

METHODS FOR IDENTIFICATION AND SELECTION OF SELF-REPLICATING RNA MOLECULES FOR BIOMEDICAL APPLICATIONS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority U.S. Provisional Application No. 63/502,219, filed on May 15, 2023. The disclosure of the above-references application is herein expressly incorporated by reference in its entirety, including any drawings.

FIELD

[0002] The present disclosure generally relates to the field of molecular virology and immunology, and particularly relates to methods for identifying and/or selecting self-replicating RNA (srRNA) delivery systems suitable for biomedical applications.

BACKGROUND

[0003] RNA technology has recently come to the forefront of innovative medicines and is being explored for a wide range of therapies, including prophylactic and therapeutic vaccines, biotherapeutic protein expression, and gene therapy. In addition to conventional mRNA platforms now approved for prophylactic SARS-CoV2 vaccines, srRNA has been increasingly used as a vaccine and therapeutic modality for the *in situ* production of proteins.

[0004] Nucleic acid vaccines, such as srRNA vaccines, represent a different approach in vaccine development. Unlike traditional vaccines, which deliver target antigens directly as proteins structures in the form of either purified or recombinant proteins or as part of attenuated or inactivated pathogens, nucleic acid-based vaccines deliver DNA or RNA encoding the target antigens (*e.g.*, immunogens). Generation of a protective immune response is dependent on cellular uptake and expression of the delivered nucleic acid preceding the immunologic presentation of target antigen. Synthetic srRNA vaccines are currently being evaluated in the clinic for infectious disease and oncology. The prototypical srRNA vectors in clinical development are derived from alphaviruses, specifically Venezuelan Equine Encephalitis Virus (VEEV).

[0005] Synthetic srRNAs have several advantages, namely increased safety profile based on the lack of potential for genomic integration or cell transformation, and simplified manufacturing. Another safety advantage of srRNAs is the reduced efficacious human dose. As srRNA amplify within the host cell, low doses still result in higher and more durable protein expression making them advantaged for biotherapeutics compared to mRNA. In addition, srRNA-based vaccines are advantaged compared to mRNA due to their ability to elicit robust cell-mediated immunity, exemplified by strong CD8+ and CD4+ T cell responses critical for efficacious oncology therapeutics. Lastly, the lack of a viral shell results in no/lower anti-vector immunity allowing repeated dosing and the ability to encode multiple larger genes of interest, normally limited by the packaging capacity of the viral particle. Despite these built-in advantages, recent clinical candidates reveal the challenges of developing fully synthetic srRNA products.

[0006] Deletion of the structural proteins in the alphavirus genome in synthetic srRNA vectors necessitates a formulation for optimal delivery *in vivo*. However, the relatively large size of srRNA molecules compared to known mRNA vectors poses unique challenges for formulating srRNA, whether as vaccines or as biotherapeutics. Such challenges necessitate the costly and time-consuming screening of many delivery systems to identify a proper optimum delivery system. Identifying the proper delivery system for srRNA products is crucial for their successful development as biomedical compositions. Delivery strategies need to be adapted to the particular use of the srRNA molecule as well as to the delivered product.

[0007] In addition, an array of quality control measures (for chemistry, control, and manufacture) is needed to identify the preferred product characteristics during in-process, release and stability evaluation of srRNA vaccine candidates or biotherapeutics to ensure a reproducible safe product for pre-clinical and clinical use. These quality control measures, in addition to safety and efficacy, are quintessential requirements for any successful therapeutic.

[0008] There is a need for efficient methods for identifying or screening effective srRNA delivery systems of prophylactic and therapeutic biomedical compositions.

SUMMARY

[0009] The present disclosure provides reliable analytical evaluation methods, based on a

newly identified combination of quality attributes that can be executed speedily to predict a suitable delivery system for biomedical applications. A suitable delivery system is important to ensure, for example, optimal *in vivo* activity of a formulated srRNA thereby ensuring effective delivery of a srRNA vaccine or a biotherapeutic (e.g. drug product.).

[0010] Accordingly, the present disclosure relates generally to methods of selecting or identifying srRNA delivery systems suitable for a biomedical application and to compositions including such srRNA delivery systems. In particular, as described in greater detail below, some embodiments of the disclosure provide methods for identifying or selecting a srRNA delivery system for a biomedical application, wherein the methods include: formulating an input srRNA composition with a non-viral delivery vehicle to generate a srRNA delivery system comprising a formulated srRNA composition, b) determining potency of the formulated srRNA composition relative to unformulated input srRNA composition, c) determining percentage change in amount of full-length srRNA molecules in the formulated srRNA composition relative to amount of full-length srRNA molecules in the input srRNA composition; and d) selecting the srRNA delivery system as being suitable for a biomedical application if the formulated srRNA composition in (b) retains at least about 25% potency relative to the potency of the unformulated input srRNA composition.

[0011] In some embodiments of the methods, the srRNA delivery system in (d) may be selected as being suitable for the biomedical application if the formulated srRNA composition in (b) retains at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55% or at least about 60% potency relative to the potency of the unformulated srRNA composition.

[0012] In some embodiments for identifying or selecting a srRNA delivery system, the percentage change in the amount of full-length srRNA molecules in the formulated srRNA composition in (c) includes a percentage decrease of less than about 40% relative to the amount of full-length srRNA molecules in the input srRNA composition. In some embodiments of the methods, the percentage decrease in (c) is less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, or less than about 10%, or less than about 5% relative to the amount of full-length srRNA molecules in the input srRNA composition.

[0013] In some embodiments, the methods of selecting or identifying a delivery system for a biomedical application, the input srRNA composition includes double-stranded RNA (dsRNA) molecules and single-stranded RNA (ssRNA) molecules. In some embodiments, the methods further include a step of determining the percentage of double-stranded RNA (dsRNA) relative to single-stranded RNA (ssRNA) in the input srRNA composition. In some embodiments, the determining the percentage of double-stranded RNA (dsRNA) relative to single-stranded RNA (ssRNA) in the input srRNA composition is by an immunoblot assay.

[0014] In some embodiments of the methods provided herein, the percentage of dsRNA relative to ssRNA is less than about 2.5%, less than about 2.0%, less than about 1.5%, less than about 1.0%, less than about 0.5%, or less than about 0.25%.

[0015] In some embodiments of the methods of the disclosure, determining potency of the formulated srRNA composition in (b) is carried out *in vivo*, or *in vitro*. In some embodiments, determining potency of the formulated srRNA composition includes detection of RNA replication, detection of viral protein expression, and/or detection of heterologous gene expression. In some embodiments, the determining potency of the formulated srRNA composition includes immunoblotting analysis, fluorescence flow cytometry analysis, enzyme-linked immunoassay analysis, immunogenicity analysis, bioactivity analysis, and/or efficacy in a disease model. In some embodiments, determining potency includes assessing RNA replication efficiency. In some embodiments, assessing RNA replication efficiency includes a monoclonal antibody. In some embodiments, the monoclonal antibody is J2. In some embodiments, the replication efficiency is determined by determining in an *in vitro* potency assay the frequency of cells having dsRNA per ng of RNA transfected.

[0016] In some embodiments, determining potency of the formulated srRNA composition in (b) includes an *in vivo* potency assay. In some embodiments, the *in vivo* potency assay is carried out in an animal cell. In some embodiments, the *in vivo* potency assay is carried out in a mammalian cell.

[0017] In some embodiments of the methods of the disclosure determining the percentage of full-length srRNA molecules in step (c) includes assaying the degradation of the full-length srRNA molecules during the formulation process of step (a). In some embodiments, determining

the percentage of full-length srRNA molecules in (c) includes gel electrophoresis and/or capillary electrophoresis.

[0018] In one aspect of the disclosure, provided herein are methods for identifying a self-replicating RNA (srRNA) delivery system for a biomedical application, wherein the methods include: (a) determining percentage of double-stranded RNA (dsRNA) molecules in an input srRNA composition relative to single-stranded RNA (ssRNA) molecules in the input srRNA composition, (b) formulating an input srRNA composition with a non-viral delivery vehicle to generate a srRNA delivery system comprising a formulated srRNA composition, (c) determining potency of the formulated srRNA composition relative to unformulated input srRNA composition; (d) determining percentage change in amount of full-length srRNA molecules in the formulated srRNA composition relative to amount of full-length srRNA molecules in the input srRNA composition; and (e) selecting the srRNA delivery system as being suitable for a biomedical application if (i) the percentage of dsRNA relative to ssRNA in (a) is less than about 2.5%, (ii) the formulated srRNA composition in (c) retains at least about 25% potency relative to the potency of the unformulated input srRNA composition, and (iii) the percentage change in (d) comprises a percentage decrease of less than about 40% relative to the amount of full-length srRNA molecules in the unformulated input srRNA composition.

[0019] In some embodiments of the methods of the disclosure, determining the percentage of dsRNA relative to ssRNA in (b) includes an immunoblot assay, determining the potency includes an *in vitro* potency assay, and determining the percentage change in the amount of full-length srRNA between the formulated srRNA and input srRNA composition in (d) includes capillary electrophoresis.

[0020] In some embodiments of the methods of the disclosure, the percentage of dsRNA relative to ssRNA is from about 0.0% to about 2.5%, from about 0.5% to about 2.0%, from about 1.0% to about 1.5%

[0021] In some embodiments, the percentage change in (d) is less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, or less than about 5%. In some embodiments, the percentage change is from about 5% to about 40%, or from about 10% to about 30% or from about 20% to about 25%.

[0022] In some embodiments of the methods provided herein, determining potency of the formulated srRNA composition in (b) is carried out *in vivo*, or *in vitro*. In some embodiments, the determining potency of the formulated srRNA composition includes detection of RNA replication, detection of viral protein expression, and/or detection of heterologous gene expression. In some embodiments, determining the potency of the formulated srRNA composition includes immunoblotting analysis, fluorescence flow cytometry analysis, enzyme-linked immunoassay analysis, immunogenicity analysis, bioactivity analysis, and/or efficacy in a disease model. In some embodiments, the determining potency includes assessing RNA replication efficiency. In some embodiments, assessing RNA replication efficiency includes a monoclonal antibody. In some embodiments, the monoclonal antibody is J2.

[0023] In some embodiments of the methods provided herein, the replication efficiency is determined by determining in an *in vitro* potency assay the frequency of cells having dsRNA per ng of RNA transfected. In some embodiments, determining potency of the formulated srRNA composition in (b) comprises an *in vivo* potency assay. In some embodiments, the *ex vivo* potency assay is carried out in an animal cell. In some embodiments, the *ex vivo* potency assay is carried out in a mammalian cell.

[0024] In some embodiments of the methods of the disclosure, determining the percentage of full-length srRNA molecules in (c) is by gel electrophoresis or by capillary electrophoresis.

[0025] Non-limiting examples of non-viral delivery vehicles include a polymer nanoparticle, or a lipid-based nanoparticle (LNP), a liposome, a microsphere, an immune stimulating complex (ISCOM), a conjugate of a bioactive ligand, a physiologic buffer, or a combination of any thereof. In some embodiments, the LNP includes a cationic lipid, an ionizable cationic lipid, an anionic lipid, or a neutral lipid. In some embodiments, the mass ratio of lipid to nucleic acid in the LNP delivery system is about 100:1 to about 3:1, about 70:1 to about 10:1, or about 16:1 to about 4:1.

[0026] In some embodiments of the methods provided herein, the non-viral delivery vehicle comprises a physical delivery system and the srRNA is formulated as a “naked” srRNA.

[0027] In some embodiments of the methods of the disclosure, the selected srRNA delivery system is formulated as an immunogenic formulation. In some embodiments, the selected srRNA

delivery system is formulated as a non-immunogenic formulation.

[0028] In some embodiments, the selected srRNA delivery system is formulated as a biotherapeutic. In some embodiments, the selected srRNA delivery system is formulated as a vaccine. In some embodiments, the vaccine is a therapeutic vaccine. In some embodiments, the vaccine is a prophylactic vaccine.

[0029] In another aspect, the disclosure provides compositions including a srRNA delivery system obtained by any one of the methods of the disclosure.

[0030] In a further aspect, the present disclosure provides pharmaceutical compositions including a srRNA delivery system obtained by any one of the methods of the disclosure and a pharmaceutically acceptable excipient.

[0031] In yet another aspect of the disclosure, provided herein are methods of preventing or treating a health condition in a subject, wherein the methods include prophylactically or therapeutically administering to the subject a composition of the disclosure. In some embodiments, the composition elicits a pro-inflammatory response or an anti-inflammatory response in the subject. In some embodiments, the composition induces production of one or more pro-inflammatory molecules in the subject. In some embodiments, the composition does not elicit an inflammatory response and/or an anti-inflammatory response in the subject.

[0032] In some embodiments, the composition elicits an immune response in the subject. In some embodiments, the composition does not elicit an immune response in the subject.

[0033] In yet another aspect, the disclosure provides methods for inducing a pharmacodynamic effect in a subject, wherein the method includes prophylactically or therapeutically administering to the subject a composition of the disclosure. In some embodiments, the composition elicits an immune response in the subject. In some embodiments, the composition does not elicit an immune or a pro-inflammatory and/or an anti-inflammatory response in the subject.

[0034] The foregoing summary is illustrative only and is not intended to be in any way limiting. In addition to the illustrative embodiments and features described herein, further aspects, embodiments, objects and features of the disclosure will become fully apparent from the drawings and the detailed description and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] FIG. 1 is a graphical representation of an example of a workflow for screening/identifying/selecting a srRNA delivery system that includes formulating an input srRNA composition with a non-viral delivery vehicle to generate an srRNA delivery system having a formulated srRNA composition, determining the potency of the formulated srRNA and selecting the srRNA delivery system as being suitable for a biomedical application if the formulated srRNA composition retains at least about 25% potency relative to the potency of the unformulated input srRNA composition.

[0036] FIG. 2 is a graphical representation of an example of a workflow for screening/identifying/selecting a srRNA delivery system that includes formulating an input srRNA composition with a non-viral delivery vehicle to generate an srRNA delivery system having a formulated srRNA composition, determining the potency of the formulated srRNA and determining a percentage change in the amount of full-length srRNA molecules in the formulated srRNA relative to the amount of full-length srRNA in the input srRNA composition and selecting the srRNA delivery system as being suitable for a biomedical application if the formulated srRNA composition retains at least about 25% potency relative to the potency of the unformulated input srRNA composition. The workflow includes confirming suitability of the srRNA delivery system if the percentage change in formulated srRNA composition has a percentage decrease of less than about 40% relative to the amount of full-length srRNA molecules in the input srRNA composition

[0037] FIG. 3 is a graphical illustration of a non-limiting example of a screening workflow in accordance with some embodiments of the methods disclosed herein, which includes (i) determining the percentage of double-stranded RNA (dsRNA) relative to single-stranded RNA (ssRNA) in the input srRNA composition (Assay 1), (ii) determining the potency of the srRNA molecule formulated in the srRNA delivery system relative to the potency of the srRNA molecule in the input srRNA composition (Assay 2), and (iii) determining a change in the amount of full-length srRNA molecules between the formulated srRNA relative to full-length srRNA in the input RNA composition (Assay 3).

[0038] FIG. 4 is a graphical representation of a sigmoidal curve of dose-dependent frequency

of dsRNA positive cells after transfection with srRNA as would be measured by an *in vitro* potency assay in accordance with an embodiment of the methods of the present disclosure.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0039] The present disclosure relates generally to methods for screening or selecting or identifying self-replicating RNA (srRNA) delivery systems suitable for biomedical applications, such as for the delivery of vaccines or biotherapeutics. These methods allow the efficient identification of suitable delivery systems during the formulation process and address the challenges of formulating large and unstable srRNA molecules, into effective and potent therapeutic or prophylactic compositions. In particular, the methods for selecting or identifying srRNA delivery systems for biomedical applications provided herein include determining a combination of quality attributes (or criteria) of the srRNA input compositions before and after formulations with delivery vehicles to generate suitable delivery systems.

[0040] The disclosure identifies three main quality attributes (QA) and the use of various combinations thereof for identifying a (potent and effective) srRNA delivery system as being suitable for a biomedical application. These QA include (1) the amount (e.g. percentage) of double stranded RNA (dsRNA) relative to single stranded RNA (ssRNA) in an input srRNA composition after the *in vitro* transcription (IVT) of the srRNA, and/or (2) the potency (e.g. the replication efficiency of the srRNA vector) of the formulated srRNA composition relative to the unformulated input srRNA composition, and/or (3) the integrity of the srRNA after formulation as measured by determining the change in the amount (e.g. percentage) of full-length srRNA molecules in the formulated srRNA composition relative to the unformulated input srRNA composition. Focusing on these three QA can allow the speedy and reliable screening of multitudes of vaccine or drug candidates.

[0041] The first quality attribute is the amount of dsRNA relative to ssRNA that is present after the *in vitro* transcription of the srRNA which can be an indicator of the quality and immunogenicity of the input srRNA composition. Enzymatic *in vitro* transcription (IVT) is typically used to generate srRNAs and can be used to generate the srRNAs of the disclosure, from a linearized DNA template. The synthesis of *in vitro* transcribed RNAs, predominantly using phage RNA polymerases (RNAPs), are robust and well-established for the large-scale

production of synthetic RNA. However, the introduction of such synthetic, *in vitro* transcribed mRNAs into cells or animal models can result in immune responses against the synthetic molecules, which can activate, for example, cytosolic sensors such as RIG-I and MDA5 that may activate the innate immune system in response to viral dsRNA. Such outcomes may be undesirable. Recent studies have identified two main types of by-products in the IVT reaction that result in the formation of dsRNA molecules. The first type of dsRNA molecules can be formed by the 3'-extension of the run-off products annealing to complementary sequences in the body of the run-off transcript either in *cis* (by folding back on the same RNA molecule) or *trans* (annealing to a second RNA molecule) to form extended duplexes. The second type of dsRNA molecules can be formed by the hybridization of an antisense RNA molecule to the run-off transcript. The anti-sense RNA molecules have been reported to be formed in a promoter- and run-off transcript-independent manner. Therefore, quantifying the percentage of dsRNA in input srRNA composition, pursuant to the present disclosure, a quality attribute that can be considered during the screening of srRNA delivery systems as suitable for biomedical applications whether to be formulated as vaccines or for biotherapeutics.

[0042] The second quality attribute is the potency (e.g. the replication efficiency of the srRNA vector) of the formulated srRNA composition relative to the unformulated input srRNA composition which can be measured by an *in vitro* potency assay. An example of *in vitro* potency assay is a transfection assay in which the ability of the srRNA to self-replicate is evaluated. The assay measures the replication efficiency by capturing intermediate dsRNA and comparable protein expression in individual cells by an antigen-specific monoclonal antibody as discussed in more details below. Evaluating the ability of the srRNA to amplify/replicate in such an *in vitro* potency assay, can verify that proper full-length sequence, RNA capping, and strand integrity are present in the srRNA delivery system.

[0043] The third quality attribute includes the measuring the integrity of the srRNA after formulation such as by determining the percentage change in the amount of full-length srRNA molecules in the formulated srRNA composition as compared to the unformulated input srRNA composition.

[0044] Accordingly, the methods of the disclosure for selecting/identifying srRNA delivery

systems may include one or more of a) determining the percentage of double stranded RNA (dsRNA) relative to single stranded RNA (ssRNA) after the *in vitro* transcription (IVT) of the srRNA, b) determining the potency of the formulated srRNA composition relative to the unformulated input srRNA composition, and c) determining a percentage change in the amount of full-length srRNA in the formulated srRNA composition relative to the amount of full-length srRNA in the unformulated srRNA input composition. In some aspects, if determining the percentage of dsRNA in step a) results in an unfavorable amount of dsRNA (as described in more details below), the synthesis of a new srRNA may be repeated or the srRNA is not formulated. If, however, the amount of dsRNA is within a suitable range (also detailed below), the input srRNA composition is formulated with a non-viral delivery system. After formulation with a non-viral delivery system and downstream processing that is necessary, the potency of the formulated srRNA composition and the percentage change in the amount of full-length srRNA molecules in the srRNA composition relative to the amount of full-length srRNA in the unformulated srRNA input composition are determined. The potency and the percentage change in the amount of full-length srRNA have been found by the inventors to be sufficient indicators and good predictors of the *in vivo* activity of the formulated srRNA composition.

[0045] An example of a workflow for the methods of the disclosure, as illustrated in FIG. 2 and detailed below can include: (a) obtaining an input srRNA composition including a srRNA generated by, for example, an *in vitro* transcription (IVT) reaction and; (b) determining the percentage of double-stranded RNA (dsRNA) relative to single-stranded (ssRNA) in the input srRNA composition. If the dsRNA percentage is below about 2.5%, then (c) proceeding to carry out the remaining steps, to formulate the srRNA with a non-viral delivery vehicle to generate a srRNA delivery system; determining the replication efficiency of the srRNA after formulation relative to the srRNA before formulation (i.e., in the input srRNA composition), and determining the percentage change in full-length RNA after formulation. In some embodiments, the workflow can include selecting the srRNA delivery system as being suitable for a biomedical application if the formulated srRNA composition in (b) retains at least about 25% potency relative to the potency of the unformulated srRNA composition.

[0046] The section headings used herein are for organizational purposes only and are not to

be construed as limiting the subject matter described.

I. DEFINITIONS

[0047] Unless otherwise defined, all terms of art, notations and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this disclosure pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. Many of the techniques and procedures described or referenced herein are well understood and commonly employed using conventional methodology by those skilled in the art.

[0048] The singular form “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a cell” includes one or more cells, including mixtures thereof. “A and/or B” is used herein to include all of the following alternatives: “A”, “B”, “A or B”, and “A and B”.

[0049] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the disclosure. The upper and lower limits of these smaller ranges can independently be included in the smaller ranges, and are also encompassed within the disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the disclosure. Certain ranges are presented herein with numerical values being preceded by the term “about” which, as used herein, has its ordinary meaning of approximately. The term “about” is used to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes. In determining whether a number is near to or approximately a specifically recited number, the near or approximating unrecited number can be a number which, in the context in which it is presented, provides the substantial equivalent of the specifically recited number. If the degree of approximation is not otherwise clear from the context, “about” means either within plus or minus

10% of the provided value, or rounded to the nearest significant figure, in all cases inclusive of the provided value.

[0050] The term “naked” as used herein references nucleic acids that are substantially free of other macromolecules, such as lipids, polymers and proteins. A “naked” nucleic acid, such as a self-replicating RNA, is not formulated with other macromolecules to improve cellular uptake. Accordingly, a naked nucleic acid is not encapsulated in, absorbed on, or bound to a liposome, a microparticle, a nanoparticle, a cationic emulsion, and the like.

[0051] The term “recombinant” when used with reference to a cell, a nucleic acid, a protein, or a vector, indicates that the cell, nucleic acid, protein or vector has been altered or produced through human intervention such as, for example, has been modified by or is the result of laboratory methods. Thus, for example, recombinant proteins and nucleic acids include proteins and nucleic acids produced by laboratory methods. Recombinant proteins can include amino acid residues not found within the native (non-recombinant or wild-type) form of the protein or can include amino acid residues that have been modified, *e.g.*, labeled. The term can include any modifications to the peptide, protein, or nucleic acid sequence. Such modifications may include the following: any chemical modifications of the peptide, protein or nucleic acid sequence, including of one or more amino acids, deoxyribonucleotides, or ribonucleotides; addition, deletion, and/or substitution of one or more of amino acids in the peptide or protein; creation of a fusion protein, *e.g.*, a fusion protein comprising an antibody fragment; and addition, deletion, and/or substitution of one or more of nucleic acids in the nucleic acid sequence. The term “recombinant” when used in reference to a cell is not intended to include naturally-occurring cells but encompass cells that have been engineered/modified to include or express a polypeptide or nucleic acid that would not be present in the cell if it was not engineered/modified.

[0052] As used herein, the term “srRNA” refers to RNA which contains all of the genetic information required for directing its own amplification or self-replication within a permissive cell. To direct its own replication, the RNA molecule 1) encodes polymerase, replicase, or other proteins which may interact with viral or host cell-derived proteins, nucleic acids or ribonucleoproteins to catalyze the RNA amplification process; and 2) contain *cis*-acting RNA

sequences required for replication and transcription of the subgenomic replicon-encoded RNA. These sequences may be bound during the process of replication to its self-encoded proteins, or non-self-encoded cell-derived proteins, nucleic acids or ribonucleoproteins, or complexes between any of these components. For the purpose of the present disclosure, an alphavirus **srRNA molecule generally contains the following ordered elements: 5' viral or defective-interfering RNA sequence(s) required in *cis* for replication, sequences coding for biologically active alphavirus non-structural proteins (*e.g.*, nsP1, nsP2, nsP3, and nsP4), promoter for the subgenomic RNA (sgRNA), 3' viral sequences required in *cis* for replication, and a polyadenylate tract (poly(A)).** Further, the term srRNA generally refers to a molecule of positive polarity, or “message” sense, and the srRNA may be of length different from that of any known, naturally-occurring alphavirus. In some embodiments of the present disclosure, the srRNA does not contain the sequences of at least one of structural viral protein; and/or sequences encoding structural genes can be substituted with heterologous sequences. In those instances, where the srRNA is to be packaged into a recombinant alphavirus particle, it can contain one or more sequences, so-called packaging signals, which serve to initiate interactions with alphavirus structural proteins that lead to particle formation.

[0053] It is understood that aspects and embodiments of the disclosure described herein include “comprising”, “consisting”, and “consisting essentially of” aspects and embodiments. As used herein, “comprising” is synonymous with “including”, “containing”, or “characterized by”, and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. As used herein, “consisting of” excludes any elements, steps, or ingredients not specified in the claimed composition or method. As used herein, “consisting essentially of” does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claimed composition or method. Any recitation herein of the term “comprising”, particularly in a description of components of a composition or in a description of steps of a method, is understood to encompass those compositions and methods consisting essentially of and consisting of the recited components or steps.

[0054] The term “effective amount”, “therapeutically effective amount”, or “pharmaceutically effective amount” of a composition of the disclosure, *e.g.*, nucleic acid

constructs, recombinant cells, recombinant polypeptides, and/or pharmaceutical compositions, generally refers to an amount sufficient for the composition to accomplish a stated purpose relative to the absence of the composition (*e.g.*, achieve the effect for which it is administered, stimulate an immune response, prevent or treat a disease, or reduce one or more symptoms of a disease, disorder, infection, or health condition). An example of an “effective amount” is an amount sufficient to contribute to the treatment, prevention, or reduction of a symptom or symptoms of a disease, which could also be referred to as a “therapeutically effective amount.” A “reduction” of a symptom means decreasing of the severity or frequency of the symptom(s), or elimination of the symptom(s). The exact amount of a composition including a “therapeutically effective amount” will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (see, *e.g.*, Lieberman, *Pharmaceutical Dosage Forms* (vols. 1-3, 1992); Lloyd, *The Art, Science and Technology of Pharmaceutical Compounding* (1999); Pickar, *Dosage Calculations* (1999); and Remington: *The Science and Practice of Pharmacy*, 20th Edition, 2003, Gennaro, Ed., Lippincott, Williams & Wilkins).

[0055] The term “pharmaceutically acceptable excipient” as used herein refers to any suitable substance that provides a pharmaceutically acceptable carrier, additive, or diluent for administration of a compound(s) of interest to a subject. As such, “pharmaceutically acceptable excipient” can encompass substances referred to as pharmaceutically acceptable diluents, pharmaceutically acceptable additives, and pharmaceutically acceptable carriers. As used herein, the term “pharmaceutically acceptable carrier” includes, but is not limited to, saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds (*e.g.*, antibiotics and additional therapeutic agents) can also be incorporated into the compositions.

[0056] It is appreciated that certain features of the disclosure, which are, for clarity, described in the context of separate embodiments, can also be provided in combination in a single embodiment. Conversely, various features of the disclosure, which are, for brevity, described in the context of a single embodiment, can also be provided separately or in any suitable sub-combination. All combinations of the embodiments pertaining to the disclosure are

specifically embraced by the present disclosure and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all subcombinations of the various embodiments and elements thereof are also specifically embraced by the present disclosure and are disclosed herein just as if each and every such subcombination was individually and explicitly disclosed herein.

[0057] The terms “cell”, “cell culture”, and “cell line” refer not only to the particular subject cell, cell culture, or cell line but also to the progeny or potential progeny of such a cell, cell culture, or cell line, without regard to the number of transfers or passages in culture. It should be understood that not all progeny are exactly identical to the parental cell. This is because certain modifications can occur in succeeding generations due to either mutation (*e.g.*, deliberate or inadvertent mutations) or environmental influences (*e.g.*, methylation or other epigenetic modifications), such that progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein, so long as the progeny retain the same functionality as that of the original cell, cell culture, or cell line.

[0058] Although various features of the disclosure can be described in the context of a single embodiment, the features can also be provided separately or in any suitable combination. Conversely, although the present disclosure can be described herein in the context of separate embodiments for clarity, the present disclosure can also be implemented in a single embodiment.

II. METHODS OF THE DISCLOSURE

A. Methods of screening or identifying a srRNA delivery system

[0059] As discussed *supra*, some embodiments of the disclosure provide methods of screening or selecting or identifying a srRNA delivery system for a biomedical application.

[0060] In one aspect, provided herein are methods for selecting or identifying a self-replicating RNA (srRNA) delivery system for a biomedical application, the methods include: a) formulating an input srRNA composition with a non-viral delivery vehicle to generate a srRNA delivery system having a formulated srRNA composition; b) determining potency of the formulated srRNA composition relative to the unformulated input srRNA composition; c) determining percentage change in the amount of full-length srRNA molecules in the formulated

srRNA composition relative to the amount of full-length srRNA molecules in the input srRNA composition; and d) selecting the srRNA delivery system as being suitable for a biomedical application if the formulated srRNA composition in (b) retains at least about 25% potency relative to the potency of the unformulated srRNA composition.

[0061] In another aspect, the disclosure provides methods for identifying a self-replicating RNA (srRNA) delivery system for a biomedical application, the methods include: (a) determining percentage of double-stranded RNA (dsRNA) molecules in an input srRNA composition relative to single-stranded RNA (ssRNA) molecules in the input srRNA composition; (b) formulating an input srRNA composition with a non-viral delivery vehicle to generate a srRNA delivery system comprising a formulated srRNA composition, (c) determining potency of the formulated srRNA composition relative to unformulated input srRNA composition, (d) determining percentage change in amount of full-length srRNA molecules in the formulated srRNA composition relative to amount of full-length srRNA molecules in the input srRNA composition; and (e) selecting the srRNA delivery system as being suitable for a biomedical application if (i) the percentage of dsRNA relative to ssRNA in (a) is less than about 2.5%, (ii) the formulated srRNA composition in (c) retains at least about 25% potency relative to the potency of the unformulated input srRNA composition, and (iii) the percentage change in (d) comprises a percentage decrease of less than about 40% relative to the amount of full-length srRNA molecules in the unformulated input srRNA composition.

[0062] Non-limiting exemplary embodiments of the disclosed methods can include one or more of the following features.

1. Input srRNA Composition

[0063] The input srRNA compositions of the disclosure is a starting material or reference material or a reference composition that includes an unformulated srRNA molecule(s) of interest. In some embodiments, the input srRNA composition that is formulated is the same as the input srRNA composition that was yielded by the *in vitro* transcription assay used to make the srRNA molecules.

[0064] In some embodiments, the srRNA molecules are intended to be used as vaccines. In some embodiments, the srRNA molecules are intended as biotherapeutics to deliver a selected

payload.

[0065] In some embodiments, the srRNA can be transcribed using an *in vitro* transcription assay as discussed below in more details. In some embodiments, the srRNA can be constructed from one or more positive-sense, single-stranded RNA (+ssRNA) viral genomes. In some embodiments, the srRNA is derived from an alphavirus. In some embodiments, the srRNA is engineered or assembled from multiple constructs or multiple genomes. In some embodiments, the srRNA is synthesized. The skilled artisan would understand that any technique to assemble nucleic acid fragments can be used. For example, by ligation or PCR-based procedures such as by Gibson-assembly techniques, or by fusion PCR, *etc.*

[0066] The input srRNA can be in any suitable buffer. Non-limiting examples include citrate buffers or other aqueous buffers. In some embodiments, the input srRNA is in a 1mM citrate buffer. In some embodiments, the input srRNA is in a 1mM citrate buffer at pH 6.5.

2. Transcription or Synthesis of srRNA

[0067] The methods of the disclosure provides srRNAs. The srRNAs can be transcribed using assays known to the skilled artisans. An example of such an assay is an enzymatic *in vitro* transcription assay (IVT) from a linearized DNA template using, for example, phage RNA polymerases (RNAPs). Such assays usually yield by-products in the IVT reaction that result in the formation of undesirable dsRNA molecules. The first type of dsRNA molecules can be formed by the 3'-extension of the run-off products annealing to complementary sequences in the body of the run-off transcript either in *cis* (by folding back on the same RNA molecule) or *trans* (annealing to a second RNA molecule) to form extended duplexes. The second type of dsRNA molecules can be formed by the hybridization of an antisense RNA molecule to the run-off transcript. Quantifying the percentage of dsRNA in input srRNA composition, as discussed in more details below, is advantageous during the identification or selection of srRNA delivery systems suitable for biomedical applications. In some embodiments, if the amount of dsRNA are greater than about 2.5% of the total RNA (dsRNA and ssRNA) in the input srRNA composition, then repeating the IVT of the srRNA would be advisable.

3. Non-viral Delivery Vehicles

[0068] One aspect of the present disclosure relates to methods for selecting/identifying

srRNA delivery systems for a biomedical application, the methods include formulating an input srRNA composition with a non-viral delivery vehicle to generate a srRNA delivery system having a formulated srRNA input composition.

[0069] Non-limiting examples of non-viral delivery vehicles that can be used in the methods of the disclosure are described, for example, in the following literature: Yan Y. *et al.* (2022). *Non-viral vectors for RNA delivery*. *J Control Release*. 2022 Feb; 342:241-279; Mirón-Barroso S, *et al.* (2021). *Nanotechnology-Based Strategies to Overcome Current Barriers in Gene Delivery*. *Int J Mol Sci*. 2021 Aug 9;22(16):8537; Chaudhary N, Weissman D, Whitehead KA. *mRNA vaccines for infectious diseases: principles, delivery and clinical translation*. *Nat Rev Drug Discov*. 2021 Nov; 20(11):817-838. Erratum in: *Nat Rev Drug Discov*; Shuai Q. *et al.* (2021). *mRNA delivery via non-viral carriers for biomedical applications*. *Int J Pharm*. 2021 Sep 25;607:121020; Ibba ML. *et al.* (2021). *Advances in mRNA non-viral delivery approaches*. *Adv Drug Deliv Rev*. 2021 Oct;177:113930; Tarach P *et al.* (2021) *Recent Advances in Preclinical Research Using PAMAM Dendrimers for Cancer Gene Therapy*. *Int J Mol Sci*. 2021 Mar 13;22(6):2912; and Ulkoski D. *et al.* (2019). Recent advances in polymeric materials for the delivery of RNA therapeutics. *Expert Opin Drug Deliv*. 2019 Nov; 16(11):1149-1167, all of which are herein incorporated by reference in their entirety.

[0070] Delivery vehicles suitable for the methods and compositions of the disclosure can be any non-viral delivery system that is able to protect the srRNA payload from degradation and be able to effectively diffuse across cellular membranes of a host cell such as, for example, by endocytosis. Exemplary delivery vehicles suitable for the methods and compositions of the disclosure include, but are not limited to, physiologic buffers, liposomes, lipid-based nanoparticles (LNPs), polymer nanoparticles, microspheres, immune stimulating complex (ISCOM), and conjugates of bioactive ligands which can facilitate delivery and/or enhance the immune response. These compounds are readily available to one skilled in the art; for example, see *Liposomes: A Practical Approach*, RCP New Ed, IRL press (1990). Adjuvants other than liposomes and the like are also used and are known in the art. Adjuvants may protect the antigen (*e.g.*, srRNA molecule) from rapid dispersal by sequestering it in a local deposit, or they may contain substances that stimulate the host to secrete factors that are chemotactic for macrophages

and other components of the immune system. An appropriate selection can be made by those skilled in the art, for example, from those described below.

[0071] In some embodiments, a delivery vehicle can include one or more of the following: physiologic buffer, a liposome, a lipid-based nanoparticle (LNP), a polymer nanoparticle, a microsphere, an immune stimulating complex (ISCOM), a conjugate of bioactive ligand, or a combination of any thereof.

[0072] Exemplary types of lipids suitable for the delivery vehicles described herein include cationic lipids, ionizable cationic lipids, anionic lipids, neutral lipids, and combinations thereof. Neutral lipids, such as the fusogenic phospholipid DOPE or the membrane component cholesterol, can be included in LNPs as ‘helper lipids’ to enhance transfection activity and nanoparticle stability. Limitations of cationic lipids include low efficacy owing to poor stability and rapid clearance, as well as the generation of inflammatory or anti-inflammatory responses. LNPs can also have hydrophobic lipids, hydrophilic lipids, or both hydrophobic and hydrophilic lipids.

[0073] In some embodiments, the LNP of the disclosure can include one or more ionizable lipids. Exemplary ionizable lipids suitable for the compositions and methods of the disclosure includes those described in PCT publications WO2020252589A1 and WO2021000041A1, and Love K.T. *et al.*, *Proc Natl Acad Sci USA*, Feb. 2, 2010 107 (5) 1864-1869, which are incorporated by reference herein in their entirety.

[0074] In some embodiments, the LNP of the disclosure includes one or more lipid compounds described in Love K.T. *et al.*, 2010 *supra*, such as C16-96, C14-110, and C12-200. In some embodiments, the LNP includes an ionizable cationic lipid selected from the group consisting of ALC-0315, C12-200, LN16, MC3, MD1, SM-102, and a combination of any thereof. In some embodiments, the LNP of the disclosure includes C12-200.

[0075] In some embodiments, the LNP of the disclosure includes one or more cationic lipids. Suitable cationic lipids include, but are not limited to, 98N12-5, C14-PEG2000, DLin-KC2-DMA (KC2), DLin-MC3-DMA (MC3), XTC, MD1, 7C1, LN16, and MD1 among others. In another example, a type of LNP includes a GalNAc moiety is attached to the outside of the LNP and acts as a ligand for uptake in to the liver via the asialyloglycoprotein receptor. Any of

these cationic lipids can be used to formulate LNP for delivery of the srRNAs of the disclosure to the liver.

[0076] In some embodiments, the LNP of the disclosure includes one or more neutral lipids. As described above, neutral lipids, also known as “structural lipids” or “helper lipids” can also be incorporated into lipid formulations and lipid particles in some embodiments. The lipid formulations and lipid particles can include one or more structural lipids at about 10 to 40 Mol% of the composition. Suitable structural lipids support the formation of particles during manufacture. Structural lipids refer to any one of a number of lipid species that exist in either in an anionic, uncharged or neutral zwitterionic form at physiological pH. Representative structural lipids include diacylphosphatidylcholines, diacylphosphatidylethanolamines, diacylphosphatidylglycerols, ceramides, sphingomyelins, dihydrosphingomyelins, cephalins, and cerebrosides.

[0077] Exemplary structural lipids include zwitterionic lipids, for example, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylethanolamine (POPE) and dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1- carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1-trans PE, 1 -stearoyl-2-oleoyl- phosphatidylethanol amine (SOPE), and 1 ,2-diacyldiacyl-sn-glycero-3- phosphoethanolamine (trans DOPE).

[0078] In another embodiment, the structural lipid can be any lipid that is negatively charged at physiological pH. These lipids include phosphatidylglycerols such as dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), palmitoyloleoylphosphatidylglycerol (POPG), cardiolipin, phosphatidylinositol, diacylphosphatidylserine, diacylphosphatidic acid, and other anionic modifying groups joined to neutral lipids. Other suitable structural lipids include glycolipids (e.g., monosialoganglioside GM1).

[0079] Non-limiting neutral lipids suitable for the compositions and methods of the

disclosure include DPSC, DPPC, POPC, DOPE, and SM. In some embodiments, the LNP of the disclosure includes one or more ionizable lipid compounds described in PCT publications WO2020252589A1 and WO2021000041A1, which are incorporated by reference herein in their entirety.

[0080] In some embodiments, the LNP of the disclosure includes C12-200, C14-PEG2000, DOPE, DMG-PEG2000, DSPC, DOTMA, DOSPA, DOTAP, DMRIE, DC-cholesterol, DOTAP-cholesterol, GAP-DMORIE-DPyPE, or GL67A-DOPE-DMPE-polyethylene glycol (PEG). In some embodiments, the lipids can be combined in any number of molar ratios to produce a LNP. In addition, the polynucleotide(s) can be combined with lipid(s) in a wide range of molar ratios to produce a LNP.

[0081] In some embodiments where the delivery vehicles described herein include an LNP, the mass ratio of lipid to nucleic acid in the LNP delivery vehicles is about 100:1 to about 3:1, about 70:1 to 10:1, or 16:1 to 4:1. In some embodiments, the mass ratio of lipid to nucleic acid in the LNP delivery system is about 16:1 to 4:1. In some embodiments, the mass ratio of lipid to nucleic acid in the LNP delivery system is about 20:1. In some embodiments, the mass ratio of lipid to nucleic acid in the LNP delivery system is about 8:1. In some embodiments, the lipid-based nanoparticles (LNPs) have an average diameter of less than about 1000 nm, about 500 nm, about 250 nm, about 200 nm, about 150 nm, about 100 nm, about 75 nm, about 50 nm, or about 25 nm. In some embodiments, the LNPs have an average diameter ranging from about 70 nm to 100 nm. In some embodiments, the LNPs have an average diameter ranging from about 88 nm to about 92 nm, from 82 nm to about 86 nm, or from about 80 nm to about 95 nm.

[0082] In some embodiments, a LNP refers to any particle having a diameter of less than 1000 nm, 500 nm, 250 nm, 200 nm, 150 nm, 100 nm, 75 nm, 50 nm, or 25 nm. Alternatively, a nanoparticle can range in size from 1-1000 nm, 1-500 nm, 1-250 nm, 25-200 nm, 25-100 nm, 35-75 nm, or 25-60 nm.

[0083] Stabilizing agents can be included in lipid formulations embodiments to ensure integrity of the mixtures. Stabilizing agents are a class of molecules which disrupt or help form the hydrophobic-hydrophilic interactions among molecules. Suitable Stabilizing agents include, but are not limited to, polysorbate 80 (also known as Tween 80, IUPAC name 2-[2-[3,4-bis(2-

hydroxyethoxy)oxolan-2-yl]-2- (2-hydroxyethoxy)ethoxy]ethyl octadec-9-enoate), Myrj52 (Polyoxyethylene (40) stearate), and Brij™ S10 (Polyoxyethylene (10) stearyl ether).

Polyethylene glycol conjugated lipids may also be used. The stabilizing agents may be used alone or in combinations with each other.

[0084] In some embodiments, the stabilizing agents comprises about .1 to 3 Mol% of the overall lipid mixture. In some embodiments, the stabilizing agents comprise about 0.5 to 2.5 Mol% of the overall lipid mixture. In some embodiments, the stabilizing agent is present at greater than 2.5Mol%. In some embodiments the stabilizing agent is present at 5 Mol%. In some embodiments the stabilizing agent is present at 10 Mol%. In some embodiments, the stabilizing agent is about 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, and so forth. In other embodiments, the stabilizing agent is 2.6-10 Mol% of the lipid mixture. In other embodiments, the stabilizing agents is present at greater than 10 Mol% of the lipid mixture.

[0085] Steroids can also be included in the lipid compositions for certain applications, and lipid particles made therefrom include sterols, such as cholesterol and phytosterol.

Polymer Delivery Vehicles

[0086] In an aspect of the disclosure, the delivery vehicle can be a polymer (polymer vector). Polymers or polymer vectors can include homopolymer and copolymer vectors. Non-limiting examples of polymer vectors include polyethyleneimine (PEI; linear PEI, branched PEI, pluronic coupled PEI, crosslinked BPEI, and PEI-lipid hybrid), polyethyleneimine hybrids, **poly(acrylates), polyesters, poly(β -amino esters), poly(amido amine)s, poly(aspartamides), polypeptides (poly(aminoacids)s (PAAs)), polyL-Lysine (PLL)), polyriboinosinic-acid stabilized with PLL (poly-ICLC), chitosan-based systems, Poly(glycoamidoamines) (PGAAs), glycopolymers, poly(d-glucaramidoamines), methacrylamide-based glycopolymers, tartarate- and glucarate-based poly(glycoamidoamine), stimuli-responsive polymers, bio-reducible polymers including bio-reducible derivatives of poly(amidoamine)s, bio-reducible derivatives of PEI, poly(2-dimethylaminoethyl methacrylate), and poly(L-lysine), pH-responsive polymers including polyesters and polyacrylates, charge-altering releasable transporters, pH-responsive gene vectors containing a combination of dimethyl aminoethyl methacrylate (DMAEMA),**

propylacrylic acid (PPAA), and butylmethacrylate (BMA), charge-altering releasable transporters (CARTs), ser-CARTs, ATP-responsive polymers, polyampholytes, and protein-based polymers.

[0087] In some embodiments, the input srRNA composition is formulated with Poly(amidoamine) (PAMAM) dendrimers (Tarach and Janaszewka, 2021). PAMAM dendrimers are repetitively branched, three-dimensional molecules, made of amide and amine subunits, possessing unique physiochemical properties.

[0088] In some embodiments, the selected srRNA delivery system is formulated as a biotherapeutic. Non-limiting examples of biotherapeutic include cytokines, chemokines, and other soluble immunomodulators, enzymes, peptide and protein agonists, peptide and protein antagonists, hormones, receptors, antibodies and antibody-derivatives, growth factors, transcription factors, and gene silencing/editing molecules.

[0089] In some embodiments, the selected srRNA delivery system is formulated as an immunogenic formulation. In some embodiments, the selected srRNA delivery system is formulated as a non-immunogenic formulation.

[0090] In some embodiments, the selected srRNA delivery system is formulated as a vaccine. In some embodiments, the vaccine is a therapeutic vaccine. In some embodiments, the vaccine is a prophylactic vaccine.

[0091] In some aspects, after formulating (mixing and complexation, **FIG. 3**) the srRNA input compositions of the disclosure with a non-viral delivery vehicle to generate a srRNA delivery system, the formulated srRNA composition may undergo downstream processing. Non-limiting examples of downstream processes include tangential flow filtration, dialysis, filtration, purification by size exclusion chromatography and affinity chromatography.

4. Determining potency

[0092] During the formulation process of an input srRNA composition, there could be loss of potency of the srRNA. Accordingly, the present disclosure, also provides methods of selecting/identifying a srRNA delivery system for a biomedical application, the method includes determining potency of the formulated srRNA composition relative to unformulated input srRNA composition.

[0093] Various methods and/or assays known to skilled artisans can be used to measure the potency of srRNA. In some embodiments, determining potency of the formulated srRNA composition includes immunoblotting analysis, fluorescence flow cytometry analysis, enzyme-linked immunoassay analysis, immunogenicity analysis, bioactivity analysis, and/or efficacy in a disease model.

[0094] In some embodiments of the methods of the disclosure, the potency of a formulated srRNA composition is determined using an *in vitro* potency assay that measures replication efficiency by capturing intermediate double-stranded RNA that are produced during srRNA replication. In some embodiments, measuring the replication efficiency includes assaying protein expression in individual cells. Assaying protein expression may involve using an antibody that detects the replication intermediates of the srRNA. In some embodiments, the antibody is a monoclonal antibody. In some embodiments, the monoclonal antibody is J2. J2 is a commercially available anti-dsRNA IgG2a mouse monoclonal antibody from English & Scientific Consulting (Scicons).

[0095] In some embodiments, the *in vitro* potency assay can be as follows. Test srRNA can be diluted and directly electroporated into cells. In some embodiments, if the test srRNA (input srRNA composition) is encapsulated or adsorbed to a non-viral delivery system, a detergent (or other extraction method) can be used to extract the srRNA from the delivery system before it is electroporated into cells. After sufficient incubation, the cells can be fixed and immunoassayed with a fluorophore-conjugated antibody (J2) that specifically detects the dsRNA replication intermediate of the vector. Signal-positive cells indicate the presence of an intact and functional srRNA, which can be quantified by fluorescence flow cytometry. The assay readout is the frequency of positive cells per ng of RNA transfected. There is a dose-responsive frequency of dsRNA+ transfected cells which results in the ability to generate a sigmoidal curve (as shown in **FIG. 3**) that is similar to the standard curve generated by the widely used enzyme-linked immunosorbent assay (ELISA) for quantification of other biological molecules as shown below. A srRNA reference standard can be used to help mitigate cell-based assay variability and enable comparison of potency across assays. A srRNA standard can be an aliquot of a large prep that is stored at -80°C and the potency of each test RNA is defined relative to the standard.

[0096] In some embodiments, a similar strategy for quantification of virus replicon particle (VRPs) titers can be employed by infection of cells with serial dilutions of the particles followed by immunoassay with the J2 antibody. The potency of the srRNA delivery systems of the disclosure can be measured by an *in vivo* assay. As would be understood by a skilled artisan, an *in vivo* assay that can be used for determining the potency of a srRNA delivery system, can be any assay that evaluates protein expression or immune responses in a cell following administration of the srRNA delivery system (formulated synthetic srRNA constructs with or without a control including unformulated vectors), as discussed in Example 4 below. In some embodiments, the potency of the srRNA delivery systems of the disclosure is evaluated by measuring expression of a reporter gene at the site of injection in a subject. In some embodiments, the reporter gene is luciferase. In some embodiments, the subject is mouse. In some embodiments, *in vivo* imaging of luciferase activity is done using an IVIS instrument at various time points. In some embodiments, for secreted proteins, such as agonists, or antagonists, or monoclonal antibodies, systemic protein levels can be assayed by sequential bleeds, followed by preparation of serum and ELISA analysis for the expressed protein.

[0097] In some embodiments, the potency of srRNA delivery systems of the present disclosure can be evaluated by measuring immune responses (*e.g.*, B cell and T cell) to encoded antigens in a subject. In some embodiments, the subject is mouse.

[0098] In some embodiments, the potency of srRNA delivery systems of the present disclosure can be evaluated by measuring encoded agonist/antagonists or monoclonal antibodies in the systemic circulation of a subject such as a mouse. In some embodiments, the potency of srRNA delivery systems of the present disclosure can be evaluated as detailed in Example 4. In some embodiments, total antigen-specific IgG in response to injections of the srRNA delivery systems are measured. In some embodiments, antigen-specific IgGs are measured using ELISA protocols known in the art.

[0099] In some embodiments of the methods of the disclosure, the srRNA delivery system is considered as being suitable for a biomedical application if the formulated srRNA composition retains at least about 25% potency relative to the potency of the unformulated srRNA composition (starting material or srRNA input composition). In some embodiments, the delivery

system is considered suitable if the composition retains at least about 26%, at least about 27%, at least about 28%, at least about 29%, at least about 30%, at least about 31%, at least about 32%, at least about 33%, at least about 34%, at least about 35%, at least about 36%, at least about 37%, at least about 38%, at least about 39%, at least about 40%, at least about 41%, at least about 42%, at least about 43%, at least about 44%, at least about 45%, at least about 50%, at least about 55% at least about 60%, at least about 65%, at least about 70%, at least about 75% or any values in between, potency relative to the potency of the unformulated input srRNA composition.

[0100] In some embodiments, the delivery system is considered suitable if the srRNA composition retains between about 25% and 100%, between about 30% and 95%, between about 35% and 85%, between about 40% and 80%, between about 45% and 75%, between about 50% and 70%, between about 55% and 65% or any ranges in between, potency relative to the potency of the unformulated input srRNA composition..

[0101] In some embodiments, the srRNA delivery system in (d) is selected as being suitable for the biomedical application if the formulated srRNA composition in (b) retains at least about 30%, about 35%, about 40%, about 45%, about 50%, about 55% or about 60% potency relative to the potency of the unformulated srRNA composition potency relative to the potency of the unformulated input srRNA composition.

5. Determining change in full-length srRNA

[0102] Provided by the disclosure are methods for selecting/identifying a srRNA delivery system for a biomedical application, the methods include determining a percentage change in amount of full-length molecules in the formulated srRNA composition relative to the amount of full-length srRNA molecules in the input srRNA composition (Assay 3 in **FIG. 2**).

[0103] Various methods and/or assays known to skilled artisans can be used for determining the amount of full-length srRNA. Determining the percentage change in amount of full-length srRNA molecules can include assaying the degradation of full-length srRNA molecules during formulation of the srRNA.

[0104] In some embodiments, determining the percentage change in amount of full-length srRNA molecules can be done by capillary electrophoresis, which allows the quantification of

full-length srRNA. Capillary electrophoresis is well known by the skilled artisan as an alternative to conventional slab electrophoresis for the separation of nucleic acid fragments.

[0105] Capillary electrophoresis (CE) separates and identifies labelled nucleic acid fragments by passing them through polymer-filled capillaries. Higher electric fields applied with CE allows faster and high through-put separation. The process allows single-base resolution of very minute sample quantities.

[0106] In some embodiments, the delivery system is selected as being suitable for a biomedical application if the amount of full-length srRNA molecules in the formulated srRNA composition includes a percentage decrease of less than about 40% relative to the amount of full-length srRNA molecules in the input srRNA composition. In some embodiments, the percentage decrease is less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, or less than about 10%, less than about 5% or any values in between, relative to the amount of full-length srRNA molecules in the input srRNA composition.

6. Determining percentage of double-stranded RNA molecules relative to single-stranded RNA molecules

[0107] Provided by the disclosure are methods for selecting/identifying a srRNA delivery system for a biomedical application, the methods include determining the amount (e.g. percentage) of double-stranded RNA (dsRNA) molecules relative to single-stranded RNA (ssRNA) molecules (Assay 1 of **FIG.2**).

[0108] The amount of dsRNA relative to ssRNA can be measured by methods known to a skilled artisan. In some embodiments, determining the amount of dsRNA relative to ssRNA can be by an immunoblot assay (Assay 1 in **FIG. 2**). Immunoblot assays are used to detect the presence of undesired dsRNA molecules in synthesized mRNA preparations. Based on the use of dsRNA-specific monoclonal antibodies, such assays allow the sensitive and selective detection of dsRNA molecules independent of their nucleotide composition and sequence. Detection is highly specific as some commercially-available kits allow the detection of dsRNA in nucleic acid extracts in the presence of 1,000-10,000-fold excess of other nucleic acids. Immunoblot assays may work on the sandwich-ELISA principle and a monoclonal antibody to dsRNA as a catcher antibody and another antibody as a detector antibody to allow detection and/or

characterization of dsRNA by MAB even in the presence of large excess of other nucleic acids. (e.g. Schönborn et al. 1991 incorporated herein by reference in its entirety).

[0109] In some embodiments, the percentage of dsRNA in the input srRNA composition relative to ssRNA may be less than about 2.5% using a dsRNA immunoblot assay. In some embodiments, the percentage of dsRNA relative to ssRNA may be less than about 2.4%, less than about 2.3%, less than about 2.2%, less than about 2.1%, less than about 2.0%, less than about 1.9%, less than about 1.8%, less than about 1.7%, less than about 1.6%, less than about 1.5%, less than about 1.4%, less than about 1.3%, less than about 1.2%, less than about 1.1%, less than about 0.9%, less than about 0.8 %, less than about 0.7 %, less than about 0.6 %, less than about 0.5 %, less than about 0.4 %, less than about 0.3 %, less than about 0.2 %, less than about 0.1 %, or any values in between.

[0110] In some embodiments, the percentage of dsRNA relative to ssRNA, using a dsRNA immunoblot assay, may range from about 0.0% to about 2.5%, from about 0.1% to about 2.4%, from about 0.2% to about 2.3%, from about 0.3% to about 2.2%, from about 0.4% to about 2.1%, from about 0.5% to about 2.0%, from about 0.6% to about 1.9%, from about 0.7% to about 1.8%, from about 0.8% to about 1.7%, from about 0.9% to about 1.6%, from about 1.0% to about 1.5%, from about 1.1% to about 1.4%, from about 1.2% to about 1.3% or any ranges in between.

[0111] In some embodiments, the percentage of dsRNA relative to ssRNA in the input srRNA composition is determined after the *in vitro* transcription/generation of the srRNA and before its formulation. In some embodiments, if the amount of dsRNA is outside the ranges described *supra*, then a new srRNA compositions may be generated.

B. Methods of preventing or treating a health condition in a subject

[0112] The present disclosure provides methods of preventing or treating a health condition in a subject, the method comprising prophylactically or therapeutically administering to the subject an effective amount of a composition including a srRNA delivery system obtained by the methods of the disclosure.

[0113] The present disclosure also provides methods for inducing a pharmacodynamic effect in a subject, the method comprising prophylactically or therapeutically administering to the subject a composition of the disclosure.

[0114] The methods of the disclosure can be useful for the treatment and/or prevention of immune diseases, autoimmune diseases, or inflammatory diseases such as, for example, glomerulonephritis, inflammatory bowel disease, nephritis, peritonitis, psoriatic arthritis, osteoarthritis, Still's disease, Familial Mediterranean Fever, systemic scleroderma and sclerosis, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, acute lung injury, meningitis, encephalitis, uveitis, multiple myeloma, glomerulonephritis, nephritis, asthma, atherosclerosis, leukocyte adhesion deficiency, multiple sclerosis, Raynaud's syndrome, Sjögren's syndrome, juvenile onset diabetes, Reiter's disease, Behcet's disease, immune complex nephritis, IgA nephropathy, IgM polyneuropathies, immune-mediated thrombocytopenias, hemolytic anemia, myasthenia gravis, lupus nephritis, lupus erythematosus, rheumatoid arthritis (RA), ankylosing spondylitis, pemphigus, Graves' disease, Hashimoto's thyroiditis, small vessel vasculitides, Omen's syndrome, chronic renal failure, autoimmune thyroid disease, acute infectious mononucleosis, HIV, herpes virus associated diseases, human virus infections, coronavirus, other enterovirus, herpes virus, influenza virus, parainfluenza virus, respiratory syncytial virus or adenovirus infection, bacteria pneumonia, wounds, sepsis, cerebral stroke/cerebral edema, ischaemia-reperfusion injury, and hepatitis C. Non-limiting examples of inflammatory diseases include asthma, inflammatory bowel disease (IBD), chronic colitis, splenomegaly, and rheumatoid arthritis.

[0115] In some embodiments of the methods of preventing or treating a health condition in a subject, the health condition is a proliferative disorder or a microbial infection (e.g., bacterial infection, micro-fungal infection, or viral infection) or cancer. In some embodiments, the subject has or is suspected of having a condition associated with proliferative disorder or a microbial infection (e.g., bacterial infection, micro-fungal infection, or viral infection).

[0116] In some embodiments of the methods of preventing or treating a health condition in a subject, the health condition is a rare disease, e.g., a disease or condition that affects less than 200,000 people in the United States, as defined by The Orphan Drug Act (www.fda.gov/patients/rare-diseases-fda) and/or an inflammatory and/or autoimmune disorder. In some embodiments, the subject has or is suspected of having a condition associated with an inflammatory and/or autoimmune disorder and/or a rare disease (e.g. including but not limited to

Familial Mediterranean Fever, adult onset Still's disease, rheumatoid arthritis, osteoarthritis). In some embodiments, the health condition is proliferative disorders (*e.g.*, cancers) and chronic infections (*e.g.*, viral infections).

[0117] As discussed *supra*, a therapeutically effective amount includes an amount of a therapeutic composition that is sufficient to promote a particular effect when administered to a subject, such as one who has, is suspected of having, or is at risk for a health condition, *e.g.*, a disease or infection. In some embodiments, an effective amount includes an amount sufficient to prevent or delay the development of a symptom of the disease or infection, alter the course of a symptom of the disease or infection (for example but not limited to, slow the progression of a symptom of the disease or infection), or reverse a symptom of the disease or infection. It is understood that for any given case, an appropriate effective amount can be determined by one of ordinary skill in the art using routine experimentation.

[0118] The efficacy of a treatment including can be determined by the skilled clinician. However, a treatment is considered effective treatment if at least any one or all of the signs or symptoms of disease or infection are improved or ameliorated. Efficacy can also be measured by failure of an individual to worsen as assessed by hospitalization or need for medical interventions (*e.g.*, progression of the disease or infection is halted or at least slowed). Methods of measuring these indicators are known to those of skill in the art and/or described herein. Treatment includes any treatment of a disease or infection in a subject or an animal (some non-limiting examples include a human, or a mammal) and includes: (1) inhibiting the disease or infection, *e.g.*, arresting, or slowing the progression of symptoms; or (2) relieving the disease or infection, *e.g.*, causing regression of symptoms; and (3) preventing or reducing the likelihood of the development of symptoms.

[0119] The discussion of the general methods given herein is intended for illustrative purposes only. Other alternative methods and alternatives will be apparent to those of skill in the art upon review of this disclosure, and are to be included within the spirit and purview of this application.

III. COMPOSITIONS

Delivery systems

[0120] The present disclosure provides, *inter alia*, compositions including srRNA delivery systems wherein the srRNA delivery systems are obtained by the methods of the disclosure that are suitable for a biomedical application.

[0121] The term “delivery system” is used herein to describe a system that is generated after an input srRNA composition is formulated with any one of the delivery vehicles described in the disclosure thereby generating a delivery system that includes the formulated srRNA composition.

[0122] Accordingly, the srRNA delivery systems of the disclosure include a srRNA molecule(s) of interest and a delivery vehicle. Examples of delivery vehicles are described in details *supra*.

[0123] In some embodiments, a delivery system is considered suitable for a biomedical application if it is capable of the eventual delivery of a target payload to an individual in need of. A delivery system can be suitable for a biomedical application if it is capable of delivering a potent and effective amount of the payload of interest to a target cell(s).

[0124] A delivery system can be a suitable delivery system for a vaccine if, for example, it can allow for, in a host cell or in a subject, an encoded antigen-specific immune response, a correction of inflammation, expression of an encoded protein(s) within a target cell, expression of an encoded protein into the systemic circulation in an organism or others.

[0125] A delivery system can be a suitable delivery system for a biotherapeutic depends on the biotherapeutic being delivered. For example, a suitable delivery system can be a system that is well tolerated by a host cell or organism (*i.e.* it generates an acceptable level of adverse events, for example), or that induces inflammation, or does not induce inflammation.

[0126] The srRNA delivery systems of the disclosure can be formulated for the delivery of a payload of interest. Non-limiting examples of payloads include small molecules, peptide, proteins and nucleic acids including small activating RNA (saRNA), long non-coding RNA (lncRNA), antisense oligonucleotides (ASOs), small interfering (siRNA), microRNA, DNA and others.

[0127] The srRNA delivery systems can be formulated as biotherapeutics. Biotherapeutics include, but are not limited to, recombinant proteins, hormones, monoclonal antibodies,

cytokines, growth factors, gene therapy products, vaccines (e.g. therapeutic or prophylactic), cell-based products, stem cell therapies, gene-silencing/editing therapies, tissue-engineered products and others.

[0128] In some embodiments, the srRNAs of the disclosure can be formulated as a prophylactic composition, or a therapeutic composition. For example, the srRNAs of the disclosure can be formulated for the amelioration, prevention, treatment, or management of a health condition such as an immune disease or a microbial infection.

Pharmaceutical compositions

[0129] Provided herein are pharmaceutical compositions including the srRNA delivery system obtained by the methods of the disclosure and a pharmaceutically acceptable excipient (e.g., carrier).

[0130] In some embodiments, the srRNAs of the disclosure are formulated with a delivery vehicle. The delivery vehicle can be a non-viral delivery vehicle. In some embodiments, the delivery vehicle is a in a lipid-based nanoparticle (LNP). Examples of LNP are discussed in more details *supra*. In some embodiments, the srRNAs of the disclosure are formulated in a polymer nanoparticle. The srRNAs of the disclosure can also be used in a naked form. In some embodiments, the srRNAs are formulated in liposomes. In some embodiments, the pharmaceutical compositions are formulated as an adjuvant.

[0131] In some embodiments, the srRNAs are formulated as a vaccine. In some embodiments, the vaccine is a prophylactic vaccine. In some embodiments, the vaccine is a therapeutic vaccine.

[0132] In some embodiments, the pharmaceutical compositions of the disclosure are immunogenic compositions, e.g., composition that can stimulate an immune response in a subject. In some embodiments, the pharmaceutical compositions do not elicit an immune response in the subject.

[0133] In some embodiments, the compositions are substantially non-immunogenic to a subject, e.g., compositions that minimally stimulate an immune response in a subject. In some embodiments, the non-immunogenic or minimally immunogenic compositions are formulated as a biotherapeutic.

[0134] In some embodiments, the compositions elicit a pro-inflammatory response or an anti-inflammatory response in a subject. In some embodiments, the compositions induce production of one or more pro-inflammatory molecules in the subject. In some embodiments, the compositions do not elicit an inflammatory response and/or an anti-inflammatory response in the subject.

[0135] In some embodiments, the disclosed pharmaceutical compositions are formulated to be compatible with its intended route of administration. In some embodiments, the pharmaceutical compositions are formulated for one or more of intranasal administration, transdermal administration, intraperitoneal administration, intramuscular administration, intranodal administration, intratumoral administration, intraarticular administration, intravenous administration, subcutaneous administration, oral administration and parenteral administration.

[0136] Examples of parenteral routes of administration include, for example, intravenous, intranodal, intradermal, intratumoral, intraarticular, subcutaneous, transdermal (topical), transmucosal, intravaginal, and rectal administration. Solutions or suspensions used for parenteral application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as mono- and/or di-basic sodium phosphate, hydrochloric acid or sodium hydroxide (*e.g.*, to a pH of about 7.2-7.8, *e.g.*, 7.5). The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0137] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™. (BASF, Parsippany, N.J.), or phosphate buffered saline (PBS). In these cases, the composition should be sterile and should be fluid to the extent that easy syringability exists. It can be stable under the conditions of

manufacture and storage, and can be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants, *e.g.*, sodium dodecyl sulfate. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be generally to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, and/or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0138] Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above.

[0139] In some embodiments, the pharmaceutical compositions of the disclosure can be administered to a subject in a composition having a pharmaceutically acceptable carrier and in an amount effective to stimulate an immune response. Generally, a subject can be immunized through an initial series of injections (or administration through one of the other routes described below) and subsequently given boosters to increase the protection afforded by the original series of administrations. The initial series of injections and the subsequent boosters are administered in such doses and over such a period of time as is necessary to stimulate an immune response in a subject. In some embodiments, the administered composition results in an increased production of interferon in the subject. In some embodiments of the disclosed methods, the subject is a mammal. In some embodiments, the mammal is human.

[0140] Pharmaceutically acceptable carriers suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the

extemporaneous preparation of sterile injectable solutions or dispersions. In these cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. The composition must further be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, *etc.*), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like.

[0141] When the pharmaceutical compositions are suitably protected, as described above, they can be orally administered, for example, with an inert diluent or an assimilable edible carrier. The pharmaceutical compositions and other ingredients can also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. For oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like.

[0142] In some embodiments, the srRNAs of the disclosure can be delivered to a cell or a subject by a lipid-based nanoparticle (LNP). LNP are generally less immunogenic than viral particles. While many humans have preexisting immunity to viral particles there is no pre-existing immunity to LNP. In addition, adaptive immune response against LNP is unlikely to occur which enables repeat dosing of LNP. LNP are discussed *supra*.

[0143] Dosage, toxicity and therapeutic efficacy of pharmaceutical compositions of the disclosure can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds that exhibit high therapeutic indices are generally suitable. While

compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0144] For example, the data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies generally within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the disclosure, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC₅₀ (*e.g.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[0145] The pharmaceutical compositions described herein, can be administered one from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors can influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the subject multivalent polypeptides and multivalent antibodies of the disclosure can include a single treatment or, can include a series of treatments. In some embodiments, the compositions are administered every 8 hours for five days, followed by a rest period of 2 to 14 days, *e.g.*, 9 days, followed by an additional five days of administration every 8 hours. With regard to nucleic acid constructs the therapeutically effective amount of a nucleic acid construct (*e.g.*, an effective dosage) depends on the nucleic acid construct selected. For instance, single dose amounts in the range of approximately 0.001 to 0.1 mg/kg of patient body weight can be administered. In some embodiments, about 0.005, 0.01, 0.05 mg/kg can be administered. In some embodiments, single dose amounts in the range of approximately 0.03 µg to 300 µg/kg of patient body weight can be

administered. In some embodiments, single dose amounts in the range of approximately 0.3 mg to 3 mg/kg of patient body weight can be administered.

[0146] In some embodiments, the pharmaceutical compositions are incorporated into therapeutic compositions for use in methods of preventing or treating a subject who has, who is suspected of having, or who can be at high risk for developing a microbial infection. In some embodiments, the microbial infection is a bacterial infection. In some embodiments, the microbial infection is a fungal infection. In some embodiments, the microbial infection is a viral infection.

[0147] In some embodiments, a composition according to the present disclosure is administered to the subject individually as a single therapy (monotherapy) or as a first therapy in combination with at least one additional therapies (e.g., second therapy). In some embodiments, the second therapy is selected from the group consisting of chemotherapy, radiotherapy, immunotherapy, hormonal therapy, toxin therapy, targeted therapy, and surgery. In some embodiments, the second therapy is selected from the group consisting of chemotherapy, radiotherapy, immunotherapy, hormonal therapy, toxin therapy or surgery. In some embodiments, the first therapy and the second therapy are administered concomitantly. In some embodiments, the first therapy is administered at the same time as the second therapy. In some embodiments, the first therapy and the second therapy are administered sequentially. In some embodiments, the first therapy is administered before the second therapy. In some embodiments, the first therapy is administered after the second therapy. In some embodiments, the first therapy is administered before and/or after the second therapy. In some embodiments, the first therapy and the second therapy are administered in rotation. In some embodiments, the first therapy and the second therapy are administered together in a single formulation.

[0148] It is appreciated that certain features of the disclosure, which are, for clarity, described in the context of separate embodiments, can also be provided in combination in a single embodiment. Conversely, various features of the disclosure, which are, for brevity, described in the context of a single embodiment, can also be provided separately or in any suitable sub-combination. All combinations of the embodiments pertaining to the disclosure are specifically embraced by the present disclosure and are disclosed herein just as if each and every

combination was individually and explicitly disclosed. In addition, all sub-combinations of the various embodiments and elements thereof are also specifically embraced by the present disclosure and are disclosed herein just as if each and every such sub combination was individually and explicitly disclosed herein.

[0149] Throughout this specification, various patents, patent applications and other types of publications (*e.g.*, journal articles, electronic database entries, *etc.*) are referenced. The disclosure of all patents, patent applications, and other publications cited herein are hereby incorporated by reference in their entirety for all purpose.

EXAMPLES

[0150] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology, biochemistry, nucleic acid chemistry, and immunology, which are well known to those skilled in the art. Such techniques are explained fully in the literature, such as Sambrook, J., & Russell, D. W. (2012). *Molecular Cloning: A Laboratory Manual* (4th ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory and Sambrook, J., & Russel, D. W. (2001). *Molecular Cloning: A Laboratory Manual* (3rd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory (jointly referred to herein as “Sambrook”); Ausubel, F. M. (1987). *Current Protocols in Molecular Biology*. New York, NY: Wiley (including supplements through 2014); Bollag, D. M. *et al.* (1996). *Protein Methods*. New York, NY: Wiley-Liss; Huang, L. *et al.* (2005). *Nonviral Vectors for Gene Therapy*. San Diego: Academic Press; Kaplitt, M. G. *et al.* (1995). *Viral Vectors: Gene Therapy and Neuroscience Applications*. San Diego, CA: Academic Press; Lefkovits, I. (1997). *The Immunology Methods Manual: The Comprehensive Sourcebook of Techniques*. San Diego, CA: Academic Press; Doyle, A. *et al.* (1998). *Cell and Tissue Culture: Laboratory Procedures in Biotechnology*. New York, NY: Wiley; Mullis, K. B., Ferré, F. & Gibbs, R. (1994). *PCR: The Polymerase Chain Reaction*. Boston: Birkhauser Publisher; Greenfield, E. A. (2014). *Antibodies: A Laboratory Manual* (2nd ed.). New York, NY: Cold Spring Harbor Laboratory Press; Beaucage, S. L. *et al.* (2000). *Current Protocols in Nucleic Acid Chemistry*. New York, NY: Wiley, (including supplements through 2014); and Makrides, S. C. (2003). *Gene Transfer and Expression in Mammalian Cells*. Amsterdam, NL: Elsevier Sciences B.V., the disclosures of

which are incorporated herein by reference.

Additional embodiments are disclosed in further detail in the following examples, which are provided by way of illustration and are not in any way intended to limit the scope of this disclosure or the claims.

EXAMPLE 1: *In vitro* Transcription (IVT)

[0151] RNA samples are prepared by *in vitro* transcription (IVT) using a plasmid DNA template linearized by enzymatic digestion. In these experiments, the DNA template is either linearized with NotI, which cuts downstream of the T7 terminator, or linearized with SapI, which cuts at the end of the polyA. Bacteriophage T7 polymerase is used for *in vitro* transcription with either a 5' ARCA cap (HiScribe™ T7 ARCA mRNA Kit, NEB) or by uncapped transcription (HiScribe™ T7 High Yield RNA Synthesis Kit, NEB) followed by addition of a 5' cap 1 (Vaccinia Capping System, mRNA Cap 2'-O-Methyltransferase, NEB). RNA products are then purified using phenol/chloroform extraction, LiCl precipitation, or column purification (Monarch® RNA Cleanup Kit, NEB). RNA concentration in the RNA samples is determined by absorbance at 260 nm (Nanodrop, Thermo Fisher Scientific). These RNA samples are used as input RNA compositions (e.g., srRNA compositions) in the assays described below.

EXAMPLE 2: Immunoblot Assay

[0152] The amount of dsRNA in an input srRNA composition can be determined by an immunoblot assay. Immunoblot assays are used to detect the presence of undesired dsRNA molecules in synthesized mRNA preparations based on the use of dsRNA-specific monoclonal antibodies. Detection is highly specific as some commercially-available kits allow the detection of dsRNA in nucleic acid extracts in the presence of 1,000-10,000-fold excess of other nucleic acids. In general, immunoblot assays work on the sandwich-ELISA principle and a monoclonal antibody to dsRNA as a catcher antibody and another antibody as a detector antibody.

EXAMPLE 3: *In vitro* Potency Assay

[0153] Replicating vectors are detected using an *in vitro* potency assay that measures replication efficiency by capturing intermediate dsRNA and comparable protein expression in individual cells by an antigen-specific monoclonal antibody. J2, Test srRNA will be diluted and directly electroporated into cells. When the test srRNA (input srRNA composition) is

encapsulated or adsorbed to a non-viral delivery system, a detergent (or other extraction method) will be used to extract the srRNA from the delivery system before it is electroporated into cells. After sufficient incubation, the cells will be fixed and immunoassayed with a fluorophore-conjugated antibody (J2) that specifically detects the dsRNA replication intermediate of the vector. Signal-positive cells indicate the presence of an intact and functional srRNA, which can be quantified by fluorescence flow cytometry. The assay readout is the frequency of positive cells per ng of RNA transfected. There is a dose-responsive frequency of dsRNA+ transfected cells which results in the ability to generate a sigmoidal curve that is similar to the standard curve generated by the widely used enzyme-linked immunosorbent assay (ELISA) for quantification of other biological molecules as shown below. A srRNA reference standard can be used to help mitigate cell-based assay variability and enable comparison of potency across assays. A srRNA standard can be an aliquot of a large prep that is stored at -80°C and the potency of each test RNA is defined relative to the standard.

[0154] In some embodiments, a similar strategy for quantification of virus replicon particle (VRPs) titers can be employed by infection of cells with serial dilutions of the particles followed by immunoassay with the J2 antibody.

EXAMPLE 4: *In vivo* Potency Assay

[0155] This Example describes *in vivo* experiments that can be performed to evaluate protein expression or immune responses following administration with the synthetic srRNA constructs (*e.g.*, both unformulated and formulated vectors).

[0156] In these experiments, synthetic srRNA constructs are designed and subsequently evaluated.

[0157] *Mice and injections.* Female C57BL/6 or BALB/c mice are purchased from Envigo, Charles River Labs or Jackson Laboratories. On day of dosing, between 0.01-40 µg of material is injected intramuscularly either unilaterally or bilaterally split into both quadricep muscles. Vectors are administered either unformulated in saline, or LNP-formulated or polymer-formulated. Animals are monitored for body weight and other general observations throughout the course of the study.

[0158] *Immunogenicity studies.* For immunogenicity studies, animals are dosed on Day 0

and Day 14 or 21 or 35 or 42 or 56. Spleens are collected at 14 days post second dosing for ELISPOT or ICS analysis, and serum is isolated at Days 14, and 14 days post second dosing for antibodies.

[0159] ELISpot. To measure the magnitude of antigen-specific T cell responses, IFN γ ELISpot analysis is performed using Mouse IFN γ ELISpot PLUS Kit (HRP) (MabTech) as per manufacturer's instructions. In brief, splenocytes are isolated and resuspended to a concentration of $1-5 \times 10^6$ cells/mL in media containing peptides representing either T cell epitopes for the encoded protein, PMA/ionomycin as a positive control, or DMSO as a mock stimulation.

[0160] Intracellular cytokine staining (ICS). To measure the magnitude and quality of antigen-specific T cell responses, intracellular cytokines produced as a result of T cell stimulation by antigens are measured by immunostaining. In brief, splenocytes are isolated and resuspended to a concentration of $1-5 \times 10^6$ cells/mL in media containing peptides representing either T cell epitopes for the encoded protein, PMA/ionomycin as a positive control, or DMSO as a mock stimulation. After an hour of activation ex vivo, golgi inhibitors are added to the cultures to trap cytokines within the cell, followed by standard immunostaining protocols to identify CD4 $^{+}$ and CD8 $^{+}$ T cells and measure cytokine expression and co-expression within the samples. Key cytokines representative of activated T cells are as IFN γ , and/or IL-2, and/or TNF. Immunostained samples are analyzed on a standard flow cytometer.

[0161] Antibodies. Antibody responses to measure total antigen-specific IgG are measured using ELISA protocols as previously published.

[0162] Protein expression. For protein expression studies, animals are dosed on Day 0, and protein expression or bioluminescence is assessed on Days 1, 3, 7, 10 and so on until loss of signal. If encoding a reporter protein such as luciferase, *in vivo* imaging of luciferase activity is done using an IVIS instrument at the indicated time points. For secreted proteins, such as agonists, or antagonists, or monoclonal antibodies, systemic protein levels can be assayed by sequential bleeds, followed by preparation of serum and ELISA analysis for the expressed protein.

EXAMPLE 5: Capillary Electrophoresis

[0163] This assay is used to measure the percentage of full length RNA in a formulated

srRNA composition, i.e., the integrity of the srRNA after formulation. This is done by measuring the amount or percentage of full-length RNA molecules in the srRNA formulation using a capillary electrophoretic (SCIEX - PA 800Plus Pharmaceutical Analysis System). Capillary electrophoresis allows the quantification of full-length srRNA after formulation.

EXAMPLE 6: Protein Expression

[0164] This assay measures the protein expression, such as proteins expressed, for example, from a srRNA. RNA is transformed by electroporation into BHK-21 or Vero cells (*e.g.* *4D-Nucleofector™*, Lonza). At 18-20 h following transformation, the cells are fixed and permeabilized (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set, Invitrogen) and stained using an APC-conjugated anti-HA mouse monoclonal antibody (2B7, Abcam) to quantify the frequency of HA protein⁺ cells and the mean fluorescence intensity (MFI) of the HA protein in individual cells by fluorescence flow cytometry.

EXAMPLE 7: Replication

[0165] This assay will assess viral replication efficiency by determining the frequency of cells having dsRNA per ng of RNA transfected. RNA is transformed by electroporation into BHK-21 or Vero cells (*e.g.* *4D-Nucleofector™*, Lonza). At 17-20 h following transformation, the cells are fixed and permeabilized (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set, Invitrogen) and stained using a PE-conjugated anti-dsRNA mouse monoclonal antibody (J2, Scicons) to quantify the frequency of dsRNA⁺ cells and the mean fluorescence intensity (MFI) of dsRNA in individual cells by fluorescence flow cytometry.

[0166] While particular alternatives of the present disclosure have been disclosed, it is to be understood that various modifications and combinations are possible and are contemplated within the true spirit and scope of the appended claims. There is no intention, therefore, of limitations to the exact abstract and disclosure herein presented

CLAIMS

WHAT IS CLAIMED IS:

1. A method of selecting/identifying a self-replicating RNA (srRNA) delivery system for a biomedical application, the method comprising:
 - a) formulating an input srRNA composition with a non-viral delivery vehicle to generate a srRNA delivery system comprising a formulated srRNA composition;
 - b) determining potency of the formulated srRNA composition relative to unformulated input srRNA composition;
 - c) determining percentage change in amount of full-length srRNA molecules in the formulated srRNA composition relative to amount of full-length srRNA molecules in the input srRNA composition; and
 - d) selecting the srRNA delivery system as being suitable for a biomedical application if the formulated srRNA composition in (b) retains at least about 25% potency relative to the potency of the unformulated input srRNA composition.
2. The method of claim 1, wherein the srRNA delivery system in (d) is selected as being suitable for the biomedical application if the formulated srRNA composition in (b) retains at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55% or at least about 60% potency relative to the potency of the unformulated input srRNA composition.
3. The method of any one of claims 1-2 wherein the percentage change in the amount of full-length srRNA molecules in the formulated srRNA composition in (c) comprises a percentage decrease of less than about 40% relative to the amount of full-length srRNA molecules in the input srRNA composition.
4. The method of claim 3, wherein the percentage decrease in (c) is less than about 35%, about 30%, less than about 25%, less than about 20%, less than about 15%, or less than about

10% or less than about 5% relative to the amount of full-length srRNA molecules in the input srRNA composition.

5. The method of any one of claims 1 to 4, wherein the input srRNA composition comprises double-stranded RNA (dsRNA) molecules and single-stranded RNA (ssRNA) molecules.

6. The method of claim 5, further comprising determining the percentage of double-stranded RNA (dsRNA) relative to single-stranded RNA (ssRNA) in the input srRNA composition.

7. The method of claim 6, wherein the determining the percentage of double-stranded RNA (dsRNA) relative to single-stranded RNA (ssRNA) in the input srRNA composition is by an immunoblot assay.

8. The method of claim 6 or claim 7, wherein the percentage of dsRNA relative to ssRNA is less than about 2.5%, less than about 2.0%, less than about 1.5%, less than about 1.0%, less than about 0.5%, or less than about 0.25%.

9. The method of any one of claims 1 to 8, wherein the determining potency of the formulated srRNA composition in (b) is carried out *in vivo*, or *in vitro*.

10. The method of claim 9, wherein the determining potency of the formulated srRNA composition comprises detection of RNA replication, detection of viral protein expression, and/or detection of heterologous gene expression.

11. The method of any one of claims 9 to 10, wherein the determining potency of the formulated srRNA composition comprises immunoblotting analysis, fluorescence flow cytometry analysis, enzyme-linked immunoassay analysis, immunogenicity analysis, bioactivity analysis, and/or efficacy in a disease model.

12. The method of claim 10 or 11, wherein the determining potency comprises assessing RNA replication efficiency.

13. The method of claim 12, wherein the assessing RNA replication efficiency comprises a monoclonal antibody.

14. The method of claim 13, wherein the monoclonal antibody is J2.
15. The method of claim 12, wherein the replication efficiency is determined by determining in an *in vitro* potency assay the frequency of cells having dsRNA per ng of RNA transfected.
16. The method of claims 1-12, wherein the determining potency of the formulated srRNA composition in (b) comprises an *in vivo* potency assay.
17. The method of claim 16, wherein the *in vivo* potency assay is carried out in an animal cell.
18. The method of claim 16, wherein the *in vivo* potency assay is carried out in a mammalian cell.
19. The method of any one of claims 1 to claim 18, wherein determining the percentage of full-length srRNA molecules in (c) comprises assaying the degradation of the full-length srRNA molecules during the formulation process of (a).
20. The method of any one of claims 1 to claim 19, wherein determining the percentage of full-length srRNA molecules in (c) comprises gel electrophoresis and/or capillary electrophoresis.
21. The method of any one of claims 1 to 20, wherein the non-viral delivery vehicle comprises a polymer nanoparticle, or a lipid-based nanoparticle (LNP), a liposome, a microsphere, an immune stimulating complex (ISCOM), a conjugate of a bioactive ligand, a physiologic buffer, or a combination of any thereof.
22. The method of claim 21, wherein the LNP comprises a cationic lipid, an ionizable cationic lipid, an anionic lipid, or a neutral lipid.
23. The method of claim 22, wherein the mass ratio of lipid to nucleic acid in the LNP delivery system is about 100:1 to about 3:1, about 70:1 to about 10:1, or about 16:1 to about 4:1.
24. The method of any of claims 1 to 20, wherein the non-viral delivery vehicle comprises a physical delivery system and the srRNA is formulated as a “naked” srRNA.

25. The method of any one of claims 1 to claim 24, wherein the selected srRNA delivery system is formulated as an immunogenic formulation.
26. The method of any one of claims 1 to claim 24, wherein the selected srRNA delivery system is formulated as a non-immunogenic formulation.
27. The method of any one of claims 1 to 26, wherein the selected srRNA delivery system is formulated as a biotherapeutic.
28. The method of any one of claims 1 to 26, wherein the selected srRNA delivery system is formulated as a vaccine.
29. The method of claim 28, wherein the vaccine is a therapeutic vaccine.
30. The method of claim 28, wherein the vaccine is a prophylactic vaccine.
31. A method for identifying a self-replicating RNA (srRNA) delivery system for a biomedical application, the method comprising:
- (a) determining percentage of double-stranded RNA (dsRNA) molecules in an input srRNA composition relative to single-stranded RNA (ssRNA) molecules in the input srRNA composition;
 - (b) formulating an input srRNA composition with a non-viral delivery vehicle to generate a srRNA delivery system comprising a formulated srRNA composition;
 - (c) determining potency of the formulated srRNA composition relative to unformulated input srRNA composition;
 - (d) determining percentage change in amount of full-length srRNA molecules in the formulated srRNA composition relative to amount of full-length srRNA molecules in the input srRNA composition; and
 - (e) selecting the srRNA delivery system as being suitable for a biomedical application if (i) the percentage of dsRNA relative to ssRNA in (a) is less than about 2.5%, (ii) the formulated srRNA composition in (c) retains at least about 25% potency relative to the potency of the unformulated input srRNA composition, and (iii) the percentage change

in (d) comprises a percentage decrease of less than about 40% relative to the amount of full-length srRNA molecules in the unformulated input srRNA composition.

32. The method claim 31, wherein the determining the percentage of dsRNA relative to ssRNA in (b) comprises an immunoblot assay, the determining the potency comprises an *in vitro* potency assay, and the determining the percentage change in the amount of full-length srRNA molecules in the formulated srRNA relative to the amount of full-length srRNA molecules in the input srRNA composition in (d) comprises capillary electrophoresis.
33. The method of claim 31 or 32, wherein the percentage of dsRNA relative to ssRNA is from about 0.0% to about 2.5%, from about 0.5% to about 2.0%, from about 1.0% to about 1.5%.
34. The method of any one of claims 31 to 33, wherein the percentage change in (d) is less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, less than about 10%, or less than about 5%.
35. The method of any one of claims 31 to 33, wherein the percentage change is from about 5% to about 40%, or from about 10% to about 30% or from about 20% to about 25%.
36. The method of any one of claims 31 to 35, wherein the determining potency of the formulated srRNA composition in (b) is carried out *in vivo*, or *in vitro*.
37. The method of claim 36, wherein the determining potency of the formulated srRNA composition comprises detection of RNA replication, detection of viral protein expression, and/or detection of heterologous gene expression.
38. The method of claim 36 or 37, wherein the determining potency of the formulated srRNA composition comprises immunoblotting analysis, fluorescence flow cytometry analysis, enzyme-linked immunoassay analysis, immunogenicity analysis, bioactivity analysis, and/or efficacy in a disease model.
39. The method of claim 36 to 38, wherein the determining potency comprises assessing RNA replication efficiency.

40. The method of claim 39, wherein the assessing RNA replication efficiency comprises a monoclonal antibody.
41. The method of claim 40, wherein the monoclonal antibody is J2.
42. The method of claim 41, wherein the replication efficiency is determined by determining in an *in vitro* potency assay the frequency of cells having dsRNA per ng of RNA transfected..
43. The method of claims 32-42, wherein the determining potency of the formulated srRNA composition in (b) comprises an *in vivo* potency assay.
44. The method of claim 37, wherein the *ex vivo* potency assay is carried out in an animal cell.
45. The method of claim 44, wherein the *ex vivo* potency assay is carried out in a mammalian cell.
46. The method of any one of claims 32 to 36, wherein determining the percentage of full-length srRNA molecules in (c) is by gel electrophoresis or by capillary electrophoresis.
47. The method of any one of claims 32 to 36, wherein the non-viral delivery vehicle comprises a polymer nanoparticle, or a lipid-based nanoparticle (LNP).
48. The method of claim 47, wherein the LNP comprises a cationic lipid, an ionizable cationic lipid, an anionic lipid, or a neutral lipid.
49. The method of claim 48, wherein the mass ratio of lipid to nucleic acid in the LNP delivery system is about 100:1 to about 3:1, about 70:1 to about 10:1, or about 16:1 to about 4:1.
50. The method of any of claims 31 to 46, wherein the non-viral delivery vehicle comprises a physical delivery system and the srRNA is formulated as a “naked” srRNA.
51. The method of any one of claims 31 to claim 50, wherein the selected srRNA delivery system is formulated as an immunogenic formulation.

52. The method of any one of claims 31 to claim 50, wherein the selected srRNA delivery system is formulated as a non-immunogenic formulation.
53. The method of any one of claims 31 to 52, wherein the selected srRNA delivery system is formulated as a biotherapeutic.
54. The method of any one of claims 31 to 52, wherein the selected srRNA delivery system is formulated as a vaccine.
55. The method of claim 54, wherein the vaccine is a therapeutic vaccine.
56. The method of claim 54, wherein the vaccine is a prophylactic vaccine.
57. A composition comprising a srRNA delivery system obtained by any one of the methods of claim 1 to claim 56.
58. A pharmaceutical composition comprising a srRNA delivery system obtained by any one of the methods of claim 1 to claim 56 and a pharmaceutically acceptable excipient.
59. A method of preventing or treating a health condition in a subject, the method comprising prophylactically or therapeutically administering to the subject a pharmaceutical composition of claim 58.
60. The method of claim 59, wherein the composition elicits a pro-inflammatory response or an anti-inflammatory response in the subject.
61. The method of claim 60, wherein the composition induces production of one or more pro-inflammatory molecules in the subject.
62. The method of claim 59, wherein the composition does not elicit an inflammatory response and/or an anti-inflammatory response in the subject.
63. The method of claim 59, wherein the composition elicits an immune response in the subject.

64. The method of claim 59, wherein the composition does not elicit an immune response in the subject.

65. A method for inducing a pharmacodynamic effect in a subject, the method comprising prophylactically or therapeutically administering to the subject the pharmaceutical composition of claim 58.

66. The method of claim 65, wherein the composition elicits an immune response in the subject.

67. The method of claim 64, wherein the composition does not elicit an immune or a pro-inflammatory and/or an anti-inflammatory response in the subject.