unit dose is divided into at least two separate unit dosages and administered simultaneously into at least two muscular locations.

24. The method of claim 22, wherein the unit dose is divided into at least two separate unit dosages and administered sequentially into the same or a different muscular locations.

25. A method of stimulating erythropoiesis in a mammalian subject in need thereof, comprising the step of administering intramuscularly the pharmaceutical composition of claim 16 to the subject.

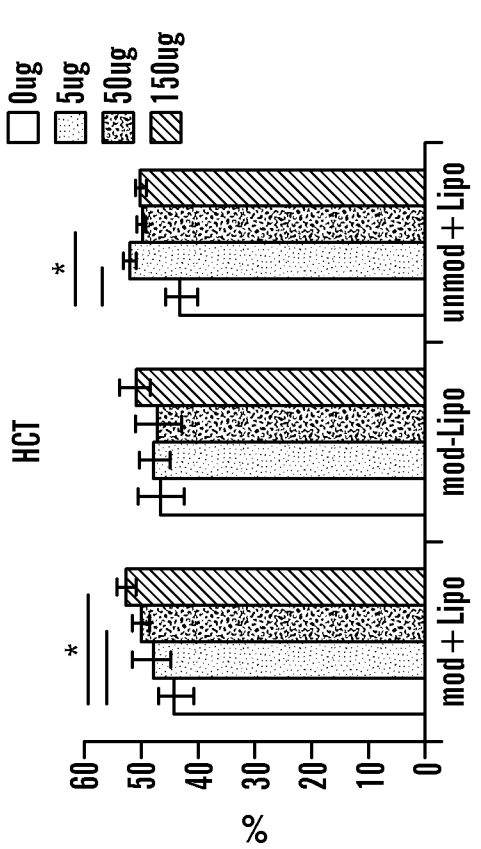
26. The method of claim 25, wherein the theapuetic agent is erythropoietin.

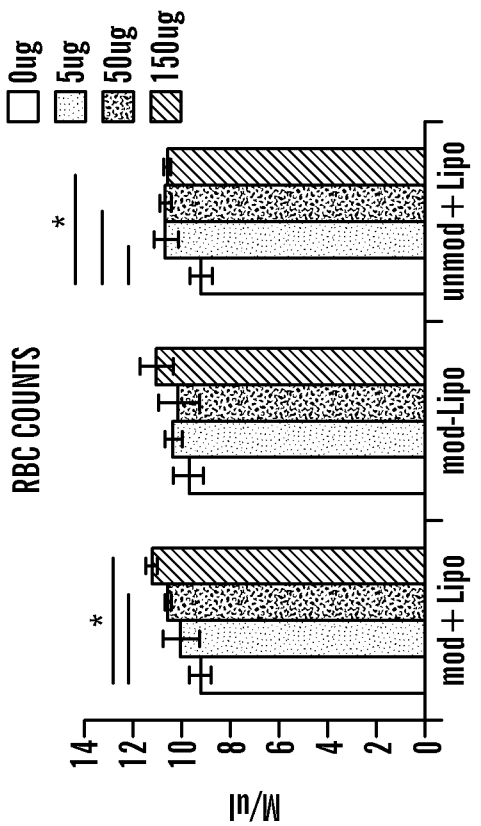


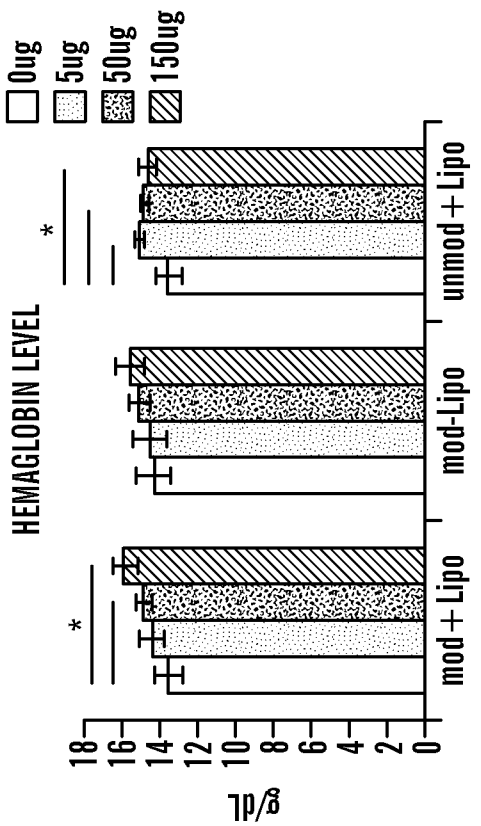
ug RNA	NUMBER OF MICE (n)	ttest
0	6	
1	1	n/a
5	3	0.118
25	4	0.083
50	4	0.015
100	1	n/a
150	4	0.003

NOTE: MALES AND FEMALES COMBINED;
0ug OUTLIER REMOVED

FIG. 1







ttest

vehicle	ug RNA	RBC	HGB	HCT
mod + Lipo	0ug			
mod-Lipo	0ug			
unmod + Lipo	0ug			
mod + Lipo	5ug	0.12863	0.18549	0.07307
mod-Lipo	5ug	0.16731	0.76719	0.70413
unmod + Lipo	**5ug**	**0.00981**	**0.02355**	**0.00510**
mod + Lipo	**50ug**	**0.00152**	**0.02456**	**0.00536**
mod-Lipo	50ug	0.52765	0.28031	0.90755
unmod + Lipo	**50ug**	**0.00385**	**0.03937**	**0.01410**
mod + Lipo	**150ug**	**0.00026**	**0.00403**	**0.00084**
mod-Lipo	150ug	0.06727	0.15037	0.16171
unmod + Lipo	**150ug**	**0.00396**	**0.09057**	**0.01161**

BOLDED BOXES ARE SIGNIFICANT

FIG. 2

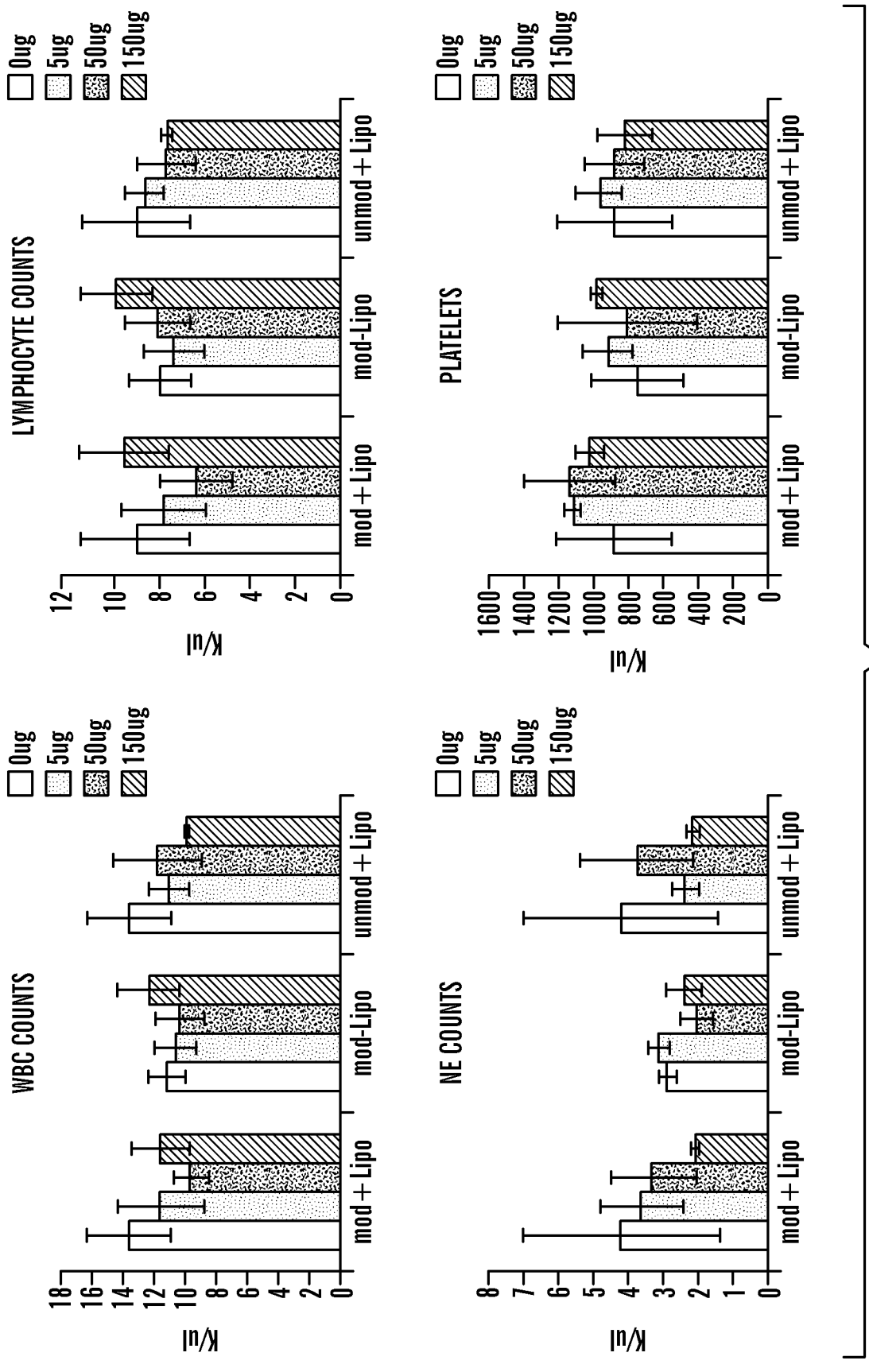

0ug
5ug
50ug
150ug
LYMPHOCYTE COUNTS
K/ul
0
2
4
6
8
10
12
mod + Lipo
mod-Lipo
unmod + Lipo
PLATELETS
K/ul
0
200
400
600
800
1000
1200
1400
1600
mod + Lipo
mod-Lipo
unmod + Lipo
WBC COUNTS
K/ul
0
2
4
6
8
10
12
14
16
18
mod + Lipo
mod-Lipo
unmod + Lipo
NE COUNTS
K/ul
0
1
2
3
4
5
6
7
8
mod + Lipo
mod-Lipo
unmod + Lipo

FIG. 3