volatile organic compounds or no volatile organic compounds and that yields mRNA of high integrity and high purity. Thus, in one example, the present disclosure provides an effective, reliable, and safer method of purifying mRNA which can be used for large-scale manufacturing process therapeutic applications without using any caustic or flammable solvents.

[0006] In one aspect, the present disclosure provides a method of purifying messenger RNA (mRNA) comprising a) precipitating the mRNA in a suspension comprising at least a 1M salt solution and an amphiphilic polymer to provide precipitated mRNA; b) capturing the precipitated mRNA; c) washing the precipitated mRNA captured in step b) with a wash solution comprising polyethylene glycol (PEG) or triethylene glycol monomethyl ether (MTEG) to purify the precipitated mRNA; and d) solubilizing the precipitated mRNA from step c) with a solubilizing solution to obtain a purified mRNA composition wherein the wash solution comprises PEG at 70% to 100% weight/volume concentration, or wherein the wash solution comprises MTEG at 75% to 100% weight/volume concentration.

[0006a] In another aspect, the present disclosure provides a method of purifying messenger RNA (mRNA) comprising: a) precipitating the mRNA in a suspension comprising at least 1M salt solution and an amphiphilic polymer; b) capturing the mRNA on a filter; and c) washing the mRNA of step b) with a triethylene glycol monomethyl ether (MTEG) solution to obtain a purified mRNA composition, wherein the MTEG solution comprises MTEG at 75% to 100% weight/volume concentration.

[0007] In some examples, the purified mRNA composition is substantially free of contaminants comprising short abortive RNA species, long abortive RNA species, double-stranded RNA (dsRNA), residual plasmid DNA, residual *in vitro* transcription enzymes, residual solvent and/or residual salt. In some examples, the purified mRNA composition is substantially free of contaminants comprising short abortive RNA species. For example, the purified mRNA composition has less than about 1% short abortive RNA species. In some examples, the purified mRNA composition is substantially free of contaminants comprising long abortive RNA species. For example, the purified mRNA composition has no greater than about 55% long abortive/degraded species as determined by capillary gel electrophoresis (CGE). In some examples, the purified mRNA composition is substantially free of contaminants comprising double-stranded RNA (dsRNA). For example, the purified mRNA

composition has less than 1 % double-stranded RNA. In some examples, the purified mRNA composition is substantially free of contaminants comprising residual plasmid DNA. For example, the purified mRNA composition has 10 pg/mg or less of residual plasmid DNA. In

[0076] In some examples, the mRNA pellet is resuspended in a buffer solution.

[0077] In some examples, the buffer solution is selected from water, TE, sodium citrate, or combinations thereof. In some examples, the buffer solution is water. In some examples the buffer solution is TE. In some examples, the buffer solution is sodium citrate.

[0078] In some examples, the precipitated mRNA comprises at least 100 mg, 1 g, 10 g, 100 g, 1 kg, 10 kg, 100 kg, one metric ton, or ten metric tons, of mRNA or any amount there between. Accordingly, in some examples, the precipitated mRNA comprises at least 100 mg. In some examples, the precipitated mRNA comprises at least 1 g. In some examples, the precipitated mRNA comprises at least 10 g. In some examples, the precipitated mRNA comprises at least 100 g. In some examples, the precipitated mRNA comprises at least 1 kg. In some examples, the precipitated mRNA comprises at least 10 kg. In some examples, the precipitated mRNA comprises at least 100 kg. In some examples, the precipitated mRNA comprises at least one metric ton. In some examples, the precipitated mRNA comprises at least ten metric tons.

[0079] In some examples, the precipitated mRNA comprises greater than 1 kg of mRNA.

[0080] In some examples, the method is ethanol free.

[0081] In another aspect, the present disclosure provides a method of purifying messenger RNA (mRNA) comprising: a) precipitating the mRNA in a guanidinium thiocyanate (GSCN) solution comprising triethylene glycol monomethyl ether (MTEG); b) centrifuging the solution to create an mRNA pellet; c) resuspending the mRNA pellet in a buffer; d) capturing the mRNA on a filter; e) washing the mRNA of step d) with an MTEG solution; and f) solubilizing the washed mRNA to obtain an mRNA composition, wherein the MTEG solution comprises MTEG at 75% to 100% weight/volume concentration.

[0082] In yet another aspect, the disclosure provides a method of manufacturing mRNA comprising the steps of a) performing *in vitro* transcription (IVT) by mixing (i) a DNA template comprising a promoter and (ii) an RNA polymerase, to generate an impure preparation comprising full-length mRNA; b) providing at least 1M salt and an amphiphilic polymer to the suspension to precipitate full-length mRNA and provide precipitated full-length

mRNA in the suspension; c) capturing the precipitated full-length mRNA by applying the

suspension to a filter; and d) washing the precipitated full-length mRNA of step c) with an aqueous solvent to obtain a purified full-length mRNA in an aqueous solution, and e) solubilizing the precipitated mRNA from step d) to obtain a purified mRNA composition, wherein the purified full-length mRNA in the aqueous solvent provided from step d) comprises less than 5% of (i) the DNA template comprising a promoter and the (ii) the RNA polymerase, and wherein in step d) the aqueous solvent comprises polyethylene glycol (PEG) present at 70% to 100% weight/volume concentration, or triethylene glycol monomethyl ether (MTEG) present at 75% to 100% weight/volume concentration.

[0083] In some examples, in step (a) the RNA polymerase is SP6 polymerase.

[0084] In some examples, the purified full-length mRNA in the aqueous solution provided from step (e) is also substantially free of (v) double-stranded RNA (dsRNA).

[0085] In some examples, the suspension comprises PEG of a molecular weight around 6000 g/mol (e.g. PEG-6000) at a final concentration of between about 5% and 20% weight/volume concentration and GSCN at a final concentration of about 2-4M. In particular examples, the suspension comprises PEG of a molecular weight around 6000 g/mol (e.g. PEG-6000) at a final concentration of about 10%, 11%, 12%, 13%, 14% or 15% weight/volume concentration and GSCN at a final concentration of about 2.5-3 M. As shown in the examples below, polymer-induced precipitation with a final concentration of PEG in the suspension of less than 20% resulted in highly pure mRNA samples after purification. Furthermore, a final concentration of PEG in the suspension of about 12% (for example a ratio of 1 of 50% PEG-6000) and a final GSCN concentration of 2.7M achieved highly effective purification of mRNA.

[0086] In some examples, MTEG can be used in place of PEG to provide a suspension of precipitated mRNA. In particular examples, MTEG is used for this purpose at a final concentration of about 15% to about 45% weight/volume. In some examples, the suspension comprises MTEG at a final concentration of about 20% to about 40% weight/volume. In some examples, the suspension comprises MTEG at a final concentration of about 20% weight/volume. In some examples, the suspension comprises MTEG at a final concentration of about 25% weight/volume. In some examples, the suspension comprises MTEG at a final concentration of about 30% weight/volume. In some examples, the suspension comprises

MTEG at a final concentration of about 35% weight/volume. In some examples, the suspension comprises MTEG at a final concentration of less than 35% weight/volume. The

CLAIMS

We claim:

1. A method of purifying messenger RNA (mRNA) comprising:
    a) precipitating the mRNA in a suspension comprising at least a 1M salt solution and an amphiphilic polymer to provide precipitated mRNA;
    b) capturing the precipitated mRNA;
    c) washing the precipitated mRNA captured in step b) with a wash solution comprising polyethylene glycol (PEG) or triethylene glycol monomethyl ether (MTEG) to purify the precipitated mRNA; and
    d) solubilizing the precipitated mRNA from step c) to obtain a purified mRNA composition
    wherein the wash solution comprises PEG at 70% to 100% weight/volume concentration, or wherein the wash solution comprises MTEG at 75% to 100% weight/volume concentration.

2. The method of claim 1, wherein the amphiphilic polymer is selected from MTEG, pluronics, polyvinyl pyrrolidone, polyvinyl alcohol, PEG, or combinations thereof.

3. The method of any one of the preceding claims, wherein the at least 1M salt solution comprises guanidinium thiocyanate (GSCN).

4. The method of any one of the preceding claims, wherein the wash solution comprises PEG or MTEG at 90% to 100% weight/volume concentration.

5. The method of any one of claims 2-4, wherein the amphiphilic polymer comprises PEG.

6. The method of claim 5, wherein the suspension comprises PEG at 5% to 20% weight/volume concentration, e.g. at 12% weight/volume concentration.

7. The method of any one of claims 2-4, wherein the amphiphilic polymer comprises MTEG.

8. The method of claim 7, wherein the suspension comprises MTEG at 15% to 45% weight/volume concentration, e.g. at 20% to 40% weight/volume concentration, e.g. at 25%, at 30%, or at 35% weight/volume concentration.

9. The method of any one of the preceding claims, wherein the molecular weight of PEG is 200 to 40,000 g/mol.

10. The method of claim 9, wherein the PEG is linear, branched, Y-shaped, or of multi-arm configuration.

11. The method of claim 10, wherein the PEG is linear.

12. The method of claim 11, wherein the suspension and/or wash solution comprises a PEG selected from triethylene glycol, tetraethylene glycol, PEG 200, PEG 300, PEG 400, PEG 600, PEG 1,000, PEG 1,500, PEG 2,000, PEG 3,000, PEG 3,350, PEG 4,000, PEG 6,000, PEG 8,000, PEG 10,000, PEG 20,000, PEG 35,000, and PEG 40,000.

13. The method of claim 12, wherein the suspension and/or wash solution comprises PEG 6,000.

14. The method of any one of claims 1-11, wherein the suspension and/or wash solution does not comprise PEG 6,000.

15. The method of claim 12, wherein the PEG is PEG 400.

16. The method of any one of the preceding claims, wherein the suspension and/or the wash solution comprises a mixture of one or more PEG polymers.

17. The method of claim 16, wherein the mixture of PEG polymers comprises polymers having distinct molecular weights.

18. The method of claim 17, wherein the mixture of PEG polymers comprises polymers having distinct geometrical configurations.

19. The method of any one of the preceding claims, wherein the wash solution is aqueous.

20. The method of any one of the preceding claims, wherein the wash solution is free of alcohol.

21. The method of claim 20, wherein the wash solution is free of ethanol, isopropyl alcohol, or benzyl alcohol.

22. The method of any one of claims 1-18, wherein the wash solution comprises a non-aqueous component.

23. The method of claim 22, wherein the non-aqueous component is ethanol, isopropyl alcohol, or benzyl alcohol.

24. The method of any one of the preceding claims, wherein capturing the precipitated mRNA occurs on a filter.

25. The method of claim 24, wherein the filter is selected from a microfiltration or ultrafiltration filter.

26. The method of claim 25, wherein the microfiltration filter has a pore size of between 0.05 µm and 1.0 µm.

27. The method of claim 26, wherein the microfiltration filter has a nominal molecular weight limit (NMWL) of greater than 1,000 kilodaltons (kDa).

28. The method of claim 25, wherein the ultrafiltration filter has a pore size of less than 0.05 µm.

29. The method of claim 28, wherein the ultrafiltration filter has a NMWL of between about 1 kDa and 1,000 kDA.

30. The method of any one of the preceding claims, wherein tangential flow filtration (TFF) or diafiltration is used to purify the precipitated mRNA in step c).

31. The method of any one of the preceding claims, wherein a filter aid is used.

32. The method of claim 31, wherein the filter aid is cellulose-based.

33. The method of claim 32, wherein the filter aid comprises diatomaceous earth, and/or volcanic ash.

34. The method of any one of the preceding claims, wherein the method does not comprise a chromatography step.

35. The method of any one of the preceding claims, wherein the precipitated mRNA is centrifuged to obtain an mRNA pellet.

36. The method of claim 35, wherein the mRNA pellet is resuspended in a buffer solution.

37. The method of claim 36, wherein the buffer solution is selected from water, Tris-EDTA (TE), sodium citrate, or combinations thereof.

38. A method of purifying messenger RNA (mRNA) comprising:
    a) precipitating the mRNA in a suspension comprising at least 1M salt solution and an amphiphilic polymer;
    b) capturing the mRNA on a filter; and
    c) washing the mRNA of step b) with a triethylene glycol monomethyl ether (MTEG) solution to obtain a purified mRNA composition,

    wherein the MTEG solution comprises MTEG at 75% to 100% weight/volume concentration.

39. The method of claim 38, wherein the amphiphilic polymer is selected from MTEG, pluronics, polyvinyl pyrrolidone, polyvinyl alcohol, polyethylene glycol (PEG), or combinations thereof.

40. The method of claim 39, wherein the amphiphilic polymer is MTEG.

41. The method of any one of claims 38-40, wherein the at least 1M salt solution comprises guanidinium thiocyanate (GSCN).

42. The method of any one of claims 38-41, wherein the method is ethanol free.

43. A method of purifying messenger RNA (mRNA) comprising:
    a) precipitating the mRNA in a guanidinium thiocyanate (GSCN) solution comprising triethylene glycol monomethyl ether (MTEG);
    b) centrifuging the solution of step a) to create an mRNA pellet;
    c) resuspending the mRNA pellet in a buffer;
    d) capturing the mRNA on a filter;
    e) washing the mRNA of step d) with an MTEG solution; and
    f) solubilizing the washed mRNA of step e) to obtain an mRNA composition,

    wherein the MTEG solution comprises MTEG at 75% to 100% weight/volume concentration.

44. A method of manufacturing mRNA comprising the steps of:
    a) performing *in vitro* transcription (IVT) by mixing (i) a DNA template comprising a promoter and (ii) an RNA polymerase to generate an impure preparation comprising full-length mRNA;
    b) providing at least 1M salt and an amphiphilic polymer to the suspension to precipitate full-length mRNA and provide precipitated full-length mRNA in the suspension;
    c) capturing the precipitated full-length mRNA by applying the suspension to a filter;
    d) washing the precipitated full-length mRNA of step c) with an aqueous solvent to obtain a purified full-length mRNA in an aqueous solution; and
    e) solubilizing the precipitated mRNA from step d) to obtain a purified mRNA composition, wherein the purified full-length mRNA in the aqueous solvent provided from step d) comprises less than 5% of (i) the DNA template comprising a promoter and the (ii) the RNA polymerase, and

    wherein in step d) the aqueous solvent comprises polyethylene glycol (PEG) present at 70% to 100% weight/volume concentration, or triethylene glycol monomethyl ether (MTEG) present at 75% to 100% weight/volume concentration.

45. The method of claim 44, wherein in step (a) the RNA polymerase is SP6 polymerase.

46. The method of claim 44 or 45, wherein in step d) the aqueous solvent comprises PEG or MTEG at 90% to 100% weight/volume concentration.

47. The method of claims 38 or 44 wherein the amphiphilic polymer is selected from MTEG, pluronics, polyvinyl pyrrolidone, polyvinyl alcohol, PEG, or combinations thereof.

48. The method of claim 47, wherein the amphiphilic polymer is PEG at 5% to 20% weight/volume concentration, e.g. at 12% weight/volume concentration.

49. The method of claim 47, wherein the amphiphilic polymer is MTEG at 15% to 45% weight/volume concentration, e.g. at 20% to 40% weight/volume concentration, e.g. at 25%, at 30%, or at 35% weight/volume concentration.

50. The method of claim 1, 38 or 44, wherein the salt is at a final concentration of between 2M to 4M and the amphiphilic polymer is PEG at a final concentration of between 5% and 20% weight/volume.

51. The method of claim 50, the salt is at a final concentration of 2.5M to 3M and the PEG is at a final concentration of between 10% and 15% weight/volume.

52. The method of claim 51, wherein the salt is at a final concentration of about 2.7M and the PEG is at a final concentration of about 12% weight/volume.

53. The method of any one of claims 47-51, wherein the salt comprises GCSN.

54. The method of any one of claims 50-53, wherein the PEG has a molecular weight of 6000 g/mol (e.g. PEG-6000).

55. The method of any one of claims 48-54, wherein the suspension further comprises a filter aid at a mass ratio with the precipitated mRNA of 2:1; 5:1; 10:1 or 15:1.

56. The method of claim 55, wherein the filter aid is at a mass ratio with the precipitated mRNA of 10:1.

57. The method of claim 55 or 56, wherein the filter aid is cellulose based.

58. The method of claim 1 or 44, wherein the PEG is present at 90% to 100% weight/volume concentration or the MTEG is present in the wash solution at 90% to 95% weight/volume concentration.

59. The method of claim 43 step a), wherein the salt comprises GSCN at a final concentration of between 2M to 4M, and the amphiphilic polymer is PEG at a final concentration of between 5% and 20% weight/volume.

60. The method of claim 59, wherein the GSCN is at a final concentration of 2.5M to 3M, e.g. 2.7M, and the PEG is at a final concentration of 10% to 15% weight/volume, e.g. 12% weight/volume.

61. The method of claim 59 or 60, wherein the PEG has a molecular weight of 6000 g/mol (e.g. PEG-6000).

62. The method of any one of claims 59-61, wherein the solution further comprises a filter aid at a mass ratio with the precipitated mRNA of 2:1; 5:1; 10:1 or 15:1.

63. The method of claim 62, wherein the filter aid is at a mass ratio with the precipitated mRNA of 10:1.

64. The method of claim 62 or 63, wherein the filter aid is cellulose based.

65. The method of claim 43 step e), wherein the MTEG in the MTEG solution is at 90 to 100% weight/volume concentration.