METHODS FOR PURIFICATION OF MESSENGER RNA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional patent application Serial No. 62/848,412, filed May 15, 2019 and 62/891,781 filed on August 26, 2019, each of which are hereby incorporated by reference in their entirety for all purposes.

BACKGROUND OF THE INVENTION

[0002] Messenger RNA (mRNA) therapy is becoming an increasingly important approach for the treatment of a variety of diseases. mRNA therapy involves administration of a drug product comprising *in vitro* transcribed (IVT) and highly pure messenger RNA (mRNA) into a patient in need of the therapy and production of the protein encoded by the mRNA within the patient's body. Thus, there is a need for efficient, large scale production of highly pure mRNA product suitable for therapeutic use.

[0003] Traditionally, mRNA generated from *in vitro* transcription is purified using commercially-available chromatography systems, e.g., HPLC, and/or by extraction into an organic mix (phenol:chloroform:isoamyl alcohol) and subsequent ethanol precipitation. However, use of column systems is expensive and challenging and the use of caustic or flammable solvents in extraction of mRNA can present safety and cost challenges, particularly in large-scale applications.

[0004] A safe and cost-effective method that produces highly pure mRNA that is acceptable for therapeutic use is currently lacking.

SUMMARY

[0005] The present disclosure provides, among other things, a highly efficient and cost-effective method of purifying messenger RNA (mRNA). The present disclosure is, in part, based on the surprising discovery of a method of purifying mRNA using low amounts of

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 a high molar 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 to purify the precipitated mRNA; and d) solubilizing the precipitated mRNA from step c) with a solubilizing solution to obtain a purified mRNA composition.

[0006a] In another aspect, the present disclosure provides a method of purifying messenger RNA (mRNA) comprising: a) precipitating the mRNA in a suspension comprising a high molar salt solution and an amphiphilic polymer; b) capturing the mRNA on a filter; and c) washing the mRNA of step b) with a PEG solution to obtain a purified mRNA composition substantially free of contaminants.

[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

some examples, the purified mRNA composition is substantially free of contaminants comprising residual *in vitro* transcription enzymes. For example, for every 15µg of purified mRNA composition there is less than 0.3 ng of polymerase. For every 15µg of purified mRNA composition there is less than 0.3 ng of cap enzymes. For every 15µg of purified mRNA composition there is less than 0.3 ng of tail enzymes. In some examples, the purified mRNA composition is substantially free of contaminants comprising residual solvent. In some examples, the purified mRNA composition is substantially free of contaminants comprising residual salt.

[0008] In some examples, the purified mRNA comprises residual plasmid DNA of 10 pg/mg purified mRNA or less.

[0009] In some examples, the amphiphilic polymer in the suspension is selected from pluronics, polyvinyl pyrrolidone, polyvinyl alcohol, polyethylene glycol (PEG), polyethers such as polypropylene glycol (PPG) or polypropylene oxide, or combinations thereof. In some examples, the amphiphilic polymer in the suspension is pluronics. In some examples, the amphiphilic polymer in the suspension is polyvinyl pyrrolidone. In some examples, the amphiphilic polymer in the suspension is polyvinyl alcohol. In some examples, the amphiphilic polymer in the suspension is polyethylene glycol (PEG). In some examples, the amphiphilic polymer in the suspension is a polyether. In some examples, the amphiphilic polymer in the suspension is polypropylene glycol (PPG). In some examples, the amphiphilic polymer in the suspension is polypropylene oxide.

[0010] In some examples, the suspension does not comprise an organic solvent. In some examples, the amphiphilic polymer in the suspension is PEG. In some examples, the suspension does not comprise an organic solvent and the mRNA is precipitated using polyethylene glycol (PEG). In some examples, the suspension comprises PEG to precipitate the mRNA. In some examples, the suspension comprises PEG at about 10% to about 100% weight/volume concentration.

[0011] In some examples, the suspension comprises PEG at about 50% weight/volume concentration.

[0012] In some examples, the suspension comprises a final concentration of PEG at less than 25% weight/volume. In some examples, the suspension comprises a final concentration of

PEG at about 5% to 20% weight/volume. In particular examples, the suspension comprises a final concentration of PEG at about 10% to 15% weight/volume, for example 12% weight/volume. In some examples, the molecular weight of the PEG is about 2000 to about 10000 g/mol. In some examples, the molecular weight of the PEG is about 4000 to about 8000 g/mol. In some examples, the molecular weight of the PEG is about 6000 g/mol (*e.g.* PEG-6000). As shown in the examples, a final concentration of PEG with a molecular weight of about 6000 g/mol (*e.g.* PEG-6000) in the suspension of about 12% weight/volume ensured effective purification and provided highly pure mRNA samples.

[0013] In some examples, the suspension does not comprise an organic solvent and does comprise triethylene glycol (TEG). In some examples, the suspension comprises TEG to precipitate the mRNA. In some examples, the suspension comprises TEG at about 10% to about 100% weight/volume concentration.

[0014] In some examples, the suspension comprises TEG at about 50% weight/volume concentration.

[0015] In some examples, the suspension does not comprise an organic solvent and does comprise triethylene glycol monomethyl ether (MTEG). In some examples, the suspension comprises MTEG to precipitate the mRNA. In some examples, the suspension comprises MTEG at about 10% to about 100% weight/volume concentration.

[0016] In some examples, the suspension comprises MTEG at about 50% weight/volume concentration.

[0017] In some examples, the suspension comprises MTEG 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.

[0018] In some examples, the high molar salt solution comprises guanidinium thiocyanate (GSCN). In some examples, the GSCN is at a final concentration of about 2-4M. In some examples, the GSCN is at a final concentration of 2.5-3 M. In particular examples, the GSCN is at a final concentration of about 2.7 M.

[0019] In some examples, the amphiphilic polymer in the wash solution is selected from pluronics, polyvinyl pyrrolidone, polyvinyl alcohol, polyethylene glycol (PEG), polyethers such as polypropylene glycol (PPG) or polypropylene oxide, or combinations thereof. In some examples, the amphiphilic polymer in the wash solution is pluronics. In some examples, the amphiphilic polymer in the wash solution is polyvinyl pyrrolidone. In some examples, the amphiphilic polymer in the wash solution is polyvinyl alcohol. In some examples, the amphiphilic polymer in the wash solution is polyethylene glycol (PEG). In some examples, the amphiphilic polymer in the wash solution is a polyether. In some examples, the amphiphilic polymer in the wash solution is polypropylene glycol (PPG). In some examples, the amphiphilic polymer in the wash solution is polypropylene oxide.

[0020] In some examples, the wash solution does not comprise an organic solvent. In some examples, the wash solution does not comprise an organic solvent and comprises polyethylene glycol (PEG). In some examples, the PEG used in the wash solution has a viscosity of 90 centistrokes or less. In some examples, the PEG used in the wash solution has a viscosity of 80 centistrokes or less. In some examples, the PEG used in the wash solution has a viscosity of 70 centistrokes or less. In some examples, the PEG used in the wash solution has a viscosity of 60 centistrokes or less. In some examples, the PEG used in the wash solution has a viscosity of 50 centistrokes or less. In some examples, the PEG used in the wash solution has a viscosity of 40 centistrokes or less. In some examples, the PEG used in the wash solution has a viscosity of 30 centistrokes or less. In some examples, the PEG used in the wash solution has a viscosity of 20 centistrokes or less. In some examples, the PEG used in the wash solution has a viscosity of 10 centistrokes or less. A PEG suitable for use in the wash solution has a viscosity of about 90 centistrokes at 25°C, such as PEG-400. Accordingly, in a particular example, the PEG used in the wash solution is PEG-400.

[0021] In some examples, the wash solution does not comprise an organic solvent and comprises triethylene glycol (TEG). In some examples, the solution comprises TEG.

[0022] In some examples, the wash solution does not comprise an organic solvent and comprises triethylene glycol monomethyl ether (MTEG). In some examples, the wash solution comprises MTEG. In some examples, MTEG is present in the wash solution at about 90% to about 100% by weight/volume concentration. In particular examples, MTEG is present in the wash solution at about 95% by weight/volume concentration.

[0023] As shown in the examples, MTEG is suitable for use in the wash solution, effectively washing the precipitated mRNA, while retaining it in precipitated form. MTEG has a viscosity of about 7 centistrokes at room temperature. MTEG therefore allows highly efficient purification and recovery of the mRNA irrespective of the process of purification used (*e.g.* flow filtration, depth filtration or centrifugation).

[0024] In some examples, the amphiphilic polymer in the wash solution is PEG. In a particular example, the molecular weight of the PEG in the wash solution is about 200 g/mol to about 600 g/mol. In particular examples, the PEG in the wash solution has a molecular weight of about 400 g/mol (for example PEG-400).

[0025] In some examples, PEG is present in the wash solution at about 10% to about 100% weight/volume concentration.

[0026] In some examples, PEG is present in the wash solution at about 50 to about 90% weight/volume concentration. In some examples, PEG is present in the wash solution at about 90% to about 100% weight/volume concentration.

[0027] In some examples, the PEG is present in the wash solution at about 90% weight/volume concentration. In particular examples, the PEG in the wash solution has a molecular weight of about 400 g/mol (for example PEG-400). In some examples, the PEG in the wash solution has a molecular weight of about 400 g/mol (for example PEG-400) and is at about 90% to about 100% weight/volume concentration. In particular examples, the PEG in the wash solution has a molecular weight of about 400 g/mol (for example PEG-400) and is present in the wash solution at about 90% weight/volume concentration.

[0028] In some examples, the molecular weight of PEG in the suspension is about 200 to about 40,000 g/mol. In some examples, the molecular weight of PEG in the wash solution is about 200 to about 40,000 g/mol. In some examples, the molecular weight of PEG both in the

suspension and the wash solution is about 200 to about 40,000 g/mol. In particular examples, the molecular weight of PEG in the suspension is about 2,000 g/mol to about 10,000 g/mol and the molecular weight of PEG in the wash solution is about 200 g/mol to about 600 g/mol. In a specific example, the PEG in the suspension is PEG-6000 and the PEG in the wash solution is PEG-400.

[0029] In some examples, the PEG in the suspension is linear. In some examples, the PEG in the suspension is branched. In some examples, the PEG in the suspension is Y-shaped. In some examples, the PEG in the suspension is of multi-arm configuration.

[0030] In some examples, the PEG in the wash solution is linear. In some examples, the PEG in the wash solution is branched. In some examples, the PEG in the wash solution is Y-shaped. In some examples, the PEG in the wash solution is of multi-arm configuration.

[0031] In some examples, the PEG both in the suspension and in the wash solution is linear. In some examples, the PEG both in the suspension and in the wash solution is branched. In some examples, the PEG both in the suspension and in the wash solution is Y-shaped. In some examples, the PEG both in the suspension and in the wash solution is of multi-arm configuration.

[0032] In some examples, the suspension 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. In some examples, the suspension comprises triethylene glycol. In some examples, the suspension comprises tetraethylene glycol. In some examples, the suspension comprises PEG 200. In some examples, the suspension comprises PEG 300. In some examples, the suspension comprises PEG 400. In some examples, the suspension comprises PEG 600. In some examples, the suspension comprises PEG 1,000. In some examples, the suspension comprises PEG 1,500. In some examples, the suspension comprises PEG 2,000. In some examples, the suspension comprises PEG 3,000. In some examples, the suspension comprises PEG 3,350. In some examples, the suspension comprises PEG 4,000. In some examples, the suspension comprises PEG 6,000. In some examples, the suspension comprises PEG 8,000. In some examples, the suspension comprises PEG 10,000. In some

examples, the suspension comprises PEG 20,000. In some examples, the suspension comprises PEG 35,000. In some examples, the suspension comprises PEG 40,000.

[0033] In some examples, the suspension comprises PEG 6,000.

[0034] In some examples, the suspension does not comprise PEG 6,000.

[0035] In some examples, the suspension is PEG 400.

[0036] In some examples, the suspension is PEG 150.

[0037] In some examples, the suspension comprises a mixture of one or more PEG polymers.

[0038] In some examples, the mixture of PEG polymers in the suspension comprises polymers having distinct molecular weights.

[0039] In some examples, the mixture of PEG polymers in the suspension comprises polymers having distinct geometrical configurations.

[0040] In some examples, the suspension is aqueous.

[0041] In some examples, the suspension has volatile organic compounds comprising less than about 50% of total volume of the suspension. For example, in some examples, the suspension has volatile organic compounds comprising less than about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 2%, 1%, 0.5%, 0.01% of the total volume of the suspension. Accordingly, in some examples, the suspension has volatile organic compounds comprising less than about 50% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 45% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 40% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 35% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 30% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 25% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 20% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising

less than 15% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 10% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 5% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 2% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 1% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 0.5% of the total volume of the suspension. In some examples, the suspension has volatile organic compounds comprising less than 0.1% of the total volume of the suspension. Many volatile organic compounds are known in the art and include, for example, ethanol, isopropyl alcohol, and benzyl alcohol.

[0042] In some examples, the suspension is free of volatile organic compounds.

[0043] In some examples, the suspension is free of alcohol.

[0044] In some examples, the suspension is free of ethanol. In some examples, the suspension is free of isopropyl alcohol. In some examples, the suspension is free of benzyl alcohol.

[0045] In some examples, the suspension comprises a non-aqueous component. In some examples, the non-aqueous component of the suspension is ethanol. In some examples, the non-aqueous component of the suspension is isopropyl alcohol. In some examples, the non-aqueous component of the suspension is benzyl alcohol.

[0046] In some examples, the 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. In some examples, the wash solution comprises triethylene glycol. In some examples, the wash solution comprises tetraethylene glycol. In some examples, the wash solution comprises PEG 200. In some examples, the wash solution comprises PEG 300. In some examples, the wash solution comprises PEG 400. In some examples, the wash solution comprises PEG 600. In some examples, the wash solution comprises PEG 1,000. In some examples, the wash solution comprises PEG 1,500. In some examples, the wash solution comprises PEG 2,000. In some examples, the wash solution

comprises PEG 3,000. In some examples, the wash solution comprises PEG 3,350. In some examples, the wash solution comprises PEG 4,000. In some examples, the wash solution comprises PEG 6,000. In some examples, the wash solution comprises PEG 8,000. In some examples, the wash solution comprises PEG 10,000. In some examples, the wash solution comprises PEG 20,000. In some examples, the wash solution comprises PEG 35,000. In some examples, the wash solution comprises PEG 40,000.

[0047] In some examples, the wash solution comprises PEG 6,000.

[0048] In some examples, the wash solution does not comprise PEG 6,000.

[0049] In some examples, the wash solution is PEG 400.

[0050] In some examples, the wash solution comprises a mixture of one or more PEG polymers.

[0051] In some examples, the mixture of PEG polymers in the wash solution comprises polymers having distinct molecular weights.

[0052] In some examples, the mixture of PEG polymers in the wash solution comprises polymers having distinct geometrical configurations.

[0053] In some examples, the wash solution is aqueous.

[0054] In some examples, the wash solution is free of volatile organic compounds.

[0055] In some examples, the wash solution is free of alcohol.

[0056] In some examples, the wash solution has volatile organic compounds comprising less than about 50% of total volume of the wash solution. For example, in some examples, the wash solution has volatile organic compounds comprising less than about 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 2%, 1%, 0.5%, 0.01% of the total volume of the wash solution. Accordingly, in some examples, the wash solution has volatile organic compounds comprising less than about 50% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 45% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 40% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 35% of the

total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 30% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 25% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 20% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 15% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 10% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 5% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 2% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 1% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 0.5% of the total volume of the wash solution. In some examples, the wash solution has volatile organic compounds comprising less than 0.1% of the total volume of the wash solution. Many volatile organic compounds are known in the art and include, for example, ethanol, isopropyl alcohol, and benzyl alcohol.

[0057] In some examples, the wash solution is free of ethanol. In some examples, the wash solution is free of isopropyl alcohol. In some examples, the wash solution is free of benzyl alcohol.

[0058] In some examples, the wash solution comprises a non-aqueous component. In some examples, the non-aqueous component of the wash solution is ethanol. In some examples, the non-aqueous component of the wash solution is isopropyl alcohol. In some examples, the non-aqueous component of the wash solution is benzyl alcohol.

[0059] In some examples, both the suspension and the wash buffer are aqueous. In some examples, both the suspension and the wash buffer comprise PEG. In some examples, both the suspension and the wash buffer are aqueous and comprise the same PEG. In some examples, both the suspension and the wash buffer are aqueous and the suspension comprises a first PEG and the wash buffer comprises a second PEG that is different from the first PEG. In some examples, the molecular weight of the PEG in the suspension is about 2000 to about

10000 g/mol and the molecular weight of the PEG in the wash buffer is about 200-600 g/mol. In some examples, the molecular weight of the PEG in the suspension is about 4000 to about 8000 g/mol and the molecular weight of the PEG in the wash buffer is about 300-500 g/mol. In some examples, the molecular weight of the PEG in the suspension is about 6000 g/mol (*e.g.* PEG-6000) and the molecular weight of the PEG in the wash buffer is about 400 g/mol (*e.g.* PEG-400).

[0060] In some examples, the capturing the precipitated mRNA occurs on a filter. In some examples, the filter is selected from a microfiltration or ultrafiltration filter. In some examples, the microfiltration filter has a pore size of between 0.05 μm and 1.0 μm. For example, in some examples, the microfiltration filter has a pore size of 0.05 μm, 0.10 μm, 0.20 μm, 0.3 μm, 0.4 μm, 0.5 μm, 0.6 μm, 0.7 μm, 0.8 μm, 0.9 μm, or 1.0 μm. Accordingly, in some examples, the microfiltration filter has a pore size of 0.05 μm. In some examples, the microfiltration filter has a pore size of 0.10 μm. In some examples, the microfiltration filter has a pore size of 0.20 μm. In some examples, the microfiltration filter has a pore size of 0.30 μm. In some examples, the microfiltration filter has a pore size of 0.40 μm. In some examples, the microfiltration filter has a pore size of 0.50 μm. In some examples, the microfiltration filter has a pore size of 0.60 μm. In some examples, the microfiltration filter has a pore size of 0.70 μm. In some examples, the microfiltration filter has a pore size of 0.80 μm. In some examples, the microfiltration filter has a pore size of 0.90 μm. In some examples, the microfiltration filter has a pore size of 1.0 μm.

[0061] In some examples, the filter will have a nominal molecular weight limit (NMWL) of between 100 kDa and 1,000 kDa. In some examples, the filter will have a NMWL of between 200 kDa and 700 kDa. In some examples, the filter will have a NMWL between 200 kDa and 500 kDa. In some examples, the filter has a NMWL of 300 kDa. In some examples, the filter has a NMWL of 500 kDa.

[0062] In some examples, the microfiltration filter has a nominal molecular weight limit (NMWL) of greater than 1,000 kilodaltons (kDa). In some examples, the ultrafiltration filter has a pore size of less than 0.05 μm. In some examples, the ultrafiltration filter has an NMWL of between about 1 kDa and 1,000 kDA. For example, in some examples, the ultrafiltration filter has an NMWL of 1 kDA, 5 kDA, 10 kDA, 15 kDA, 20 kDA, 25 kDA, 50

kDA, 100 kDA, 150 kDA, 200 kDA, 250 kDA, 300 kDA, 350 kDA, 400 kDA, 450 kDA, 500 kDA, 550 kDA, 600 kDA, 650 kDA, 700 kDA, 750 kDA, 800 kDA, 850 kDA, 900 kDA, 950 kDA, or 1,000 kDA. Accordingly, in some examples, the ultrafiltration filter has an NMWL of 1 kDA. In some examples, the ultrafiltration filter has an NMWL of 5 kDA. In some examples, the ultrafiltration filter has an NMWL of 10 kDA. In some examples, the ultrafiltration filter has an NMWL of 15 kDA. In some examples, the ultrafiltration filter has an NMWL of 20 kDA. In some examples, the ultrafiltration filter has an NMWL of 25 kDA. In some examples, the ultrafiltration filter has an NMWL of 50 kDA. In some examples, the ultrafiltration filter has an NMWL of 100 kDA. In some examples, the ultrafiltration filter has an NMWL of 150 kDA. In some examples, the ultrafiltration filter has an NMWL of 200 kDA. In some examples, the ultrafiltration filter has an NMWL of 250 kDA. In some examples, the ultrafiltration filter has an NMWL of 300 kDA. In some examples, the ultrafiltration filter has an NMWL of 350 kDA. In some examples, the ultrafiltration filter has an NMWL of 400 kDA. In some examples, the ultrafiltration filter has an NMWL of 450 kDA. In some examples, the ultrafiltration filter has an NMWL of 500 kDA. In some examples, the ultrafiltration filter has an NMWL of 550 kDA. In some examples, the ultrafiltration filter has an NMWL of 600 kDA. In some examples, the ultrafiltration filter has an NMWL of 650 kDA. In some examples, the ultrafiltration filter has an NMWL of 700 kDA. In some examples, the ultrafiltration filter has an NMWL of 750 kDA. In some examples, the ultrafiltration filter has an NMWL of 800 kDA. In some examples, the ultrafiltration filter has an NMWL of 850 kDA. In some examples, the ultrafiltration filter has an NMWL of 900 kDA. In some examples, the ultrafiltration filter has an NMWL of 950 kDA. In some examples, the ultrafiltration filter has an NMWL of 1,000 kDA.

[0063] In some examples, tangential flow filtration (TFF) or diafiltration is used to purify the precipitated mRNA in step c). Accordingly, in some examples, TFF is used to purify the precipitated mRNA in step c). In some examples, diafiltration is used to purify the precipitated mRNA in step c).

[0064] In some examples, a filter aid is used.

[0065] In some examples, the filter aid is cellulose-based. In particular examples, the cellulose-based filter aid is added to the suspension at a mass ratio of precipitated mRNA to

filter aid of 1:10. In some examples, the cellulose-based filter aid comprises purified cellulose fibres of about 5 to about 500 µm in length. In some examples, the cellulose fibres are of about 10 to about 100 µm in length. In some examples, the cellulose fibres are about 20 µm, 30 µm, 40 µm or 50 µm in length. In particular examples, the cellulose-based filter aid comprises purified cellulose fibres of about 20 µm in length (*e.g.* Solka-Floc® or Sigmacell Cellulose 20).

[0066] In some examples, the filter aid comprises diatomaceous earth, and/or volcanic ash. In some examples, the filter aid comprises diatomaceous earth. In some examples, the filter aid comprises volcanic ash. In some examples, the filter aid comprises diatomaceous earth and volcanic ash.

[0067] In some examples, the solubilizing solution is selected from water, Tris- EDTA (TE), sodium citrate, or combinations thereof. In some examples, the solubilizing solution is water. In some examples the solubilizing solution is TE. In some examples, the solubilizing solution is sodium citrate.

[0068] In some examples, yield of the purified mRNA is about 50% to about 100%.

[0069] In some examples, the yield of the purified mRNA is about 70% to about 99%.

[0070] In some examples, the yield of the purified mRNA is between about 90 and about 99%. In particular examples, the yield of the purified mRNA is more than about 93%, e.g., more than about 94%, particularly more than about 95%.

[0071] In some examples, purity of the purified mRNA is between about 60% and about 100%.

[0072] In some examples, the purity of the purified mRNA is between about 80% and 99%.

[0073] In some examples, the purity of the purified mRNA is between about 90% and about 99%.

[0074] In some examples, the method does not comprise a chromatography step.

[0075] In some examples, the precipitated mRNA is centrifuged to obtain an mRNA pellet.

[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 PEG; 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 a PEG solution; and f) solubilizing the washed mRNA to obtain an mRNA composition substantially free of contaminants.

[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 high molar 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 solution provided from step (d) is substantially free of (i) the DNA template comprising a promoter and the (ii) the RNA polymerase.

[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

rest of the conditions used in MTEG-induced precipitation are the same as those used in the PEG-induced precipitation. As shown in the examples, a suspension comprising mRNA, GSCN and MTEG, with MTEG at a final concentration of less than 35% weight/volume ensured efficient recovery of mRNA without unwanted precipitation of process enzymes. Particularly suitable for efficient recovery of mRNA without unwanted precipitation of process enzymes is a suspension comprising mRNA, GSCN and MTEG, with MTEG at a final concentration of about 25%, in addition to a filter aid (for example cellulose-based filtering aid) at a mass ratio with the precipitated mRNA of about 10:1.

BRIEF DESCRIPTION OF THE DRAWING

[0087] The following figures are for illustration purposes only and not for limitation.

[0088] **Figure 1** shows the yield of mRNA purified with different precipitation conditions from 5 mg of IVT reactions.

[0089] **Figure 2** shows a silver stain gel of mRNA purified with different precipitation conditions. The silver stain gel was used to show the presence of contaminating process