

COMPOSITIONS AND METHODS FOR PURIFYING POLYRIBONUCLEOTIDES

Sequence Listing

This application contains a Sequence Listing which has been filed electronically in Extensible Markup Language (XML) format and is hereby incorporated by reference in its entirety. Said XML copy, created on December 22, 2022, is named 51509-056WO2_Sequence_Listing_12_21_22.XML and is 3,683 bytes in size.

Background

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

Summary

In one example, the disclosure features a method of separating a linear polyribonucleotide from a plurality of polyribonucleotides. The plurality of polyribonucleotides include a mixture of linear polyribonucleotides and circular polyribonucleotides that include an open reading frame (ORF) encoding a polypeptide. The method includes (a) providing a sample with the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides include the linear polyribonucleotide; (b) attaching a target region to the linear polyribonucleotide; and (c) contacting the sample with an oligonucleotide that hybridizes to the target region. The method further includes (d) separating the linear polyribonucleotide with the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample. In one aspect, the present disclosure refers to a method of separating a linear polyribonucleotide from a plurality of polyribonucleotides comprising a mixture of linear polyribonucleotides and circular polyribonucleotides comprising an open reading frame (ORF) encoding a polypeptide, the method comprising: (a) providing a sample comprising the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides comprise the linear polyribonucleotide; (b) attaching a target region to the linear polyribonucleotide; (c) contacting the sample with an oligonucleotide that hybridizes to the target region; and (d) separating the linear polyribonucleotide comprising the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample.

In some examples, the oligonucleotide is conjugated (e.g., directly or indirectly) to a particle. The particle may be, for example, a magnetic bead. In some examples, the oligonucleotide is conjugated to a resin that includes a plurality of the particles. The resin may include, for example, cross-linked poly[styrene-divinylbenzene], agarose, or SEPHAROSE® agarose. In some examples, a column includes the resin.

In some examples, separating the linear polyribonucleotide includes immobilizing the oligonucleotide.

In some examples, separating the linear polyribonucleotide includes collecting a portion of the sample that is not hybridized to the oligonucleotide. For example, the portion of the sample that is not hybridized to the oligonucleotide may include the circular polyribonucleotide.

In some examples, a level of expression from the ORF of the circular polyribonucleotide after purification is increased at least 10% relative to a level of expression from the ORF prior to purification.

In another example, the disclosure features a method of separating a linear polyribonucleotide from a plurality of polyribonucleotides. The plurality of polyribonucleotides include a mixture of linear polyribonucleotides and circular polyribonucleotides. The method includes (a) providing a sample with the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides include the linear polyribonucleotide; (b) attaching a target region to the linear polyribonucleotide; and (c) contacting the sample with a column that includes a resin with a plurality of particles conjugated to an oligonucleotide that hybridizes to the target region. The method further includes (d) collecting an eluate

that includes a portion of the sample that is not hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample. In one aspect, the present disclosure refers to a method of separating a linear polyribonucleotide 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; (b) attaching a target region to the linear polyribonucleotide; (c) contacting the sample with a column comprising a resin comprising a plurality of particles conjugated to an oligonucleotide that hybridizes to the target region; and (d) collecting an eluate comprising a portion of the sample that is not hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample.

5 In some examples, the portion of the sample that is not hybridized to the oligonucleotide includes the circular polyribonucleotide.

In some examples, the resin includes cross-linked poly[styrene-divinylbenzene], agarose, or SEPHAROSE® agarose.

10 In some examples of any of the above aspects, the method includes a step of circularizing the circular polyribonucleotide from a linear precursor prior to step (a). For example, the linear precursor may include a 5' self-splicing intron fragment and a 3' self-splicing intron fragment, and the circular polyribonucleotide is produced by self-splicing of the linear precursor. The 5' self-splicing intron fragment and the 3' self-splicing intron fragment may each be, for example, a Group I or Group II self-splicing intron fragment.

15 In another example, the disclosure features a method of separating a linear polyribonucleotide from a plurality of polyribonucleotides. The plurality of polyribonucleotides include a mixture of linear polyribonucleotides and circular polyribonucleotides. The method includes (a) circularizing a linear precursor to form the circular polyribonucleotide; (b) providing a sample that includes the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides include the linear polyribonucleotide; (c) attaching a target region to the linear polyribonucleotide; and (d) contacting the sample with an oligonucleotide that hybridizes to the target region. The method further includes (e) separating the linear polyribonucleotide with the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample. In one aspect, the present disclosure refers to a method of separating a linear polyribonucleotide from a plurality of polyribonucleotides comprising a mixture of linear polyribonucleotides and circular polyribonucleotides, the method comprising: (a) circularizing a linear precursor to form the circular polyribonucleotide; (b) providing a sample comprising the plurality of polyribonucleotides, wherein a subset of the plurality of polyribonucleotides comprise the linear polyribonucleotide; (c) attaching a target region to the linear polyribonucleotide; (d) contacting the sample with an oligonucleotide that hybridizes to the target region; and (e) separating the linear polyribonucleotide comprising the target region that is hybridized to the oligonucleotide from the plurality of polyribonucleotides in the sample.

In some examples, circularizing the circular polyribonucleotide is produced by splint-ligation of the linear precursor.

35 In some examples, the linear precursor includes a 5' self-splicing intron fragment and a 3' self-splicing intron fragment, and the circular polyribonucleotide is produced by self-splicing of the linear precursor.

In some examples, the 5' self-splicing intron fragment and the 3' self-splicing intron fragment are each a Group I or Group II self-splicing intron fragment.

40 In some examples of any of the above aspects, the circular polyribonucleotide includes an ORF. The ORF may encode, for example, a polypeptide.

In some examples of any of the above aspects, the circular polyribonucleotide includes an internal ribosome entry site (IRES). The ORF may be operably linked to the IRES.

45 In some examples, the method includes attaching the target region to a 3' or 5' terminus of the linear polyribonucleotide. For example, the method may include attaching the target region to the 3' terminus of the linear polyribonucleotide. The attaching step may include polyadenylating the 3' terminus of the linear polyribonucleotide. The polyadenylation may include providing a polyA polymerase, e.g., *E. coli* polyA polymerase. In another example, the attaching step includes ligating the target region to the 3' terminus of the linear polyribonucleotide.

50 In some examples, the linear polyribonucleotide of step (a) comprises a target region (e.g., prior to attaching the target region in step (b)). Accordingly, such examples provide a further method of purification wherein a first copy of a target region is present in the linear polyribonucleotide prior to step

(b), and a second copy of the target region is attached to the linear polyribonucleotide during step (b). The target region present in the linear polyribonucleotide of step (a) may be the same or different (e.g., in sequence and length) than the target region attached to the linear polyribonucleotide in step (b). In some examples, the linear precursor includes, operably linked in the following 5' to 3' order: a target region, a first circularization element (e.g., a first self-splicing intron fragment), a polyribonucleotide cargo (e.g., optionally including one or more of a spacer, an IRES, and an open reading frame), and a second circularization element (e.g., a second self-splicing intron fragment).

5 In some examples, the circular polyribonucleotide does not include a polyA sequence (e.g., a polyA sequence of at least 10, e.g., at least 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25, e.g., at least 15, at least 20, at least 25 or at least 30) contiguous adenosine nucleotides.

10 In some examples, the target region includes a polyA sequence (e.g., a polyA sequence of at least 10 contiguous adenosine nucleotides).

In some examples, the oligonucleotide that binds the target includes a polyU or polydT sequence. The polyU or polydT sequence may include at least 10 (e.g., at least 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, e.g., at least 15, at least 20, at least 25 or at least 30) uridine or thymidine residues. In some examples, the polydT includes (dT)₂₅.

15 In some examples, the oligonucleotide is conjugated to a particle. The particle may be, for example, a magnetic bead. In some examples, the oligonucleotide is conjugated to a resin that includes a plurality of the particles. The resin may include, for example, cross-linked poly[styrene-divinylbenzene], agarose, or SEPHAROSE® agarose. In some examples, a column includes the resin.

20 In some examples, separating the linear polyribonucleotide includes immobilizing the oligonucleotide.

In some examples, separating the linear polyribonucleotide includes collecting a portion of the sample that is not hybridized to the oligonucleotide. For example, the portion of the sample that is not hybridized to the oligonucleotide may include the circular polyribonucleotide.

25 In some examples of any of the above aspects, the method further includes washing the linear polyribonucleotide with the target region that is hybridized to the oligonucleotide one or more times.

In some examples of any of the above aspects, the method further includes eluting the linear polyribonucleotide with the target region from the oligonucleotide.

30 In some examples, the method includes providing a plurality of oligonucleotides, wherein each oligonucleotide hybridizes to a distinct target region.

In some examples, 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 examples, 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 examples, the oligonucleotide is 23 nucleotides in length. In some examples, 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.

35 In some examples, the method includes providing the oligonucleotide at a molar ratio of 10:1 to 1:10 to the linear polyribonucleotide with the target region.

In some examples, a level of expression from the ORF of the circular polyribonucleotide after purification is increased at least 10% relative to a level of expression from the ORF prior to purification.

In some examples of any of the above aspects, the method separates at least 500 µg (e.g., at least 600 µg, 700 µg, 800 µg, 900 µg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60mg, 70 mg, 80 mg, 90 mg, 100 mg, 200 mg, 300 mg, 400 mg, 500 mg, 600 mg, 700 mg, 800 mg, 900 mg, 1,000 mg, or more) of the linear polyribonucleotide with the target region. In some examples, the method separates from 500 µg to 1,000 mg of the linear polyribonucleotide including the target region.

In another example, the disclosure features a population of polyribonucleotides produced by the method as described herein (e.g., of any of the aspects). In one aspect, the present disclosure refers to a population of polyribonucleotides produced by the methods as described herein, wherein, optionally the population comprises a circular polyribonucleotide lacking the target region and the circular polyribonucleotide comprises at least 40% (mol/mol) of the total polyribonucleotides in the composition and/or wherein the population comprises less than 40% (mol/mol) linear polyribonucleotides of the total polyribonucleotides in the composition.

In some examples, the population includes a circular polyribonucleotide lacking the target region and the circular polyribonucleotide includes at least 40% (e.g., at least 50%, 60%, 70%, 80%, 85%, 90%, 95%, 97%, 99%, or 100%) (mol/mol) of the total polyribonucleotides in the composition.

In some examples, the population includes less than 40% (e.g., less than 35%, 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, or 1%) (mol/mol) linear polyribonucleotides of the total polyribonucleotides in the composition.

In some examples, a total weight of polyribonucleotides in the population of polyribonucleotides at least 500 µg (e.g., at least 600 µg, 700 µg, 800 µg, 900 µg, 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg, 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60mg, 70 mg, 80 mg, 90 mg, 100 mg, 200 mg, 300 mg, 400 mg, 500 mg, 600 mg, 700 mg, 800 mg, 900 mg, 1,000 mg, or more). In some examples, the total weight of polyribonucleotides in the population of polyribonucleotides is from 500 µg to 1000 mg.

In another example, the disclosure features a pharmaceutical composition that includes the population of polyribonucleotides of any of the above examples (e.g., that is produced by a method as described herein) and a diluent, carrier, or excipient.

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 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.

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 "adjuvant" refers to a composition (e.g., a compound, polypeptide, nucleic acid, or lipid) that increases an immune response, for example, increases a specific immune