

CIRCULAR POLYRIBONUCLEOTIDES ENCODING ANTIFUSOGENIC POLYPEPTIDES**Background**

Delivery of polynucleotides and proteins is important for a wide variety of therapeutic fields.

5 However, current delivery modalities are often ineffective. For example, delivery of short polypeptides, such as polypeptides encoding an antifusogenic polypeptide, often results in short half-life and rapid clearance of the polypeptides. Accordingly, a need exists for improved compositions and methods for delivering an antifusogenic polypeptide, e.g., to treat or prevent a viral infection.

10 **Summary of the Invention**

The disclosure provides compositions and methods for producing, purifying, and using circular RNA encoding an antifusogenic polypeptide.

In one aspect, the invention features a circular polyribonucleotide that includes a polyribonucleotide cargo encoding the antifusogenic polypeptide. In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding the antifusogenic polypeptide.

In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding a polypeptide of Table 1. In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding a polypeptide having at least 85% (e.g., at least 90%, 95%, 97%, 99%, or 100%) sequence identity to a polypeptide of Table 1. In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding a polypeptide having at least 85% (e.g., at least 90%, 95%, 97%, 99%, or 100%) sequence identity to any one of SEQ ID NOs: 1-324.

In some embodiments, the circular polyribonucleotide includes a splice junction joining a 5' exon fragment and a 3' exon fragment.

In some embodiments, the polyribonucleotide cargo includes an IRES operably linked to the expression sequence encoding the antifusogenic polypeptide. The circular polyribonucleotide may further include a spacer region between the IRES and the 3' exon fragment or the 5' exon fragment. The spacer region may be at least 5 ribonucleotides in length. For example, the spacer region may be at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, or more ribonucleotides in length. In some embodiments, the spacer region is from 5 to 500 ribonucleotides in length. The spacer region may include a polyA, a polyA-C, polyA-U, or polyA-G sequence. The spacer region may be a random sequence.

In some embodiments, the circular polyribonucleotide is at least 500 ribonucleotides in length. For example, the circular polyribonucleotide may be at least 500, 600, 700, 800, 900, 1,000, 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 15,000, 20,000, 25,000, 30,000, 35,000, 40,000, 45,000, 50,000, or more polyribonucleotides. In some embodiments, the circular polyribonucleotide is from 500 to 20,000 ribonucleotides in length.

In another aspect, featured is a linear polyribonucleotide including, from 5' to 3', (A) a 3' intron fragment; (B) a 3' splice site; (C) a 3' exon fragment; (D) a polyribonucleotide cargo encoding the antifusogenic polypeptide; (E) a 5' exon fragment; (F) a 5' splice site; and (G) a 5' intron fragment.

40 In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding the antifusogenic polypeptide.

In some embodiments, the polyribonucleotide cargo includes an IRES operably linked to the expression sequence encoding the antifusogenic polypeptide (e.g., a polypeptide of Table 1). The circular polyribonucleotide may further include a spacer region between the IRES and the 3' exon fragment or the 5' exon fragment. The circular polyribonucleotide may further include a spacer region
5 between one or more of (A), (B), (C), (D), (E), (F), and (G).

The spacer region may be at least 5 ribonucleotides in length. For example, the spacer region may be at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, or more ribonucleotides in length. In some embodiments, the spacer region is from 5 to 500 ribonucleotides in length. The spacer region may include a polyA, a polyA-C, polyA-U, or polyA-G sequence. The spacer
10 region may be a random sequence.

In some embodiments, the circular polyribonucleotide lacks an IRES. In some embodiments, the circular polyribonucleotide lacks one or both of a 5' cap and a polyA sequence.

In some embodiments, the circular polyribonucleotide comprises a protein translation initiation site. In some embodiments, the protein translation initiation site comprises a Kozak sequence.

15 In some embodiments, the linear polyribonucleotide is at least 500 ribonucleotides in length. For example, the linear polyribonucleotide may be at least 500, 600, 700, 800, 900, 1,000, 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 15,000, 20,000, 25,000, 30,000, 35,000, 40,000, 45,000, 50,000, or more polyribonucleotides. In some embodiments, the linear polyribonucleotide is from 500 to 20,000 ribonucleotides in length.

20 In another aspect, featured is a DNA vector encoding a polyribonucleotide (e.g., a linear or circular polyribonucleotide) as described herein.

In another aspect, featured is a method of expressing an antifusogenic polypeptide (e.g., a polypeptide of Table 1) in a cell. The method includes providing a circular, a linear polyribonucleotide, or the DNA vector as described herein to the cell under conditions suitable to express the antifusogenic
25 polypeptide.

In another aspect, featured is a method of producing a circular polyribonucleotide from a linear polyribonucleotide as described herein. The method includes providing the linear polyribonucleotide under conditions suitable for self-splicing of the linear polyribonucleotide to produce the circular polyribonucleotide.

30 In another aspect, featured is a pharmaceutical composition that includes the circular polyribonucleotide, the linear polyribonucleotide, or the DNA vector of any of the above embodiments, and a diluent, carrier, or excipient.

In another aspect, featured is a method of expressing the antifusogenic polypeptide (e.g., a polypeptide of Table 1) in a subject. The method includes administering a first dose of the
35 pharmaceutical composition in an amount sufficient to produce a serum concentration of at least 500 ng/mL (e.g., at least 600 ng/mL, 700 ng/mL, 800 ng/mL, 900 ng/mL, 1,000 ng/mL, 1,100 ng/mL, 1,200 ng/mL, 1,300 ng/mL, 1,400 ng/mL, 1,500 ng/mL, 1,600 ng/mL, 1,700 ng/mL, 1,800 ng/mL, 1,900 ng/mL, 2,000 ng/mL, 2,100 ng/mL, 2,200 ng/mL, 2,300 ng/mL, 2,400 ng/mL, 2,500 ng/mL, 2,600 ng/mL, 2,700 ng/mL, 2,800 ng/mL, 2,900 ng/mL, 3,000 ng/mL, or more) of the antifusogenic polypeptide (e.g., a
40 polypeptide of Table 1) in the subject.

In some embodiments, the method may further include administering a second dose of the pharmaceutical composition. The method may further include administering a third, fourth, fifth, sixth, seventh, eighth, ninth, tenth, or more doses of the pharmaceutical composition.

In some embodiments, the second dose is administered at least one hour (e.g., at least two
5 hours, three hours, four hours, five hours, six hours, seven hours, eight hours, nine hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, one day, two days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, one year, or longer) after the first dose
10 of the pharmaceutical composition.

In some embodiments, the second dose is administered from 1 hour to 1 year (e.g., from 1 hour to 1 day, e.g., one hour, two hours, three hours, four hours, five hours, six hours, seven hours, eight hours, nine hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, or one day, e.g., from one day to one week,
15 e.g., two days, three days, four days, five days, six days, or one week, e.g., from one week to one month, e.g., two weeks, three weeks, or one month, e.g., from one month to one year, e.g., one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, or one year) after the first dose of the pharmaceutical composition. In some embodiments, the second dose is administered from 1 days to 180 days (e.g., from 1 day to 90 days, from 1 day to 45 days, from one day to 30 days, from 1 day to 14 days, from 1 day to 7 days, from 2 days to 45 days, from 2 days to 30 days, from 2 days to 14 days, from 2 days to 7 days, from 3 days to 90 days, from 3 days to 45 days, from 3 days to 30 days, from 3 days to 14 days, from 3 days to 7 days, from 4 days to 90 days, from 4 days to 45 days, from 4 days to 30 days, from 4 days to 14 days, from 4 days to 7 days, from 5 days to 90 days, from 5 days to 45 days, from 5 days to 30 days, from 5 days to 14 days, from 5 days to 7 days, from 6 days to 90 days, from 6 days to 45 days, from 6 days to 30 days, from 6 days to 14 days, from 6 days to 7 days, from 7 days to 90 days, from 7 days to 45 days, from 7 days to 30 days, from 7 days to 14 days, from 14 days to 90 days, from 14 days to 45 days, from 14 days to 30 days, from 21 days to 90 days, from 21 days to 60 days, from 21 days to 45 days, from 21 days to 30 days, from 30 days to 90 days, from 30 days to 60 days, from 30 days to 45 days, from 45 to 180 days, from 45 to 120 days, from 45 to 100 days, from 45 to 90 days, from 45 to 60 days, from 60 to 180 days, from 60 to 120 days, from 60 to 100 days, from 60 to 90 days, from 90 to 100 days, from 90 to 120 days, or from 90 to 180 days) after the first dose of the pharmaceutical composition.

In some embodiments, the third dose is administered at least one hour (e.g., at least two hours, three hours, four hours, five hours, six hours, seven hours, eight hours, nine hours, 10 hours, 11 hours,
35 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, one day, two days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, one year, or longer) after the second dose of the pharmaceutical composition.

In some embodiments, the third dose is administered from 1 hour to 1 year (e.g., from 1 hour to 1 day, e.g., one hour, two hours, three hours, four hours, five hours, six hours, seven hours, eight hours,

nine hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, or one day, e.g., from one day to one week, e.g., two days, three days, four days, five days, six days, or one week, e.g., from one week to one month, e.g., two weeks, three weeks, or one month, e.g., from one month to one year, e.g., one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, or one year) after the second dose of the pharmaceutical composition. In some embodiments, the third dose is administered from 1 days to 180 days (e.g., from 1 day to 90 days, from 1 day to 45 days, from one day to 30 days, from 1 day to 14 days, from 1 day to 7 days, from 2 days to 45 days, from 2 days to 30 days, from 2 days to 14 days, from 2 days to 7 days, from 3 days to 90 days, from 3 days to 45 days, from 3 days to 30 days, from 3 days to 14 days, from 3 days to 7 days, from 4 days to 90 days, from 4 days to 45 days, from 4 days to 30 days, from 4 days to 14 days, from 4 days to 7 days, from 5 days to 90 days, from 5 days to 45 days, from 5 days to 30 days, from 5 days to 14 days, from 5 days to 7 days, from 6 days to 90 days, from 6 days to 45 days, from 6 days to 30 days, from 6 days to 14 days, from 6 days to 7 days, from 7 days to 90 days, from 7 days to 45 days, from 7 days to 30 days, from 7 days to 14 days, from 14 days to 90 days, from 14 days to 45 days, from 14 days to 30 days, from 21 days to 90 days, from 21 days to 60 days, from 21 days to 45 days, from 21 days to 30 days, from 30 days to 90 days, from 30 days to 60 days, from 30 days to 45 days, from 45 to 180 days, from 45 to 120 days, from 45 to 100 days, from 45 to 90 days, from 45 to 60 days, from 60 to 180 days, from 60 to 120 days, from 60 to 100 days, from 60 to 90 days, from 90 to 100 days, from 90 to 120 days, or from 90 to 180 days) after the second dose of the pharmaceutical composition.

In some embodiments, the second dose is administered before a serum concentration of the antifusogenic polypeptide is less than about 500 ng/mL in serum of the subject.

In some embodiments, the method maintains a serum concentration of at least 500 ng/mL (e.g., at least 600 ng/mL, 700 ng/mL, 800 ng/mL, 900 ng/mL, 1,000 ng/mL, 1,100 ng/mL, 1,200 ng/mL, 1,300 ng/mL, 1,400 ng/mL, 1,500 ng/mL, 1,600 ng/mL, 1,700 ng/mL, 1,800 ng/mL, 1,900 ng/mL, 2,000 ng/mL, 2,100 ng/mL, 2,200 ng/mL, 2,300 ng/mL, 2,400 ng/mL, 2,500 ng/mL, 2,600 ng/mL, 2,700 ng/mL, 2,800 ng/mL, 2,900 ng/mL, 3,000 ng/mL, or more) of the antifusogenic polypeptide in the subject, e.g., for at least one hour (e.g., at least two hours, three hours, four hours, five hours, six hours, seven hours, eight hours, nine hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, one day, two days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, one year, or longer).

In some embodiments, the method treats or prevents a viral infection in the subject. For example, the pharmaceutical composition may be administered to the subject in an amount and for a duration sufficient to treat or prevent a viral infection. The pharmaceutical composition may be administered to the subject to reduce the risk of a viral infection.

In some embodiments, the method treats or prevents a human immunodeficiency virus (HIV) infection.

In some embodiments, the method treats or prevents a coronavirus infection (e.g., a betacoronavirus infection, e.g., SARS-CoV-2 infection, such as a SARS-CoV-2 infection that produces symptoms of COVID-19).

In some embodiments, the method treats or prevents a Hepatitis C Virus (HCV) infection.

5 In some embodiments, a circular polynucleotide encoding the antifusogenic polypeptide (e.g., a polypeptide of Table 1) is used for reducing viral entry.

In another aspect, featured is a circular polyribonucleotide that includes a polyribonucleotide cargo encoding multiple antifusogenic polypeptides. The polyribonucleotide cargo may include expression sequences encoding the antifusogenic polypeptides. In some embodiments, the
10 antifusogenic polypeptides are directed to the same virus. Alternatively, the antifusogenic polypeptides may be directed to more than one virus.

Definitions

To facilitate the understanding of this disclosure, a number of terms are defined below. Terms
15 defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the disclosure. Terms such as "a", "an," and "the" are not intended to refer to only a singular entity but include the general class of which a specific example may be used for illustration. The term "or" is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternative are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." The
20 terminology herein is used to describe specific embodiments, but their usage is not to be taken as limiting, except as outlined in the claims.

As used herein, any values provided in a range of values include both the upper and lower bounds, and any values contained within the upper and lower bounds.

As used herein, the term "about" refers to a value that is within $\pm 10\%$ of a recited value.

25 As used herein, the term "carrier" is a compound, composition, reagent, or molecule that facilitates the transport or delivery of a composition (e.g., a circular polyribonucleotide) into a cell by a covalent modification of the circular polyribonucleotide, via a partially or completely encapsulating agent, or a combination thereof. Non-limiting examples of carriers include carbohydrate carriers (e.g., an anhydride-modified phyto glycogen or glycogen-type material), nanoparticles (e.g., a nanoparticle that
30 encapsulates or is covalently linked binds to the circular polyribonucleotide), liposomes, fusosomes, ex vivo differentiated reticulocytes, exosomes, protein carriers (e.g., a protein covalently linked to the circular polyribonucleotide), or cationic carriers (e.g., a cationic lipopolymer or transfection reagent).

As used herein, the terms "circular polyribonucleotide," "circular RNA," and "circRNA" are used interchangeably and mean a polyribonucleotide molecule that has a structure having no free ends (i.e., no
35 free 3' or 5' ends), for example a polyribonucleotide molecule that forms a circular or end-less structure through covalent or non-covalent bonds. The circular polyribonucleotide may be, e.g., a covalently closed polyribonucleotide.

As used herein, the term "circularization efficiency" is a measurement of resultant circular polyribonucleotide versus its non-circular starting material.

40 The term "diluent" means a vehicle including an inactive solvent in which a composition described herein (e.g., a composition including a circular polyribonucleotide) may be diluted or dissolved. A diluent

can be an RNA solubilizing agent, a buffer, an isotonic agent, or a mixture thereof. A diluent can be a liquid diluent or a solid diluent. Non-limiting examples of liquid diluents include water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3- butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and 1,3-butanediol. Non-limiting examples of solid diluents include calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, or powdered sugar.

As used herein, the terms “disease,” “disorder,” and “condition” each refer to a state of sub-optimal health, for example, a state that is or would typically be diagnosed or treated by a medical professional.

As used herein, the term “expression sequence” is a nucleic acid sequence that encodes a product, e.g., a peptide or polypeptide (e.g., an antifusogenic polypeptide). An exemplary expression sequence that codes for a peptide or polypeptide can include a plurality of nucleotide triads, each of which can code for an amino acid and is termed as a “codon.”

As used herein, the term “fragment” with respect to a polypeptide or a nucleic acid sequence, e.g., an antifusogenic polypeptide or a nucleic acid sequence encoding an antifusogenic polypeptide, refers to a continuous, less than a whole portion of a sequence of the polypeptide or the nucleic acid. A fragment of a polypeptide or a nucleic acid sequence encoding a polypeptide, for instance, refers to continuous, less than a whole fraction (e.g., at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% of the entire length) of the sequence such as a sequence disclosed herein. It is understood that all the present disclosure contemplates fragments of any antifusogenic polypeptide disclosed herein.

As used herein, the term “Fc domain” refers to a polypeptide chain that includes at least a hinge domain and second and third antibody constant domains (C_H2 and C_H3) or functional fragments thereof (e.g., fragments that that capable of dimerizing and binding to an Fc receptor). The Fc domain can be any immunoglobulin antibody isotype, including IgG, IgE, IgM, IgA, or IgD (e.g., IgG). Additionally, the Fc domain can be an IgG subtype (e.g., IgG1, IgG2a, IgG2b, IgG3, or IgG4) (e.g., IgG1). An Fc domain does not include any portion of an immunoglobulin that can act as an antigen-recognition region, e.g., a variable domain or a complementarity determining region (CDR). Fc domains in the conjugates as described herein can contain one or more changes from a wild-type Fc domain sequence (e.g., 1-10, 1-8, 1-6, 1-4 amino acid substitutions, additions, or deletions) that alter the interaction between an Fc domain and an Fc receptor. Examples of suitable changes are known in the art. Unless otherwise specified herein, numbering of amino acid residues in the IgG or Fc domain is according to the EU numbering system for antibodies, also called the Kabat EU index, as described, for example, in Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991

As used herein, the term “GC content” refers to the percentage of guanine (G) and cytosine (C) in a nucleic acid sequence. The formula for calculation of the GC content is $(G+C) / (A+G+C+U) \times 100\%$ (for RNA) or $(G+C) / (A+G+C+T) \times 100\%$ (for DNA). Likewise, the term “uridine content” refers to the

percentage of uridine (U) in a nucleic acid sequence. The formula for calculation of the uridine content is $U / (A+G+C+U) \times 100\%$. Likewise, the term “thymidine content” refers to the percentage of thymidine (T) in a nucleic acid sequence. The formula for calculation of the thymidine content is $T / (A+G+C+T) \times 100\%$.

5 By “heterologous” is meant to occur in a context other than in the naturally occurring (native) context. A “heterologous” polynucleotide sequence indicates that the polynucleotide sequence is being used in a way other than what is found in that sequence’s native genome. For example, a “heterologous promoter” is used to drive transcription of a sequence that is not one that is natively transcribed by that promoter; thus, a “heterologous promoter” sequence is often included in an expression construct by
10 means of recombinant nucleic acid techniques. The term “heterologous” is also used to refer to a given sequence that is placed in a non-naturally occurring relationship to another sequence; for example, a heterologous coding or non-coding nucleotide sequence is commonly inserted into a genome by genomic transformation techniques, resulting in a genetically modified or recombinant genome.

As used herein, the term “intron fragment” refers to a portion of an intron, where a first intron
15 fragment and a second intron fragment together form an intron, such as a catalytic intron. An intron fragment may be a 5’ portion of an intron (e.g., a 5’ portion of a catalytic intron) or a 3’ portion of an intron (e.g., a 3’ portion of a catalytic intron), such that the 5’ intron fragment and the 3’ intron fragment, together, form a functional intron, such as a functional intron capable of catalytic self-splicing. The term intron fragment is meant to refer to an intron split into two portions. The term intron fragment is not meant
20 to state, imply, or suggest that the two portion or halves are equal in length. The term intron fragment is used synonymously with the term split-intron and may be used instead of the term “half-intron.”

As used herein, the term “linear counterpart” is a polyribonucleotide molecule (and its fragments) having the same or similar nucleotide sequence (e.g., 100%, 95%, 90%, 85%, 80%, 75%, or any percentage therebetween sequence identity) as a circular polyribonucleotide and having two free ends
25 (i.e., the uncircularized version (and its fragments) of the circularized polyribonucleotide). In some embodiments, the linear counterpart (e.g., a pre-circularized version) is a polyribonucleotide molecule (and its fragments) having the same or similar nucleotide sequence (e.g., 100%, 95%, 90%, 85%, 80%, 75%, or any percentage therebetween sequence identity) and same or similar nucleic acid modifications as a circular polyribonucleotide and having two free ends (i.e., the uncircularized version (and its
30 fragments) of the circularized polyribonucleotide). In some embodiments, the linear counterpart is a polyribonucleotide molecule (and its fragments) having the same or similar nucleotide sequence (e.g., 100%, 95%, 90%, 85%, 80%, 75%, or any percentage therebetween sequence identity) and different or no nucleic acid modifications as a circular polyribonucleotide and having two free ends (i.e., the uncircularized version (and its fragments) of the circularized polyribonucleotide). In some embodiments, a
35 fragment of the polyribonucleotide molecule that is the linear counterpart is any portion of linear counterpart polyribonucleotide molecule that is shorter than the linear counterpart polyribonucleotide molecule. In some embodiments, the linear counterpart further includes a 5’ cap. In some embodiments, the linear counterpart further includes a poly adenosine tail. In some embodiments, the linear counterpart further includes a 3’ UTR. In some embodiments, the linear counterpart further includes a 5’ UTR.

40 As used herein, the terms “linear RNA,” “linear polyribonucleotide,” and “linear polyribonucleotide molecule” are used interchangeably and mean polyribonucleotide molecule having a 5’ and 3’ end. One

or both of the 5' and 3' ends may be free ends or joined to another moiety. Linear RNA includes RNA that has not undergone circularization (e.g., is pre-circularized) and can be used as a starting material for circularization through, for example, splint ligation, or chemical, enzymatic, ribozyme- or splicing-catalyzed circularization methods.

5 As used herein, the term "modified ribonucleotide" means a nucleotide with at least one modification to the sugar, the nucleobase, or the internucleoside linkage.

 As used herein, the term "naked delivery" is a formulation for delivery to a cell without the aid of a carrier and without covalent modification to a moiety that aids in delivery to a cell. A naked delivery formulation is free from any transfection reagents, cationic carriers, carbohydrate carriers, nanoparticle
10 carriers, or protein carriers. For example, naked delivery formulation of a circular polyribonucleotide is a formulation that includes a circular polyribonucleotide without covalent modification and is free from a carrier.

 As used herein, the terms "nicked RNA," "nicked linear polyribonucleotide," and "nicked linear polyribonucleotide molecule" are used interchangeably and mean a polyribonucleotide molecule having a
15 5' and 3' end that results from nicking or degradation of a circular RNA.

 The term "pharmaceutical composition" is intended to also disclose that the circular or linear polyribonucleotide included within a pharmaceutical composition can be used for the treatment of the human or animal body by therapy. It is thus meant to be equivalent to "a polyribonucleotide for use in therapy."

20 The term "polynucleotide," as used herein, means a molecule including one or more nucleic acid subunits, or nucleotides, and can be used interchangeably with "nucleic acid" or "oligonucleotide". A polynucleotide can include one or more nucleotides selected from adenosine (A), cytosine (C), guanine (G), thymine (T) and uracil (U), or variants thereof. A nucleotide can include a nucleoside and at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphate (PO₃) groups. A nucleotide can include a nucleobase, a five-
25 carbon sugar (either ribose or deoxyribose), and one or more phosphate groups. Ribonucleotides are nucleotides in which the sugar is ribose. Polyribonucleotides or ribonucleic acids, or RNA, can refer to macromolecules that include multiple ribonucleotides that are polymerized via phosphodiester bonds. Deoxyribonucleotides are nucleotides in which the sugar is deoxyribose. As used herein, a polyribonucleotide sequence that recites thymine (T) is understood to represent uracil (U).

30 As used herein, the term "polyribonucleotide cargo" herein includes any sequence including at least one polyribonucleotide. In embodiments, the polyribonucleotide cargo includes one or multiple expression sequences, wherein each expression sequence encodes a polypeptide. In embodiments, the polyribonucleotide cargo includes one or multiple noncoding sequences, such as a polyribonucleotide having regulatory or catalytic functions. In embodiments, the polyribonucleotide cargo includes a
35 combination of expression and noncoding sequences. In embodiments, the polyribonucleotide cargo includes one or more polyribonucleotide sequence described herein, such as one or multiple regulatory elements, internal ribosomal entry site (IRES) elements, or spacer sequences.

 As used interchangeably herein, the terms "polyA" and "polyA sequence" refer to an untranslated, contiguous region of a nucleic acid molecule of at least 5 nucleotides in length and consisting of
40 adenosine residues. In some embodiments, a polyA sequence is at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 nucleotides in length. In some embodiments, a polyA sequence is

located 3' to (e.g., downstream of) an open reading frame (e.g., an open reading frame encoding a polypeptide), and the polyA sequence is 3' to a termination element (e.g., a stop codon) such that the polyA is not translated. In some embodiments, a polyA sequence is located 3' to a termination element and a 3' untranslated region.

- 5 As used herein, the elements of a nucleic acid are “operably connected” or “operably linked” if they are positioned on the vector such that they can be transcribed to form a linear RNA that can then be circularized into a circular RNA using the methods provided herein.

“Polydeoxyribonucleotides,” “deoxyribonucleic acids,” and “DNA” mean macromolecules that include multiple deoxyribonucleotides that are polymerized via phosphodiester bonds. A nucleotide can be a nucleoside monophosphate or a nucleoside polyphosphate. A nucleotide means a
 10 deoxyribonucleoside polyphosphate, such as, e.g., a deoxyribonucleoside triphosphate (dNTP), which can be selected from deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), uridine triphosphate (dUTP) and deoxythymidine triphosphate (dTTP) dNTPs, that include detectable tags, such as luminescent tags or markers (e.g., fluorophores). A
 15 nucleotide can include any subunit that can be incorporated into a growing nucleic acid strand. Such subunit can be an A, C, G, T, or U, or any other subunit that is specific to one or more complementary A, C, G, T or U, or complementary to a purine (i.e., A or G, or variant thereof) or a pyrimidine (i.e., C, T or U, or variant thereof). In some examples, a polynucleotide is deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or derivatives or variants thereof. In some cases, a polynucleotide is a short interfering RNA
 20 (siRNA), a microRNA (miRNA), a plasmid DNA (pDNA), a short hairpin RNA (shRNA), small nuclear RNA (snRNA), messenger RNA (mRNA), precursor mRNA (pre-mRNA), antisense RNA (asRNA), to name a few, and encompasses both the nucleotide sequence and any structural embodiments thereof, such as single-stranded, double-stranded, triple-stranded, helical, hairpin, etc. In some cases, a polynucleotide molecule is circular. A polynucleotide can have various lengths. A nucleic acid molecule can have a
 25 length of at least about 10 bases, 20 bases, 30 bases, 40 bases, 50 bases, 100 bases, 200 bases, 300 bases, 400 bases, 500 bases, 1 kilobase (kb), 2 kb, 3 kb, 4 kb, 5 kb, 10 kb, 50 kb, or more. A polynucleotide can be isolated from a cell or a tissue. Embodiments of polynucleotides include isolated and purified DNA/RNA molecules, synthetic DNA/RNA molecules, and synthetic DNA/RNA analogs.

Embodiments of polynucleotides, e.g., polyribonucleotides or polydeoxyribonucleotides, may
 30 include one or more nucleotide variants, including nonstandard nucleotide(s), non-natural nucleotide(s), nucleotide analog(s), or modified nucleotides. Examples of modified nucleotides include, but are not limited to diaminopurine, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxymethyl)uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-
 35 isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-D46-isopentenyladenine, uracil-5-oxyacetic acid, wybutosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-
 40 thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid(v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, 2,6-diaminopurine and the like. In some

cases, nucleotides may include modifications in their phosphate moieties, including modifications to a triphosphate moiety. Non-limiting examples of such modifications include phosphate chains of greater length (e.g., a phosphate chain having, 4, 5, 6, 7, 8, 9, 10 or more phosphate moieties) and modifications with thiol moieties (e.g., alpha-thiotriphosphate and beta-thiotriphosphates). In embodiments, nucleic acid molecules are modified at the base moiety (e.g., at one or more atoms that typically are available to form a hydrogen bond with a complementary nucleotide or at one or more atoms that are not typically capable of forming a hydrogen bond with a complementary nucleotide), sugar moiety or phosphate backbone. In embodiments, nucleic acid molecules contain amine -modified groups, such as amino allyl 1-dUTP (aa-dUTP) and aminohexylacrylamide-dCTP (aha-dCTP) to allow covalent attachment of amine reactive moieties, such as N-hydroxysuccinimide esters (NHS). Alternatives to standard DNA base pairs or RNA base pairs in the oligonucleotides of the present disclosure can provide higher density in bits per cubic mm, higher safety (resistant to accidental or purposeful synthesis of natural toxins), easier discrimination in photo-programmed polymerases, or lower secondary structure. Such alternative base pairs compatible with natural and mutant polymerases for de novo or amplification synthesis are described in Betz K, Malyshev DA, Laverigne T, Welte W, Diederichs K, Dwyer TJ, Ordoukhanian P, Romesberg FE, Marx A. Nat. Chem. Biol. 2012 Jul;8(7):612-4, which is herein incorporated by reference for all purposes.

As used herein, "polypeptide" means a polymer of amino acid residues (natural or unnatural) linked together most often by peptide bonds. The term, as used herein, refers to proteins, polypeptides, and peptides of any size, structure, or function. Polypeptides can include gene products, naturally occurring polypeptides, synthetic polypeptides, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing. A polypeptide can be a single molecule or a multi-molecular complex such as a dimer, trimer, or tetramer. They can also include single chain or multichain polypeptides such as antibodies or insulin and can be associated or linked. Most commonly disulfide linkages are found in multichain polypeptides. The term polypeptide can also apply to amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid.

As used herein, the term "prevent" means to reduce the likelihood of developing a disease, disorder, or condition (e.g., a viral infection, e.g., HIV, SARS-CoV-2, HCV, influenza, or RSV), or alternatively, to reduce the severity or frequency of symptoms in a subsequently developed disease or disorder. A therapeutic agent can be administered to a subject who is at increased risk of developing a viral infection relative to a member of the general population in order to prevent the development of, or lessen the severity of, the disease or condition. A therapeutic agent can be administered as a prophylactic, e.g., before development of any symptom or manifestation of a viral infection.

As used herein, the term "regulatory element" is a moiety, such as a nucleic acid sequence, that modifies expression of an expression sequence within the circular or linear polyribonucleotide.

As used herein, a "spacer" refers to any contiguous nucleotide sequence (e.g., of one or more nucleotides) that provides distance or flexibility between two adjacent polynucleotide regions.

A "signal sequence" refers to a polypeptide sequence, e.g., between 10 and 45 amino acids in length, that is present at the N-terminus of a polypeptide sequence of a nascent protein which targets the polypeptide sequence to the secretory pathway.

As used herein, the term "sequence identity" is determined by alignment of two peptide or two nucleotide sequences using a global or local alignment algorithm. Sequences are referred to as "substantially identical" or "essentially similar" when they share at least a certain minimal percentage of sequence identity when optimally aligned (e.g., when aligned by programs such as GAP or BESTFIT using default parameters). GAP uses the Needleman and Wunsch global alignment algorithm to align two sequences over their entire length, maximizing the number of matches and minimizes the number of gaps. Generally, the GAP default parameters are used, with a gap creation penalty = 50 (nucleotides) / 8 (proteins) and gap extension penalty = 3 (nucleotides) / 2 (proteins). For nucleotides the default scoring matrix used is nwsgapdna, and for proteins the default scoring matrix is Blosum62 (Henikoff & Henikoff, 1992, PNAS 89, 915-919). Sequence alignments and scores for percentage sequence identity are determined, e.g., using computer programs, such as the GCG Wisconsin Package, Version 10.3, available from Accelrys Inc., 9685 Scranton Road, San Diego, CA 92121-3752 USA, or EmbossWin version 2.10.0 (using the program "needle"). Alternatively, or additionally, percent identity is determined by searching against databases, e.g., using algorithms such as FASTA, BLAST, etc. Sequence identity refers to the sequence identity over the entire length of the sequence.

As used herein, the term "subject" refers to an organism, such as an animal, plant, or microbe. In embodiments, the subject is a vertebrate animal (e.g., mammal, bird, fish, reptile, or amphibian). In embodiments, the subject is a human. In embodiments, the subject is a non-human mammal. In embodiments, the subject is a non-human mammal such as a non-human primate (e.g., monkeys, apes), ungulate (e.g., cattle, buffalo, bison, sheep, goat, pig, camel, llama, alpaca, deer, horses, donkeys), carnivore (e.g., dog, cat), rodent (e.g., rat, mouse), or lagomorph (e.g., rabbit). In embodiments, the subject is a bird, such as a member of the avian taxa Galliformes (e.g., chickens, turkeys, pheasants, quail), Anseriformes (e.g., ducks, geese), Paleognathae (e.g., ostriches, emus), Columbiformes (e.g., pigeons, doves), or Psittaciformes (e.g., parrots). In embodiments, the subject is an invertebrate such as an arthropod (e.g., insects, arachnids, crustaceans), a nematode, an annelid, a helminth, or a mollusc. In embodiments, the subject is an invertebrate agricultural pest or an invertebrate that is parasitic on an invertebrate or vertebrate host. In embodiments, the subject is a plant, such as an angiosperm plant (which can be a dicot or a monocot) or a gymnosperm plant (e.g., a conifer, a cycad, a gnetophyte, a Ginkgo), a fern, horsetail, clubmoss, or a bryophyte. In embodiments, the subject is a eukaryotic alga (unicellular or multicellular). In embodiments, the subject is a plant of agricultural or horticultural importance, such as row crop plants, fruit-producing plants and trees, vegetables, trees, and ornamental plants including ornamental flowers, shrubs, trees, groundcovers, and turf grasses.

As used herein, the term "antifusogenic polypeptide" refers to a polypeptide, such as a polypeptide of between 10 and 200 amino acids, which inhibits viral fusion-associated events such as viral entry or viral fusion. An antifusogenic polypeptide includes, for example, a polypeptide of Table 1. An antifusogenic polypeptide includes a polypeptide as well as any biologically active fragments thereof (e.g., a fragment of at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, or 500 amino acids). An antifusogenic polypeptide includes, for example, a polypeptide that targets HIV, SARS-CoV-2, HCV, or RSV. In some embodiments, an antifusogenic polypeptide includes a polypeptide having at least 70%, e.g., at least 80%, e.g., at least 85% (e.g., at least 90%, 95%, 97%, 99%, or 100%) sequence identity to any one of SEQ ID NOs: 1-324. An antifusogenic polypeptide also refers to a

polynucleotide (e.g., polyribonucleotide, e.g., circular polyribonucleotide encoding an antifusogenic polypeptide (e.g., a polypeptide of Table 1) or a biologically active fragment thereof.

As used herein, the terms “treat” and “treating” refer to a prophylactic or therapeutic treatment of a viral infection, e.g., HIV, SARS-CoV-2, HCV, influenza, or RSV, in a subject. The effect of treatment can include reversing, alleviating, reducing severity of, curing, inhibiting the progression of, reducing the likelihood of recurrence of the disease or one or more symptoms or manifestations of the viral infection, stabilizing (i.e., not worsening) the state of the viral infection, or preventing the spread of the viral infection as compared to the state or the condition of the viral infection in the absence of the therapeutic treatment.

As used herein, the term “termination element” is a moiety, such as a nucleic acid sequence, that terminates translation of the expression sequence in the circular or linear polyribonucleotide.

As used herein, the term “translation efficiency” is a rate or amount of protein or peptide production from a ribonucleotide transcript. In some embodiments, translation efficiency can be expressed as amount of protein or peptide produced per given amount of transcript that codes for the protein or peptide, e.g., in a given period of time, e.g., in a given translation system, e.g., a cell-free translation system like rabbit reticulocyte lysate.

As used herein, the term “translation initiation sequence” is a nucleic acid sequence that initiates translation of an expression sequence in the circular or linear polyribonucleotide.

As used herein, a “vector” means a piece of DNA that is synthesized (e.g., using PCR), or that is taken from a virus, plasmid, or cell of a higher organism into which a foreign DNA fragment can be or has been inserted for cloning or expression purposes. In some embodiments, a vector can be stably maintained in an organism. A vector can include, for example, an origin of replication, a selectable marker or reporter gene, such as antibiotic resistance or GFP, or a multiple cloning site (MCS). The term includes linear DNA fragments (e.g., PCR products, linearized plasmid fragments), plasmid vectors, viral vectors, cosmids, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), and the like. In one embodiment, the vectors provided herein include a multiple cloning site (MCS). In another embodiment, the vectors provided herein do not include an MCS.

Brief Description of the Drawings

FIG. 1 is a schematic drawing showing the protein domains or regions of various Coronavirus S proteins and sequences of the HR1 and HR2 regions.

FIG. 2 is a schematic drawing showing various antifusogenic polypeptides and sequences derived from the HR2 region of SARS CoV-2.

FIG. 3 is a schematic drawing showing exemplary embodiments of multi ORF antifusogenic polypeptide constructs.

FIGS. 4A and **4B** are graphs showing inhibitory efficacy on fusion using Omicron and Delta pseudo viruses. **FIG. 4A** shows % inhibition of Delta and Omicron using either HR2 Full length or HR2 Full length with a HiBiT tag. **FIG. 4B** shows relative expression of the antifusogenic polypeptide as compared to a mock control.

FIG. 5 is a graph showing in vivo expression of HR2 full length antifusogenic polypeptides with and without HiBiT tag at 6 hours and 24 hours.

FIGS. 6A and 6B are graphs showing in vitro neutralization of pseudovirus of the Wuhan and Omicron strains of SARS CoV-2 using the HR2A polypeptide. **FIG. 6A** shows neutralization of the Wuhan strain of SARS CoV-2 using HR2A. **FIG. 6B** shows neutralization of the Omicron strain of SARS CoV-2 using HR2A.

5 **FIGS. 7A and 7B** are graphs showing the inhibition rate (%) of SARS CoV-2 Pseudovirus Omicron BA4 and BA.5 (**FIG. 7A**) or SARS CoV-1 Pseudovirus (**FIG. 7B**) strains.

FIGS. 8A-8D are graphs showing the inhibition rate (%) of SARS CoV-2 Pseudovirus using full length HR2 (HR2Complete). **FIG. 8A** shows inhibition of Wuhan strain. **FIG. 8B** shows inhibition of Omicron BA.4 and BA. 5 strain. **FIG. 8C** shows inhibition of Omicron BA.1 strain. **FIG. 8D** shows
10 inhibition of SARS CoV-1 Pseudovirus.

FIG. 9 is a schematic drawing showing construct designs and sequences for various HIV antifusogenic polypeptides.

FIGS. 10A and 10B are a graph (**FIG. 10A**) and a table (**FIG. 10B**) showing expression of various HIV antifusogenic polypeptides from circular RNA.

15 **FIGS. 11A and 11B** are graphs showing expression of various HIV antifusogenic polypeptides from circular RNA (**FIG. 11A**) or plasmid DNA (**FIG. 11B**)

FIG. 12 is a table showing expression of various HIV antifusogenic polypeptides from circular RNA.

20 Detailed Description

The present invention features compositions containing a circular polyribonucleotide (circular RNA) encoding an antifusogenic polypeptide and methods of use thereof. Circular polyribonucleotides described herein are particularly useful for delivering a polynucleotide cargo encoding an antifusogenic polypeptide to a target cell.

25 The circular polyribonucleotide may include a polyribonucleotide cargo encoding a polypeptide of Table 1. In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding a polypeptide having at least 85% (e.g., at least 90%, 95%, 97%, 99%, or 100%) sequence identity to a polypeptide of Table 1. In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding a polypeptide having at least 85% (e.g., at least 90%, 95%, 97%, 99%, or 100%)
30 sequence identity to any one of SEQ ID NOs: 1-324.

The circular polyribonucleotide may be produced from a precursor, such as a linear deoxyribonucleotide, a circular deoxyribonucleotide, or a circular polyribonucleotide.

A circular polyribonucleotide may include, for example, a splice junction joining a 5' exon fragment and a 3' exon fragment.

35 The linear RNA molecules described herein a polyribonucleotide encoding an antifusogenic polypeptide. In some embodiments, the linear RNA molecules include, from 5' to 3', (A) a 3' catalytic intron fragment; (B) a 3' splice site; (C) a 3' exon fragment; (D) a polyribonucleotide cargo encoding an antifusogenic polypeptide (e.g., polyribonucleotide cargo encoding an IRES operably linked to an expression sequence encoding an antifusogenic polypeptide); (E) a 5' exon fragment; (F) a 5' splice site;
40 and (G) a 5' catalytic intron fragment. The catalytic intron fragments and splice sites may then allow the

linear polyribonucleotide to self-splice, thus forming a circular polyribonucleotide encoding an antifusogenic polypeptide.

Also featured are methods of using a circular polyribonucleotide as described herein. For example, the circular polyribonucleotide can be formulated as a composition (e.g., a pharmaceutical composition) for administration to a subject, e.g., a human subject. The pharmaceutical composition may be administered in one or more doses of the composition. The composition may be administered to the subject to treat or prevent a viral infection (e.g., HIV, SARS-CoV-2, HCV, influenza, or RSV).

Polynucleotides

The disclosure features circular polyribonucleotide compositions encoding an antifusogenic polypeptide, uses thereof, and methods of making circular polyribonucleotides encoding an antifusogenic polypeptide. In some embodiments, a circular polyribonucleotide is produced from a linear polyribonucleotide (e.g., by self-splicing compatible ends of the linear polyribonucleotide). In some embodiments, a linear polyribonucleotide is transcribed from a deoxyribonucleotide template (e.g., a vector, a linearized vector, or a cDNA). Accordingly, the disclosure features deoxyribonucleotides, linear polyribonucleotides, and circular polyribonucleotides and compositions thereof useful in the production of circular polyribonucleotides encoding an antifusogenic polypeptide.

Template Deoxyribonucleotides

The present invention features a template deoxyribonucleotide for making a circular RNA as described herein. In embodiments, the deoxyribonucleotide includes the following, operably linked in a 5'-to-3' orientation: (A) a 3' catalytic intron fragment; (B) a 3' splice site; (C) a 3' exon fragment; (D) a polyribonucleotide cargo encoding an antifusogenic polypeptide; (E) a 5' exon fragment; (F) a 5' splice site; and (G) a 5' catalytic intron fragment. In embodiments, the deoxyribonucleotide includes further elements, e.g., outside of or between any of elements (A), (B), (C), (D), (E), (F), or (G). In embodiments, any of the elements (A), (B), (C), (D), (E), (F), or (G) is separated from each other by a spacer sequence, as described herein.

In embodiments, the deoxyribonucleotide is, for example, a circular DNA vector, a linearized DNA vector, or a linear DNA (e.g., a cDNA, e.g., produced from a DNA vector).

In some embodiments, the deoxyribonucleotide further includes an RNA polymerase promoter operably linked to a sequence encoding a linear RNA described herein. In embodiments, the RNA polymerase promoter is heterologous to the sequence encoding the linear RNA. In some embodiments, the RNA polymerase promoter is a T7 promoter, a T6 promoter, a T4 promoter, a T3 promoter, an SP6 virus promoter, or an SP3 promoter.

In some embodiments, the deoxyribonucleotide includes a multiple-cloning site (MCS).

In some embodiments, the deoxyribonucleotide is used to produce circular RNA with the size range of about 100 to about 20,000 nucleotides. In some embodiments, the circular RNA is at least 100, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, 3,000, 3,500, 4,000, 4,500 or 5,000 nucleotides in size. In some embodiments, the circular RNA is no more than 20,000, 15,000, 10,000, 9,000, 8,000, 7,000, 6,000, 5,000 or 4,000 nucleotides in size.

Linear Polyribonucleotides

The present invention also features linear polyribonucleotides encoding an antifusogenic polypeptide. The linear polyribonucleotide may be used to create a circular polyribonucleotide, e.g., by ligating or splicing (e.g., self-splicing) the linear polyribonucleotide to produce the circular polyribonucleotide. In embodiments, the linear polyribonucleotide includes the following, operably linked in a 5'-to-3' orientation: (A) a 3' catalytic intron fragment; (B) a 3' splice site; (C) a 3' exon fragment; (D) a polyribonucleotide cargo encoding an antifusogenic polypeptide; (E) a 5' exon fragment; (F) a 5' splice site; and (G) a 5' catalytic intron fragment. In embodiments, the linear polyribonucleotide includes further elements, e.g., outside of or between any of elements (A), (B), (C), (D), (E), (F), or (G). For example, any of elements (A), (B), (C), (D), (E), (F), or (G) may be separated by a spacer sequence, as described herein.

In certain embodiments, provided herein is a method of generating linear RNA encoding an antifusogenic polypeptide by performing transcription in a cell-free system (e.g., in vitro transcription) using a deoxyribonucleotide (e.g., a vector, linearized vector, or cDNA) encoding an antifusogenic polypeptide provided herein as a template (e.g., a vector, linearized vector, or cDNA provided herein with an RNA polymerase promoter positioned upstream of the region that codes for the linear RNA).

In embodiments, a deoxyribonucleotide template is transcribed to produce a linear RNA containing the components described herein. Upon expression, the linear polyribonucleotide produces a splicing-compatible polyribonucleotide, which may be self-spliced in order to produce a circular polyribonucleotide.

In some embodiments, the linear polyribonucleotide is from 50 to 20,000, 100 to 20,000, 200 to 20,000, 300 to 20,000 (e.g., 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, 3,000, 3,500, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 11,000, 12,000, 13,000, 14,000, 15,000, 16,000, 17,000, 18,000, 19,000, or 20,000) ribonucleotides in length. In embodiments, the linear polyribonucleotide is, e.g., at least 500, at least 1,000, at least 2,000, at least 3,000, at least 4,000, or at least 5,000 ribonucleotides in length.

Circular Polyribonucleotides

In some embodiments, the invention features a circular polyribonucleotide including an expression sequence encoding an antifusogenic polypeptide. In embodiments, the polyribonucleotide includes an IRES operably linked to an expression sequence encoding an antifusogenic polypeptide. The circular polyribonucleotide may include a splice junction, e.g., joining a 5' exon fragment and a 3' exon fragment. The circular polyribonucleotide may include any one or more of the elements described herein. In some embodiments, the circular polyribonucleotide includes any feature or any combination of features as disclosed in International Patent Publication No. WO2019/118919, which is hereby incorporated by reference in its entirety.

In embodiments, the circular polynucleotide further includes a polyribonucleotide cargo. In embodiments, the polyribonucleotide cargo includes an expression (or coding) sequence, a non-coding sequence, or a combination of an expression (coding) sequence and a non-coding sequence. In embodiments, the polyribonucleotide cargo includes an expression (coding) sequence encoding a polypeptide. In embodiments, the polyribonucleotide includes an IRES operably linked to an expression

sequence encoding a polypeptide. In some embodiments, the IRES is located upstream of the expression sequence. In some embodiments, the IRES is located downstream of the expression sequence. In some embodiments, the circular polyribonucleotide further includes a spacer region between the IRES and the 3' exon fragment or the 5' exon fragment. The spacer region may be, *e.g.*, at least 5 (*e.g.*, at least 10, at least 15, at least 20) ribonucleotides in length. The spacer region may be, *e.g.*, from 5 to 500 (*e.g.*, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, or 500) ribonucleotides. In some embodiments, the spacer region includes a polyA sequence. In some embodiments, the spacer region includes a polyA-C sequence. In some embodiments, the spacer region includes a polyA-G sequence. In some embodiments, the spacer region includes a polyA-T sequence. In some embodiments, the spacer region includes a random sequence. In some embodiments, the first annealing region and the second annealing region are joined, thereby forming a circular polyribonucleotide.

In some embodiments, the circular RNA is produced by a deoxyribonucleotide template or a linear RNA described herein. In some embodiments, the circular RNA is produced by any of the methods described herein.

In some embodiments, the circular polyribonucleotide is at least about 20 nucleotides, at least about 30 nucleotides, at least about 40 nucleotides, at least about 50 nucleotides, at least about 75 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, at least about 300 nucleotides, at least about 400 nucleotides, at least about 500 nucleotides, at least about 1,000 nucleotides, at least about 2,000 nucleotides, at least about 5,000 nucleotides, at least about 6,000 nucleotides, at least about 7,000 nucleotides, at least about 8,000 nucleotides, at least about 9,000 nucleotides, at least about 10,000 nucleotides, at least about 12,000 nucleotides, at least about 14,000 nucleotides, at least about 15,000 nucleotides, at least about 16,000 nucleotides, at least about 17,000 nucleotides, at least about 18,000 nucleotides, at least about 19,000 nucleotides, or at least about 20,000 nucleotides.

In some embodiments, the circular polyribonucleotide is between 500 nucleotides and 20,000 nucleotides, between 1,000 and 20,000 nucleotides, between 2,000 and 20,000 nucleotides, or between 5,000 and 20,000 nucleotides. In some embodiments, the circular polyribonucleotide is between 500 nucleotides and 10,000 nucleotides, between 1,000 and 10,000 nucleotides, between 2,000 and 10,000 nucleotides, or between 5,000 and 10,000 nucleotides.

As a result of its circularization, the circular polyribonucleotide may include certain characteristics that distinguish it from linear RNA. For example, the circular polyribonucleotide is less susceptible to degradation by exonuclease as compared to linear RNA. As such, the circular polyribonucleotide is more stable than a linear RNA, especially when incubated in the presence of an exonuclease. The increased stability of the circular polyribonucleotide compared with linear RNA makes circular polyribonucleotide more useful as a cell transforming reagent to produce polypeptides and can be stored more easily and for longer than linear RNA. The stability of the circular polyribonucleotide treated with exonuclease can be tested using methods standard in art which determine whether RNA degradation has occurred (*e.g.*, by gel electrophoresis). Moreover, unlike linear RNA, the circular polyribonucleotide is less susceptible to dephosphorylation when the circular polyribonucleotide is incubated with phosphatase, such as calf intestine phosphatase.

The circular polyribonucleotides described herein and compositions or pharmaceutical compositions thereof may be used in therapeutic and veterinary methods of dosing to produce a level of circular polyribonucleotide, a level of binding to a target, or a level of protein in a plurality of cells after providing the plurality with at least two doses of circular polyribonucleotide. In some embodiments, the circular polyribonucleotide is non-immunogenic in a mammal, e.g., a human. In some embodiments, the circular polyribonucleotide is capable of replicating or replicates in a cell from an aquaculture animal (fish, crabs, shrimp, oysters etc.), a mammalian cell, e.g., a cell from a pet or zoo animal (cats, dogs, lizards, birds, lions, tigers and bears etc.), a cell from a farm or working animal (horses, cows, pigs, chickens etc.), a human cell, cultured cells, primary cells or cell lines, stem cells, progenitor cells, differentiated cells, germ cells, cancer cells (e.g., tumorigenic, metastatic), non-tumorigenic cells (normal cells), fetal cells, embryonic cells, adult cells, mitotic cells, non-mitotic cells, or any combination thereof. In some embodiments, the invention includes a cell that includes the circular polyribonucleotide described herein, wherein the cell is a cell from an aquaculture animal (fish, crabs, shrimp, oysters etc.), a mammalian cell, e.g., a cell from a pet or zoo animal (cats, dogs, lizards, birds, lions, tigers and bears etc.), a cell from a farm or working animal (horses, cows, pigs, chickens etc.), a human cell, a cultured cell, a primary cell or a cell line, a stem cell, a progenitor cell, a differentiated cell, a germ cell, a cancer cell (e.g., tumorigenic, metastatic), a non-tumorigenic cell (normal cells), a fetal cell, an embryonic cell, an adult cell, a mitotic cell, a non-mitotic cell, or any combination thereof. In some embodiments, the cell is modified to include the circular polyribonucleotide.

In some embodiments, the circular polyribonucleotide includes sequences for expression products. In some embodiments, the circular polyribonucleotide includes a binding site for binding to a target. In some embodiments, the circular polyribonucleotide is provided to a plurality of cells via any a dosing regimen described herein. In some embodiments, the circular polyribonucleotide as described herein induces a response or response level in a subject. In some embodiments, the expression products encoded by the sequences included in the circular polyribonucleotide are expressed in one or more of cells in the plurality of cells.

In some embodiments, the circular polyribonucleotide has a half-life of at least that of a linear counterpart, e.g., linear expression sequence, or linear polyribonucleotide. In some embodiments, the circular polyribonucleotide has a half-life that is increased over that of a linear counterpart. In some embodiments, the half-life is increased by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, or more. In some embodiments, the circular polyribonucleotide has a half-life or persistence in a cell for at least about 1 hour, e.g., at least 2 hours, 3 hours, 4 hours, 5 hours 6 hours, 12 hours, 24 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 2 weeks, 3 weeks, 4 weeks, 2 months, 3 months, 6 months, or longer. In some embodiments, the circular polyribonucleotide has a half-life or persistence in a cell for from about 1 hour to about 60 days, e.g., about 1 hour, 2 hours, 6 hours, 12 hours, 18 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 35 days, 40 days, 45 days, 50 days, 55 days, or 60 days. In some embodiments, the circular polyribonucleotide has a half-life or persistence in a cell while the cell is dividing. In some embodiments, the circular polyribonucleotide has a half-life or persistence in a cell post division. In certain embodiments, the circular polyribonucleotide has a half-life or persistence

in a dividing cell for at least about 10 minutes, e.g., at least about 1 hour, e.g., at least 2 hours, 3 hours, 4 hours, 5 hours 6 hours, 12 hours, 24 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 2 weeks, 3 weeks, 4 weeks, 2 months, 3 months, 6 months, or longer. In certain embodiments, the circular polyribonucleotide has a half-life or persistence in a dividing cell of from about 10 minutes to about 60 days, e.g., about 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, or 60 days.

In some embodiments, the circular polyribonucleotide modulates a cellular function, e.g., transiently, or long term. In certain embodiments, the cellular function is stably altered, such as a modulation that persists for at least about 10 minutes, e.g., at least about 1 hour, e.g., at least 2 hours, 3 hours, 4 hours, 5 hours 6 hours, 12 hours, 24 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 2 weeks, 3 weeks, 4 weeks, 2 months, 3 months, 6 months, or longer. In certain embodiments, the cellular function is stably altered, such as a modulation that persists for from about 1 hour to about 60 days, e.g., from about 1 hour to about 30 days, e.g., for at least about 2 hours, 6 hours, 12 hours, 18 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, or 60 days.

Elements of Polynucleotides

The polynucleotides (e.g., circular polyribonucleotides) described herein may include any one or more of the elements described herein and an expression sequence encoding an antifusogenic polypeptide.

Antifusogenic Polypeptides

The disclosure provides circular polyribonucleotides that encode at least one expression sequence encoding an antifusogenic polypeptide. In some embodiments, the antifusogenic polypeptide inhibits viral entry. In some embodiments, the antifusogenic polypeptide inhibits viral fusion.

In some embodiments, the antifusogenic polypeptide is a polypeptide or a variant thereof including an amino acid sequence selected from a sequence of Table 1. In some embodiments, the antifusogenic polypeptide is a polypeptide including a contiguous stretch of at least 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acids of a sequence in Table 1. In some embodiments, the antifusogenic polypeptide is a polypeptide including a contiguous stretch of at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the amino acids of a sequence in Table 1. In some embodiments, the antifusogenic polypeptide is a polypeptide including a sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% sequence identity to a sequence of Table 1. In some embodiments, the antifusogenic polypeptide is a variant of a sequence in Table 1 that includes no more than one, two, three, four, five, six, seven, eight, nine, or ten mutations (e.g., substitutions, deletions, or insertions). In some embodiments, the circular polyribonucleotide includes an expression sequence encoding more than one antifusogenic polypeptide.

In some embodiments, the circular polyribonucleotide encoding more than one fusion protein reduces the chance for viral escape.

- 5 In some embodiments, the antifusogenic polypeptide targets one or more (e.g., two, three, four, or five) viruses. Viruses that may be targeted by the antifusogenic polypeptide include, but are not limited to, all strains of viruses listed in Table 1. In some embodiments, the virus does not infect humans. In some embodiments, the virus infects humans.

Table 1. Exemplary antifusogenic polypeptides

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Golden oyster mushroom, <i>Pleurotus citrinopileatus</i>	Antiviral protein Y3	Tobacco mosaic virus (TMV), Influenza hemagglutinin (HA), Human immunodeficiency virus (HIV) pseudovirions	AACARFIDDFCDLTPNIYR PRDNGQRCYAVNGHRCDF TVFNTNNGGNPIRASTPNC KTVLRTAANRCPTGGRGKI NPNAPFLFAIDPNDGDCST NF	1
Daruma pond frog, <i>Rana brevipoda porsa</i>	Brevinin-1	Herpes simplex virus-1 (HSV-1), Herpes simplex virus-2 (HSV-2)	FLPVLAGIAAKVVPALFCKIT KKC	2
European common frog, <i>Rana temporaria</i>	Temporin B	CCV, FV3	LLPIVGNLLKSL	3
European common frog, <i>Rana temporaria</i>	Temporin G	Influenza A virus (IAV), Parainfluenza respiratory viruses (PIV)	FFPVIGRILNGIL	4
North American frog, <i>Rana pipiens</i> and Oregon spotted frog, <i>Rana pretiosa</i>	Ranateurin 3P	HIV	GLMDTVKKNVAKNLAGHMLD KLKCKITGC	5
Human, <i>Homo sapiens</i>	Human neutrophil peptide-1 (HNP-1)	Pseudotyped viruses expressing SARS-CoV-2 spike proteins	ACYCRIPACIAGERRYGTCT YQGRLWAFCC	6

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Human, <i>Homo sapiens</i>	HNP-2	Pseudotyped viruses expressing SARS-CoV-2 spike proteins	CYCRIPACIAGERRYGTCIY QGRLWAFCC	7
Human, <i>Homo sapiens</i>	HNP-3	Pseudotyped viruses expressing SARS-CoV-2 spike proteins	DCYCRIPACIAGERRYGTCTI YQGRLWAFCC	8
Human, <i>Homo sapiens</i>	HNP-4	Pseudotyped viruses expressing SARS-CoV-2 spike proteins	VCSCRLVFCRRELTVGNC LIGGVSTFYCCTRV	9
Human, <i>Homo sapiens</i>	Human defensin (HD-5)	BK virus (BKV)	ATCYCRTGRCATRESLSGV CEISGRLYRLCCR	10
Human, <i>Homo sapiens</i>	HD-6	Respiratory syncytial virus (RSV), human PIV (HPIV), HIV, HSV, influenza A virus (IAV), BKV, human adenoviruses (HAdV), human papilloma virus (HPV)	AFTCHCRRSCYSTEYSYGT CTVMGINHRFCCL	11
Rabbit, <i>Oryctolagus cuniculus</i>	Rabbit neutrophil peptide 1 (NP-1)	HSV, enveloped viruses	VVCACRRALCLPRERRAGF CRIRGRIHPLCCRR	12
Rabbit, <i>Oryctolagus cuniculus</i>	Rabbit neutrophil defensin 2 (NP-2)	RSV, HIV, HSV	VVCACRRALCLPLERRAGF CRIRGRIHPLCCRR	13
Porcine neutrophil, <i>Sus scrofa</i>	Protegrin 1 (PG-1)	HIV, HSV-1, HSV-2, Porcine reproductive and respiratory syndrome virus (PRRSV)	RGGRLCYCRRRFCVCVGR	14
Atlantic horseshoe crab, <i>Limulus polyphemus</i>	Polyphemusin I	HIV	RRWCFRVCYRGFCYRKCR	15

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Atlantic horseshoe crab, <i>Limulus polyphemus</i>	Polyphemusin II	HIV	RRWCFRVCYKGFCYRKCR	16
Southeast Asian hemocytes, <i>Tachypleus tridentatus</i> , <i>Tachypleus gigas</i> , <i>Carcinoscorpius rotundicauda</i>	Tachyplesin I (TP1)	HIV, HSV-1, HSV-2, Singapore grouper iridovirus (SGIV)	KWCFRVCYRGICYRRCR	17
Rabbit, <i>Oryctolagus cuniculus</i>	Rabbit neutrophil defensin 3a (NP-3a)	RSK, HIV, HSV	GICACRRRFCPNSERFSGY CRVNGARYVRCCSRR	18
Porcine neutrophil, <i>Sus scrofa</i>	PG-2	Pseudorabies virus (PRV), PRRSV	RGGRLCYCRRRFCICV	19
Porcine neutrophil, <i>Sus scrofa</i>	PG-3	PRRSV	RGGGLCYCRRRFCVCVGR	20
Porcine neutrophil, <i>Sus scrofa</i>	PG-4	PRRSV	RGGRLCYCRGWICFCVGR	21
Rat, <i>Rattus norvegicus</i>	Rat NP-1, rat defensin	HIV, HSV	VTICYRRTRCGFRERLSGA CGYRGRIYRLCCR	22
Rat, <i>Rattus norvegicus</i>	Rat NP-2	HIV, HSV	VTICYRSTRCGFRERLSGA CGYRGRIYRLCCR	23
Rat, <i>Rattus norvegicus</i>	Rat NP-3	HIV, HSV	CSCRTSSCRFGERLSGACR LNGRIYRLCC	24
Rat, <i>Rattus norvegicus</i>	Rat NP-4	HIV, HSV	ACYCRIGACVSGERLTGAC GLNGRIYRLCCR	25
Australian green tree frog, <i>Litoria splendida</i> , <i>Litoria rothii</i>	Caerin 1.1	HIV, Murine leukemia virus	GLLSVLGSVAKHVLPVVP VIAEHL	26

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Australian green tree frog, <i>Litoria caerulea</i>	Caerin 1.2	HIV	GLLGVLGSAKHVLPVVPVIAEHL	27
Australian green tree frog, <i>Litoria caerulea</i>	Caerin 1.3	HIV	GLLSVLGSAQHVLPVVPVIAEHL	28
Australian green tree frog, <i>Litoria caerulea</i>	Caerin 1.4	HIV	GLLSSLSSVAKHVLPVVPVIAEHL	29
Australian green tree frog, <i>Litoria caerulea</i>	Caerin 1.5	HIV	GLLSVLGSVVKHVLPVVPVIAEHL	30
Australian orange-thighed frog, <i>Litoria xanthomera</i>	Caerin 1.6	HIV	GLFSVLGAVAKHVLPVVPVIAEK	31
Australian orange-thighed frog, <i>Litoria xanthomera</i>	Caerin 1.7	HIV	GLFKVLGSAKHLLPHVAPVIAEK	32
Australian green tree frog, <i>Litoria caerulea</i>	Caerin 1.9	HIV	GLFGVLGSIKHVLPVVPVIAEKL-NH ₂	33
Australian green tree frog, <i>Litoria caerulea</i>	Caerin 1.10	HIV	GLLSVLGSAKHVLPVVOVIAEKL-NH ₂	34
Australian green tree frog, <i>Litoria caerulea</i>	Caerin 1.19	HIV	GLFKVLGSAKHLLPHVAPIIAEKL-NH ₂	35
Australian green tree frog, <i>Litoria caerulea</i>	Caerin 1.20	HIV	GLFGILGSAKHVLPVVPVIAEHL-NH ₂	36
Australian green tree frog, <i>Litoria caerulea</i>	Caerin 4.1	HIV	GLWQKIKSAAGDLASGIVEGIKS	37
Mouse, <i>Mus musculus</i>	Murine beta-defensin 1 (mBD-1)	HIV, IAV	DQYKCLQHGGFCLRSSCP SNTKLQGTCKPDKPNCKS	38

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Mouse, <i>Mus musculus</i>	Mouse cathelin-related antimicrobial peptide (mCRAMP)	RSV	GLLRKGGEKIGEKLLKIGQK IKNFFQKLVPQPEQ	39
Human, <i>Homo sapiens</i>	Human beta defensin 3 (hBD-3)	HIV	GIINTLQKYCYCRVRGGRCA VLSCLPKKEEQIGKCSTRGR KCCRRKK	40
Southern brown tree frog, <i>Litoria ewingii</i>	Ewingiin 2.1 (formerly named Caeridin 7.1)	HIV	GLLDMVTGLLGNL-NH2	41
Australian tree frog, <i>Litoria genimaculata</i>	Maculatin 1.1	HIV, PRV	GLFGVLAKVAAHVPAIAEH F-NH2	42
Hybrid striped bass, <i>Morone saxatilis</i> , <i>Morone chrysops</i>	Piscidin 1H (Pis-1H)	HIV	FFHHIFRGIVHVGKTIHRLVT G	43
Hybrid striped bass, <i>Morone saxatilis</i> , <i>Morone chrysops</i>	Piscidin 1N (Pis-1N)	HIV	FFHHIFRGIVHVGKTIHRLVT G	44
Hybrid striped bass, <i>Morone saxatilis</i> , <i>Morone chrysops</i>	Piscidin 2 (Pis-2)	HIV	FFHHIFRGIVHVGKTIHKLVT G	45
Hybrid striped bass, <i>Morone saxatilis</i> , <i>Morone chrysops</i>	Piscidin 3 (Pis-3)	HIV	FIHHIFRGIVHAGRSIGRFLT G	46
Blue mussel, <i>Mytilus edulis</i>	Mytilin B	Viral hemorrhagic septicemia virus (VHSV) VHSV, IPNV	SCASRCKGHCRARRCGYY VSVLYRGRCYCKCLRC	47

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Mediterranean mussel, <i>Mytilus galloprovincialis</i>	Mytilin C	VHSV, IPNV	SCASRCKSRCRARRCRY VSVRYGWFCYCRCLRC	48
Fungus growing termite, <i>Pseudocanthotermes spiniger</i>	Spinigerin	HIV	HVDKKVADKVLKQLRIMR LLTRL	49
North American frog, <i>Rana catesbeiana</i>	Ranateurin 6	HIV	FISAIASMLGKFL	50
North American frog, <i>Rana catesbeiana</i>	Ranateurin 9	HIV	FLFPKITSFLSKVL	51
Rhesus macaque, <i>Macaca mulatta</i>	Rhesus theta defensin-1 (RTD-1)	HIV	GFCRCLCRRGVCRCICTR	52
Human, <i>Homo sapiens</i>	Human beta-defensin 1 (hBD-1)	HIV, HSV	DHYNCVSSGGQCLYSACPI FTKIQGTCTYRGKAKCK	53
Human, <i>Homo sapiens</i>	hBD-2	HIV, HSV	GIGDPVTCLKSGAICHPVFC PRRYKQIGTCGLPGTKCK KP	54
<i>Streptomyces</i> strain AA3891	Siamycin II	HIV	CLGIGSCNDFAGCGYAIVCF W	55
North American mink frog, <i>Rana septentrionalis</i>	Brevinin-2-related peptide	HIV	GIWDTIKSMGKVFAGKILQN L	56
Guinea pig, <i>Cavia porcellus</i>	CAP11	IAV	GLRKKFRKTRKRIQKLGRKI GKTGRKVWKAWREYQGIP YPCRI	57
Rhesus macaque, <i>Macaca mulatta</i>	RTD-2	HIV	GVCRCCLCRRGVCRCCLCRR	58
Rhesus macaque, <i>Macaca mulatta</i>	RTD-3	HIV	GFCRCICTRGFCRCICTR	59

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
African clawed frog, <i>Xenopus laevis</i>	Magainin 1	HSV-1, HSV-2	GIGKFLHSAGKFGKAFVGEI MKS	60
African herb, <i>Oldenlandia affinis</i>	Kalata B1 (KB1)	HIV	GLPVCGETCVGGTCNTPG CTCSWPVCTRN	61
Chicken, <i>Gallus gallus domesticus</i> , Duck, <i>Anas platyrhynchos</i>	Chicken avian beta defensin 6 (AvBD-6)	IAV	SPIHACRYQRGVCIPGPCR WPYYRVGSCGSLKSCCV RNRWA	62
Chicken, <i>Gallus gallus domesticus</i>	AvBD-5	IAV	GLPQDCERRGGFCSHKSC PPGIGRIGLCSKEDFCCRSR WYS	63
Chinese red belly toad, <i>Bombina maxima</i>	Maximin H1	Nipah virus (NiV)	ILGPVISTIGGVLGGLLKNL	64
Sahara frog, <i>Pelophylax saharicus</i>	Temporin-Sha	HSV	FLSGIVGMLGKLF	65
<i>Leonia cymosa</i>	Cycloviolin A	HIV	GVIPCGESCVFIPCISAAIGC SCKNKVCYRN	66
<i>Leonia cymosa</i>	Cycloviolin B	HIV	GTACGESCVLPCFTVGCT CTSSQCFKN	67
<i>Leonia cymosa</i>	Cycloviolin C	HIV	GIPCGESCVFIPCLTTVAGC SCKNKVCYRN	68
<i>Leonia cymosa</i>	Cycloviolin D	HIV	GFPCGESCVFIPCISAAIGC SCKNKVCYRN	69
European field pansy, <i>Viola arvensis</i>	Varv peptide E (Varv E)	HIV	GLPICGETCVGGTCNTPGC SCSWPVCTRN	70
African herb, <i>Oldenlandia affinis</i>	KB8	HIV	GSVLNCGETCLLGTCYTTG CTCNKYRVCTKD	71
<i>Palicourea condensata</i>	Palicourein	HIV	GDPTFCGETCRVIPVCTYS AALGCTCDDRSGLCKRN	72

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Arrowhead violet, <i>Viola betonicifolia</i>	KB2	HIV	GLPVCGETCFGGTCNTPG CSCTWPICTRD	73
Australian violet, <i>Viola hederacea</i>	Vhl-1	HIV	SISCGESCAMISFCFTEVIG CSCKNKVCYLN	74
Tropical tree, <i>Chassalia parvifolia</i>	Circulin C	HIV	GIPCGESCVFIPCITSVAGC SCKSKVCYRN	75
Tropical tree, <i>Chassalia parvifolia</i>	Circulin D	HIV	KIPCGESCVWIPCVTSIFNC KCKENKVCYHD	76
Tropical tree, <i>Chassalia parvifolia</i>	Circulin E	HIV	KIPCGESCVWIPCLTSVFNC KCENKVCYHD	77
Tropical tree, <i>Chassalia parvifolia</i>	Circulin F	HIV	KVCYRAIPCGESCVWIPCIS AAIGCSCKN	78
Tilapia, <i>Oreochromis mossambicus</i>	Hepcidin 1-5 (TH1-5)	Nervous necrosis virus (NNV)	GIKCRFCCGCCTPGICGVC CRF	79
Sweet violet, <i>Viola odorata</i>	Cycloviolacin O13	HIV	GIPCGESCVWIPCISAAIGC SCKSKVCYRN	80
Sweet violet, <i>Viola odorata</i>	Cycloviolacin O14	HIV	GSIPACGESCFKGKCYTPG CSCSKYPLCAKN	81
Sweet violet, <i>Viola odorata</i>	Cycloviolacin O24	HIV	GLPTCGETCFGGTCNTPGC TCDPWPVCTHN	82
Chinese herb, <i>Viola yedoensis</i>	Cycloviolacin Y1	HIV	GGTIFDCGETCFLGTCYTP GCSCGNYGFCYGTN	83
Chinese herb, <i>Viola yedoensis</i>	Cycloviolacin Y4	HIV	GVPCGESCVFIPCITGVIGC SCSSNVCYLN	84
Chinese herb, <i>Viola yedoensis</i>	Cycloviolacin Y5	HIV	GIPCAESCVWIPCTVTALVG CSCSDKVCYN	85
Chinese herb, <i>Viola yedoensis</i>	Cycloviolacin VY1	IAV	CGESCVFIPCITTVLGCS CSI KVCYKNGSIP	86
<i>Streptomyces</i> strain AA6532	Siamycin I	HIV	CLGVGSCNDFAGCGYAVV CFW	87
<i>Streptomyces</i> strain AA6532	NP-06	HIV	CLGVGSCNDFAGCGYAIVC FW	88

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Human, <i>Homo sapiens</i>	Retrocyclin-1 (RC1)	HIV	GICRCICGRGICRCICGR	89
Human, <i>Homo sapiens</i>	RC2	HIV	GICRCICGRRICRCICGR	90
Human, <i>Homo sapiens</i>	RC3	HIV	RICRCICGRRICRCICGR	91
Red sea turtle, <i>Caretta caretta</i>	Turtle egg white protein (TEWP)	Rhabdovirus Chandipura virus	QKKCPGRCTLKCGKHERPT LPYNCCKYICCVPVKVK	92
Mediterranean mussel, <i>Mytilus galloprovincialis</i>	Myticin C (Myt C)	VHSV	QEAQSVACTSYYCSKFCGS AGCSLYGCYLLHPGKICYCL HCSR	93
Australian white-lipped tree frog, <i>Litoria infrafrenata</i>	Frenatin 2	Yellow fever virus (YFV)	GLLGTLGNLLNGLGL	94
Coconut, <i>Cocos nucifera</i>	Coconut antifungal peptide	HIV	EQCREEDDR	95
Human, <i>Homo sapiens</i>	Secretory leukocyte protease inhibitor (SLPI)	HIV	SGKSFKAGVCPPKSAQCL RYKKPECQSDWQCPGKKR CCPDTCGIKCLDPVDTPNP TRRKPGKCPVTYGGQCLMLN PP	96
Cyanobacterium, <i>Scytonema varium</i>	Antiviral lectin scytovirin (SVN)	HIV-1	GSGPTYCWNEANNPGGPN RCSNNKQCDGARTCSSSG FCQGTSRKPDGPGKGPTY CWDEAKNPGGPNRCSNSK QCD	97
Cyanobacterium, <i>Nostoc ellipsosporum</i>	Cyanovirin-N (CVN)	HIV-1	LGKFSQTCYNIAIQGSVLTS TCERTNGGYNTSSIDLNSVI ENVDGSLKWQPSNFIETCR NTQLAGSSELAAECKTRAQ QF	98

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Cyanobacterium , <i>Microcystis viridis</i>	Microcystis viridis lectin (MVL)	HIV-1	MASYKVNIPAGPLWSNAEA QQVGPKIAAAHQGNFTGQ WTTVVESAMSVVEVELQVE NTGIHEFKTDVLAGPLWSN D	99
Red algae, <i>Griffithsia sp</i>	Griffithsin (GRFT)	HIV-1	SLTHRKFGGSGGSPFSGLS SIAVRSGSYLDAIIDGVHHG GSGGNLSPTFTFGSGEYIS NMTIRSGDYIDNISFETNMG RR	100
Asian common mud crab, <i>Scylla paramamosain</i>	Scylla paramamosain anti-lipopolysaccharide factor isoform 1 (Sp-ALF1)	White spot syndrome virus (WSSV)	YETLIASVLGKLTGLWHNNS VDFMGHTCHFRRRPKVRK FKLYHEGKFWCPGWAPFE GRSRTKSRSGSSREAIKDF VRK	101
Asian common mud crab, <i>Scylla paramamosain</i>	Sp-ALF2	WSSV	YEALVASILGKLSGLWHSDT VDFMGHTCHIRRRPKFRKF KLYHEGKFWCPGWTHLEG NSRTKSRSGSARDAIKDFV YKA	102
Peking duck, <i>Anas platyrhynchos</i>	Apl-AvBD-16	Duck hepatitis virus (DHV)	FFLLFLQGAAGNSVLCRIRG GRCHVGSGHFPERHIGRCS GFQACCIRTWG	103
Asian forest scorpion, <i>Heterometrus petersii</i>	Hp1090	Hepatitis C virus (HCV)	IFKAIWSGIKSLF	104
Red swamp crayfish, <i>Procambarus clarkii</i>	Procambarin	WSSV	HRPYCGSKGGIGGGHGGG SGGFGGGGGFGGGGLGG GKPIGIGGGGFGGGSGFG GGVGLKPNVGGGGFGGG GGGFGGGIGLKNVGGGG GFGGGIGLKNVGGGGGF GGGGGGFGGGGGFGGG FGGGKLIGGGIGWRRWWL CRKQRLRKVNHL	105

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Scorpion, <i>Heterometrus petersii</i>	Hp1036	HSV-1	ILGKIWEGIKSIF	106
Scorpion, <i>Heterometrus petersii</i>	Hp1239	HSV-1	ILSYLWNGIKSIF	107
Mouse, <i>Mus musculus</i>	mBD-3	IAV	KINNPVSCLRKGGRCWNR CIGNTRQIGSCGVPFLKCK RK	108
<i>Staphylococcus epidermidis</i> strain 115, <i>Staphylococcus equorum</i> strain WS 2733	Micrococcin P1 (MP1)	HCV	SCTTCVCTCSCCTT	109
Turbot, <i>Scophthalmus maximus</i>	<i>S. maximus</i> hepcidin-1 (SmHep1P)	Megalocytivirus (MCV)	QSHISLCRWCCNCKANK GCGFCKKF	110
Turbot, <i>Scophthalmus maximus</i>	SmHep2P	MCV	GMKCKFCCNCCNLNGCGV CCRF	111
<i>Actinomadura namibiensis</i> DSM 6313	Labyrinthopeptin A1 (LabyA1)	HIV, HSV, RSV, DENV, Zika virus (ZIKV), HCV, Chikungunya virus (CHIKV), KSHV, CMV	SNASVWECCSTGSWVPFT CC	112
<i>Actinomadura namibiensis</i>	LabyA2	HIV, HSV, RSV, DENV, ZIKV, HCV, CHIKV, KSHV, CMV	SDWSLWECCSTGSLFACC	113
Spotted scat, <i>Scatophagus argus</i>	SA-hepcidin2	<i>Siniperca chuatsi</i> rhabdovirus (SCRV), <i>Micropterus salmoides</i> reovirus (MsReV)	NPAGCRFCGCGCPNMIGC GVCCRF	114
Hemolymph, <i>Haemaphysalis longicornis</i>	HEdefensin	Langat virus (LGTV)	EESEVAHLRVRRGFGCPL NQGACHRHCRSIRRRGGY CSGIKQTCTCYRN	115

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Scorpion, <i>Mesobuthus martensii</i> Karsch	BmKDfsin4	Hepatitis B virus (HBV)	GFGCPFNQGGQCHKHCQSI RRRGGYCDGFLKTRCVCY R	116
Tiger shrimp, <i>Penaeus monodon</i>	Cyclic shrimp anti- lipopolysacchar ide factor (cSALF)	NNV	ECKFTVKPYLKRQVYYKG RMWCP	117
Human, <i>Homo sapiens</i>	Heparin- binding EGF- like growth factor (HB- EGF)	IAV	GKRKKKGKGLGKKRDPCL RKYK	118
Severe acute respiratory syndrome coronavirus (SARS-CoV) Class I fusion protein S2	SARS _{WW-I}	SARS-CoV	MWKTPTLKYFGGFNFSQIL	119
SARS-CoV Class I fusion protein S2	SARS _{WW-II}	SARS-CoV	ATAGWTFGAGAALQIPFAM QMay	120
SARS-CoV Class I fusion protein S2	SARS _{WW-III}	SARS-CoV	GYHLMSFPQAAPHGVVFLH VTW	121
SARS-CoV Class I fusion protein S2	SARS _{WW-IV}	SARS-CoV	GVFVFNGTSWFITQRNFFS	122
SARS-CoV Class I fusion protein S2	SARS _{WW-Vb}	SARS-CoV	AACEVAKNLNLSIDLQELG KYEQYIKW	123
CMV Class III fusion protein G _b	174-200	CMV	WEIHHINKFAQAYSSYSRVI GGTVFA	124
CMV Class III fusion protein G _b	233-263	CMV	WHSRGSTWLLYRETANLN AMLTITTARSKYPY	125

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
CMV Class III fusion protein G _b	264-291	CMV	HFFATSTGDVVYISPFYNGT NRNASYFG	126
CMV Class III fusion protein G _b	297-315	CMV	FFIFPNYTIVSDFGRPNAA	127
Rift Valley fever virus (RVFY) Class I fusion protein G _n	RVFY-6	RVFY	WNFFDWFSGLMWFGGPLK	128
RVFY Class I fusion protein G _n	RVFY-6	RVFY	WNFFDWFSGLMSWFGGPL KTI	129
RVFY Class I fusion protein G _n	RVFY-6	RVFY	SWNFFDWFSGLMSWFGGP LK	130
RVFY Class I fusion protein G _n	RVFY-6	RVFY	SGSWNFFDWFSGLMSWFG G	131
RVFY Class I fusion protein G _n	RVFY-6	RVFY	SGSWNFFDWFSGLMSWFG GPLKPL	132
IAV Class I fusion protein Hemagglutinin (HA)	Residues 84-99 of A/H1	IAV	VDDGFLDIWTYNAELL	133
IAV Class I fusion protein HA	Residues 84-99 of A/H3, A/H4, and A/H14	IAV	VEDTKIDLWSYNAELL	134
IAV Class I fusion protein HA	Residues 84-99 of A/H5	IAV	MEDGFLDVWTYNAELL	135
Pichinde virus fusion protein complex GP1 protein	Peptide 4.6 (GP1 ₁₄₃₋₁₇₀)	Pichinde virus (PICV)	GHTLKWLLELHFNVLVHTR HIGARCKT	136
Pichinde virus fusion protein complex GP1 protein	Peptide 5.0 (GP1 ₁₉₄₋₂₁₂)	PICV	HLIASLAQIIGDPKIAWVGK	137
Pichinde virus fusion protein complex GP1 protein	Peptide 6.0 (GP1 ₂₃₃₋₂₅₁)	PICV	HYNFLIIQNTTWENHCTYT	138

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Pichinde virus fusion protein complex GP2 protein	Peptide 7.0 (GP2 ₂₉₁₋₃₀₇)	PICV	PGGYCLEQWAIWAGIKCF	139
Pichinde virus fusion protein complex GP2 protein	Peptide 8.0 (GP2 ₃₄₈₋₃₆₆)	PICV	LNLFKKTINGLISDSLVR	140
HCV fusion protein E1	E1 ₁₉₇₋₂₁₄	HCV	VSGIYHVTNDCSNSSIVY	141
HCV fusion protein E2	E2 ₄₀₇₋₄₂₄	HCV	PSQKIQLVNTNGSWHINR	142
HCV fusion protein E2	E2 ₆₁₀₋₆₂₇	HCV	DYPYRLWHYPCTVNFTVF	143
HCV fusion protein E2	E2 ₇₀₁₋₇₁₈	HCV	YLYGIGSAVVVSFAIKWEY	144
Human, <i>Homo sapiens</i> (Fibroblast growth factor 4 signal sequence)	EB	HSV, IAV, Vaccinia virus	RRKKAVALLPVLLALLAP	145
Human, <i>Homo sapiens</i>	FluPep 1 (FP1), Tkip	IAV	WLVFFVIFYFFR	146
Human, <i>Homo sapiens</i>	FP2	IAV	WLVFFVIAYFAR	147
Human, <i>Homo sapiens</i>	FP3	IAV	WLVFFVIFYFFRRRKK	148
Human, <i>Homo sapiens</i>	FP4	IAV	RRKKWLVFFVIFYFFR	149
Human, <i>Homo sapiens</i>	FP7	IAV	RRKKIFYFFR	150
Human, <i>Homo sapiens</i>	FP8	IAV	WLVFFVRRKK	151
Human, <i>Homo sapiens</i>	FP9	IAV	FFVIFYRRKK	152

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Human, <i>Homo sapiens</i> GTPase RhoA	RhoA	RSV, HIV	ILMCFSIDSPDSLEN	153
HSV fusion protein G _b	gB64	HSV	TTPKFTVAWDWVPKR	154
HSV fusion protein G _b	gB94	HSV	KTTSSIEFARLQFTY	155
HSV fusion protein G _b	gB122	HSV	GHRRYFTFGGGYVYF	156
HSV fusion protein G _b	gB131	HSV	HEVVPLEVYTRHEIK	157
HCV nonstructural protein NS5A	C5A	HCV	SWLRDIWDWICEVLSDFK	158
Human, <i>Homo sapiens</i> Claudin 1	CL-58	HCV	MANAGLQLLGFI AFLGW	159
Human, <i>Homo sapiens</i> Claudin 1	CL-59	HCV	AFLGWIGAIVSTALPQWR	160
Murine brain cDNA phage display library	P1	West Nile Virus (WNV)	DTRACDVIAL LCHLNT	161
Murine brain cDNA phage display library	P9	WNV	CDVIAL LACHLNT	162
GB virus C (GBV-C) surface glycoprotein E2	P4-7	HIV	WDRGNVTLLCDCP NGPWV WV	163
GBV-C surface glycoprotein E2	P6-2	HIV	LCDP NGPWVWVPAFCQA VG	164
Human, <i>Homo sapiens</i>	P5	SARS-CoV-2	GGGYSKAQK AQAKQAK QAQKAQK AQAKQAKQ	165
Human, <i>Homo sapiens</i>	P5+14	SARS-CoV-2	GGGYSKAQK AQAKQAK QAQKAQK AQAKQAK QAQKAQK AQAKQ	166
Human, <i>Homo sapiens</i>	(P5R) _D	SARS-CoV-2	GGGY SRAQRAQARQAR QAQRAQRAQARQARQ	167

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Human, <i>Homo sapiens</i> and Domestic cow, <i>Bos taurus</i>	Lactoferricin	CMV, HIV, HSV, HPV	KCFQWQRNMRKVRGPPVS CIKRDS	168
Human, <i>Homo sapiens</i> and Domestic cow, <i>Bos taurus</i>	Lactoferrin	CMV, HIV, HSV, HPV	MKLVFLVLLFLGALGLCLAG RRRRSVQWCAVSQPEATK CFQWQRNMRKVRGPPVSC IKRDSPIQCIQAIENRADAV TLDGGFIYEAGLAPYKLRPV AAEVYGTERQPRTHYYAVA VVKKGGSFQLNELQGLKSC HTGLRRTAGWNVPIGTLRP FLNWTGPPEPIEAAVARFFS ASCVPGADKGQFPNLCRLC AGTGENKCAFSSQEPYFSY SGAFKCLRDGAGDVAFIRE STV	169
Human, <i>Homo sapiens</i>	CAP37	HSV-1, Adenovirus	GRHFCGGALIHARFVMTAA SCFQ	170
HIV-1	HIV-1 Tat	HIV-1	GRKKRRQRRR	171
HIV	C46	HIV	WMEWDREINNYTSLIHSLIE ESQNQQEKNEQEELLELDK WASLWNWF	172
HIV	C46mutGlyco	HIV	WMEWDREINNYASLIHSLIE ESQNQQEKNEQEELLELDK WASLWNWF	173
HIV	C34	HIV	WMEWDREINNYTSLIHSLIE ESQNQQEKNEQEELL	174
HIV	C36	HIV	YTSLIHSLIEESQNQQEKNE QELLELDKWASLWNWF	175
HIV	T-1249	HIV	WQEWKITALLEQAQIQQ EKNEYELQKLDKWASLWE WF	176
HIV	T-649	HIV	WMEWDREINNYTSLIHSLIE ESQNQQEKNEQEELLE	177
HIV	SC35EK	HIV	WEEWDKKIEEYTKKIEELIK KSEEQKKNEEELKK	178

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
HIV	T-2635	HIV	TTWEAWDRAIAEYAARIEAL IRAAQEQQEKNEAALREL	179
HIV	HIV-2EHO-C46	HIV	WQQWERQVRFLDANITKLL EEAQIQQEKKNMYELQELDK WASLWNWF	180
HIV	DP178 analog derived from gp41 peptide region of isolate HIV-1 S _{F2}	HIV	TNTIYTLLEESQNQQEKNE QELLELDKWASLWNWF	181
HIV	DP178 analog derived from gp41 peptide region of isolate HIV-1 _{RF}	HIV	YTGIYNLLEESQNQQEKNE QELLELDKW ANLWNWF	182
HIV	DP178 analog derived from gp41 peptide region of isolate HIV-1 MN	HIV	YTSLIYSLLEKSQIQQEKNE QELLELDKWASLWNWF	183
HIV	Partial C34 peptide (originally derived from gp41)	HIV	WMEWDREINNYTSLIHSLIE ESQNQQEKNEQELL	184
RSV	DP178 and/or DP107 analog	RSV	YTSVITIELSNIKENKCNGAK VKLIKQELDKYK	185
RSV	DP178 and/or DP107 analog	RSV	TSVITIELSNIKENKCNGAKV KLIKQELDKYKN	186
RSV	DP178 and/or DP107 analog	RSV	VITIELSNIKENKCNGAKVKL IKQELDKYKNAV	187
RSV	DP178 and/or DP107 analog	RSV	IALLSTNKAVVSLSNQGVSVL TSKVLDLKNYIDK	188
HPIV	DP178 and/or DP107 analog	HPIV	VEAKQARSDIEKLKEAIRD NKAVQSVQSSIGNLI	189

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
HPIV	DP178 and/or DP107 analog	HPIV	RSDIEKLKEAIRD TNKAVQS VQSSIGNLIVA IKS V	190
HPIV	DP178 and/or DP107 analog	HPIV	NSVALDPIDISIELNKA KSDL EESKEWIRRSNQKL	191
HPIV	DP178 and/or DP107 analog	HPIV	ALDPIDISIELNKA KSDLEES KEWIRRSNQKLDSI	192
HPIV	DP178 and/or DP107 analog	HPIV	LDPIDISIELNKA KSDLEESK EWIRRSNQKLDSIG	193
HPIV	DP178 and/or DP107 analog	HPIV	DPIDISIELNKA KSDLEESKE WIRRSNQKLDSIGN	194
HPIV	DP178 and/or DP107 analog	HPIV	PIDISIELNKA KSDLEESKEW IRRSNQKLDSIGNW	195
HPIV	DP178 and/or DP107 analog	HPIV	IDISIELNKA KSDLEESKEWI RRSNQKLDSIGNWH	196
MeV	DP178 and/or DP107 analog	MeV	HRIDLGPPI SLERLDVGTNL GNAIAKLEAKELLE	197
MeV	DP178 and/or DP107 analog	MeV	IDLGPPI SLERLDVGTNLGN AIAKLEAKELLESS	198
MeV	DP178 and/or DP107 analog	MeV	LGPPISLERLDVGTNLGNAI AKLEAKELLESSDQ	199
MeV	DP178 and/or DP107 analog	MeV	PISLERLDVGTNLGNIAKL EAKELLESSDQILR	200
SIV	DP178 and/or DP107 analog	SIV	WQEWERKVDFLEENITALL EEAQIQQEKNMYELQK	201
SIV	DP178 and/or DP107 analog	SIV	QEWERKVDFLEENITALLEE AQIQQEKNMYELQKL	202
SIV	DP178 and/or DP107 analog	SIV	EWERKVDFLEENITALLEEA QIQQEKNMYELQKLN	203
SIV	DP178 and/or DP107 analog	SIV	WERKVDFLEENITALLEEAQ IQQEKNMYELQKLNS	204
SIV	DP178 and/or DP107 analog	SIV	ERKVDFLEENITALLEEAQI QQEKNMYELQKLNSW	205
SIV	DP178 and/or DP107 analog	SIV	RKVDFLEENITALLEEAQIQ QEKNMYELQKLNSWD	206
SIV	DP178 and/or DP107 analog	SIV	KVDFLEENITALLEEAQIQQ EKNMYELQKLNSWDV	207
SIV	DP178 and/or DP107 analog	SIV	VDFLEENITALLEEAQIQQE KNMYELQKLNSWDVF	208

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
SIV	DP178 and/or DP107 analog	SIV	DFLEENITALLEEAIQQEK NMYELQKLNSWDVFG	209
SIV	DP178 and/or DP107 analog	SIV	FLEENITALLEEAIQQEKN MYELQKLNSWDVFGN	210
HIV	DP178 peptide (AA 638-673 of gp41 from the HIV-1 _{LAI} isolate)	HIV	YTSLIHSLEEESQNQQEKNE QELLELDKWASLWNWF	211
HIV	DP107 peptide (AA 558-595 of gp41 from the HIV-1 _{LAI} isolate)	HIV	NNLLRAIEAQQHLLQLTVW QIKQLQARILAVEYLKDKQ	212
HIV	DP178 analog derived from gp41 peptide region of HIV-1 _{SF2}	HIV	YTNTIYTLLEESQNQQEKNE QELLELDKWASLWNWF	213
HIV	DP178 analog derived from gp41 peptide region of HIV-1 _{RF}	HIV	YTGIIYNLLEESQNQQEKNE QELLELDKWANLWNWF	214
HIV	DP178 analog derived from gp41 peptide region of HIV-1 _{MN}	HIV	YTSLIYSLLEKSQIQQEKNE QELLELDKWASLWNWF	215
RSV	DP178 and/or DP107 analog	RSV	YTSWITIELSNIKENKCNGA KWKLKQELDKYK	216
RSV	DP178 and/or DP107 analog	RSV	TSWITIELSNIKENKCNGAK WKLKQELDKYKN	217
RSV	DP178 and/or DP107 analog	RSV	WITIELSNIKENKCNGAKWK LIKQELDKYKNAW	218
RSV	DP178 and/or DP107 analog	RSV	IALSTNKAWWSLSNGWS WLTSKWLDLKNYIDK	219

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
HPIV	DP178 and/or DP107 analog	HPIV	VEAKQARSDIEKLKEAIRD TNKAVQSVQSSIGNLI	220
HPIV	DP178 and/or DP107 analog	HPIV	RSDIEKLKEAIRD TNKAVQSVQSSIGNLIVAIKSV	221
HPIV	DP178 and/or DP107 analog	HPIV	NSVALDPIDISIELNKA KSDL EESKEWIRRSNOKL	222
HPIV	DP178 and/or DP107 analog	HPIV	ALDPIDISIELNKA KSDL EES KEWIRRSNOKLDSI	223
HPIV	DP178 and/or DP107 analog	HPIV	LDPIDISIELNKA KSDL EES KEWIRRSNOKLDSIG	224
HPIV	DP178 and/or DP107 analog	HPIV	DPIDISIELNKA KSDL EES KEWIRRSNOKLDSIGN	225
HPIV	DP178 and/or DP107 analog	HPIV	PIDISIELNKA KSDL EES KEWIRRSNOKLDSIGNW	226
HPIV	DP178 and/or DP107 analog	HPIV	IDISIELNKA KSDL EES KEWIRRSNOKLDSIGNWE	227
MeV	DP178 and/or DP107 analog	MeV	HRIDLGPPI SLERLDWGTNL GNIAKLEAKELLE	228
MeV	DP178 and/or DP107 analog	MeV	IDLGPPI SLERLDWGTNLGN AIAKLEAKELLESS	229
MeV	DP178 and/or DP107 analog	MeV	LGPPISLER LDWGTNLGNAI AKLEAKELLESSDQ	230
MeV	DP178 and/or DP107 analog	MeV	PISLERLDW GTNLGNIAKL EAKELLESSDQILR	231
SIV	DP178 and/or DP107 analog	SIV	WQEWERK VDFLEENITALL EEAQIQQEKNMYELQK	232
SIV	DP178 and/or DP107 analog	SIV	QEWERK VDFLEENITALL EEAQIQQEKNMYELQKL	233
SIV	DP178 and/or DP107 analog	SIV	EWERK VDFLEENITALL EEAQIQQEKNMYELQKLN	234
SIV	DP178 and/or DP107 analog	SIV	WERK VDFLEENITALL EEAQIQQEKNMYELQKLNS	235
SIV	DP178 and/or DP107 analog	SIV	ERK VDFLEENITALL EEAQIQQEKNMYELQKLNSW	236
SIV	DP178 and/or DP107 analog	SIV	RK VDFLEENITALL EEAQIQQEKNMYELQKLNSWD	237
SIV	DP178 and/or DP107 analog	SIV	K VDFLEENITALL EEAQIQQEKNMYELQKLNSWDV	238

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
SIV	DP178 and/or DP107 analog	SIV	WDFLEENITALLEEAIQQE KNMYELQKLNSWDVF	239
SIV	DP178 and/or DP107 analog	SIV	DFLEENITALLEEAIQQEK NMYELQKLNSWDVFG	240
SIV	DP178 and/or DP107 analog	SIV	FLEENITALLEEAIQQEKN MYELQKLNSWDVFGN	241
HIV	Enfuvirtide (T-1249)	HIV	YTSLIHSLIEESQNQQEKNE QELLELNKWA SLWNWF	242
HIV		HIV	CLLLGTEVSEALGGAGLT	243
RSV		RSV	DEFDASISQVNEKINQSLAFI RKSDHELLHNVNAGK	244
Hendra Virus; Human parainfluenza virus type 3		Hendra Virus; Human parainfluenza virus type 3	DITLNNSVALDPIDISIELNKA KSDLEESKEWIRRSNQKLD SIGN	245
RSV		RSV	DPLVFPSDEFDASISQVNEK INQSLAFIRKSDCELL	246
HIV; Influenza A		HIV; Influenza A	DQYKCLQHGGFCLRSSCP SNTKLQGTCKPDKPNCCKS	247
RSV		RSV	EFDASISQVNEKINQSLAFIR KSDCELLHNVNAGKS	248
RSV		RSV	FDASISQVNEKINQSLAFIRK SDELLHNVNAGKST	249
Chikungunya virus; Dengue virus		Chikungunya virus; Dengue virus	FLGAILKIGHALAKTVLPMVT NAFKPKQ	250
RSV		RSV	FPSDEFDASISQVNEKINQS LAFIRKSDCELLHNVN	251
RSV		RSV	FYDPLVFPSDEFDASISQVN EKINQSLAFIRKSDE	252
Influenza B virus; Hepatitis E virus		Influenza B virus; Hepatitis E virus	GADDVVDSSKSFVMENFSS YHGTPGYVDSIQKGIQKP KSGTQGNYYDDWKEFYST DKNYDAAGYSVDNENPLSG KAGGVVKVTPGLTKVLAL KVDNAETIKKELGLSLTEPL MEQVGT	253

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
Dengue virus:Zika virus		Dengue virus; Zika virus	GFGCPLDQMQQCHNHCQSV RYRGGYCTNFLKMTCKCY	254
HIV		HIV	GIFPKIIGKGIVNGIKSLAKG VGMKVFKAGLNNIGNTGCN NRDEC	255
HIV		HIV	HSLIEESQNQQEKNEQELL ELDKWASLWNWFNITNW	256
RSV		RSV	IINFYDPLVFPSPDEFDASISQ VNEKINQSLAFIRK	257
HIV		HIV	IKKEIEAIKKEQEAIKKKIEAIE KEISGIVQQQNNLLRAIEAQ QHLLQLTVWGIKQLQARIL	258
RSV		RSV	INFYDPLVFPSPDEFDASISQ VNEKINQSLAFIRKS	259
Hendra Virus:Nipah virus		Hendra Virus:Nipah virus	KVDISSQISSMNQSLQQSK DYIKEAQRLDVTNPSL	260
HIV		HIV	LGTEVSEALGGAGLTGGF	261
Measles virus:Human parainfluenza virus type 3		Measles virus:Human parainfluenza virus type 3	LHRIDLGPPISLERLDVGTN LGNAIAKLEDAKELL	262
HIV		HIV	LNNCLLLGTEVSEALGGA	263
RSV		RSV	LVFPSPDEFDASISQVNEKIN QSLAFIRKSDELLHN	264
HIV		HIV	MTWMEWDREINNYTSLIHS LIEESQNQQEKNEQELL	265
RSV		RSV	NFYDPLVFPSPDEFDASISQV NEKINQSLAFIRKSD	266
HIV		HIV	NNLLRAIEAQQHLLQLTVW GIKQLQARILAVEERYLKDQ	267
RSV		RSV	PLVFPSPDEFDASISQVNEKI NQSLAFIRKSDELLH	268
Hendra Virus; Human parainfluenza virus type 3		Hendra Virus; Human parainfluenza virus type 3	PPVYTKDVIDISSQISSMNQS LQQSKDYIKEAQKILDTVNP SL	269

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
RSV		RSV	PSDEFDASISQVNEKINQSL AFIRKSDELLHNVNA	270
HIV		HIV	RFPFHRCGAGPKLTKDLE	271
HIV		HIV	RSQKEGLHYTCSSHPYSQ YQFWK	272
RSV		RSV	SDEFDASISQVNEKINQSLA FIRKSDELLHNVNAG	273
HIV		HIV	SGIVQQQNNLLRAIEAQQHL LQLTVWGIKQLQARIL	274
Hendra Virus; Human parainfluenza virus type 3		Hendra Virus; Human parainfluenza virus type 3	SIELNKAQSDLEESKEWIRR SNQKLDSE	275
HIV		HIV	TTWEAWDRAIAEYAARIEAL IRAAQEQQEKLEAALREL	276
Hendra Virus; Human parainfluenza virus type 3		Hendra Virus; Human parainfluenza virus type 3	VALDPIDISIELNKAQSDLEE SKEWIRR	277
Hendra Virus; Human parainfluenza virus type 3		Hendra Virus; Human parainfluenza virus type 3	VALDPIDISIELNKAQSDLEE SKEWIRRSNQKLDSD	278
Hendra Virus; Human parainfluenza virus type 3		Hendra Virus; Human parainfluenza virus type 3	VALDPIDISIELNKAQSDLEE SKEWIRRSNQKLDSE	278
Hendra Virus; Human parainfluenza virus type 3		Hendra Virus; Human parainfluenza virus type 3	VANDPIDISIELNKAQSDLEE SKEWIRRSNQKLDSD	280
Hendra Virus; Human parainfluenza virus type 3		Hendra Virus; Human parainfluenza virus type 3	VANDPIDISIELNKAQSDLEE SKEWIRRSNQKLDSE	281

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
RSV		RSV	VFPSDEFDASISQVNEKINQ SLAFIRKSDELLHNV	282
Hendra Virus; Human parainfluenza virus type 3		Hendra Virus; Human parainfluenza virus type 3	VYTDKVDISSQISSMNQSLQ QSKDYIKEAQKILDTV	283
HCV		HCV	WVAVTPTVATRDGKLPTT	284
RSV		RSV	YDPLVFPSDEFDASISQVNE KINQSLAFIRKSDEL	285
HIV		HIV	YTSLIHSLEESQNQQEKNE QQLLELDKWASLWNWF	286
HIV-1 gp41	Albuviritide	HIV	Ac- WEEWDREINNYT(Mpa)LIH ELIEESQNQQEKNEQELL- CONH ₂ (Mpa=3- maleimidopropionic acid)	287
CoV-OC43	EK1	CoV	SLDQINVTFDLLEYEMKKLE EAIKKLEESYIDLKEL	288
CoV-OC43	OC43-HR2P	CoV	SLDYINVTFDLQDEMNRLO EAIKVLNQSYINLKDI	289
SARS-CoV-2	Wuhan-Hu-1 HR1	CoV	NVLYENQKLIANQFNSAIGKI QDSLSTASALGKLQDVVN QNAQALNTLVKQLSSNFGAI SSVLNDILSRDKVEAEVQI DRLITGRLQSLQTYVTQQLI RAAEIRASAN	290
SARS-CoV-2	Alpha HR1	CoV	NVLYENQKLIANQFNSAIGKI QDSLSTASALGKLQDVVN QNAQALNTLVKQLSSNFGAI SSVLNDILARLDKVEAEVQI DRLITGRLQSLQTYVTQQLI RAAEIRASAN	291
SARS-CoV-2	Beta HR1	CoV	NVLYENQKLIANQFNSAIGKI QDSLSTASALGKLQDVVN QNAQALNTLVKQLSSNFGAI SSVLNDILSRDKVEAEVQI DRLITGRLQSLQTYVTQQLI RAAEIRASAN	292

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
SARS-CoV-2	Gamma HR1	CoV	NVLYENQKLIANQFNSAIGKI QDSLSTASALGKLQDVVN QNAQALNTLVKQLSSNFGAI SSVLNDILSRDKVEAEVQI DRLITGRLQSLQTYVTQQLI RAAEIRASAN	293
SARS-CoV-2	Delta HR1	CoV	NVLYENQKLIANQFNSAIGKI QDSLSTASALGKLQNVVN QNAQALNTLVKQLSSNFGAI SSVLNDILSRDKVEAEVQI DRLITGRLQSLQTYVTQQLI RAAEIRASAN	294
SARS-CoV-2	Omicron HR1	CoV	NVLYENQKLIANQFNSAIGKI QDSLSTASALGKLQDVVN HNAQALNTLVKQLSSKFGAI SSVLNDIFSRLDKVEAEVQI DRLITGRLQSLQTYVTQQLI RAAEIRASAN	295
SARS-CoV-1	SARS-CoV1 HR1	CoV	NVLYENQKLIANQFNKAISQ IQESLTTTSTALGKLQDVVN QNAQALNTLVKQLSSNFGAI SSVLNDILSRDKVEAEVQI DRLITGRLQSLQTYVTQQLI RAAEIRASAN	296
MERS	MERS HR1	CoV	QVLSNQKLIANKFNQALG AMQTGFTTTNEAFQKVQDA VNNNAQALSKLASELSNTF GAISASIGDIIQRLDVLEQDA QIDRLINGRLTTLNAFVAQQ LVRSESAALSAQ	297
SARS-CoV-2	Wuhan-Hu-1 HR2	CoV	VVIGIVNNTVYDPLQPELDS FKEELDKYFKNHTSPDVL GDISGINASVVNIQKEIDRLN EVAKNLNESLIDLQEL	298
SARS-CoV-2	Alpha HR2	CoV	VVIGIVNNTVYDPLQPELDS FKEELDKYFKNHTSPDVL GDISGINASVVNIQKEIDRLN EVAKNLNESLIDLQEL	299

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
SARS-CoV-2	Beta HR2	CoV	VVIGIVNNTVYDPLQPELDS FKEELDKYFKNHTSPDVDL GDISGINASVVNIQKEIDRLN EVAKNLNESLIDLQEL	300
SARS-CoV-2	Gamma HR2	CoV	VVIGIVNNTVYDPLQPELDS FKEELDKYFKNHTSPDVDL GDISGINASFVNIQKEIDRLN EVAKNLNESLIDLQEL	301
SARS-CoV-2	Delta HR2	CoV	VVIGIVNNTVYDPLQPELDS FKEELDKYFKNHTSPDVDL GDISGINASVVNIQKEIDRLN EVAKNLNESLIDLQEL	302
SARS-CoV-2	Omicron HR2	CoV	VVIGIVNNTVYDPLQPELDS FKEELDKYFKNHTSPDVDL GDISGINASVVNIQKEIDRLN EVAKNLNESLIDLQEL	303
SARS-CoV-1	SARS-CoV1 HR2	CoV	VVIGIINNTVYDPLQPELDSF KEELDKYFKNHTSPDVDLG DISGINASVVNIQKEIDRLNE VAKNLNESLIDLQEL	304
MERS	MERS HR2	CoV	VTYQNISTNLPPLLGNGSTG IDFQDELDEFFKNVSTSIPN FGSLTQINTTLLDLTYEMLS LQQVVKALNESYIDLKEL	305
SARS-CoV-2	HR2A	CoV	DISGINASVVNIQKEIDRLNE VAKNLNESLIDLQEL	306
SARS-CoV-2	HR2B	CoV	VVIGIVNNTVYDPLQPELDS FKEELDKYFKNHTSPD	307
SARS-CoV-2	HR2C	CoV	FKNHTSPDVLGDISGINAS VVNIQKEIDRLNEVAK	308
SARS-CoV-2	HR2 Full Length	CoV	GIVNNTVYDPLQPELDSFKE ELDKYFKNHTSPDVLGDIS GINASVVNIQKEIDRLNEVA KNL NESLIDLQEL	309

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
SARS-CoV-2	HR2 Full Length Fc fusion	CoV	MGWSCIIILFLVATATGVHS GIVNNTVYDPLQPELDSFKE ELDKYFKNHTSPDVLGDIS GINASVVNIQKEIDRLNEVA KNLNEGLIDLQELGGGGSG GGGSGGGGSAESKYGPPC PPCAPEAAGGPSVFLFPP KPKDTLMISRTPEVTCVVVD VSQEDPEVQFNWYVDGVE VHNAKTKPREEQFNSTYRV VSVLTVLHQDWLNGKEYKC KVSNGKLPSSIEKTISKAKG QPREPQVYTLPPSQEEMTK NQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVL DSDGSFFLYSRLTVDKSRW QEGNVFSCSVMEALHNH YTQKSLSLSLG	310
SARS-CoV-2	HR2 Full Length Fc fusion without signal peptide	CoV	GIVNNTVYDPLQPELDSFKE ELDKYFKNHTSPDVLGDIS GINASVVNIQKEIDRLNEVA KNLNEGLIDLQELGGGGSG GGGSGGGGSAESKYGPPC PPCAPEAAGGPSVFLFPP KPKDTLMISRTPEVTCVVVD VSQEDPEVQFNWYVDGVE VHNAKTKPREEQFNSTYRV VSVLTVLHQDWLNGKEYKC KVSNGKLPSSIEKTISKAKG QPREPQVYTLPPSQEEMTK NQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVL DSDGSFFLYSRLTVDKSRW QEGNVFSCSVMEALHNH YTQKSLSLSLG	311
HIV	HIV_CHR	HIV	TTWMEWDREINNYTSLIHS LIEESQNQQEKNEQELLELD KWASLWNWF	312

Source	Peptide	Target	Amino Acid Sequence	SEQ ID NO:
HIV	T20	HIV	YTSLIHS LIEESQNQQEKNE QELLELDKWASLWNWF	313
HIV	T2410	HIV	MTWMEWDREINNYTSLIHS LIEESQNQQEKNEQELLEL	314
HIV	T2410_2.0	HIV	MTWMEWDREINNYTSLIHS LIEESQNQQEKNEQELLELD KWASLWNWF	315
HIV	T1144	HIV	TTWEAWDRAIAEYAARIEAL LRALQEQQEKNEAALREL	316
HIV	T144_2.0	HIV	TTWEAWDRAIAEYAARIEAL LRALQEQQEKNEAALRELD KWASLWNWF	317
HIV	T1249	HIV	WQEWQKITALLEQAQIQQ EKNEYELQKLDKWASLWE WF	318
HIV	T1249_2.0	HIV	TTWQEWQKITALLEQAQI QQEKNEYELQKLDKWASL WEWF	319
HIV	T2635	HIV	TTWEAWDRAIAEYAARIEAL IRAAQEQQEKNEAALREL	320
HIV	T2635_2.0	HIV	TTWEAWDRAIAEYAARIEAL IRAAQEQQEKNEAALRELD KWASLWNWF	321
HIV	T2635_3.0	HIV	MTWEAWDRAIAEYAARIEA LIRAAQEQQEKNEAALREL DKWASLWNWF	322
HIV	T290676	HIV	TTWEAWDRAIAEYAARIEAL IRASQEQQEKNEAELREL	323
HIV	T290676_2.0	HIV	TTWEAWDRAIAEYAARIEAL IRASQEQQEKNEAELRELD KWASLWNWF	324

In some embodiments, the virus is human immunodeficiency virus (HIV) (e.g., the antifusogenic polypeptide inhibits viral entry of HIV). In some embodiments, the HIV is HIV-1. In some embodiments, the HIV is a strain of HIV-1 (e.g., HIV-1_{HE}, HIV-1_{IIIB}, HIV-1_{MN}, HIV-1_{NDK}, HIV-1_{NL4-3}, HIV-1_{RF}, or HIV-1_{SF2}). In some embodiments, the HIV is HIV-2. In some embodiments, the HIV is a strain of HIV-2 (e.g., HIV-2_{EHO} or HIV-2_{ROD}). In some embodiments, the HIV is an HIV pseudovirion. In some embodiments, the antifusogenic polypeptide prevents HIV viral fusion by specifically binding to HIV glycoprotein 120 (gp120). In some embodiments, the antifusogenic polypeptide prevents binding of gp120 to the cluster of

differentiation 4 (CD4) co-receptor. In some embodiments, the antifusogenic polypeptide reduces the affinity of gp120 for a co-receptor (e.g., C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4)). In some embodiments, the antifusogenic polypeptide prevents binding of gp120 to a co-receptor (e.g., CCR5 and CXCR4). In some embodiments, the antifusogenic polypeptide prevents HIV viral fusion by specifically binding to HIV glycoprotein 41 (gp41). In some embodiments, the antifusogenic polypeptide inhibits entry of the HIV viral core into the cell. In some embodiments, the polynucleotide cargo includes an expression sequence encoding a polypeptide (e.g., a polypeptide that inhibits viral entry of HIV) of any one of SEQ ID NOs: 1, 5, 11, 13-18, 22-38, 40-46, 49-56, 58, 59, 61, 66-78, 80-85, 87-91, 95-100, 112, 113, 153, 163, 64, 168, 169, 171-184, 211-215, 242, 243, 247, 255, 256, 258, 261, 261, 265, 267, 271, 272, 274, 276, 286, 287, or 312-324. In some embodiments, the polynucleotide cargo includes an expression sequence encoding a polypeptide having at least 85% (e.g., at least 90%, 95%, 97%, 99%, or 100%) sequence identity to a polypeptide of any one of SEQ ID NOs: 1, 5, 11, 13-18, 22-38, 40-46, 49-56, 58, 59, 61, 66-78, 80-85, 87-91, 95-100, 112, 113, 153, 163, 64, 168, 169, 171-184, 211-215, 242, 243, 247, 255, 256, 258, 261, 261, 265, 267, 271, 272, 274, 276, 286, 287, or 312-324.

In some embodiments, the virus is hepatitis virus (e.g., the antifusogenic polypeptide inhibits viral entry of a hepatitis virus). In some embodiments, the hepatitis virus is hepatitis A virus (HAV). In some embodiments, the hepatitis virus is hepatitis B virus (HBV). In some embodiments, the hepatitis virus is hepatitis C virus (HCV). In some embodiments, the hepatitis virus is hepatitis D virus (HDV). In some embodiments, the hepatitis virus is hepatitis E virus (HEV). In some embodiments, the hepatitis virus is duck hepatitis virus (DHV). In some embodiments, the polynucleotide cargo includes an expression sequence encoding a polypeptide (e.g., a polypeptide that inhibits viral entry of a hepatitis virus such as HCV) of any one of SEQ ID NOs: 104, 109, 112, 113, 141-145, 158-160, or 284. In some embodiments, the polynucleotide cargo includes an expression sequence encoding a polypeptide having at least 85% (e.g., at least 90%, 95%, 97%, 99%, or 100%) sequence identity to a polypeptide of any one of SEQ ID NOs: 104, 109, 112, 113, 141-145, 158-160, or 284.

In some embodiments, the virus is a coronavirus, such as a betacoronavirus (e.g., the antifusogenic polypeptide inhibits viral entry of a coronavirus). In some embodiments, the betacoronavirus is SARS-CoV type-1 (SARS-CoV-1). In some embodiments, the betacoronavirus is SARS-CoV type 2 (SARS-CoV-2). In some embodiments, the betacoronavirus is a pseudotyped virus expressing the SARS-CoV-2 spike protein. In some embodiments, the polynucleotide cargo includes an expression sequence encoding a polypeptide (e.g., a polypeptide that inhibits viral entry of a betacoronavirus such as SARS-CoV-1 or SARS-CoV-2) of any one of SEQ ID NOs: 6-9, 119-123, 165-167, or 288-311. In some embodiments, the polynucleotide cargo includes an expression sequence encoding a polypeptide having at least 85% (e.g., at least 90%, 95%, 97%, 99%, or 100%) sequence identity to a polypeptide of any one of SEQ ID NOs: 6-9, 119-123, 165-167, or 288-311.

In some embodiments, the virus is respiratory syncytial virus (RSV) (e.g., the antifusogenic polypeptide inhibits viral entry of RSV). In some embodiments, the RSV is RSV subtype A (RSVA). In some embodiments, the RSV is RSV subtype B (RSVB). In some embodiments, the polynucleotide cargo includes an expression sequence encoding a polypeptide (e.g., a polypeptide that inhibits viral entry of RSV) of any one of SEQ ID NOs: 11, 13, 39, 112, 113, 153, 185-188, 216-219, 244, 246, 247-

249, 251, 252, 257, 259, 264, 266, 268, 270, 273, 282, or 285. In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding a polypeptide having at least 85% (e.g., at least 90%, 95%, 97%, 99%, or 100%) sequence identity to a polypeptide of any one of SEQ ID NOs: 11, 13, 39, 112, 113, 153, 185-188, 216-219, 244, 246, 247-249, 251, 252, 257, 259, 264, 266, 268, 270, 273, 282, or 285.

In some embodiments, the virus is influenza (e.g., seasonal influenza, pandemic influenza, influenza A, influenza H1N1 subtype, influenza B, influenza C, influenza D). In some embodiments, the influenza is influenza A. In some embodiments, the influenza is influenza B. In some embodiments, the influenza is influenza C. In some embodiments, the influenza is influenza D. In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding a polypeptide (e.g., a polypeptide that inhibits viral entry of influenza) of any one of SEQ ID NOs: 1, 4, 11, 245, 247, 253, 262, 269, 275, 277, 278, 279, 280, 281, or 283. In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding a polypeptide having at least 85% (e.g., at least 90%, 95%, 97%, 99%, or 100%) sequence identity to a polypeptide of any one of SEQ ID NOs: 1, 4, 11, 245, 247, 253, 262, 269, 275, 277, 278, 279, 280, 281, or 283.

In some embodiments, the virus is influenza virus (e.g., the antifusogenic polypeptide inhibits viral entry of influenza). In some embodiments, the influenza virus is influenza A virus (IAV). In some embodiments, the influenza virus is influenza B virus (IBV). In some embodiments, the influenza virus is an influenza virus expressing hemagglutinin (HA).

In some embodiments, the virus is herpes simplex virus (HSV) (e.g., the antifusogenic polypeptide inhibits viral entry of HSV). In some embodiments, the HSV is HSV-1. In some embodiments, the HSV is HSV-2.

In some embodiments, the virus is human papilloma virus (HPV) (e.g., the antifusogenic polypeptide inhibits viral entry of HPV). In some embodiments, the HPV is a high-risk HPV strain (e.g., HPV 16, HPV 18, HPV 31, HPV 33, HPV 45, HPV 52, or HPV 58). In some embodiments, the HPV is a low-risk HPV strain (e.g., HPV 6, HPV 11, HPV 42, HPV 43, HPV or 44).

In some embodiments, the virus is any virus listed in Table 1.

In some embodiments, the GC content of a nucleic acid sequence encoding an antifusogenic polypeptide is at least 51% (e.g., at least 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, or 60%). In some embodiments, the GC content of a nucleic acid sequence encoding an antifusogenic polypeptide is at most 52%, 53%, 54%, 55%, 56%, 57%, 58% or 59%, or 60%. In some embodiments, the GC content of a nucleic acid sequence encoding an antifusogenic polypeptide is 51% to 60%, 52% to 60%, 53% to 60%, 54% to 60%, 55% to 60%, 52% to 58%, 53% to 58%.

In some embodiments, the uridine content (for RNA) or the thymidine content (for DNA) of a nucleic acid sequence encoding an antifusogenic polypeptide is more than 10% (e.g., more than 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, or 25%). In some embodiments, the uridine content (for RNA) or the thymidine content (for DNA) of a nucleic acid sequence encoding an antifusogenic polypeptide is at most 30% (e.g., at most 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, or 20%). In some embodiments, the uridine content (for RNA) or the thymidine content (for DNA) of a nucleic acid sequence encoding an antifusogenic polypeptide is 20% to

28%, 21% to 26%, 10% to 24%, 15% to 24%, 20% to 24%, 21% to 24%, 22% to 24%, 23% to 24%, 10% to 23%, 15% to 23%, 20% to 23%, 21% to 23%, or 22% to 23%.

The GC content of an expression sequence encoding the antifusogenic polypeptide refers to the GC content of the expression sequence that exclusively encodes the antifusogenic polypeptide with no other coding regions that encode polypeptides other than the antifusogenic polypeptide. Likewise, the uridine content or thymidine of an expression sequence encoding the antifusogenic polypeptide refers to the uridine content of the expression sequence that exclusively encodes the antifusogenic polypeptide with no other coding regions that encode polypeptides other than the antifusogenic polypeptide. In some embodiments, the calculation of the GC content or the uridine (or thymidine) content of the expression sequence encoding the antifusogenic polypeptide only takes into account the continuous nucleic acid sequence that starts in a 5' to 3' direction from the first nucleoside of the start codon of the open reading frame that encodes the antifusogenic polypeptide to the last nucleoside of the stop codon of the same open reading frame. In other embodiments, the calculation of the GC content or the uridine (or thymidine) content of the expression sequence encoding the antifusogenic polypeptide only takes into account the continuous nucleic acid sequence that starts in a 5' to 3' direction from the first nucleoside of the codon that encodes the N-terminal end amino acid residue of the antifusogenic polypeptide to the last nucleoside of the codon that encodes the C-terminal end amino acid residue of the antifusogenic polypeptide.

In some embodiments, the nucleic acid sequence encoding the antifusogenic polypeptide has a uridine content of more than 20%. In some embodiments, the uridine content of a nucleic acid sequence encoding an antifusogenic polypeptide is more than 10% (e.g., more than 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, or 25%). In some embodiments, the uridine content of a nucleic acid sequence encoding an antifusogenic polypeptide is at most 30% (e.g., at most 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, or 20%). In some embodiments, the uridine content of a nucleic acid sequence encoding an antifusogenic polypeptide is 20% to 28%, 21% to 26%, 10% to 24%, 15% to 24%, 20% to 24%, 21% to 24%, 22% to 24%, 23% to 24%, 10% to 23%, 15% to 23%, 20% to 23%, 21% to 23%, or 22% to 23%. In some embodiments, the nucleic acid sequence encoding the antifusogenic polypeptide has a uridine content of 20% to 28%.

30 *Multiple Antifusogenic Polypeptides*

In some embodiments, the circular polyribonucleotide encodes multiple expression sequences each encoding an antifusogenic polypeptide (e.g., two or more, such as 2 to 100, 2 to 50, 2 to 20, 2 to 10, 5 to 100, 5 to 50, 5 to 20, or 5 to 10 expression sequences).

In some embodiments, the circular polyribonucleotide encodes two or more (e.g., 2 to 100, 2 to 50, 2 to 20, 2 to 10, 5 to 100, 5 to 50, 5 to 20, or 5 to 10) copies of the same antifusogenic polypeptide.

In some embodiments, the circular polyribonucleotide encodes two or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, or 10) different (e.g., sharing less than 100% sequence identity) antifusogenic polypeptides. In some embodiments, the two or more different antifusogenic polypeptides are each selected from an antifusogenic polypeptide of Table 1. In some embodiments, the two or more different antifusogenic polypeptides each inhibit a different virus. For example, a circular polyribonucleotide may encode a first antifusogenic polypeptide that inhibits influenza and a second an antifusogenic polypeptide that inhibits

RSV. A circular polyribonucleotide may include a first antifusogenic polypeptide that inhibits influenza and a second antifusogenic polypeptide that inhibits SARS-CoV-2. A circular polyribonucleotide may include a first antifusogenic polypeptide that inhibits HIV and a second antifusogenic polypeptide that inhibits SARS-CoV-2. A circular polyribonucleotide may include a first antifusogenic polypeptide that inhibits HIV and a second antifusogenic polypeptide that inhibits HCV. Where either the first or second antifusogenic polypeptide inhibits a plurality of viruses, the first and second antifusogenic polypeptides may have different virus specificity.

Wherein a circular polyribonucleotide encodes two or more antifusogenic polypeptides, the antifusogenic polypeptides may be encoded in a single open reading frame or multiple open reading frames.

In some embodiments, the disclosure provides a circular polyribonucleotide including an open reading frame (e.g., an open reading frame operably linked to an IRES) that includes two or more expression sequences, where each expression sequence encodes an antifusogenic polypeptide. In some embodiments, translation of the open reading frame produces a polypeptide fusion including the two or more antifusogenic polypeptides. The antifusogenic polypeptides may be linked, e.g., by a linker described herein (e.g., a peptide linker encoded by the open reading frame, such as a glycine-serine linker described below with respect to peptide-Fc fusions). In some embodiments, the antifusogenic polypeptides may be separated by a cleavage domain (e.g., a stagger sequence), for example as described herein.

In some embodiments, the disclosure provides a circular polyribonucleotide including a first open reading frame encoding a first antifusogenic polypeptide (e.g., operably linked to a first IRES) and a second open reading frame encoding a second antifusogenic polypeptide (e.g., operably linked to a second IRES).

25 *Antifusogenic Polypeptide-Fc Fusions*

In some embodiments, the circular polyribonucleotide includes an expression sequence encoding a fusion protein including an antifusogenic polypeptide. In some embodiments, the fusion protein includes an antifusogenic polypeptide fused to an Fc domain (e.g., a single chain of an Fc domain) of an immunoglobulin. In some embodiments, the antifusogenic polypeptide is selected from Table 1. In some embodiments, the circular polyribonucleotide includes an expression sequence encoding more than one fusion protein including an antifusogenic polypeptide. In some embodiments, the circular polyribonucleotide includes an expression sequence encoding more than one antifusogenic polypeptide. In some embodiments, the circular polyribonucleotide encoding more than one fusion protein reduces the chance for viral escape.

In some embodiments, the Fc domain is an IgG4 Fc domain or a fragment thereof. In some embodiments, the Fc domain is an IgG1 Fc domain or a fragment thereof. In some embodiments, the Fc domain is an IgG2 Fc domain or a fragment thereof. In some embodiments, the Fc domain is an IgG2a Fc domain or a fragment thereof. In some embodiments, the Fc domain is an IgG2b Fc domain or a fragment thereof. In some embodiments, the Fc domain is an IgG3 Fc domain or a fragment thereof.

In some embodiments, the C-terminal amino acid residue of the antifusogenic polypeptide is fused to the N-terminal amino acid residue of the Fc domain, optionally via a peptide linker. In some

embodiments, the N-terminal amino acid residue of the antifusogenic polypeptide is fused to the C-terminal amino acid residue of the Fc domain, optionally via a peptide linker. In some embodiments, the peptide linker between the antifusogenic polypeptide and the Fc domain includes at least two amino acid residues (e.g., at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, or at least 20 amino acid residues). In some embodiments, the peptide linker between the antifusogenic polypeptide and the Fc domain includes 2-200 amino acids residue (e.g., 2-200, 2-180, 2-160, 2-140, 2-120, 2-100, 2-90, 2-80, 2-70, 2-60, 2-50, 2-45, 2-40, 2-35, 2-30, 2-25, 2-20, 2-15, 2-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 4-200, 5-200, 6-200, 7-200, 8-200, 9-200, 10-200, 15-200, 20-200, 25-200, 30-200, 35-200, 40-200, 45-200, 50-200, 60-200, 70-200, 80-200, 90-200, 100-200, 120-200, 140-200, 160-200, or 180-200 amino acids residues). In some embodiments, the peptide linker consists of glycine (Gly) and serine (Ser) residues. In some embodiments, the peptide linker includes the amino acid sequence of any one of (GS)_x, (GGS)_x, (GGGS)_x, (GGSG)_x, or (SGGG)_x, wherein x is an integer from 1 to 50 (e.g., 1-40, 1-30, 1-20, 1-10, or 1-5). In some embodiments, the peptide linker includes the amino acid sequence of any one of (GS)_x, (GGS)_x, (GGGS)_x, (GGSG)_x, or (SGGG)_x, wherein x is an integer from 1 to 10 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10). In some embodiments, the peptide linker includes 6 to 36 amino acids. In some embodiments, the peptide linker includes 21 to 31 amino acids.

Polyribonucleotide Cargo

A polyribonucleotide cargo described herein includes any sequence including at least one polyribonucleotide. In some embodiments, the polyribonucleotide cargo includes an expression sequence, a non-coding sequence, or an expression sequence and a non-coding sequence. In some embodiments, the polyribonucleotide cargo includes an expression sequence encoding an antifusogenic polypeptide. In some embodiments, the polyribonucleotide cargo includes an IRES operably linked to an expression sequence encoding an antifusogenic polypeptide. In some embodiments, the polyribonucleotide cargo includes an expression sequence that encodes an antifusogenic polypeptide that has a biological effect on a subject.

A polyribonucleotide cargo may, for example, include at least about 40 nucleotides, at least about 50 nucleotides, at least about 75 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, at least about 300 nucleotides, at least about 400 nucleotides, at least about 500 nucleotides, at least about 1,000 nucleotides, at least about 2,000 nucleotides, at least about 5,000 nucleotides, at least about 6,000 nucleotides, at least about 7,000 nucleotides, at least about 8,000 nucleotides, at least about 9,000 nucleotides, at least about 10,000 nucleotides, at least about 12,000 nucleotides, at least about 14,000 nucleotides, at least about 15,000 nucleotides, at least about 16,000 nucleotides, at least about 17,000 nucleotides, at least about 18,000 nucleotides, at least about 19,000 nucleotides, or at least about 20,000 nucleotides. In some embodiments, the polyribonucleotide cargo includes from 1-20,000 nucleotides, 1-10,000 nucleotides, 1-5,000 nucleotides, 100-20,000 nucleotide, 100-10,000 nucleotides, 100-5,000 nucleotides, 500-20,000 nucleotides, 500-10,000 nucleotides, 500-5,000 nucleotides, 1,000-20,000 nucleotides, 1,000-10,000 nucleotides, or 1,000-5,000 nucleotides.

In embodiments, the polyribonucleotide cargo includes one or multiple expression (or coding) sequences, wherein each expression (or coding) sequence encodes a polypeptide (e.g., an antifusogenic polypeptide). In embodiments, the polyribonucleotide cargo includes one or multiple noncoding

sequences. In embodiments, the polyribonucleotide cargo consists entirely of non-coding sequence(s). In embodiments, the polyribonucleotide cargo includes a combination of expression (or coding) and noncoding sequences.

5 In some embodiments, the polyribonucleotide includes any feature, or any combination of features as disclosed in International Patent Publication No. WO2019/118919, which is hereby incorporated by reference in its entirety.

Polypeptide expression sequences

10 In some embodiments, the polyribonucleotide described herein (*e.g.*, the polyribonucleotide cargo of the circular polyribonucleotide) includes one or more expression (or coding) sequences, wherein each expression sequence encodes an antifusogenic polypeptide. In some embodiments, the circular polyribonucleotide includes two, three, four, five, six, seven, eight, nine, ten or more expression (or coding) sequences.

Each encoded polypeptide may be linear or branched. In various embodiments, the polypeptide 15 has a length from about 5 to about 40,000 amino acids, about 15 to about 35,000 amino acids, about 20 to about 30,000 amino acids, about 25 to about 25,000 amino acids, about 50 to about 20,000 amino acids, about 100 to about 15,000 amino acids, about 200 to about 10,000 amino acids, about 500 to about 5,000 amino acids, about 1,000 to about 2,500 amino acids, or any range therebetween. In some 20 embodiments, the polypeptide has a length of less than about 40,000 amino acids, less than about 35,000 amino acids, less than about 30,000 amino acids, less than about 25,000 amino acids, less than about 20,000 amino acids, less than about 15,000 amino acids, less than about 10,000 amino acids, less than about 9,000 amino acids, less than about 8,000 amino acids, less than about 7,000 amino acids, less than about 6,000 amino acids, less than about 5,000 amino acids, less than about 4,000 amino acids, less than about 3,000 amino acids, less than about 2,500 amino acids, less than about 2,000 25 amino acids, less than about 1,500 amino acids, less than about 1,000 amino acids, less than about 900 amino acids, less than about 800 amino acids, less than about 700 amino acids, less than about 600 amino acids, less than about 500 amino acids, less than about 400 amino acids, less than about 300 amino acids, or less may be useful.

Polypeptides included herein may include naturally occurring polypeptides or non-naturally 30 occurring polypeptides. In some embodiments, the polypeptide is or includes a functional fragment or variant of a reference polypeptide (*e.g.*, a biologically active fragment or variant of an antifusogenic polypeptide). For example, the polypeptide may be a functionally active variant of any of the polypeptides described herein with at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% 35 identity, *e.g.*, over a specified region or over the entire sequence, to a sequence of a polypeptide described herein or a naturally occurring polypeptide. In some instances, the polypeptide may have at least 50% (*e.g.*, at least 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, or greater) identity to a protein of interest.

In embodiments, polypeptides include multiple polypeptides, *e.g.*, multiple copies of one 40 polypeptide sequence, or multiple different polypeptide sequences. In embodiments, multiple polypeptides are connected by linker amino acids or spacer amino acids.

In embodiments, the polynucleotide cargo includes a sequence encoding a signal peptide. Many signal peptide sequences have been described, for example, the Tat (Twin-arginine translocation) signal sequence is typically an N-terminal peptide sequence containing a consensus SRRxFLK "twin-arginine" motif, which serves to translocate a folded protein containing such a Tat signal peptide across a lipid bilayer. See also, *e.g.*, the Signal Peptide Database publicly available at [www\[dot\]signalpeptide\[dot\]de](http://www.signalpeptide.de). Signal peptides are also useful for directing a protein to specific organelles; see, *e.g.*, the experimentally determined and computationally predicted signal peptides disclosed in the Spdb signal peptide database, publicly available at proline.bic.nus.edu.sg/spdb.

In some embodiments, the expression (or coding) sequence includes a poly-A sequence (*e.g.*, at the 3' end of an expression sequence). In some embodiments, the length of a poly-A sequence is greater than 10 nucleotides in length. In one embodiment, the poly-A sequence is greater than 15 nucleotides in length (*e.g.*, at least or greater than about 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000 nucleotides). In some embodiments, the poly-A sequence is designed according to the descriptions of the poly-A sequence in [0202]-[0204] of International Patent Publication No. WO2019/118919A1, which is incorporated herein by reference in its entirety. In some embodiments, the expression sequence lacks a poly-A sequence (*e.g.*, at the 3' end of an expression sequence).

In some embodiments, a circular polyribonucleotide includes a polyA, lacks a polyA, or has a modified polyA to modulate one or more characteristics of the circular polyribonucleotide. In some embodiments, the circular polyribonucleotide lacking a polyA or having modified polyA improves one or more functional characteristics, *e.g.*, immunogenicity (*e.g.*, the level of one or more marker of an immune or inflammatory response), half-life, and/or expression efficiency.

25 *Internal Ribosomal Entry Sites*

In some embodiments, a circular polyribonucleotide described herein includes one or more internal ribosome entry site (IRES) elements. In some embodiments, the IRES is operably linked to one or more expression sequences (*e.g.*, each IRES is operably linked to one or more expression sequences). In embodiments, the IRES is located between a heterologous promoter and the 5' end of a coding sequence.

A suitable IRES element to include in a polyribonucleotide includes an RNA sequence capable of engaging a eukaryotic ribosome. In some embodiments, the IRES element is at least about 5 nt, at least about 8 nt, at least about 9 nt, at least about 10 nt, at least about 15 nt, at least about 20 nt, at least about 25 nt, at least about 30 nt, at least about 40 nt, at least about 50 nt, at least about 100 nt, at least about 200 nt, at least about 250 nt, at least about 350 nt, or at least about 500 nt.

In some embodiments, the IRES element is derived from the DNA of an organism including, but not limited to, a virus, a mammal, and a *Drosophila*. Such viral DNA may be derived from, but is not limited to, picomavirus complementary DNA (cDNA), with encephalomyocarditis virus (EMCV) cDNA and poliovirus cDNA. In one embodiment, *Drosophila* DNA from which an IRES element is derived includes, but is not limited to, an Antennapedia gene from *Drosophila melanogaster*.

In some embodiments, the IRES sequence is an IRES sequence of Taura syndrome virus, Triatoma virus, Theiler's encephalomyelitis virus, simian Virus 40, Solenopsis invicta virus 1, Rhopalosiphum padi virus, Reticuloendotheliosis virus, fuman poliovirus 1, Plautia stall intestine virus, Kashmir bee virus, Human rhinovirus 2 (HRV-2), Homalodisca coagulata virus-1, Human

5 Immunodeficiency Virus type 1, Homalodisca coagulata virus- 1, Himetobi P virus, Hepatitis C virus, Hepatitis A virus, Hepatitis GB virus, foot and mouth disease virus, Human enterovirus 71, Equine rhinitis virus, Ectropis obliqua picorna-like virus, Encephalomyocarditis virus (EMCV), Drosophila C Virus, Crucifer tobamo virus, Cricket paralysis virus, Bovine viral diarrhea virus 1, Black Queen Cell Virus, Aphid lethal paralysis virus, Avian encephalomyelitis virus (AEV), Acute bee paralysis virus, Hibiscus chlorotic

10 ringspot virus, Classical swine fever virus, Human FGF2, Human SFTPA1, Human AML1/RUNX1, Drosophila antennapedia, Human AQP4, Human AT1R, Human BAG-I, Human BCL2, Human BiP, Human c-IAP1, Human c-myc, Human eIF4G, Mouse NDST4L, Human LEF1, Mouse HIF1 alpha, Human n.myc, Mouse Gtx, Human p27kipl, Human PDGF2/c-sis, Human p53, Human Pim-I, Mouse Rbm3, Drosophila reaper, Canine Scamper, Drosophila Ubx, Human UNR, Mouse UtrA, Human VEGF-A,

15 Human XIAP, Salivirus, Cosavirus, Parechovirus, Drosophila hairless, S. cerevisiae TFIIID, S. cerevisiae YAP1, Human c-src, Human FGF-I, Simian picomavirus, Turnip crinkle virus, Aichivirus, Crohivirus, Echovirus 11, an aptamer to eIF4G, Coxsackievirus B3 (CVB3) or Coxsackievirus A (CVB1/2). In yet another embodiment, the IRES is an IRES sequence of Coxsackievirus B3 (CVB3). In a further embodiment, the IRES is an IRES sequence of Encephalomyocarditis virus. In a further embodiment, the

20 IRES is an IRES sequence of Theiler's encephalomyelitis virus.

The IRES sequence may have a modified sequence in comparison to the wild-type IRES sequence. In some embodiments, when the last nucleotide of the wild-type IRES is not a cytosine nucleic acid residue, the last nucleotide of the wild-type IRES sequence may be modified such that it is a cytosine residue. For example, the IRES sequence may be a CVB3 IRES sequence wherein the terminal

25 adenosine residue is modified to cytosine residue. In some embodiments, the modified CVB3 IRES may have the nucleic acid sequence of:

TTAAACAGCCTGTGGGTTGATCCCACCCACAGGCCCATTTGGGCGCTAGCACTCTGGTATCACGGT
ACCTTTGTGCGCCTGTTTTATACCCCTCCCCCACTGTAACCTAGAAGTAACACACACCGATCAACA
GTCAGCGTGGCACACCAGCCACGTTTTGATCAAGCACTTCTGTTACCCCGGACTGAGTATCAATAGA
30 CTGCTCACGCGGTTGAAGGAGAAAGCGTTTCGTTATCCGGCCAACTACTTCGAAAAACCTAGTAACAC
CGTGGAAGTTGCAGAGTGTTCGCTCAGCACTACCCAGTGTAGATCAGGTGATGAGTCACCGCA
TTCCCCACGGGCGACCGTGGCGGTGGCTGCGTTGGCGGCCTGCCCATGGGGAAACCCATGGGAC
GCTCTAATACAGACATGGTGCGAAGAGTCTATTGAGCTAGTTGGTAGTCCTCCGGCCCCTGAATGCG
GCTAATCCTAACTGCGGAGCACACACCCCTCAAGCCAGAGGGCAGTGTGTCGTAACGGGCAACTCTG
35 CAGCGGAACCGACTACTTTGGGTGTCCGTGTTTCATTTATTCCTATACTGGCTGCTTATGGTGACAA
TTGAGAGATCGTTACCATATAGCTATTGGATTGGCCATCCGGTGACTAATAGAGCTATTATATATCCC
TTTGTGGGTTTATACCACTTAGCTTGAAAGAGGTTAAACATTACAATTCATTGTTAAGTTGAATACA
GCAAC (SEQ ID NO: 325).

In some embodiments, the IRES sequence is an Enterovirus 71 (EV17) IRES. In some

40 embodiments, the terminal guanosine residue of the EV17 IRES sequence is modified to a cytosine residue. In some embodiments, the modified EV71 IRES may have the nucleic acid sequence of:

ACGTTACTGGCCGAAGCCGCTTGAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCACCAT
 ATTGCCGTCTTTTGGCAATGTGAGGGCCCCGAAACCTGGCCCTGTCTTCTTGACGAGCATTTCCTAGG
 GGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGG
 AAGCTTCTTGAAGACAAACAACGTCTGTAGCGACCCCTTTCAGGCAGCGGAACCCCCCACCTGGCG
 5 ACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCCAGT
 GCCACGTTGTGAGTTGGATAGTTGTGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTCAACAAGGG
 GCTGAAGGATGCCAGAAGGTACCCCATTTGTATGGGATCTGATCTGGGGCCTCGGTGCACATGCTT
 TACATGTGTTTAGTCGAGGTTAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTT
 GAAAAACACGATGATAATA (SEQ ID NO: 326).

10 In some embodiments, the polyribonucleotide includes at least one IRES flanking at least one
 (e.g., 2, 3, 4, 5 or more) expression sequence. In some embodiments, the IRES flanks both sides of at
 least one (e.g., 2, 3, 4, 5 or more) expression sequence. In some embodiments, the polyribonucleotide
 includes one or more IRES sequences on one or both sides of each expression sequence, leading to
 separation of the resulting peptide(s) and or polypeptide(s). For example, a polyribonucleotide described
 15 herein may include a first IRES operably linked to a first expression sequence and a second IRES
 operably linked to a second expression sequence.

In some embodiments, a polyribonucleotide described herein includes an IRES (e.g., an IRES
 operably linked to a coding region). For example, the polyribonucleotide may include any IRES as
 described in Chen et al. MOL. CELL 81(20):4300-18, 2021; Jopling et al. ONCOGENE 20:2664-70, 2001;
 20 Baranick et al. PNAS 105(12):4733-38, 2008; Lang et al. MOLECULAR BIOLOGY OF THE CELL 13(5):1792-
 1801, 2002; Dorokhov et al. PNAS 99(8):5301-06, 2002; Wang et al. NUCLEIC ACIDS RESEARCH
 33(7):2248-58, 2005; Petz et al. NUCLEIC ACIDS RESEARCH 35(8):2473-82, 2007; Chen et al. SCIENCE
 268:415-417, 1995; Fan et al. NATURE COMMUNICATION 13(1):3751-3765, 2022, and International
 Publication No. WO2021/263124, each of which is hereby incorporated by reference in their entirety.

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Signal Sequences

In some embodiments, an antifusogenic polypeptide expressed from a circular polyribonucleotide
 disclosed herein includes a secreted protein, for example, a protein that naturally includes a signal
 sequence, or one that does not usually encode a signal sequence but is modified to contain one. In some
 30 embodiments, the antifusogenic polypeptide encoded by the circular polyribonucleotide includes a
 secretion signal. For example, the secretion signal may be the naturally encoded secretion signal for a
 secreted protein. In another example, the secretion signal may be a modified secretion signal for a
 secreted protein. In other embodiments, the antifusogenic polypeptide encoded by the circular
 polyribonucleotide does not include a secretion signal.

35 In some embodiments, the signal sequence is selected from SecSP38
 (MWWRLWWLLLLLLLWPMVWA; SEQ ID NO: 327); SecD4 (MWWLLLLLLLWPMVWA; SEQ ID NO:
 328), gLuc (MGVKVLFALICIAVAEAK; SEQ ID NO: 329); INHC1 (MASRLTLLTLLLLLAGDRASS; SEQ
 ID NO: 330); Epo (MGVHECPAWLWLLSLSLPLGLPVLG; SEQ ID NO: 331); and IL-2
 (MYRMQLLSIALSLALVTNS; SEQ ID NO: 332).

40 In some embodiments, a circular polyribonucleotide encodes multiple copies of the same
 antifusogenic polypeptide (e.g., one, two, three, four, five, six, seven, eight, nine, ten, or more). In some

embodiments, at least one copy of the antifusogenic polypeptide includes a signal sequence and at least one copy of the antifusogenic polypeptide does not include a signal sequence. In some embodiments, a circular polyribonucleotide encodes plurality of an antifusogenic polypeptides (e.g., a plurality of different antifusogenic polypeptides or a plurality of an antifusogenic polypeptides having less than 100% sequence identity), where at least one of the plurality of an antifusogenic polypeptides includes a signal sequence and at least one copy of the plurality of an antifusogenic polypeptides does not include a signal sequence.

In some embodiments, the signal sequence is a wild-type signal sequence that is present on the N-terminus of the corresponding wild-type antifusogenic polypeptide, e.g., when expressed endogenously. In some embodiments, the signal sequence is heterologous to the antifusogenic polypeptide, e.g., is not present when the wild-type antifusogenic polypeptide is expressed endogenously. A polyribonucleotide sequence encoding an antifusogenic polypeptide may be modified to remove the nucleotide sequence encoding a wild-type signal sequence and/or add a sequence encoding a heterologous signal sequence.

A polypeptide encoded by a polyribonucleotide (e.g., an antifusogenic polypeptide) may include a signal sequence that directs the antifusogenic polypeptide to the secretory pathway. In some embodiments, the signal sequence may direct the antifusogenic polypeptide to reside in certain organelles (e.g., the endoplasmic reticulum, Golgi apparatus, or endosomes). In some embodiments, the signal sequence directs the antifusogenic polypeptide to be secreted from the cell. For secreted proteins, the signal sequence may be cleaved after secretion, resulting in a mature protein. In other embodiments, the signal sequence may become embedded in the membrane of the cell or certain organelles, creating a transmembrane segment that anchors the protein to the membrane of the cell, endoplasmic reticulum, or Golgi apparatus. In certain embodiments, the signal sequence of a transmembrane protein is a short sequence at the N-terminal of the polypeptide. In other embodiments, the first transmembrane domain acts as the first signal sequence, which targets the protein to the membrane.

In some embodiments, the secretion signal is a human interleukin-2 (IL-2) secretion signal. In some embodiments, the IL-2 secretion signal has an amino acid sequence of at least 90% sequence identity to MYRMQLLSIALSLALVTNS (SEQ ID NO: 332). In some embodiments, the IL-2 secretion signal has an amino acid sequence of at least 95% sequence identity to SEQ ID NO: 332. In some embodiments, the IL-2 secretion signal has an amino acid sequence of at least 99% sequence identity to SEQ ID NO: 332. In some embodiments, the IL-2 secretion signal has an amino acid sequence of 100% sequence identity to SEQ ID NO: 332.

In some embodiments, the secretion signal is Gaussia luciferase secretion signal. In some embodiments, the Gaussia luciferase secretion signal has an amino acid sequence of at least 90% sequence identity of MGVKVLFALICIAVAEAK (SEQ ID NO: 329). In some embodiments, the Gaussia luciferase secretion signal has an amino acid sequence of at least 95% sequence identity of SEQ ID NO: 329. In some embodiments, the Gaussia luciferase secretion signal has an amino acid sequence of at least 99% sequence identity of SEQ ID NO: 329. In some embodiments, the Gaussia luciferase secretion signal has an amino acid sequence of 100% sequence identity of SEQ ID NO: 329.

In some embodiments, the secretion signal is an EPO (e.g., a human EPO) secretion signal. In some embodiments, the EPO secretion signal has an amino acid sequence of at least 90% sequence

identity of MGVHECPAWLWLLLSLLSLPLGLPVLGA (SEQ ID NO: 333). In some embodiments, the EPO secretion signal has an amino acid sequence of at least 95% sequence identity of SEQ ID NO: 333. In some embodiments, the EPO secretion signal has an amino acid sequence of at least 99% sequence identity of SEQ ID NO: 333. In some embodiments, the EPO secretion signal has an amino acid sequence of 100% sequence identity of SEQ ID NO: 333.

In some embodiments, the secretion signal is a wildtype SARS-CoV-2 secretion signal. In some embodiments, the wildtype SARS-CoV-2 secretion signal has an amino acid sequence of at least 90% sequence identity of MFVFLVLLPLVSS (SEQ ID NO: 334). In some embodiments, the wildtype SARS-CoV-2 secretion signal has an amino acid sequence of at least 95% sequence identity of SEQ ID NO: 334. In some embodiments, the wildtype SARS-CoV-2 secretion signal has an amino acid sequence of at least 99% sequence identity of SEQ ID NO: 334. In some embodiments, the wildtype SARS-CoV-2 secretion signal has an amino acid sequence of 100% sequence identity of SEQ ID NO: 334.

In some embodiments, an antifusogenic polypeptide encoded by a polyribonucleotide includes either a secretion signal sequence, a transmembrane insertion signal sequence, or does not include a signal sequence.

Regulatory Elements

In some embodiments, the polyribonucleotide described herein (e.g., the polyribonucleotide cargo of the polyribonucleotide) includes one or more regulatory elements. In some embodiments, the polyribonucleotide includes a regulatory element, e.g., a sequence that modifies expression of an expression sequence within the polyribonucleotide.

A regulatory element may include a sequence that is located adjacent to an expression sequence that encodes an expression product. A regulatory element may be linked operatively to the adjacent sequence. A regulatory element may increase an amount of product expressed as compared to an amount of the expressed product when no regulatory element exists. In addition, one regulatory element can increase an amount of products expressed for multiple expression sequences attached in tandem. Hence, one regulatory element can enhance the expression of one or more expression sequences. Multiple regulatory elements are well-known to persons of ordinary skill in the art.

In some embodiments, the regulatory element is a translation modulator. A translation modulator can modulate translation of the expression sequence in the polyribonucleotide. A translation modulator can be a translation enhancer or suppressor. In some embodiments, the polyribonucleotide includes at least one translation modulator adjacent to at least one expression sequence. In some embodiments, the polyribonucleotide includes a translation modulator adjacent each expression sequence. In some embodiments, the translation modulator is present on one or both sides of each expression sequence, leading to separation of the expression products, e.g., peptide(s) and or polypeptide(s).

In some embodiments, the regulatory element is a microRNA (miRNA) or a miRNA binding site.

Further examples of regulatory elements are described, e.g., in paragraphs [0154] – [0161] of International Patent Publication No. WO2019/118919, which is hereby incorporated by reference in its entirety.

Cleavage Domains

A circular polyribonucleotide of the disclosure can include a cleavage domain (e.g., a stagger element or a cleavage sequence).

The term "stagger element" refers to a moiety, such as a nucleotide sequence, that induces ribosomal pausing during translation. In some embodiments, the stagger element is a non-conserved sequence of amino-acids with a strong alpha-helical propensity followed by the consensus sequence -D(V/I)ExNPGP, where x= any amino acid (SEQ ID NO: 335). In some embodiments, the stagger element may include a chemical moiety, such as glycerol, a non-nucleic acid linking moiety, a chemical modification, a modified nucleic acid, or any combination thereof.

In some embodiments, the circular polyribonucleotide includes at least one stagger element adjacent to an expression sequence. In some embodiments, the circular polyribonucleotide includes a stagger element adjacent to each expression sequence. In some embodiments, the stagger element is present on one or both sides of each expression sequence, leading to separation of the expression products, e.g., peptide(s) and or polypeptide(s). In some embodiments, the stagger element is a portion of the one or more expression sequences. In some embodiments, the circular polyribonucleotide includes one or more expression sequences, and each of the one or more expression sequences is separated from a succeeding expression sequence by a stagger element on the circular polyribonucleotide. In some embodiments, the stagger element prevents generation of a single polypeptide (a) from two rounds of translation of a single expression sequence or (b) from one or more rounds of translation of two or more expression sequences. In some embodiments, the stagger element is a sequence separate from the one or more expression sequences. In some embodiments, the stagger element includes a portion of an expression sequence of the one or more expression sequences.

In some embodiments, the circular polyribonucleotide includes a stagger element. To avoid production of a continuous expression product, e.g., peptide or polypeptide, while maintaining rolling circle translation, a stagger element may be included to induce ribosomal pausing during translation. In some embodiments, the stagger element is at 3' end of at least one of the one or more expression sequences. The stagger element can be configured to stall a ribosome during rolling circle translation of the circular polyribonucleotide. The stagger element may include, but is not limited to a 2A-like, or CHYSEL (SEQ ID NO: 336) (cis-acting hydrolase element) sequence. In some embodiments, the stagger element encodes a sequence with a C-terminal consensus sequence that is $X_1X_2X_3EX_5NPGP$ (SEQ ID NO: 337), where X_1 is absent or G or H, X_2 is absent or D or G, X_3 is D or V or I or S or M, and X_5 is any amino acid. In some embodiments, this sequence includes a non-conserved sequence of amino-acids with a strong alpha-helical propensity followed by the consensus sequence -D(V/I)EXNPGP (SEQ ID NO: 338), where x= any amino acid. Some nonlimiting examples of stagger elements includes GDVESNPGP (SEQ ID NO: 339), GDIEENPGP (SEQ ID NO: 340), VEPNPGP (SEQ ID NO: 341), IETNPGP (SEQ ID NO: 342), GDIESNPGP (SEQ ID NO: 343), GDVELNPGP (SEQ ID NO: 344), GDIETNPGP (SEQ ID NO: 345), GDVENPGP (SEQ ID NO: 346), GDVEENPGP (SEQ ID NO: 347), GDVEQNPGP (SEQ ID NO: 348), IESNPGP (SEQ ID NO: 349), GDIELNPGP (SEQ ID NO: 350), HDIETNPGP (SEQ ID NO: 351), HDVETNPGP (SEQ ID NO: 352), HDVEMNPGP (SEQ ID NO: 353), GDMESNPGP (SEQ ID NO: 354), GDVETNPGP (SEQ ID NO: 355), GDIEQNPGP (SEQ ID NO: 356), and DSEFNPGP (SEQ ID NO: 357).

In some embodiments, the stagger element described herein cleaves an expression product, such as between G and P of the consensus sequence described herein. As one non-limiting example, the circular polyribonucleotide includes at least one stagger element to cleave the expression product. In some embodiments, the circular polyribonucleotide includes a stagger element adjacent to at least one expression sequence. In some embodiments, the circular polyribonucleotide includes a stagger element after each expression sequence. In some embodiments, the circular polyribonucleotide includes a stagger element is present on one or both sides of each expression sequence, leading to translation of individual peptide(s) and or polypeptide(s) from each expression sequence.

In some embodiments, a stagger element includes one or more modified nucleotides or unnatural nucleotides that induce ribosomal pausing during translation. Unnatural nucleotides may include peptide nucleic acid (PNA), Morpholino and locked nucleic acid (LNA), as well as glycol nucleic acid (GNA) and threose nucleic acid (TNA). Examples such as these are distinguished from naturally occurring DNA or RNA by changes to the backbone of the molecule. Exemplary modifications can include any modification to the sugar, the nucleobase, the internucleoside linkage (e.g., to a linking phosphate / to a phosphodiester linkage / to the phosphodiester backbone), and any combination thereof that can induce ribosomal pausing during translation. Some of the exemplary modifications provided herein are described elsewhere herein.

In some embodiments, the stagger element is present in the circular polyribonucleotide in other forms. For example, in some exemplary circular polyribonucleotides, a stagger element includes a termination element of a first expression sequence in the circular polyribonucleotide, and a nucleotide spacer sequence that separates the termination element from a first translation initiation sequence of an expression succeeding the first expression sequence. In some examples, the first stagger element of the first expression sequence is upstream of (5' to) a first translation initiation sequence of the expression succeeding the first expression sequence in the circular polyribonucleotide. In some cases, the first expression sequence and the expression sequence succeeding the first expression sequence are two separate expression sequences in the circular polyribonucleotide. The distance between the first stagger element and the first translation initiation sequence can enable continuous translation of the first expression sequence and its succeeding expression sequence.

In some embodiments, the first stagger element includes a termination element and separates an expression product of the first expression sequence from an expression product of its succeeding expression sequences, thereby creating discrete expression products. In some cases, the circular polyribonucleotide including the first stagger element upstream of the first translation initiation sequence of the succeeding sequence in the circular polyribonucleotide is continuously translated, while a corresponding circular polyribonucleotide including a stagger element of a second expression sequence that is upstream of a second translation initiation sequence of an expression sequence succeeding the second expression sequence is not continuously translated. In some cases, there is only one expression sequence in the circular polyribonucleotide, and the first expression sequence and its succeeding expression sequence are the same expression sequence. In some exemplary circular polyribonucleotides, a stagger element includes a first termination element of a first expression sequence in the circular polyribonucleotide, and a nucleotide spacer sequence that separates the termination element from a downstream translation initiation sequence. In some such examples, the first stagger

element is upstream of (5' to) a first translation initiation sequence of the first expression sequence in the circular polyribonucleotide. In some cases, the distance between the first stagger element and the first translation initiation sequence enables continuous translation of the first expression sequence and any succeeding expression sequences.

5 In some embodiments, the first stagger element separates one round expression product of the first expression sequence from the next round expression product of the first expression sequences, thereby creating discrete expression products. In some cases, the circular polyribonucleotide including the first stagger element upstream of the first translation initiation sequence of the first expression sequence in the circular polyribonucleotide is continuously translated, while a corresponding circular
10 polyribonucleotide including a stagger element upstream of a second translation initiation sequence of a second expression sequence in the corresponding circular polyribonucleotide is not continuously translated. In some cases, the distance between the second stagger element and the second translation initiation sequence is at least 2x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, or 10x greater in the corresponding circular polyribonucleotide than a distance between the first stagger element and the first translation initiation in
15 the circular polyribonucleotide. In some cases, the distance between the first stagger element and the first translation initiation is at least 2 nt, 3 nt, 4 nt, 5 nt, 6 nt, 7 nt, 8 nt, 9 nt, 10 nt, 11 nt, 12 nt, 13 nt, 14 nt, 15 nt, 16 nt, 17 nt, 18 nt, 19 nt, 20 nt, 25 nt, 30 nt, 35 nt, 40 nt, 45 nt, 50 nt, 55 nt, 60 nt, 65 nt, 70 nt, 75 nt, or greater. In some embodiments, the distance between the second stagger element and the second translation initiation is at least 2 nt, 3 nt, 4 nt, 5 nt, 6 nt, 7 nt, 8 nt, 9 nt, 10 nt, 11 nt, 12 nt, 13 nt, 14 nt, 15
20 nt, 16 nt, 17 nt, 18 nt, 19 nt, 20 nt, 25 nt, 30 nt, 35 nt, 40 nt, 45 nt, 50 nt, 55 nt, 60 nt, 65 nt, 70 nt, 75 nt, or greater than the distance between the first stagger element and the first translation initiation. In some embodiments, the circular polyribonucleotide includes more than one expression sequence.

Examples of stagger elements are described in paragraphs [0172] – [0175] of International Patent Publication No. WO2019/118919, which is hereby incorporated by reference in its entirety.

25 In some embodiments, a plurality of an antifusogenic polypeptides encoded by a circular ribonucleotide may be separated by an IRES between each antifusogenic polypeptide (e.g., each antifusogenic polypeptide is operably linked to a separate IRES). For example, a circular polyribonucleotide may include a first IRES operably linked to a first expression sequence and a second IRES operably linked to a second expression sequence. The IRES may be the same IRES between all
30 antifusogenic polypeptides. The IRES may be different between different antifusogenic polypeptides.

In some embodiments, the plurality of an antifusogenic polypeptides may be separated by a 2A self-cleaving peptide. For example, a circular polyribonucleotide may encode an IRES operably linked to an open reading frame encoding a first antifusogenic polypeptide, a 2A, and a second antifusogenic polypeptide.

35 In some embodiments, the plurality of an antifusogenic polypeptides may be separated by a protease cleavage site (e.g., a furin cleavage site). For example, a circular polyribonucleotide may encode an IRES operably linked to an open reading frame encoding a first antifusogenic polypeptide, a protease cleavage site (e.g., a furin cleavage site), and a second antifusogenic polypeptide.

40 In some embodiments, the plurality of an antifusogenic polypeptides may be separated by a 2A self-cleaving peptide and a protease cleavage site (e.g., a furin cleavage site). For example, a circular polyribonucleotide may encode an IRES operably linked to an open reading frame encoding a first

antifusogenic polypeptide, a 2A, a protease cleavage site (e.g., a furin cleavage site), and a second antifusogenic polypeptide. A circular polyribonucleotide may also encode an IRES operably linked to an open reading frame encoding a first antifusogenic polypeptide, a protease cleavage site (e.g., a furin cleavage site), a 2A, and a second antifusogenic polypeptide. A tandem 2A and furin cleavage site may
 5 be referred to as a furin-2A (which includes furin-2A or 2A-furin, arranged in either orientation).

Furthermore, the plurality of an antifusogenic polypeptides encoded by the circular ribonucleotide may be separated by both IRES and 2A sequences. For example, an IRES may be between one antifusogenic polypeptide and a second antifusogenic polypeptide while a 2A peptide may be between the second antifusogenic polypeptide and the third antifusogenic polypeptide. The selection of a
 10 particular IRES or 2A self-cleaving peptide may be used to control the expression level of an antifusogenic polypeptide under control of the IRES or 2A sequence. For example, depending on the IRES and or 2A peptide selected, expression on the polypeptide may be higher or lower.

In some embodiments, a circular polyribonucleotide includes at least one cleavage sequence. In some embodiments, the cleavage sequence is adjacent to an expression sequence. In some
 15 embodiments, the cleavage sequence is between two expression sequences. In some embodiments, cleavage sequence is included in an expression sequence. In some embodiments, the circular polyribonucleotide includes between 2 and 10 cleavage sequences. In some embodiments, the circular polyribonucleotide includes between 2 and 5 cleavage sequences. In some embodiments, the multiple cleavage sequences are between multiple expression sequences; for example, a circular
 20 polyribonucleotide may include three expression sequences two cleavage sequences such that there is a cleavage sequence in between each expression sequence. In some embodiments, the circular polyribonucleotide includes a cleavage sequence, such as in an immolating circRNA or cleavable circRNA or self-cleaving circRNA. In some embodiments, the circular polyribonucleotide includes two or more cleavage sequences, leading to separation of the circular polyribonucleotide into multiple products,
 25 e.g., miRNAs, linear RNAs, smaller circular polyribonucleotide, etc.

In some embodiments, a cleavage sequence includes a ribozyme RNA sequence. A ribozyme (from ribonucleic acid enzyme, also called RNA enzyme or catalytic RNA) is an RNA molecule that catalyzes a chemical reaction. Many natural ribozymes catalyze either the hydrolysis of one of their own phosphodiester bonds, or the hydrolysis of bonds in other RNA, but they have also been found to
 30 catalyze the aminotransferase activity of the ribosome. Catalytic RNA can be “evolved” by in vitro methods. Similar to riboswitch activity discussed above, ribozymes and their reaction products can regulate gene expression. In some embodiments, a catalytic RNA or ribozyme can be placed within a larger non-coding RNA such that the ribozyme is present at many copies within the cell for the purposes of chemical transformation of a molecule from a bulk volume. In some embodiments, aptamers and
 35 ribozymes can both be encoded in the same non-coding RNA.

In some embodiments, the cleavage sequence encodes a cleavable polypeptide linker. For example, a polyribonucleotide may encode two or more antifusogenic polypeptides, e.g., where the two or more antifusogenic polypeptides are encoded by a single open-reading frame (ORF). For example, two or more antifusogenic polypeptides may be encoded by a single open-reading frame, the expression of
 40 which is controlled by an IRES. In some embodiments, the ORF further encodes a polypeptide linker, e.g., such that the expression product of the ORF encodes two or more antifusogenic polypeptides each

separated by a sequence encoding a polypeptide linker (e.g., a linker of 5-200, 5 to 100, 5 to 50, 5 to 20, 50 to 100, or 50 to 200 amino acids). The polypeptide linker may include a cleavage site, for example, a cleavage site recognized and cleaved by a protease (e.g., an endogenous protease in a subject following administration of the polyribonucleotide to that subject). In such embodiments, a single expression product including the amino acid sequence of two or more antifusogenic polypeptides is cleaved upon expression, such that the two or more antifusogenic polypeptides are separated following expression. Exemplary protease cleavage sites are known to those of skill in the art, for example, amino acid sequences that act as protease cleavage sites recognized by a metalloproteinase (e.g., a matrix metalloproteinase (MMP), such as any one or more of MMPs 1-28), a disintegrin and metalloproteinase (ADAM, such as any one or more of ADAMs 2, 7-12, 15, 17-23, 28-30 and 33), a serine protease (e.g., furin), urokinase-type plasminogen activator, matrilysin, a cysteine protease, an aspartic protease, or a cathepsin protease. In some embodiments, the protease is MMP9 or MMP2. In some embodiments, the protease is matrilysin.

In some embodiments, a circular polyribonucleotide described herein is an immolating circular polyribonucleotide, a cleavable circular polyribonucleotide, or a self-cleaving circular polyribonucleotide. A circular polyribonucleotide can deliver cellular components including, for example, RNA, lncRNA, lincRNA, miRNA, tRNA, rRNA, snoRNA, ncRNA, siRNA, or shRNA. In some embodiments, a circular polyribonucleotide includes miRNA separated by (i) self-cleavable elements; (ii) cleavage recruitment sites; (iii) degradable linkers; (iv) chemical linkers; and/or (v) spacer sequences. In some embodiments, circRNA includes siRNA separated by (i) self-cleavable elements; (ii) cleavage recruitment sites (e.g., ADAR); (iii) degradable linkers (e.g., glycerol); (iv) chemical linkers; and/or (v) spacer sequences. Non-limiting examples of self-cleavable elements include hammerhead, splicing element, hairpin, hepatitis delta virus (HDV), Varkud Satellite (VS), and *glmS* ribozymes.

25 *Translation Initiation Sequences*

In some embodiments, the polyribonucleotide described herein (e.g., the polyribonucleotide cargo of the polyribonucleotide) includes at least one translation initiation sequence. In some embodiments, the polyribonucleotide includes a translation initiation sequence operably linked to an expression sequence.

In some embodiments, the polyribonucleotide encodes a polypeptide and may include a translation initiation sequence, e.g., a start codon. In some embodiments, the translation initiation sequence includes a Kozak or Shine-Dalgarno sequence. In some embodiments, the polyribonucleotide includes the translation initiation sequence, e.g., Kozak sequence, adjacent to an expression sequence. In some embodiments, the translation initiation sequence is a non-coding start codon. In some embodiments, the translation initiation sequence, e.g., Kozak sequence, is present on one or both sides of each expression sequence, leading to separation of the expression products. In some embodiments, the polyribonucleotide includes at least one translation initiation sequence adjacent to an expression sequence. In some embodiments, the translation initiation sequence provides conformational flexibility to the polyribonucleotide. In some embodiments, the translation initiation sequence is within a substantially single stranded region of the polyribonucleotide. Further examples of translation initiation sequences are described in paragraphs [0163] – [0165] of International Patent Publication No. WO2019/118919, which is hereby incorporated by reference in its entirety.

The polyribonucleotide may include more than 1 start codon such as, but not limited to, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, at least 30, at least 35, at least 40, at least 50, at least 60 or more than 60 start codons. Translation
5 may initiate on the first start codon or may initiate downstream of the first start codon.

In some embodiments, the polyribonucleotide may initiate at a codon which is not the first start codon, e.g., AUG. Translation of the polyribonucleotide may initiate at an alternative translation initiation sequence, such as, but not limited to, ACG, AGG, AAG, CTG/CUG, GTG/GUG, ATA/AUA, ATT/AUU, TTG/UUG. In some embodiments, translation begins at an alternative translation initiation sequence
10 under selective conditions, e.g., stress induced conditions. As a non-limiting example, the translation of the polyribonucleotide may begin at alternative translation initiation sequence, such as ACG. As another non-limiting example, the polyribonucleotide translation may begin at alternative translation initiation sequence, CTG/CUG. As another non-limiting example, the polyribonucleotide translation may begin at alternative translation initiation sequence, GTG/GUG. As another non-limiting example, the
15 polyribonucleotide may begin translation at a repeat-associated non-AUG (RAN) sequence, such as an alternative translation initiation sequence that includes short stretches of repetitive RNA e.g., CGG, GGGGCC, CAG, CTG.

Termination Elements

20 In some embodiments, the polyribonucleotide described herein (e.g., the polyribonucleotide cargo of the polyribonucleotide) includes least one termination element. In some embodiments, the polyribonucleotide includes a termination element operably linked to an expression sequence. In some embodiments, the polynucleotide lacks a termination element.

In some embodiments, the polyribonucleotide includes one or more expression sequences, and
25 each expression sequence may or may not have a termination element. In some embodiments, the polyribonucleotide includes one or more expression sequences, and the expression sequences lack a termination element, such that the polyribonucleotide is continuously translated. Exclusion of a termination element may result in rolling circle translation or continuous expression of expression product.

In some embodiments, the circular polyribonucleotide includes one or more expression
30 sequences, and each expression sequence may or may not have a termination element. In some embodiments, the circular polyribonucleotide includes one or more expression sequences, and the expression sequences lack a termination element, such that the circular polyribonucleotide is continuously translated. Exclusion of a termination element may result in rolling circle translation or continuous expression of expression product, e.g., peptides or polypeptides, due to lack of ribosome
35 stalling or fall-off. In such an embodiment, rolling circle translation expresses a continuous expression product through each expression sequence. In some other embodiments, a termination element of an expression sequence can be part of a stagger element. In some embodiments, one or more expression sequences in the circular polyribonucleotide includes a termination element. However, rolling circle translation or expression of a succeeding (e.g., second, third, fourth, fifth, etc.) expression sequence in
40 the circular polyribonucleotide is performed. In such instances, the expression product may fall off the ribosome when the ribosome encounters the termination element, e.g., a stop codon, and terminates

translation. In some embodiments, translation is terminated while the ribosome, e.g., at least one subunit of the ribosome, remains in contact with the circular polyribonucleotide.

In some embodiments, the circular polyribonucleotide includes a termination element at the end of one or more expression sequences. In some embodiments, one or more expression sequences
5 includes two or more termination elements in succession. In such embodiments, translation is terminated and rolling circle translation is terminated. In some embodiments, the ribosome completely disengages with the circular polyribonucleotide. In some such embodiments, production of a succeeding (e.g., second, third, fourth, fifth, etc.) expression sequence in the circular polyribonucleotide may require the ribosome to reengage with the circular polyribonucleotide prior to initiation of translation. Generally,
10 termination elements include an in-frame nucleotide triplet that signals termination of translation, e.g., UAA, UGA, UAG. In some embodiments, one or more termination elements in the circular polyribonucleotide are frame-shifted termination elements, such as but not limited to, off-frame or -1 and + 1 shifted reading frames (e.g., hidden stop) that may terminate translation. Frame-shifted termination elements include nucleotide triples, TAA, TAG, and TGA that appear in the second and third reading
15 frames of an expression sequence. Frame-shifted termination elements may be important in preventing misreads of mRNA, which is often detrimental to the cell. In some embodiments, the termination element is a stop codon.

Further examples of termination elements are described in paragraphs [0169] – [0170] of International Patent Publication No. WO2019/118919, which is hereby incorporated by reference in its
20 entirety.

Untranslated Regions

In some embodiments, a circular polyribonucleotide includes untranslated regions (UTRs). UTRs of a genomic region including a gene may be transcribed but not translated. In some embodiments, a
25 UTR may be included upstream of the translation initiation sequence of an expression sequence described herein. In some embodiments, a UTR may be included downstream of an expression sequence described herein. In some instances, one UTR for first expression sequence is the same as or continuous with or overlapping with another UTR for a second expression sequence. In some embodiments, the intron is a human intron. In some embodiments, the intron is a full-length human
30 intron, e.g., ZKSCAN1.

Exemplary untranslated regions are described in paragraphs [0197] – [201] of International Patent Publication No. WO2019/118919, which is hereby incorporated by reference in its entirety.

In some embodiments, a circular polyribonucleotide includes a polyA sequence. Exemplary polyA sequences are described in paragraphs [0202] – [0205] of International Patent Publication No.
35 WO2019/118919, which is hereby incorporated by reference in its entirety. In some embodiments, a circular polyribonucleotide lacks a polyA sequence.

In some embodiments, a circular polyribonucleotide includes a UTR with one or more stretches of Adenosines and Uridines embedded within. These AU rich signatures may increase turnover rates of the expression product.

40 Introduction, removal, or modification of UTR AU rich elements (AREs) may be useful to modulate the stability, or immunogenicity (e.g., the level of one or more marker of an immune or

inflammatory response) of the circular polyribonucleotide. When engineering specific circular polyribonucleotides, one or more copies of an ARE may be introduced to the circular polyribonucleotide and the copies of an ARE may modulate translation and/or production of an expression product.

Likewise, AREs may be identified and removed or engineered into the circular polyribonucleotide to
 5 modulate the intracellular stability and thus affect translation and production of the resultant protein.

It should be understood that any UTR from any gene may be incorporated into the respective flanking regions of the circular polyribonucleotide.

In some embodiments, a circular polyribonucleotide lacks a 5'-UTR and is competent for protein expression from its one or more expression sequences. In some embodiments, the circular
 10 polyribonucleotide lacks a 3'-UTR and is competent for protein expression from its one or more expression sequences. In some embodiments, the circular polyribonucleotide lacks a polyA sequence and is competent for protein expression from its one or more expression sequences. In some embodiments, the circular polyribonucleotide lacks a termination element and is competent for protein expression from its one or more expression sequences. In some
 15 polyribonucleotide lacks an internal ribosomal entry site and is competent for protein expression from its one or more expression sequences. In some embodiments, the circular polyribonucleotide lacks a cap and is competent for protein expression from its one or more expression sequences. In some embodiments, the circular polyribonucleotide lacks a 5'-UTR, a 3'-UTR, and an IRES, and is competent for protein expression from its one or more expression sequences. In some
 20 polyribonucleotide includes one or more of the following sequences: a sequence that encodes one or more miRNAs, a sequence that encodes one or more replication proteins, a sequence that encodes an exogenous gene, a sequence that encodes a therapeutic, a regulatory element (e.g., translation modulator, e.g., translation enhancer or suppressor), a translation initiation sequence, one or more regulatory nucleic acids that targets endogenous genes (e.g., siRNA, lncRNAs, shRNA), and a sequence
 25 that encodes a therapeutic mRNA or protein.

In some embodiments, a circular polyribonucleotide lacks a 5'-UTR. In some embodiments, the circular polyribonucleotide lacks a 3'-UTR. In some embodiments, the circular polyribonucleotide lacks a polyA sequence. In some embodiments, the circular polyribonucleotide lacks a termination element. In some
 30 embodiments, the circular polyribonucleotide lacks an internal ribosomal entry site. In some embodiments, the circular polyribonucleotide lacks degradation susceptibility by exonucleases. In some embodiments, the fact that the circular polyribonucleotide lacks degradation susceptibility can mean that the circular polyribonucleotide is not degraded by an exonuclease, or only degraded in the presence of an exonuclease to a limited extent, e.g., that is comparable to or similar to in the absence of exonuclease. In some
 35 embodiments, the circular polyribonucleotide is not degraded by exonucleases. In some embodiments, the circular polyribonucleotide has reduced degradation when exposed to exonuclease. In some embodiments, the circular polyribonucleotide lacks binding to a cap-binding protein. In some embodiments, the circular polyribonucleotide lacks a 5' cap.

Protein-Binding Sequences

40 In some embodiments, a circular polyribonucleotide includes one or more protein binding sites that allow a protein, e.g., a ribosome, to bind to an internal site in the RNA sequence. By engineering

protein binding sites, *e.g.*, ribosome binding sites, into the circular polyribonucleotide, the circular polyribonucleotide may evade or have reduced detection by the host's immune system, have modulated degradation, or modulated translation, by masking the circular polyribonucleotide from components of the host's immune system.

5 In some embodiments, a circular polyribonucleotide includes at least one immunoprotein binding site, for example to evade immune responses, *e.g.*, CTL (cytotoxic T lymphocyte) responses. In some embodiments, the immunoprotein binding site is a nucleotide sequence that binds to an immunoprotein and aids in masking the circular polyribonucleotide as exogenous. In some embodiments, the immunoprotein binding site is a nucleotide sequence that binds to an immunoprotein and aids in hiding
10 the circular polyribonucleotide as exogenous or foreign.

Traditional mechanisms of ribosome engagement to linear RNA involve ribosome binding to the capped 5' end of an RNA. From the 5' end, the ribosome migrates to an initiation codon, whereupon the first peptide bond is formed. According to the present disclosure, internal initiation (*i.e.*, cap-independent) of translation of the circular polyribonucleotide does not require a free end or a capped end. Rather, a
15 ribosome binds to a non-capped internal site, whereby the ribosome begins polypeptide elongation at an initiation codon. In some embodiments, the circular polyribonucleotide includes one or more RNA sequences including a ribosome binding site, *e.g.*, an initiation codon.

Natural 5' UTRs bear features which play roles in translation initiation. They harbor signatures like Kozak sequences which are commonly known to be involved in the process by which the ribosome
20 initiates translation of many genes. Kozak sequences have the consensus CCR(A/G)CCAUGG (SEQ ID NO: 358), where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. 5' UTRs also have been known to form secondary structures which are involved in elongation factor binding.

In some embodiments, a circular polyribonucleotide encodes a protein binding sequence that
25 binds to a protein. In some embodiments, the protein binding sequence targets or localizes the circular polyribonucleotide to a specific target. In some embodiments, the protein binding sequence specifically binds an arginine-rich region of a protein.

In some embodiments, the protein binding site includes, but is not limited to, a binding site to the protein such as ACIN1, AGO, APOBEC3F, APOBEC3G, ATXN2, AUH, BCCIP, CAPRIN1, CELF2,
30 CPSF1, CPSF2, CPSF6, CPSF7, CSTF2, CSTF2T, CTCF, DDX21, DDX3, DDX3X, DDX42, DGCR8, EIF3A, EIF4A3, EIF4G2, ELAVL1, ELAVL3, FAM120A, FBL, FIP1L1, FKBP4, FMR1, FUS, FXR1, FXR2, GNL3, GTF2F1, HNRNPA1, HNRNPA2B1, HNRNPC, HNRNPK, HNRNPL, HNRNPM, HNRNPU, HNRNPUL1, IGF2BP1, IGF2BP2, IGF2BP3, ILF3, KHDRBS1, LARP7, LIN28A, LIN28B, m6A, MBNL2, METTL3, MOV10, MSI1, MSI2, NONO, NONO-, NOP58, NPM1, NUDT21, PCBP2, POLR2A, PRPF8,
35 PTBP1, RBFOX2, RBM10, RBM22, RBM27, RBM47, RNPS1, SAFB2, SBDS, SF3A3, SF3B4, SIRT7, SLBP, SLTM, SMNDC1, SND1, SRRM4, SRSF1, SRSF3, SRSF7, SRSF9, TAF15, TARDBP, TIA1, TNRC6A, TOP3B, TRA2A, TRA2B, U2AF1, U2AF2, UNK, UPF1, WDR33, XRN2, YBX1, YTHDC1, YTHDF1, YTHDF2, YWHAG, ZC3H7B, PDK1, AKT1, and any other protein that binds RNA.

Spacer Sequences

In some embodiments, the polyribonucleotide described herein includes one or more spacer sequences. A spacer refers to any contiguous nucleotide sequence (e.g., of one or more nucleotides) that provides distance or flexibility between two adjacent polynucleotide regions. Spacers may be present in between any of the nucleic acid elements described herein. Spacer may also be present within a nucleic acid element described herein.

For example, wherein a nucleic acid includes any two or more of the following elements: (A) a 3' catalytic intron fragment; (B) a 3' splice site; (C) a 3' exon fragment; (D) a polyribonucleotide cargo; (E) a 5' exon fragment; (F) a 5' splice site; and (G) a 5' catalytic intron fragment; a spacer region may be present between any one or more of the elements. Any of elements (A), (B), (C), (D), (E), (F), or (G) may be separated by a spacer sequence, as described herein. For example, there may be a spacer between (A) and (B), between (B) and (C), between (C) and (D), between (D) and (E), between (E) and (F), or between (F) and (G).

In some embodiments, the polyribonucleotide further includes a first spacer region between the 5' exon fragment of (C) and the polyribonucleotide cargo of (D). The spacer may be, e.g., at least 5 (e.g., at least 10, at least 15, at least 20) ribonucleotides in length. In some embodiments, the polyribonucleotide further includes a second spacer region between the polyribonucleotide cargo of (D) and the 5' exon fragment of (E).

A spacer sequences may be used to separate an IRES from adjacent structural elements to maintain the structure and function of the IRES or the adjacent element. A spacer can be specifically engineered depending on the IRES. In some embodiments, an RNA folding computer software, such as RNAFold, can be utilized to guide designs of the various elements of the vector, including the spacers.

The spacer may be, e.g., at least 5 (e.g., at least 10, at least 15, at least 20) ribonucleotides in length. In some embodiments, each spacer region is at least 5 (e.g., at least 10, at least 15, at least 20) ribonucleotides in length. Each spacer region may be, e.g., from 5 to 500 (e.g., 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, or 500) ribonucleotides in length. The first spacer region, the second spacer region, or the first spacer region and the second spacer region may include a polyA sequence. The first spacer region, the second spacer region, or the first spacer region and the second spacer region may include a polyA-C sequence. In some embodiments, the first spacer region, the second spacer region, or the first spacer region and the second spacer region includes a polyA-G sequence. In some embodiments, the first spacer region, the second spacer region, or the first spacer region and the second spacer region includes a polyA-T sequence. In some embodiments, the first spacer region, the second spacer region, or the first spacer region and the second spacer region includes a random sequence.

Spacers may also be present within a nucleic acid region described herein. For example, a polynucleotide cargo region may include one or multiple spacers. Spacers may separate regions within the polynucleotide cargo.

In some embodiments, the spacer sequence can be, for example, at least 10 nucleotides in length, at least 15 nucleotides in length, or at least 30 nucleotides in length. In some embodiments, the spacer sequence is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or 30 nucleotides in length. In some embodiments, the spacer sequence is no more than 100, 90, 80, 70, 60, 50, 45, 40, 35

or 30 nucleotides in length. In some embodiments the spacer sequence is from 20 to 50 nucleotides in length. In certain embodiments, the spacer sequence is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides in length.

5 The spacer sequences can be polyA sequences, polyA-C sequences, polyC sequences, or poly-U sequences.

In some embodiments, the spacer sequences can be polyA-T, polyA-C, polyA-G, or a random sequence.

10 Exemplary spacer sequences are described in paragraphs [0293] – [0302] of International Patent Publication No. WO2019/118919, which is hereby incorporated by reference in its entirety.

In some embodiments, the polyribonucleotide includes a 5' spacer sequence (*e.g.*, between the 5' annealing region and the polyribonucleotide cargo). In some embodiments, the 5' spacer sequence is at least 10 nucleotides in length. In another embodiment, the 5' spacer sequence is at least 15 nucleotides in length. In a further embodiment, the 5' spacer sequence is at least 30 nucleotides in length. In some embodiments, the 5' spacer sequence is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or 30 nucleotides in length. In some embodiments, the 5' spacer sequence is no more than 100, 90, 80, 70, 60, 50, 45, 40, 35 or 30 nucleotides in length. In some embodiments the 5' spacer sequence is between 20 and 50 nucleotides in length. In certain embodiments, the 5' spacer sequence is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides in length. In one embodiment, the 5' spacer sequence is a polyA sequence. In another embodiment, the 5' spacer sequence is a polyA-C sequence. In some embodiments, the 5' spacer sequence includes a polyA-G sequence. In some embodiments, the 5' spacer sequence includes a polyA-T sequence. In some embodiments, the 5' spacer sequence includes a random sequence.

25 In some embodiments, the polyribonucleotide includes a 3' spacer sequence (*e.g.*, between the 3' annealing region and the polyribonucleotide cargo). In some embodiments, the 3' spacer sequence is at least 10 nucleotides in length. In another embodiment, the 3' spacer sequence is at least 15 nucleotides in length. In a further embodiment, the 3' spacer sequence is at least 30 nucleotides in length. In some embodiments, the 3' spacer sequence is at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or 30 nucleotides in length. In some embodiments, the 3' spacer sequence is no more than 100, 90, 80, 70, 60, 50, 45, 40, 35 or 30 nucleotides in length. In some embodiments the 3' spacer sequence is from 20 to 50 nucleotides in length. In certain embodiments, the 3' spacer sequence is 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 nucleotides in length. In one embodiment, the 3' spacer sequence is a polyA sequence. In another embodiment, the 5' spacer sequence is a polyA-C sequence. In some embodiments, the 5' spacer sequence includes a polyA-G sequence. In some embodiments, the 5' spacer sequence includes a polyA-T sequence. In some embodiments, the 5' spacer sequence includes a random sequence.

40 In one embodiment, the polyribonucleotide includes a 5' spacer sequence, but not a 3' spacer sequence. In another embodiment, the polyribonucleotide includes a 3' spacer sequence, but not a 5' spacer sequence. In another embodiment, the polyribonucleotide includes neither a 5' spacer sequence,

nor a 3' spacer sequence. In another embodiment, the polyribonucleotide does not include an IRES sequence. In a further embodiment, the polyribonucleotide does not include an IRES sequence, a 5' spacer sequence or a 3' spacer sequence.

In some embodiments, the spacer sequence includes at least 3 ribonucleotides, at least 4
 5 ribonucleotides, at least 5 ribonucleotides, at least about 8 ribonucleotides, at least about 10
 ribonucleotides, at least about 12 ribonucleotides, at least about 15 ribonucleotides, at least about 20
 ribonucleotides, at least about 25 ribonucleotides, at least about 30 ribonucleotides, at least about 40
 ribonucleotides, at least about 50 ribonucleotides, at least about 60 ribonucleotides, at least about 70
 ribonucleotides, at least about 80 ribonucleotides, at least about 90 ribonucleotides, at least about 100
 10 ribonucleotides, at least about 120 ribonucleotides, at least about 150 ribonucleotides, at least about 200
 ribonucleotides, at least about 250 ribonucleotides, at least about 300 ribonucleotides, at least about 400
 ribonucleotides, at least about 500 ribonucleotides, at least about 600 ribonucleotides, at least about 700
 ribonucleotides, at least about 800 ribonucleotides, at least about 900 ribonucleotides, or at least about
 100 ribonucleotides.

15

Modifications

A polyribonucleotide (e.g., circular polyribonucleotide) as described herein may include one or
 more substitutions, insertions and/or additions, deletions, and covalent modifications with respect to
 reference sequences, in particular, the parent polyribonucleotide, are included within the scope of this
 20 disclosure.

In some embodiments, a circular polyribonucleotide includes one or more post-transcriptional
 modifications (e.g., capping, cleavage, polyadenylation, splicing, polyA sequence, methylation, acylation,
 phosphorylation, methylation of lysine and arginine residues, acetylation, and nitrosylation of thiol groups
 and tyrosine residues, etc.). The one or more post-transcriptional modifications can be any post-
 25 transcriptional modification, such as any of the more than one hundred different nucleoside modifications
 that have been identified in RNA (Rozenski, J, Crain, P, and McCloskey, J. (1999). *The RNA Modification
 Database: 1999 update. Nucl Acids Res* 27: 196-197). In some embodiments, the first isolated nucleic
 acid includes messenger RNA (mRNA). In some embodiments, the polyribonucleotide includes at least
 one nucleoside selected from the group such as those described in [0311] of International Patent
 30 Publication No. WO2019/118919A1, which is incorporated herein by reference in its entirety.

A polyribonucleotide may include any useful modification, such as to the sugar, the nucleobase,
 or the internucleoside linkage (e.g., to a linking phosphate / to a phosphodiester linkage / to the
 phosphodiester backbone). One or more atoms of a pyrimidine nucleobase may be replaced or
 substituted with optionally substituted amino, optionally substituted thiol, optionally substituted alkyl (e.g.,
 35 methyl or ethyl), or halo (e.g., chloro or fluoro). In certain embodiments, modifications (e.g., one or more
 modifications) are present in each of the sugar and the internucleoside linkage. Modifications may be
 modifications of ribonucleic acids (RNAs) to deoxyribonucleic acids (DNAs), threose nucleic acids
 (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs) or hybrids
 thereof). Additional modifications are described herein.

40 In some embodiments, a polyribonucleotide includes at least one N(6)methyladenosine (m6A)
 modification to increase translation efficiency. In some embodiments, the m6A modification can reduce

immunogenicity (e.g., reduce the level of one or more marker of an immune or inflammatory response) of the circular polyribonucleotide.

In some embodiments, a modification may include a chemical or cellular induced modification. For example, some non-limiting examples of intracellular RNA modifications are described by Lewis and Pan in "RNA modifications and structures cooperate to guide RNA-protein interactions" from Nat Reviews Mol Cell Biol, 2017, 18:202-210.

In some embodiments, chemical modifications to the ribonucleotides of a circular polyribonucleotide may enhance immune evasion. The circular polyribonucleotide may be synthesized and/or modified by methods well established in the art, such as those described in "Current protocols in nucleic acid chemistry," Beaucage, S.L. et al. (Eds.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference. Modifications include, for example, end modifications, e.g., 5' end modifications (phosphorylation (mono-, di- and tri-), conjugation, inverted linkages, etc.), 3' end modifications (conjugation, DNA nucleotides, inverted linkages, etc.), base modifications (e.g., replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners), removal of bases (abasic nucleotides), or conjugated bases. The modified ribonucleotide bases may also include 5-methylcytidine and pseudouridine. In some embodiments, base modifications may modulate expression, immune response, stability, subcellular localization, to name a few functional effects, of the circular polyribonucleotide. In some embodiments, the modification includes a bi-orthogonal nucleotide, e.g., an unnatural base. See for example, Kimoto et al, Chem Commun (Camb), 2017, 53:12309, DOI: 10.1039/c7cc06661a, which is hereby incorporated by reference.

In some embodiments, sugar modifications (e.g., at the 2' position or 4' position) or replacement of the sugar one or more ribonucleotides of the circular polyribonucleotide may, as well as backbone modifications, include modification or replacement of the phosphodiester linkages. Specific examples of circular polyribonucleotide include, but are not limited to, circular polyribonucleotide including modified backbones or no natural internucleoside linkages such as internucleoside modifications, including modification or replacement of the phosphodiester linkages. Circular polyribonucleotides having modified backbones include, among others, those that do not have a phosphorus atom in the backbone. For the purposes of this application, and as sometimes referenced in the art, modified RNAs that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. In particular embodiments, the circular polyribonucleotide will include ribonucleotides with a phosphorus atom in its internucleoside backbone.

Modified polyribonucleotide backbones may include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates such as 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates such as 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included. In some embodiments, the circular polyribonucleotide may be negatively or positively charged.

The modified nucleotides, which may be incorporated into the polyribonucleotide, can be modified on the internucleoside linkage (e.g., phosphate backbone). Herein, in the context of the polynucleotide backbone, the phrases "phosphate" and "phosphodiester" are used interchangeably. Backbone phosphate groups can be modified by replacing one or more of the oxygen atoms with a different substituent. Further, the modified nucleosides and nucleotides can include the wholesale replacement of an unmodified phosphate moiety with another internucleoside linkage as described herein. Examples of modified phosphate groups include, but are not limited to, phosphorothioate, phosphoroselenates, boranophosphates, boranophosphate esters, hydrogen phosphonates, phosphoramidates, phosphorodiamidates, alkyl or aryl phosphonates, and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. The phosphate linker can also be modified by the replacement of a linking oxygen with nitrogen (bridged phosphoramidates), sulfur (bridged phosphorothioates), and carbon (bridged methylenephosphonates).

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

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

Other internucleoside linkages that may be employed according to the present disclosure, including internucleoside linkages which do not contain a phosphorous atom, are described herein.

In some embodiments, a circular polyribonucleotide may include one or more cytotoxic nucleosides. For example, cytotoxic nucleosides may be incorporated into circular polyribonucleotide, such as bifunctional modification. Cytotoxic nucleoside may include, but are not limited to, adenosine arabinoside, 5-azacytidine, 4'-thio-aracytidine, cyclopentenylcytosine, cladribine, clofarabine, cytarabine, cytosine arabinoside, 1-(2-C-cyano-2-deoxy-beta-D-arabino-pentofuranosyl)-cytosine, decitabine, 5-fluorouracil, fludarabine, floxuridine, gemcitabine, a combination of tegafur and uracil, tegafur ((RS)-5-fluoro-1-(tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione), troxacitabine, tezacitabine, 2'-deoxy-2'-methylidenecytidine (DMDC), and 6-mercaptopurine. Additional examples include fludarabine phosphate, N4-behenoyl-l-beta-D-arabinofuranosylcytosine, N4-octadecyl-1-beta-D-arabinofuranosylcytosine, N4-palmitoyl-l-(2-C-cyano-2-deoxy-beta-D-arabino-pentofuranosyl) cytosine, and P-4055 (cytarabine 5'-elaidic acid ester).

A polyribonucleotide may or may not be uniformly modified along the entire length of the molecule. For example, one or more or all types of nucleotides (e.g., naturally occurring nucleotides, purine or pyrimidine, or any one or more or all of A, G, U, C, I, pU) may or may not be uniformly modified in the circular polyribonucleotide, or in a given predetermined sequence region thereof. In some embodiments, the circular polyribonucleotide includes a pseudouridine. In some embodiments, the circular polyribonucleotide includes an inosine, which may aid in the immune system characterizing the circular polyribonucleotide as endogenous versus viral RNAs. The incorporation of inosine may also mediate improved RNA stability/reduced degradation. See for example, Yu, Z. et al. (2015) RNA editing

by ADAR1 marks dsRNA as “self”. Cell Res. 25, 1283–1284, which is incorporated by reference in its entirety.

In some embodiments, all nucleotides in a polyribonucleotide (or in a given sequence region thereof) are modified. In some embodiments, the modification may include an m6A, which may augment expression; an inosine, which may attenuate an immune response; pseudouridine, which may increase RNA stability, or translational readthrough (stagger element), an m5C, which may increase stability; and a 2,2,7-trimethylguanosine, which aids subcellular translocation (e.g., nuclear localization).

Different sugar modifications, nucleotide modifications, and/or internucleoside linkages (e.g., backbone structures) may exist at various positions in a circular polyribonucleotide. One of ordinary skill in the art will appreciate that the nucleotide analogs or other modification(s) may be located at any position(s) of the circular polyribonucleotide, such that the function of the circular polyribonucleotide is not substantially decreased. A modification may also be a non-coding region modification. The circular polyribonucleotide may include from about 1% to about 100% modified nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, i.e. any one or more of A, G, U or C) or any intervening percentage (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100%).

Methods of Circularization

The disclosure provides methods for producing circular polyribonucleotides encoding an antifusogenic polypeptide (e.g., a polypeptide of Table 1), including, e.g., recombinant technology or chemical synthesis. For example, a DNA molecule used to produce an RNA circle can include a DNA sequence of a naturally occurring original nucleic acid sequence, a modified version thereof, or a DNA sequence encoding a synthetic polypeptide not normally found in nature (e.g., chimeric molecules or fusion proteins). DNA and RNA molecules can be modified using a variety of techniques including, but not limited to, classic mutagenesis techniques and recombinant techniques, such as site-directed mutagenesis, chemical treatment of a nucleic acid molecule to induce mutations, restriction enzyme cleavage of a nucleic acid fragment, ligation of nucleic acid fragments, polymerase chain reaction (PCR) amplification or mutagenesis of selected regions of a nucleic acid sequence, synthesis of oligonucleotide mixtures and ligation of mixture groups to “build” a mixture of nucleic acid molecules and combinations thereof.

In some embodiments, a linear polyribonucleotide for circularization may be cyclized, or concatemerized. In some embodiments, the linear polyribonucleotide for circularization may be cyclized in vitro prior to formulation and/or delivery. In some embodiments, the circular polyribonucleotide may be in a mixture with linear polyribonucleotides. In some embodiments, the linear polyribonucleotides have the same nucleic acid sequence as the circular polyribonucleotides.

In some embodiments, a linear polyribonucleotide for circularization is cyclized, or concatemerized using a chemical method to form a circular polyribonucleotide. In some chemical methods, the 5'-end and the 3'-end of the nucleic acid (e.g., a linear polyribonucleotide for circularization) includes chemically reactive groups that, when close together, may form a new covalent linkage between the 5'-end and the 3'-end of the molecule. The 5'-end may contain an NHS-ester reactive group and the 3'-end may contain a 3'-amino-terminated nucleotide such that in an organic solvent the 3'-amino-terminated nucleotide on the 3'-end of a linear RNA molecule will undergo a nucleophilic attack on the 5'-NHS-ester moiety forming a new 5'-/3'-amide bond.

In some embodiments, a DNA or RNA ligase is used to enzymatically link a 5'-phosphorylated nucleic acid molecule (e.g., a linear polyribonucleotide for circularization) to the 3'-hydroxyl group of a nucleic acid (e.g., a linear nucleic acid) forming a new phosphodiester linkage. In an example reaction, a linear polyribonucleotide for circularization is incubated at 37°C for 1 hour with 1-10 units of T4 RNA ligase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. The ligation reaction may occur in the presence of a linear nucleic acid capable of base-pairing with both the 5'- and 3'- region in juxtaposition to assist the enzymatic ligation reaction. In some embodiments, the ligation is splint ligation. For example, a splint ligase, like SplintR® ligase, can be used for splint ligation, RNA ligase II, T4 RNA ligase, or T4 DNA ligase. For splint ligation, a single stranded polynucleotide (splint), like a single stranded RNA, can be designed to hybridize with both termini of a linear polyribonucleotide, so that the two termini can be juxtaposed upon hybridization with the single-stranded splint. Splint ligase can thus catalyze the ligation of the juxtaposed two termini of the linear polyribonucleotide, generating a circular polyribonucleotide.

In some embodiments, a DNA or RNA ligase is used in the synthesis of the circular polynucleotides. In some embodiments, either the 5'-or 3'-end of the linear polyribonucleotide for circularization can encode a ligase ribozyme sequence such that during in vitro transcription, the resultant linear polyribonucleotide for circularization includes an active ribozyme sequence capable of ligating the 5'-end of the linear polyribonucleotide for circularization to the 3'-end of the linear polyribonucleotide for circularization. The ligase ribozyme may be derived from the Group I Intron, Hepatitis Delta Virus, Hairpin ribozyme or may be selected by SELEX (systematic evolution of ligands by exponential enrichment). The ribozyme ligase reaction may take 1 to 24 hours at temperatures between 0 and 37°C.

In some embodiments, a linear polyribonucleotide for circularization is cyclized or concatemerized by using at least one non-nucleic acid moiety. In one aspect, the at least one non-nucleic acid moiety may react with regions or features near the 5' terminus and/or near the 3' terminus of the linear polyribonucleotide for circularization in order to cyclize or concatemerized the linear polyribonucleotide for circularization. In another aspect, the at least one non-nucleic acid moiety may be located in or linked to or near the 5' terminus and/or the 3' terminus of the linear polyribonucleotide for circularization. The non-nucleic acid moieties contemplated may be homologous or heterologous. As a non-limiting example, the non-nucleic acid moiety may be a linkage such as a hydrophobic linkage, ionic linkage, a biodegradable linkage, and/or a cleavable linkage. As another non-limiting example, the non-nucleic acid moiety is a ligation moiety. As yet another non-limiting example, the non-nucleic acid moiety may be an oligonucleotide or a peptide moiety, such as an aptamer or a non-nucleic acid linker as described herein.

In some embodiments, the linear polyribonucleotide for circularization is synthesized using IVT and an RNA polymerase, where the nucleotide mixture used for IVT may contain an excess of guanosine monophosphate relative to guanosine triphosphate to preferentially produce RNA with a 5' monophosphate; the purified IVT product may be circularized using a splint DNA.

5 In some embodiments, a linear polyribonucleotide for circularization is cyclized or concatemerized due to a non-nucleic acid moiety that causes an attraction between atoms, molecular surfaces at, near or linked to the 5' and 3' ends of the linear polyribonucleotide for circularization. As a non-limiting example, one or more linear polyribonucleotides for circularization may be cyclized or concatemerized by intermolecular forces or intramolecular forces. Non-limiting examples of
10 intermolecular forces include dipole-dipole forces, dipole-induced dipole forces, induced dipole-induced dipole forces, Van der Waals forces, and London dispersion forces. Non-limiting examples of intramolecular forces include covalent bonds, metallic bonds, ionic bonds, resonant bonds, agnostic bonds, dipolar bonds, conjugation, hyperconjugation and antibonding.

In some embodiments, a linear polyribonucleotide for circularization may include a ribozyme RNA
15 sequence near the 5' terminus and near the 3' terminus. The ribozyme RNA sequence may covalently link to a peptide when the sequence is exposed to the remainder of the ribozyme. In one aspect, the peptides covalently linked to the ribozyme RNA sequence near the 5' terminus and the 3' terminus may associate with each other causing a linear polyribonucleotide for circularization to cyclize or concatemerized. In another aspect, the peptides covalently linked to the ribozyme RNA near the 5'
20 terminus and the 3' terminus may cause the linear primary construct or linear mRNA to cyclize or concatemerized after being subjected to ligated using various methods known in the art such as, but not limited to, protein ligation. Non-limiting examples of ribozymes for use in the linear primary constructs or linear RNA of the present invention or a non-exhaustive listing of methods to incorporate and/or covalently link peptides are described in US patent application No. US20030082768, the contents of
25 which is here incorporated by reference in its entirety.

In some embodiments, a linear polyribonucleotide for circularization may include a 5' triphosphate of the nucleic acid converted into a 5' monophosphate, e.g., by contacting the 5' triphosphate with RNA 5' pyrophosphohydrolase (RppH) or an ATP diphosphohydrolase (apyrase). In some embodiments, the 5' end of at least a portion of the linear polyribonucleotides includes a monophosphate moiety. In some
30 embodiments, the population of polyribonucleotides including circular and linear polyribonucleotides is contacted with RppH prior to digesting at least a portion of the linear polyribonucleotides with a 5' exonuclease and/or a 3' exonuclease. Alternately, converting the 5' triphosphate of the linear polyribonucleotide for circularization into a 5' monophosphate may occur by a two-step reaction including: (a) contacting the 5' nucleotide of the linear polyribonucleotide for circularization with a phosphatase (e.g.,
35 Antarctic Phosphatase, Shrimp Alkaline Phosphatase, or Calf Intestinal Phosphatase) to remove all three phosphates; and (b) contacting the 5' nucleotide after step (a) with a kinase (e.g., Polynucleotide Kinase) that adds a single phosphate.

In some embodiments, circularization efficiency of the circularization methods provided herein is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at
40 least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or 100%. In some embodiments,

the circularization efficiency of the circularization methods provided herein is at least about 40%. In some embodiments, the circularization method provided has a circularization efficiency of between about 10% and about 100%; for example, the circularization efficiency may be about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, and about 99%. In some
 5 embodiments, the circularization efficiency is between about 20% and about 80%. In some
 embodiments, the circularization efficiency is between about 30% and about 60%. In some embodiments the circularization efficiency is about 40%.

10 In some embodiments, the circular polyribonucleotide includes an internal splicing element that
 when replicated the spliced ends are joined together. Some examples may include miniature introns
 (<100 nt) with splice site sequences and short inverted repeats (30–40 nt) such as AluSq2, AluJr, and
 AluSz, inverted sequences in flanking introns, *Alu* elements in flanking introns, and motifs found in
 (suptable4 enriched motifs) *cis*-sequence elements proximal to back splice events such as sequences in
 the 200 bp preceding (upstream of) or following (downstream from) a back splice site with flanking exons.
 15 In some embodiments, the linear polyribonucleotide includes at least one repetitive nucleotide sequence
 described elsewhere herein as an internal splicing element. In such embodiments, the repetitive
 nucleotide sequence may include repeated sequences from the *Alu* family of introns. In some
 embodiments, a splicing-related ribosome binding protein can regulate circular polyribonucleotide
 biogenesis (e.g., the Muscle blind and Quaking (QKI) splicing factors).

20 In some embodiments, the linear polyribonucleotide may include canonical splice sites that flank
 head-to-tail junctions of the circular polyribonucleotide.

In some embodiments, the linear polyribonucleotide may include a bulge-helix-bulge motif,
 including a 4-base pair stem flanked by two 3-nucleotide bulges. Cleavage occurs at a site in the bulge
 region, generating characteristic fragments with terminal 5'-hydroxyl group and 2', 3'-cyclic phosphate.
 25 Circularization proceeds by nucleophilic attack of the 5'-OH group onto the 2', 3'-cyclic phosphate of the
 same molecule forming a 3', 5'-phosphodiester bridge.

In some embodiments, the linear polyribonucleotide may include a multimeric repeating RNA
 sequence that harbors a HPR element. The HPR includes a 2',3'-cyclic phosphate and 5'-OH termini.
 The HPR element self-processes the 5'- and 3'-ends of the linear linear polyribonucleotide, thereby
 30 ligating the ends together.

In some embodiments, the linear polyribonucleotide may include a sequence that mediates self-ligation.
 In one embodiment, the linear polyribonucleotide may include a HDV sequence, e.g., HDV replication
 domain conserved sequence,

GGCUCAUCUCGACAAGAGGCGGCAGUCCUCAGUACUCUUACUCUUUUCUGUAAAGAGGAGACUG
 35 CUGGACUCGCCGCCCAAGUUCGAGCAUGAGCC (Beeharry et al 2004) (SEQ ID NO: 359) or
 GGCUAGAGGCGGCAGUCCUCAGUACUCUUACUCUUUUCUGUAAAGAGGAGACUGCUGGACUCGC
 CGCCCGAGCC (SEQ ID NO: 360), to self-ligate. In one embodiment, the linear polyribonucleotide may
 include loop E sequence (e.g., in PSTVd) to self-ligate. In another embodiment, the linear
 polyribonucleotide may include a self-circularizing intron, e.g., a 5' and 3' splice junction, or a self-
 40 circularizing catalytic intron such as a Group I, Group II or Group III Introns. Nonlimiting examples of

group I intron self-splicing sequences may include self-splicing permuted intron-exon sequences derived from T4 bacteriophage gene *td*, and the intervening sequence (IVS) rRNA of *Tetrahymena*.

In some embodiments, linear polyribonucleotides for circularization may include complementary sequences, including either repetitive or nonrepetitive nucleic acid sequences within individual introns or across flanking introns. Repetitive nucleic acid sequences are sequences that occur within a segment of the linear polyribonucleotide. In some embodiments, the linear polyribonucleotide includes a repetitive nucleic acid sequence. In some embodiments, the repetitive nucleotide sequence includes poly CA or poly UG sequences. In some embodiments, the linear polyribonucleotide includes at least one repetitive nucleic acid sequence that hybridizes to a complementary repetitive nucleic acid sequence in another segment of the linear polyribonucleotide, with the hybridized segment forming an internal double strand. In some embodiments, the linear polyribonucleotide includes between 1 and 10 (e.g., 2, 3, 4, 5, 6, 7, 8, 9, and 10) repetitive nucleic acid sequences that hybridize to a complementary repetitive nucleic acid sequence in another segment of the linear polyribonucleotide, with the hybridized segment forming an internal double strand. In some embodiments, the linear polyribonucleotide includes 2 repetitive nucleic acid sequences that hybridize to a complementary repetitive nucleic acid sequence in another segment of the linear polyribonucleotide, with the hybridized segment forming an internal double strand. In some embodiments, repetitive nucleic acid sequences and complementary repetitive nucleic acid sequences from two separate linear polyribonucleotides hybridize to generate a single circularized polyribonucleotide, with the hybridized segments forming internal double strands. In some embodiments, the complementary sequences are found at the 5' and 3' ends of the linear polyribonucleotides for circularization. In some embodiments, the complementary sequences include about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more paired nucleotides.

In some embodiments, chemical methods of circularization may be used to generate the circular polyribonucleotide. Such methods may include, but are not limited to click chemistry (e.g., alkyne and azide-based methods, or clickable bases), olefin metathesis, phosphoramidate ligation, hemiaminal-imine crosslinking, base modification, and any combination thereof.

In some embodiments, enzymatic methods of circularization may be used to generate the circular polyribonucleotide. In some embodiments, a ligation enzyme, e.g., DNA or RNA ligase, may be used to generate a template of the circular polyribonucleotide or complement, a complementary strand of the circular polyribonucleotide, or the circular polyribonucleotide.

Circularization of the linear polyribonucleotide may be accomplished by methods known in the art, for example, those described in "RNA circularization strategies in vivo and in vitro" by Petkovic and Muller from *Nucleic Acids Res*, 2015, 43(4): 2454-2465, and "In vitro circularization of RNA" by Muller and Appel, from *RNA Biol*, 2017, 14(8):1018-1027.

The circular polyribonucleotide may encode a sequence and/or motif useful for replication. Exemplary replication elements are described in paragraphs [0280] – [0286] of International Patent Publication No. WO2019/118919, which is hereby incorporated by reference in its entirety.

In some embodiments, linear polyribonucleotides may include complementary sequences, including either repetitive or nonrepetitive nucleic acid sequences within individual introns or across flanking introns. Repetitive nucleic acid sequences are sequences that occur within a segment of the

circular polyribonucleotide. In some embodiments, the linear polyribonucleotide includes a repetitive nucleic acid sequence. In some embodiments, the repetitive nucleotide sequence includes poly CA or poly UG sequences. In some embodiments, the linear polyribonucleotide includes at least one repetitive nucleic acid sequence that hybridizes to a complementary repetitive nucleic acid sequence in another segment of the linear polyribonucleotide, with the hybridized segment forming an internal double strand. In some embodiments, repetitive nucleic acid sequences and complementary repetitive nucleic acid sequences from two separate linear polyribonucleotides hybridize to generate a single circularized polyribonucleotide, with the hybridized segments forming internal double strands. In some embodiments, the complementary sequences are found at the 5' and 3' ends of the linear polyribonucleotides. In some embodiments, the complementary sequences include about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, or more paired nucleotides.

In some embodiments, chemical methods of circularization may be used to generate the circular polyribonucleotide. Such methods may include, but are not limited to click chemistry (e.g., alkyne and azide-based methods, or clickable bases), olefin metathesis, phosphoramidate ligation, hemiaminal-imine crosslinking, base modification, and any combination thereof.

Methods of making the circular polyribonucleotides described herein are described in, for example, Khudyakov & Fields, *Artificial DNA: Methods and Applications*, CRC Press (2002); in Zhao, *Synthetic Biology: Tools and Applications*, (First Edition), Academic Press (2013); Muller and Appel, *RNA Biol*, 2017, 14(8):1018-1027; and Egli & Herdewijn, *Chemistry and Biology of Artificial Nucleic Acids*, (First Edition), Wiley-VCH (2012). Other methods of making circular polyribonucleotides are described, for example, in International Publication No. WO2022/247943, US Patent No. US11000547, International Publication No. 2018/191722, International Publication No. WO2019/236673, International Publication No. WO2020/023595, International Publication No. WO2022/204460, International Publication No. WO2022/204464, and International Publication No. WO2022/204466.

Various methods of synthesizing circular polyribonucleotides are also described elsewhere (see, e.g., US Patent No. US6210931, US Patent No. US5773244, US Patent No. US5766903, US Patent No. US5712128, US Patent No. US5426180, US Publication No. US20100137407, International Publication No. WO1992001813, International Publication No. WO2010084371, and Petkovic et al., *Nucleic Acids Res.* 43:2454-65 (2015); the contents of each of which are herein incorporated by reference in their entirety).

In some embodiments, the circular polyribonucleotide is purified, e.g., free ribonucleic acids, linear or nicked RNA, DNA, proteins, etc. are removed. In some embodiments, the circular polyribonucleotides may be purified by any known method commonly used in the art. Examples of nonlimiting purification methods include, column chromatography, gel excision, size exclusion, etc.

Methods of Production

Methods of production in a cell-free system

The disclosure also provides methods of producing a circular RNA. For example, a deoxyribonucleotide template may be transcribed in a cell-free system (e.g., by in vitro transcription) to a

produce a linear RNA. The linear polyribonucleotide produces a splicing-compatible polyribonucleotide, which may be self-spliced to produce a circular polyribonucleotide.

In some embodiments, the disclosure provides a method of producing a circular polyribonucleotide (*e.g.*, in a cell-free system) by providing a linear polyribonucleotide; and self-splicing linear polyribonucleotide under conditions suitable for splicing of the 3' and 5' splice sites of the linear polyribonucleotide; thereby producing a circular polyribonucleotide.

In some embodiments, the disclosure provides a method of producing a circular polyribonucleotide by providing a deoxyribonucleotide encoding the linear polyribonucleotide; transcribing the deoxyribonucleotide in a cell-free system to produce the linear polyribonucleotide; optionally purifying the splicing-compatible linear polyribonucleotide; and self-splicing the linear polyribonucleotide under conditions suitable for splicing of the 3' and 5' splice sites of the linear polyribonucleotide, thereby producing a circular polyribonucleotide.

In some embodiments, the disclosure provides a method of producing a circular polyribonucleotide by providing a deoxyribonucleotide encoding a linear polyribonucleotide; transcribing the deoxyribonucleotide in a cell-free system to produce the linear polyribonucleotide, wherein the transcribing occurs in a solution under conditions suitable for splicing of the 3' and 5' splice sites of the linear polyribonucleotide, thereby producing a circular polyribonucleotide. In some embodiments, the linear polyribonucleotide comprises a 5' split-intron and a 3' split-intron (*e.g.*, a self-splicing construct for producing a circular polyribonucleotide). In some embodiments, the linear polyribonucleotide comprises a 5' annealing region and a 3' annealing region.

Suitable conditions for *in vitro* transcriptions and or self-splicing may include any conditions (*e.g.*, a solution or a buffer, such as an aqueous buffer or solution) that mimic physiological conditions in one or more respects. In some embodiments, suitable conditions include between 0.1-100mM Mg²⁺ ions or a salt thereof (*e.g.*, 1-100mM, 1-50mM, 1-20mM, 5- 50mM, 5-20 mM, or 5-15mM). In some embodiments, suitable conditions include between 1-1000mM K⁺ ions or a salt thereof such as KCl (*e.g.*, 1-1000mM, 1-500mM, 1-200mM, 50- 500mM, 100-500mM, or 100-300mM). In some embodiments, suitable conditions include between 1-1000mM Cl⁻ ions or a salt thereof such as KCl (*e.g.*, 1-1000mM, 1-500mM, 1-200mM, 50- 500mM, 100-500mM, or 100-300mM). In some embodiments, suitable conditions include between 0.1-100mM Mn²⁺ ions or a salt thereof such as MnCl₂ (*e.g.*, 0.1-100mM, 0.1-50mM, 0.1-20mM, 0.1-10mM, 0.1-5mM, 0.1-2mM, 0.5- 50mM, 0.5-20 mM, 0.5-15mM, 0.5-5mM, 0.5-2mM, or 0.1-10mM). In some embodiments, suitable conditions include dithiothreitol (DTT) (*e.g.*, 1-1000 μM, 1-500 μM, 1-200μM, 50- 500μM, 100-500μM, 100-300μM, 0.1-100mM, 0.1-50mM, 0.1-20mM, 0.1-10mM, 0.1-5mM, 0.1-2mM, 0.5- 50mM, 0.5-20 mM, 0.5-15mM, 0.5-5mM, 0.5-2mM, or 0.1-10mM). In some embodiments, suitable conditions include between 0.1mM and 100mM ribonucleoside triphosphate (NTP) (*e.g.*, 0.1-100 mM, 0.1-50mM, 0.1-10mM, 1- 100mM, 1-50mM, or 1-10mM). In some embodiments, suitable conditions include a pH of 4 to 10 (*e.g.*, pH of 5 to 9, pH of 6 to 9, or pH of 6.5 to 8.5). In some embodiments, suitable conditions include a temperature of 4°C to 50°C (*e.g.*, 10°C to 40°C, 15 °C to 40°C, 20°C to 40°C, or 30°C to 40°C),

In some embodiments the linear polyribonucleotide is produced from a deoxyribonucleic acid, *e.g.*, a deoxyribonucleic acid described herein, such as a DNA vector, a linearized DNA vector, or a

cDNA. In some embodiments, the linear polyribonucleotide is transcribed from the deoxyribonucleic acid by transcription in a cell-free system (*e.g.*, in vitro transcription).

Methods of production in a cell

5 The disclosure also provides methods of producing a circular RNA in a cell, *e.g.*, a prokaryotic cell or a eukaryotic cell. In some embodiments, an exogenous polyribonucleotide is provided to a cell (*e.g.*, a linear polyribonucleotide described herein or a DNA molecule encoding for the transcription of a linear polyribonucleotide described here). The linear polyribonucleotides may be transcribed in the cell from an exogenous DNA molecule provided to the cell. The linear polyribonucleotide may be transcribed
10 in the cell from an exogenous recombinant DNA molecule transiently provided to the cell. In some embodiments, the exogenous DNA molecule does not integrate into the cell's genome. In some embodiments, the linear polyribonucleotide is transcribed in the cell from a recombinant DNA molecule that is incorporated into the cell's genome.

 In some embodiments, the cell is a prokaryotic cell. In some embodiments, the prokaryotic cell
15 including the polyribonucleotides described herein may be a bacterial cell or an archaeal cell. For example, the prokaryotic cell including the polyribonucleotides described herein may be *E. coli*, halophilic archaea (*e.g.*, *Haloferax volcanii*), *Sphingomonas*, cyanobacteria (*e.g.*, *Synechococcus elongatus*, *Spirulina* (*Arthrospira*) spp., and *Synechocystis* spp.), *Streptomyces*, actinomycetes (*e.g.*, *Nonomuraea*, *Kitasatospora*, or *Thermobifida*), *Bacillus* spp. (*e.g.*, *Bacillus subtilis*, *Bacillus anthracis*, *Bacillus cereus*),
20 betaproteobacteria (*e.g.*, *Burkholderia*), alphaproteobacterial (*e.g.*, *Agrobacterium*), *Pseudomonas* (*e.g.*, *Pseudomonas putida*), and enterobacteria. The prokaryotic cells may be grown in a culture medium. The prokaryotic cells may be contained in a bioreactor.

 In some embodiments, the cell is a eukaryotic cell. In some embodiments, the eukaryotic cell including the polyribonucleotides described herein is a unicellular eukaryotic cell. In some embodiments,
25 the unicellular eukaryotic is a unicellular fungal cell such as a yeast cell (*e.g.*, *Saccharomyces cerevisiae* and other *Saccharomyces* spp., *Brettanomyces* spp., *Schizosaccharomyces* spp., *Torulaspora* spp, and *Pichia* spp.). In some embodiments, the unicellular eukaryotic cell is a unicellular animal cell. A unicellular animal cell may be a cell isolated from a multicellular animal and grown in culture, or the daughter cells thereof. In some embodiments, the unicellular animal cell may be dedifferentiated. In
30 some embodiments, the unicellular eukaryotic cell is a unicellular plant cell. A unicellular plant cell may be a cell isolated from a multicellular plant and grown in culture, or the daughter cells thereof. In some embodiments, the unicellular plant cell may be dedifferentiated. In some embodiments, the unicellular plant cell is from a plant callus. In embodiments, the unicellular cell is a plant cell protoplast. In some
35 embodiments, the unicellular eukaryotic cell is a unicellular eukaryotic algal cell, such as a unicellular green alga, a diatom, a euglenid, or a dinoflagellate. Non-limiting examples of unicellular eukaryotic algae of interest include *Dunaliella salina*, *Chlorella vulgaris*, *Chlorella zofingiensis*, *Haematococcus pluvialis*, *Neochloris oleoabundans* and other *Neochloris* spp., *Protosiphon botryoides*, *Botryococcus braunii*, *Cryptococcus* spp., *Chlamydomonas reinhardtii* and other *Chlamydomonas* spp. In some
40 embodiments, the unicellular eukaryotic cell is a protist cell. In some embodiments, the unicellular eukaryotic cell is a protozoan cell.

In some embodiments, the eukaryotic cell is a cell of a multicellular eukaryote. For example, the multicellular eukaryote may be selected from the group consisting of a vertebrate animal, an invertebrate animal, a multicellular fungus, a multicellular alga, and a multicellular plant. In some embodiments, the eukaryotic organism is a human. In some embodiments, the eukaryotic organism is a non-human vertebrate animal. In some embodiments, the eukaryotic organism is an invertebrate animal. In some embodiments, the eukaryotic organism is a multicellular fungus. In some embodiments, the eukaryotic organism is a multicellular plant. In embodiments, the eukaryotic cell is a cell of a human or a cell of a non-human mammal such as a non-human primate (*e.g.*, monkeys, apes), ungulate (*e.g.*, bovids including cattle, buffalo, bison, sheep, goat, and musk ox; pig; camelids including camel, llama, and alpaca; deer, antelope; and equids including horse and donkey), carnivore (*e.g.*, dog, cat), rodent (*e.g.*, rat, mouse, guinea pig, hamster, squirrel), or lagomorph (*e.g.*, rabbit, hare). In embodiments, the eukaryotic cell is a cell of a bird, such as a member of the avian taxa Galliformes (*e.g.*, chickens, turkeys, pheasants, quail), Anseriformes (*e.g.*, ducks, geese), Paleaognathae (*e.g.*, ostriches, emus), Columbiformes (*e.g.*, pigeons, doves), or Psittaciformes (*e.g.*, parrots). In embodiments, the eukaryotic cell is a cell of an arthropod (*e.g.*, insects, arachnids, crustaceans), a nematode, an annelid, a helminth, or a mollusc. In embodiments, the eukaryotic cell is a cell of a multicellular plant, such as an angiosperm plant (which can be a dicot or a monocot) or a gymnosperm plant (*e.g.*, a conifer, a cycad, a gnetophyte, a Ginkgo), a fern, horsetail, clubmoss, or a bryophyte. In embodiments, the eukaryotic cell is a cell of a eukaryotic multicellular alga.

The eukaryotic cells may be grown in a culture medium. The eukaryotic cells may be contained in a bioreactor.

Methods of purification

One or more purification steps may be included in the methods described herein. For example, in some embodiments, the linear polyribonucleotide is substantively enriched or pure (*e.g.*, purified) prior to self-splicing the linear polyribonucleotide. In other embodiments, the linear polyribonucleotide is not purified prior to self-splicing the linear polyribonucleotide. In some embodiments, the resulting circular RNA is purified.

Purification may include separating or enriching the desired reaction product from one or more undesired components, such as any unreacted starting material, byproducts, enzymes, or other reaction components. For example, purification of linear polyribonucleotide following transcription in a cell-free system (*e.g.*, in vitro transcription) may include separation or enrichment from the DNA template prior to self-splicing the linear polyribonucleotide. Purification of the circular RNA product following splicing may be used to separate or enrich the circular RNA from its corresponding linear RNA. Methods of purification of RNA are known to those of skill in the art and include enzymatic purification or by chromatography.

In some embodiments, the methods of purification result in a circular polyribonucleotide that has less than 50% (*e.g.*, less than 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1%) linear polyribonucleotides.

Bioreactors

In some embodiments, any method of producing a circular polyribonucleotide described herein may be performed in a bioreactor. A bioreactor refers to any vessel in which a chemical or biological

process is carried out which involves organisms or biochemically active substances derived from such organisms. Bioreactors may be compatible with the cell-free methods for production of circular RNA described herein. A vessel for a bioreactor may include a culture flask, a dish, or a bag that may be single use (disposable), autoclavable, or sterilizable. A bioreactor may be made of glass, or it may be polymer-based, or it may be made of other materials.

Examples of bioreactors include, without limitation, stirred tank (*e.g.*, well mixed) bioreactors and tubular (*e.g.*, plug flow) bioreactors, airlift bioreactors, membrane stirred tanks, spin filter stirred tanks, vibromixers, fluidized bed reactors, and membrane bioreactors. The mode of operating the bioreactor may be a batch or continuous processes. A bioreactor is continuous when the reagent and product streams are continuously being fed and withdrawn from the system. A batch bioreactor may have a continuous recirculating flow, but no continuous feeding of reagents or product harvest.

Some methods of the present disclosure are directed to large-scale production of circular polyribonucleotides. For large-scale production methods, the method may be performed in a volume of 1 liter (L) to 50 L, or more (*e.g.*, 5 L, 10 L, 15 L, 20 L, 25 L, 30 L, 35 L, 40 L, 45 L, 50 L, or more). In some embodiments, the method may be performed in a volume of 5 L to 10 L, 5 L to 15 L, 5 L to 20 L, 5 L to 25 L, 5 L to 30 L, 5 L to 35 L, 5 L to 40 L, 5 L to 45 L, 10 L to 15 L, 10 L to 20 L, 10 L to 25 L, 20 L to 30 L, 10 L to 35 L, 10 L to 40 L, 10 L to 45 L, 10 L to 50 L, 15 L to 20 L, 15 L to 25 L, 15 L to 30 L, 15 L to 35 L, 15 L to 40 L, 15 L to 45 L, or 15 to 50 L.

In some embodiments, a bioreactor may produce at least 1g of circular RNA. In some embodiments, a bioreactor may produce 1-200g of circular RNA (*e.g.*, 1-10g, 1-20g, 1-50g, 10-50g, 10-100g, 50-100g, or 50-200g of circular RNA). In some embodiments, the amount produced is measured per liter (*e.g.*, 1-200g per liter), per batch or reaction (*e.g.*, 1-200g per batch or reaction), or per unit time (*e.g.*, 1-200g per hour or per day).

In some embodiments, more than one bioreactor may be utilized in series to increase the production capacity (*e.g.*, one, two, three, four, five, six, seven, eight, or nine bioreactors may be used in series).

Methods of Use

In some embodiments, a circular polyribonucleotide encoding an antifusogenic polypeptide (*e.g.*, a polypeptide of Table 1) is used for the treatment or prevention of a viral infection (*e.g.*, HIV, SARS-CoV-2, HCV, influenza, or RSV).

In some embodiments, a circular polynucleotide encoding an antifusogenic polypeptide (*e.g.*, a polypeptide of Table 1) is used for reducing viral entry.

In some embodiments, a circular polynucleotide encoding an antifusogenic polypeptide (*e.g.*, a polypeptide of Table 1) may be administered to a subject to reduce the risk of a viral infection (*e.g.*, HIV, SARS-CoV-2, HCV, influenza, or RSV).

For example, a circular polyribonucleotide as described herein may be administered to a subject (*e.g.*, in a pharmaceutical composition). In some embodiments, the subject is a vertebrate animal (*e.g.*, mammal, bird, fish, reptile, or amphibian). In some embodiments, the subject is a human. In some embodiments, the subject is a non-human mammal. In some embodiments, the subject is a non-human mammal is such as a non-human primate (*e.g.*, monkeys, apes), ungulate (*e.g.*, cattle, buffalo, sheep,

goat, pig, camel, llama, alpaca, deer, horses, donkeys), carnivore (e.g., dog, cat), rodent (e.g., rat, mouse), or lagomorph (e.g., rabbit). In embodiments, the subject is a bird, such as a member of the avian taxa Galliformes (e.g., chickens, turkeys, pheasants, quail), Anseriformes (e.g., ducks, geese),
 5 Palearognathae (e.g., ostriches, emus), Columbiformes (e.g., pigeons, doves), or Psittaciformes (e.g., parrots). In embodiments, the subject is an invertebrate such as an arthropod (e.g., insects, arachnids, crustaceans), a nematode, an annelid, a helminth, or a mollusk.

In some embodiments, the disclosure provides a method of modifying a subject by providing to the subject a composition or formulation described herein. In some embodiments, the composition or formulation is or includes a nucleic acid molecule (e.g., a DNA molecule or an RNA molecule described
 10 herein), and the polynucleotide is provided to a eukaryotic subject. In some embodiments, the composition or formulation is or includes or a eukaryotic or prokaryotic cell including a nucleic acid described herein.

In some embodiments, the disclosure provides a method of treating a viral infection in a subject in need thereof by providing to the subject a composition or formulation described herein. In some
 15 embodiments, the composition or formulation is or includes a nucleic acid molecule (e.g., a DNA molecule or an RNA molecule described herein), and the polynucleotide is provided to a eukaryotic subject. In some embodiments, the composition or formulation is or includes a eukaryotic or prokaryotic cell including a nucleic acid described herein. In some embodiments, the polyribonucleotide is provided in an amount and for a duration sufficient to treat a viral infection in a subject, e.g., in need thereof.

20 In some embodiments, the method may be used to treat or prevent HIV. For example, in some embodiments, the circular polyribonucleotide encodes an antifusogenic polypeptide that targets HIV, and the composition may be used to treat or prevent HIV.

In some embodiments, the method may be used to treat or prevent SARS-CoV-2. For example, in some embodiments, the circular polyribonucleotide encodes an antifusogenic polypeptide that targets
 25 SARS-CoV-2, and the composition may be used to treat or prevent SARS-CoV-2.

In some embodiments, the method may be used to treat or prevent HCV. For example, in some embodiments, the circular polyribonucleotide encodes an antifusogenic polypeptide that targets HCV, and the composition may be used to treat or prevent HCV.

30 In some embodiments, the method may be used to treat or prevent RSV. For example, in some embodiments, the circular polyribonucleotide encodes an antifusogenic polypeptide that targets RSV, and the composition may be used to treat or prevent RSV.

Methods of Dosing

A method of dosing to produce a level of circular polyribonucleotide encoding an antifusogenic
 35 polypeptide (e.g., a polypeptide of Table 1) or express a level of an antifusogenic polypeptide (e.g., a polypeptide of Table 1) in a cell after providing the cell with at least two doses or compositions of circular polyribonucleotide is disclosed herein. A method of dosing to produce a level of circular polyribonucleotide or express a level of an antifusogenic polypeptide (e.g., a polypeptide of Table 1, e.g., a polypeptide of any one of Tables 2-4) in a subject (e.g., a mammal, e.g., a human) after providing (e.g.,
 40 administering to) the subject with at least two doses or compositions of circular polyribonucleotide is disclosed herein. The composition includes a circular polyribonucleotide encoding an antifusogenic

polypeptide as described herein. A method of dosing can include administering two or more doses of a composition of circular polyribonucleotides, e.g., over short time period or over an extended period. In some embodiments, the composition containing a circular polyribonucleotide further includes a pharmaceutically acceptable carrier or excipient. The circular polyribonucleotide encodes an
 5 antifusogenic polypeptide, which can be expressed in a cell, e.g., following administration.

The methods described herein may include administering a first dose of the pharmaceutical composition in an amount sufficient to produce a serum concentration of at least 500 ng/mL (e.g., at least 600 ng/mL, 700 ng/mL, 800 ng/mL, 900 ng/mL, 1,000 ng/mL, 1,100 ng/mL, 1,200 ng/mL, 1,300 ng/mL, 1,400 ng/mL, 1,500 ng/mL, 1,600 ng/mL, 1,700 ng/mL, 1,800 ng/mL, 1,900 ng/mL, 2,000 ng/mL, 2,100
 10 ng/mL, 2,200 ng/mL, 2,300 ng/mL, 2,400 ng/mL, 2,500 ng/mL, 2,600 ng/mL, 2,700 ng/mL, 2,800 ng/mL, 2,900 ng/mL, 3,000 ng/mL, or more) of an antifusogenic polypeptide in the subject.

In some embodiments, the method may further include administering a second dose of the pharmaceutical composition. The method may further include administering a third, fourth, fifth, sixth, seventh, eighth, ninth, tenth, or more doses of the pharmaceutical composition. In some embodiments, a
 15 subsequent dose helps maintain a serum concentration of at least 500 ng/mL (e.g., at least 600 ng/mL, 700 ng/mL, 800 ng/mL, 900 ng/mL, 1,000 ng/mL, 1,100 ng/mL, 1,200 ng/mL, 1,300 ng/mL, 1,400 ng/mL, 1,500 ng/mL, 1,600 ng/mL, 1,700 ng/mL, 1,800 ng/mL, 1,900 ng/mL, 2,000 ng/mL, 2,100 ng/mL, 2,200 ng/mL, 2,300 ng/mL, 2,400 ng/mL, 2,500 ng/mL, 2,600 ng/mL, 2,700 ng/mL, 2,800 ng/mL, 2,900 ng/mL, 3,000 ng/mL, or more) of an antifusogenic polypeptide in the subject. In some embodiments, a
 20 subsequent dose is administered before the serum concentration drops below 500 ng/mL of an antifusogenic polypeptide in the subject.

In some embodiments, multiple doses are provided to produce a level of the composition or express a level of the antifusogenic polypeptide in a cell, tissue or subject. In some embodiments, multiple doses are provided to produce or maintain a level of the composition, or to produce or maintain a
 25 level of the antifusogenic polypeptide, in a cell, tissue or subject for a period of time, for instance, for at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150 days, or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18, 21, or 24 months, or at least 1, 2, 3, 4, or 5 years.

In some embodiments, the second dose is administered at least one hour (e.g., at least two hours, three hours, four hours, five hours, six hours, seven hours, eight hours, nine hours, 10 hours, 11
 30 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, one day, two days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, one year, or longer) after the first dose of the pharmaceutical composition.

In some embodiments, the second dose is administered from 1 hour to 1 year (e.g., from 1 hour to 1 day, e.g., one hour, two hours, three hours, four hours, five hours, six hours, seven hours, eight hours, nine hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18
 35 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, or one day, e.g., from one day to one week, e.g., two days, three days, four days, five days, six days, or one week, e.g., from one week to one month, e.g., two weeks, three weeks, or one month, e.g., from one month to one year, e.g., one month, two
 40 months, three months, four months, five months, six months, seven months, eight months, nine months,

ten months, eleven months, or one year) after the first dose of the pharmaceutical composition. In some embodiments, the second dose is administered from 1 days to 180 days (e.g., from 1 day to 90 days, from 1 day to 45 days, from one day to 30 days, from 1 day to 14 days, from 1 day to 7 days, from 2 days to 45 days, from 2 days to 30 days, from 2 days to 14 days, from 2 days to 7 days, from 3 days to 90 days, from 3 days to 45 days, from 3 days to 30 days, from 3 days to 14 days, from 3 days to 7 days, from 4 days to 90 days, from 4 days to 45 days, from 4 days to 30 days, from 4 days to 14 days, from 4 days to 7 days, from 5 days to 90 days, from 5 days to 45 days, from 5 days to 30 days, from 5 days to 14 days, from 5 days to 7 days, from 6 days to 90 days, from 6 days to 45 days, from 6 days to 30 days, from 6 days to 14 days, from 6 days to 7 days, from 7 days to 90 days, from 7 days to 45 days, from 7 days to 30 days, from 7 days to 14 days, from 14 days to 90 days, from 14 days to 45 days, from 14 days to 30 days, from 21 days to 90 days, from 21 days to 60 days, from 21 days to 45 days, from 21 days to 30 days, from 30 days to 90 days, from 30 days to 60 days, from 30 days to 45 days, from 45 to 180 days, from 45 to 120 days, from 45 to 100 days, from 45 to 90 days, from 45 to 60 days, from 60 to 180 days, from 60 to 120 days, from 60 to 100 days, from 60 to 90 days, from 90 to 100 days, from 90 to 120 days, or from 90 to 180 days) after the first dose of the pharmaceutical composition.

In some embodiments, the third dose is administered at least one hour (e.g., at least two hours, three hours, four hours, five hours, six hours, seven hours, eight hours, nine hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, one day, two days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, one year, or longer) after the second dose of the pharmaceutical composition.

In some embodiments, the third dose is administered from 1 hour to 1 year (e.g., from 1 hour to 1 day, e.g., one hour, two hours, three hours, four hours, five hours, six hours, seven hours, eight hours, nine hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, or one day, e.g., from one day to one week, e.g., two days, three days, four days, five days, six days, or one week, e.g., from one week to one month, e.g., two weeks, three weeks, or one month, e.g., from one month to one year, e.g., one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, or one year) after the second dose of the pharmaceutical composition. In some embodiments, the third dose is administered from 1 days to 180 days (e.g., from 1 day to 90 days, from 1 day to 45 days, from one day to 30 days, from 1 day to 14 days, from 1 day to 7 days, from 2 days to 45 days, from 2 days to 30 days, from 2 days to 14 days, from 2 days to 7 days, from 3 days to 90 days, from 3 days to 45 days, from 3 days to 30 days, from 3 days to 14 days, from 3 days to 7 days, from 4 days to 90 days, from 4 days to 45 days, from 4 days to 30 days, from 4 days to 14 days, from 4 days to 7 days, from 5 days to 90 days, from 5 days to 45 days, from 5 days to 30 days, from 5 days to 14 days, from 5 days to 7 days, from 6 days to 90 days, from 6 days to 45 days, from 6 days to 30 days, from 6 days to 14 days, from 6 days to 7 days, from 7 days to 90 days, from 7 days to 45 days, from 7 days to 30 days, from 7 days to 14 days, from 14 days to 90 days, from 14 days to 45 days, from 14 days to 30 days, from 21 days to 90 days, from 21 days to 60 days, from 21 days to 45 days, from 21 days to 30 days, from 30 days to 90 days, from 30 days to 60 days, from 30 days to 45 days, from 45 to 180 days, from 45

to 120 days, from 45 to 100 days, from 45 to 90 days, from 45 to 60 days, from 60 to 180 days, from 60 to 120 days, from 60 to 100 days, from 60 to 90 days, from 90 to 100 days, from 90 to 120 days, or from 90 to 180 days) after the second dose of the pharmaceutical composition.

In some embodiments, the second dose is administered before a serum concentration of an antifusogenic polypeptide is less than about 500 ng/mL in serum of the subject.

In some embodiments, the method maintains a serum concentration of at least 500 ng/mL (e.g., at least 600 ng/mL, 700 ng/mL, 800 ng/mL, 900 ng/mL, 1,000 ng/mL, 1,100 ng/mL, 1,200 ng/mL, 1,300 ng/mL, 1,400 ng/mL, 1,500 ng/mL, 1,600 ng/mL, 1,700 ng/mL, 1,800 ng/mL, 1,900 ng/mL, 2,000 ng/mL, 2,100 ng/mL, 2,200 ng/mL, 2,300 ng/mL, 2,400 ng/mL, 2,500 ng/mL, 2,600 ng/mL, 2,700 ng/mL, 2,800 ng/mL, 2,900 ng/mL, 3,000 ng/mL, or more) of an antifusogenic polypeptide in the subject, e.g., for at least one hour (e.g., at least two hours, three hours, four hours, five hours, six hours, seven hours, eight hours, nine hours, 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17 hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, one day, two days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, one year, or longer).

A method of administering multiple doses of a composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide) includes providing two or more compositions over a period of time, to a cell, tissue or subject (e.g., a mammal). According to certain embodiments, multiple doses of a composition of a nucleic acid molecule described herein may be administered to a subject over a defined time course. The methods according to this aspect of the invention include sequentially administering to a subject multiple doses of a composition of a nucleic acid molecule described herein (e.g., a circular polyribonucleotide, a linear polyribonucleotide, a circular polydeoxyribonucleotide, a linear polydeoxyribonucleotide) (e.g., in a pharmaceutical or veterinary composition). As used herein, “sequentially administering” means that each dose of composition of a nucleic acid molecule described herein is administered to the subject at a different point in time, e.g., on different days separated by a predetermined interval (e.g., hours, days, weeks or months). In some embodiments, the present invention provides methods which include sequentially administering to the subject a single initial dose of a composition of a nucleic acid molecule described herein, followed by one or more secondary doses of the composition, and optionally followed by one or more tertiary doses of the composition.

The terms “initial dose,” “secondary doses,” and “tertiary doses,” refer to the temporal sequence of administration of a composition of a nucleic acid molecule described herein. Thus, the “initial dose” is the dose which is administered at the beginning of the treatment regimen; the “secondary doses” are the doses which are administered after the initial dose; and the “tertiary doses” are the doses which are administered after the secondary doses. The initial, secondary, and tertiary doses may all contain the same amount of a composition of a nucleic acid molecule described herein, and in certain embodiments, may differ from one another in terms of frequency of administration. In certain embodiments, the amount of a composition of a nucleic acid molecule described herein contained in the initial, secondary and/or tertiary doses varies from one another (e.g., adjusted up or down as appropriate) during the course of treatment. In certain embodiments, one or more (e.g., 2, 3, 4, or 5) doses are administered at the

beginning of the treatment regimen as "loading doses" followed by subsequent doses that are administered on a less frequent basis (e.g., "maintenance doses").

In certain embodiments, each secondary and/or tertiary dose is administered after the immediately preceding dose. The phrase "the immediately preceding dose," as used herein, means, in a sequence of multiple administrations, the dose of the composition of a nucleic acid molecule described herein which is administered to a subject prior to the administration of the very next dose in the sequence with no intervening doses. In certain embodiments, each secondary and/or tertiary dose is administered every day, every 2 days, 3 days, 4 days, 5 days, 6 days, or 7 days after the immediately preceding dose. In certain embodiments, each secondary and/or tertiary dose is administered every 0.5 weeks, 1 week, 2 weeks, 3 weeks, or 4 weeks after the immediately preceding dose.

The methods according to this aspect of the invention may include administering to a subject any number of secondary and/or tertiary doses of a composition of a nucleic acid molecule described herein. For example, in certain embodiments, only a single secondary dose is administered to the subject. In other embodiments, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, or more) secondary doses are administered to the subject. Likewise, in certain embodiments, only a single tertiary dose is administered to the subject. In other embodiments, two or more (e.g., 2, 3, 4, 5, 6, 7, 8, or more) tertiary doses are administered to the subject.

In certain embodiments, the frequency at which the secondary and/or tertiary doses are administered to a subject can vary over the course of the treatment regimen. The frequency of administration may also be adjusted during the course of treatment.

In some embodiments, the method includes providing (e.g., administering) at least a first composition and a second composition to the cells, tissue, or subject (e.g., a mammal, e.g., a human). In some embodiments, the method further includes providing (e.g., administering) a third composition, fourth composition, fifth composition, sixth composition, seventh composition, eighth composition, ninth composition, tenth composition, or more. In some embodiments, additional compositions are provided for the duration of the life of the cell. In some embodiments, additional compositions are provided (e.g., administered) while the cell, tissue or subject obtains a benefit from the composition.

In some embodiments, a first composition in a multiple dosing regimen includes a first amount of the nucleic acid molecule (e.g., circular polyribonucleotide) disclosed herein. In some embodiments, a second composition in a multiple dosing regimen includes a second amount of the nucleic acid molecule (e.g., circular polyribonucleotide) disclosed herein. In some embodiments, a third composition, a fourth composition, a fifth composition, a sixth composition, a seventh composition, an eighth composition, a ninth composition, a tenth composition, or more in a multiple dosing regimen includes a third, fourth, fifth, sixth, seventh, eighth, ninth, tenth or more amount of the nucleic acid molecule (e.g., circular polyribonucleotide) disclosed herein. In some embodiments, the second amount of the nucleic acid molecule (e.g., circular polyribonucleotide) is the same as the first amount of the nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, the third amount of the nucleic acid molecule (e.g., circular polyribonucleotide) is the same as the first amount of the nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, the fourth, fifth, sixth, seventh, eighth, ninth, tenth, or more amount of the nucleic acid molecule (e.g., circular polyribonucleotide) is the same as the first amount of the nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, the second

amount of the nucleic acid molecule (e.g., circular polyribonucleotide) is less than the first amount of the nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, the third amount of the nucleic acid molecule (e.g., circular polyribonucleotide) is less than the first amount of the nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, the fourth, fifth, sixth, seventh, eighth, ninth, tenth, or more amount of the nucleic acid molecule (e.g., circular polyribonucleotide) is less than the first amount of the nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, the second amount of the nucleic acid molecule (e.g., circular polyribonucleotide) is greater than the first amount of the nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, the third amount of the nucleic acid molecule (e.g., circular polyribonucleotide) is greater than the first amount of the nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, the fourth, fifth, sixth, seventh, eighth, ninth, tenth, or more amount of the nucleic acid molecule (e.g., circular polyribonucleotide) is greater than the first amount of the nucleic acid molecule (e.g., circular polyribonucleotide). In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of the second composition varies by no more than 1%, 5%, 10%, 15%, 20%, or 25% of an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of the first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of the second composition is no more than 1%, 5%, 10%, 15%, 20%, or 25% less than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of the first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a second composition is from 0.1-fold to 1000-fold higher than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a second composition is 0.1-fold, 1-fold, 5-fold, 10-fold, 100-fold, or 1000-fold higher than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a subsequent composition (e.g., a composition administered after a first composition) is 0.1-fold, 1-fold, 5-fold, 10-fold, 100-fold, or 1000-fold higher than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a second composition is from 0.1-fold to 1000-fold lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a second composition is 0.1-fold, 1-fold, 5-fold, 10-fold, 100-fold, or 1000-fold lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a subsequent composition (e.g., a composition administered after a first composition) is 0.1-fold, 1-fold, 5-fold, 10-fold, 100-fold, or 1000-fold lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a subsequent composition (e.g., after a first composition of an amount of nucleic acid molecule (e.g., circular polyribonucleotide)) is from 0.1-fold to 1000-fold higher or lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. In some embodiments, an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a subsequent composition (e.g., after a first composition of an amount of nucleic acid molecule (e.g., circular polyribonucleotide)) is from 0.1-fold to 1000-fold higher or lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition.

circular polyribonucleotide)) is 0.1-fold, 1-fold, 5-fold, 10-fold, 100-fold, or 1000-fold higher or lower than an amount of the nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition. For example, a first composition includes 1-fold nucleic acid molecule (e.g., circular polyribonucleotide), a second composition includes 5-fold nucleic acid molecule (e.g., circular polyribonucleotide) compared to the first composition, and a third composition includes 0.2-fold nucleic acid molecule (e.g., circular polyribonucleotide) compared to the first composition. In some embodiments, the second composition includes at least 5-fold nucleic acid molecule (e.g., circular polyribonucleotide) compared to an amount of nucleic acid molecule (e.g., circular polyribonucleotide) of a first composition.

In some embodiments, the first composition includes a higher amount of the nucleic acid molecule (e.g., circular polyribonucleotide) than the second composition. In some embodiments, the first composition includes a higher amount of the nucleic acid molecules (e.g., circular polyribonucleotides) than the third, fourth, fifth, sixth, seventh, eighth, ninth, or tenth composition.

In some embodiments, the plurality (e.g., two or more) of compositions of a nucleic acid molecule (e.g., circular polyribonucleotide) encoding an antifusogenic polypeptide, which are administered in a multiple dosing regimen as described herein, are the same compositions. In some embodiments, the plurality (e.g., two or more) of compositions of a nucleic acid molecule (e.g., circular polyribonucleotide) encoding an antifusogenic polypeptide, which are administered in a multiple dosing regimen as described herein, are different compositions. In some embodiments, the same compositions include the nucleic acid molecules (e.g., circular polyribonucleotides) encoding the same antifusogenic polypeptide. In some embodiments, the different compositions include the nucleic acid molecules (e.g., circular polyribonucleotides) encoding different antifusogenic polypeptides, or a combination thereof.

In some embodiments, in a multiple dosing regimen, the method of administering the nucleic acid molecule (e.g., circular polyribonucleotide) provided herein includes administering to a subject in need thereof the nucleic acid molecule for multiple times (multiple doses), e.g., at least 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 30, 40, 50, 60, 100, 150, 200, or 500 times, with an interval of from 1 day to 56 days, such as about 49 days, 42 days, 35 days, 28 days, 21 days, 14 days, or 7 days. In some embodiments, in a multiple dosing regimen, the method provided herein includes administering to a subject in need thereof the nucleic acid molecule for at least 3 times, with an interval of about 7 days. In some embodiments, in a subject that receives administration of multiple doses of the nucleic acid molecule (e.g., at least 3, 4, 5, 6, 7, 8, or 9 doses) provided herein, a level of the antifusogenic polypeptide (e.g., a plasma antifusogenic polypeptide) is maintained at a level with variation of less than 50%, 40%, 30%, 20%, or 10% for a period of longer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, or 20 weeks after the last dose. In some embodiments, in a subject that receives administration of multiple doses of the nucleic acid molecule (e.g., at least 3, 4, 5, 6, 7, 8, or 9 doses) provided herein, a level of the antifusogenic polypeptide (e.g., a plasma antifusogenic polypeptide level) is maintained at a first level for a period of longer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 19, or 20 weeks after the second, third, fourth, fifth, sixth, seventh, eighth, or the last dose, wherein the first level is higher than a level of the antifusogenic polypeptide measured shortly after the first dose (e.g., measured about 12, 24, 36, or 48 hours after the first dose). In some embodiments, in a subject that receives administration of multiple doses of the nucleic acid molecule (e.g., at least 3 doses) provided herein with an interval of about 7 days, a level of the antifusogenic polypeptide (e.g., a plasma antifusogenic polypeptide level) is

maintained at a first level for a period of longer than 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 weeks after the second, third, fourth, fifth, sixth, seventh, eighth, or the last dose, wherein the first level is higher than a level of the antifusogenic polypeptide measured shortly after the first dose (e.g., measured about 12, 24, 36, or 48 hours after the first dose).

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Methods of Delivery

A circular polyribonucleotide encoding an antifusogenic polypeptide (e.g., a polypeptide of Table 1) described herein may be included in pharmaceutical compositions with a carrier or without a carrier.

Pharmaceutical compositions described herein may be formulated for example including a carrier, such as a pharmaceutical carrier and/or a polymeric carrier, e.g., a liposome, and delivered by known methods to a subject in need thereof (e.g., a human or non-human agricultural or domestic animal, e.g., cattle, dog, cat, horse, poultry). Such methods include, but not limited to, transfection (e.g., lipid-mediated, cationic polymers, calcium phosphate, dendrimers); electroporation or other methods of membrane disruption (e.g., nucleofection), viral delivery (e.g., lentivirus, retrovirus, adenovirus, AAV), microinjection, microprojectile bombardment ("gene gun"), fugene, direct sonic loading, cell squeezing, optical transfection, protoplast fusion, impalefection, magnetofection, exosome-mediated transfer, lipid nanoparticle-mediated transfer, and any combination thereof. Methods of delivery are also described, e.g., in Gori et al., *Delivery and Specificity of CRISPR/Cas9 Genome Editing Technologies for Human Gene Therapy*. Human Gene Therapy. July 2015, 26(7): 443-451. doi:10.1089/hum.2015.074; and Zuris et al. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing *in vitro* and *in vivo*. Nat Biotechnol. 2014 Oct 30;33(1):73–80.

In some embodiments, circular polyribonucleotides may be delivered in a "naked" delivery formulation. A naked delivery formulation delivers a circular polyribonucleotide to a cell without the aid of a carrier and without covalent modification of the circular polyribonucleotide or partial or complete encapsulation of the circular polyribonucleotide.

A naked delivery formulation is a formulation that is free from a carrier and wherein the circular polyribonucleotide is without a covalent modification that binds a moiety that aids in delivery to a cell and the circular polyribonucleotide is not partially or completely encapsulated. In some embodiments, a circular polyribonucleotide without covalent modification that binds to a moiety that aids in delivery to a cell may be a polyribonucleotide that is not covalently bound to a moiety, such as a protein, small molecule, a particle, a polymer, or a biopolymer that aids in delivery to a cell. In some embodiments, circular polyribonucleotides may be delivered in a delivery formulation with protamine or a protamine salt (e.g., protamine sulfate).

A polyribonucleotide without covalent modification that binds to a moiety that aids in delivery to a cell may not contain a modified phosphate group. For example, a polyribonucleotide without covalent modification that binds to a moiety that aids in delivery to a cell may not contain phosphorothioate, phosphoroselenates, boranophosphates, boranophosphate esters, hydrogen phosphonates, phosphoramidates, phosphorodiamidates, alkyl or aryl phosphonates, or phosphotriesters.

In some embodiments, a naked delivery formulation may be free of any or all of: transfection reagents, cationic carriers, carbohydrate carriers, nanoparticle carriers, or protein carriers. For example, a naked delivery formulation may be free from phytoglycogen octenyl succinate, phytoglycogen beta-

dextrin, anhydride-modified phytyglycogen beta-dextrin, lipofectamine, polyethylenimine, poly(trimethylenimine), poly(tetramethylenimine), polypropylenimine, aminoglycoside-polyamine, dideoxy-diamino-b-cyclodextrin, spermine, spermidine, poly(2-dimethylamino)ethyl methacrylate, poly(lysine), poly(histidine), poly(arginine), cationized gelatin, dendrimers, chitosan, 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM), 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 3B-[N-(N,N'-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HCl), diheptadecylamidoglycyl spermidine (DOGS), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin.

A naked delivery formulation may include a non-carrier excipient. In some embodiments, a non-carrier excipient may include an inactive ingredient that does not exhibit an active cell-penetrating effect. In some embodiments, a non-carrier excipient may include a buffer, for example PBS. In some embodiments, a non-carrier excipient may be a solvent, a non-aqueous solvent, a diluent, a suspension aid, a surface-active agent, an isotonic agent, a thickening agent, an emulsifying agent, a preservative, a polymer, a peptide, a protein, a cell, a hyaluronidase, a dispersing agent, a granulating agent, a disintegrating agent, a binding agent, a buffering agent, a lubricating agent, or an oil.

In some embodiments, a naked delivery formulation may include a diluent, such as a parenterally acceptable diluent. A diluent (e.g., a parenterally acceptable diluent) may be a liquid diluent or a solid diluent. In some embodiments, a diluent (e.g., a parenterally acceptable diluent) may be an RNA solubilizing agent, a buffer, or an isotonic agent. Examples of an RNA solubilizing agent include water, ethanol, methanol, acetone, formamide, and 2-propanol. Examples of a buffer include 2-(N-morpholino)ethanesulfonic acid (MES), Bis-Tris, 2-[(2-amino-2-oxoethyl)-(carboxymethyl)amino]acetic acid (ADA), N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES), piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES), 3-(N-morpholino)propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tris, Tricine, Gly-Gly, Bicine, or phosphate. Examples of an isotonic agent include glycerin, mannitol, polyethylene glycol, propylene glycol, trehalose, or sucrose.

In some embodiments, the formulation includes a cell-penetrating agent. In some embodiments, the formulation is a topical formulation and includes a cell-penetrating agent. The cell-penetrating agent can include organic compounds such as alcohols having one or more hydroxyl function groups. In some cases, the cell-penetrating agent includes an alcohol such as, but not limited to, monohydric alcohols, polyhydric alcohols, unsaturated aliphatic alcohols, and alicyclic alcohols. The cell-penetrating agent can include one or more of methanol, ethanol, isopropanol, phenoxyethanol, triethanolamine, phenethyl alcohol, butanol, pentanol, cetyl alcohol, ethylene glycol, propylene glycol, denatured alcohol, benzyl alcohol, specially denatured alcohol, glycol, stearyl alcohol, cetearyl alcohol, menthol, polyethylene glycols (PEG)-400, ethoxylated fatty acids, or hydroxyethylcellulose. In certain embodiments, the cell-penetrating agent includes ethanol. The cell-penetrating agents can include any cell-penetrating agent in

any amount or in any formulation as described in WO 2020/180751 or WO 2020/180752, which are hereby incorporated by reference in their entirety.

In some embodiments, the pharmaceutical preparation as disclosed herein, the pharmaceutical composition as disclosed herein, the pharmaceutical drug substance of as disclosed, or the pharmaceutical drug product as disclosed herein is in parenteral nucleic acid delivery system. The parental nucleic acid delivery system may include the pharmaceutical preparation as disclosed herein, the pharmaceutical composition as disclosed herein, the pharmaceutical drug substance of as disclosed, or the pharmaceutical drug product as disclosed herein, and a parenterally acceptable diluent. In some embodiments, the pharmaceutical preparation as disclosed herein, the pharmaceutical composition as disclosed herein, the pharmaceutical drug substance of as disclosed, or the pharmaceutical drug product as disclosed herein in the parenteral nucleic acid delivery system is free of any carrier.

The disclosure is further directed to a host or host cell including the circular polyribonucleotide described herein. In some embodiments, the host or host cell is a vertebrate, mammal (e.g., human), or other organism or cell.

In some embodiments, the circular polyribonucleotide has a decreased, or fails to produce a, undesired response by the host's immune system as compared to the response triggered by a reference compound, e.g., a linear polynucleotide corresponding to the described circular polyribonucleotide. In embodiments, the circular polyribonucleotide is non-immunogenic in the host. Some immune responses include, but are not limited to, humoral immune responses (e.g., production of immunogen-specific antibodies) and cell-mediated immune responses (e.g., lymphocyte proliferation).

In some embodiments, a host or a host cell is contacted with (e.g., delivered to or administered to) the circular polyribonucleotide. In some embodiments, the host is a mammal, such as a human. The amount of the circular polyribonucleotide or linear, expression product, or both in the host can be measured at any time after administration. In certain embodiments, a time course of host growth in a culture is determined. If the growth is increased or reduced in the presence of the circular polyribonucleotide or linear, the circular polyribonucleotide or expression product or both is identified as being effective in increasing or reducing the growth of the host.

A method of delivering a circular polyribonucleotide molecule as described herein to a cell, tissue, or subject, includes administering the pharmaceutical composition, pharmaceutical drug substance or pharmaceutical drug product as described herein to the cell, tissue, or subject.

In some embodiments, the cell is a eukaryotic cell. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is an ungulate cell. In some embodiments, the cell is an animal cell. In some embodiments, the cell is an immune cell. In some embodiments, the tissue is a connective tissue, a muscle tissue, a nervous tissue, or an epithelial tissue. In some embodiments, the tissue is an organ (e.g., liver, lung, spleen, kidney, etc.).

In some embodiments, the method of delivering is an *in vivo* method. For example, a method of delivery of a circular polyribonucleotide as described herein includes parenterally administering to a subject in need thereof, the pharmaceutical composition, pharmaceutical drug substance or pharmaceutical drug product as described herein to the subject in need thereof. As another example, a method of delivering a circular polyribonucleotide to a cell or tissue of a subject, includes administering parenterally to the cell or tissue the pharmaceutical composition, pharmaceutical drug substance or

pharmaceutical drug product as described herein. In some embodiments, the circular polyribonucleotide is in an amount effective to elicit a biological response in the subject. In some embodiments, the circular polyribonucleotide is an amount effective to have a biological effect on the cell or tissue in the subject. In some embodiments, the pharmaceutical composition, pharmaceutical drug substance or pharmaceutical drug product as described herein includes a carrier. In some embodiments the pharmaceutical composition, pharmaceutical drug substance or pharmaceutical drug product as described herein includes a diluent and is free of any carrier.

In some embodiments the pharmaceutical composition, the pharmaceutical drug substance, or the pharmaceutical drug product is administered parenterally. In some embodiments the pharmaceutical composition, the pharmaceutical drug substance, or the pharmaceutical drug product is administered intravenously, intraarterially, intraperitoneally, intradermally, intracranially, intrathecally, intralymphatically, subcutaneously, or intramuscularly. In some embodiments, parenteral administration is intravenously, intramuscularly, ophthalmically, subcutaneously, intradermally or topically.

In some embodiments, the pharmaceutical composition, pharmaceutical drug substance or pharmaceutical drug product as described herein is administered intramuscularly. In some embodiments, the pharmaceutical composition, pharmaceutical drug substance or pharmaceutical drug product as described herein is administered subcutaneously. In some embodiments, the pharmaceutical composition, pharmaceutical drug substance or pharmaceutical drug product as described herein is administered topically. In some embodiments, the pharmaceutical composition, the pharmaceutical drug substance, or the pharmaceutical drug product is administered intratracheally.

In some embodiments the pharmaceutical composition, pharmaceutical drug substance or pharmaceutical drug product is administered by injection. The administration can be systemic administration or local administration. In some embodiments, any of the methods of delivery as described herein are performed with a carrier. In some embodiments, any methods of delivery as described herein are performed without the aid of a carrier or cell penetrating agent.

In some embodiments, the circular polyribonucleotide or a product translated from the circular polyribonucleotide is detected in the cell, tissue, or subject at least 1 day, at least 2 days, at least 3 days, at least 4 days, or at least 5 days after the administering step. In some embodiments, the presence of the circular polyribonucleotide or a product translated from the circular polyribonucleotide is evaluated in the cell, tissue, or subject before the administering step. In some embodiments, the presence of the circular polyribonucleotide or a product translated from the circular polyribonucleotide is evaluated in the cell, tissue, or subject after the administering step.

Formulations

In some embodiments of the present disclosure a circular polyribonucleotide described herein may be formulated in composition, e.g., a composition for delivery to a cell, a plant, an invertebrate animal, a non-human vertebrate animal, or a human subject, e.g., an agricultural, veterinary, or pharmaceutical composition. In some embodiments, the circular polyribonucleotide is formulated in a pharmaceutical composition. In some embodiments, a composition includes a circular polyribonucleotide and a diluent, a carrier, an adjuvant, or a combination thereof. In a particular embodiment, a composition includes a circular polyribonucleotide described herein and a carrier or a diluent free of any carrier. In

some embodiments, a composition including a circular polyribonucleotide with a diluent free of any carrier is used for naked delivery of the circular polyribonucleotide to a subject.

Pharmaceutical compositions may optionally include one or more additional active substances, e.g., therapeutically and/or prophylactically active substances. Pharmaceutical compositions may optionally include an inactive substance that serves as a vehicle or medium for the compositions described herein (e.g., compositions including circular polyribonucleotides, such as any one of the inactive ingredients approved by the United States Food and Drug Administration (FDA) and listed in the Inactive Ingredient Database). Pharmaceutical compositions of the present invention may be sterile and/or pyrogen-free. General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in Remington: The Science and Practice of Pharmacy 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference). Non-limiting examples of an inactive substance include solvents, aqueous solvents, non-aqueous solvents, dispersion media, diluents, dispersions, suspension aids, surface active agents, isotonic agents, thickening agents, emulsifying agents, preservatives, polymers, peptides, proteins, cells, hyaluronidases, dispersing agents, granulating agents, disintegrating agents, binding agents, buffering agents (e.g., phosphate buffered saline (PBS)), lubricating agents, oils, and mixtures thereof.

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

Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product.

In some embodiments, the reference criterion for the amount of circular polyribonucleotide molecules present in the preparation is at least 30% (w/w), 40% (w/w), 50% (w/w), 60% (w/w), 70% (w/w), 80% (w/w), 85% (w/w), 90% (w/w), 91% (w/w), 92% (w/w), 93% (w/w), 94% (w/w), 95% (w/w), 96% (w/w), 97% (w/w), 98% (w/w), 99% (w/w), 99.1% (w/w), 99.2% (w/w), 99.3% (w/w), 99.4% (w/w), 99.5% (w/w), 99.6% (w/w), 99.7% (w/w), 99.8% (w/w), 99.9% (w/w), or 100% (w/w) molecules of the total ribonucleotide molecules in the pharmaceutical preparation.

In some embodiments, the reference criterion for the amount of linear polyribonucleotide molecules present in the preparation is the presence of no more than 1 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml, 20 ng/ml, 25 ng/ml, 30 ng/ml, 35 ng/ml, 40 ng/ml, 50 ng/ml, 60 ng/ml, 70 ng/ml, 80 ng/ml, 90 ng/ml,

100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml, 500 ng/ml, 600 ng/ml, 1 µg/ml, 10 µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml, 600 µg/ml, 700 µg/ml, 800 µg/ml, 900 µg/ml, 1 mg/ml, 1.5 mg/ml, or 2 mg/ml of linear polyribonucleotide molecules.

In some embodiments, the reference criterion for the amount of linear polyribonucleotide molecules present in the preparation is no more than 0.5% (w/w), 1% (w/w), 2% (w/w), 5% (w/w), 10% (w/w), 15% (w/w), 20% (w/w), 25% (w/w), 30% (w/w), 40% (w/w), 50% (w/w) linear polyribonucleotide molecules of the total ribonucleotide molecules in the pharmaceutical preparation.

In some embodiments, the reference criterion for the amount of nicked polyribonucleotide molecules present in the preparation is no more than 0.5% (w/w), 1% (w/w), 2% (w/w), 5% (w/w), 10% (w/w), or 15% (w/w) nicked polyribonucleotide molecules of the total ribonucleotide molecules in the pharmaceutical preparation.

In some embodiments, the reference criterion for the amount of combined nicked and linear polyribonucleotide molecules present in the preparation is no more than 0.5% (w/w), 1% (w/w), 2% (w/w), 5% (w/w), 10% (w/w), 15% (w/w), 20% (w/w), 25% (w/w), 30% (w/w), 40% (w/w), 50% (w/w) combined nicked and linear polyribonucleotide molecules of the total ribonucleotide molecules in the pharmaceutical preparation. In some embodiments, a pharmaceutical preparation is an intermediate pharmaceutical preparation of a final circular polyribonucleotide drug product. In some embodiments, a pharmaceutical preparation is a drug substance or active pharmaceutical ingredient (API). In some embodiments, a pharmaceutical preparation is a drug product for administration to a subject.

In some embodiments, a preparation of circular polyribonucleotides is (before, during or after the reduction of linear RNA) further processed to substantially remove DNA, protein contamination (e.g., cell protein such as a host cell protein or protein process impurities), endotoxin, mononucleotide molecules, and/or a process-related impurity.

In some embodiments, a pharmaceutical formulation disclosed herein can include: (i) a compound (e.g., circular polyribonucleotide) disclosed herein; (ii) a buffer; (iii) a non-ionic detergent; (iv) a tonicity agent; and/or (v) a stabilizer. In some embodiments, the pharmaceutical formulation disclosed herein is a stable liquid pharmaceutical formulation. In some embodiments, the pharmaceutical formulation disclosed herein includes protamine or a protamine salt (e.g., protamine sulfate).

30 *Preservatives*

A composition or pharmaceutical composition provided herein can include material for a single administration, or can include material for multiple administrations (e.g., a "multidose" kit). The polyribonucleotide can be present in either linear or circular form. The composition or pharmaceutical composition can include one or more preservatives such as thiomersal or 2-phenoxyethanol. Preservatives can be used to prevent microbial contamination during use. Suitable preservatives include: benzalkonium chloride, thimerosal, chlorobutanol, methyl paraben, propyl paraben, phenylethyl alcohol, edetate disodium, sorbic acid, Onamer M, or other agents known to those skilled in the art. In ophthalmic products, e.g., such preservatives can be employed at a level of from 0.004% to 0.02%. In the compositions described herein the preservative, e.g., benzalkonium chloride, can be employed at a level of from 0.001% to less than 0.01%, e.g., from 0.001% to 0.008%, preferably about 0.005% by weight.

Polyribonucleotides can be susceptible to RNase that can be abundant in ambient environment. Compositions provided herein can include reagents that inhibit RNase activity, thereby preserving the polyribonucleotide from degradation. In some cases, the composition or pharmaceutical composition includes any RNase inhibitor known to one skilled in the art. Alternatively or additionally, the
5 polyribonucleotide, and cell-penetrating agent and/or pharmaceutically acceptable diluents or carriers, vehicles, excipients, or other reagents in the composition provided herein can be prepared in RNase-free environment. The composition can be formulated in RNase-free environment.

In some cases, a composition provided herein can be sterile. The composition can be formulated as a sterile solution or suspension, in suitable vehicles, known in the art. The composition can be
10 sterilized by conventional, known sterilization techniques, e.g., the composition can be sterile filtered.

Salts

In some cases, a composition or pharmaceutical composition provided herein includes one or more salts. For controlling the tonicity, a physiological salt such as sodium salt can be included a
15 composition provided herein. Other salts can include potassium chloride, potassium dihydrogen phosphate, disodium phosphate, and/or magnesium chloride, or the like. In some cases, the composition is formulated with one or more pharmaceutically acceptable salts. The one or more pharmaceutically acceptable salts can include those of the inorganic ions, such as, for example, sodium, potassium, calcium, magnesium ions, and the like. Such salts can include salts with inorganic or organic acids, such
20 as hydrochloric acid, hydrobromic acid, phosphoric acid, nitric acid, sulfuric acid, methanesulfonic acid, p-toluenesulfonic acid, acetic acid, fumaric acid, succinic acid, lactic acid, mandelic acid, malic acid, citric acid, tartaric acid, or maleic acid. The polyribonucleotide can be present in either linear or circular form.

Buffers/pH

25 A composition or pharmaceutical composition provided herein can include one or more buffers, such as a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer (e.g., with an aluminum hydroxide adjuvant); or a citrate buffer. Buffers, in some cases, are included in the 5-20 mM range.

A composition or pharmaceutical composition provided herein can have a pH between about 5.0 and about 8.5, between about 6.0 and about 8.0, between about 6.5 and about 7.5, or between about 7.0
30 and about 7.8. The composition or pharmaceutical composition can have a pH of about 7. The polyribonucleotide can be present in either linear or circular form.

Detergents/surfactants

A composition or pharmaceutical composition provided herein can include one or more
35 detergents and/or surfactants, depending on the intended administration route, e.g., polyoxyethylene sorbitan esters surfactants (commonly referred to as "Tweens"), e.g., polysorbate 20 and polysorbate 80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, e.g., octoxynol-9 (Triton X-100, or t-
40 octylphenoxypolyethoxyethanol); (octylphenoxy)polyethoxyethanol (IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); nonylphenol ethoxylates, such as the Tergitol™ NP

series; polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); and sorbitan esters (commonly known as "SPANs"), such as sorbitan trioleate (Span 85) and sorbitan monolaurate, an octoxynol (such as octoxynol-9 (Triton X-100) or t-octylphenoxypolyethoxyethanol), a cetyl trimethyl ammonium bromide ("CTAB"), or sodium deoxycholate. The one or more detergents and/or surfactants can be present only at trace amounts. In some cases, the composition can include less than 1 mg/ml of each of octoxynol-10 and polysorbate 80. Non-ionic surfactants can be used herein. Surfactants can be classified by their "HLB" (hydrophile/lipophile balance). In some cases, surfactants have a HLB of at least 10, at least 15, and/or at least 16. The polyribonucleotide can be present in either linear or circular form.

Diluents

In some embodiments, a composition of the disclosure includes a circular polyribonucleotide and a diluent. In some embodiments, a composition of the disclosure includes a linear polyribonucleotide and a diluent.

A diluent can be a non-carrier excipient. A non-carrier excipient serves as a vehicle or medium for a composition, such as a circular polyribonucleotide as described herein. A non-carrier excipient serves as a vehicle or medium for a composition, such as a linear polyribonucleotide as described herein. Non-limiting examples of a non-carrier excipient include solvents, aqueous solvents, non-aqueous solvents, dispersion media, diluents, dispersions, suspension aids, surface active agents, isotonic agents, thickening agents, emulsifying agents, preservatives, polymers, peptides, proteins, cells, hyaluronidases, dispersing agents, granulating agents, disintegrating agents, binding agents, buffering agents (e.g., phosphate buffered saline (PBS)), lubricating agents, oils, and mixtures thereof. A non-carrier excipient can be any one of the inactive ingredients approved by the United States Food and Drug Administration (FDA) and listed in the Inactive Ingredient Database that does not exhibit a cell-penetrating effect. A non-carrier excipient can be any inactive ingredient suitable for administration to a non-human animal, for example, suitable for veterinary use. Modification of compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation.

In some embodiments, the circular polyribonucleotide may be delivered as a naked delivery formulation, such as including a diluent. A naked delivery formulation delivers a circular polyribonucleotide, to a cell without the aid of a carrier and without modification or partial or complete encapsulation of the circular polyribonucleotide, capped polyribonucleotide, or complex thereof.

A naked delivery formulation is a formulation that is free from a carrier and wherein the circular polyribonucleotide is without a covalent modification that binds a moiety that aids in delivery to a cell or without partial or complete encapsulation of the circular polyribonucleotide. In some embodiments, a circular polyribonucleotide without a covalent modification that binds a moiety that aids in delivery to a cell is a polyribonucleotide that is not covalently bound to a protein, small molecule, a particle, a polymer, or a biopolymer. A circular polyribonucleotide without covalent modification that binds a moiety that aids in delivery to a cell does not contain a modified phosphate group. For example, a circular polyribonucleotide without a covalent modification that binds a moiety that aids in delivery to a cell does

not contain phosphorothioate, phosphoroselenates, boranophosphates, boranophosphate esters, hydrogen phosphonates, phosphoramidates, phosphorodiamidates, alkyl or aryl phosphonates, or phosphotriesters.

In some embodiments, a naked delivery formulation is free of any or all of: transfection reagents, cationic carriers, carbohydrate carriers, nanoparticle carriers, or protein carriers. In some embodiments, a naked delivery formulation is free from phytoglycogen octenyl succinate, phytoglycogen beta-dextrin, anhydride-modified phytoglycogen beta-dextrin, lipofectamine, polyethylenimine, poly(trimethylenimine), poly(tetramethylenimine), polypropylenimine, aminoglycoside-polyamine, dideoxy-diamino-b-cyclodextrin, spermine, spermidine, poly(2-dimethylamino)ethyl methacrylate, poly(lysine), poly(histidine), poly(arginine), cationized gelatin, dendrimers, chitosan, 1,2-Dioleoyl-3-Trimethylammonium-Propane(DOTAP), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM), 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 3B-[N—(N,N'-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HCl), diheptadecylamidoglycyl spermidine (DOGS), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC), human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin.

In certain embodiments, a naked delivery formulation includes a non-carrier excipient. In some embodiments, a non-carrier excipient includes an inactive ingredient that does not exhibit a cell-penetrating effect. In some embodiments, a non-carrier excipient includes a buffer, for example PBS. In some embodiments, a non-carrier excipient is a solvent, a non-aqueous solvent, a diluent, a suspension aid, a surface-active agent, an isotonic agent, a thickening agent, an emulsifying agent, a preservative, a polymer, a peptide, a protein, a cell, a hyaluronidase, a dispersing agent, a granulating agent, a disintegrating agent, a binding agent, a buffering agent, a lubricating agent, or an oil.

In some embodiments, a naked delivery formulation includes a diluent. A diluent may be a liquid diluent or a solid diluent. In some embodiments, a diluent is an RNA solubilizing agent, a buffer, or an isotonic agent. Examples of an RNA solubilizing agent include water, ethanol, methanol, acetone, formamide, and 2-propanol. Examples of a buffer include 2-(N-morpholino)ethanesulfonic acid (MES), Bis-Tris, 2-[(2-amino-2-oxoethyl)-(carboxymethyl)amino]acetic acid (ADA), N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES), piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES), 3-(N-morpholino)propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Tris, Tricine, Gly-Gly, Bicine, or phosphate. Examples of an isotonic agent include glycerin, mannitol, polyethylene glycol, propylene glycol, trehalose, or sucrose.

Carriers

In some embodiments, a composition of the disclosure includes a circular polyribonucleotide and a carrier. In some embodiments, a composition of the disclosure includes a linear polyribonucleotide and a carrier.

In certain embodiments, a composition includes a circular polyribonucleotide as described herein in a vesicle or other membrane-based carrier. In certain embodiments, a composition includes a linear polyribonucleotide as described herein in a vesicle or other membrane-based carrier.

In other embodiments, a composition includes the circular polyribonucleotide in or via a cell,
5 vesicle or other membrane-based carrier. In other embodiments, a composition includes the linear polyribonucleotide in or via a cell, vesicle or other membrane-based carrier. In one embodiment, a composition includes the circular polyribonucleotide in liposomes or other similar vesicles. In one embodiment, a composition includes the linear polyribonucleotide in liposomes or other similar vesicles. Liposomes are spherical vesicle structures composed of a uni- or multilamellar lipid bilayer surrounding
10 internal aqueous compartments and a relatively impermeable outer lipophilic phospholipid bilayer. Liposomes may be anionic, neutral, or cationic. Liposomes are biocompatible, nontoxic, can deliver both hydrophilic and lipophilic drug molecules, protect their cargo from degradation by plasma enzymes, and transport their load across biological membranes and the blood brain barrier (BBB) (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011.
15 doi:10.1155/2011/469679 for review).

Vesicles can be made from several different types of lipids; however, phospholipids are most commonly used to generate liposomes as drug carriers. Methods for preparation of multilamellar vesicle lipids are known in the art (see for example U.S. Pat. No. 6,693,086, the teachings of which relating to multilamellar vesicle lipid preparation are incorporated herein by reference). Although vesicle formation
20 can be spontaneous when a lipid film is mixed with an aqueous solution, it can also be expedited by applying force in the form of shaking by using a homogenizer, sonicator, or an extrusion apparatus (see, e.g., Spuch and Navarro, Journal of Drug Delivery, vol. 2011, Article ID 469679, 12 pages, 2011. doi:10.1155/2011/469679 for review). Extruded lipids can be prepared by extruding through filters of decreasing size, as described in Templeton et al., Nature Biotech, 15:647-652, 1997, the teachings of
25 which relating to extruded lipid preparation are incorporated herein by reference.

In certain embodiments, a composition of the disclosure includes a circular polyribonucleotide and lipid nanoparticles, for example lipid nanoparticles described herein. In certain embodiments, a composition of the disclosure includes a linear polyribonucleotide and lipid nanoparticles. Lipid nanoparticles are another example of a carrier that provides a biocompatible and biodegradable delivery
30 system for a circular polyribonucleotide molecule as described herein. Lipid nanoparticles are another example of a carrier that provides a biocompatible and biodegradable delivery system for a linear polyribonucleotide molecule as described herein. Nanostructured lipid carriers (NLCs) are modified solid lipid nanoparticles (SLNs) that retain the characteristics of the SLN, improve drug stability and loading capacity, and prevent drug leakage. Polymer nanoparticles (PNPs) are an important component of drug
35 delivery. These nanoparticles can effectively direct drug delivery to specific targets and improve drug stability and controlled drug release. Lipid-polymer nanoparticles (PLNs), a new type of carrier that combines liposomes and polymers, may also be employed. These nanoparticles possess the complementary advantages of PNPs and liposomes. A PLN is composed of a core-shell structure; the polymer core provides a stable structure, and the phospholipid shell offers good biocompatibility. As
40 such, the two components increase the drug encapsulation efficiency rate, facilitate surface modification,

and prevent leakage of water-soluble drugs. For a review, see, e.g., Li et al. 2017, *Nanomaterials* 7, 122; doi:10.3390/nano7060122.

Additional non-limiting examples of carriers include carbohydrate carriers (e.g., an anhydride-modified phytoglycogen or glycogen-type material), protein carriers (e.g., a protein covalently linked to the circular polyribonucleotide or a protein covalently linked to the linear polyribonucleotide), or cationic carriers (e.g., a cationic lipopolymer or transfection reagent). Non-limiting examples of carbohydrate carriers include phytoglycogen octenyl succinate, phytoglycogen beta-dextrin, and anhydride-modified phytoglycogen beta-dextrin. Non-limiting examples of cationic carriers include lipofectamine, polyethylenimine, poly(trimethylenimine), poly(tetramethylenimine), polypropylenimine, aminoglycoside-polyamine, dideoxy-diamino-b-cyclodextrin, spermine, spermidine, poly(2-dimethylamino)ethyl methacrylate, poly(lysine), poly(histidine), poly(arginine), cationized gelatin, dendrimers, chitosan, 1,2-Dioleoyl-3-Trimethylammonium-Propane(DOTAP), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM), 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 3B-[N-(N,N'-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HCl), diheptadecylamidoglycyl spermidine (DOGS), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), and N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC). Non-limiting examples of protein carriers include human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin.

Exosomes can also be used as drug delivery vehicles for a circular RNA composition or preparation described herein. Exosomes can be used as drug delivery vehicles for a linear polyribonucleotide composition or preparation described herein. For a review, see Ha et al. July 2016. *Acta Pharmaceutica Sinica B*. Volume 6, Issue 4, Pages 287-296; doi.org/10.1016/j.apsb.2016.02.001. Ex vivo differentiated red blood cells can also be used as a carrier for a circular RNA composition or preparation described herein. Ex vivo differentiated red blood cells can also be used as a carrier for a linear polyribonucleotide composition or preparation described herein. See, e.g., International Patent Publication Nos. WO2015/073587; WO2017/123646; WO2017/123644; WO2018/102740; WO2016/183482; WO2015/153102; WO2018/151829; WO2018/009838; Shi et al. 2014. *Proc Natl Acad Sci USA*. 111(28): 10131–10136; US Patent 9,644,180; Huang et al. 2017. *Nature Communications* 8: 423; Shi et al. 2014. *Proc Natl Acad Sci USA*. 111(28): 10131–10136. Fusosome compositions, e.g., as described in International Patent Publication No. WO2018/208728, can also be used as carriers to deliver a circular polyribonucleotide molecule described herein. Fusosome compositions, e.g., as described in WO2018/208728, can also be used as carriers to deliver a linear polyribonucleotide molecule described herein.

Virosomes and virus-like particles (VLPs) can also be used as carriers to deliver a circular polyribonucleotide molecule described herein to targeted cells. Virosomes and virus-like particles (VLPs) can also be used as carriers to deliver a linear polyribonucleotide molecule described herein to targeted cells.

Plant nanovesicles and plant messenger packs (PMPs), e.g., as described in International Patent Publication Nos. WO2011/097480, WO2013/070324, WO2017/004526, or WO2020/041784 can also be

used as carriers to deliver the circular RNA composition or preparation described herein. Plant nanovesicles and plant messenger packs (PMPs) can also be used as carriers to deliver a linear polyribonucleotide composition or preparation described herein.

Microbubbles can also be used as carriers to deliver a circular polyribonucleotide molecule described herein. Microbubbles can also be used as carriers to deliver a linear polyribonucleotide molecule described herein. See, e.g., US7115583; Beeri, R. et al., *Circulation*. 2002 Oct 1;106(14):1756-1759; Bez, M. et al., *Nat Protoc*. 2019 Apr; 14(4): 1015–1026; Hernot, S. et al., *Adv Drug Deliv Rev*. 2008 Jun 30; 60(10): 1153–1166; Rychak, J.J. et al., *Adv Drug Deliv Rev*. 2014 Jun; 72: 82–93. In some embodiments, microbubbles are albumin-coated perfluorocarbon microbubbles.

The carrier including the circular polyribonucleotides described herein may include a plurality of particles. The particles may have median particle size of 30 to 700 nanometers (e.g., 30 to 50, 50 to 100, 100 to 200, 200 to 300, 300 to 400, 400 to 500, 500 to 600, 600 to 700, 100 to 500, 50 to 500, or 200 to 700 nanometers). The size of the particle may be optimized to favor deposition of the payload, including the circular polyribonucleotide into a cell. Deposition of the circular polyribonucleotide into certain cell types may favor different particle sizes. For example, the particle size may be optimized for deposition of the circular polyribonucleotide into antigen presenting cells. The particle size may be optimized for deposition of the circular polyribonucleotide into dendritic cells. Additionally, the particle size may be optimized for depositions of the circular polyribonucleotide into draining lymph node cells.

Lipid Nanoparticles

The compositions, methods, and delivery systems provided by the present disclosure may employ any suitable carrier or delivery modality described herein, including, in certain embodiments, lipid nanoparticles (LNPs). Lipid nanoparticles, in some embodiments, include one or more ionic lipids, such as non-cationic lipids (e.g., neutral or anionic, or zwitterionic lipids); one or more conjugated lipids (such as PEG-conjugated lipids or lipids conjugated to polymers described in Table 5 of WO2019217941; incorporated herein by reference in its entirety); one or more sterols (e.g., cholesterol).

Lipids that can be used in nanoparticle formations (e.g., lipid nanoparticles) include, for example those described in Table 4 of WO2019217941, which is incorporated by reference—e.g., a lipid-containing nanoparticle can include one or more of the lipids in Table 4 of WO2019217941. Lipid nanoparticles can include additional elements, such as polymers, such as the polymers described in Table 5 of WO2019217941, incorporated by reference.

In some embodiments, conjugated lipids, when present, can include one or more of PEG-diacylglycerol (DAG) (such as 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG)), PEG-dialkylolxypropyl (DAA), PEG-phospholipid, PEG-ceramide (Cer), a pegylated phosphatidylethanolamine (PEG-PE), PEG succinate diacylglycerol (PEGs-DAG) (such as 4-0-(2',3'-di(tetradecanoyloxy)propyl)-1-0-(ω-methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG)), PEG dialkoxypolypropylcarbam, N-(carbonyl-methoxypoly ethylene glycol 2000)- 1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt, and those described in Table 2 of WO2019051289 (incorporated by reference), and combinations of the foregoing.

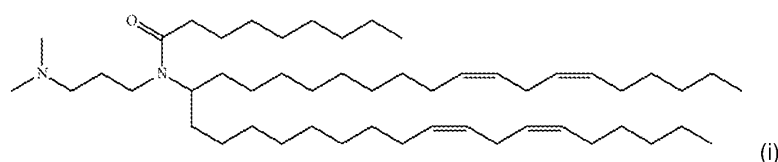
In some embodiments, sterols that can be incorporated into lipid nanoparticles include one or more of cholesterol or cholesterol derivatives, such as those in WO2009/127060 or US2010/0130588,

which are incorporated by reference. Additional exemplary sterols include phytosterols, including those described in Eygeris et al. (2020), [dx.doi.org/10.1021/acs.nanolett.0c01386](https://doi.org/10.1021/acs.nanolett.0c01386), incorporated herein by reference.

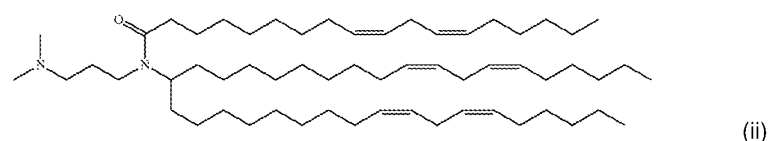
In some embodiments, the lipid particle includes an ionizable lipid, a non-cationic lipid, a conjugated lipid that inhibits aggregation of particles, and a sterol. The amounts of these components can be varied independently and to achieve desired properties. For example, in some embodiments, the lipid nanoparticle includes an ionizable lipid in an amount from about 20 mol % to about 90 mol % of the total lipids (in other embodiments it may be 20-70% (mol), 30-60% (mol) or 40-50% (mol); about 50 mol % to about 90 mol % of the total lipid present in the lipid nanoparticle), a non-cationic lipid in an amount from about 5 mol % to about 30 mol % of the total lipids, a conjugated lipid in an amount from about 0.5 mol % to about 20 mol % of the total lipids, and a sterol in an amount from about 20 mol % to about 50 mol % of the total lipids. The ratio of total lipid to nucleic acid can be varied as desired. For example, the total lipid to nucleic acid (mass or weight) ratio can be from about 10: 1 to about 30: 1.

In some embodiments, the lipid to nucleic acid ratio (mass/mass ratio; w/w ratio) can be in the range of from about 1:1 to about 25:1, from about 10:1 to about 14:1, from about 3:1 to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1. The amounts of lipids and nucleic acid can be adjusted to provide a desired N/P ratio, for example, N/P ratio of 3, 4, 5, 6, 7, 8, 9, 10 or higher. Generally, the lipid nanoparticle formulation's overall lipid content can range from about 5 mg/ml to about 30 mg/mL.

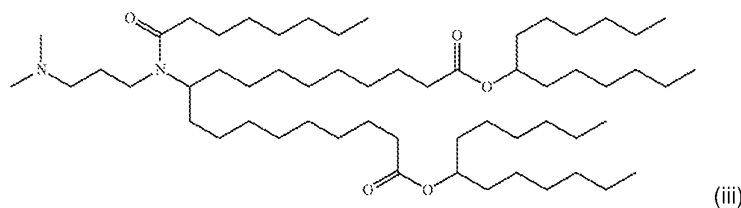
Some non-limiting example of lipid compounds that may be used (e.g., in combination with other lipid components) to form lipid nanoparticles for the delivery of compositions described herein, e.g., nucleic acid (e.g., RNA (e.g., circular polyribonucleotide, linear polyribonucleotide)) described herein includes,



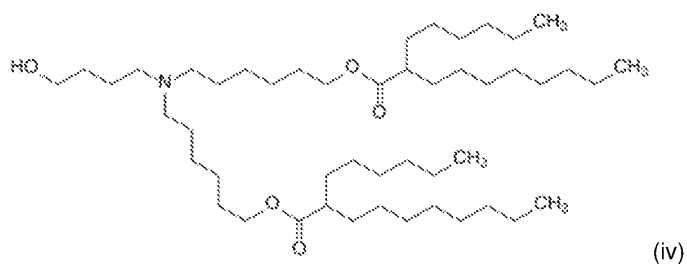
In some embodiments an LNP including Formula (i) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.



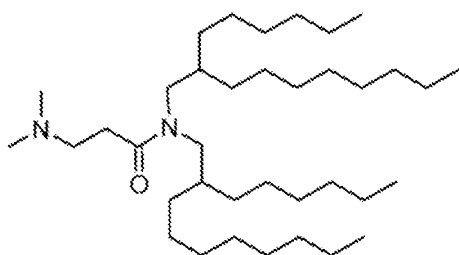
In some embodiments an LNP including Formula (ii) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.



In some embodiments an LNP including Formula (iii) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.

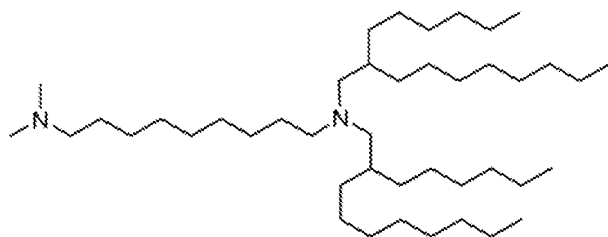


(iv)



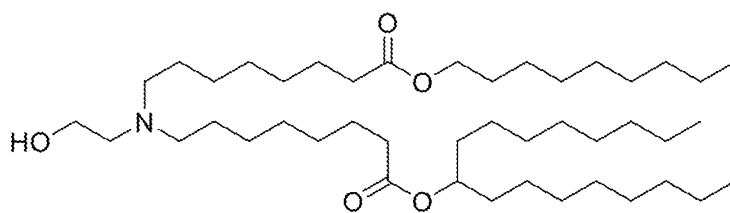
(v)

5 In some embodiments an LNP including Formula (v) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.



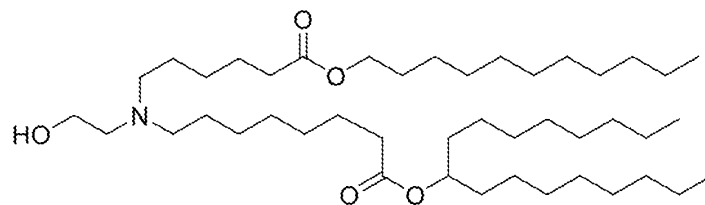
(vi)

In some embodiments an LNP including Formula (vi) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.



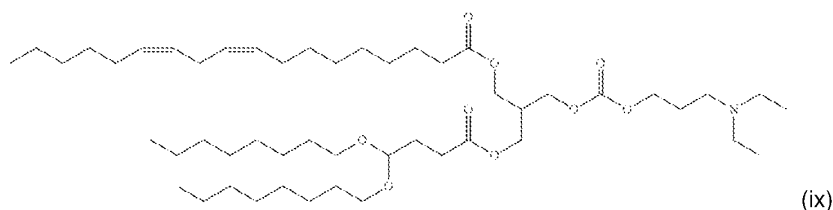
(vii)

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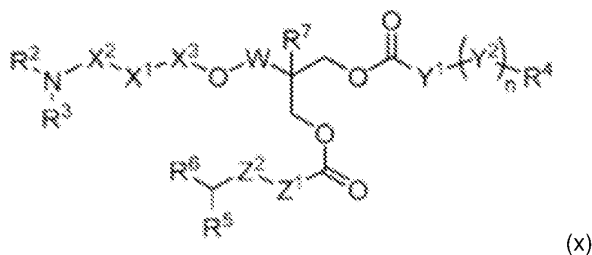


(viii)

In some embodiments an LNP including Formula (viii) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.

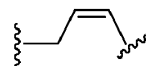


In some embodiments an LNP including Formula (ix) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.

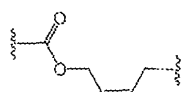


5 wherein

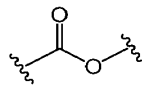
X¹ is O, NR¹, or a direct bond, X² is C2-5 alkylene, X³ is C(=O) or a direct bond, R¹ is H or Me, R³ is C1-3 alkyl, R² is C1-3 alkyl, or R² taken together with the nitrogen atom to which it is attached and 1-3 carbon atoms of X² form a 4-, 5-, or 6-membered ring, or X¹ is NR¹, R¹ and R² taken together with the nitrogen atoms to which they are attached form a 5- or 6-membered ring, or R² taken together with R³ and the nitrogen atom to which they are attached form a 5-, 6-, or 7-membered ring, Y¹ is C2-12 alkylene, Y² is selected from



(in either orientation),



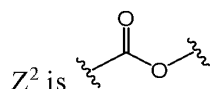
(in either orientation),



(in either orientation),

n is 0 to 3, R⁴ is C1-15 alkyl, Z¹ is C1-6 alkylene or a direct bond,

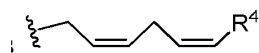
15



(in either orientation) or absent, provided that if Z¹ is a direct bond, Z² is absent;

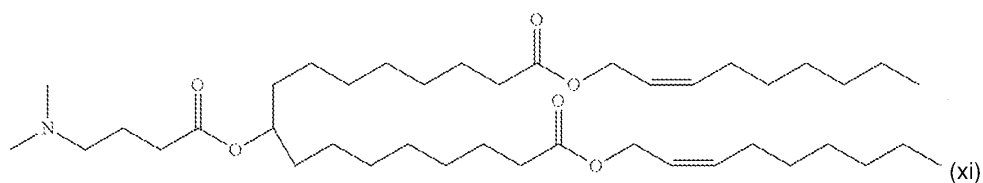
R⁵ is C5-9 alkyl or C6-10 alkoxy, R⁶ is C5-9 alkyl or C6-10 alkoxy, W is methylene or a direct bond, and R⁷ is H or Me, or a salt thereof, provided that if R³ and R² are C2 alkyls, X¹ is O, X² is linear C3 alkylene, X³ is C(=O), Y¹ is linear C₆ alkylene, (Y²)_n-R⁴ is

20



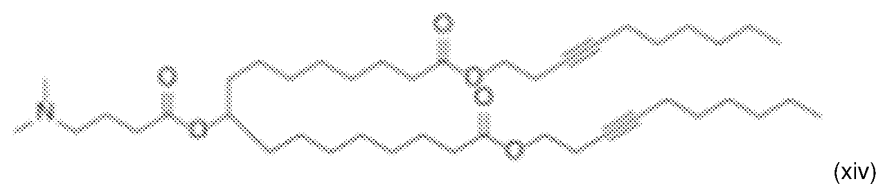
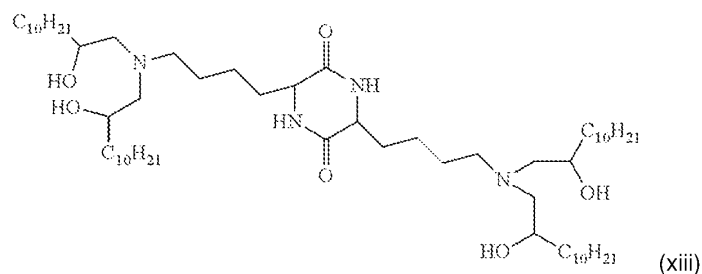
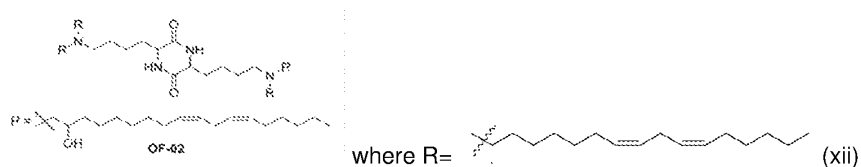
, R⁴ is linear C5 alkyl, Z¹ is C2 alkylene, Z² is absent, W is methylene, and R⁷ is H, then R⁵ and R⁶ are not C_x alkoxy.

In some embodiments an LNP including Formula (xii) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.



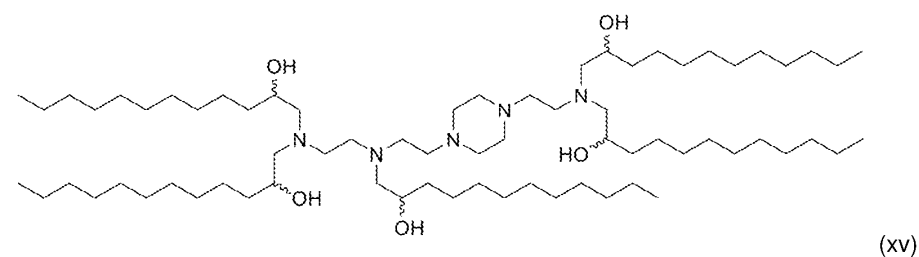
In some embodiments an LNP including Formula (xi) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.

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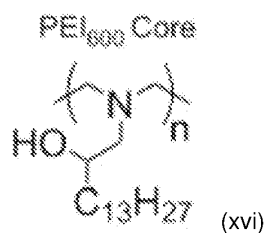
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In some embodiments an LNP includes a compound of Formula (xiii) and a compound of Formula (xiv).



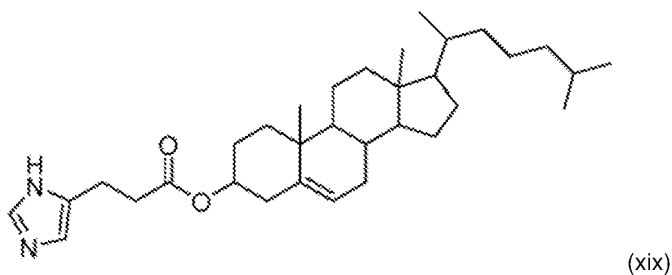
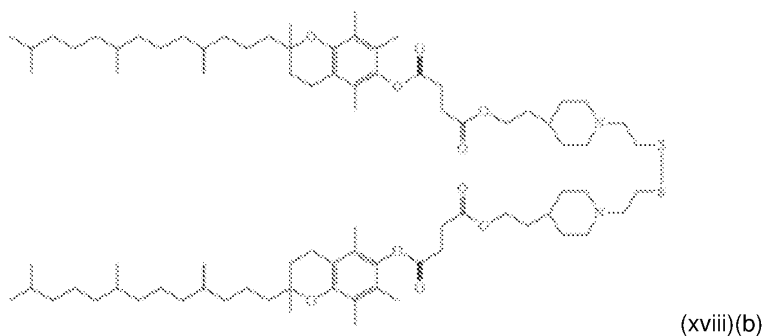
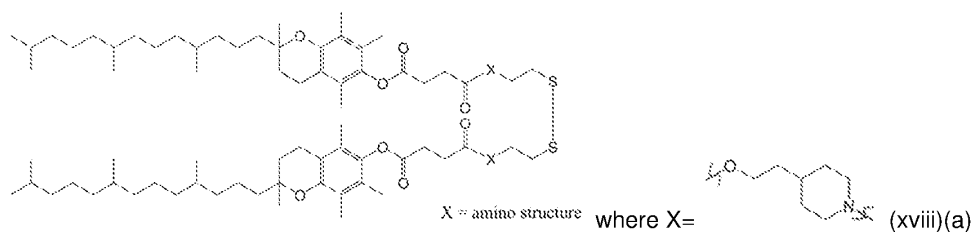
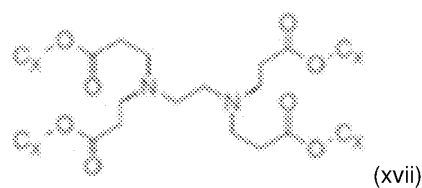
In some embodiments an LNP including Formula (xv) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.

15



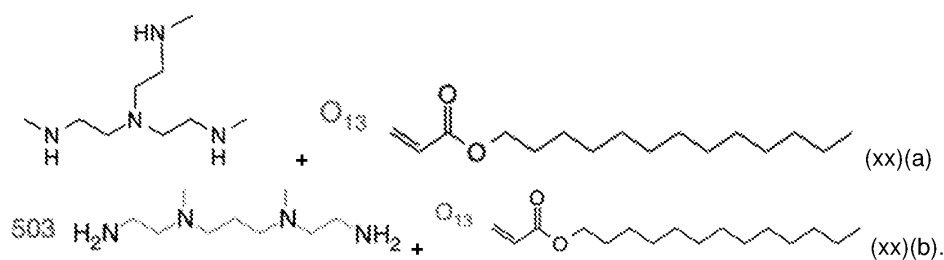
In some embodiments an LNP including a formulation of Formula (xvi) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.

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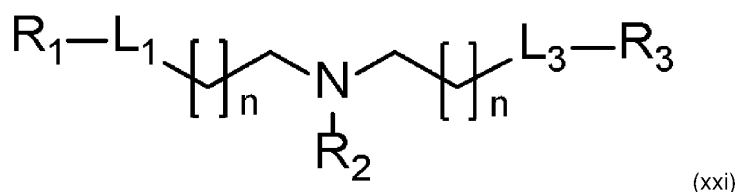


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In some embodiments, a lipid compound used to form lipid nanoparticles for the delivery of compositions described herein, e.g., nucleic acid (e.g., RNA (e.g., circular polyribonucleotide, linear polyribonucleotide)) described herein is made by one of the following reactions:

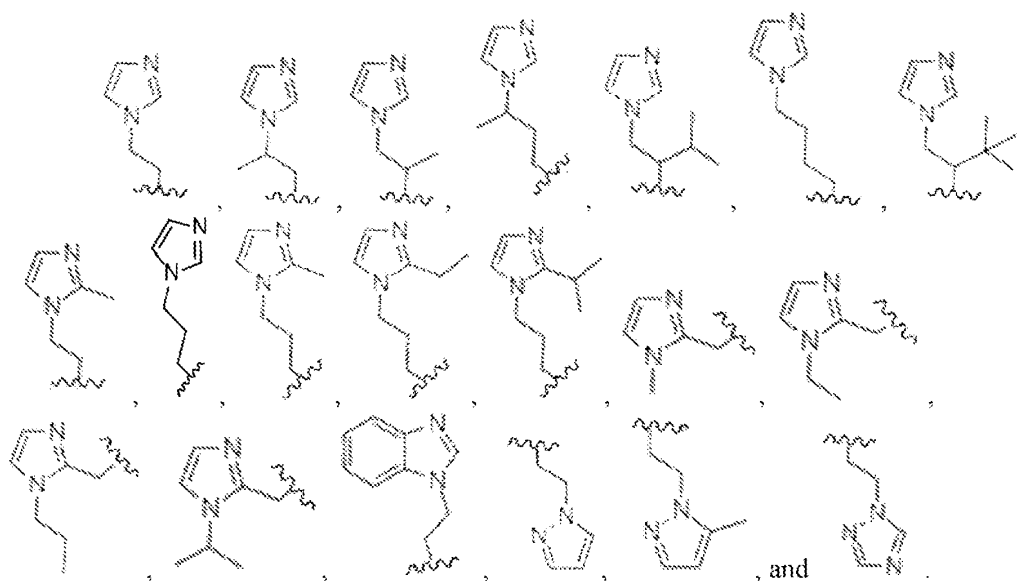


- In some embodiments an LNP including Formula (xxi) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells. In some embodiments the LNP of Formula (xxi) is an LNP described by WO2021113777 (e.g., a lipid of Formula (1) such as a lipid of Table 1 of WO2021113777).



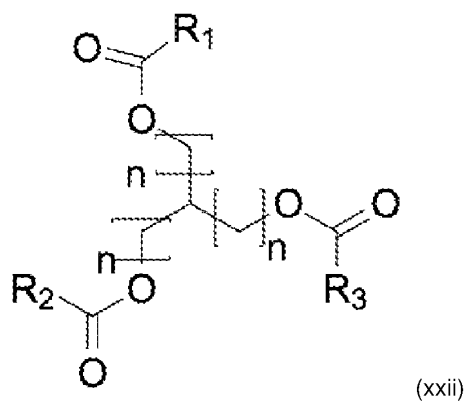
wherein

- each n is independently an integer from 2-15; L₁ and L₃ are each independently -OC(O)-* or -C(O)O-*, wherein "*" indicates the attachment point to R₁ or R₃;
- R₁ and R₃ are each independently a linear or branched C₉-C₂₀ alkyl or C₉-C₂₀ alkenyl, optionally substituted by one or more substituents selected from a group consisting of oxo, halo, hydroxy, cyano, alkyl, alkenyl, aldehyde, heterocyclalkyl, hydroxyalkyl, dihydroxyalkyl, hydroxyalkylaminoalkyl, aminoalkyl, alkylaminoalkyl, dialkylaminoalkyl, (heterocycl)(alkyl)aminoalkyl, heterocycl, heteroaryl, alkylheteroaryl, alkynyl, alkoxy, amino, dialkylamino, aminoalkylcarbonylamino, aminocarbonylalkylamino, (aminocarbonylalkyl)(alkyl)amino, alkenylcarbonylamino, hydroxycarbonyl, alkyloxycarbonyl, aminocarbonyl, aminoalkylaminocarbonyl, alkylaminoalkylaminocarbonyl, dialkylaminoalkylaminocarbonyl, heterocyclalkylaminocarbonyl, (alkylaminoalkyl)(alkyl)aminocarbonyl, alkylaminoalkylcarbonyl, dialkylaminoalkylcarbonyl, heterocyclcarbonyl, alkenylcarbonyl, alkynylcarbonyl, alkylsulfoxide, alkylsulfoxidealkyl, alkyl sulfonyl, and alkyl sulfonealkyl; and
- R₂ is selected from a group consisting of:



In some embodiments an LNP including Formula (xxii) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells. In some embodiments the LNP of Formula (xxii) is an LNP described by WO2021113777 (e.g., a lipid of

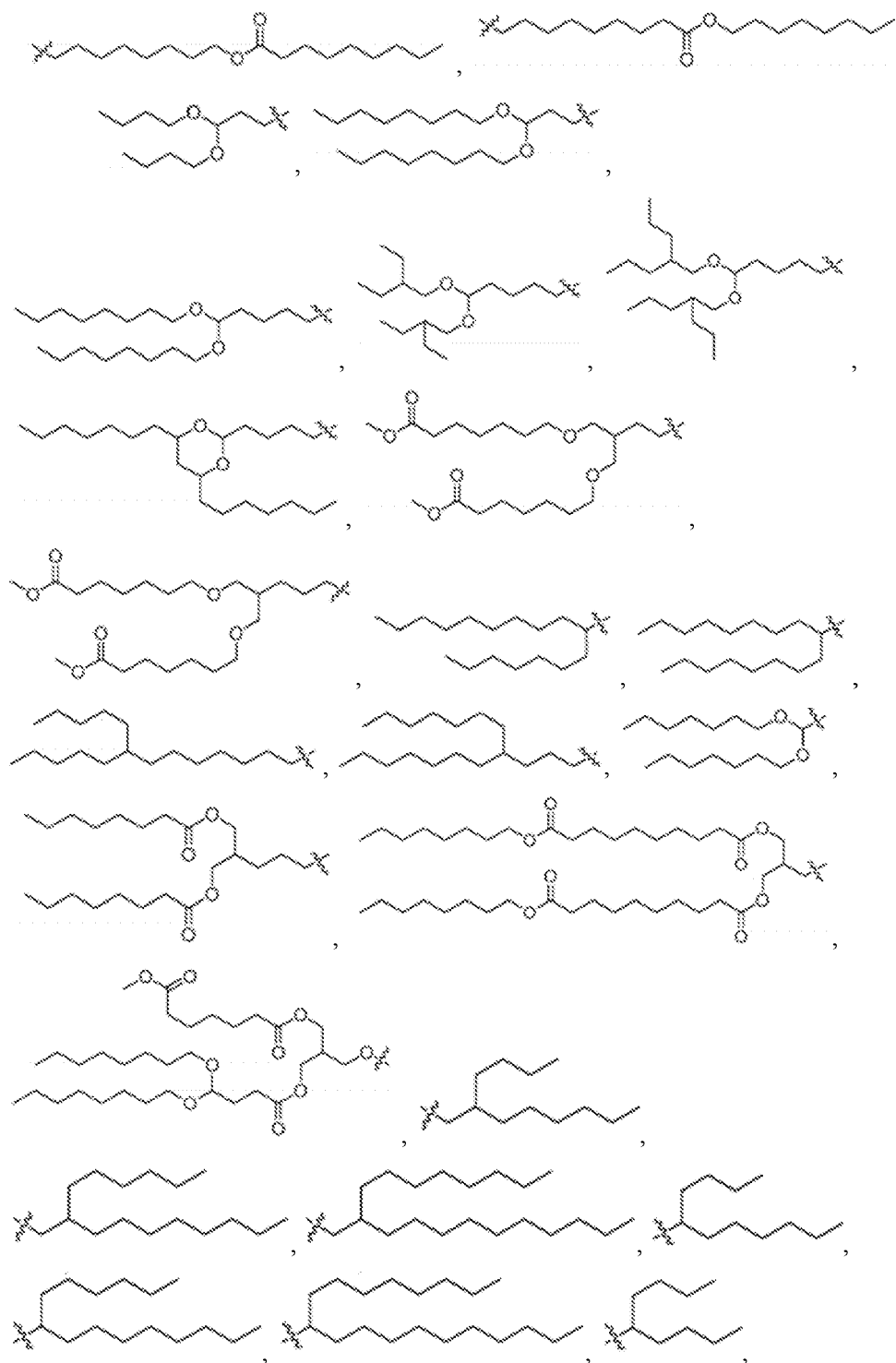
5 Formula (2) such as a lipid of Table 2 of WO2021113777).



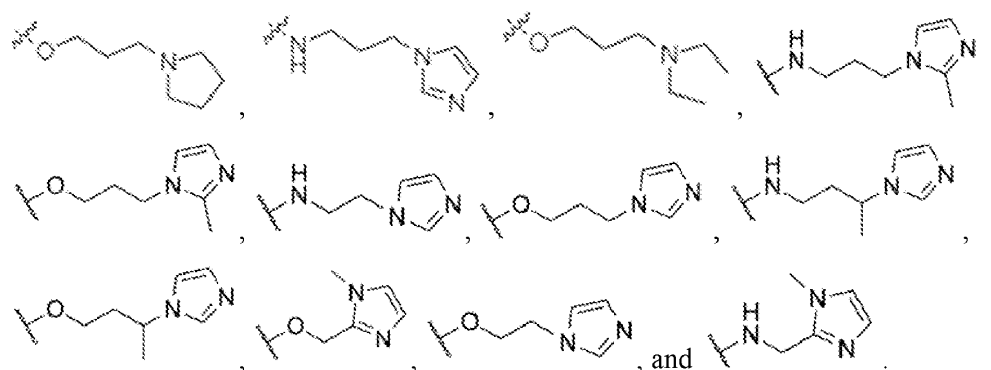
wherein

each n is independently an integer from 1-15;

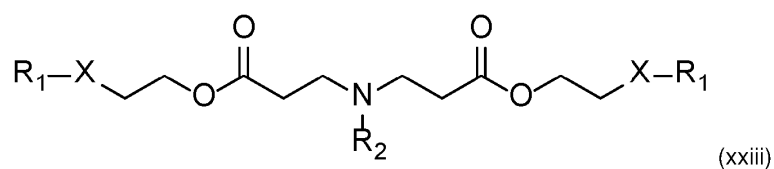
R₁ and R₂ are each independently selected from a group consisting of:



R₃ is selected from a group consisting of:



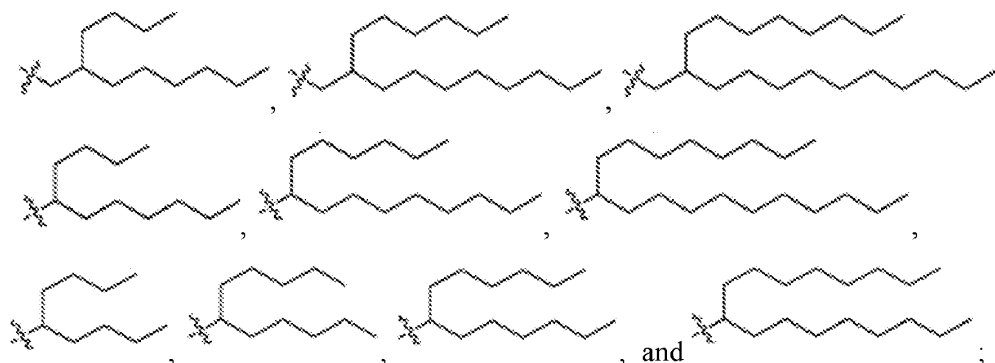
- In some embodiments an LNP including Formula (xxiii) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells. In some embodiments the LNP of Formula (xxiii) is an LNP described by WO2021113777 (e.g., a lipid of Formula (3) such as a lipid of Table 3 of WO2021113777).



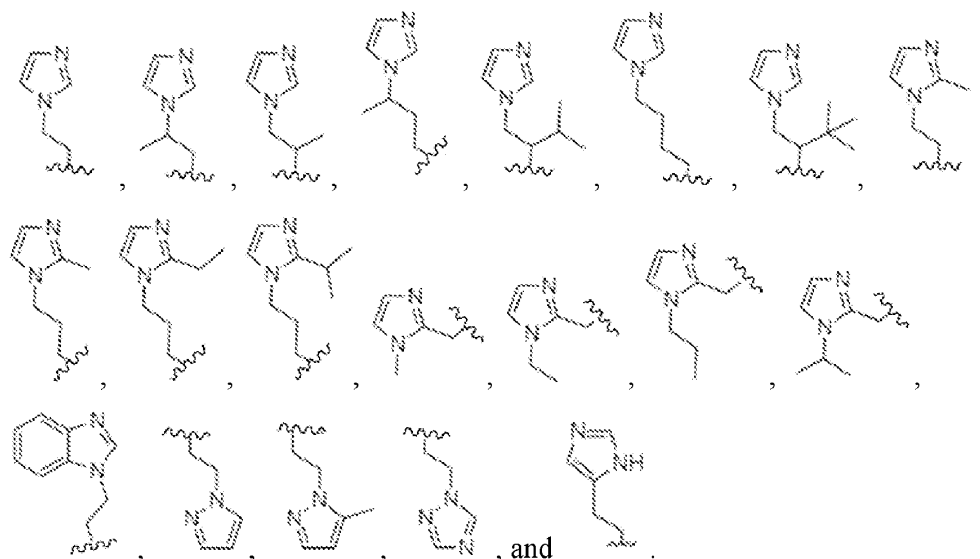
wherein

X is selected from -O-, -S-, or -OC(O)-*, wherein * indicates the attachment point to R₁;

- R₁ is selected from a group consisting of:



and R₂ is selected from a group consisting of:



In some embodiments, a composition described herein (e.g., a nucleic acid (e.g., a circular polyribonucleotide, a linear polyribonucleotide) or a protein) is provided in an LNP that includes an ionizable lipid. In some embodiments, the ionizable lipid is heptadecan-9-yl 8-((2-hydroxyethyl)(6-oxo-6-(undecyloxy)hexyl)amino)octanoate (SM-102); e.g., as described in Example 1 of US9,867,888 (incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is 9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-((((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate (LP01), e.g., as synthesized in Example 13 of WO2015/095340 (incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is Di((Z)-non-2-en-1-yl) 9-((4-dimethylamino)butanoyl)oxy)heptadecanedioate (L319), e.g., as synthesized in Example 7, 8, or 9 of US2012/0027803 (incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is 1,1'-((2-(4-(2-((2-(Bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl) amino)ethyl)piperazin-1-yl)ethyl)azanediyl)bis(dodecan-2-ol) (C12-200), e.g., as synthesized in Examples 14 and 16 of WO2010/053572 (incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is Imidazole cholesterol ester (ICE) lipid (3S, 10R, 13R, 17R)-10, 13-dimethyl-17- ((R)-6-methylheptan-2-yl)-2, 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl 3-(1H-imidazol-4-yl)propanoate, e.g., Structure (I) from WO2020/106946 (incorporated by reference herein in its entirety).

In some embodiments, an ionizable lipid may be a cationic lipid, an ionizable cationic lipid, e.g., a cationic lipid that can exist in a positively charged or neutral form depending on pH, or an amine-containing lipid that can be readily protonated. In some embodiments, the cationic lipid is a lipid capable of being positively charged, e.g., under physiological conditions. Exemplary cationic lipids include one or more amine group(s) which bear the positive charge. In some embodiments, the lipid particle includes a cationic lipid in formulation with one or more of neutral lipids, ionizable amine-containing lipids, biodegradable alkyne lipids, steroids, phospholipids including polyunsaturated lipids, structural lipids (e.g., sterols), PEG, cholesterol, and polymer conjugated lipids. In some embodiments, the cationic lipid may

be an ionizable cationic lipid. An exemplary cationic lipid as disclosed herein may have an effective pKa over 6.0. In embodiments, a lipid nanoparticle may include a second cationic lipid having a different effective pKa (e.g., greater than the first effective pKa), than the first cationic lipid. A lipid nanoparticle may include between 40 and 60 mol percent of a cationic lipid, a neutral lipid, a steroid, a polymer
 5 conjugated lipid, and a therapeutic agent, e.g., a nucleic acid (e.g., RNA (e.g., a circular polyribonucleotide, a linear polyribonucleotide)) described herein, encapsulated within or associated with the lipid nanoparticle. In some embodiments, the nucleic acid is co-formulated with the cationic lipid. The nucleic acid may be adsorbed to the surface of an LNP, e.g., an LNP including a cationic lipid. In some
 10 embodiments, the nucleic acid may be encapsulated in an LNP, e.g., an LNP including a cationic lipid. In some embodiments, the lipid nanoparticle may include a targeting moiety, e.g., coated with a targeting agent. In embodiments, the LNP formulation is biodegradable. In some embodiments, a lipid nanoparticle including one or more lipid described herein, e.g., Formula (i), (ii), (iii), (vii) and/or (ix)
 15 encapsulates at least 1%, at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 92%, at least 95%, at least 97%, at least 98% or 100% of an RNA molecule.

Exemplary ionizable lipids that can be used in lipid nanoparticle formulations include, without limitation, those listed in Table 1 of WO2019051289, incorporated herein by reference. Additional exemplary lipids include, without limitation, one or more of the following formulae: X of US2016/0311759; I of US20150376115 or in US2016/0376224; I, II or III of US20160151284; I, IA, II, or IIA of
 20 US20170210967; I-c of US20150140070; A of US2013/0178541; I of US2013/0303587 or US2013/0123338; I of US2015/0141678; II, III, IV, or V of US2015/0239926; I of US2017/0119904; I or II of WO2017/117528; A of US2012/0149894; A of US2015/0057373; A of WO2013/116126; A of US2013/0090372; A of US2013/0274523; A of US2013/0274504; A of US2013/0053572; A of WO2013/016058; A of WO2012/162210; I of US2008/042973; I, II, III, or IV of US2012/01287670; I or II
 25 of US2014/0200257; I, II, or III of US2015/0203446; I or III of US2015/0005363; I, IA, IB, IC, ID, II, IIA, IIB, IIC, IID, or III-XXIV of US2014/0308304; of US2013/0338210; I, II, III, or IV of WO2009/132131; A of US2012/01011478; I or XXXV of US2012/0027796; XIV or XVII of US2012/0058144; of US2013/0323269; I of US2011/0117125; I, II, or III of US2011/0256175; I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII of US2012/0202871; I, II, III, IV, V, VI, VII, VIII, X, XII, XIII, XIV, XV, or XVI of US2011/0076335; I
 30 or II of US2006/008378; I of US2013/0123338; I or X-A-Y-Z of US2015/0064242; XVI, XVII, or XVIII of US2013/0022649; I, II, or III of US2013/0116307; I, II, or III of US2013/0116307; I or II of US2010/0062967; I-X of US2013/0189351; I of US2014/0039032; V of US2018/0028664; I of US2016/0317458; I of US2013/0195920; 5, 6, or 10 of US10,221,127; III-3 of WO2018/081480; I-5 or I-8 of WO2020/081938; 18 or 25 of US9,867,888; A of US2019/0136231; II of WO2020/219876; 1 of
 35 US2012/0027803; OF-02 of US2019/0240349; 23 of US10,086,013; cKK-E12/A6 of Miao et al (2020); C12-200 of WO2010/053572; 7C1 of Dahlman et al (2017); 304-O13 or 503-O13 of Whitehead et al; TS-P4C2 of US9,708,628; I of WO2020/106946; I of WO2020/106946; and (1), (2), (3), or (4) of WO2021/113777. Exemplary lipids further include a lipid of any one of Tables 1-16 of WO2021/113777.

In some embodiments, the ionizable lipid is MC3 (6Z,9Z,28Z,3 IZ)-heptatriaconta- 6,9,28,3 I-tetraen-19-yl-4-(dimethylamino) butanoate (DLin-MC3-DMA or MC3), e.g., as described in Example 9 of
 40 WO2019051289A9 (incorporated by reference herein in its entirety). In some embodiments, the ionizable

lipid is the lipid ATX-002, e.g., as described in Example 10 of WO2019051289A9 (incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is (I3Z,I6Z)-A,A-dimethyl-3-nonyldocosa-I3, I6-dien-I-amine (Compound 32), e.g., as described in Example 11 of WO2019051289A9 (incorporated by reference herein in its entirety). In some embodiments, the ionizable lipid is Compound 6 or Compound 22, e.g., as described in Example 12 of WO2019051289A9 (incorporated by reference herein in its entirety).

Exemplary non-cationic lipids include, but are not limited to, distearoyl-sn-glycero-phosphoethanolamine, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine (such as 16-O-monomethyl PE), dimethyl-phosphatidylethanolamine (such as 16-O-dimethyl PE), 18-1-trans PE, 1-stearoyl-2-oleoyl-phosphatidylethanolamine (SOPE), hydrogenated soy phosphatidylcholine (HSPC), egg phosphatidylcholine (EPC), dioleoylphosphatidylserine (DOPS), sphingomyelin (SM), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), distearoylphosphatidylglycerol (DSPG), dierycylphosphatidylcholine (DEPC), palmitoyloleoylphosphatidylglycerol (POPG), dielaidoyl-phosphatidylethanolamine (DEPE), lecithin, phosphatidylethanolamine, lysolecithin, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebrosides, dicetylphosphate, lysophosphatidylcholine, dilinoleoylphosphatidylcholine, or mixtures thereof. It is understood that other diacylphosphatidylcholine and diacylphosphatidylethanolamine phospholipids can also be used. The acyl groups in these lipids are preferably acyl groups derived from fatty acids having C10-C24 carbon chains, e.g., lauroyl, myristoyl, palmitoyl, stearoyl, or oleoyl. Additional exemplary lipids, in certain embodiments, include, without limitation, those described in Kim et al. (2020) [dx.doi.org/10.1021/acs.nanolett.0c01386](https://doi.org/10.1021/acs.nanolett.0c01386), incorporated herein by reference. Such lipids include, in some embodiments, plant lipids found to improve liver transfection with mRNA (e.g., DGTS).

Other examples of non-cationic lipids suitable for use in the lipid nanoparticles include, without limitation, nonphosphorous lipids such as, e.g., stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerol ricinoleate, hexadecyl stearate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethyloxylated fatty acid amides, dioctadecyl dimethyl ammonium bromide, ceramide, sphingomyelin, and the like. Other non-cationic lipids are described in WO2017/099823 or US patent publication US2018/0028664, the contents of which is incorporated herein by reference in their entirety.

In some embodiments, the non-cationic lipid is oleic acid or a compound of Formula I, II, or IV of US2018/0028664, incorporated herein by reference in its entirety. The non-cationic lipid can include, for example, 0-30% (mol) of the total lipid present in the lipid nanoparticle. In some embodiments, the non-cationic lipid content is 5-20% (mol) or 10-15% (mol) of the total lipid present in the lipid nanoparticle. In

embodiments, the molar ratio of ionizable lipid to the neutral lipid ranges from about 2:1 to about 8:1 (e.g., about 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, or 8:1).

In some embodiments, the lipid nanoparticles do not include any phospholipids.

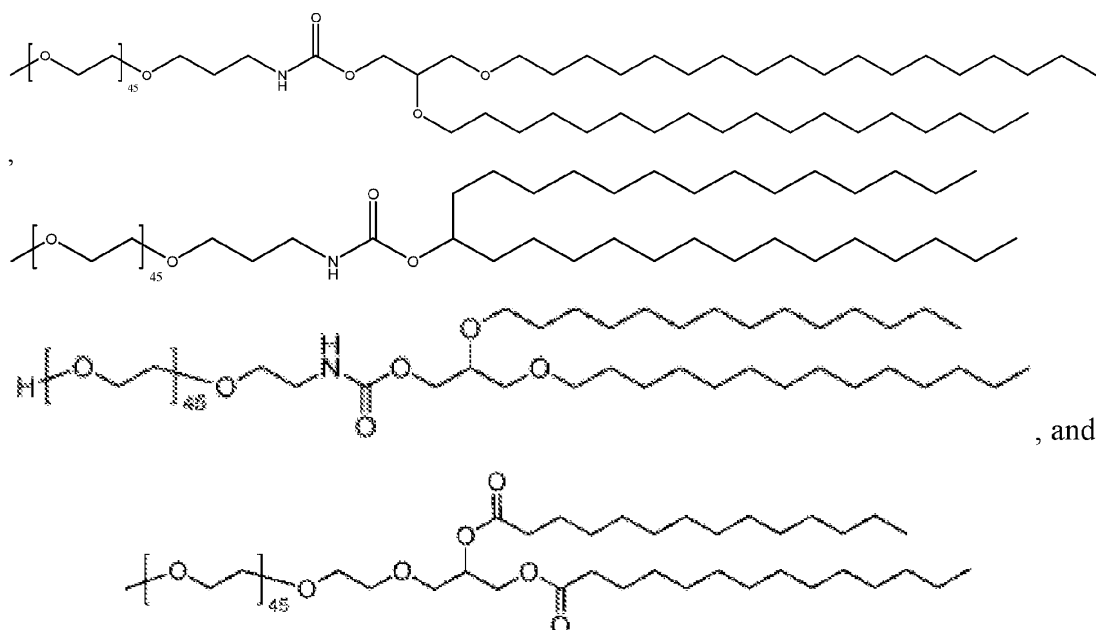
In some aspects, the lipid nanoparticle can further include a component, such as a sterol, to provide membrane integrity. One exemplary sterol that can be used in the lipid nanoparticle is cholesterol and derivatives thereof. Non-limiting examples of cholesterol derivatives include polar analogues such as 5 α -cholestanol, 5 β -coprostanol, cholesteryl-(2'-hydroxy)-ethyl ether, cholesteryl-(4'-hydroxy)-butyl ether, and 6-ketocholestanol; non-polar analogues such as 5 α -cholestane, cholestenone, 5 α -cholestanone, 5 β -cholestanone, and cholesteryl decanoate; and mixtures thereof. In some embodiments, the cholesterol derivative is a polar analogue, e.g., cholesteryl-(4'-hydroxy)-butyl ether. Exemplary cholesterol derivatives are described in PCT publication WO2009/127060 and US patent publication US2010/0130588, each of which is incorporated herein by reference in its entirety.

In some embodiments, the component providing membrane integrity, such as a sterol, can include 0-50% (mol) (e.g., 0-10%, 10-20%, 20-30%, 30-40%, or 40-50%) of the total lipid present in the lipid nanoparticle. In some embodiments, such a component is 20-50% (mol) 30-40% (mol) of the total lipid content of the lipid nanoparticle.

In some embodiments, the lipid nanoparticle can include a polyethylene glycol (PEG) or a conjugated lipid molecule. Generally, these are used to inhibit aggregation of lipid nanoparticles and/or provide steric stabilization. Exemplary conjugated lipids include, but are not limited to, PEG-lipid conjugates, polyoxazoline (POZ)-lipid conjugates, polyamide-lipid conjugates (such as ATTA-lipid conjugates), cationic-polymer lipid (CPL) conjugates, and mixtures thereof. In some embodiments, the conjugated lipid molecule is a PEG-lipid conjugate, for example, a (methoxy polyethylene glycol)-conjugated lipid.

Exemplary PEG-lipid conjugates include, but are not limited to, PEG-diacylglycerol (DAG) (such as 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG)), PEG-dialkylloxypropyl (DAA), PEG-phospholipid, PEG-ceramide (Cer), a pegylated phosphatidylethanolamine (PEG-PE), PEG succinate diacylglycerol (PEGS-DAG) (such as 4-O-(2',3'-di(tetradecanoyloxy)propyl)-1-O-(w-methoxy(polyethoxy)ethyl) butanedioate (PEG-S-DMG)), PEG dialkoxypolypropylcarbam, N-(carbonyl-methoxypolyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine sodium salt, or a mixture thereof. Additional exemplary PEG-lipid conjugates are described, for example, in US5,885,613, US6,287,591, US2003/0077829, US2003/0077829, US2005/0175682, US2008/0020058, US2011/0117125, US2010/0130588, US2016/0376224, US2017/0119904, US2018/0028664, and WO2017/099823, the contents of all of which are incorporated herein by reference in their entirety. In some embodiments, a PEG-lipid is a compound of Formula III, III-a-1, III-a-2, III-b-1, III-b-2, or V of US2018/0028664, the content of which is incorporated herein by reference in its entirety. In some embodiments, a PEG-lipid is of Formula II of US20150376115 or US2016/0376224, the content of both of which is incorporated herein by reference in its entirety. In some embodiments, the PEG-DAA conjugate can be, for example, PEG-dilauryloxypropyl, PEG-dimyristyloxypropyl, PEG-dipalmitoyloxypropyl, or PEG-distearoyloxypropyl. The PEG-lipid can be one or more of PEG-DMG, PEG-dilaurylglycerol, PEG-dipalmitoylglycerol, PEG-disteryl-glycerol, PEG-dilaurylglycamide, PEG-dimyristylglycamide, PEG-dipalmitoylglycamide, PEG-disteryl-glycamide, PEG-cholesterol (1-[8'-(Cholest-5-en-3[β]-

oxy)carboxamido-3',6'-dioxaoctanyl] carbamoyl-[omega]-methyl-poly(ethylene glycol), PEG- DMB (3,4-Ditetradecoxybenzyl- [omega]-methyl-poly(ethylene glycol) ether), and 1,2- dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]. In some embodiments, the PEG-lipid includes PEG-DMG, 1,2- dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]. In some embodiments, the PEG-lipid includes a structure selected from:



In some embodiments, lipids conjugated with a molecule other than a PEG can also be used in place of PEG-lipid. For example, polyoxazoline (POZ)-lipid conjugates, polyamide-lipid conjugates (such as ATTA-lipid conjugates), and cationic-polymer lipid (GPL) conjugates can be used in place of or in addition to the PEG-lipid.

Exemplary conjugated lipids, i.e., PEG-lipids, (POZ)-lipid conjugates, ATTA-lipid conjugates and cationic polymer-lipids are described in the PCT and LIS patent applications listed in Table 2 of WO2019051289A9, the contents of all of which are incorporated herein by reference in their entirety.

In some embodiments, the PEG or the conjugated lipid can include 0-20% (mol) of the total lipid present in the lipid nanoparticle. In some embodiments, PEG or the conjugated lipid content is 0.5- 10% or 2-5% (mol) of the total lipid present in the lipid nanoparticle. Molar ratios of the ionizable lipid, non-cationic-lipid, sterol, and PEG-conjugated lipid can be varied as needed. For example, the lipid particle can include 30-70% ionizable lipid by mole or by total weight of the composition, 0-60% cholesterol by mole or by total weight of the composition, 0-30% non-cationic lipid by mole or by total weight of the composition and 1-10% conjugated lipid by mole or by total weight of the composition. Preferably, the composition includes 30-40% ionizable lipid by mole or by total weight of the composition, 40-50% cholesterol by mole or by total weight of the composition, and 10- 20% non-cationic-lipid by mole or by total weight of the composition. In some other embodiments, the composition is 50-75% ionizable lipid by mole or by total weight of the composition, 20-40% cholesterol by mole or by total weight of the composition, and 5 to 10% non-cationic lipid, by mole or by total weight of the composition and 1-10% conjugated lipid by mole or by total weight of the composition. The composition may contain 60-70%

ionizable lipid by mole or by total weight of the composition, 25-35% cholesterol by mole or by total weight of the composition, and 5-10% non-cationic lipid by mole or by total weight of the composition. The composition may also contain up to 90% ionizable lipid by mole or by total weight of the composition and 2 to 15% non-cationic lipid by mole or by total weight of the composition. The formulation may also be a lipid nanoparticle formulation, for example including 8-30% ionizable lipid by mole or by total weight of the composition, 5-30% non-cationic lipid by mole or by total weight of the composition, and 0-20% cholesterol by mole or by total weight of the composition; 4-25% ionizable lipid by mole or by total weight of the composition, 4-25% non-cationic lipid by mole or by total weight of the composition, 2 to 25% cholesterol by mole or by total weight of the composition, 10 to 35% conjugate lipid by mole or by total weight of the composition, and 5% cholesterol by mole or by total weight of the composition; or 2-30% ionizable lipid by mole or by total weight of the composition, 2-30% non-cationic lipid by mole or by total weight of the composition, 1 to 15% cholesterol by mole or by total weight of the composition, 2 to 35% conjugate lipid by mole or by total weight of the composition, and 1-20% cholesterol by mole or by total weight of the composition; or even up to 90% ionizable lipid by mole or by total weight of the composition and 2-10% non-cationic lipids by mole or by total weight of the composition, or even 100% cationic lipid by mole or by total weight of the composition. In some embodiments, the lipid particle formulation includes ionizable lipid, phospholipid, cholesterol and a PEG-ylated lipid in a molar ratio of 50:10:38.5:1.5. In some other embodiments, the lipid particle formulation includes ionizable lipid, cholesterol and a PEG-ylated lipid in a molar ratio of 60:38.5: 1.5.

In some embodiments, the lipid particle includes ionizable lipid, non-cationic lipid (e.g., phospholipid), a sterol (e.g., cholesterol) and a PEG-ylated lipid, where the molar ratio of lipids ranges from 20 to 70 mole percent for the ionizable lipid, with a target of 40-60, the mole percent of non-cationic lipid ranges from 0 to 30, with a target of 0 to 15, the mole percent of sterol ranges from 20 to 70, with a target of 30 to 50, and the mole percent of PEG-ylated lipid ranges from 1 to 6, with a target of 2 to 5.

In some embodiments, the lipid particle includes ionizable lipid / non-cationic- lipid / sterol / conjugated lipid at a molar ratio of 50:10:38.5:1.5.

In an aspect, the disclosure provides a lipid nanoparticle formulation including phospholipids, lecithin, phosphatidylcholine and phosphatidylethanolamine.

In some embodiments, one or more additional compounds can also be included. Those compounds can be administered separately, or the additional compounds can be included in the lipid nanoparticles of the invention. In other words, the lipid nanoparticles can contain other compounds in addition to the nucleic acid or at least a second nucleic acid, different than the first. Without limitations, other additional compounds can be selected from the group consisting of small or large organic or inorganic molecules, monosaccharides, disaccharides, trisaccharides, oligosaccharides, polysaccharides, peptides, proteins, peptide analogs and derivatives thereof, peptidomimetics, nucleic acids, nucleic acid analogs and derivatives, an extract made from biological materials, or any combinations thereof.

In some embodiments, the LNPs include biodegradable, ionizable lipids. In some embodiments, the LNPs include (9Z,12Z)-3-((4,4-bis(octyloxy)butanoyl)oxy)-2-(((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl octadeca-9,12-dienoate, also called 3-((4,4-bis(octyloxy)butanoyl)oxy)-2-(((3-(diethylamino)propoxy)carbonyl)oxy)methyl)propyl (9Z,12Z)-octadeca-9,12-dienoate) or another ionizable lipid. See, e.g., lipids of WO2019/067992, WO/2017/173054,

WO2015/095340, and WO2014/136086, as well as references provided therein. In some embodiments, the term cationic and ionizable in the context of LNP lipids is interchangeable, e.g., wherein ionizable lipids are cationic depending on the pH.

In some embodiments, the average LNP diameter of the LNP formulation may be between 10s of nm and 100s of nm, e.g., measured by dynamic light scattering (DLS). In some embodiments, the average LNP diameter of the LNP formulation may be from about 40 nm to about 150 nm, such as about 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm. In some embodiments, the average LNP diameter of the LNP formulation may be from about 50 nm to about 100 nm, from about 50 nm to about 90 nm, from about 50 nm to about 80 nm, from about 50 nm to about 70 nm, from about 50 nm to about 60 nm, from about 60 nm to about 100 nm, from about 60 nm to about 90 nm, from about 60 nm to about 80 nm, from about 60 nm to about 70 nm, from about 70 nm to about 100 nm, from about 70 nm to about 90 nm, from about 70 nm to about 80 nm, from about 80 nm to about 100 nm, from about 80 nm to about 90 nm, or from about 90 nm to about 100 nm. In some embodiments, the average LNP diameter of the LNP formulation may be from about 70 nm to about 100 nm. In a particular embodiment, the average LNP diameter of the LNP formulation may be about 80 nm. In some embodiments, the average LNP diameter of the LNP formulation may be about 100 nm. In some embodiments, the average LNP diameter of the LNP formulation ranges from about 1 mm to about 500 mm, from about 5 mm to about 200 mm, from about 10 mm to about 100 mm, from about 20 mm to about 80 mm, from about 25 mm to about 60 mm, from about 30 mm to about 55 mm, from about 35 mm to about 50 mm, or from about 38 mm to about 42 mm.

A LNP may, in some instances, be relatively homogenous. A polydispersity index may be used to indicate the homogeneity of a LNP, e.g., the particle size distribution of the lipid nanoparticles. A small (e.g., less than 0.3) polydispersity index generally indicates a narrow particle size distribution. A LNP may have a polydispersity index from about 0 to about 0.25, such as 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.20, 0.21, 0.22, 0.23, 0.24, or 0.25. In some embodiments, the polydispersity index of a LNP may be from about 0.10 to about 0.20.

The zeta potential of an LNP may be used to indicate the electrokinetic potential of the composition. In some embodiments, the zeta potential may describe the surface charge of an LNP. Lipid nanoparticles with relatively low charges, positive or negative, are generally desirable, as more highly charged species may interact undesirably with cells, tissues, and other elements in the body. In some embodiments, the zeta potential of a LNP may be from about -10 mV to about +20 mV, from about -10 mV to about +15 mV, from about -10 mV to about +10 mV, from about -10 mV to about +5 mV, from about -10 mV to about 0 mV, from about -10 mV to about -5 mV, from about -5 mV to about +20 mV, from about -5 mV to about +15 mV, from about -5 mV to about +10 mV, from about -5 mV to about +5 mV, from about -5 mV to about 0 mV, from about 0 mV to about +20 mV, from about 0 mV to about +15 mV, from about 0 mV to about +10 mV, from about 0 mV to about +5 mV, from about +5 mV to about +20 mV, from about +5 mV to about +15 mV, or from about +5 mV to about +10 mV.

The efficiency of encapsulation of a protein and/or nucleic acid describes the amount of protein and/or nucleic acid that is encapsulated or otherwise associated with a LNP after preparation, relative to the initial amount provided. The encapsulation efficiency is desirably high (e.g., close to 100%). The

encapsulation efficiency may be measured, for example, by comparing the amount of protein or nucleic acid in a solution containing the lipid nanoparticle before and after breaking up the lipid nanoparticle with one or more organic solvents or detergents. An anion exchange resin may be used to measure the amount of free protein or nucleic acid (e.g., RNA) in a solution. Fluorescence may be used to measure the amount of free protein and/or nucleic acid (e.g., RNA) in a solution. For the lipid nanoparticles described herein, the encapsulation efficiency of a protein and/or nucleic acid may be at least 50%, for example 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%. In some embodiments, the encapsulation efficiency may be at least 80%. In some embodiments, the encapsulation efficiency may be at least 90%. In some embodiments, the encapsulation efficiency may be at least 95%.

A LNP may optionally include one or more coatings. In some embodiments, a LNP may be formulated in a capsule, film, or tablet having a coating. A capsule, film, or tablet including a composition described herein may have any useful size, tensile strength, hardness or density.

Additional exemplary lipids, formulations, methods, and characterization of LNPs are taught by WO2020/061457 and WO2021/113777, each of which is incorporated herein by reference in its entirety. Further exemplary lipids, formulations, methods, and characterization of LNPs are taught by Hou et al. Lipid nanoparticles for mRNA delivery. *Nat Rev Mater* (2021). doi.org/10.1038/s41578-021-00358-0, which is incorporated herein by reference in its entirety (see, for example, exemplary lipids and lipid derivatives of Figure 2 of Hou et al.).

In some embodiments, in vitro or ex vivo cell lipofections are performed using Lipofectamine MessengerMax (Thermo Fisher) or TransIT-mRNA Transfection Reagent (Mirus Bio). In certain embodiments, LNPs are formulated using the GenVoy_ILM ionizable lipid mix (Precision NanoSystems). In certain embodiments, LNPs are formulated using 2,2-dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (DLin-KC2-DMA) or dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA or MC3), the formulation and in vivo use of which are taught in Jayaraman et al. *Angew Chem Int Ed Engl* 51(34):8529-8533 (2012), incorporated herein by reference in its entirety.

LNP formulations optimized for the delivery of CRISPR-Cas systems, e.g., Cas9-gRNA RNP, gRNA, Cas9 mRNA, are described in WO2019067992 and WO2019067910, both incorporated by reference, and are useful for delivery of circular polyribonucleotides and linear polyribonucleotides described herein.

Additional specific LNP formulations useful for delivery of nucleic acids (e.g., circular polyribonucleotides, linear polyribonucleotides) are described in US8158601 and US8168775, both incorporated by reference, which include formulations used in patisiran, sold under the name ONPATTRO.

In embodiments, a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) encoding at least a portion (e.g., an antigenic portion) of a protein or polypeptide described herein is formulated in an LNP, wherein: (a) the LNPs comprise a cationic lipid, a neutral lipid, a cholesterol, and a PEG lipid, (b) the LNPs have a mean particle size of between 80 nm and 160 nm, and (c) the polyribonucleotide. In embodiments, the polyribonucleotide (e.g., circular polyribonucleotide, linear polyribonucleotide) formulated in an LNP is a vaccine.

Exemplary dosing of polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) LNP may include about 0.1, 0.25, 0.3, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, or 100 mg/kg (RNA). In some embodiments, a dose of a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) antigenic composition described herein is between 30-200 mcg, e.g., 30 mcg, 50 mcg, 75 mcg, 100 mcg, 150 mcg, or 200 mcg.

Kits

In some aspects, the disclosure provides a kit. In some embodiments, the kit includes (a) a circular polyribonucleotide encoding an antifusogenic polypeptide (e.g., a polypeptide of Table 1) or a pharmaceutical composition described herein, and optionally (b) informational material. The informational material may be descriptive, instructional, marketing or other material that relates to the methods described herein and/or the use of the pharmaceutical composition or circular polyribonucleotide for the methods described herein. The pharmaceutical composition or circular polyribonucleotide may include material for a single administration (e.g., single dosage form), or may include material for multiple administrations (e.g., a "multidose" kit).

The informational material of the kits is not limited in its form. In one embodiment, the informational material may include information about production of the pharmaceutical composition, the pharmaceutical drug substance, or the pharmaceutical drug product, molecular weight of the pharmaceutical composition, the pharmaceutical drug substance, or the pharmaceutical drug product, concentration, date of expiration, batch or production site information, and so forth. In one embodiment, the informational material relates to methods for administering a dosage form of the pharmaceutical composition. In one embodiment, the informational material relates to methods for administering a dosage form of the circular polyribonucleotide.

In addition to a dosage form of the pharmaceutical composition and circular polyribonucleotide described herein, the kit may include other ingredients, such as a solvent or buffer, a stabilizer, a preservative, a flavoring agent (e.g., a bitter antagonist or a sweetener), a fragrance, a dye or coloring agent, for example, to tint or color one or more components in the kit, or other cosmetic ingredient, and/or a second agent for treating a condition or disorder described herein. Alternatively, the other ingredients may be included in the kit, but in different compositions or containers than a pharmaceutical composition or circular polyribonucleotide described herein. In such embodiments, the kit may include instructions for admixing a pharmaceutical composition or nucleic acid molecule (e.g., a circular polyribonucleotide) described herein and the other ingredients, or for using a pharmaceutical composition or nucleic acid molecule (e.g., a circular polyribonucleotide) described herein together with the other ingredients.

In some embodiments, the components of the kit are stored under inert conditions (e.g., under Nitrogen or another inert gas such as Argon). In some embodiments, the components of the kit are stored under anhydrous conditions (e.g., with a desiccant). In some embodiments, the components are stored in a light blocking container such as an amber vial.

A dosage form of a pharmaceutical composition or nucleic acid molecule (e.g., a circular polyribonucleotide) described herein may be provided in any form, e.g., liquid, dried or lyophilized form. It is preferred that a pharmaceutical composition or nucleic acid molecule (e.g., a circular polyribonucleotide) described herein be substantially pure and/or sterile. When a pharmaceutical

composition or nucleic acid molecule (e.g., a circular polyribonucleotide) described herein is provided in a liquid solution, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being preferred. When a pharmaceutical composition or nucleic acid molecule (e.g., a circular polyribonucleotide) described herein is provided as a dried form, reconstitution generally is by the addition of a suitable solvent. The solvent, e.g., sterile water or buffer, can optionally be provided in the kit.

The kit may include one or more containers for the composition containing a dosage form described herein. In some embodiments, the kit contains separate containers, dividers or compartments for the composition and informational material. For example, the pharmaceutical composition or circular polyribonucleotide may be contained in a bottle, vial, or syringe, and the informational material may be contained in a plastic sleeve or packet. In other embodiments, the separate elements of the kit are contained within a single, undivided container. For example, the dosage form of a pharmaceutical composition or nucleic acid molecule (e.g., a circular polyribonucleotide) described herein is contained in a bottle, vial or syringe that has attached thereto the informational material in the form of a label. In some embodiments, the kit includes a plurality (e.g., a pack) of individual containers, each containing one or more unit dosage forms of a pharmaceutical composition or circular polyribonucleotide described herein. For example, the kit includes a plurality of syringes, ampules, foil packets, or blister packs, each containing a single unit dose of a dosage form described herein.

The containers of the kits can be airtight, waterproof (e.g., impermeable to changes in moisture or evaporation), and/or light-tight.

The kit optionally includes a device suitable for use of the dosage form, e.g., a syringe, pipette, forceps, measured spoon, swab (e.g., a cotton swab or wooden swab), or any such device.

The kits of the invention may include dosage forms of varying strengths to provide a subject with doses suitable for one or more of the initiation phase regimens, induction phase regimens, or maintenance phase regimens described herein. Alternatively, the kit may include a scored tablet to allow the user to administer divided doses, as needed.

Examples

The following examples are put forth so as to provide those of ordinary skill in the art with a description of how the compositions and methods described herein may be used, made, and evaluated, and are intended to be purely exemplary of the disclosure and are not intended to limit the scope of what the inventors regard as their invention.

Example 1: Expression of antifusogenic polypeptides from RNA in mammalian cells

This example demonstrates expression of one or more open reading frames (ORFs) encoding one or more OC43-HR2P and EK1 peptides in Huh-7 cells.

In this Example, circular RNAs encoding one OC43-HR2P peptide (SEQ ID NO: 289), one EK1 peptide (SEQ ID NO: 288), multiple OC43-HR2P peptides, multiple EK1 peptides, and a combination of OC43-HR2P peptides, peptide analogs, and EK1 peptides are designed. Circular RNAs are designed to include an IRES, a secretion signal, a furin site, one or more OC43-HR2P peptides, analogs, and/or EK1 sequences, and a spacer element. The circular RNAs are transfected into Huh-7 cells using Lipofectamine MessengerMax (Invitrogen LMRNA001) according to the manufacturer's instructions.

In one study, peptide expression is monitored in vitro over a time course.

Example 2: Inhibition of MERS-CoV S protein-mediated cell–cell fusion

To determine inhibition of MERS-CoV fusion with the 293T target cell, a MERS-CoV S protein-mediated cell–cell fusion assay is used. This example uses 293T cells that are transfected with the plasmid pAAV-IRES-EGFP encoding the EGFP (293T/EGFP) or pAAV-IRES-MERS-EGFP encoding the MERS-CoV S protein and EGFP (293T/MERS/EGFP) and cultured in DMEM containing 10% FBS at 37 °C for 48 h. Huh-7 cells (5×10^4) expressing the MERS-CoV receptor DPP4, prepared according to Example 1, are incubated in 96-well plates at 37 °C for 5 h, followed by the addition of 1×10^4 293T/EGFP or 293T/MERS/EGFP cells, respectively.

After co-culture at 37 °C for 4 h, the 293T/MERS/EGFP cells (293T/EGFP cells are used as the negative control) fused or unfused with Huh-7 cells are counted under an inverted fluorescence microscope (Nikon Eclipse Ti-S). The fused cell is seen as one that is 2-fold or more larger than the unfused cell, and the differences of intensity of fluorescence in the fused cell is compared to that of the unfused cell. The percent inhibition of cell–cell fusion can be calculated using the following formula: $(1 - (E - N) / (P - N)) \times 100$. 'E' represents the % cell–cell fusion in the experimental group. 'P' represents the % cell–cell fusion in the positive control group, to which no circRNA was added. 'N' is the % cell–cell fusion in negative control group, in which 293T/MERS/EGFP cells are replaced by 293T/EGFP cells. The concentration for 50% inhibition (IC_{50}) can be calculated using the CalcuSyn software. Co-culture can continue at 37 °C for 48 h and measurements may be taken, for example of syncytium formation. In-cell S protein ELISA can be adapted to measure antiviral activities two days following viral challenge.

Example 3: Inhibition of pseudotyped SARS-CoV-2 and MERS-CoV infection

SARS or MERS pseudovirus bearing SARS-CoV-2 or MERS-CoV S protein, respectively, and a defective HIV-1 genome that expresses luciferase as reporter are prepared by co-transfecting 293T cells with the plasmid pNL4-3.luc.RE (encoding Env-defective, luciferase-expressing HIV-1) and pcDNA3.1-MERS-CoV-S plasmid. To detect the inhibitory activity of the expressed peptides on infection by SARS or MERS pseudovirus, ACE2-transfected 293T (293T/ACE2) cells and Huh-7 cells (10^4 per well in 96-well plates) that have and have not been transfected with circRNAs of the present invention are respectively infected with SARS or MERS-CoV pseudovirus. Following infection, the culture is re-fed with fresh medium 12 h post-infection and incubated for an additional 72 h. Cells are washed with PBS and lysed using lysis reagent included in a luciferase kit (Promega). Aliquots of cell lysates are transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar), followed by the addition of luciferase substrate (Promega). Relative light units are determined immediately in the Ultra 384 luminometer (Tecan US).

Example 4: Expression of SARS-CoV-2 antifusogenic polypeptides

This example demonstrates expression of SARS-CoV-2 antifusogenic polypeptides from circular RNAs. Several SARS-CoV-2 antifusogenic polypeptides were designed (FIGS. 2 and 3) based on the HR2 region show in FIG. 1. Circular RNAs were designed to include an IRES and a nucleotide sequence

encoding a SARS-CoV-2 antifusogenic polypeptide. In this example, DNA constructs were designed to include a spacer element and a polynucleotide cargo. The constructs were designed to include a combination of an IRES and an ORF as the polynucleotide cargo. The ORF was designed to include an IL-2 secretion signal sequence, a nucleotide sequence encoding a SARS-CoV-2 antifusogenic polypeptide, and a nucleotide sequence encoding a HiBiT tag with a GGGGS peptide linker. The IRES was EMCV.

Amino acid and nucleic acid sequences for all constructs used are shown below.

10 N-terminal IL-2 secretion signal shown in uppercase (20 AA or 60 nucleotides)

Bold = Furin

Bold and italics = HiBiT tag with G4S peptide linker

HR2 Full Length

15 ATGTATAGAATGCAGCTGCTGTCTTGTATTGCTCTTTCTCTGGCTCTTGTGACTAATTCT**agactgaggag**
aggtattgtaataataactgtttacgatcctcttcagcctgaacttgattcttttaagaagaactggataaataatttaagaatcatacttctcctgacgttga
tctgggtgatatttctggtattaacgcttctgttgaatattcagaaagaaattgatagactgaacgaagttgctaagaatctgaacgaatctcttattgatc
ttcaggaacttggaggaggaggaagcgctcagcggctggcggctgttcaagaagatcagc (SEQ ID NO: 378)

20 MYRMQLLSICIALSLALVTNS**RLRR**GIVNNTVYDPLQPELDSFKEELDKYFKNHTSPDVLGDISGINASVV
 NIQKEIDRLNEVAKNLNESLIDLQEL**GGGGSVSGWRLFKKIS** (SEQ ID NO: 379)

HR2A

25 ATGTATAGAATGCAGCTTCTTTCTTGTATTGCTCTTTCTCTTGGTCTGTTACTAATTCT**agactgaggag**
agatatttctggtattaacgcttctgttgaatattcagaaagaaattgatagacttaacgaagttgctaaaaatctgaacgaatctctgattgatctgcag
gaactggaggaggaggaagcgctcagcggctggcggctgttcaagaagatcagc (SEQ ID NO: 380)

MYRMQLLSICIALSLALVTNS**RLRR**DISGINASVVNIQKEIDRLNEVAKNLNESLIDLQEL**GGGGSVSGWRL**
FKKIS (SEQ ID NO: 381)

30

HR2C

ATGTATAGAATGCAGCTTCTGTCTTGTATTGCTCTGTCTTGTCTTGTACTAATTCT**agactgaggag**
atttaaaatcatacttctcctgacgttgatctgggtgatatttctggtattaacgcttctgttgaatattcagaaagaaattgatagactgaacgaagttg
ctaaaaggaggaggaggaagcgctcagcggctggcggctgttcaagaagatcagc (SEQ ID NO: 382)

35

MYRMQLLSICIALSLALVTNS**RLRR**FKNHTSPDVLGDISGINASVVNIQKEIDRLNEVAK**GGGGSVSGWR**
LFKKIS (SEQ ID NO: 383)

HR2B

ATGTATAGAAATGCAGCTTCTGTCTTGTATTGCTCTGTCTCTTGCTCTGGTTACTAATTCT**aggctgagaag**
agttgtattggtattgtaataactgtttacgaccccttcagcctgaacttgattctttaaggaagaactggataagtat
gatggaggaggaggaagcgtcagcggctggcggctgttcaagaagatcagc (SEQ ID NO: 384)

MYRMQLLSICIALSLALVTNS**RLRR**VVIGIVNNTVYDPLQPELDSFKEELDKYFKNHTSPD**GGGGSVSGWR**
LFFKIS (SEQ ID NO: 385)

EK1

ATGTATAGAAATGCAGCTTCTTTCTTGTATTGCTCTGTCTCTGGCTCTTGTTACTAATTCT**agactgaggag**
atctctgacagattaacgttactttctggatctggaatacgaaatgaaaaagctggaagaagctattaaaaagctgaagaatcttatattgatctga
aagaactggaggaggaggaagcgtcagcggctggcggctgttcaagaagatcagc (SEQ ID NO: 386)

MYRMQLLSICIALSLALVTNS**RLRR**SLDQINVTFDLEYEMKKLEEAIAKKLEESYIDLKEL**GGGGSVSGWRL**
FKKIS (SEQ ID NO: 387)

Circular RNAs were generated by self-splicing using a method described herein. Unmodified linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template including the motifs listed above in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription. Circular RNAs encoding an antifusogenic peptide were purified by urea polyacrylamide gel electrophoresis (Urea-PAGE) or by reversed phase column chromatography.

To measure the expression of the SARS-CoV-2 antifusogenic polypeptides, 0.4 pmol of circular RNA was delivered to HEK293 cells using lipofectamine. Expression was measured after 48 hours. Various polypeptides were expressed in HEK293 cells. Total expression (ng/ml and nM) is shown in Table 2.

Table 2: HR2 construct expression

	Expression (ng/mL)	Expression (nM)
HR2 Full Length	69.2	8.4
HR2A	5	1.3
HR2C	1.4	0.4
HR2B	0	0
EK1	0.1	0

Example 5: Inhibition of pseudotyped SARS-CoV-2 infection

This example demonstrates inhibition of pseudotyped SARS-CoV2- infection by antifusogenic polypeptides expressed from circular RNAs.

Circular RNAs were designed to include an internal ribosome entry site (IRES) and a nucleotide sequence encoding an anitfusogenic polypeptide of SARS-CoV-2. In this example, DNA constructs were

designed to include a spacer element and a combination of an EMCV IRES and an ORF as the polynucleotide cargo. The ORF was designed to include an IL-2 secretion signal sequence and a nucleotide sequence encoding an HR2 full length antifusogenic polypeptide, and a nucleotide sequence encoding a HiBiT peptide tag. An ORF was also designed to include an IL-2 secretion signal sequence and a nucleotide sequence encoding an HR2 full length antifusogenic polypeptide without a nucleotide sequence encoding a HiBiT peptide tag.

Circular RNAs were generated by self-splicing using a method described herein. Unmodified linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template including the motifs listed above in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription. Circular RNAs encoding the HR2 full length antifusogenic polypeptide were purified by urea polyacrylamide gel electrophoresis (Urea-PAGE) or by reversed phase column chromatography.

To detect the inhibitory activity of the expressed polypeptides on infection by SARS pseudovirus, ACE2-transfected 293T (293T/ACE2) cells (10^4 per well in 96-well plates) that have and have not been transfected with circular RNAs were respectively infected with SARS-CoV-2 pseudovirus. Transfection reagent alone (with no circular RNA) was used as a control ("Mock"). Following infection, the culture was re-fed with fresh medium 12 hours post-infection and incubated for an additional 72 hours. Cells were washed with PBS and lysed using lysis reagent included in a luciferase kit (Promega). Aliquots of cell lysates were transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar), followed by the addition of luciferase substrate (Promega). Relative light units were determined immediately in the Ultra 384 luminometer (Tecan US).

Efficacy of fusion inhibition in vitro using Omicron and Delta pseudoviruses was determined using circular polyribonucleotides encoding antifusogenic polypeptides described herein. HR2 full length antifusogenic polypeptide expressed in cells (**FIG. 4A**), and HR2 full length and HR2 full length conjugated to a HiBiT tag antifusogenic polypeptides were shown to inhibit fusion (**FIG. 4A**) of Delta and Omicron strains. There was no change in cell viability (measured by cellTiter glo) (**FIG. 4B**) suggesting that the decrease in luciferase signaling (**FIG. 4A**) was due to inhibition of viral fusion.

To measure the expression of the polypeptide in vivo, 120 pmol of circular RNAs formulated in lipid nanoparticles were delivered to mice via intravenous injection. Expression was measured by Nano-Glo® HiBiT Extracellular Detection System (#N3030, Promega) 10% Serum. The antifusogenic polypeptide was highly expressed at 6 hours and significantly decreased by 24 hours as shown in Table 3 and **FIG. 5**.

Table 3: HR2 expression

	6 hours (ng/mL)	24 hours (ng/mL)
HR2 Full Length HiBiT	24.8	0.2
No HiBiT	0.02	0.01

Example 6: Inhibition of pseudotyped SARS-CoV-2 infection

This example demonstrates pseudotyped SARS-CoV2- infection using antifusogenic polypeptides.

Antifusogenic polypeptides of SARS-CoV-2 were created based on the HR2, HR2A, HR2B, and HR2C regions of SARS-CoV-2 Spike polypeptide and the EK1 polypeptide (**FIG. 2**).

A functional assay was performed to measured pseudoviral neutralization by EK-1 and HR2A polypeptides in vitro. HR2A polypeptides showed efficacy against Wuhan and Omicron strains (**FIGS. 6A** and **6B**).

Further experiments with polypeptides were performed using HR2A-C and and HR2 full length polypeptides. For a negative control, IPB19, a HIV peptide, was used, and for a positive control, ACE-Fc, an antibody that binds the receptor directly, was used. Polypeptides (starting dilution 10 μ M) were prepared using 4-fold serial dilution (8 dilutions) and HEK293 ACE2 cells. All polypeptides were shown to inhibit Omicron and WT strains, with IC50 values shown in Table 4.

Table 4: IC50 Values

	IC50 (μ M)		IC50 (nM)		IC50 (ng/ml)	
	Omicron	Wildtype	Omicron	Wildtype	Omicron	Wildtype
HR2 Full Length	0.02625	0.1742	26.3	174.2	215.3	1428.4
HR2A	-	-	37.7	614.2	158.1	2579.6
EK-1	-	-	64.8	329.3	272.2	1383.1
HR2B	-	-	Neg.	Neg.	Neg.	Neg.
HR2C	-	-	Neg.	Neg.	Neg.	Neg.
IPB19	-	-	1046.0	Neg.	4393.2	Neg.
ACE-Fc	-	-	4.3	8.4		

Full length HR2 Fc fusions were also tested and shown to maintain inhibitory activity against Omicron and Wuhan strains.

HR2 full length polypeptide ("HR2Complete") was shown to successfully inhibit the fusion of Omicron BA.4/BA.5, SARS CoV-1, and Wuhan strains (**FIGS. 7A, 7B, and 8A-8D**).

Example 7: Expression of HIV antifusogenic polypeptides

This example demonstrates expression of HIV antifusogenic polypeptides from circular RNAs.

Several HIV antifusogenic polypeptides were designed (**FIG. 9**). Circular RNAs were designed to include an IRES and a nucleotide sequence encoding an HIV antifusogenic polypeptide. In this example, DNA constructs were designed to include a spacer element and a polynucleotide cargo (**FIG. 9**). The constructs were designed to include a combination of an IRES and an ORF as the polynucleotide cargo. The ORF was designed to include an IL-2 secretion signal sequence (SEQ ID NO: 332), a nucleotide sequence encoding an HIV antifusogenic polypeptide, and a nucleotide sequence encoding a HiBiT tag (having sequence VSGWRLFKKIS (SEQ ID NO: 362) with a GGS or GGGS peptide linker. The IRES was either EMCV or a modified CVB3.

Circular RNAs were generated by self-splicing using a method described herein. Unmodified linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template including the motifs listed above in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription. Circular RNAs encoding an antifusogenic peptide were purified by urea polyacrylamide gel electrophoresis (Urea-PAGE) or by reversed phase column chromatography.

To measure the expression of the HIV antifusogenic polypeptides, 0.4 pmol of circular RNA was delivered to HEK293 cells using lipofectamine. Expression was measured after 48 hours. As shown in **FIGS. 10A** and **10B**, the polypeptides were expressed in HEK293 cells. As shown in **FIGS. 11A** and **11B**, expression was comparable between circular RNA and DNA plasmid. Total expression (ng/mL and nM) is shown in **FIG. 12** for the various polypeptides.

HIV polypeptide and nucleic acid sequences

15

T20

YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF (SEQ ID NO: 313)

tatacttctctgatccactctctgatcgaggaaatcagaaccagcaggagaagaacgaacaggaactgctggaactggataagtgggctctctgtggaactggttc (with EMCV IRES) (SEQ ID NO: 363)

20

OR

tacaccagcctgatccacagcctgatcgaggaaagccagaaccagcaagagaagaacgagcaggagctgctggagctggacaagtgggcca gctgtggaactggttc (with modified CVB3 IRES) (SEQ ID NO: 364)

T1249

25

WQEWKITALLEQAQIQKEKNEYELQKLDKWASLWEWF (SEQ ID NO: 318)

tggcaggagtgggaacagaagatcactgctctgctggaacaggctcagattcagcaggaaaagaacgaatacgaactgcagaagctggataa gtgggctctctgtgggagtggttc (with EMCV IRES) (SEQ ID NO: 365)

OR

30

tggcaggagtgggagcagaagatcaccgccctgctggagcaggccagatccagcaagagaagaacgagtagcagctgcagaagctggac aagtgggccagcctgtgggagtggttc (with modified CVB3 IRES) (SEQ ID NO: 366)

T1144

TTWEAWDRAIAEYAARIEALLRALQEQKEKNEAALREL (SEQ ID NO: 316)

35

actacttggaagcttggaatagagctatcgctgaatacgtgctagaattgaagctctgctgagagctctgcaggaacagcaggaaaagaacga agctgctctgagagaactg (with EMCV IRES) (SEQ ID NO: 367)

OR

accacctgggaggcctgggaccgggccatcgccgagtagccgctcgatcgaggccctgctgcgggccctgcaggagcagcaagagaaga acgaggccgccctgcgggagctg (with modified CVB3 IRES) (SEQ ID NO: 368)

40

1144-2-0

TTWEAWDRAIAEYAARIEALLRALQEQQEKNEAALRELDKWASLWNWF (SEQ ID NO: 317)
accacctgggaggcctgggaccgggccatcgccgagtagccgctcgatcgaggccctgctgcgggccctgcaggagcagcaagagaaga
acgaggccgacctgcgggagctggacaagtgggccagcctgtggaactggttc (with modified CVB3 IRES) (SEQ ID NO:
5 369)

T_2410

MTWMEWDREINNYTSLIHSLEESQNQQEKNEQELLE (SEQ ID NO: 314)
atgacctggatggagtgggaccgggagatcaacaattaccagcctgatccacagcctgatcgaggaaagccagaaccagcaagagaaga
10 acgagcaggagctgctggagctg (with modified CVB3 IRES) (SEQ ID NO: 370)

T_2410_2.0

MTWMEWDREINNYTSLIHSLEESQNQQEKNEQELLELDKWASLWNWF (SEQ ID NO: 315)
atgacctggatggagtgggaccgggagatcaacaattaccagcctgatccacagcctgatcgaggaaagccagaaccagcaagagaaga
15 acgagcaggagctgctggagctggacaagtgggccagcctgtggaactggttc (with modified CVB3 IRES) (SEQ ID NO:
371)

1249_2.0

TTWQEWQKITALLEQAQIQQEKNEYELQKLDKWASLWEWF (SEQ ID NO: 319)
20 accacctggcaggagtgggagcagaagatcacccctgctggagcaggcccatccagcaagagaagaacgagtagcagctgcagaag
ctggacaagtgggccagcctgtgggagtggttc (with modified CVB3 IRES) (SEQ ID NO: 372)

290676

TTWEAWDRAIAEYAARIEALIRASQEQQEKNEAELREL (SEQ ID NO: 323)
25 accacctgggaggcctgggaccgggccatcgccgagtagccgctcgatcgaggccctgatccgggccagccaggagcagcaagagaaga
acgaggccgagctgcgggagctg (with modified CVB3 IRES) (SEQ ID NO: 373)

290676-2-0

TTWEAWDRAIAEYAARIEALIRASQEQQEKNEAELRELDKWASLWNWF (SEQ ID NO: 324)
30 accacctgggaggcctgggaccgggccatcgccgagtagccgctcgatcgaggccctgatccgggccagccaggagcagcaagagaaga
acgaggccgagctgcgggagctggacaagtgggccagcctgtggaactggttc (with modified CVB3 IRES) (SEQ ID NO:
374)

2635

TTWEAWDRAIAEYAARIEALIRAAQEQQEKNEAALREL (SEQ ID NO: 320)
35 accacctgggaggcctgggaccgggccatcgccgagtagccgctcgatcgaggccctgatccgggccagccaggagcagcaagagaaga
acgaggccgcactgcgggagctg (with modified CVB3 IRES) (SEQ ID NO: 375)

2635_2.0

40 TTWEAWDRAIAEYAARIEALIRAAQEQQEKNEAALRELDKWASLWNWF (SEQ ID NO: 321)

accacctgggaggcctgggaccgggccatgccgagtagccgctcgatcgaggccctgatccgggccgcccaggagcagcaagagaaga
acgaggccgcactgcgggagctggacaagtgggccagcctgtggaactggttc (with modified CVB3 IRES) (SEQ ID NO:
376)

5 **2635_3.0**

MTWEAWDRAIAEYAARIEALIRAAQEQKEKNEAALRELDKWASLWNWF (SEQ ID NO: 322)

atgacctgggaggcctgggaccgggccatgccgagtagccgctcgatcgaggccctgatccgggccgcccaggagcagcaagagaaga
acgaggccgcactgcgggagctggacaagtgggccagcctgtggaactggttc (SEQ ID NO: 377)

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Other Embodiments

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the invention that come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the claims. Other embodiments are within the claims.

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CLAIMS

1. A circular polyribonucleotide comprising a polyribonucleotide cargo encoding an antifusogenic polypeptide.
2. The circular polyribonucleotide of claim 1, wherein the polyribonucleotide cargo comprises an expression sequence encoding the antifusogenic polypeptide.
3. The circular polyribonucleotide of claim 1 or 2, wherein the circular polyribonucleotide comprises a splice junction joining a 5' exon fragment and a 3' exon fragment.
4. The circular polyribonucleotide claim 2 or 3, wherein the polyribonucleotide cargo comprises an IRES operably linked to the expression sequence encoding the antifusogenic polypeptide.
5. The circular polyribonucleotide of claim 4, wherein the circular polyribonucleotide further comprises a spacer region between the IRES and the 3' exon fragment or the 5' exon fragment.
6. The circular polyribonucleotide of claim 5, wherein the spacer region is at least 5 ribonucleotides in length.
7. The circular polyribonucleotide of claim 6, wherein the spacer region is from 5 to 500 ribonucleotides in length.
8. The circular polyribonucleotide of any one of claims 5-7, wherein the spacer region comprises a polyA, a polyA-C, polyA-U, or polyA-G sequence.
9. The circular polyribonucleotide of any one of claims 1-8, wherein the circular polyribonucleotide is at least 500 ribonucleotides in length.
10. The circular polyribonucleotide of claim 9, wherein the circular polyribonucleotide is from 500 to 20,000 ribonucleotides in length.
11. A linear polyribonucleotide comprising, from 5' to 3', (A) a 3' intron fragment; (B) a 3' splice site; (C) a 3' exon fragment; (D) a polyribonucleotide cargo encoding the antifusogenic polypeptide; (E) a 5' exon fragment; (F) a 5' splice site; and (G) a 5' intron fragment.
12. The linear polyribonucleotide of claim 11, wherein the polyribonucleotide cargo comprises an expression sequence encoding the antifusogenic polypeptide.
13. The linear polyribonucleotide claim 12, wherein the polyribonucleotide cargo comprises an IRES operably linked to the expression sequence encoding the antifusogenic polypeptide.

14. The linear polyribonucleotide of any one of claims 11-13, wherein the circular polyribonucleotide further comprises a spacer region between one or more of (A), (B), (C), (D), (E), (F), and (G).
15. The linear polyribonucleotide of claim 14, wherein the spacer region is at least 5 ribonucleotides in length.
16. The linear polyribonucleotide of claim 15, wherein the spacer region is from 5 to 500 ribonucleotides in length.
17. The linear polyribonucleotide of any one of claims 14-16, wherein the spacer region comprises a polyA, a polyA-C, polyA-U, or polyA-G sequence.
18. The linear polyribonucleotide of any one of claims 11-17, wherein the linear polyribonucleotide is at least 500 ribonucleotides in length.
19. The linear polyribonucleotide of claim 18, wherein the linear polyribonucleotide is from 500 to 20,000 ribonucleotides in length.
20. A DNA vector encoding the linear polyribonucleotide of any one of claims 11-19.
21. A method of expressing an antifusogenic polypeptide in a cell, the method comprising providing the circular polyribonucleotide of any one of claims 1-10, the linear polyribonucleotide of any one of claims 11-19, or the DNA vector of claim 20 to the cell under conditions suitable to express the antifusogenic polypeptide.
22. A method of producing a circular polyribonucleotide from the linear polyribonucleotide of any one of claim 11-20, the method comprising providing the linear polyribonucleotide under conditions suitable for self-splicing of the linear polyribonucleotide to produce the circular polyribonucleotide.
23. A pharmaceutical composition comprising the circular polyribonucleotide of any one of claims 1-10, the linear polyribonucleotide of any one of claims 11-19, or the DNA vector of claim 20 and a diluent, carrier, or excipient.
24. A method of expressing an antifusogenic polypeptide in a subject comprising administering a first dose of the pharmaceutical composition of claim 23 in an amount sufficient to produce a serum concentration of at least 500 ng/mL of the antifusogenic polypeptide in the subject.
25. The method of claim 24, further comprising administering a second dose of the pharmaceutical composition.

26. The method of claim 25, wherein the second dose is administered at least 1 day after the first dose of the pharmaceutical composition.
27. The method of claim 26, wherein the second dose is administered from 1 day to 90 days after the first dose of the pharmaceutical composition.
28. The method of any one of claims 25-27, wherein the second dose is administered before a serum concentration of the antifusogenic polypeptide is less than about 500 ng/mL in serum of the subject.
29. The method of claim 28, wherein the method maintains a serum concentration of at least 500 ng/mL of the antifusogenic polypeptide in the subject.
30. The method of any one of claims 24-29, wherein the method treats or prevents a viral infection in the subject.
31. The method of claim 30, wherein the pharmaceutical composition is administered to the subject in an amount and for a duration sufficient to treat or prevent the viral infection.
32. The method of any one of claims 24-31, wherein the method reduces viral entry.
33. A circular polyribonucleotide comprising a polyribonucleotide cargo encoding multiple antifusogenic polypeptides.
34. The circular polyribonucleotide of claim 33, wherein the polyribonucleotide cargo comprises expression sequences encoding the antifusogenic polypeptides.
35. The circular polyribonucleotide of claim 33 or 34, wherein the antifusogenic polypeptides are directed to the same virus.
36. The circular polyribonucleotide of claim 33 or 34, wherein the antifusogenic polypeptides are directed to more than one virus.