[0009] To gain a better understanding of these structures and to ultimately harness the therapeutic potential of G-rich oligonucleotides that form quadruplex structures, researchers need to be able to detect, characterize, isolate, and purify these molecules. Generally, most approaches for purifying guanine-rich oligonucleotides or separating them from associated impurities aim to disrupt secondary interactions, such as quadruplex formation, by using elevated temperatures, high pH buffers, or introducing chaotropic agents or organic modifiers. Such strongly denaturing conditions promote single-strand formation. The single strand could then be purified, but the quadruplex would then need to be assembled from the purified single strands. From an analytical perspective, strong denaturing conditions may affect accurate quantitation of the quadruplex structure or other impurities of higher order structure present in an analytical sample.

[0010] In view of the foregoing, there remains a need for efficient methods of purifying or separating guanine-rich oligonucleotides from the quadruplexes formed therefrom and other impurities.

SUMMARY

[0011] The present disclosure relates to a separation method for guanine-rich oligonucleotides that tend to form quadruplex structures. The disclosure is based, in part, on the discovery that the formation of quadruplex structures from the guanine-rich oligonucleotides can be chromatographically separated by the presently disclosed methods which employ a chromatographic matrix comprising a hydrophobic ligand comprising C4 to C8 alkyl chains and a mobile phase comprising a gradient of acetate and a gradient of acetonitrile. Such methods, as demonstrated herein, allow high resolution separation between not only the guanine-rich oligonucleotide and the quadruplex, but between other predominant molecular species of the guanine-rich oligonucleotide. Advantageously, the methods of the present disclosure may be used to achieve high resolution separation for the guanine-rich oligonucleotide, its complementary strand, the quadruplex and the duplex comprising the guanine-rich oligonucleotide and its complementary strand.

[0012] The present inventors have surprisingly found that by excluding a cationic ion pairing agent, such as triethylamine (TEA), from the mobile phase, high resolution among the peaks corresponding to the molecular species of the guanine-rich oligonucleotide may be achieved using a reverse-phase (i.e. hydrophobic) stationary phase.

[0013] Accordingly, the present disclosure provides methods of separating molecular species of a guanine-rich oligonucleotide from a mixture of molecular species. In exemplary examples, at least one molecular species of the mixture is a quadruplex formed from the guanine-rich oligonucleotide. In exemplary examples, the method comprises (a) applying the mixture to a chromatographic matrix comprising a hydrophobic ligand, wherein said hydrophobic ligand comprises C4 to C8 alkyl chains, wherein molecular species bind to the hydrophobic ligand; and (b) applying a mobile phase which comprises a gradient of acetate and a gradient of acetonitrile to the chromatographic matrix to elute molecular species of the guanine-rich oligonucleotide. In exemplary examples, each molecular species elutes at a time distinct from the time at which a different molecular species elutes. For example, in exemplary instances, the guanine-rich oligonucleotide elutes at a distinct time at which the quadruplex elutes. In various examples, the guanine-rich oligonucleotide elutes in a first set of elution fractions and the quadruplex elutes in a second set of elution fractions.

[0014] In exemplary examples, the guanine-rich oligonucleotide is a sense strand or an antisense strand of a small interfering RNA (siRNA). In exemplary instances, the mixture comprises single-stranded molecular species and/or double-stranded molecular species. Optionally, the mixture comprises one or more molecular species selected from the group consisting of: an antisense single strand, a sense single strand, a duplex, and a quadruplex. In various examples, the guanine-rich oligonucleotide is the antisense single strand. In various instances, the duplex comprises the antisense single strand and the sense single strand. In exemplary examples, the mixture comprises all of the following molecular species: an antisense single strand, a sense single strand, a duplex, and a quadruplex. Optionally, each molecular species elutes in a fraction separate from that of another molecular species. In exemplary examples, the duplex elutes in a first set of elution fractions, the sense strand elutes in a second set of elution fractions, the antisense strand elutes in third set of elution fractions, and the quadruplex elutes in a fourth set of elution fractions. In various examples, the resolution of the separation of the peaks of each molecular species (e.g., the resolution of the separation between the peak of the duplex and the peak of the sense single strand) is at least or about 1.0, optionally, at least or about 1.1, at least or about 1.2, at least or about 1.3, or at least or about 1.4. In various examples, the resolution of the separation of the peaks of each molecular species is at least or about 1.5, optionally, at least or about 1.6, at least or about 1.7, at least or about 1.8, or at least or about 1.9. Optionally, the resolution of the separation of the peaks corresponding to each molecular species (e.g., the resolution of the separation between the peak of the duplex and the peak of the sense single strand) is at least or about 2.0 (e.g., at least

or about 2.1, at least or about 2.2, at least or about 2.3, at least or about 2.4. In various examples, the resolution of the separation is at least or about 2.4. In exemplary instances, the resolution is at least or about 2.5, at least or about 3.0, or at least or about 4.0. Optionally, the resolution of the separation between the peak of the duplex and the peak of the sense strand is at least 4.0. In various examples, the limit of quantitation (LOQ) of each molecular species is about 0.03 mg/mL to about 0.08 mg/mL, when the signal-to-noise ratio is greater than or equal to 10.0. In various instance, the LOQ is about 0.08 mg/ml when the signal-to-noise ratio is greater than or equal to 10.0.

[0015] In various instances, the mixture is prepared in a solution comprising one or more of: water, a source of acetate, a source of potassium, and sodium chloride. The source of acetate is ammonium acetate, sodium acetate, potassium acetate in certain examples. Optionally, the source of potassium is potassium phosphate. In various examples, the solution comprises about 50 mM to about 150 mM acetate or potassium. In various instances, the solution comprises about 75 mM to about 100 mM of ammonium acetate, sodium acetate, or potassium acetate. In exemplary examples, the solution comprises potassium phosphate and sodium chloride.

[0016] In certain examples, the chromatographic matrix comprises a hydrophobic ligand comprising C4 alkyl chains, C6 alkyl chains, or C8 alkyl chains. Optionally, the hydrophobic ligand comprises C4 alkyl chains. In exemplary examples, the chromatographic matrix is housed in a chromatographic column having an internal diameter of 2.1 mm and/or a column length of about 50 mm. In exemplary instances, the column temperature is about 20 °C to about 35 °C, optionally, about 30 °C. In various instances, the chromatographic matrix comprises ethylene bridged hybrid (BEH) particles. Optionally, the BEH particles have a particle diameter of about 1.7 μm or about 3.5 μm

[0017] In some examples, the gradient of acetate in the mobile phase is made with an acetate stock solution comprising about 50 mM to about 150 mM acetate. Optionally, the acetate stock solution comprises about 70 mM to about 80 mM acetate, optionally, about 75 mM acetate. In various examples, the acetate stock solution comprises about 90 mM to about 110 mM acetate, optionally, about 100 mM acetate. In various instances, the acetate is ammonium acetate, sodium acetate or potassium acetate. In exemplary examples, the pH of the acetate stock solution is about 6.5 to about 7.0, optionally, about 6.7, about 6.8, about 6.9, or about 7.0. In exemplary examples of the present disclosure, the mobile phase comprises a decreasing gradient of the acetate and an increasing gradient of acetonitrile. In exemplary instances, the gradient of acetate starts with a maximum concentration and gradually decreases to a minimum

concentration over a first time period. Optionally, the first time period is about 18 to about 19 minutes, and, alternatively, the first time period is about 22 minutes to about 26 minutes. In various examples, after the first time period, the mobile phase increases to the maximum concentration of acetate, optionally, about 0.1 to about 3 minutes after the gradient reaches the minimum concentration of acetate. In various examples, the gradient of acetonitrile starts with a minimum concentration and gradually increases to a maximum concentration over the first time period. Optionally, after the first time period, the mobile phase decreases to the minimum concentration of acetonitrile. Optionally, the mobile phase decreases to the minimum concentration of acetonitrile about 0.1 to about 3 minutes after the gradient of acetonitrile reaches the maximum concentration of acetonitrile. In certain examples, the method of the present disclosure comprises applying the mobile phase to the chromatographic matrix according to the following conditions:

Time (min)	Acetate (%)	Acetonitrile
0	93	7
5	88	12
8	88	12
11	86	14
18	70	30
19	70	30
21	93	7
26	93	7

In alternative or additional examples, the method comprises applying the mobile phase to the chromatographic matrix according to the following conditions:

Time (min)	Acetate (%)	Acetonitrile (%)
0	92	8
2	90	10
18	86	14
26	70	30
26.1	92	8
30	92	8

In alternative or additional examples, the method comprises applying the mobile phase to the chromatographic matrix according to the following conditions:

Time (min)	Acetate (%)	Acetonitrile (%)
0.0	92	8
2.0	90	10

18.0	86	14
22.0	78	22
22.1	20	80
24.0	20	80
24.1	92	8
30.0	92	8

[0018] In exemplary examples, the mobile phase does not comprise a cationic ion pairing agent, e.g., TEA. The total run time is, in various instances, at least about 25 minutes and less than 40 minutes, optionally, less than 35 minutes, optionally, less than or equal to 30 minutes. In various instances, the run time is about 22 minutes to about 26 minutes. In various examples, the flow rate of the mobile phase is about 0.5 ml/min to about 1.0 ml/min, optionally, about 0.7 ml/min to about 0.8 ml/min.

[0019] In exemplary examples, the guanine-rich oligonucleotide comprises about 19 to about 23 nucleotides. In exemplary instances, the guanine-rich oligonucleotide and one or more of the molecular species thereof in the mixture comprises one or more modified nucleotides. Optionally, the one or more modified nucleotides are 2'-modified nucleotides, such as 2'-O-methyl modified nucleotides, 2'-fluoro modified nucleotides, deoxynucleotides, or combinations thereof. In various examples, the guanine-rich oligonucleotide and one or more of the molecular species thereof in the mixture comprises synthetic internucleotide linkages, such as phosphorothioate linkages.

[0020] The present disclosure also provides a method of determining the purity of a sample comprising a guanine-rich oligonucleotide drug substance or drug product. In exemplary examples, the method comprises separating molecular species of the guanine-rich oligonucleotide in accordance with the presently disclosed methods of separating molecular species of the guanine-rich oligonucleotide. In various examples, the sample is an in-process sample and the method is used as part of an in-process control assay or as an assay for ensuring the manufacture of the G-rich oligonucleotide is being carried out without substantial impurities. In various instances, the sample is a lot sample and the method is used as part of a lot release assay. In various examples, the sample is a stressed sample or a sample that has been exposed to one or more stresses, and the method is a stability assay. Accordingly, the present disclosure provides a method of testing stability of a guanine-rich oligonucleotide drug substance or drug product, comprising applying stress to a sample comprising the guanine-rich oligonucleotide drug substance or drug product and determining the purity of the sample

WHAT IS CLAIMED IS:

1. A method of separating molecular species of a guanine-rich oligonucleotide from a mixture of molecular species, wherein at least one molecular species of the mixture is a quadruplex formed from the guanine-rich oligonucleotide, said method comprising:
    a. applying the mixture to a chromatographic matrix comprising a hydrophobic ligand, wherein said hydrophobic ligand comprises C4 to C8 alkyl chains, wherein molecular species bind to the hydrophobic ligand;
    b. applying a mobile phase which comprises a gradient of acetate and a gradient of acetonitrile to the chromatographic matrix to elute molecular species of the guanine-rich oligonucleotide, wherein the guanine-rich oligonucleotide elutes in a first set of elution fractions and the quadruplex elutes in a second set of elution fractions.
2. The method of claim 1, wherein the guanine-rich oligonucleotide is a sense strand or an antisense strand of a small interfering RNA (siRNA).
3. The method of claim 1 or 2, wherein the mixture comprises a single-stranded molecular species and/or a double-stranded molecular species.
4. The method of claim 3, wherein the mixture comprises one or more molecular species selected from the group consisting of: an antisense single strand, a sense single strand, a duplex, and a quadruplex.
5. The method of claim 4, wherein the guanine-rich oligonucleotide is the antisense single strand and/or the duplex comprises the antisense single strand and the sense single strand.
6. The method of claim 4 or 5, wherein the mixture comprises the following molecular species: an antisense single strand, a sense single strand, a duplex, and a quadruplex.
7. The method of any one of the preceding claims, wherein each molecular species elutes in a fraction separate from that of another molecular species, optionally, wherein the mixture comprises an antisense single strand, a sense single strand, a duplex, and a quadruplex and the duplex elutes in a first set of elution fractions, the sense strand

elutes in a second set of elution fractions, the antisense strand elutes in third set of elution fractions, and the quadruplex elutes in a fourth set of elution fractions.

8. The method of any one of the preceding claims, wherein the LOQ of each molecular species is about 0.03 mg/mL to about 0.08 mg/mL.

9. The method of claim 7 or 8, wherein the resolution of the separation of the peaks of each molecular species is at least or about 1.0, optionally, at least or about 1.2.

10. The method of any one of the preceding claims, wherein the mixture is prepared in a solution comprising one or more of: water, a source of acetate, a source of potassium, and sodium chloride, optionally, source of acetate is ammonium acetate, sodium acetate, or potassium acetate or potassium phosphate.

11. The method of claim 10, wherein the solution comprises about 50 mM to about 150 mM acetate or potassium optionally about 75 mM to about 100 mM of ammonium acetate, sodium acetate, or potassium acetate.

12. The method of any one of the preceding claims, wherein the hydrophobic ligand comprises C4 alkyl chains, C6 alkyl chains, or C8 alkyl chains.

13. The method of claim 12, wherein the hydrophobic ligand comprises C4 alkyl chains.

14. The method of any one of the preceding claims, wherein (A) the chromatographic matrix is housed in a chromatographic column having an internal diameter of 2.1 mm and/or a column length of about 50 mm, (B) the column temperature is about 20 °C to about 35 °C, and/or (C) the matrix comprises 1.7 ethylene bridged hybrid (BEH) particles.

15. The method of any one of the preceding claims, wherein the gradient of acetate is made with an acetate stock solution comprising about 50 mM to about 150 mM acetate.

16. The method of claim 15, wherein the acetate stock solution comprises about 70 mM to about 80 mM acetate, optionally, about 75 mM acetate.

17. The method of claim 15, wherein the acetate stock solution comprises about 90 mM to about 110 mM acetate, optionally, about 100 mM acetate.

18. The method of any one of the preceding claims, wherein the acetate is ammonium acetate, sodium acetate or potassium acetate.

19. The method of any one of claims 15 to 18, wherein the pH of the acetate stock solution is about 6.5 to about 7.0.

20. The method of any one of claims 16 to 19, wherein the acetate stock solution is 75 mM ammonium acetate in water having a pH of 6.7 ± 0.1.

21. The method of any one of the preceding claims, wherein (A) the gradient of acetonitrile is made with an acetonitrile stock solution and the acetonitrile stock solution is 100% acetonitrile, (B) the mobile phase comprises a decreasing concentration gradient of the acetate and an increasing concentration gradient of acetonitrile, and/or (C) the gradient of acetate starts with a maximum concentration and gradually decreases to a minimum concentration over a first time period, optionally, wherein the first time period is about 18 to about 19 minutes or about 22 to about 26 minutes.

22. The method of any one of the preceding claims, wherein after the first time period, the acetate concentration in the mobile phase increases to the maximum concentration of acetate, optionally, wherein the acetate increases to the maximum concentration of acetate about 0.1 to about 3 minutes after the gradient reaches the minimum concentration of acetate.

23. The method of any one of the preceding claims, wherein the gradient of acetonitrile starts with a minimum concentration and gradually increases to a maximum concentration over the first time period.

24. The method of claim 23, wherein after the first time period, the acetonitrile concentration in the mobile phase decreases to the minimum concentration of acetonitrile, optionally, wherein the concentration of acetonitrile decreases to the minimum concentration about 0.1 to about 3 minutes after the gradient of acetonitrile reaches the maximum concentration of acetonitrile.

25. The method of any one of claims 1 to 24, comprising applying the mobile phase to the chromatographic matrix according to the following conditions:

Time (min)	Acetate (%)	Acetonitrile (%)
0	93	7
5	88	12
8	88	12
11	86	14
18	70	30
19	70	30
21	93	7
26	93	7

or

Time (min)	Acetate (%)	Acetonitrile (%)
0	92	8
2	90	10
18	86	14
26	70	30
26.1	92	8
30	92	8

or

Time (min)	Acetate (%)	Acetonitrile (%)
0.0	92	8
2.0	90	10
18.0	86	14
22.0	78	22
22.1	20	80
24.0	20	80
24.1	92	8
30.0	92	8

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26. The method of any one of the preceding claims, wherein the mobile phase does not comprise a cationic ion pairing agent.

27. The method of any one of the preceding claims, wherein (A) the total run time is at least about 25 minutes and less than 40 minutes, optionally, about 26 minutes, (B) the method comprises monitoring elution of molecular species using an ultraviolet detector, and/or (C) the method is a non-denaturing method.

28. The method of any one of the preceding claims, wherein the flow rate of the mobile phase is about 0.5 ml/min to about 1 ml/min, optionally, about 0.7 ml/min to about 0.8 ml/min.

29. The method of any one of the preceding claims, further comprising collecting the elution fractions into separate containers over a time period.

30. The method of any one of the preceding claims, wherein the guanine-rich oligonucleotide comprises about 19 to about 23 nucleotides.

31. The method of any one of the preceding claims, wherein the guanine-rich oligonucleotide and one or more of the molecular species thereof in the mixture comprises one or more modified nucleotides and/or synthetic internucleotide linkages, optionally, wherein the one or more modified nucleotides are 2'-modified nucleotides and/or the synthetic internucleotide linkage is a phosphorothioate linkage.

32. The method of any one of the preceding claims, wherein the guanine-rich oligonucleotide comprises the sequence of SEQ ID NO: 2 or SEQ ID NO: 4.

33. A method of separating molecular species of a guanine-rich oligonucleotide from a mixture of molecular species, wherein the molecular species of the mixture are a quadruplex formed from the guanine-rich oligonucleotide, the guanine-rich oligonucleotide, a duplex comprising the guanine-rich oligonucleotide and the complement strand thereof, and the complement strand, said method comprising:
    a. applying the mixture to a chromatographic matrix comprising a hydrophobic ligand, wherein said hydrophobic ligand comprises C4 to C8 alkyl chains, wherein molecular species bind to the hydrophobic ligand;
    b. applying a mobile phase which comprises a decreasing concentration gradient of acetate and an increasing concentration gradient of acetonitrile to the chromatographic matrix to elute molecular species of the guanine-rich oligonucleotide, wherein each of the quadruplex, the guanine-rich oligonucleotide, the duplex and the complement strand separately elute from the chromatographic matrix.

34. The method of claim 33, comprising applying the mobile phase to the chromatographic matrix according to the following conditions:

Time (min)	Acetate (%)	Acetonitrile (%)
0.0	92	8
2.0	90	10
18.0	86	14
22.0	78	22
22.1	20	80
24.0	20	80
24.1	92	8
30.0	92	8

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35. The method of claim 33 or 34, wherein (A) the resolution of the separation of the peaks of each molecular species is (i) at least or about 2.0, optionally, at least or about 2.4, or (ii) at least or about 3.0 or at least or about 4.0 and/or (B) the LOQ of each molecular species is about 0.03 mg/mL to about 0.08 mg/mL.