

COMPOSITIONS AND METHODS FOR PURIFYING POLYRIBONUCLEOTIDES**Background**

Polyribonucleotides are useful for a variety of therapeutic and engineering applications. Thus,
5 new compositions and methods for separating and purifying polyribonucleotides are useful.

Summary of the Invention

In one aspect, the invention features a method of separating a polyribonucleotide having a target
region from a plurality of polyribonucleotides. In this method, a sample that includes the plurality of
10 polyribonucleotides is provided. A subset of the plurality of polyribonucleotides in the sample have the
target region. The method further includes contacting the sample with an oligonucleotide that hybridizes
to the target region and separating the polyribonucleotide having the target region that is hybridized to the
oligonucleotide from the plurality of polyribonucleotides in the sample.

In another aspect, the invention features a method of separating a linear polyribonucleotide
15 having a target region from a plurality of polyribonucleotides that includes a mixture of linear
polyribonucleotides and circular polyribonucleotides. In this method, a sample that includes the plurality
of polyribonucleotides is provided. A subset of the plurality of linear polyribonucleotides in the sample
have the target region. The method further includes contacting the sample with an oligonucleotide that
hybridizes to the target region and separating the linear polyribonucleotide having the target region that is
20 hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample. In some
embodiments, polyribonucleotide having the target region lacks a polyA sequence (*i.e.*, a polyA tail). In
some embodiments, the target region is not located at a 3' or 5' terminus of the polyribonucleotide having
the target region. The circular polyribonucleotides or a subset thereof may lack the target region.

In another aspect, the invention features a method of separating a polyribonucleotide having a
25 target region from a plurality of polyribonucleotides. The method includes providing a sample that
includes the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides have
the target region. The target region is not located at a 3' or 5' terminus of the polyribonucleotide having
the target region (*i.e.*, the target region is located internally on the polyribonucleotide). The method
further includes contacting the sample with an oligonucleotide that hybridizes to the target region and
30 separating the polyribonucleotide having the target region that is hybridized to the oligonucleotide from
the plurality of polyribonucleotides in the sample.

In another aspect, the invention features a method of separating a linear polyribonucleotide
having a target region from a plurality of circular polyribonucleotides having the target region. The
method includes providing a sample that includes the plurality of polyribonucleotides. The method further
35 includes contacting the sample with an oligonucleotide. The oligonucleotide hybridizes to the target
region of the linear polyribonucleotide with a first binding affinity, and the oligonucleotide hybridizes to the
target region of the circular polynucleotide with a second binding affinity that is different from the first
binding affinity and separating the linear polyribonucleotide having the target region that is hybridized to
the oligonucleotide from the plurality of circular polyribonucleotides in the sample. In some embodiments,
40 the first binding affinity is less than the second binding affinity, and the oligonucleotide preferentially binds
the linear polyribonucleotide.

In another aspect, the invention features a method of separating a polyribonucleotide in a first conformation having a target region from a plurality of polyribonucleotides in a second conformation having the target region. The method includes providing a sample that includes the plurality of polyribonucleotides. The method further includes contacting the sample with an oligonucleotide. The oligonucleotide hybridizes to the target region of the polynucleotide in the first conformation with a first binding affinity, and the oligonucleotide hybridizes to the target region of the polynucleotide in the second conformation with a second binding affinity that is different from the first binding affinity and separating the polyribonucleotide in the first conformation having the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the second conformation in the sample. In some embodiments, the first binding affinity is less than the second binding affinity, and the oligonucleotide preferentially binds the polyribonucleotide in the first conformation.

In some embodiments of any of the aspects described herein, separating includes immobilizing the oligonucleotide.

In some embodiments of any of the methods described herein, the target region is not located at a 5' or 3' terminus of the polyribonucleotide having the target region. For example, the target region may be internally located on the polyribonucleotide. In some embodiments, the target region is not located at a 3' terminus of the polyribonucleotide.

In some embodiments of any of the methods described herein, the target region is located at a 5' or 3' terminus of the polyribonucleotide, and the target region does not contain a polyA sequence. In some embodiments, the target region is located at a 3' terminus of the polyribonucleotide and does not contain a polyA sequence.

In some embodiments of any of the methods described herein, the target region does not contain a polyA sequence.

In some embodiments of any of the above aspect, the oligonucleotide is conjugated to a particle. The particle may be, for example, a magnetic particle or a bead. The bead may be, *e.g.*, a crosslinked agarose, *e.g.*, a SEPHAROSE® bead.

In some embodiments of any of the above aspects, the oligonucleotide is conjugated to a first capture agent.

In some embodiments, the oligonucleotide is conjugated to the first capture agent or the particle by way of a chemical linker. The chemical linker may include, *e.g.*, triethylene glycol. In some embodiments, the chemical linker is conjugated to a 3' end or a 5' end of the oligonucleotide.

In some embodiments, the first capture agent includes an antigen (*e.g.*, biotin).

In some embodiments, the method further includes contacting the sample with a second capture that binds to the first capture agent. The second capture agent may include, for example, an antibody or antigen-binding fragment thereof (*e.g.*, streptavidin).

In some embodiments, the second capture agent is conjugated to a particle. The particle may be, for example, a magnetic particle or a bead. The bead may be, *e.g.*, a crosslinked agarose, *e.g.*, a SEPHAROSE® bead. In some embodiments, the second capture agent is conjugated to the particle with a chemical linker. The chemical linker may include, *e.g.*, triethylene glycol.

In some embodiments, the polyribonucleotide having the target region includes an intron or portion thereof (*e.g.*, a half-intron). The target region may include an intron or portion thereof. In some

embodiments, the intron or portion thereof is a catalytic intron (*e.g.*, a Group I catalytic intron or a Group II catalytic intron) or a portion thereof. In some embodiments the intron or portion thereof has a length of at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100 nucleotides. In some embodiments the intron or portion thereof has a length of 20 to 500, 20 to 400, 20 to 300, 20 to 200, 20 to 100, 50 to 500, 50 to 400, 50 to 300, 50 to 200, 100 to 500, 100 to 400, 100 to 300, or 100 to 200 nucleotides.

In some embodiments, the target region includes a half-intron (*e.g.*, a 5' or 3' portion of a catalytic intron). In some embodiments, the target region includes a 5' half-intron (*e.g.*, a 5' half-intron corresponding to a 5' portion of a catalytic intron). In some embodiments, the target region includes a 3' half-intron (*e.g.*, a 3' half-intron corresponding to a 3' portion of a catalytic intron). In some embodiments, the target region includes a 5' half of a Group I catalytic intron. In some embodiments, the 5' half of the Group I catalytic intron is from a 5' portion of a cyanobacterium *Anabaena* pre-tRNA-Leu gene, a *Tetrahymena* pre-rRNA, a T4 phage td gene, or a variant thereof. In some embodiments, the target region includes a 3' half of a Group I catalytic intron. In some embodiments, the 3' half of the Group I catalytic intron is selected from a 3' portion from a cyanobacterium *Anabaena* pre-tRNA-Leu gene, a *Tetrahymena* pre-rRNA, a T4 phage td gene, or a variant thereof.

The target region may be located 5' or 3' to the intron or portion thereof or may include a portion of the region located 5' or 3' to the intron or portion thereof.

In some embodiments, the method includes separating a spliced polyribonucleotide from a non-spliced or partially spliced polyribonucleotide. In some embodiments, the spliced polyribonucleotide is a circular polyribonucleotide. In some embodiments, the spliced polyribonucleotide is a linear polyribonucleotide. In some embodiments, the spliced polyribonucleotide lacks an intron or portion thereof (*e.g.*, the spliced polyribonucleotide lacks a catalytic intron, such as a Group I catalytic intron, or a portion thereof).

In some embodiments, the method enriches an amount of the spliced polyribonucleotide by at least 10% (*e.g.*, by at least 20%, 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 99%, or more) relative to the sample.

In some embodiments, the polyribonucleotide having the intron or portion thereof is a linear polyribonucleotide.

In some embodiments, the oligonucleotide has at least 5 nucleotides (*e.g.*, at least 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 nucleotides) in length. In some embodiments, the oligonucleotide is, *e.g.*, from 5-100, 5-95, 10-90, 10-80, 12-60, 15-50, 15-40, 15-30, 18-30, 20-25, or 20-22 nucleotides in length. In some embodiments, the oligonucleotide is 23 nucleotides in length.

In some embodiments, the oligonucleotide may have a melting temperature (T_m) of, for example, from about 45 °C to about 75 °C, *e.g.*, about 46 °C, 47 °C, 48 °C, 49 °C, 50 °C, 51 °C, 52 °C, 53 °C, 54 °C, 55 °C, 56 °C, 57 °C, 58 °C, 59 °C, 60 °C, 61 °C, 62 °C, 63 °C, 64 °C, 65 °C, 66 °C, 67 °C, 68 °C, 69 °C, 70 °C, 71 °C, 72 °C, 73 °C, 74 °C, or 75 °C. In some embodiments, the oligonucleotide has a T_m of about 45 °C to about 65 °C.

In some embodiments, the method further includes the step of washing the captured polyribonucleotide having the target region (*e.g.*, after the contacting and/or after separating step) one or

more (*e.g.*, two, three, four, five, or more) times. In some embodiments, the method further includes washing the first and/or second capture agent one or more (*e.g.*, two, three, four, five, or more) times.

In some embodiments, the method further includes the step of performing a first elution step to release the captured polyribonucleotide having a target region. The first elution step may include, *e.g.*,
5 adding a first buffer and/or heating the sample, *e.g.*, to at least 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, or higher.

In some embodiments, the method further includes performing a second elution step. The second elution step may include adding a second buffer and/or heating the sample, *e.g.*, to at least 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, or higher. In some embodiments, the second buffer includes a
10 denaturing agent, *e.g.*, formamide or urea. The second buffer may include, *e.g.*, from about 40% to about 60% formamide (*e.g.*, about 40%, 45%, 50%, 55%, or 60% formamide).

In some embodiments, the method includes incubating the sample with the oligonucleotide (*e.g.*, conjugated to the first capture agent) for at least ten (*e.g.*, at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, or more) minutes.

15 In some embodiments, the method includes collecting a portion of the sample that is not bound by the oligonucleotide. In some embodiments, the method includes collecting a portion of the sample that is not bound by the capture agent (*e.g.*, the first and/or second capture agent).

In some embodiments, the method includes providing a plurality of oligonucleotides, wherein each oligonucleotide hybridizes to a distinct target region. In some embodiments, each oligonucleotide is
20 conjugated to a first capture agent.

In some embodiments, the method includes providing an oligonucleotide that hybridizes to multiple target regions. In some embodiments, the method includes providing a single oligonucleotide that hybridizes to a 3' half intron and a 5' half intron.

In some embodiments, the method includes providing a first oligonucleotide that hybridizes to a 3' half-intron and a second oligonucleotide that hybridizes to a 5' half-intron. In some embodiments, the method includes providing a plurality of oligonucleotides that each hybridize to a distinct region on the 3' half-intron and/or the 5' half-intron.

In some embodiments, the oligonucleotide has at least 80% (*e.g.*, at least 85%, 90%, 95%, 97%, 99%, or 100%) complementarity an equal length portion of the target region.

30 In some embodiments, the method includes providing the oligonucleotide at a molar ratio of 10:1 to 1:10 (*e.g.*, 10:1, 5:1, 2:1, 1:2, 1:5, or 1:10) to the polyribonucleotide.

In another aspect, the invention features a population of polyribonucleotides produced by the method of any of the embodiments described herein. The population may include, *e.g.*, a circular polyribonucleotide lacking a target region and the circular polyribonucleotide includes at least 1% (*e.g.*, at
35 least 5%, *e.g.*, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, or more) (mol/mol) of the total polyribonucleotides in the composition.

In some embodiments, the population has less than 50% (*e.g.*, less than 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1%) (mol/mol) linear polyribonucleotides.

In another aspect, the invention features a composition that includes a mixture of
40 polyribonucleotides. A first subset of the mixture includes a circular polyribonucleotide lacking a target region, and a second subset of the plurality of the polyribonucleotides includes a linear polyribonucleotide

having a target region. The first subset includes at least 1%, (*e.g.*, at least 5%, *e.g.*, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, or more) (mol/mol) of the total polyribonucleotides in the composition.

In another aspect, the invention features a composition that includes a polyribonucleotide having
 5 a target region and an oligonucleotide configured to hybridize to the target region, wherein the oligonucleotide is conjugated to a first capture agent (*e.g.*, an antigen, such as biotin). The composition may further include, *e.g.*, a polyribonucleotide lacking the target region. The polyribonucleotide lacking the target region may be, *e.g.*, a circular polyribonucleotide. The composition may further include a second capture agent (*e.g.*, an antibody or antigen binding fragment thereof, such as streptavidin)
 10 configured to bind the first capture agent. The second capture agent may be conjugated to a particle.

In some embodiments, the composition is produced by a method as described herein.

In some embodiments of any of the compositions as described herein, the linear polyribonucleotide includes an intron or portion thereof. The target region may include the intron or portion thereof. The target region may be located 5' or 3' to the intron or portion thereof.

15 In some embodiments of any of the above aspects, the polyribonucleotide and/or oligonucleotide may be modified.

In another aspect, the invention features a pharmaceutical composition that includes a composition as described herein, *e.g.*, produced by a method as described above, and a diluent, carrier, or excipient.

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Definitions

To facilitate the understanding of this disclosure, a number of terms are defined below. Terms 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
 25 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 terminology herein is used to describe specific embodiments, but their usage is not to be taken as limiting, except as outlined in the claims.

30 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.

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
 35 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 phytoglycogen or glycogen-type material), nanoparticles (*e.g.*, a nanoparticle that 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
 40 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 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 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.

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

As used herein, the term “impurity” is an undesired substance present in a composition, *e.g.*, a pharmaceutical composition as described herein. In some embodiments, an impurity is a process-related impurity. In some embodiments, an impurity is a product-related substance other than the desired product in the final composition, *e.g.*, other than the active drug ingredient, *e.g.*, circular polyribonucleotide, as described herein. As used herein, the term “process-related impurity” is a substance used, present, or generated in the manufacturing of a composition, preparation, or product that is undesired in the final composition, preparation, or product other than the linear polyribonucleotides described herein. In some embodiments, the process-related impurity is an enzyme used in the synthesis or circularization of polyribonucleotides. As used herein, the term “product-related substance” is a substance or byproduct produced during the synthesis of a composition, preparation, or product, or any intermediate thereof. In some embodiments, the product-related substance is deoxyribonucleotide fragments. In some embodiments, the product-related substance is deoxyribonucleotide monomers. In some embodiments, the product-related substance is one or more of: derivatives or fragments of polyribonucleotides described herein, *e.g.*, fragments of 10, 9, 8, 7, 6, 5, or 4 ribonucleic acids, monoribonucleic acids, diribonucleic acids, or triribonucleic acids.

As used herein, "increasing fitness" or "promoting fitness" of a subject refers to any favorable alteration in physiology, or of any activity carried out by a subject organism, as a consequence of administration of a peptide or polypeptide described herein, including, but not limited to, any one or more of the following desired effects: (1) increased tolerance of biotic or abiotic stress; (2) increased yield or biomass; (3) modified flowering time; (4) increased resistance to pests or pathogens; (4) increased resistance to herbicides; (5) increasing a population of a subject organism (*e.g.*, an agriculturally important insect); (6) increasing the reproductive rate of a subject organism (*e.g.*, insect, *e.g.*, bee or silkworm); (7) increasing the mobility of a subject organism (*e.g.*, insect, *e.g.*, bee or silkworm); (8) increasing the body weight of a subject organism (*e.g.*, insect, *e.g.*, bee or silkworm); (9) increasing the metabolic rate or activity of a subject organism (*e.g.*, insect, *e.g.*, bee or silkworm); (10) increasing pollination (*e.g.*, number of plants pollinated); (11) increasing production of subject organism (*e.g.*, insect, *e.g.*, bee or silkworm) byproducts (*e.g.*, honey from honeybee or silk from silkworm); (12) increasing nutrient content of the subject organism (*e.g.*, insect) (*e.g.*, protein, fatty acids, or amino acids); (13) increasing a subject organism's resistance to pesticides (*e.g.*, a neonicotinoid (*e.g.*, imidacloprid) or an organophosphorus insecticide (*e.g.*, a phosphorothioate, *e.g.*, fenitrothion)); or (14) increasing health or reducing disease of a subject organism such as a human or non-human animal. An increase in host fitness can be determined in comparison to a subject organism to which the modulating agent has not been administered. Conversely, "decreasing fitness" of a subject refers to any unfavorable alteration in physiology, or of any activity carried out by a subject organism, as a consequence of administration of a peptide or polypeptide described herein, including, but not limited to, any one or more of the following intended effects: (1) decreased tolerance of biotic or abiotic stress; (2) decreased yield or biomass; (3) modified flowering time; (4) decreased resistance to pests or pathogens, (4) decreased resistance to herbicides; (5) decreasing a population of a subject organism (*e.g.*, an agriculturally important insect); (6) decreasing the reproductive rate of a subject organism (*e.g.*, insect, *e.g.*, bee or silkworm); (7) decreasing the mobility of a subject organism (*e.g.*, insect, *e.g.*, bee or silkworm); (8) decreasing the body weight of a subject organism (*e.g.*, insect, *e.g.*, bee or silkworm); (9) decreasing the metabolic rate or activity of a subject organism (*e.g.*, insect, *e.g.*, bee or silkworm); (10) decreasing pollination (*e.g.*, number of plants pollinated in a given amount of time) by a subject organism (*e.g.*, insect, *e.g.*, bee or silkworm); (11) decreasing production of subject organism (*e.g.*, insect, *e.g.*, bee or silkworm) byproducts (*e.g.*, honey from a honeybee or silk from a silkworm); (12) decreasing nutrient content of the subject organism (*e.g.*, insect) (*e.g.*, protein, fatty acids, or amino acids); (13) decreasing a subject organism's resistance to pesticides (*e.g.*, a neonicotinoid (*e.g.*, imidacloprid) or an organophosphorus insecticide (*e.g.*, a phosphorothioate, *e.g.*, fenitrothion)); or (14) decreasing health or reducing disease of a subject organism such as a human or non-human animal. A decrease in host fitness can be determined in comparison to a subject organism to which the modulating agent has not been administered. It will be apparent to one of skill in the art that certain changes in the physiology, phenotype, or activity of a subject, *e.g.*, modification of flowering time in a plant, can be considered to increase fitness of the subject or to decrease fitness of the subject, depending on the context (*e.g.*, to adapt to a change in climate or other environmental conditions). For example, a delay in flowering time (*e.g.*, about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, 100% fewer plants in a population flowering at a given calendar date) can be a beneficial adaptation to later or cooler Spring times and thus be considered to increase a plant's fitness;

conversely, the same delay in flowering time in the context of earlier or warmer Spring times can be considered to decrease a plant's fitness.

As used herein, the terms "linear polyribonucleotide," "linear RNA," and "linear polyribonucleotide molecule" are used interchangeably and mean a polyribonucleotide molecule having a 5' and 3' end.

- 5 One or both the 5' and 3' ends may be free ends or joined to another moiety. A linear polyribonucleotide may be a polyribonucleotide 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.

- 10 As used herein, the term "modified oligonucleotide" means an oligonucleotide containing a nucleotide with at least one modification to the sugar, nucleobase, or internucleotide linkage.

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

- 15 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 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.

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

- 25 The term "optionally substituted X," as used herein, is intended to be equivalent to "X, wherein X is optionally substituted" (*e.g.*, "alkyl, wherein said alkyl is optionally substituted"). It is not intended to mean that the feature "X" (*e.g.*, alkyl) per se is optional. The term "optionally substituted," as used herein, refers to having 0, 1, or more substituents (*e.g.*, 0-25, 0-20, 0-10, or 0-5 substituents). For example, a C₁ alkyl group, *i.e.*, methyl, may be substituted with oxo to form a formyl group and further substituted with -OH or -NH₂ to form a carboxyl group or an amido group.

- 30 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.

- 35 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-carbon sugar (either ribose or deoxyribose), and one or more phosphate groups. Ribonucleotides are nucleotides in which the sugar is ribose. Polyribonucleotides, ribonucleic acids, or RNA, can refer to macromolecules that include multiple ribonucleotides that are polymerized by way of phosphodiester bonds. Deoxyribonucleotides are nucleotides in which the sugar is deoxyribose.
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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 (or coding) sequences, wherein each expression (or coding) 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 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 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 reason 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.

As used herein, the elements of a nucleic acid are “operably connected” or “operably linked” if they are positioned in the vector such that they can be transcribed to form a linear polyribonucleotide that can then be circularized into a circular polyribonucleotide 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 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, which include detectable tags, such as luminescent tags or markers (*e.g.*, fluorophores). A 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 (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 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, include polynucleotides that contain 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 diamino purine, 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-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 (v), butoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-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 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 aminohexyl acrylamide-dCTP (aha-dCTP) to allow covalent attachment of amine reactive moieties, such as N-hydroxy succinimide 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; 8:612-4, 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 “plant-modifying polypeptide” refers to a polypeptide that can alter the genetic properties (*e.g.*, increase gene expression, decrease gene expression, or otherwise alter the nucleotide sequence of DNA or RNA), epigenetic properties, or biochemical or physiological properties of a plant in a manner that results in a change in the plant’s physiology or phenotype, *e.g.*, an increase or a decrease in plant fitness.

As used herein, the terms “purify,” “purifying,” and “purification” refer to one or more steps or processes of removing impurities (*e.g.*, a process-related impurity (*e.g.*, an enzyme), a process-related substance (*e.g.*, a deoxyribonucleotide fragment, a deoxyribonucleotide monomer)) or by-products (*e.g.*, linear RNA) from a sample containing a mixture circular RNA and linear RNA, among other substances, to produce a composition containing an enriched population of circular RNA with a reduced level of an impurity (*e.g.*, a process-related impurity (*e.g.*, an enzyme), a process-related substance (*e.g.*, deoxyribonucleotide fragment, deoxyribonucleotide monomer)) or by-product (*e.g.*, linear RNA) as compared to the original mixture or in which the linear RNA or substances have been reduced by 40% or more by mass (*e.g.*, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 97%, or 99% or more) relative to a starting mixture.

As used herein, the terms “pure” and “purity” refer to the extent to which an analyte (*e.g.*, circular RNA) has been isolated and is free of other components. In the context of nucleic acids (*e.g.*, polyribonucleotides), purity of an isolated nucleic acid (*e.g.*, circular RNA) can be expressed with regard to the population of nucleic acids that is free of any contaminants, impurities, or by-products (*e.g.*, linear RNA and other substances). For example, purity of a population of circular RNA indicates how much of the population is circular RNA by total mass of the isolated material, which may be determined using, *e.g.*, pure circular RNA as a reference. A level of purity found in the disclosure can be 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, greater than 95%, or greater than 99% (w/w). In some embodiments, the level of contaminants or impurities or by-products is no more than about 20%, 15%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% (w/w). Purity can be determined by detecting a level of a specific analyte (*e.g.*, circular RNA) or a specific impurity or by-product (*e.g.*, linear RNA) using gel electrophoresis, spectrophotometry (*e.g.*, NanoDrop by ThermoFisher Scientific), or other technique suitable for measuring purity of a population of nucleic acids and calculating a percentage of the analyte (w/w) relative to the total nucleic acid content (*e.g.*, as determined by an assay known in the art).

As used herein, the phrase “substantially free of one or more impurities or by-products” refers to a property of a sample, such as a sample containing an enriched population of circular RNA, that is free of one or more impurities or by-products (*e.g.*, one or more impurities or by-products disclosed herein) or contains a minimal amount of the one or more impurities or by-products. A minimal amount of the one or more impurities or by-products may be no more than 20% (w/w) (*e.g.*, no more than 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% (w/w), or less). In another example, the sample or the enriched population of circular RNA is substantially free of one or more impurities or by-products if the one or more impurities or by-products are present in an amount that is less than 15% (w/w) (*e.g.*, no more than 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% (w/w), or less). In another example, the sample or the enriched population of circular RNA is substantially free of one or more impurities or by-products if the one or more impurities or by-products are

present in an amount that is less than 10% (w/w) (*e.g.*, no more than 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% (w/w), or less). In another example, the sample or the enriched population of circular RNA is substantially free of one or more impurities or by-products if the one or more impurities or by-products are present in an amount that is less than 5% (w/w) (*e.g.*, no more than 4%, 3%, 2%, 1% (w/w) or less). In yet another example, the sample or the enriched population of circular RNA is substantially free of one or more impurities or by-products if the one or more impurities or by-products are present in an amount that is less than 1% (no more than 0.9%, 0.8%, 0.7%, 0.6%, 0.5%, 0.4%, 0.3%, 0.2%, 0.1% (w/w), or less).

As used herein, a "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.

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 nws gapdna, and for proteins the default scoring matrix is Blosum62 (Henikoff & Henikoff, 1992, PNAS 89, 915-19). 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, "structured" with regard to RNA refers to an RNA sequence that is predicted by the RNAFold software or similar predictive tools to form a structure (*e.g.*, a hairpin loop) with itself or other sequences in the same RNA molecule.

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), *Paleaognathae* (*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 mollusca. 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 terms "treat" and "treating" refer to a prophylactic or therapeutic treatment of a disease or disorder (*e.g.*, an infectious disease, a cancer, a toxicity, or an allergic reaction) 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 disease or disorder, stabilizing (*i.e.*, not worsening) the state of the disease or disorder, or preventing the spread of the disease or disorder as compared to the state or the condition of the disease or disorder in the absence of the therapeutic treatment. Embodiments include treating plants to control a disease or adverse condition caused by or associated with an invertebrate pest or a microbial (*e.g.*, bacterial, fungal, oomycete, or viral) pathogen. Embodiments include treating a plant to increase the plant's innate defense or immune capability to tolerate pest or pathogen pressure.

As used herein, a "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, "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, a "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 "therapeutic polypeptide" refers to a polypeptide that when administered to or expressed in a subject provides some therapeutic benefit. In embodiments, a therapeutic polypeptide is used to treat or prevent a disease, disorder, or condition in a subject by administration of the therapeutic peptide to a subject or by expression in a subject of the therapeutic polypeptide. In alternative embodiments, a therapeutic polypeptide is expressed in a cell and the cell is administered to a subject to provide a therapeutic benefit.

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.

As used herein, the term "yield" refers to the relative amount of an analyte (*e.g.*, a population of circular polyribonucleotides) obtained after a purification step or process as compared to the amount of

analyte in the starting material (*e.g.*, a mixed population of polyribonucleotides, such as, *e.g.*, circular and linear polyribonucleotides) (w/w). The yield may be expressed as a percentage. In the context of the disclosure, the amount of analyte (*e.g.*, circular polyribonucleotides) in the starting material and analyte obtained after the purification step can be measured using an assay (*e.g.*, gel electrophoresis or spectrophotometry). The methods of the disclosure can be used to produce a yield of an enriched population of circular polyribonucleotides of about 20% (w/w) or greater relative to the amount present in the starting material, *e.g.*, mixed population of polyribonucleotides. For example, the methods can be used to produce a yield of purified circular polyribonucleotides of about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 65%, 70%, 75%, 80%, 85%, or 90% (w/w) or greater.

Brief Description of the Drawings

FIG. 1 is a schematic drawing showing a method as described herein. On the left is a mixture of linear and circular polyribonucleotides, some of which contain a target region. An oligonucleotide conjugated to a first capture agent is added to the mixture of polyribonucleotides and hybridizes to the polyribonucleotides that contain the target region. A second capture agent that is conjugated to a particle binds to the first capture agent, thereby separating the polyribonucleotides containing the target region from polyribonucleotides that lack the target region.

FIG. 2 is a gel showing linear byproducts in an in vitro transcription (IVT) mixture in which circular RNA was generated by self-splicing. The gel shows the desired circular RNA product, unspliced linear RNA, partly spliced linear RNA, nicked circular RNA, and spliced introns.

FIG. 3 is a schematic drawing showing a design of the method for removing linear RNA byproducts from circular RNA generated by self-splicing.

FIG. 4 is a gel showing enrichment of circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions. The construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo, an exon fragment 1 (E1), and a 5' half of a catalytic intron. The open reading frame (ORF) is a model protein: gaussia luciferase (Gluc).

FIG. 5 is a gel showing enrichment of circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions. The construct was designed to include, from 5' to 3': a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo, an exon fragment 1 (E1), and a 5' half of a catalytic intron. The ORF is firefly luciferase (Fluc).

FIG. 6 is a gel showing enrichment of circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions.

FIG. 7 is a gel showing two different approaches for enrichment of circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions. One method is incubating RNA-oligo mixture with Streptavidin-SEPHAROSE® bead in a tube (Batch method), the other is prepacking bead in a column and passing through RNA-oligomer mixture by gravity (Column method).

FIG. 8 is a gel showing further enrichment of circular RNA by consecutive linear RNA pull down.

FIGS. 9A and 9B are gels showing the effect of salt concentration in binding buffer for circular RNA enrichment by capturing linear byproducts *via* oligo-streptavidin interactions. **FIG. 9A** shows a construct designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide

cargo including an ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron. The ORF is hEPO. **FIG. 9B** shows a similar construct as **FIG. 9A** but the ORF is a SARS-CoV-2 spike protein (Spike).

FIG. 10 is a gel showing enhanced expression from circular RNA purified by linear RNA pull down method for enriching circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions.

5 The construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo including an ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron. The ORF is a model protein: gaussia luciferase (Gluc)

FIG. 11 is a gel showing enhanced expression from circular RNA purified by linear RNA pull down method for enriching circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions.

10 The construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo including an ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron. The ORF is hEPO.

FIG. 12 is a schematic drawing showing exemplary oligomer designs for the 3' half-intron (#1 to #4) and for the 5' half-intron (#5 and #6) that are used in linear pull-down methods described herein.

15 **FIGS. 13A** and **13B** are gels showing the effect on circular RNA enrichment by linear RNA pull down by capturing linear byproducts *via* oligo-streptavidin interactions using a reduced number of oligomers.

FIGS. 14A and **14B** are gels showing the effect on circular RNA enrichment by linear RNA pull down by capturing linear byproducts *via* oligo-streptavidin interactions using a combination of two oligos (i.e., an oligomer for 3' half-intron and an oligomer for 5' half-intron).

FIG. 15 is a bar graph showing expression from circular RNAs purified by linear RNA pull down method for enriching circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions.

FIG. 16 is a schematic drawing showing exemplary single oligomers that target both the 3' half-intron and the 5' half-intron.

25 **FIGS. 17A** and **17B** are gels showing the effect on circular RNA enrichment by linear RNA pull down by capturing linear byproducts *via* oligo-streptavidin interactions using a single oligomer that targets both the 3' half-intron and the 5' half-intron.

Detailed Description

30 The present disclosure describes compositions and methods for processing, *e.g.*, purifying, ribonucleotides. Polyribonucleotides, such as linear or circular polyribonucleotides may be used for a variety of engineering or therapeutic purposes. However, when polyribonucleotides are generated *via* certain biological reactions, various impurities, by-products, or incomplete products may be present. The present invention features methods useful to reduce or remove these impurities, byproducts, or
35 incomplete products from a sample in order to produce compositions with a desired polyribonucleotide composition, amount, and/or purity, or a population containing a plurality of polyribonucleotides with a desired polyribonucleotide composition, amount, and/or purity.

In certain embodiments, the methods are useful for purifying a polyribonucleotide that has undergone a splicing reaction. In such an embodiment, the methods may be used to separate spliced
40 polyribonucleotides from non-spliced polyribonucleotides or non-spliced polyribonucleotides from spliced polyribonucleotides. In some embodiments, the methods may be used to separate circular

polyribonucleotides (*e.g.*, that have been spliced) from linear polyribonucleotides or linear polyribonucleotides from circular polyribonucleotides. Such purified compositions containing a desired polyribonucleotide may be useful for various downstream applications, such as delivering a polynucleotide cargo (*e.g.*, encoding a gene or protein) to a target cell. The compositions and methods are described in more detail below.

Methods

In some embodiments, the methods described herein include separating a polyribonucleotide having a target region from a plurality of polyribonucleotides. The method includes providing a sample that includes the plurality of polyribonucleotides. A subset of the plurality of polyribonucleotides have the target region. The method further includes contacting the sample with an oligonucleotide that hybridizes to the target region and separating the polyribonucleotide having the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample (**FIG. 1**).

In some embodiments, the methods described herein include the step of separating a linear polyribonucleotide having a target region from a plurality of polyribonucleotides that includes a mixture of linear polyribonucleotides and circular polyribonucleotides. In this method, a sample that includes the plurality of polyribonucleotides is provided. A subset of the plurality of linear polyribonucleotides in the sample have the target region. The method further includes the step of contacting the sample with an oligonucleotide that hybridizes to the target region and separating the linear polyribonucleotide having the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample. In some embodiments, polyribonucleotide having the target region lacks a polyA sequence (*e.g.*, polyA tail). In some embodiments, the target region is not located at a 3' or 5' terminus of the polyribonucleotide having the target region. The circular polyribonucleotides or a subset thereof may lack the target region.

In some embodiments, the methods described herein include the step of separating a polyribonucleotide having a target region from a plurality of polyribonucleotides. The method includes providing a sample that includes the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides have the target region. The target region is not located at a 3' or 5' terminus of the polyribonucleotide having the target region (*e.g.*, the target region is located internally on the polyribonucleotide). The method further includes contacting the sample with an oligonucleotide that hybridizes to the target region and separating the polyribonucleotide having the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample.

In some embodiments, the methods described herein include separating polyribonucleotides by differential binding affinity to a target region. Differential binding may be achieved when the target binding region is present within differential secondary structural elements that modulate access to a target site for binding. For example, an oligonucleotide may bind to a target region of a polyribonucleotide in a first structural conformation with a first affinity and to a target region of the polyribonucleotide in a second structural conformation with a second affinity, *e.g.*, that is different from the first affinity. In some embodiments, differential binding or secondary structural elements may occur when the target region is present within a linear versus a circular polyribonucleotide, or in a polyribonucleotide that exists in two or more distinct structural conformations. For example, an oligonucleotide may bind to a target region of a

linear polyribonucleotide with a first binding affinity and to the target region of a circular polyribonucleotide with a second binding affinity. The first binding affinity may be greater than the second binding affinity. Alternatively, the first binding affinity may be less than the second binding affinity. A lower binding affinity allows the oligonucleotide to preferentially bind the target, *e.g.*, over the target in a conformation with the greater binding affinity.

In some embodiments, the methods described herein include separating a polyribonucleotide in a first conformation having a target region from a plurality of polyribonucleotides in a second conformation having the target region. The method includes providing a sample including the plurality of polyribonucleotides. The method further includes contacting the sample with an oligonucleotide. The oligonucleotide hybridizes to the target region of the polynucleotide in the first conformation with a first binding affinity, and the oligonucleotide hybridizes to the target region of the polynucleotide in the second conformation with a second binding affinity that is different from the first binding affinity and separating the polyribonucleotide in the first conformation having the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the second conformation in the sample. In some embodiments, the first binding affinity is less than the second binding affinity, and the oligonucleotide preferentially binds the polyribonucleotide in the first conformation.

In some embodiments, the method includes the step of contacting the polyribonucleotides with an oligonucleotide conjugated to a particle (*e.g.*, with or without a capture agent).

In some embodiments, the methods described herein include the step of separating a linear polyribonucleotide having a target region from a plurality of circular polyribonucleotides having the target region. The method includes providing a sample including the plurality of polyribonucleotides. The method further includes contacting the sample with an oligonucleotide. The oligonucleotide hybridizes to the target region of the linear polynucleotide with a first binding affinity, and the oligonucleotide hybridizes to the target region of the circular polynucleotide with a second binding affinity that is different from the first binding affinity and separating the linear polyribonucleotide having the target region that is hybridized to the oligonucleotide from the plurality of circular polyribonucleotides in the sample. In some embodiments, the method includes the step of contacting the polyribonucleotides with an oligonucleotide conjugated to a particle (*e.g.*, with or without a capture agent).

In some embodiments of the methods described herein, the oligonucleotide is conjugated to a first capture agent. In some embodiments, the method further includes the step of providing a second capture agent that binds the first capture agent. The second capture agent may be conjugated to a particle. In some embodiments, the first capture agent is conjugated to a particle.

In some embodiments, the oligonucleotide is conjugated to a particle.

In some embodiments, the methods described herein include the step of separating a polyribonucleotide having a target region from a plurality of polyribonucleotides. The method includes providing a sample that includes the plurality of polyribonucleotides and an oligonucleotide conjugated to a first capture agent. A subset of the plurality of polyribonucleotides have the target region, and the oligonucleotide hybridizes to the target region. The method further includes the step of contacting the sample with a second capture agent that binds to the first capture agent and separating the polyribonucleotide having the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample.

In some embodiments, the methods described herein include the step of separating a polyribonucleotide in a first conformation having a target region from a plurality of polyribonucleotides having the target region in a second conformation. The method includes providing a sample including the plurality of polyribonucleotides and an oligonucleotide conjugated to a first capture agent. The
5 oligonucleotide hybridizes to the target region of the polynucleotide in the first conformation with a first binding affinity, and the oligonucleotide hybridizes to the target region of the polynucleotide in the second conformation with a second binding affinity that is different from the first binding affinity. The method further includes the step of contacting the sample with a second capture agent that binds to the first capture agent and separating the polyribonucleotide in the first conformation having the target region that
10 is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the second conformation in the sample. In some embodiments, the first binding affinity is less than the second binding affinity, and the oligonucleotide preferentially binds the polyribonucleotide in the first conformation.

In some embodiments, the methods described herein include the step of separating a linear polyribonucleotide having a target region from a plurality of circular polyribonucleotides including the
15 target region. The method includes providing a sample including the plurality of polyribonucleotides and an oligonucleotide conjugated to a first capture agent. The oligonucleotide hybridizes to the target region of the linear polynucleotide with a first binding affinity, and the oligonucleotide hybridizes to the target region of the circular polynucleotide with a second binding affinity that is different from the first binding affinity. The method further includes the step of contacting the sample with a second capture agent that
20 binds to the first capture agent and separating the linear polyribonucleotide having the target region that is hybridized to the oligonucleotide from the plurality of circular polyribonucleotides in the sample.

In some embodiments, the methods described herein include the step of separating a polyribonucleotide having a target region from a plurality of polyribonucleotides, *e.g.*, using an oligonucleotide without a first or second capture agent. The oligonucleotide may be directly conjugated to
25 a particle. The method includes providing a sample including the plurality of polyribonucleotides and an oligonucleotide conjugated to the particle. A subset of the plurality of polyribonucleotides have a target region, and the oligonucleotide hybridizes to the target region.

In some embodiments of any of the methods described herein, the target region is not located at a 5' or 3' terminus of the polyribonucleotide having the target region. For example, the target region may
30 be internally located on the polyribonucleotide. In some embodiments, the target region is not located at a 3' terminus of the polyribonucleotide.

In some embodiments of any of the methods described herein, the target region is located at a 5' or 3' terminus of the polyribonucleotide and the target region does not contain a polyA sequence. In
some embodiments, the target region is located at a 3' terminus of the polyribonucleotide and does not
35 contain a polyA sequence.

In some embodiments of any of the methods described herein, the target region does not contain a polyA sequence (*e.g.*, polyA tail).

In some embodiments of any of the methods described herein, separating includes immobilizing the oligonucleotide. The method may include, for example, immobilizing the oligonucleotide, the first
40 capture agent, the second capture agent, the particle, or a combination thereof.

In some embodiments, the particle is a magnetic particle. The method may include applying a force to the magnetic particle, such as a magnetic force. The particle or bead may be, *e.g.*, a crosslinked agarose, *e.g.*, SEPHAROSE®, bead. The method may include applying a force to the bead or particle, such as a mechanical, optical, centrifugal, or acoustic force.

5 In some embodiments, the polyribonucleotide having the target region includes an intron or portion thereof (*e.g.*, a half-intron). The target region may include an intron or portion thereof. In some embodiments, the intron or portion thereof is a catalytic intron (*e.g.*, a Group I catalytic intron or a Group II catalytic intron) or a portion thereof. In some embodiments the intron or portion thereof has a length of at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100
10 nucleotides. In some embodiments the intron or portion thereof has a length of 20 to 500, 20 to 400, 20 to 300, 20 to 200, 20 to 100, 50 to 500, 50 to 400, 50 to 300, 50 to 200, 100 to 500, 100 to 400, 100 to 300, or 100 to 200 nucleotides.

In some embodiments, the target region includes a half-intron (*e.g.*, a 5' or 3' portion of a catalytic intron). In some embodiments, the target region includes a 5' half-intron (*e.g.*, a 5' half-intron
15 corresponding to a 5' portion of a catalytic intron). In some embodiments, the target region includes a 3' half-intron (*e.g.*, a 3' half-intron corresponding to a 3' portion of a catalytic intron). In some embodiments, the target region includes a 5' half of a Group I catalytic intron. In some embodiments, the 5' half of the Group I catalytic intron is from a 5' portion of a cyanobacterium *Anabaena* pre-tRNA-Leu gene, a *Tetrahymena* pre-rRNA, a T4 phage td gene, or a variant thereof. In some embodiments, the target
20 region includes a 3' half of a Group I catalytic intron. In some embodiments, the 3' half of the Group I catalytic intron is selected from a 3' portion from a cyanobacterium *Anabaena* pre-tRNA-Leu gene, a *Tetrahymena* pre-rRNA, a T4 phage td gene, or a variant thereof.

As described herein, the methods may be used to separate, *e.g.*, spliced from non-spliced polyribonucleotides. In some embodiments, the methods described herein include separating a spliced
25 polyribonucleotide from a non-spliced or partially spliced polyribonucleotide. In some embodiments, the spliced polyribonucleotide is a circular polyribonucleotide. In some embodiments, the spliced polyribonucleotide is a linear polyribonucleotide. In some embodiments, the spliced polyribonucleotide lacks an intron or portion thereof, *e.g.*, following a splicing (*e.g.*, self-splicing) event during generation. In some embodiments, the polyribonucleotide having the intron or portion thereof is a linear
30 polyribonucleotide.

In some embodiments, the method further includes washing the captured polyribonucleotide having the target region and/or the capture agent (*e.g.*, first and/or second capture agent) one or more (*e.g.*, two, three, four five, or more) times. The washing may occur after the contacting and/or after separating step.

35 In some embodiments, the method further includes performing a first elution step to release the captured polyribonucleotide includes the target region and/or the capture agent (*e.g.*, first and/or second capture agent) from the polyribonucleotide having the target region. The first elution step may include adding a first buffer and/or heating the sample, *e.g.*, to at least 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, or higher.

40 In some embodiments, the method further includes performing a second elution step. The second elution step may include adding a second buffer and/or heating the sample, *e.g.*, to at least 50 °C,

55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C, or higher. In some embodiments, the second buffer includes a denaturing agent, *e.g.*, formamide or urea. The second buffer may include, *e.g.*, from about 40% to about 60% formamide (*e.g.*, about 40%, 45%, 50%, 55%, or 60% formamide).

5 In some embodiments, the method includes incubating the sample with the oligonucleotide and/or capture agent (*e.g.*, first and/or second capture agent) for at least ten (*e.g.*, at least 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, or more) minutes.

In some embodiments, the method includes collecting a portion of the sample that is not bound by the capture agent (*e.g.*, first and/or second capture agent) and/or the oligonucleotide.

10 In some embodiments, the method includes providing a plurality of oligonucleotides, wherein each oligonucleotide hybridizes to a distinct target region. Each oligonucleotide may be, *e.g.*, conjugated to a first capture agent or a particle, *e.g.*, a magnetic particle or a bead.

In some embodiments, the method includes providing an oligonucleotide that hybridizes to multiple target regions. In some embodiments, the method includes providing an oligonucleotide that hybridizes to a 3' half intron and a 5' half intron.

15 In some embodiments, the method includes providing a first oligonucleotide that hybridizes to a 3' half-intron and a second oligonucleotide that hybridizes to a 5' half-intron. In some embodiments, the method includes providing a plurality of oligonucleotides that each hybridize to a distinct region on the 3' half-intron and/or the 5' half-intron.

20 In some embodiments, the method includes providing the oligonucleotide at a molar ratio of 10:1 to 1:10 (*e.g.*, 10:1, 5:1, 2:1, 1:2, 1:5, or 1:10) to the polyribonucleotide.

25 In some embodiments, the method includes providing a sample of particles, *e.g.*, beads, *e.g.*, magnetic beads. The particles may be present in a vessel, *e.g.*, a microcentrifuge tube, or packed in a column. The particles may be conjugated to the oligonucleotide. The method may include flowing the mixture of polyribonucleotides (*e.g.*, with the oligonucleotide conjugated to the first capture agent) over the column containing the particles. As such, the polyribonucleotides bound by the oligonucleotide will bind the column. In some embodiments, the particles are conjugated to the second capture agent, *e.g.*, which is configured to bind the first capture agent conjugated to the oligonucleotide. In other embodiments, the particles are conjugated directly to an oligonucleotide, *e.g.*, configured to hybridize to the target region of the polyribonucleotide.

30 In some embodiments, *e.g.*, when using a magnetic particle, the method may include pelleting the magnetic particles, *e.g.*, in a vessel (*e.g.*, microcentrifuge tube) by providing a permanent magnet.

35 In some embodiments, the methods described herein enrich an amount of the desired polyribonucleotide in the sample. For example, the method may enrich the amount of the desired (*e.g.*, spliced) polyribonucleotide by at least 10%, (*e.g.*, at least 20%, 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 99%, or more) relative to the sample prior to purification.

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

Oligonucleotides

The oligonucleotides described herein are configured to hybridize to a target region of a polyribonucleotide. In some embodiments, the oligonucleotide has at least 80% (*e.g.*, at least 85%, 90%, 95%, 97%, 99%, or 100%) complementarity an equal length portion of the target region. In some
 5 embodiments, the oligonucleotide has at most 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 mismatch to the target region of the polyribonucleotide. In some embodiments, the oligonucleotide has no mismatches to the target region. In some embodiments the oligonucleotide may be a modified oligonucleotide (*e.g.*, having modified phosphate, sugar, or base). In some embodiments, the oligonucleotide contains a portion configured to hybridize to the target region and a portion that does not hybridize to the target region (*e.g.*,
 10 a terminal region).

The oligonucleotide may be, for example, at least 5 nucleotides (*e.g.*, at least 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 nucleotides) in length. In some embodiments, the oligonucleotide is, *e.g.*, from 5-100, 5-95, 10-90, 10-80, 12-60, 15-50, 15-40, 15-30, 18-30, 20-25, or 20-22 nucleotides in length.

The oligonucleotide may have, for example, a GC content of from 30-70%, *e.g.*, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 70%. The oligonucleotide may have a melting temperature (T_m) of, for example, from about 45 °C to about 75 °C, *e.g.*, about 46 °C, 47 °C, 48 °C, 49 °C, 50 °C, 51 °C, 52 °C, 53 °C, 54 °C, 55 °C, 56 °C, 57 °C, 58 °C, 59 °C, 60 °C, 61 °C, 62 °C, 63 °C, 64 °C, 65 °C, 66 °C, 67 °C, 68 °C, 69 °C, 70 °C, 71 °C, 72 °C, 73 °C, 74 °C, or 75 °C.

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Capture Agents

As described herein, an oligonucleotide and/or a particle may be conjugated to a capture agent. A capture agent is configured to bind to, *e.g.*, capture, a target molecule. A capture agent or set of capture agents, *e.g.*, a first and/or second capture agent, may include any affinity pair, such as an
 25 antibody or antigen-binding fragment thereof and an antigen. Similarly, an affinity pair may include a receptor or fragment thereof and its cognate ligand. The first capture agent and second capture agent can be any two molecules configured to interact with sufficient binding energy to allow a covalent or noncovalent (*e.g.*, ionic or van der Waals force) interaction.

An oligonucleotide used in a composition or method described herein may be conjugated to a first
 30 capture agent. In some embodiments, the first capture agent includes an antigen. The antigen may be, for example, biotin.

As described herein, a capture agent or an oligonucleotide (*e.g.*, oligonucleotide conjugated to a capture agent) may be bound or configured to be bound by a second capture agent. In some
 35 embodiments, the second capture agent includes an antibody or antigen-binding fragment thereof, *e.g.*, configured to bind to an antigen. The second capture agent may be, for example, streptavidin. In some embodiments, the oligonucleotide is not conjugated to or does not include a first capture agent, and the second capture agent binds directly to the oligonucleotide.

In some embodiments, the first capture agent is an antibody or antigen-binding fragment thereof. In some embodiments, the second capture agent is an antigen.

40

Particles

The oligonucleotides and/or capture agents described herein may be conjugated to a particle, *e.g.*, a magnetic particle or a bead. In some embodiments, the oligonucleotide and/or capture agent is conjugated to a plurality of particles. In some embodiments, a particle is conjugated to a plurality of capture agents and/or oligonucleotides.

Magnetic particles include at least one component that is responsive to a magnetic force. A magnetic particle may be entirely magnetic or may contain components that are non-magnetic. A magnetic particle may be a magnetic bead, *e.g.*, a substantially spherical magnetic bead. The magnetic particle may be entirely magnetic or may contain one or more magnetic cores surrounded by one or more additional materials, such as, for example, one or more functional groups and/or modifications for binding one or more target molecules. In some examples, a magnetic particle may contain a magnetic component and a surface modified with one or more silanol groups. Magnetic particles of this type may be used for binding target nucleic acid molecules.

A particle, *e.g.*, a magnetic particle or a bead, may be porous, non-porous, hollow, solid, semi-solid, semi-fluidic, fluidic, and/or a combination thereof. In some instances, a particle, *e.g.*, a bead, may be dissolvable or degradable. In some cases, a particle, *e.g.*, a bead, may not be degradable. In some embodiments, the bead is composed of crosslinked agarose, *e.g.*, SEPHAROSE®.

A particle, *e.g.*, a magnetic particle or a bead, may include natural and/or synthetic materials. For example, a particle, *e.g.*, a bead, can include a natural polymer, a synthetic polymer or both natural and synthetic polymers. Examples of natural polymers include proteins and sugars such as deoxyribonucleic acid, rubber, cellulose, starch (*e.g.*, amylose, amylopectin), proteins, enzymes, polysaccharides, silks, polyhydroxyalkanoates, chitosan, dextran, collagen, carrageenan, ispaghula, acacia, agar, gelatin, shellac, sterculia gum, xanthan gum, corn sugar gum, guar gum, gum karaya, agarose, alginic acid, alginate, or natural polymers thereof. Examples of synthetic polymers include acrylics, nylons, silicones, spandex, viscose rayon, polycarboxylic acids, polyvinyl acetate, polyacrylamide, polyacrylate, polyethylene glycol, polyurethanes, polylactic acid, silica, polystyrene, polyacrylonitrile, polybutadiene, polycarbonate, polyethylene, polyethylene terephthalate, poly(chlorotrifluoroethylene), poly(ethylene oxide), poly(ethylene terephthalate), polyethylene, polyisobutylene, poly(methyl methacrylate), poly(oxyethylene), polyformaldehyde, polypropylene, polystyrene, poly(tetrafluoroethylene), poly(vinyl acetate), poly(vinyl alcohol), poly(vinyl chloride), poly(vinylidene dichloride), poly(vinylidene difluoride), poly(vinyl fluoride) and/or combinations (*e.g.*, co-polymers) thereof. Beads may also be formed from materials other than polymers, including lipids, micelles, ceramics, glass-ceramics, material composites, metals, other inorganic materials, and others.

Cross-linking may be permanent or reversible, depending upon the particular cross-linker used. Reversible cross-linking may allow for the polymer to linearize or dissociate under appropriate conditions. In some cases, reversible cross-linking may also allow for reversible attachment of a material bound to the surface of a bead.

Particles, *e.g.*, beads or magnetic particles, may be of uniform size or heterogeneous size. In some cases, the diameter of a particle, *e.g.*, a bead, may be at least about 1 μm , 5 μm , 10 μm , 20 μm , 30 μm , 40 μm , 50 μm , 60 μm , 70 μm , 80 μm , 90 μm , 100 μm , 250 μm , 500 μm , 1 mm, 2 mm, 3 mm, 4 mm, 5 mm, 6 mm, 7 mm, 8 mm, 9 mm, 10 mm, or greater. In some cases, a particle, *e.g.*, a bead, may have a

diameter of less than about 1 μm , 5 μm , 10 μm , 20 μm , 30 μm , 40 μm , 50 μm , 60 μm , 70 μm , 80 μm , 90 μm , 100 μm , 250 μm , 500 μm , 1 mm, 2 mm, 3 mm, 4 mm, 5 mm, 6 mm, 7 mm, 8 mm, 9 mm, 10 mm or less. In some cases, a particle, *e.g.*, a bead, may have a diameter in the range of about 40-75 μm , 30-75 μm , 20-75 μm , 40-85 μm , 40-95 μm , 20-100 μm , 10-100 μm , 1-100 μm , 20-250 μm , or 20-500 μm , 500 μm -1 mm, 1 mm-2 mm, 1-5 mm, or 1-10 mm.

Particles may be of any suitable shape. Examples of particles, *e.g.*, magnetic particles or beads, shapes include, but are not limited to, spherical, non-spherical, oval, oblong, amorphous, circular, cylindrical, and variations thereof.

10 Linkers

In some embodiments, a linker is used to conjugate two or more components used in a composition or method described herein. For example, a linker may be used to conjugate an oligonucleotide to a capture agent, a capture agent to a particle (*e.g.*, a bead), an oligonucleotide to a particle (*e.g.*, a bead), or any combination or variation thereof. In some embodiments, the oligonucleotide is conjugated to the first capture agent with a chemical linker. The chemical linker may be conjugated to a 3' end or a 5' end of the oligonucleotide. Alternatively, the chemical linker may be conjugated to an interior region of the oligonucleotide.

In some embodiments, the second capture agent is conjugated to a particle. The particle may be, for example, a magnetic particle or a bead. The bead may be, *e.g.*, a crosslinked agarose, *e.g.*, a SEPHAROSE®, bead. In some embodiments, an oligonucleotide is conjugated directly to a particle (*e.g.*, a bead, *e.g.*, a magnetic bead or crosslinked agarose, *e.g.*, SEPHAROSE® bead).

A chemical linker provides space, rigidity, and/or flexibility between, for example, an oligonucleotide and a capture agent (*e.g.*, first capture agent) and/or a capture agent (*e.g.*, second capture agent) and a particle. In some embodiments, a linker may be a bond, *e.g.*, a covalent bond, *e.g.*, an amide bond, a disulfide bond, a C-O bond, a C-N bond, a N-N bond, a C-S bond, or any kind of bond created from a chemical reaction, *e.g.*, chemical conjugation. In some embodiments, a linker includes no more than 250 atoms (*e.g.*, 1-2, 1-4, 1-6, 1-8, 1-10, 1-12, 1-14, 1-16, 1-18, 1-20, 1-25, 1-30, 1-35, 1-40, 1-45, 1-50, 1-55, 1-60, 1-65, 1-70, 1-75, 1-80, 1-85, 1-90, 1-95, 1-100, 1-110, 1-120, 1-130, 1-140, 1-150, 1-160, 1-170, 1-180, 1-190, 1-200, 1-210, 1-220, 1-230, 1-240, or 1-250 atom(s); 250, 240, 230, 220, 210, 200, 190, 180, 170, 160, 150, 140, 130, 120, 110, 100, 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 28, 26, 24, 22, 20, 18, 16, 14, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 atom(s)). In some embodiments, a linker includes no more than 250 non-hydrogen atoms (*e.g.*, 1-2, 1-4, 1-6, 1-8, 1-10, 1-12, 1-14, 1-16, 1-18, 1-20, 1-25, 1-30, 1-35, 1-40, 1-45, 1-50, 1-55, 1-60, 1-65, 1-70, 1-75, 1-80, 1-85, 1-90, 1-95, 1-100, 1-110, 1-120, 1-130, 1-140, 1-150, 1-160, 1-170, 1-180, 1-190, 1-200, 1-210, 1-220, 1-230, 1-240, or 1-250 non-hydrogen atom(s); 250, 240, 230, 220, 210, 200, 190, 180, 170, 160, 150, 140, 130, 120, 110, 100, 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 28, 26, 24, 22, 20, 18, 16, 14, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 non-hydrogen atom(s)). In some embodiments, the backbone of a linker includes no more than 250 atoms (*e.g.*, 1-2, 1-4, 1-6, 1-8, 1-10, 1-12, 1-14, 1-16, 1-18, 1-20, 1-25, 1-30, 1-35, 1-40, 1-45, 1-50, 1-55, 1-60, 1-65, 1-70, 1-75, 1-80, 1-85, 1-90, 1-95, 1-100, 1-110, 1-120, 1-130, 1-140, 1-150, 1-160, 1-170, 1-180, 1-190, 1-200, 1-210, 1-220, 1-230, 1-240, or 1-250 atom(s); 250, 240, 230, 220, 210, 200, 190, 180, 170, 160, 150, 140, 130, 120, 110, 100, 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30,

28, 26, 24, 22, 20, 18, 16, 14, 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 atom(s)). The “backbone” of a linker refers to the atoms in the linker that together form the shortest path from one part of the conjugate to another part of the conjugate. The atoms in the backbone of the linker are directly involved in linking one part of the conjugate to another part of the conjugate. For examples, hydrogen atoms attached to carbons in the backbone of the linker are not considered as directly involved in linking one part of the conjugate to another part of the conjugate.

In some embodiments, a linker may include a synthetic group derived from, *e.g.*, a synthetic polymer (*e.g.*, a polyethylene glycol (PEG) polymer). The chemical linker may include, *e.g.*, triethylene glycol (TEG). In some embodiments, a linker may include one or more amino acid residues. In some embodiments, a linker may be an amino acid sequence (*e.g.*, a 1-25 amino acid, 1-10 amino acid, 1-9 amino acid, 1-8 amino acid, 1-7 amino acid, 1-6 amino acid, 1-5 amino acid, 1-4 amino acid, 1-3 amino acid, 1-2 amino acid, or 1 amino acid sequence). In some embodiments, a linker may include one or more optionally substituted C₁-C₂₀ alkylene, optionally substituted C₁-C₂₀ heteroalkylene (*e.g.*, a PEG unit), optionally substituted C₂-C₂₀ alkenylene (*e.g.*, C₂ alkenylene), optionally substituted C₂-C₂₀ heteroalkenylene, optionally substituted C₂-C₂₀ alkynylene, optionally substituted C₂-C₂₀ heteroalkynylene, optionally substituted C₃-C₂₀ cycloalkylene (*e.g.*, cyclopropylene, cyclobutylene), optionally substituted C₂-C₂₀ heterocycloalkylene, optionally substituted C₄-C₂₀ cycloalkenylene, optionally substituted C₄-C₂₀ heterocycloalkenylene, optionally substituted C₈-C₂₀ cycloalkynylene, optionally substituted C₈-C₂₀ heterocycloalkynylene, optionally substituted C₅-C₁₅ arylene (*e.g.*, C₆ arylene), optionally substituted C₃-C₁₅ heteroarylene (*e.g.*, imidazole, pyridine), O, S, NRi (Ri is H, optionally substituted C₁-C₂₀ alkyl, optionally substituted C₁-C₂₀ heteroalkyl, optionally substituted C₂-C₂₀ alkenyl, optionally substituted C₂-C₂₀ heteroalkenyl, optionally substituted C₂-C₂₀ alkynyl, optionally substituted C₂-C₂₀ heteroalkynyl, optionally substituted C₃-C₂₀ cycloalkyl, optionally substituted C₂-C₂₀ heterocycloalkyl, optionally substituted C₄-C₂₀ cycloalkenyl, optionally substituted C₄-C₂₀ heterocycloalkenyl, optionally substituted C₈-C₂₀ cycloalkynyl, optionally substituted C₈-C₂₀ heterocycloalkynyl, optionally substituted C₅-C₁₅ aryl, or optionally substituted C₃-C₁₅ heteroaryl), P, carbonyl, thiocarbonyl, sulfonyl, phosphate, phosphoryl, or imino.

Covalent conjugation of two or more components in a conjugate using a linker may be accomplished using well-known organic chemical synthesis techniques and methods. Complementary functional groups on two components may react with each other to form a covalent bond. Examples of complementary reactive functional groups include, but are not limited to, *e.g.*, maleimide and cysteine, amine and activated carboxylic acid, thiol and maleimide, activated sulfonic acid and amine, isocyanate and amine, azide and alkyne, and alkene and tetrazine. Site-specific conjugation to a polypeptide may be accomplished using techniques known in the art.

Compositions

As described herein, the invention features a composition that includes a population of polyribonucleotides produced by a method as described herein. The population may include, *e.g.*, a circular polyribonucleotide lacking a target region, and the circular polyribonucleotide includes at least 1% (*e.g.*, at least 5%, *e.g.*, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, or more) (mol/mol) of the total polyribonucleotides in the composition. In

some embodiments, the population has less than 50% (*e.g.*, less than 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1%) (mol/mol) linear polyribonucleotides in the composition.

In other embodiments, the population may include, *e.g.*, a polyribonucleotide in a first conformation having the target region and the polyribonucleotide in a second conformation having the target region, and the polyribonucleotide in the first conformation includes at least 1%, *e.g.*, at least 5%, *e.g.*, at least 10%, at least 20%, at least 30%, or at least 40% (*e.g.*, at least 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, or more) (mol/mol) of the total polyribonucleotides in the composition.

In some embodiments as described herein, the invention features a composition that includes a mixture of polyribonucleotides. A first subset of the mixture includes a circular polyribonucleotide lacking a target region, and a second subset of the plurality of the polyribonucleotides includes a linear polyribonucleotide having the target region. The first subset includes at least 1%, *e.g.*, at least 5%, *e.g.*, at least 10%, at least 20%, at least 30%, or at least 40% (*e.g.*, at least 50%, 60%, 70%, 80%, 90%, 95%, 97%, 99%, or more) (mol/mol) of the total polyribonucleotides in the composition.

In some embodiments as described herein, the invention features a composition that includes a polyribonucleotide having a target region and an oligonucleotide configured to hybridize to the target region, wherein the oligonucleotide is conjugated to a first capture agent (*e.g.*, an antigen, such as biotin). The composition may further include, *e.g.*, a polyribonucleotide lacking the target region. The polyribonucleotide lacking the target region may be, *e.g.*, a circular polyribonucleotide. The composition may further include a second capture agent (*e.g.*, an antibody or antigen binding fragment thereof, such as streptavidin) configured to bind the first capture agent. The second capture agent may be conjugated to a particle, *e.g.*, via a linker.

In some embodiments of any of the compositions as described herein, the linear polyribonucleotide includes an intron or portion thereof. The target region may include the intron or portion thereof. The target region may be located 5' or 3' to the intron or portion thereof.

In some embodiments, the polyribonucleotide may be a modified polyribonucleotide.

In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide 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), or 100% (w/w) pure on a mass basis. Purity may be measured by any one of a number of analytical techniques known to one skilled in the art, such as, but not limited to, the use of separation technologies such as chromatography (using a column, using a paper, using a gel, using HPLC, using UHPLC, etc., or by IC, by SEC, by reverse phase, by anion exchange, by mixed mode, etc.) or electrophoresis (UREA PAGE, chip-based, polyacrylamide gel, RNA, capillary, c-IEF, etc.) with or without pre- or post-separation derivatization methodologies using detection techniques based on mass spectrometry, UV-visible, fluorescence, light scattering, refractive index, or that use silver or dye stains or radioactive decay for detection. Alternatively, purity may be determined without the use of a separation technology by mass spectrometry, by microscopy, by circular dichroism (CD) spectroscopy, by UV or UV-vis spectrophotometry, by fluorometry (*e.g.*, Qubit), by RNase H analysis, by surface plasmon resonance (SPR), or by methods that utilize silver or dye stains or radioactive decay for detection.

In some embodiments, purity can be measured by biological test methodologies (*e.g.*, cell-based or receptor-based tests). In some embodiments, 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) or 100% (w/w) of the total of mass ribonucleotide in the a preparation described herein is contained in circular polyribonucleotide molecules. The percent may be measured by any one of a number of analytical techniques known to one skilled in the art such as, but not limited to, the use of a separation technology such as chromatography (using a column, using a paper, using a gel, using HPLC, using UHPLC, etc., or by IC, by SEC, by reverse phase, by anion exchange, by mixed mode, etc.) or electrophoresis (UREA PAGE, chip-based, polyacrylamide gel, RNA, capillary, c-IEF, etc.) with or without pre- or post-separation derivatization methodologies using detection techniques based on mass spectrometry, UV-visible, fluorescence, light scattering, refractive index, or that use silver or dye stains or radioactive decay for detection. Alternatively, purity may be determined without the use of separation technologies by mass spectrometry, by microscopy, by circular dichroism (CD) spectroscopy, by UV or UV-vis spectrophotometry, by fluorometry (*e.g.*, Qubit), by RNase H analysis, by surface plasmon resonance (SPR), or by methods that utilize silver or dye stains or radioactive decay for detection.

In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a circular polyribonucleotide concentration of at least 0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL, 0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, 50 µg/mL, 60 µg/mL, 70 µg/mL, 80 µg/mL, 100 µg/mL, 200 µg/mL, 300 µg/mL, 500 µg/mL, 1000 µg/mL, 5000 µg/mL, 10,000 µg/mL, 100,000 µg/mL, 200 mg/mL, 300 mg/mL, 400 mg/mL, 500 mg/mL, 600 mg/mL, 650 mg/mL, 700 mg/mL, or 750 mg/mL. In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) is substantially free of mononucleotide or has a mononucleotide content of no more than 1 pg/ml, 10 pg/ml, 0.1 ng/ml, 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, 1000 µg/mL, 5000 µg/mL, 10,000 µg/mL, or 100,000 µg/mL. In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a mononucleotide content from the limit of detection up to 1 pg/ml, 10 pg/ml, 0.1 ng/ml, 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, 1000 µg/mL, 5000 µg/mL, 10,000 µg/mL, or 100,000 µg/mL.

In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has mononucleotide content no more than 0.1% (w/w), 0.2% (w/w), 0.3% (w/w), 0.4% (w/w), 0.5% (w/w), 0.6% (w/w), 0.7% (w/w), 0.8% (w/w), 0.9% (w/w), 1% (w/w), 2% (w/w), 3% (w/w), 4% (w/w), 5% (w/w), 6% (w/w), 7% (w/w), 8% (w/w), 9% (w/w), 10% (w/w), 15% (w/w), 20% (w/w),

25% (w/w), 30% (w/w), or any percentage therebetween of total nucleotides on a mass basis, wherein total nucleotide content is the total mass of deoxyribonucleotide molecules and ribonucleotide molecules.

In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a linear RNA content, *e.g.*, linear RNA counterpart or RNA fragments, 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, 2 mg/ml, 5 mg/ml, 10 mg/ml, 50 mg/ml, 100 mg/ml, 200 mg/ml, 300 mg/ml, 400 mg/ml, 500 mg/ml, 600 mg/ml, 650 mg/ml, 700 mg/ml, or 750 mg/ml. In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a linear RNA content, *e.g.*, linear RNA counterpart or RNA fragments, from the limit of detection of up to 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, 2 mg/ml, 5 mg/ml, 10 mg/ml, 50 mg/ml, 100 mg/ml, 200 mg/ml, 300 mg/ml, 400 mg/ml, 500 mg/ml, 600 mg/ml, 650 mg/ml, 700 mg/ml, or 750 mg/ml.

In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a nicked RNA content of no more than 10% (w/w), 9.9% (w/w), 9.8% (w/w), 9.7% (w/w), 9.6% (w/w), 9.5% (w/w), 9.4% (w/w), 9.3% (w/w), 9.2% (w/w), 9.1% (w/w), 9% (w/w), 8% (w/w), 7% (w/w), 6% (w/w), 5% (w/w), 4% (w/w), 3% (w/w), 2% (w/w), 1% (w/w), 0.5% (w/w), or 0.1% (w/w), or percentage therebetween. In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a nicked RNA content that is as low as zero or is substantially free of nicked RNA.

In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a combined linear RNA and nicked RNA content of no more than 30% (w/w), 25% (w/w), 20% (w/w), 15% (w/w), 10% (w/w), 9% (w/w), 8% (w/w), 7% (w/w), 6% (w/w), 5% (w/w), 4% (w/w), 3% (w/w), 2% (w/w), 1% (w/w), 0.5% (w/w), or 0.1% (w/w), or percentage therebetween. In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a combined nicked RNA and linear RNA content that is as low as zero or is substantially free of nicked and linear RNA.

In some embodiments, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a linear RNA content, *e.g.*, linear RNA counterpart or RNA fragments, of no more than the detection limit of analytical methodologies, such as methods utilizing

mass spectrometry, UV spectroscopic or fluorescence detectors, light scattering techniques, surface plasmon resonance (SPR) with or without the use of methods of separation including HPLC, by HPLC, chip or gel based electrophoresis with or without using either pre or post separation derivatization methodologies, methods of detection that use silver or dye stains or radioactive decay, or microscopy, visual methods or a spectrophotometer.

In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has no more than 0.1% (w/w), 1% (w/w), 2% (w/w), 3% (w/w), 4% (w/w), 5% (w/w), 6% (w/w), 7% (w/w), 8% (w/w), 9% (w/w), 10% (w/w), 15% (w/w), 20% (w/w), 25% (w/w), 30% (w/w), 35% (w/w), 40% (w/w), 45% (w/w), 50% (w/w) of linear RNA, *e.g.*, as measured by the methods in Example 2.

In some embodiments, the linear polyribonucleotide molecules of the circular polyribonucleotide preparation include the linear counterpart or a fragment thereof of the circular polyribonucleotide molecule. In some embodiments, the linear polyribonucleotide molecules of the circular polyribonucleotide preparation include the linear counterpart (*e.g.*, a pre-circularized version). In some embodiments, the linear polyribonucleotide molecules of the circular polyribonucleotide preparation include a non-counterpart or fragment thereof to the circular polyribonucleotide. In some embodiments, the linear polyribonucleotide molecules of the circular polyribonucleotide preparation include a non-counterpart to the circular polyribonucleotide. In some embodiments, the linear polyribonucleotide molecules include a combination of the counterpart of the circular polyribonucleotide and a non-counterpart or fragment thereof of the circular polyribonucleotide. In some embodiments, the linear polyribonucleotide molecules include a combination of the counterpart of the circular polyribonucleotide and a non-counterpart of the circular polyribonucleotide. In some embodiments, a linear polyribonucleotide molecule fragment is a fragment that is at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 11000, 12000, or more nucleotides in length, or any nucleotide number therebetween.

In some embodiments, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has an A260/A280 absorbance ratio from about 1.6 to about 2.3, *e.g.*, as measured by spectrophotometer. In some embodiments, the A260/A280 absorbance ratio is about 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, or any number therebetween. In some embodiments, a circular polyribonucleotide (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide) has an A260/A280 absorbance ratio greater than about 1.8, *e.g.*, as measured by spectrophotometer. In some embodiments, the A260/A280 absorbance ratio is about 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, or greater.

In some embodiments, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) is substantially free of an impurity or byproduct. In various embodiments, the level of at least one impurity or byproduct in a composition including the circular polyribonucleotide is reduced by at least 30% (w/w), at least 40% (w/w), at least 50% (w/w), at least 60%

(w/w), at least 70% (w/w), at least 80% (w/w), at least 90% (w/w), or at least 95% (w/w) as compared to that of the composition prior to purification or treatment to remove the impurity or byproduct. In some embodiments, the level of at least one process-related impurity or byproduct is reduced by at least 30% (w/w), at least 40% (w/w), at least 50% (w/w), at least 60% (w/w), at least 70% (w/w), at least 80% (w/w), at least 90% (w/w), or at least 95% (w/w) as compared to that of the composition prior to purification or treatment to remove the impurity or byproduct. In some embodiments, the level of at least one product-related substance is reduced by at least 30% (w/w), at least 40% (w/w), at least 50% (w/w), at least 60% (w/w), at least 70% (w/w), at least 80% (w/w), at least 90% (w/w), or at least 95% (w/w) as compared to that of the composition prior to purification or treatment to remove the impurity or byproduct. In some embodiments, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) is further substantially free of a process-related impurity or byproduct. In some embodiments, the process-related impurity or byproduct includes a protein (*e.g.*, a cell protein, such as a host cell protein), a deoxyribonucleic acid (*e.g.*, a cell deoxyribonucleic acid, such as a host cell deoxyribonucleic acid), monodeoxyribonucleotide or dideoxyribonucleotide molecules, an enzyme (*e.g.*, a nuclease, such as an endonuclease or exonuclease, or ligase), a reagent component, a gel component, or a chromatographic material. In some embodiments, the impurity or byproduct is selected from: a buffer reagent, a ligase, a nuclease, RNase inhibitor, RNase R, deoxyribonucleotide molecules, acrylamide gel debris, and monodeoxyribonucleotide molecules. In some embodiments, the pharmaceutical preparation includes protein (*e.g.*, cell protein, such as a host cell protein) contamination, impurities, or by-products of less than 0.1 ng, 1 ng, 5 ng, 10 ng, 15 ng, 20 ng, 25 ng, 30 ng, 35 ng, 40 ng, 50 ng, 60 ng, 70 ng, 80 ng, 90 ng, 100 ng, 200 ng, 300 ng, 400 ng, or 500 ng of protein contamination, impurities, or by-products per milligram (mg) of the circular polyribonucleotide molecules.

In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) is substantially free of DNA content *e.g.*, template DNA or cell DNA (*e.g.*, host cell DNA), has a DNA content, as low as zero, or has a DNA content of no more than 1 pg/ml, 10 pg/ml, 0.1 ng/ml, 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, 1000 µg/mL, 5000 µg/mL, 10,000 µg/mL, or 100,000 µg/mL.

In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) is substantially free of DNA content, has a DNA content as low as zero, or has DNA content no more than 0.001% (w/w), 0.01% (w/w), 0.1% (w/w), 1% (w/w), 2% (w/w), 3% (w/w), 4% (w/w), 5% (w/w), 6% (w/w), 7% (w/w), 8% (w/w), 9% (w/w), 10% (w/w), 15% (w/w), 20% (w/w), 25% (w/w), 30% (w/w), 35% (w/w), 40% (w/w), 45% (w/w), 50% (w/w) of total nucleotides on a mass basis, wherein total nucleotide molecules is the total mass of deoxyribonucleotide content and ribonucleotide molecules. In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) is substantially free of DNA content, has DNA content as low as zero, or has DNA content no more than 0.001% (w/w), 0.01% (w/w), 0.1% (w/w), 1% (w/w), 2% (w/w), 3%

(w/w), 4% (w/w), 5% (w/w), 6% (w/w), 7% (w/w), 8% (w/w), 9% (w/w), 10% (w/w), 15% (w/w), 20% (w/w), 25% (w/w), 30% (w/w), 35% (w/w), 40% (w/w), 45% (w/w), 50% (w/w) of total nucleotides on a mass basis as measured after a total DNA digestion by enzymes that digest nucleosides by quantitative liquid chromatography-mass spectrometry (LC-MS), in which the content of DNA is back calculated from a standard curve of each base (i.e., A, C, G, T) as measured by LC-MS.

In an embodiment, a circular polyribonucleotide preparation (e.g., a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a protein (e.g., cell protein (CP), e.g., enzyme, a production-related protein, e.g., carrier protein) contamination, impurities, or by-products of no more than 0.1 ng/ml, 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, or 500 ng/ml. In an embodiment, a circular polyribonucleotide (e.g., a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide) has a protein (e.g., production-related protein such as a cell protein (CP), e.g., enzyme) contamination, impurities, or by-products from the limit of detection of up to 0.1 ng/ml, 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, or 500 ng/ml.

In an embodiment, a circular polyribonucleotide preparation (e.g., a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has a protein (e.g., production-related protein such as a cell protein (CP), e.g., enzyme) contamination, impurities, or by-products of less than 0.1 ng, 1 ng, 5 ng, 10 ng, 15 ng, 20 ng, 25 ng, 30 ng, 35 ng, 40 ng, 50 ng, 60 ng, 70 ng, 80 ng, 90 ng, 100 ng, 200 ng, 300 ng, 400 ng, or 500 ng per milligram (mg) of the circular polyribonucleotide. In an embodiment, a circular polyribonucleotide (e.g., a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide) has a protein (e.g., production-related protein such as a cell protein (CP), e.g., enzyme) contamination, impurities, or by-products from the level of detection up to 0.1 ng, 1 ng, 5 ng, 10 ng, 15 ng, 20 ng, 25 ng, 30 ng, 35 ng, 40 ng, 50 ng, 60 ng, 70 ng, 80 ng, 90 ng, 100 ng, 200 ng, 300 ng, 400 ng, or 500 ng per milligram (mg) of the circular polyribonucleotide.

In an embodiment, a circular polyribonucleotide preparation (e.g., a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) has low levels or is substantially absent of endotoxins, e.g., as measured by the Limulus amoebocyte lysate (LAL) test. In some embodiments, the pharmaceutical preparation or compositions or an intermediate in the production of the circular polyribonucleotides includes less than 20 EU/kg (weight), 10 EU/kg, 5 EU/kg, 1 EU/kg endotoxin, or lacks endotoxin as measured by the Limulus amoebocyte lysate test. In an embodiment, a circular polyribonucleotide composition has low levels or absence of a nuclease or a ligase.

In some embodiments, a circular polyribonucleotide preparation (e.g., a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) includes no greater than about 50% (w/w), 45% (w/w), 40% (w/w), 35% (w/w), 30% (w/w), 25% (w/w), 20% (w/w), 19% (w/w), 18% (w/w), 17% (w/w), 16% (w/w), 15% (w/w), 14% (w/w), 13% (w/w), 12% (w/w), 11% (w/w), 10% (w/w), 9% (w/w), 8% (w/w), 7% (w/w), 6% (w/w), 5%

(w/w), 4% (w/w), 3% (w/w), 2% (w/w), 1% (w/w) of at least one enzyme, *e.g.*, polymerase, *e.g.*, RNA polymerase.

In an embodiment, a circular polyribonucleotide preparation (*e.g.*, a circular polyribonucleotide pharmaceutical preparation or composition or an intermediate in the production of the circular polyribonucleotide preparation) is sterile or substantially free of microorganisms, *e.g.*, the composition or preparation supports the growth of fewer than 100 viable microorganisms as tested under aseptic conditions, the composition or preparation meets the standard of USP <71>, and/or the composition or preparation meets the standard of USP <85>. In some embodiments, the pharmaceutical preparation includes a bioburden of less than 100 CFU/100 ml, 50 CFU/100 ml, 40 CFU/100 ml, 30 CFU/100 ml, 200 CFU/100 ml, 10 CFU/100 ml, or 10 CFU/100 ml before sterilization.

In some embodiments, the circular polyribonucleotide preparation can be further purified using known techniques in the art for removing impurities or byproduct, such as column chromatography or pH, vial inactivation.

Polynucleotides

The present invention features polyribonucleotides that are used in methods of separation and/or purification and present in compositions described herein. The polyribonucleotides described herein may be linear polyribonucleotides, circular polyribonucleotides or a combination thereof. In some embodiments, a circular polyribonucleotide is produced from a linear polyribonucleotide (*e.g.*, by 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 invention features linear deoxyribonucleotides, circular deoxyribonucleotides, linear polyribonucleotides, and circular polyribonucleotides and compositions thereof useful in the production of polyribonucleotides.

Linear polyribonucleotides

The present invention features linear polyribonucleotides that may include one or more of the following: a 3' half-intron; a 3' splice site; a 3' exon; a polyribonucleotide cargo; a 5' exon; a 5' splice site; and a 5' half-intron. In some embodiments, the 3' half-intron corresponds to a 3' portion of a catalytic Group I intron, for example, a catalytic Group I intron from a cyanobacterium *Anabaena* pre-tRNA-Leu gene, a *Tetrahymena* pre-rRNA, a T4 phage td gene, or a variant thereof. In some embodiments, the 5' half-intron corresponds to a 5' portion of a catalytic Group I intron, for example, a catalytic Group I intron from a cyanobacterium *Anabaena* pre-tRNA-Leu gene, a *Tetrahymena* pre-rRNA, a T4 phage td gene, or a variant thereof.

The linear polyribonucleotide may include additional elements, *e.g.*, outside of or between any of elements described above. For example, any of the above elements may be separated by a spacer sequence, as described herein. A target region as described herein may be present in any region of the linear polyribonucleotide as described herein. In some embodiments, the target region is present within an intron or portion thereof.

In certain embodiments, provided herein is a method of generating a linear polyribonucleotide by performing transcription in a cell-free system (*e.g.*, in vitro transcription) using a deoxyribonucleotide (*e.g.*,

a vector, linearized vector, or cDNA) 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 polyribonucleotide).

- 5 A deoxyribonucleotide template may be transcribed to produce a linear polyribonucleotide containing the components described herein. Upon expression, the linear polyribonucleotide may produce a splicing-compatible polyribonucleotide, which may be spliced in order to produce a circular polyribonucleotide, *e.g.*, for subsequent use.

In some embodiments, the linear polyribonucleotide is from 50 to 20,000, *e.g.*, 300 to 20,000 (*e.g.*, 50, 60, 70, 80, 90, 100, 150, 200, 250, 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. The linear polyribonucleotide may be, *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

- 10 In some embodiments, the invention features a circular polyribonucleotide. The circular polyribonucleotide may include a splice junction joining a 5' exon and a 3' exon. A target region as described herein may be present in any region of the circular polyribonucleotide as described herein. The circular polyribonucleotide may lack an intron, *e.g.*, after splicing.

- The circular polynucleotide may further include a polyribonucleotide cargo. The polyribonucleotide cargo may include an expression sequence, a non-coding sequence, or a combination of an expression sequence and a non-coding sequence. The polyribonucleotide cargo may include an expression sequence encoding a polypeptide. The polyribonucleotide may include an IRES operably linked to an expression sequence encoding a polypeptide. In some embodiments, the circular polyribonucleotide further includes a spacer region between the IRES and the 5' exon fragment or the 3' 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, polyA-G, polyA-U, or other heterogeneous or random sequence.

- 25 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 may be of a sufficient size to accommodate a binding site for a ribosome. In some embodiments, the size of a circular polyribonucleotide is a length sufficient to encode useful polypeptides, and thus, lengths of at least 20,000 nucleotides, at least 15,000 nucleotides, at least 10,000 nucleotides, at least 7,500 nucleotides, or at least 5,000 nucleotides, at least 4,000 nucleotides, at least 3,000 nucleotides, at least 2,000 nucleotides, at least 1,000 nucleotides, at least 500 nucleotides, at least 1400 nucleotides, at least 300 nucleotides, at least 200 nucleotides, at least 100 nucleotides may be produced.

In some embodiments, the circular polyribonucleotide includes one or more elements described herein. In some embodiments, the elements may be separated from one another by a spacer sequence. In some embodiments, the elements may be separated from one another by 1 ribonucleotide, 2 nucleotides, about 5 nucleotides, about 10 nucleotides, about 15 nucleotides, about 20 nucleotides, about 30 nucleotides, about 40 nucleotides, about 50 nucleotides, about 60 nucleotides, about 80 nucleotides, about 100 nucleotides, about 150 nucleotides, about 200 nucleotides, about 250 nucleotides, about 300 nucleotides, about 400 nucleotides, about 500 nucleotides, about 600 nucleotides, about 700 nucleotides, about 800 nucleotides, about 900 nucleotides, about 1000 nucleotides, up to about 1 kb, at least about 1000 nucleotides, or any amount of nucleotides therebetween. In some embodiments, one or more elements are contiguous with one another, *e.g.*, lacking a spacer element.

In some embodiments, the circular polyribonucleotide may include one or more repetitive elements. In some embodiments, the circular polyribonucleotide includes one or more modifications described herein. In one embodiment, the circular polyribonucleotide contains at least one nucleoside modification. In one embodiment, up to 100% of the nucleosides of the circular polyribonucleotide are modified. In one embodiment, at least one nucleoside modification is a uridine modification or an adenosine modification.

As a result of its circularization, the circular polyribonucleotide may include certain characteristics that distinguish it from a linear polyribonucleotide. For example, the circular polyribonucleotide may contain a target region that is more or less accessible than a linear polyribonucleotide. In some embodiments, the circular polyribonucleotide may be less susceptible to degradation by exonuclease as compared to a linear polyribonucleotide. As such, the circular polyribonucleotide may be more stable than a linear polyribonucleotide, especially when incubated in the presence of an exonuclease. The increased stability of the circular polyribonucleotide compared with a linear polyribonucleotide makes circular polyribonucleotide more useful as a cell transforming reagent to produce polypeptides and can be stored more easily and for longer than a linear polyribonucleotide. 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 a linear polyribonucleotide, the circular polyribonucleotide may be less susceptible to dephosphorylation when the circular polyribonucleotide is incubated with phosphatase, such as calf intestine phosphatase.

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 a polypeptide. In some embodiments, the polyribonucleotide cargo includes an IRES operably linked to an expression sequence encoding a polypeptide. In some embodiments, the polyribonucleotide cargo includes an expression sequence that encodes a 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 polyribonucleotides 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. In embodiments, the polyribonucleotide cargo includes ones 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.

In some embodiments, polyribonucleotides made as described herein are used as effectors in therapy or agriculture. For example, a circular polyribonucleotide made by the methods described herein (*e.g.*, the cell-free methods described herein) may be administered to a subject (*e.g.*, in a pharmaceutical, veterinary, or agricultural composition). In another example, a circular polyribonucleotide made by the methods described herein (*e.g.*, the cell-free methods described herein) may be delivered to a cell.

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

In some embodiments, the polyribonucleotide cargo includes an open reading frame. In some embodiments, the open reading frame is operably linked to an IRES. The open reading frame may encode an RNA or a polypeptide. In some embodiments, the open reading frame encodes a polypeptide and the polyribonucleotide (*e.g.*, circular polyribonucleotide) provides increased expression (*e.g.*, by at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or more) of the polypeptide, *e.g.*, as compared to a linear polyribonucleotide encoding the polypeptide. In some embodiments, increased purity of the polyribonucleotide, *e.g.*, a circular polyribonucleotide, results in increased expression (*e.g.*, by at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or more) of the polypeptide, *e.g.*, as compared to a population of circular and linear polyribonucleotides.

Polypeptide expression sequences

In some embodiments, the polyribonucleotide described herein (*e.g.*, the polyribonucleotide cargo of a circular polyribonucleotide) includes one or more expression (or coding) sequences, wherein each expression sequence encodes a 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 embodiments, the polypeptide 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 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 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 occurring polypeptides. In some embodiments, the polypeptide is or includes a functional fragment or variant of a reference polypeptide (*e.g.*, an enzymatically active fragment or variant of an enzyme). 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% 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.

Some examples of a polypeptide include, but are not limited to, a fluorescent tag or marker, an antigen, a therapeutic polypeptide, or a polypeptide for agricultural applications.

A therapeutic polypeptide may be a hormone, a neurotransmitter, a growth factor, an enzyme (*e.g.*, oxidoreductase, metabolic enzyme, mitochondrial enzyme, oxygenase, dehydrogenase, ATP - independent enzyme, lysosomal enzyme, desaturase), a cytokine, an antigen binding polypeptide (*e.g.*, antigen binding antibody or antibody-like fragments, such as single chain antibodies, nanobodies or other Ig heavy chain or light chain containing polypeptides), an Fc fusion protein, an anticoagulant, a blood factor, a bone morphogenetic protein, an interferon, an interleukin, and a thrombolytic.

A polypeptide for agricultural applications may be a bacteriocin, a lysin, an antimicrobial polypeptide, an antifungal polypeptide, a nodule C-rich peptide, a bacteriocyte regulatory peptide, a peptide toxin, a pesticidal polypeptide (*e.g.*, insecticidal polypeptide or nematocidal polypeptide), an antigen binding polypeptide (*e.g.*, antigen binding antibody or antibody-like fragments, such as single

chain antibodies, nanobodies or other Ig heavy chain or light chain containing polypeptides), an enzyme (*e.g.*, nuclease, amylase, cellulase, peptidase, lipase, chitinase), a peptide pheromone, and a transcription factor.

In some cases, the polyribonucleotide expresses a non-human protein.

5 In some embodiments, the polyribonucleotide expresses an antibody, *e.g.*, an antibody fragment, or a portion thereof. In some embodiments, the antibody expressed by the circular polyribonucleotide can be of any isotype, such as IgA, IgD, IgE, IgG, IgM. In some embodiments, the circular polyribonucleotide expresses a portion of an antibody, such as a light chain, a heavy chain, a Fc fragment, a CDR (complementary determining region), a Fv fragment, or a Fab fragment, a further portion thereof. In some
10 embodiments, the circular polyribonucleotide expresses one or more portions of an antibody. For instance, the circular polyribonucleotide can include more than one expression sequence, each of which expresses a portion of an antibody, and the sum of which can constitute the antibody. In some cases, the circular polyribonucleotide includes one expression sequence coding for the heavy chain of an antibody, and another expression sequence coding for the light chain of the antibody. In some cases, when the
15 circular polyribonucleotide is expressed in a cell or a cell-free environment, the light chain and heavy chain can be subject to appropriate modification, folding, or other post-translation modification to form a functional antibody.

In embodiments, polypeptides include multiple polypeptides, *e.g.*, multiple copies of one polypeptide sequence, or multiple different polypeptide sequences. In embodiments, multiple
20 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
25 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 embodiments, the polynucleotide cargo includes sequence encoding a cell-penetrating peptide (CPP). Hundreds of CPP sequences have been described; see, *e.g.*, the database of cell-penetrating peptides, CPPsite, publicly available at crdd.osdd.net/raghava/cppsite/. An example of a commonly used CPP sequence is a poly-arginine sequence, *e.g.*, octoarginine or nonoarginine, which can be fused to the C-terminus of the CGI peptide.

In embodiments, the polynucleotide cargo includes sequence encoding a self-assembling
35 peptide; see, *e.g.*, Miki et al. (2021) Nature Communications, 21:3412, DOI: 10.1038/s41467-021-23794-6.

In some embodiments, the expression 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
40 (*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.

Therapeutic polypeptides

In some embodiments, a polyribonucleotide described herein (*e.g.*, the polyribonucleotide cargo of the circular polyribonucleotide) includes at least one expression sequence encoding a therapeutic polypeptide. A therapeutic polypeptide is a polypeptide that when administered to or expressed in a subject provides some therapeutic benefit. Administration to a subject or expression in a subject of a therapeutic polypeptide may be used to treat or prevent a disease, disorder, or condition or a symptom thereof. In some embodiments, the circular polyribonucleotide encodes two, three, four, five, six, seven, eight, nine, ten or more therapeutic polypeptides.

In some embodiments, the polyribonucleotide includes an expression sequence encoding a therapeutic protein. The protein may treat the disease in the subject in need thereof. In some embodiments, the therapeutic protein can compensate for a mutated, under-expressed, or absent protein in the subject in need thereof. In some embodiments, the therapeutic protein can target, interact with, or bind to a cell, tissue, or virus in the subject in need thereof.

A therapeutic polypeptide can be a polypeptide that can be secreted from a cell, or localized to the cytoplasm, nucleus, or membrane compartment of a cell.

A therapeutic polypeptide may be a hormone, a neurotransmitter, a growth factor, an enzyme (*e.g.*, oxidoreductase, metabolic enzyme, mitochondrial enzyme, oxygenase, dehydrogenase, ATP-independent enzyme, lysosomal enzyme, desaturase), a cytokine, a transcription factor, an antigen binding polypeptide (*e.g.*, antigen binding antibody or antibody-like fragments, such as single chain antibodies, nanobodies or other Ig heavy chain or light chain containing polypeptides), an Fc fusion protein, an anticoagulant, a blood factor, a bone morphogenetic protein, an interferon, an interleukin, a thrombolytic, an antigen (*e.g.*, a tumor, viral, or bacterial antigen), a nuclease (*e.g.*, an endonuclease such as a Cas protein, *e.g.*, Cas9), a membrane protein (*e.g.*, a chimeric antigen receptor (CAR), a transmembrane receptor, a G-protein-coupled receptor (GPCR), a receptor tyrosine kinase (RTK), an antigen receptor, an ion channel, or a membrane transporter), a secreted protein, a gene editing protein (*e.g.*, a CRISPR-Cas, TALEN, or zinc finger), or a gene writing protein (see, *e.g.*, International Patent Publication No. WO2020/047124, incorporated in its entirety herein by reference).

In some embodiments, the therapeutic polypeptide is an antibody, *e.g.*, a full-length antibody, an antibody fragment, or a portion thereof. In some embodiments, the antibody expressed by the polyribonucleotide (*e.g.*, circular polyribonucleotide) can be of any isotype, such as IgA, IgD, IgE, IgG,

IgM. In some embodiments, the polyribonucleotide expresses a portion of an antibody, such as a light chain, a heavy chain, a Fc fragment, a CDR (complementary determining region), a Fv fragment, or a Fab fragment, a further portion thereof. In some embodiments, the polyribonucleotide expresses one or more portions of an antibody. For instance, the polyribonucleotide can include more than one expression
 5 sequence, each of which expresses a portion of an antibody, and the sum of which can constitute the antibody. In some cases, the polyribonucleotide includes one expression sequence coding for the heavy chain of an antibody, and another expression sequence coding for the light chain of the antibody. When the polyribonucleotide is expressed in a cell, the light chain and heavy chain can be subject to appropriate modification, folding, or other post-translation modification to form a functional antibody.

10 In some embodiments, polyribonucleotides made as described herein (*e.g.*, circular polyribonucleotides) are used as effectors in therapy or agriculture. For example, a polyribonucleotide made by the methods described herein may be administered to a subject (*e.g.*, in a pharmaceutical, veterinary, or agricultural composition). 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
 15 method 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, 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
 20 subject is an invertebrate such as an arthropod (*e.g.*, insects, arachnids, crustaceans), a nematode, an annelid, a helminth, or a *mollusca*. 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,
 25 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.

30 *Plant-modifying polypeptides*

In some embodiments, the polyribonucleotide described herein (*e.g.*, the polyribonucleotide cargo of the polyribonucleotide) includes at least one expression sequence encoding a plant-modifying polypeptide. A plant-modifying polypeptide refers to a polypeptide that can alter the genetic properties
 35 (*e.g.*, increase gene expression, decrease gene expression, or otherwise alter the nucleotide sequence of DNA or RNA), epigenetic properties, or physiological or biochemical properties of a plant in a manner that results in a change in the plant's physiology or phenotype, *e.g.*, an increase or decrease in the plant's fitness. In some embodiments, the polyribonucleotide encodes two, three, four, five, six, seven, eight, nine, ten or more different plant-modifying polypeptides, or multiple copies of one or more plant-modifying
 40 polypeptides. A plant-modifying polypeptide may change the physiology or phenotype of, or increase or

decrease the fitness of, a variety of plants, or can be one that effects such change(s) in one or more specific plants (*e.g.*, a specific species or genera of plants).

Examples of polypeptides that can be used herein can include an enzyme (*e.g.*, a metabolic recombinase, a helicase, an integrase, a RNase, a DNase, or a ubiquitination protein), a pore-forming protein, a signaling ligand, a cell penetrating peptide, a transcription factor, a receptor, an antibody, a nanobody, a gene editing protein (*e.g.*, CRISPR-Cas endonuclease, TALEN, or zinc finger), riboprotein, a protein aptamer, or a chaperone.

Agricultural polypeptides

In some embodiments, the polyribonucleotide described herein (*e.g.*, the polyribonucleotide cargo of the polyribonucleotide) includes at least one expression sequence encoding an agricultural polypeptide. An agricultural polypeptide is a polypeptide that is suitable for an agricultural use. In embodiments, an agricultural polypeptide is applied to a plant or seed (*e.g.*, by foliar spray, dusting, injection, or seed coating) or to the plant's environment (*e.g.*, by soil drench or granular soil application), resulting in an alteration of the plant's physiology, phenotype, or fitness. Embodiments of an agricultural polypeptide include polypeptides that alter a level, activity, or metabolism of one or more microorganisms that are resident in or on a plant or non-human animal host, the alteration resulting in an increase in the host's fitness. In some embodiments the agricultural polypeptide is a plant polypeptide. In some embodiments, the agricultural polypeptide is an insect polypeptide. In some embodiments, the agricultural polypeptide has a biological effect when contacted with a non-human vertebrate animal, invertebrate animal, microbial, or plant cell.

In some embodiments, the polyribonucleotide encodes two, three, four, five, six, seven, eight, nine, ten or more agricultural polypeptides, or multiple copies of one or more agricultural polypeptides.

Embodiments of polypeptides useful in agricultural applications include, for example, bacteriocins, lysins, antimicrobial peptides, nodule C-rich peptides, and bacteriocyte regulatory peptides. Such polypeptides can be used to alter the level, activity, or metabolism of target microorganisms for increasing the fitness of insects, such as honeybees and silkworms. Embodiments of agriculturally useful polypeptides include peptide toxins, such as those naturally produced by entomopathogenic bacteria (*e.g.*, *Bacillus thuringiensis*, *Photorhabdus luminescens*, *Serratia entomophila*, or *Xenorhabdus nematophila*), as is known in the art. Embodiments of agriculturally useful polypeptides include polypeptides (including small peptides such as cyclodipeptides or diketopiperazines) for controlling agriculturally important pests or pathogens, *e.g.*, antimicrobial polypeptides or antifungal polypeptides for controlling diseases in plants, or pesticidal polypeptides (*e.g.*, insecticidal polypeptides or nematocidal polypeptides) for controlling invertebrate pests such as insects or nematodes. Embodiments of agriculturally useful polypeptides include antibodies, nanobodies, and fragments thereof, *e.g.*, antibody or nanobody fragments that retain at least some (*e.g.*, at least 10%) of the specific binding activity of the intact antibody or nanobody. Embodiments of agriculturally useful polypeptides include transcription factors, *e.g.*, plant transcription factors; *see, e.g.*, the "AtTFDB" database listing the transcription factor families identified in the model plant *Arabidopsis thaliana*), publicly available at [knowledgebase\[dot\]org/AtTFDB/](https://knowledgebase[dot]org/AtTFDB/). Embodiments of agriculturally useful polypeptides include nucleases, for example, exonucleases or endonucleases (*e.g.*, Cas nucleases such as Cas9 or Cas12a).

Embodiments of agriculturally useful polypeptides further include cell-penetrating peptides, enzymes (e.g., amylases, cellulases, peptidases, lipases, chitinases), peptide pheromones (for example, yeast mating pheromones, invertebrate reproductive and larval signaling pheromones, see, e.g., Altstein (2004) Peptides, 25:1373–76).

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Internal Ribosomal Entry Site (IRES)

In some embodiments, the polyribonucleotide described herein (e.g., the polyribonucleotide cargo of the polyribonucleotide) 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, picornavirus 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, if present, 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, Homalodisca coagulata virus- 1, Human 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, Acute bee paralysis virus, Hibiscus chlorotic 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, Human XIAP, Salivirus, Cosavirus, Parechovirus, *Drosophila* hairless, *S. cerevisiae* TFIID, *S. cerevisiae* YAP1, Human c-src, Human FGF-I, Simian picomavirus, Turnip crinkle virus, 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 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 peptides and or polypeptides.

In some embodiments, the polyribonucleotide cargo includes an IRES. For example, the
5 polyribonucleotide cargo may include a circular RNA IRES, *e.g.*, as described in Chen et al. Mol. Cell 81:1-19, 2021, which is hereby incorporated by reference in its entirety.

Regulatory elements

In some embodiments, the polyribonucleotide described herein (*e.g.*, the polyribonucleotide cargo
10 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
15 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 the amount or number 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
20 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
25 embodiments, the translation modulator is present on one or both sides of each expression sequence, leading to separation of the expression products, *e.g.*, peptides and or polypeptides.

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
30 entirety.

Translation initiation sequences

In some embodiments, the polyribonucleotide described herein (*e.g.*, the polyribonucleotide cargo
35 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.
40 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 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 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 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 (SEQ ID NO: 1), CAG, CTG.

Termination elements

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 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 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

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 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 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, 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 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 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 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.

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

Introduction, removal, or modification of UTR AU rich elements (AREs) may be useful to
5 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
10 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
15 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 poly-A 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 embodiments, the circular
20 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 embodiments, the circular
25 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
30 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 poly-A sequence. In some embodiments, the circular polyribonucleotide lacks a termination element. In some
35 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
40 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.

Stagger elements

5 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: 2) (cis-acting hydrolase element) sequence. In some embodiments, the stagger element encodes a sequence with a C-terminal consensus sequence that is $X_1X_2X_3EX_5NPGP$ 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 (SEQ ID NO: 83). 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, where x= any amino acid (SEQ ID NO: 4). Some nonlimiting examples of stagger elements includes GDVESNPGP (SEQ ID NO: 5), GDIEENPGP (SEQ ID NO: 6), VEPNPGP (SEQ ID NO: 7), IETNPGP (SEQ ID NO: 8), GDIESNPGP (SEQ ID NO: 9), GDVELNPGP (SEQ ID NO: 10), GDIETNPGP (SEQ ID NO: 11), GDVENPGP (SEQ ID NO: 12), GDVEENPGP (SEQ ID NO: 13), GDVEQNPGP (SEQ ID NO: 14), IESNPGP (SEQ ID NO: 15), GDIELNPGP (SEQ ID NO: 16), HDIETNPGP (SEQ ID NO: 17), HDVETNPGP (SEQ ID NO: 18), HDVEMNPGP (SEQ ID NO: 19), GDMESNPGP (SEQ ID NO: 20), GDVETNPGP (SEQ ID NO: 21) GDIEQNPGP (SEQ ID NO: 22), and DSEFNPGP (SEQ ID NO: 23).

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 peptides and or polypeptides 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
5 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. 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
10 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
15 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
20 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
25 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
30 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
35 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.

40 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 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 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 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.

20 *Non-coding sequences*

In some embodiments, the polyribonucleotide described herein (*e.g.*, the polyribonucleotide cargo of the polyribonucleotide) includes one or more non-coding sequence, *e.g.*, a sequence that does not encode the expression of polypeptide. In some embodiments, the polyribonucleotide includes two, three, four, five, six, seven, eight, nine, ten or more than ten non-coding sequences. In some embodiments, the polyribonucleotide does not encode a polypeptide expression sequence.

Noncoding sequences can be natural or synthetic sequences. In some embodiments, a noncoding sequence can alter cellular behavior, such as *e.g.*, lymphocyte behavior. In some embodiments, the noncoding sequences are antisense to cellular RNA sequences.

In some embodiments, the polyribonucleotide includes regulatory nucleic acids that are RNA or RNA-like structures typically from about 5-500 base pairs (bp) (depending on the specific RNA structure (*e.g.*, miRNA 5-30 bp, lncRNA 200-500 bp) and may have a nucleobase sequence identical (complementary) or nearly identical (substantially complementary) to a coding sequence in an expressed target gene within the cell. In embodiments, the circular polyribonucleotide includes regulatory nucleic acids that encode an RNA precursor that can be processed to a smaller RNA, *e.g.*, a miRNA precursor, which can be from about 50 to about 1000 bp, that can be processed to a smaller miRNA intermediate or a mature miRNA.

Long non-coding RNAs (lncRNA) are defined as non-protein coding transcripts longer than 100 nucleotides. Many lncRNAs are characterized as tissue specific. Divergent lncRNAs that are transcribed in the opposite direction to nearby protein-coding genes include a significant proportion (*e.g.*, about 20% of total lncRNAs in mammalian genomes) and possibly regulate the transcription of the nearby gene. In

one embodiment, the polyribonucleotide provided herein includes a sense strand of a lncRNA. In one embodiment, the polyribonucleotide provided herein includes an antisense strand of a lncRNA.

In embodiments, the polyribonucleotide encodes a regulatory nucleic acid that is substantially complementary, or fully complementary, to all or to at least one fragment of an endogenous gene or gene product (*e.g.*, mRNA). In embodiments, the regulatory nucleic acids complement sequences at the boundary between introns and exons, in between exons, or adjacent to an exon, to prevent the maturation of newly generated nuclear RNA transcripts of specific genes into mRNA for transcription. The regulatory nucleic acids that are complementary to specific genes can hybridize with the mRNA for that gene and prevent its translation. The antisense regulatory nucleic acid can be DNA, RNA, or a derivative or hybrid thereof. In some embodiments, the regulatory nucleic acid includes a protein-binding site that can bind to a protein that participates in regulation of expression of an endogenous gene or an exogenous gene.

In embodiments, the length of the polyribonucleotide encodes a regulatory nucleic acid that hybridizes to a transcript of interest wherein the regulatory RNA has a length of from about 5 to 30 nucleotides, from about 10 to 30 nucleotides, or about 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more than 30 nucleotides. In embodiments, the degree of sequence identity of the regulatory RNA to the targeted transcript is at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%.

In embodiments, the polyribonucleotide encodes a microRNA (miRNA) molecule identical to about 5 to about 25 contiguous nucleotides of a target gene or encodes a precursor to that miRNA. In some embodiments, the miRNA sequence has a that allows the mRNA to recognize and bind to a specific target mRNA. In embodiments, miRNA sequence commences with the dinucleotide AA, includes a GC - content of about 30-70% (about 30-60%, about 40-60%, or about 45%-55%), and does not have a high percentage identity to any nucleotide sequence other than the target in the genome of the subject (*e.g.*, a mammal) in which it is to be introduced, for example as determined by standard BLAST search.

In some embodiments, the polyribonucleotide includes at least one miRNA (or miRNA precursor), *e.g.*, 2, 3, 4, 5, 6, or more miRNAs or miRNA precursors. In some embodiments, the polyribonucleotide includes a sequence that encodes a miRNA (or its precursor) having at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or 100% nucleotide sequence complementarity to a target sequence.

siRNAs and shRNAs resemble intermediates in the processing pathway of the endogenous microRNA (miRNA) genes. In some embodiments, siRNAs can function as miRNAs and vice versa. MicroRNAs, like siRNAs, use RISC to downregulate target genes, but unlike siRNAs, most animal miRNAs do not cleave the mRNA. Instead, miRNAs reduce protein output through translational suppression or polyA removal and mRNA degradation. Known miRNA binding sites are within mRNA 3' UTRs; miRNAs seem to target sites with near-perfect complementarity to nucleotides 2-8 from the miRNA's 5' end. This region is known as the seed region. Because mature siRNAs and miRNAs are interchangeable, exogenous siRNAs downregulate mRNAs with seed complementarity to the siRNA.

Lists of known miRNA sequences can be found in databases maintained by research organizations, such as Wellcome Trust Sanger Institute, Penn Center for Bioinformatics, Memorial Sloan Kettering Cancer Center, and European Molecule Biology Laboratory, among others. Known effective siRNA sequences and cognate binding sites are also well represented in the relevant literature. RNAi

molecules are readily designed and produced by technologies known in the art. In addition, there are computational tools that increase the chance of finding effective and specific sequence motifs.

Protein-binding sequences

5 In some embodiments, a circular polyribonucleotide includes one or more protein binding sites that enable 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
10 host's immune system.

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
15 immunoprotein binding site is a nucleotide sequence that binds to an immunoprotein and aids in hiding 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)
20 of translation of the circular polyribonucleotide does not require a free end or a capped end. Rather, a 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 for translation initiation. They harbor signatures
25 like Kozak sequences which are commonly known to be involved in the process by which the ribosome initiates translation of many genes. Kozak sequences have the consensus CCR(A/G)CCAUGG (SEQ ID NO: 24), where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. 5'UTR also have been known to form secondary structures which are involved in elongation factor binding.

30 In some embodiments, a circular polyribonucleotide encodes a protein binding sequence that 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
35 protein such as ACIN1, AGO, APOBEC3F, APOBEC3G, ATXN2, AUH, BCCIP, CAPRIN1, CELF2, 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,
40 METTL3, MOV10, MSI1, MSI2, NONO, NONO-, NOP58, NPM1, NUDT21, PCBP2, POLR2A, PRPF8, 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.

5 *Spacer Sequences*

In some embodiments, a 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. Spacers may also be present within a nucleic acid element described herein.

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.

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.

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.

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.

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.

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 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 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.

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Bioreactors

In some embodiments, any method of purifying a polyribonucleotide (*e.g.*, 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 purifying or producing 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 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 RNA. In some embodiments, a bioreactor may produce 1-200g of RNA (*e.g.*, 1-10g, 1-20g, 1-50g, 10-50g, 10-100g, 50-100g, of 50-200g of 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).

35 **Methods of Use**

In some embodiments, the polyribonucleotides (*e.g.*, circular polyribonucleotides) made as described herein are used as effectors in therapy or agriculture.

For example, a polyribonucleotide purified by the methods described herein may be administered to a subject (*e.g.*, in a pharmaceutical, veterinary, or agricultural 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 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), *Paleaognathae* (*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 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.

15 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 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
20 described herein.

In some embodiments, the disclosure provides a method of treating a condition in a subject in need thereof 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 a polyribonucleotide described herein), and the polynucleotide is provided to a eukaryotic subject. In
25 some embodiments, the composition or formulation is or includes a eukaryotic or prokaryotic cell including a nucleic acid described herein.

In some embodiments, the disclosure provides a method of providing a polyribonucleotide (*e.g.*, circular polyribonucleotide) to a subject by providing a eukaryotic or prokaryotic cell include a polynucleotide described herein to the subject.

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Formulations

In some embodiments of the present disclosure a polyribonucleotide (*e.g.*, 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
35 agricultural, veterinary, or pharmaceutical composition. In some embodiments, the polyribonucleotide is formulated in a pharmaceutical composition. In some embodiments, a composition includes a polyribonucleotide and a diluent, a carrier, an adjuvant, or a combination thereof. In a particular embodiment, a composition includes a polyribonucleotide described herein and a carrier or a diluent free of any carrier. In some embodiments, a composition including a polyribonucleotide with a diluent free of
40 any carrier is used for naked delivery of the polyribonucleotide (*e.g.*, 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

5 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

10 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.

15 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 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

20 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,

25 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.

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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.

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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%

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(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 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, impurities, or by-products (*e.g.*, cell protein such as a host cell protein or protein process impurities), endotoxin, mononucleotide molecules, and/or a process-related impurity.

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 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 as hydrochloric acid, hydrobromic acid, phosphoric acid, nitric acid, sulfuric acid, methane sulfonic 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

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

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

5 A composition or pharmaceutical composition provided herein can include one or more 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
 10 number of repeating ethoxy (oxy-1,2-ethanediyl) groups, *e.g.*, octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol); (octyl phenoxy)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
 15 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
 20 “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 polyribonucleotide and a
 25 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.
 30 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
 35 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
 40 the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation.

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

5 A naked delivery formulation is a formulation that is free from a carrier and wherein the polyribonucleotide (*e.g.*, 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 polyribonucleotide. In some embodiments, a 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
10 particle, a polymer, or a biopolymer. A 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 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
15 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),
20 poly(tetra ethylenimine), 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)imidazolium chloride (DOTIM), 2,3-dioleoyloxy-N-
25 [2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 3B-[N-(N,N'-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HC1), 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),
30 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
35 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
40 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)propane sulfonic 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, 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 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. 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 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 Biotechnol., 15:647-52, 1997, the teachings of which relating to extruded lipid preparation are incorporated herein by reference.

In certain embodiments, a composition of the disclosure includes a 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 system for a 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 delivery. These

5 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 such, the two

10 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

15 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(tetra ethylenimine), polypropylenimine, aminoglycoside-polyamine, dideoxy-

20 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),

25 3B-[N-(N,N'-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HC1), 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.

30 Exosomes can also be used as drug delivery vehicles for a 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 287-96; doi.org/10.1016/j.apsb.2016.02.001.

Ex vivo differentiated red blood cells can also be used as a carrier for a composition or preparation

35 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 polyribonucleotide molecule described herein.

5 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 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
10 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 composition or preparation described herein. Plant nanovesicles and plant messenger packs (PMPs) can also be used as carriers to deliver a linear polyribonucleotide composition
15 or preparation described herein.

Microbubbles can also be used as carriers to deliver a 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
20 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 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
25 nanometers). The size of the particle may be optimized to favor deposition of the payload, including the polyribonucleotide into a cell. Deposition of the polyribonucleotide into certain cell types may favor different particle sizes. For example, the particle size may be optimized for deposition of the polyribonucleotide into antigen presenting cells. The particle size may be optimized for deposition of the polyribonucleotide into dendritic cells. Additionally, the particle size may be optimized for depositions of
30 the 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
35 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
40 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.

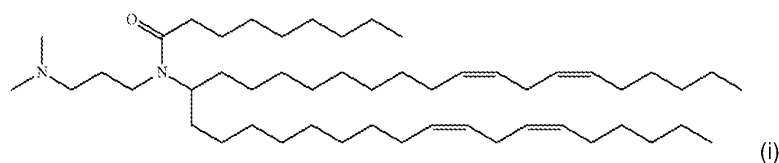
In some embodiments, conjugated lipids, when present, can include one or more of PEG-diacylglycerol (DAG) (such as 1-(monomethoxy-polyethyleneglycol)-2,3- dimyristoylglycerol (PEG-DMG)), PEG-dialkylxypropyl (DAA), PEG-phospholipid, PEG- ceramide (Cer), a pegylated
 5 phosphatidylethanolamine (PEG-PE), PEG succinate diacylglycerol (PEGS-DAG) (such as 4-0-(2',3'- di(tetradecanoyloxy)propyl)-l-0-(w- methoxy(polyethoxy)ethyl) butanedioate (PEG-S-DMG)), PEG dialkoxypyrrolcarbam, 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
 10 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 W02009/127060 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, incorporated herein by
 15 reference.

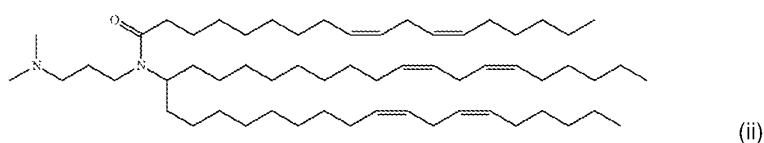
In some embodiments, the lipid particle includes an ionizable lipid, a non-cationic lipid, a conjugated lipid that inhibits aggregation of particles, and a sterol. The amounts of these components can be varied independently and to achieve desired properties. For example, in some embodiments, the lipid nanoparticle includes an ionizable lipid is in an amount from about 20 mol % to about 90 mol % of
 20 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
 25 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
 30 or higher. Generally, the lipid nanoparticle formulation's overall lipid content can range from about 5 mg/ml to about 30 mg/mL.

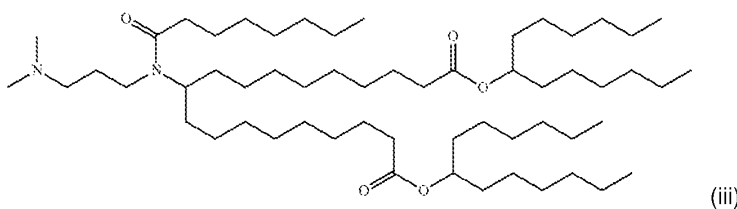
Some non-limiting example of lipid compounds that may be used (*e.g.*, in combination with other lipid components) to form lipid nanoparticles for the delivery of compositions described herein, *e.g.*, nucleic acid (*e.g.*, RNA (*e.g.*, circular polyribonucleotide, linear polyribonucleotide)) described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



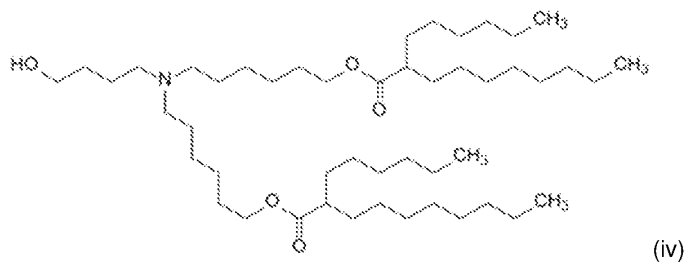
In some embodiments an LNP including Formula (i) is used to deliver a polyribonucleotide (*e.g.*, a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.



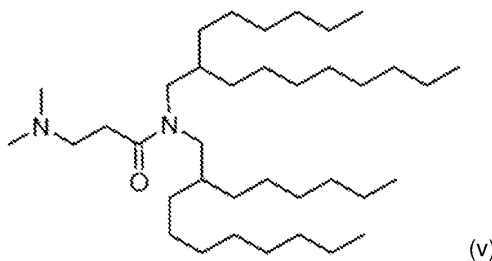
In some embodiments an LNP including Formula (ii) is used to deliver a polyribonucleotide (*e.g.*, a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.



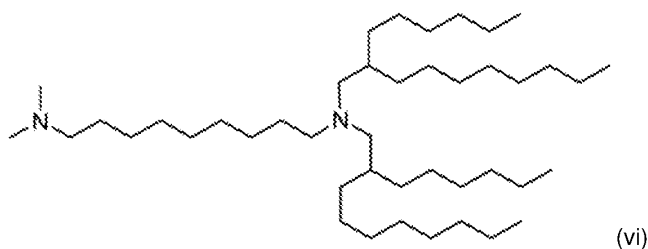
In some embodiments an LNP including Formula (iii) is used to deliver a polyribonucleotide (*e.g.*, a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.



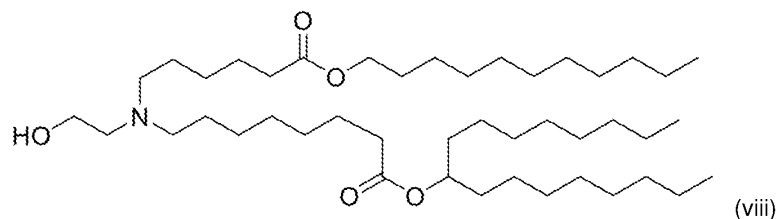
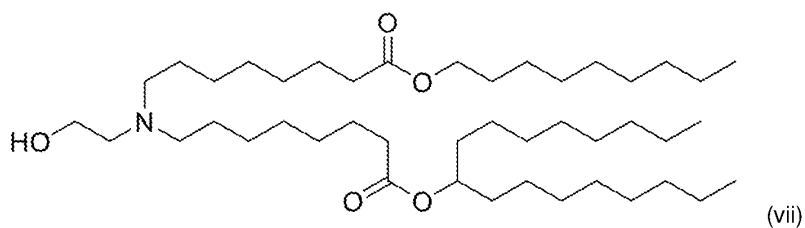
10



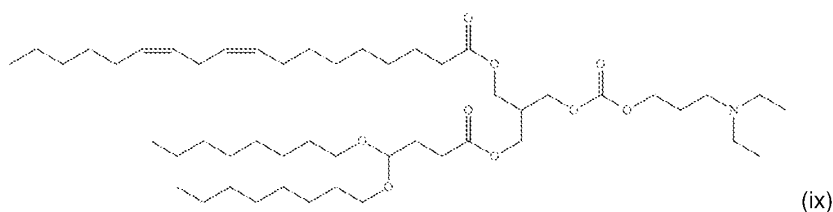
In some embodiments an LNP including Formula (v) is used to deliver a polyribonucleotide (*e.g.*, a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.



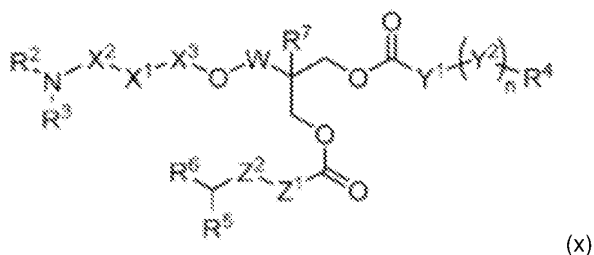
In some embodiments an LNP including Formula (vi) is used to deliver a polyribonucleotide (*e.g.*, a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.



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.

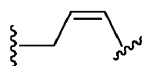


In some embodiments an LNP including Formula (ix) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.

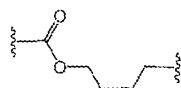


wherein

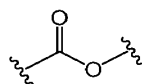
10 X¹ is O, NR¹, or a direct bond, X² is C2-5 alkylene, X³ is C(=O) or a direct bond, R¹ is H or Me, R³ is C1-3
alkyl, R² is C1-3 alkyl, or R² taken together with the nitrogen atom to which it is attached and 1-3 carbon
atoms of X² form a 4-, 5-, or 6-membered ring, or X¹ is NR¹, R¹ and R² taken together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



(in either orientation),

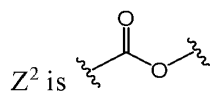


(in either orientation),



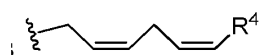
(in either orientation),

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



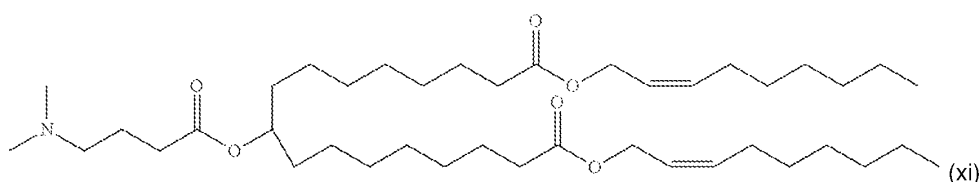
(in either orientation) or absent, provided that if Z^1 is a direct bond, Z^2 is absent;

R^5 is C5-9 alkyl or C6-10 alkoxy, R^6 is C5-9 alkyl or C6-10 alkoxy, W is methylene or a direct bond, and R^7 is H or Me, or a salt thereof, provided that if R^3 and R^2 are C2 alkyls, X^1 is O, X^2 is linear C3 alkylene, X^3 is C(=O), Y^1 is linear C_e alkylene, (Y^2)_n- R^4 is

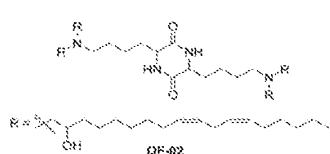


, R^4 is linear C5 alkyl, Z^1 is C2 alkylene, Z^2 is absent, W is methylene, and R^7 is H, then R^5 and R^6 are not C_x alkoxy.

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.

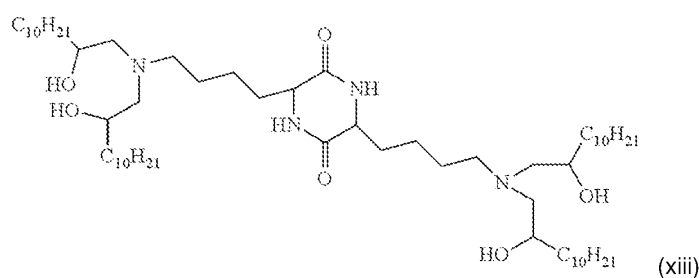


In some embodiments an LNP including Formula (xi) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.

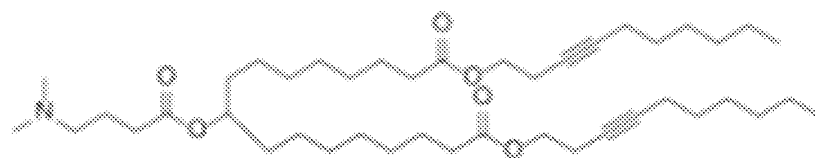


where R=

(xii)

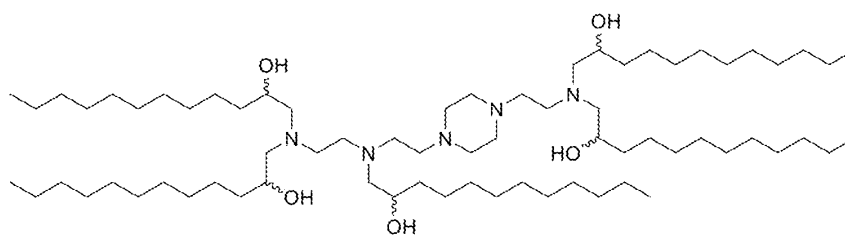


(xiii)



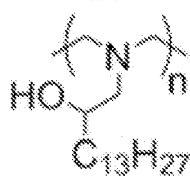
(xiv)

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



(xv)

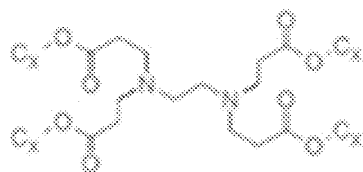
In some embodiments an LNP including Formula (xv) is used to deliver a polyribonucleotide (*e.g.*, a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells.

PEI₆₀₀ Core

(xvi)

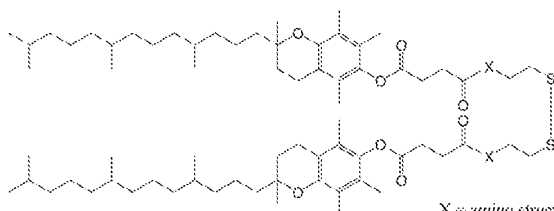
5

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.



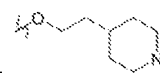
(xvii)

10

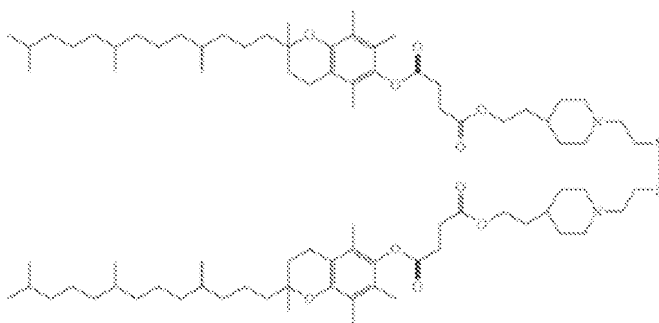


X = amino structure

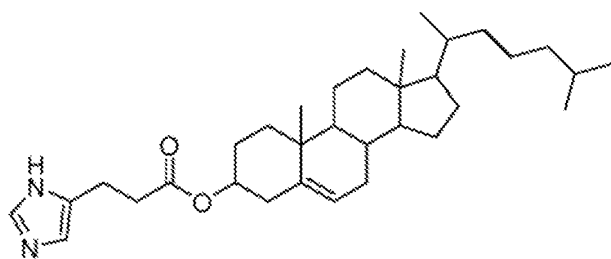
where X=



(xviii)(a)

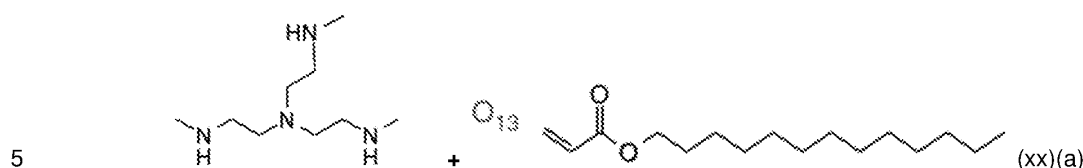


(xviii)(b)

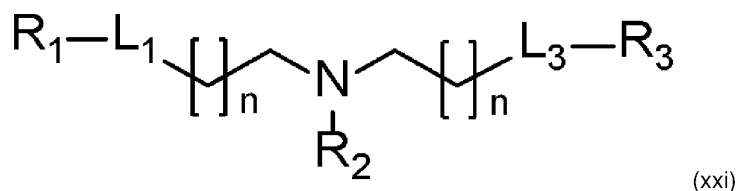


(xix)

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:



10 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).

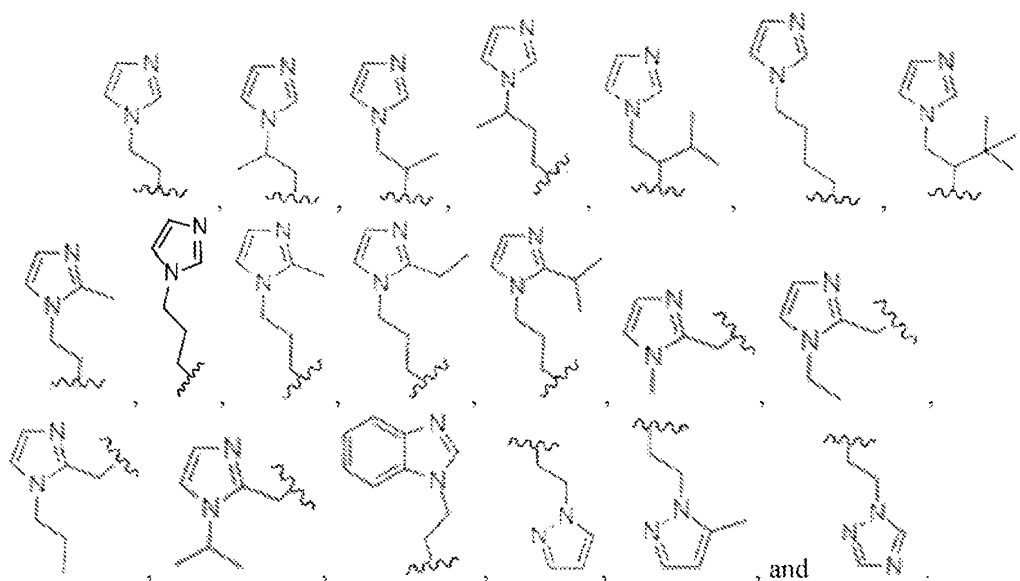


wherein

15 each *n* is independently an integer from 2-15; *L*₁ and *L*₃ are each independently -OC(O)-* or -C(O)O-*, wherein "*" indicates the attachment point to *R*₁ or *R*₃;

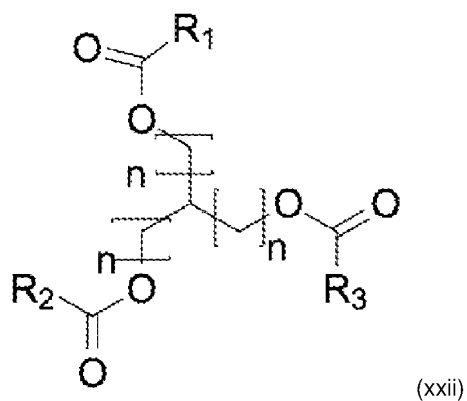
20 *R*₁ and *R*₃ are each independently a linear or branched C₉-C₂₀ alkyl or C₉-C₂₀ alkenyl, optionally substituted by one or more substituents selected from a group consisting of oxo, halo, hydroxy, cyano, alkyl, alkenyl, aldehyde, heterocyclalkyl, hydroxyalkyl, dihydroxy alkyl, hydroxyalkylaminoalkyl, amino alkyl, alkylaminoalkyl, dialkylamino alkyl, (heterocycl)(alkyl)aminoalkyl, heterocycl, heteroaryl, alkyl heteroaryl, alkynyl, alkoxy, amino, dialkylamino, aminoalkylcarbonylamino, aminocarbonyl alkylamino, (aminocarbonylalkyl)(alkyl)amino, alkenylcarbonylamino, hydroxycarbonyl, alkyloxy carbonyl, aminocarbonyl, aminoalkylaminocarbonyl, alkylaminoalkylaminocarbonyl, dialkylaminoalkylaminocarbonyl, heterocyclalkylaminocarbonyl, (alkylaminoalkyl)(alkyl)aminocarbonyl, alkylaminoalkylcarbonyl, dialkylaminoalkylcarbonyl, heterocyclcarbonyl, alkenyl carbonyl, alkynyl carbonyl, alkyl sulfoxide, alkylsulfoxidealkyl, alkyl sulfonyl, and alkyl sulfone alkyl; and

25 *R*₂ 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

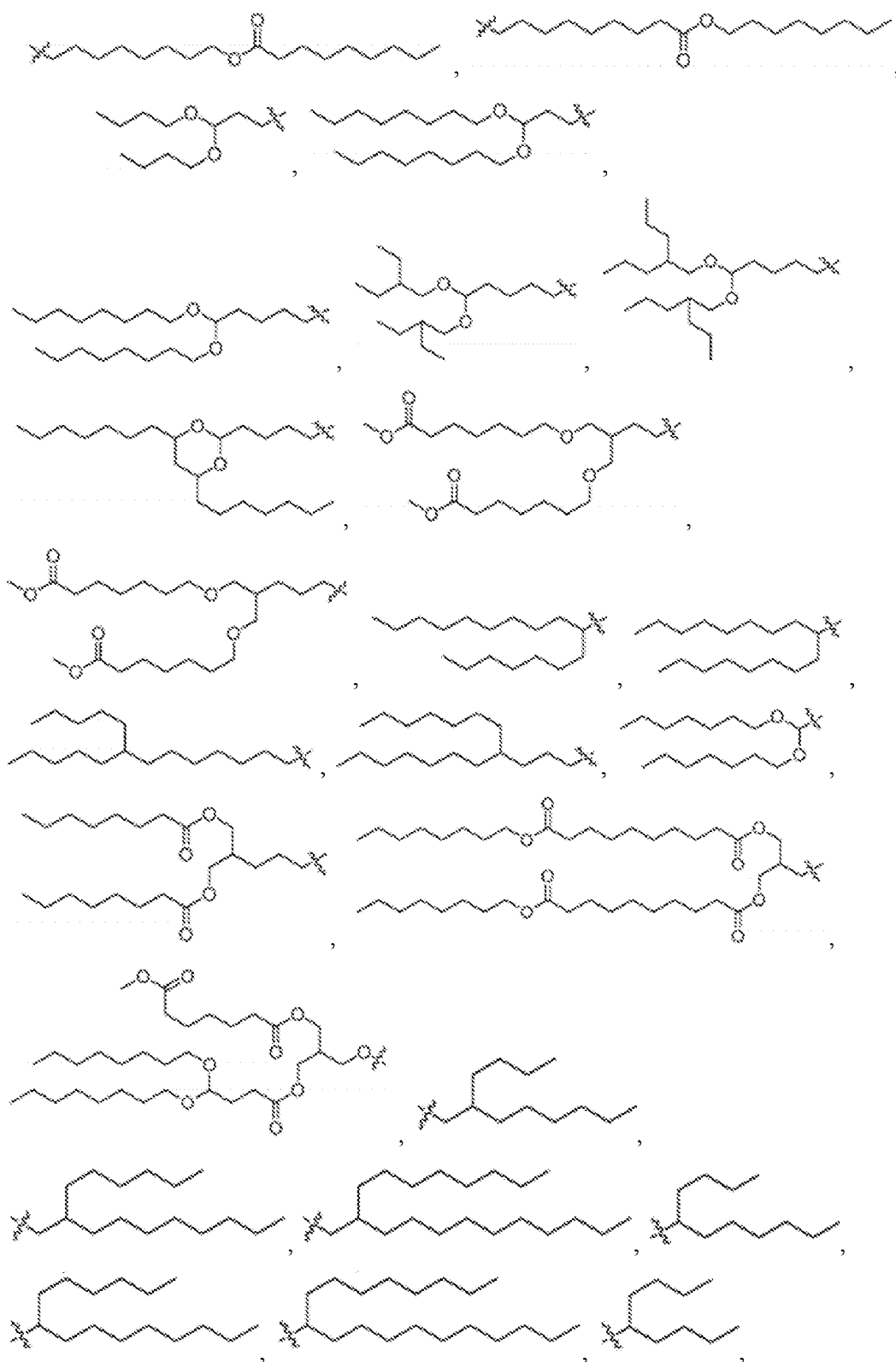
5 Formula (2) such as a lipid of Table 2 of WO2021113777).



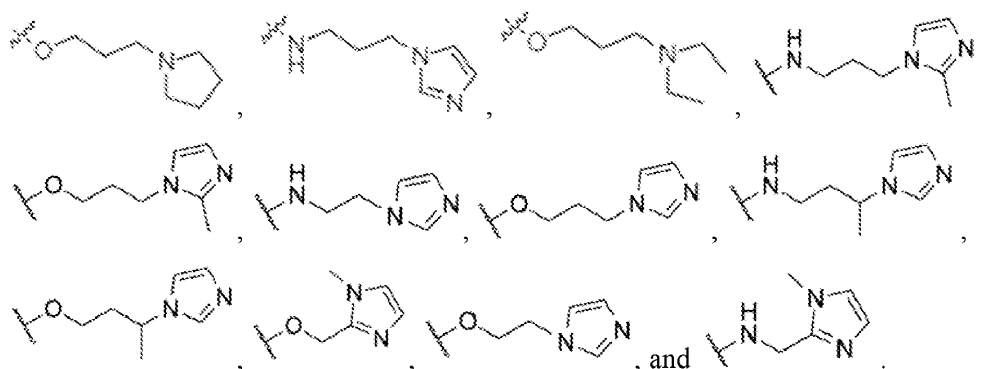
wherein

each n is independently an integer from 1-15;

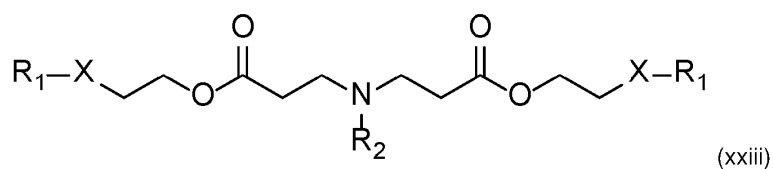
R₁ and R₂ are each independently selected from a group consisting of:



R₃ is selected from a group consisting of:



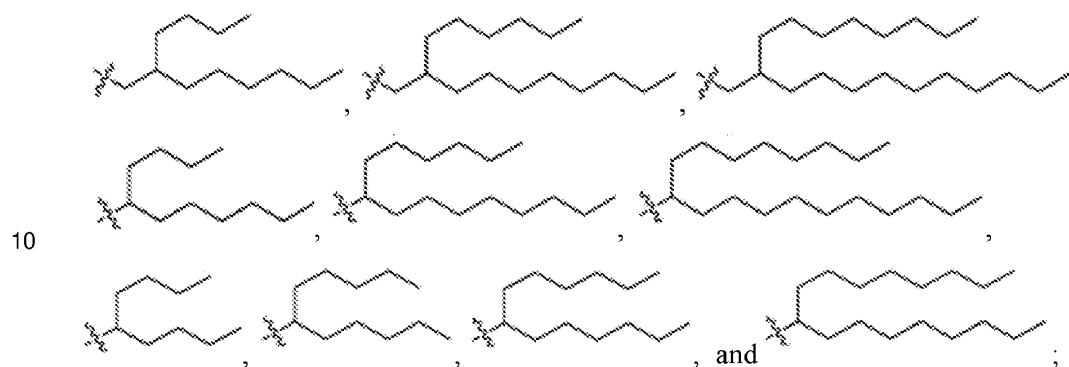
In some embodiments an LNP including Formula (xxiii) is used to deliver a polyribonucleotide (e.g., a circular polyribonucleotide, a linear polyribonucleotide) composition described herein to cells. In some embodiments the LNP of Formula (xxiii) is an LNP described by WO2021113777 (e.g., a lipid of Formula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



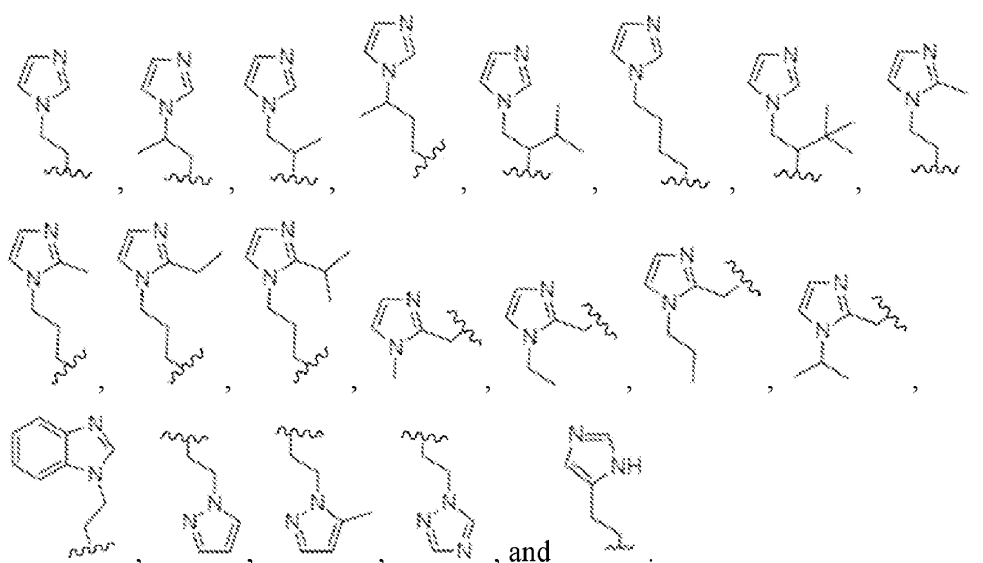
wherein

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

R_1 is selected from a group consisting of:



and R_2 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 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 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) 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 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 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 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 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 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

5 herein in its entirety).

Exemplary non-cationic lipids include, but are not limited to, distearoyl-sn-glycero-phosphoethanolamine, distearoylphosphatidylcholine (DSPC), dioleoyl phosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoyl phosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE),

10 palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl-phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal), dipalmitoylphosphatidylethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidylethanolamine (DSPE), monomethyl-phosphatidylethanolamine (such as 16-O-monomethyl PE), dimethyl-phosphatidylethanolamine (such as 16-O-dimethyl PE), I8-I-trans PE, I-stearoyl-2-oleoyl-

15 phosphatidylethanolamine (SOPE), hydrogenated soy phosphatidylcholine (HSPC), egg phosphatidylcholine (EPC), dioleoyl phosphatidylserine (DOPS), sphingomyelin (SM), dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), distearoylphosphatidylglycerol (DSPG), dierythroylphosphatidylcholine (DEPC), palmitoyloleoylphosphatidylglycerol (POPG), dielaidoyl-phosphatidylethanolamine (DEPE), lecithin, phosphatidylethanolamine, lysolecithin, Lys

20 phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, sphingomyelin, egg sphingomyelin (ESM), cephalin, cardiolipin, phosphatidic acid, cerebroside, dicetylphosphate, Lys phosphatidylcholine, dilinoleoylphosphatidylcholine, or mixtures thereof. It is understood that other diacyl phosphatidylcholine and diacyl phosphatidylethanolamine 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,

25 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

30 limitation, non-phosphorous lipids such as, *e.g.*, stearylamine, dodecylamine, hexadecylamine, acetyl palmitate, glycerol ricin oleate, hexadecyl stearate, isopropyl myristate, amphoteric acrylic polymers, triethanolamine-lauryl sulfate, alkyl-aryl sulfate polyethoxylated 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 are incorporated herein

35 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

40 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.

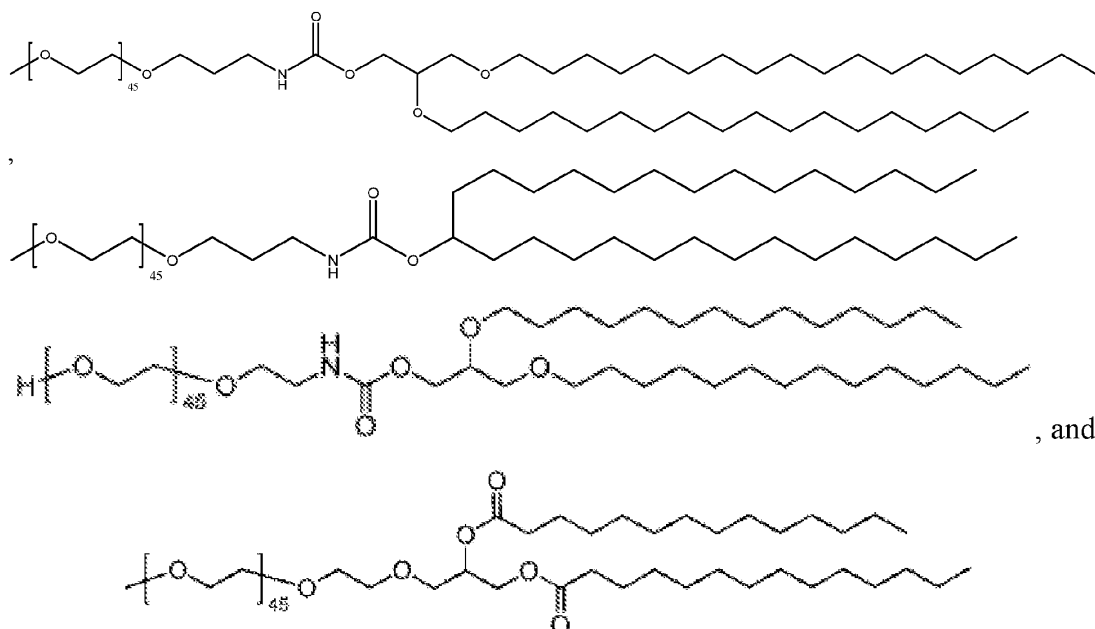
In some aspects, the lipid nanoparticle can further include a component, such as a sterol, to provide membrane integrity. One exemplary sterol that can be used in the lipid nanoparticle is cholesterol and derivatives thereof. Non-limiting examples of cholesterol derivatives include polar analogues such as 5a-cholestanol, 5 β -coprostanol, cholesteryl-(2-hydroxy)-ethyl ether, cholesteryl-(4'-hydroxy)-butyl ether, and 6-ketocholestanol; non-polar analogues such as 5a-cholestane, cholestenone, 5a-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 W02009/127060 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-polyethylene glycol)-2,3-dimyristoylglycerol (PEG-DMG)), PEG-dialkoxypentyl (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 dialkoxypentylcarbam, 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-Ditetradecoxylbenzyl- [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:



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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.

10 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

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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 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 a 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 include 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.

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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.

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Example 1: Linear RNA pulldown method for enrichment of circular RNA

This example describes the design of the method for removing linear RNA byproducts from circular RNA generated by self-splicing.

There may be several major linear impurities or byproducts in the *in vitro* transcription (IVT) mixture when circular RNA is generated by self-splicing: unspliced linear RNA, partly spliced linear RNA, nicked circular RNA, and spliced introns (**FIG. 2**).

Oligomers are designed to have complementary sequences against intron regions. Intron regions are the sequences that are not present in the circular RNA product but only in linear byproducts as part of linear RNA or spliced-out form (**FIGS. 2 and 3**). The oligomers are designed to have biotin connected by a TEG linker that can bind streptavidin protein with high affinity. Once the oligomers bind to linear byproducts that contain intronic sequences, the byproducts can be captured by streptavidin-bead and circular RNA can be enriched in the unbound fraction.

Example 2: Linear RNA pull down specifically captures linear byproducts and enriches circular RNA

This example demonstrates enrichment of circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions.

In this example, the construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo including an open reading frame (ORF), an exon fragment 1 (E1), and a 5' half of a catalytic intron. The ORF was either *gaussia* luciferase (Gluc, for **FIG. 4**) or firefly luciferase (Fluc, for **FIG. 5**). The length of linear RNA was around 1.4 Kb with the Gluc ORF and around 2.6 Kb with the Fluc ORF.

For linear RNA pull down (LP) to remove linear byproducts that contain intronic sequences, six different oligomers against the intronic region were designed, four oligomers for 3' half-intron, and two oligomers for 5' half-intron. Each oligomer was 23 nucleotides and had biotin that was linked by TEG.

Linear RNA was synthesized by *in vitro* transcription using T7 RNA polymerase from a DNA template in the presence of 7.5mM of NTP. Template DNA was removed by treating with DNase for 20 minutes. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription; no further or additional reaction was required.

Self-spliced RNA (200 pmol) was mixed with the same amount of each oligomer (200 pmol of each oligomer, total 1.2 nmol of oligomers) in the presence of 1X binding buffer (150 mM NaCl, 15 mM

sodium citrate, 0.5 mM EDTA) (final RNA concentration was 400 nM) in 500 µl total. To test the effect of RNA:oligomer ratio on LP efficiency, two times (800 nM) and five times more oligomers (2 µM) were used. As a negative control, RNA without oligomer was used. The RNA-oligomer mixtures were incubated for 30 minutes at room temperature (RT), then mixed with 100 µl of Streptavidin-
 5 SEPHAROSE® (Sigma). The mixture was incubated at RT for 1 hour on rotor mix and unbound fraction was collected by spinning down Streptavidin-SEPHAROSE® bead by centrifugation. The bead was washed three times with 1X binding buffer. RNA bound to bead was eluted by heating the resin for 10 minutes at 75 °C in the presence of 1X binding buffer ('Eluted' in **FIGS. 4 and 5**). RNA still bound to bead was eluted by heating the bead for 5 minutes at 95 °C in the presence of 95% formamide ('Resin' in
 10 **FIGS. 4 and 5**). The concentration of unbound and eluted RNA was measured by Qubit and 200 ng of RNA was separated by Urea polyacrylamide gel electrophoresis (Urea PAGE), stained using gel stain and visualized using an imaging system.

In both the 1.4 Kb RNA (**FIG. 4**) and the 2.6 Kb RNA (**FIG. 5**), there was significant enrichment of circular RNA in unbound fractions (around 90% enrichment in 1.4 Kb RNA (**FIG. 4**) and 50% enrichment
 15 in 2.6Kb RNA (**FIG. 5**)) compared with input. The majority of RNA bound to the resin was linear RNA that contains the intronic region (linear RNA, **FIGS. 4 and 5**), indicating that the oligomers specifically captured intron-containing RNA. Increase of oligomer concentration did not affect circular RNA enrichment yield in both 1.4 Kb RNA and 2.6 Kb RNA.

20 **Example 3: Single oligomer can capture linear RNA byproducts and enrich circular RNA**

This example demonstrates enrichment of circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions. In this example, linear RNA included an extended region at the 5' end of 3' half-intron that is spliced out during self-splicing.

In this example, the construct was designed to include a 3' half of a catalytic intron, an exon
 25 fragment 2 (E2), a polyribonucleotide cargo including an ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron. The construct included 36 nucleotides of extended sequences at the 5' end.

To pull down linear byproducts that contain extended sequences, an oligomer against the extended region was designed. The oligomer was 36 nucleotides and had biotin that was linked by TEG.

Linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA
 30 template in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase for 20 minutes. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription; no further/additional reaction was required.

Self-spliced RNA (200 pmol) was mixed with 400 pmol of oligo in the presence of 1X binding buffer (150 mM NaCl, 15 mM sodium citrate, 0.5 mM EDTA) (final RNA concentration was 400 nM and
 35 oligomer concentration was 800 nM). To test the effect of RNA:oligomer ratio on linear RNA pull down efficiency, 2.5 times (1 µM) or 5 times more (2 µM) oligomer was used. As a negative control RNA without oligomer was used. The RNA-oligomer mixtures were incubated for 30 minutes at RT, then mixed with 100 µl of Streptavidin-SEPHAROSE® (Sigma). The mixture was incubated at RT for 1 hour on rotor mix and unbound fraction was collected by spinning down SEPHAROSE® bead by
 40 centrifugation. The bead was washed three times with 1X binding buffer. RNA bound to resin was eluted by heating the resin for 10 minutes at 75 °C in the presence of 1X binding buffer ('Eluted' in **FIG. 6**). RNA

still bound to bead was eluted by heating the resin for 5 minutes at 95 °C in the presence of 95% formamide ('Resin' in **FIG. 6**). The concentration of unbound and eluted RNA was measured by Qubit and 200 ng of RNA were separated by urea PAGE, stained with a gel stain and visualized by using an imaging system.

There was significant enrichment of circular RNA in unbound fractions (50% enrichment in 800 nM oligo (**FIG. 6**)) compared with input. The majority of RNA bound to the resin was linear RNA that contained the extended region, indicating that oligomer specifically captured the linear RNA with the extended region (linear RNA, **FIG. 6**). Increase of oligomer concentration significantly affected circular RNA enrichment yield from 50% in the case of 800 nM oligomer to 96% for 4 μM oligomer.

Example 4: Comparing batch-purification and column-packing method

This example demonstrates two different approaches for enrichment of circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions. One method was incubating RNA-oligo mixture with Streptavidin-SEPHAROSE® bead in a tube (Batch method), the other method was prepacking bead in the column and passing through RNA-oligo mixture by gravity (Column method).

In this example, the construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo including an ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron.

To pull down linear byproducts that contain intronic sequences, six different oligomers against intronic region were designed, four oligomers for the 3' half-intron, and two oligomers for the 5' half-intron. Each oligomer was 23 nucleotides and had biotin that was linked by TEG.

Linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase for 20 minutes. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050).

Self-splicing occurred during transcription; no further/additional reaction was required.

Self-spliced RNA (1 nmol) was mixed with 2 nmol of each oligo in the presence of 1X binding buffer (150 mM NaCl, 15 mM sodium citrate, 0.5 mM EDTA) in 2.5 ml total (final RNA concentration was 400 nM and oligomer concentration was 800 nM per each oligomer). The RNA-oligo mixtures were incubated for 30 minutes at RT with or without heating at 75 °C for 3 minutes. RNA mixture without oligomers was used as negative control. For batch purification method, 500 μl of Streptavidin-SEPHAROSE® bead was added to the mixture. The mixture was then incubated at RT for 1 hour on rotor mix and unbound fraction was collected by spinning down SEPHAROSE® bead by centrifugation. For column purification, 500 μl of bead was pre-packed in empty column and RNA-oligo mixture was put on the packed resin. The RNA-oligo mixture was passed through the resin by gravity and flow through was collected. The concentration RNA of unbound or flow through was measured by Qubit and 200 ng of RNA were separated by Urea-PAGE, stained using a gel stain and visualized using an imaging system.

No significant differences were found in enrichment of circular RNA between the batch purification and column purification methods (see, *e.g.*, lanes 3 and 4 versus lanes 6 and 7, respectively) compared with input (**FIG. 7**). Heating and cooling of the RNA-oligo mixture did not increase circular RNA

enrichment. This data indicate that the linear RNA pull-down method (LP) method can be scaled up through column packing of SEPHAROSE® bead.

Example 5: Consecutive linear RNA pull downs can further enrich circular RNA when the circularization efficiency is low

This example demonstrates enrichment of circular RNA by consecutive linear RNA pull down.

In this example, the construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo including an ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron.

To pull down linear byproducts that contain intronic sequences, six different oligomers against intronic region were designed, four for 3' half-intron, and two for 5' half-intron. Each oligomer was 23 nucleotides and had biotin that was linked by TEG.

Linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase for 20 minutes. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription; no further/additional reaction was required. Self-spliced RNA (1 nmol) was mixed with 2 nmol of each oligo in the presence of 1X binding buffer (150 mM NaCl, 15 mM sodium citrate, 0.5 mM EDTA) in 2.5 ml total (final RNA concentration was 400 nM and oligomer concentration was 800 nM per each oligomer). The RNA-oligomer mixtures were incubated for 30 minutes at RT. For column purification, 500 µl of bead was pre-packed in an empty column and RNA-oligomer mixture was placed on the packed resin. The RNA-oligomer mixture was passed through the resin by gravity and flow through was collected. The collected flow through was mixed with additional oligomers (final 800 nM), then passed through freshly pre-packed resin. The flow through was collected and the concentration of first and second round of flow through was measured by Qubit. 200 ng of RNA were separated by Urea-PAGE, stained using gel stain and visualized using an imaging system.

In the first round of linear RNA pull down, there was 130% of circular RNA enrichment (**FIG. 8**, first LP; circular RNA purity from 23% to 54%). With the second round of pull down, there was additional enrichment, then finally more than 200% enrichment was achieved (**FIG. 8**, second LP; circular RNA purity from 23% to 72%).

Example 6: Optimization of linear RNA pull down with different salt concentration

This example demonstrates the effect of salt concentration in the binding buffer for circular RNA enrichment by capturing linear byproducts *via* oligo-streptavidin interactions.

In this example, the construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo including an ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron. The ORF was either hEPO (hEPO, for **FIG. 9A**) or SARS-CoV-2 spike protein (Spike, for **FIG. 9B**). The length of linear RNA was around 1.2 Kb with the hEPO ORF and around 4.5 Kb with the SARS-CoV-2 Spike ORF (running with 2 Kb ladder on 6% Urea PAGE even though the size of the RNA was 4.5 Kb).

To pull down linear byproducts that contain intronic sequences, six different oligomers against intronic region were designed, four for 3' half-intron, and two for 5' half-intron. Each oligomer was 23 nucleotides and had biotin that was linked by TEG.

Linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase for 20 minutes. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription; no further/additional reaction was required.

Self-spliced RNA (1 nmol) was mixed with 2 nmol of each oligo in the presence of 1X binding buffer (150 mM NaCl, 15 mM sodium citrate, 0.5 mM EDTA) in 2.5 ml total (final RNA concentration was 400 nM and oligomer concentration was 800 nM per each oligomer). To test the effect of salt concentration on circular RNA enrichment, 500 mM, 1000 mM and 2000 mM NaCl concentrations were also tested. The RNA-oligomer mixtures were incubated for 30 minutes at RT. For column purification, 500 µl of bead was pre-packed in empty column and RNA-oligomer mixture was placed on the packed resin. The RNA-oligomer mixture was passed through the resin by gravity and flow through was collected. The flow through was collected and the concentration of RNA in flow through was measured by Qubit. 200 ng of RNA was separated by urea-PAGE, stained with a gel stain and visualized using an imaging system.

In the case of the 1.2 Kb RNA, there were no significant differences on circular RNA enrichment in different salt conditions (**FIG. 9A**). However, circular RNA enrichment of the 4.5 Kb RNA was increased when the salt concentration was increased (**FIG. 9B**). There was 60% enrichment in 150 mM NaCl (20% of circular RNA purity from input to 32% of circular RNA purity) but 90% enrichment in 2000 mM NaCl (20% of circular RNA purity from input to 38% of circular RNA purity). This data indicates that higher salt can improve circular RNA enrichment of long RNA in linear RNA pull down (LP).

Example 7: Linear RNA pull down-mediated circular RNA enrichment enhances circular RNA expression after downstream process purification

This example demonstrates enhanced expression from circular RNA purified by the linear RNA pull down method.

In this example, the construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo including an ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron. The ORF was either gaussian luciferase (Gluc, for **FIG. 10**) or hEPO (for **FIG. 11**).

To pull down linear byproducts that contain intronic sequences, six different oligomers against intronic region were designed, four oligomers for the 3' half-intron, and two oligomers for the 5' half-intron. Each oligomer was 23 nucleotides and had biotin that was linked by TEG.

Linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template in the presence of 7.5mM of NTP. Template DNA was removed by treating with DNase for 20 minutes. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription; no further/additional reaction was required.

Self-spliced RNA (1 nmol) was mixed with 2 nmol of each oligo in the presence of 1X binding buffer (150 mM NaCl, 15 mM sodium citrate, 0.5 mM EDTA) in 2.5 ml total (final RNA concentration was 400 nM and oligomer concentration was 800nM per each oligomer). The RNA-oligomer mixtures were

incubated for 30 minutes at RT. For column purification, 500 µl of bead was pre-packed in empty column and RNA-oligomer mixture was placed on the packed resin. The RNA-oligomer mixture was passed through the resin by gravity and flow through was collected. The collected flow through was buffer exchanged with water by Amicon ultra centrifugal filter (100K cut off).

5 Enriched circular RNA was purified with different downstream processes. In vitro transcribed RNA without enrichment was purified in parallel for control.

For circular RNA that encoded Gluc, four different purification methods were tested. First method was column purification, enriched circular RNA was column-purified (Monarch). Second method was gel-purification. Enriched circular RNA encoding Gluc was purified by Urea-PAGE, eluted in a buffer (0.5 M Sodium Acetate, 0.1% SDS, 1 mM EDTA), ethanol precipitated and resuspended in RNase-free water. Third method was reverse phase chromatography (RP). Circular RNA encoding Gluc was purified by reverse phase chromatography and the fractions were buffer exchanged with sodium citrate then water in Amicon Ultra Centrifugation filter (100K cut off). Fourth method was anionic exchange chromatography (AEX). Circular RNA encoding Gluc was purified by anionic exchange chromatograph (AEX) and the fractions were buffer exchanged with water in Amicon Ultra Centrifugation filter (100K cut off). In vitro transcribed RNA was subjected to RP and AEX purification for comparison.

For circular RNA that encoded hEPO, four different purification methods were tested. First method was without purification, only buffer exchanged circular RNA was used (BE). Second method was gel-purification. Enriched circular RNA encoding hEPO was purified by Urea-PAGE, eluted in a buffer (0.5 M Sodium Acetate, 0.1% SDS, 1 mM EDTA), ethanol precipitated and resuspended in RNase-free water. Third method was reverse phase chromatography (RP). Circular RNA encoding hEPO was purified by reverse phase chromatography and the fractions were buffer exchanged with sodium citrate then water in Amicon Ultra Centrifugation filter (100K cut off). Fourth method was anionic exchange chromatography (AEX). Circular RNA encoding hEPO was purified by anionic exchange chromatograph (AEX) and the fractions were buffer exchanged with water in Amicon Ultra Centrifugation filter (100K cut off). In vitro transcribed RNA was subjected to gel-purification, RP and AEX purification for comparison.

To compare expression of Gluc from enriched (LP) or un-enriched (IVT) circular RNA, 0.1 pmol of purified circular RNAs were transfected HeLa cells (10,000 cells per well in a 96 well plate) using LIPOFECTAMINE® MessengerMax transfection reagent (Invitrogen). Cell culture media was harvested at 6 hours, 24 hours or 48 hours. To measure Gluc activity, 10 µl of harvested cell media was transferred to a white 96 well plate, and a bioluminescent reporter assay system was used according to the manufacturer's instruction (Pierce Gaussia Luciferase Flash Assay Kit, 16158, Thermo Scientific). The plate was read in a luminometer instrument (Promega).

To compare expression of hEPO from enriched (LP) or un-enriched (IVT) circular RNA, 0.25 pmol of purified circular RNAs were transfected in HeLa cells (10,000 cells per well in a 96 well plate) using LIPOFECTAMINE® MessengerMax transfection reagent (Invitrogen). Cell culture media was harvested at 24 hours or 48 hours. The amount of secreted hEPO protein was measured by hEPO ELISA kit (Invitrogen) according to the manufacturer's instruction.

In both Gluc RNA and hEPO RNA, circular RNA purified *via* linear RNA pull down (LP) showed better expression than without enrichment if the circular RNA was purified using the same method (*e.g.*, LP-RP showed more than 4-fold higher expression than IVT-RP in the case of Gluc, and 2-fold higher

expression in the case of hEPO). This data indicates that circular RNA enrichment using the methods described herein enhances expression.

Example 8: Linear RNA pull down with reduced number of oligomers enriches circular RNA

5 This example demonstrates enrichment of circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions with smaller number of oligomers.

In this example, the construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo including an ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron. For this example, the ORF is hEPO, and the length of linear RNA with the hEPO ORF is
10 around 1.4 Kb.

For linear pull down to remove linear byproducts that contain intronic sequences, six different oligomers against the intronic region were designed, four oligomers for 3' half-intron (#1 to #4), and two oligomers for 5' half-intron (#5 and #6) (**FIG. 12**). Each oligomer was 23 nucleotides and had biotin that was linked by TEG. In this example, the pull-down efficiency with a decreased number of oligomers was
15 examined.

In a first study, oligomers against 3' half-intron were tested (#1 to #4). Linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase for 20 minutes. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during
20 transcription; no further or additional reaction was required.

Self-spliced RNA (200 pmol) was mixed with each oligomer (#1 to #4) against the 3' half-intron at two different concentrations (2.4 μ M or 4.8 μ M) in the presence of 1X binding buffer (150 mM NaCl, 15 mM sodium citrate, 0.5 mM EDTA) (final RNA concentration was 400 nM) in 500 μ l total. As a negative control, RNA without oligomer was used. For positive control, a mixture of all six oligomers (#1 to #6)
25 was used. The RNA-oligomer mixtures were incubated for 30 minutes at room temperature (RT). For column purification, 500 μ l of resin was pre-packed in an empty column and the RNA-oligomer mixture was placed on the packed resin. The RNA-oligomer mixture was passed through the resin by gravity and the flow through was collected. The concentration of RNA of the flow through was measured by Qubit and 200 ng of RNA was separated by Urea PAGE, stained using a gel stain solution and visualized using
30 an imaging system.

The #1 and #2 oligomers showed similar enrichment efficiency at both 2.4 μ M and 4.8 μ M concentrations as compared to the positive control where all six oligomers for pull down were used (**FIG. 13A**, 6% gel) and removed 3' half-introns (**FIG. 13B**, 10% gel).

In a second study, combinations of oligos against both the 3' half-intron (#1 and #2) and 5' half-intron (#5 and #6) were tested. Linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase for 20 minutes. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription; no further or additional reaction was
35 required.

40 Self-spliced RNA (200 pmol) was mixed with the #1 oligomer in combination with the #5 or #6 oligo, or the #2 oligomer in combination with the #5 or #6 oligo, in the presence of 1X binding buffer (150

mM NaCl, 15 mM sodium citrate, 0.5 mM EDTA) (final RNA concentration was 400 nM) in 500 µl total (final oligomer concentration is 2.4 µM each). As a negative control, RNA without oligomer was used. As a further control, the #1 oligo only or the #5 oligomer only was used. For a positive control, a mixture of all six oligomers (#1 to #6) was used. The RNA-oligomer mixtures were incubated for 30 minutes at room temperature (RT). For column purification, 500 µl of resin was pre-packed in an empty column and the RNA-oligomer mixture was placed on the packed resin. The RNA-oligomer mixture was passed through the resin by gravity and flow through was collected. The concentration RNA of flow through was measured by Qubit and 200 ng of RNA were separated by Urea PAGE, stained using a gel stain and visualized using an imaging system.

The combination of two oligos against 3' half-intron or 5' half-intron showed similar circular RNA enrichment efficiency (FIG. 14A, 6% gel) and better intron removal than using a single oligomer against the 3' half-intron (FIG. 14B, 10% gel). The #1 and #5 oligo combination showed similar circular RNA enrichment efficiency (FIG. 14A) and intron removal efficiency (FIG. 14B) as compared to the positive control where all six oligomers for pull down were used. These data demonstrate that the use of two oligomers have equivalent circular RNA enrichment and intron removal efficiencies with the use of six oligomers.

Example 9: Linear RNA pull down mediated circular RNA enrichment using reduced number of oligomers results in circular RNA expression

This example demonstrates expression from circular RNA purified by a linear RNA pull down method using two oligomers.

In this example, the construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo including an hEPO ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron. Linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase for 20 minutes. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription; no additional reaction was required.

For linear pull down to remove linear byproducts that contain intronic sequences, six different oligomers against the intronic region (#1 to #6) were designed as described in Example 8.

Self-spliced RNA (200 pmol) was mixed with the #1 oligomer and the #5 or #6 oligomer, or the #2 oligomer and the #5 or #6 oligomer, in the presence of 1X binding buffer (150 mM NaCl, 15 mM sodium citrate, 0.5 mM EDTA) (final RNA concentration was 400 nM) in 500 µl total (final oligomer concentration is 2.4 µM each). As a negative control, RNA without oligomer was used. As another control, the #1 oligomer only or the #5 oligomer only was used. For a positive control, a mixture of all six oligomers (#1 to #6) was used. The RNA-oligomer mixtures were incubated for 30 minutes at room temperature (RT). For column purification, 500 µl of resin was pre-packed in an empty column and the RNA-oligomer mixture was placed on the packed resin. The RNA-oligomer mixture was passed through the resin by gravity and the flow through was collected. The collected flow through was buffer exchanged with water by Amicon ultra centrifugal filter (100K cut off).

To compare expression of hEPO from enriched (LP) circular RNA with different oligomers, 0.25 pmol of purified circular RNAs were transfected in HeLa cells (10,000 cells per well in a 96 well plate)

using LIPOFECTAMINE® MessengerMax transfection reagent (Invitrogen). Cell culture media was harvested at 24 hours. The amount of secreted hEPO protein was measured using by hEPO ELISA kit (Invitrogen) according to the manufacturer's instruction.

5 Circular RNA purified *via* linear pull down (LP) using two oligomers showed comparable expression with circular RNA purified *via* LP using all six oligomers (#1 to #6) (**FIG. 15**). In particular, the data show that circular RNA expression is obtained from circular RNA purified *via* LP using a combination of two oligomers (i.e., an oligomer for 3' half-intron and an oligomer for 5' half-intron) and is comparable in expression observed from circular RNA purified *via* LP using all six oligomers.

10 **Example 10: Linear RNA pull down with single oligomer that targets both 3' half-intron and 5' half-intron**

This example demonstrates enrichment of circular RNA by capturing linear byproducts *via* oligo-streptavidin interactions with a single oligomer that targets both the 3' half-intron and the 5' half-intron.

15 In this example, the construct was designed to include a 3' half of a catalytic intron, an exon fragment 2 (E2), a polyribonucleotide cargo including an ORF, an exon fragment 1 (E1), and a 5' half of a catalytic intron. The ORF is hEPO, and the length of linear RNA was around 1.4 Kb.

20 In this example, two oligomers were designed: oligo 5-Bio_1A5 consists of the #1 oligo (an oligomer against the 3' half-intron as described in Example 8) followed by the #5 oligo (an oligomer against the 5' half-intron as described in Example 8); oligo 5-Bio_5A1 consists of the #5 oligo followed by the #1 oligo. Both the 5-Bio_1A5 and 5-Bio_5A1 oligomers have eight nucleotides of adenosine linker between different oligomer sequences and 5' end biotin that was linked by TEG to oligomer. A schematic of the 5-Bio_1A5 and 5-Bio_5A1 oligomers is provided in **FIG. 16**.

25 Linear RNA was synthesized by in vitro transcription using T7 RNA polymerase from a DNA template in the presence of 7.5 mM of NTP. Template DNA was removed by treating with DNase for 20 minutes. Synthesized linear RNA was purified with an RNA clean up kit (New England Biolabs, T2050). Self-splicing occurred during transcription; no additional reaction was required.

30 Self-spliced RNA (200 pmol) was mixed with oligomer 5-Bio_1A5 or a 5-Bio_5A1 which targets both the 3' half-intron and the 5' half-intron (2.4 μ M concentration) in the presence of 1X binding buffer (150 mM NaCl, 15 mM sodium citrate, 0.5 mM EDTA) (final RNA concentration was 400 nM) in 500 μ l total volume. As a negative control, RNA without an oligomer was used. For a positive control, a mixture of all six oligomers (#1 to #6) was used. The RNA-oligomer mixtures were incubated for 30 minutes at room temperature (RT). For column purification, 500 μ l of resin was pre-packed in an empty column and the RNA-oligomer mixture was placed on the packed resin. The RNA-oligomer mixture was passed through the resin by gravity and flow through was collected. The concentration of the RNA of flow
35 through was measured by Qubit and 200 ng of RNA were separated by Urea PAGE, stained using a gel stain and visualized using an imaging system.

40 Circular RNA purified *via* the linear pull-down method using 5-Bio_1A5 or 5-Bio_5A1 efficiently removed linear RNA (**FIG. 17A**) and intron RNA (**FIG. 17B**). The circular RNA enrichment efficiency (**FIG. 17A**) and intron removal efficiency (**FIG. 17B**) were similar to the positive control where all six oligomers for pull down were used. These data demonstrate that a single oligomer designed to target

both the 3' half-intron and the 5' half-intron can be used for linear pull down to enrich circular RNA, with comparable circular RNA enrichment and intron removal efficiencies using LP with six oligomers.

Other Embodiments

- 5 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.

CLAIMS

1. A method of separating a linear polyribonucleotide comprising a target region from a plurality of polyribonucleotides comprising a mixture of linear polyribonucleotides and circular polyribonucleotides, the method comprising:
 - (a) providing a sample comprising the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides comprise the linear polyribonucleotide comprising the target region;
 - (b) contacting the sample with an oligonucleotide that hybridizes to the target region; and
 - (c) separating the linear polyribonucleotide comprising the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample.
2. The method of claim 1, wherein the circular polyribonucleotides lack the target region.
3. A method of separating a polyribonucleotide comprising a target region from a plurality of polyribonucleotides, the method comprising:
 - (a) providing a sample comprising the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides comprise the target region, wherein the target region is not located at a 3' or 5' terminus of the polyribonucleotide comprising the target region;
 - (b) contacting the sample with an oligonucleotide that hybridizes to the target region; and
 - (c) separating the polyribonucleotide comprising the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample.
4. A method of separating a linear polyribonucleotide comprising a target region from a plurality of circular polyribonucleotides comprising the target region, the method comprising:
 - (a) providing a sample comprising the plurality of polyribonucleotides;
 - (b) contacting the sample with an oligonucleotide, wherein the oligonucleotide hybridizes to the target region of the linear polyribonucleotide with a first binding affinity, and wherein the oligonucleotide hybridizes to the target region of the circular polynucleotide with a second binding affinity that is different from the first binding affinity; and
 - (c) separating the linear polyribonucleotide comprising the target region that is hybridized to the oligonucleotide from the plurality of circular polyribonucleotides in the sample.
5. The method of claim 4, wherein the first binding affinity is less than the second binding affinity, and wherein the oligonucleotide preferentially binds the linear polyribonucleotide.
6. A method of separating a polyribonucleotide in a first conformation comprising a target region from a plurality of polyribonucleotides in a second conformation comprising the target region, the method comprising:
 - (a) providing a sample comprising the plurality of polyribonucleotides;
 - (b) contacting the sample with an oligonucleotide, wherein the oligonucleotide hybridizes to the target region of the polynucleotide in the first conformation with a first binding affinity, and wherein the

oligonucleotide hybridizes to the target region of the polynucleotide in the second conformation with a second binding affinity that is different from the first binding affinity; and

(c) separating the polyribonucleotide in the first conformation comprising the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the second conformation in the sample.

7. The method of claim 6, wherein the first binding affinity is less than the second binding affinity, and wherein the oligonucleotide preferentially binds the polyribonucleotide in the first conformation.
8. The method of any one of claims 1-7, wherein step (c) comprises immobilizing the oligonucleotide.
9. The method of any one of claims 1-8, wherein the target region lacks a polyA sequence.
10. The method of any one of claims 1-9, wherein the oligonucleotide is conjugated to a particle.
11. The method of any one of claims 1-9, wherein the oligonucleotide is conjugated to a first capture agent.
12. The method of claim 11, wherein the oligonucleotide is conjugated to the first capture agent with a chemical linker.
13. The method of claim 12, wherein the chemical linker comprises triethylene glycol.
14. The method of claim 12 or 13, wherein the chemical linker is conjugated to a 3' end or a 5' end of the oligonucleotide.
15. The method of any one of claims 11-14, wherein the first capture agent comprises an antigen.
16. The method of claim 15, wherein the antigen is biotin.
17. The method of any one of claims 11-16, wherein the method further comprises providing a second capture agent that binds to the first capture agent.
18. The method of claim 17, wherein the second capture agent comprises an antibody or antigen-binding fragment thereof.
19. The method of claim 18, wherein the antibody or antigen-binding fragment thereof is streptavidin.
20. The method of any one of claims 17-19, wherein the second capture agent is conjugated to a particle.

21. The method of claim 20, wherein the particle is a magnetic particle or a bead.
22. The method of claim any one of claims 1-21, wherein the polyribonucleotide comprising the target region comprises an intron or portion thereof.
23. The method of claim 22, wherein the target region comprises an intron or portion thereof.
24. The method of claim 22, wherein the target region is located 5' or 3' to the intron or portion thereof.
25. The method of claim 23 or 24, wherein the method comprises separating a spliced polyribonucleotide from a non-spliced or partially spliced polyribonucleotide.
26. The method of claim 25, wherein the spliced polyribonucleotide is a circular polyribonucleotide.
27. The method of claim 25, wherein the spliced polyribonucleotide is a linear polyribonucleotide.
28. The method of any one of claims 25-27, wherein the spliced polyribonucleotide lacks an intron or portion thereof.
29. The method of claim 28, wherein the method enriches an amount of the spliced polyribonucleotide by at least 50% relative to the sample.
30. The method of any one of claims 22-29, wherein the polyribonucleotide comprising the intron or portion thereof is a linear polyribonucleotide.
31. The method of any one of claims 1-30, further comprising washing the captured polyribonucleotide comprising the target region one or more times.
32. The method of any one of claims 1-31, further comprising, after step (c), performing a first elution step to release the captured polyribonucleotide comprising the target region.
33. The method of claim 32, wherein the first elution step comprises adding a first buffer and/or heating the sample.
34. The method of claim 33, wherein the first elution step comprises heating the sample to at least 50 °C.
35. The method of any one of claims 32-34, further comprising, after the first elution step, performing a second elution step.
36. The method of claim 34, wherein the second elution step comprises adding a second buffer and/or heating the sample.

37. The method of claim 36, wherein the second elution step comprises heating the sample to at least 50°C.
38. The method of claim 36 or 37, wherein the second buffer comprises a denaturing agent.
39. The method of claim 38, wherein the denaturing agent comprises formamide or urea.
40. The method of any one of claims 1-39, wherein step (a) comprises incubating the sample with the oligonucleotide for at least ten minutes.
41. The method of any one of claims 1-40, wherein step (b) comprises collecting a portion of the sample that is not bound by the oligonucleotide.
42. The method of any one of claims 1-41, wherein the method comprises providing a plurality of oligonucleotides, wherein each oligonucleotide hybridizes to a distinct target region.
43. The method of claim 42, wherein each oligonucleotide is conjugated to a first capture agent.
44. The method of any one of claim 1-43, wherein the oligonucleotide has at least 80% complementarity an equal length portion of the target region.
45. The method of claim 44, wherein the oligonucleotide has at least 85%, 90%, 95%, 97%, 99%, or 100% complementarity to the equal length portion of the target region.
46. The method of any one of claims 1-45, wherein step (a) comprises providing the oligonucleotide at a molar ratio of 10:1 to 1:10 to the polyribonucleotide comprising the target region.
47. A population of polyribonucleotides produced by the method of any one of claims 1-46.
48. The population of polyribonucleotides of claim 47, wherein the population comprises a circular polyribonucleotide lacking a target region and the circular polyribonucleotide comprises at least 40% (mol/mol) of the total polyribonucleotides in the composition.
49. A composition comprising a mixture of polyribonucleotides, wherein a first subset of the mixture comprises a circular polyribonucleotide lacking a target region, and a second subset of the plurality of the polyribonucleotides comprises a linear polyribonucleotide comprising the target region, wherein the first subset comprises at least 40% (mol/mol) of the total polyribonucleotides in the composition.
50. The composition of claim 49, wherein the population comprises less than 50% (mol/mol) linear polyribonucleotides.

51. A composition comprising a polyribonucleotide comprising a target region and an oligonucleotide configured to hybridize to the target region, wherein the oligonucleotide is conjugated to a first capture agent.
52. The composition of claim 51, further comprising a polyribonucleotide lacking the target region.
53. The composition of claim 52, wherein the polyribonucleotide lacking the target region is a circular polyribonucleotide.
54. The composition of any one of claims 51-53, further comprising a second capture agent configured to bind the first capture agent.
55. The composition of claim 54, wherein the second capture agent is conjugated to a particle.
56. The composition of any one of claims 51-55, wherein the first capture agent is biotin.
57. The composition of any one of claims 51-56, wherein the second capture agent is streptavidin.
58. The composition of any one of claims 49-57, wherein the composition is produced by the method of any one of claims 1-46.
59. The composition of any one of claims 49-58, wherein the linear polyribonucleotide comprises an intron or portion thereof.
60. The composition of claim 59, wherein the target region comprises the intron or portion thereof.
61. The composition of claim 60, wherein the target region is located 5' or 3' to the intron or portion thereof.
62. A pharmaceutical composition comprising the composition of any one of claims 49-61 and a diluent, carrier, or excipient.