Chapter 5

Large Scale Purification of RNA Nanoparticles by Preparative Ultracentrifugation

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Abstract

Purification of large quantities of supramolecular RNA complexes is of paramount importance due to the large quantities of RNA needed and the purity requirements for in vitro and in vivo assays. Purification is generally carried out by liquid chromatography (HPLC), polyacrylamide gel electrophoresis (PAGE), or agarose gel electrophoresis (AGE). Here, we describe an efficient method for the large-scale purification of RNA prepared by in vitro transcription using T7 RNA polymerase by cesium chloride (CsCl) equilibrium density gradient ultracentrifugation and the large-scale purification of RNA nanoparticles by sucrose gradient rate-zonal ultracentrifugation or cushioned sucrose gradient rate-zonal ultracentrifugation.

Key words RNA, Nanoparticles, Ultracentrifugation, Nanotechnology, Nanobiotechnology, RNA nanotechnology, RNA therapeutics, Large-scale purification

1 Introduction

Since the first example of RNA nanotechnology in 1998 [1], RNA has shown to be a promising therapeutic delivery system to target cancers, viral infections, and genetic diseases [2, 3]. Recent advances in RNA nanotechnology have led to the construction of diverse nanoparticles with varying sizes and structural features [4–11]. Many of these nanoparticles have the potential for imaging, disease diagnosis, and drug and therapeutic RNA delivery [2, 3, 12–15]. RNA is a polymer made up of four different nucleotides: adenine (A), cytosine (C), guanine (G), and uracil (U). Much like DNA, RNA can be easily manipulated to form precise structures, while at the same time retaining flexibility in structure and diversity in function much like proteins [16].

RNA nanoparticles assemble with high efficiencies; however, fully-assembled and contamination-free nanocomplexes are required for most in vitro and in vivo applications. Besides HPLC, in many cases, purification is performed by polyacrylamide gel electrophoresis (PAGE) or agarose gel electrophoresis (AGE),

through which the correctly folded structures migrate in a sharp and distinct band and are separated from the faster migrating single stranded (ss) RNA and the slower migrating misfolded and aggregate structures [8, 17]. While these methods have proven to be effective, there are downsides to using PAGE and AGE. After electrophoresis, the RNA must be extracted from the gel, which is labor-intensive and often requires an additional purification step to remove contaminating gel residue. Also, some nanoparticles do not enter PAGE and AGE gels efficiently due to the large branched structures of these complexes. Finally, PAGE and AGE lack the scalability needed to purify large quantities of nanostructures. HPLC is often used for the purification of chemically synthesized and chemically modified oligonucleotides; however, HPLC is not conducive to large RNA nanoparticles [18]. Ultracentrifugation is a useful method for the scalable and efficient purification of RNA supramolecular complexes on the large scale [19–21]. Here, we describe a scalable, cost-effective, and contamination-free method to purify RNA synthesized by in vitro transcription and supramolecular RNA nanoparticles by preparative ultracentrifugation [22–24].

While analytical ultracentrifugation is used to study molecular interactions and define the properties of the analyte, such as molecular weight, sedimentation coefficient, shape, and conformation, preparative ultracentrifugation is primarily used to isolate and purify specific particles. There are three main types of preparative ultracentrifugation: differential (also known as pelleting), rate-zonal, and density-equilibrium (also known as isopycnic). Differential centrifugation separates based on size or molecular weight in a process in which successive pelleting steps allows researchers to recover the particle of interest. Rate-zonal centrifugation again resolves particles by size and shape dependent on run time but the particle of interest remains isolated in the gradient and is typically fractionated and recovered. Equilibrium density centrifugation, however, separates entirely on density, independent of run time. In the methods introduced in this chapter, equilibrium density and rate zonal gradients are employed. Equilibrium density gradient ultracentrifugation is used in the CsCl gradient purification of ssRNA oligomers and rate-zonal is employed in the sucrose gradient purification of supramolecular RNA nanoparticles.

Post in vitro transcription, RNA is separated from unincorporated nucleotides and protein impurities by CsCl Equilibrium density gradient ultracentrifugation (Fig. 1). Exploiting the differences in density between proteins, RNA nucleotides, and synthesized RNA oligonucleotides enables the large-scale purification of ssRNA, which are then used to form multi-strand supramolecular RNA nanoparticles.

RNA nanoparticles are then constructed from the purified ssRNA oligonucleotides. The RNA nanoparticles are purified from misfolded and aggregated complexes and unincorporated ssRNA by sucrose gradient rate-zonal ultracentrifugation (Fig. 2). Post centrifugation,
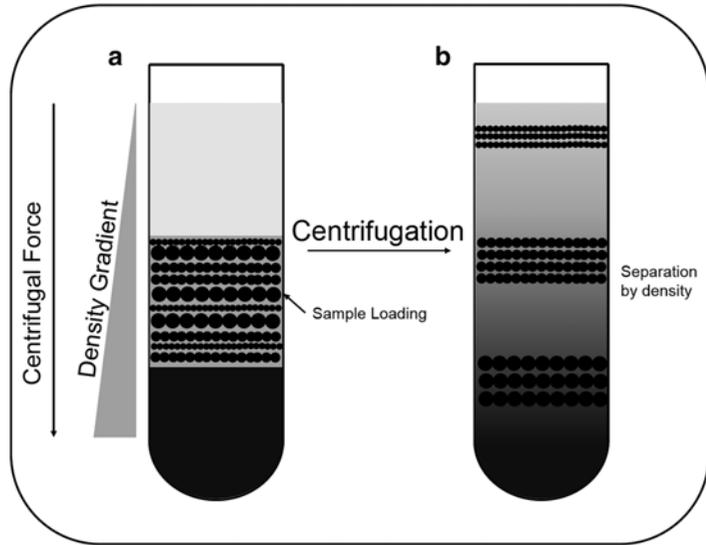
Fig. 1 Mechanism for CsCl equilibrium density gradient. (**a**) Different densities of CsCl salt solution are loaded into centrifuge tube with sample in medium density solution. (**b**) Post centrifugation, when equilibrium is reached, a gradient is formed with the migration of particles separating by their density (specific gravity)
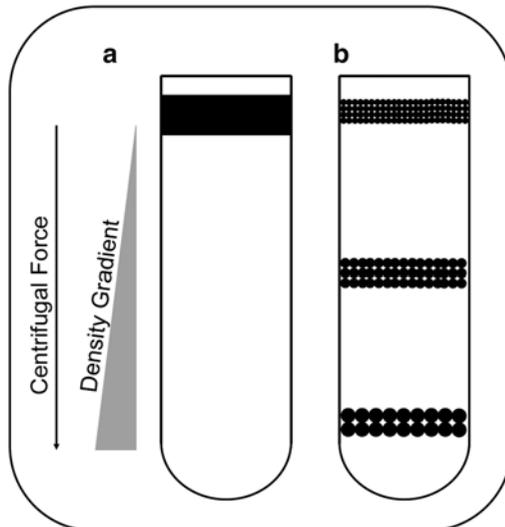

Fig. 2 Mechanism of separation for sucrose gradient rate-zonal ultracentrifugation. (**a**) Before centrifugation, the sample is loaded on the top of the gradient in a thin band. (**b**) After centrifugation, the particles separate based on shape and mass. Higher mass particles migrate faster to the higher density portion of the gradient and lower mass particles stay in the lower density portion at the top of the gradient

fractions are collected and examined on non-denaturing AGE and stained with ethidium bromide (EtBr) (Fig. 3).

To demonstrate the expected results utilizing this procedure, synthesis and purification of RNA nanoparticles utilizing the
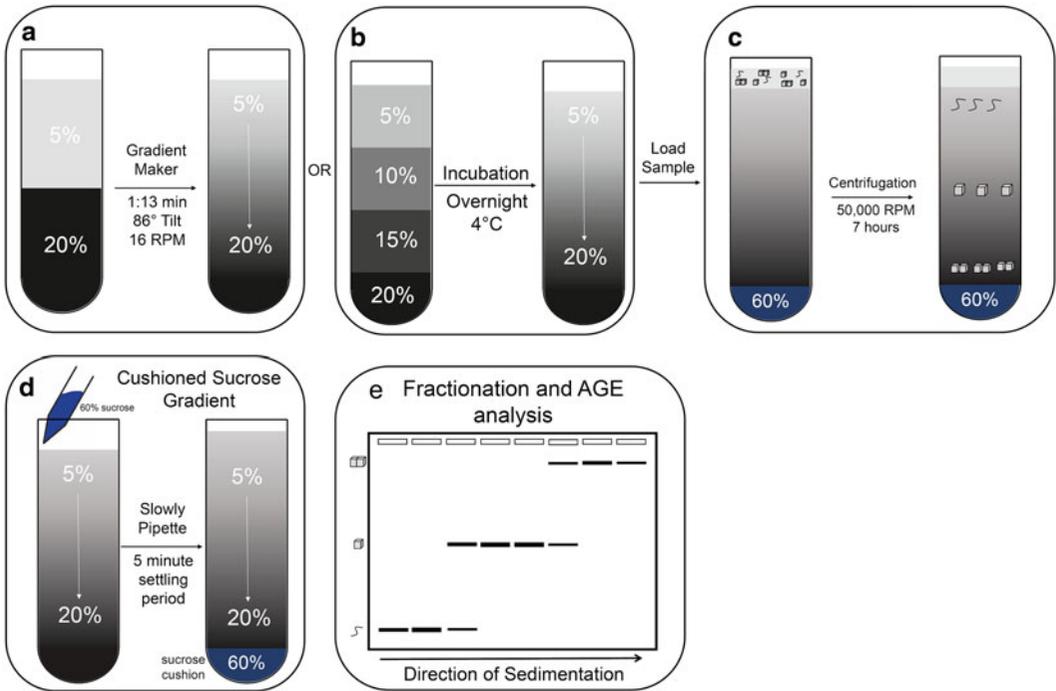
Fig. 3 Gradient formation, sample loading and analysis scheme of sucrose gradient rate-zonal ultracentrifugation and expected results. (**a**) Formation of gradient by automated gradient maker. (**b**) Formation of gradient by manual layering. (**c**) Sample is loaded in a thin band on top of the gradient. After centrifugation the particles are separated by size. (**d**) Alternate method for the formation a cushioned sucrose rate-zonal density gradient using 60 % sucrose solution pipetted to the bottom of a preformed sucrose gradient. (**e**) Expected AGE analysis results. AGE analysis reveals the identity of each fraction. After AGE analysis the target particle fractions are combined and concentrated

packaging RNA (pRNA) [25] from the phi29 bacteriophage DNA packaging motor was performed. The native pRNA structure has previously been engineered to assemble into many nanostructures including dimers, trimers, tetramers, hexamers, and larger supramolecular complexes utilizing hand-in-hand and foot-to-foot interactions [1, 7, 8, 26, 27]. As an example, we show here the purification of pRNA monomer and dimer constructs from two distinct pRNA monomers, Ab′ and Ba′ [28]. Monomers were purified by CsCl equilibrium density gradient ultracentrifugation and monomer and dimer constructs were purified by 5–20 % sucrose gradient rate-zonal ultracentrifugation. The Ab′ and Ba′ pRNA monomers assemble to form dimer constructs, a size difference that can be confirmed by traditional native PAGE analysis. Despite a size difference of only 120 nucleotides between monomer and dimer, the two species can be resolved by sucrose gradient ultracentrifugation. Furthermore, the recovered fractions exhibited comparable dimer assembly to RNA nanoparticles purified by PAGE (Fig. 4).
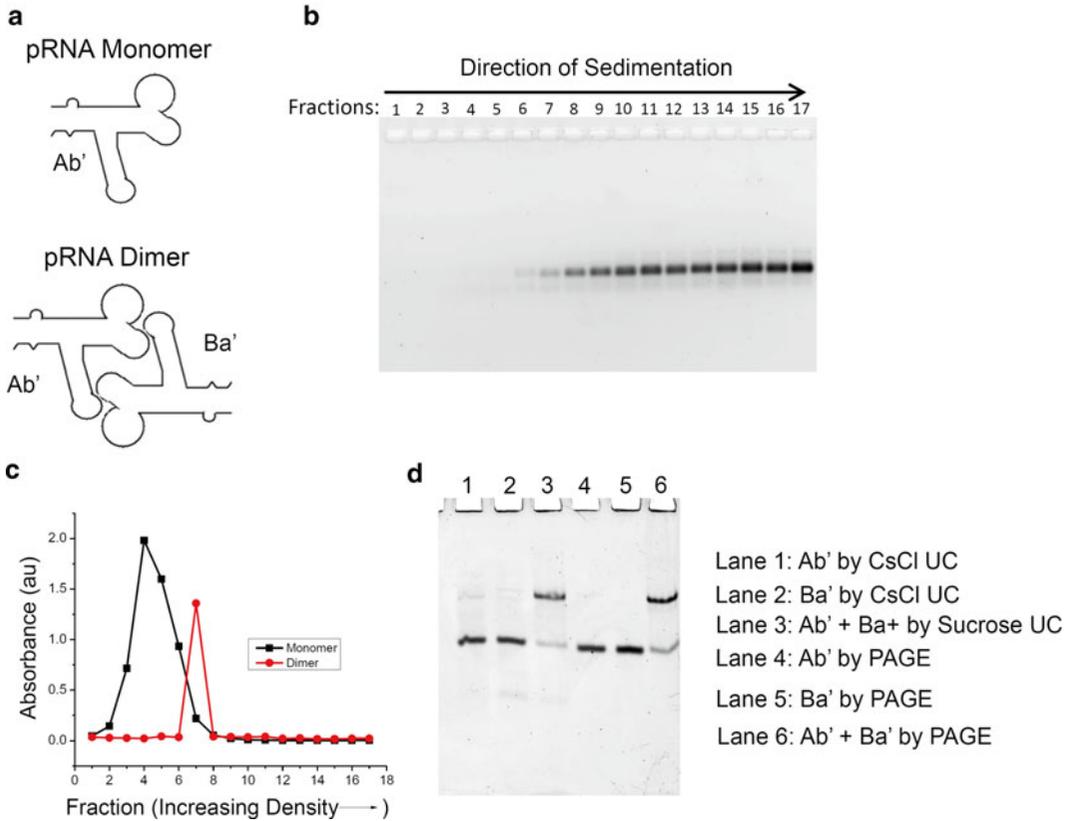
Fig. 4 pRNA monomer and dimer purification. (**a**) Schematic Representation of pRNA monomer (Ab′ and Ba′) and dimer complex (Ab′–Ba′). The pRNA dimer is formed by two distinct pRNA monomers, termed Ab′ and Ba′ (**b**) The two pRNA monomers are purified by CsCl density gradient ultracentrifugation and analyzed by agarose gel electrophoresis. The monomer sedimented toward the bottom of the tube, an example of differential centrifugation. (**c**) 5–20 % sucrose gradient was used to purify the pRNA dimer complex (*red*). As a reference, pRNA monomer was also examined under the same conditions (*black*). (**d**) Post centrifugation, fraction 7 was recovered and native PAGE was used to examine the assembly of pRNA dimers purified by ultracentrifugation (UC, *Lane 3*) compared to those purified by PAGE (*Lane 6*). *Lanes 1* and *2* are pRNA monomers purified by CsCl ultracentrifugation, used as size controls in the gel; *Lanes 4 and 5* were purified by PAGE as a comparison to results obtained by purification using ultracentrifugation. Comparable assembly of the pRNA dimer complex is observed between each method

2 Materials

Prepare all solutions using Millipore water (prepared by purifying deionized water to attain a sensitivity of 18.2 MΩ cm^{-1} at 25 °C) that has been incubated at 37 °C overnight with diethylpyrocarbonate (DEPC) and then autoclaved. All reagents used should be analytical grade and RNase and DNase free as RNA is extremely sensitive to RNase degradation. All glassware, tubes, and pipette tips used in preparation of reagents and buffers should be autoclaved to ensure sterilization. Gloves and lab coats should be worn at all times. All waste disposal regulations should be carefully

followed. Prepare and store reagents at room temperature (RT) unless otherwise noted. All reagents were purchased from Fisher Scientific unless otherwise specified.

2.1 For Gradient Preparation

1. Diethylpyrocarbonate (DEPC) aqueous solution: 0.05 % solution in millipore water (v/v): Add 5 mL DEPC to 995 mL millipore water and shake the solution vigorously. Incubate the solution overnight at 37 °C and then autoclave to remove DEPC.

2. CsCl Ultracentrifugation Buffer: 1× Tris–EDTA (TE) Buffer (10 mM Tris–HCl, 1 mM ethylenediaminetetraacetate (EDTA)). Add about 100 mL water to a 1-L glass beaker with a magnetic stir bar (*see* **Note 1**). Weigh 1.21 g Tris–HCl, and 0.000292 g EDTA (*see* **Note 2**) and transfer to the glass beaker. Bring the volume to 900 mL with water and then adjust the pH to 8.0 with HCl (*see* **Note 4**). Bring the final volume to 1 L and autoclave.

3. Sucrose Ultracentrifugation Buffer: 1× Tris–magnesium saline (TMS) Buffer (50 mM Tris-HCl, 100 mM NaCl, and 10 mM $MgCl_2$). Add about 100 mL water to a 1-L glass beaker with a magnetic stir bar. Weigh 6.05 g Tris base, 5.844 g sodium chloride, and 2.03 g $MgCl_2$ hexahydrate and transfer to the glass beaker. Bring the volume to 900 mL with water and then adjust the pH to 8.0 with HCl. Bring the final volume to 1 L and autoclave.

4. CsCl Solutions: $d = 1.65$ (0.790 g/mL). Dissolve 79.0 g molecular biology grade CsCl in 50 mL of 1× TE buffer in a glass beaker and mix with a magnetic stir bar. Once dissolved, dilute to 100 mL with 1× TE buffer in a graduated cylinder. $D = 1.95$ (1.230 mg/mL). Follow same procedure as before with 116.73 g of CsCl. Additional 1.745 g CsCl for sample preparation (*see* **Note 5**).

5. Sucrose solution: 5, 10, 15, 20 % (w/v). To prepare sucrose solutions, dilute respective amount of sucrose (e.g., 5 g for 5 % w/v) to 100 mL final volume with 1× TMS Buffer. Mix in glass beakers and stir with a magnetic beaker (*see* **Note 5**).

6. Ultracentrifugation tubes: Beckman #326819, 5-mL.

7. Automated Gradient Maker: BioComp Gradient Master Model 106 (BioComp Instruments) gradient maker.

8. Long Neck Pipette tips: Bio-Rad 223-9916, 1–200 μL pipette tip for gel loading.

9. Syringe and Needle: 5-mL BD Syringe, Fisher Scientific. Reusable non-sterile hypodermic needle, 3.5 in., Fisher Scientific.

10. Mild detergent: Alconox Detergent, Fisher Scientific #NC9003111.

11. Pipette: 1,000 and 200 μL pipette, Denville Scientific Inc.

12. Vortexer: VWR Analog Vortex Mixer.

2.2 For Ultracen-trifugation

1. Analytical Balance: Mettler Toledo AB204-S Analytic Balance.

2. Ultracentrifuge: Beckman Coulter L-80 Ultracentrifuge (Beckman #392051) (*see* **Note 6**).

3. Ultracentrifuge Rotor: Beckman Coulter SW 55 Ti Rotor (Beckman #342194) (*see* **Note 7**).

4. Ultracentrifugation Bucket: Swinging Bucket for SW 55 Ti Rotor (Beckman #342194) (*see* **Note 8**).

5. Ultracentrifugation Bucket Rack: Bucket rack for swinging bucket SW 55 Ti Rotor (Beckman #331313).

6. Ultracentrifugation Spinkote Lubricant: Lubricant for ultra-centrifuge (Beckman #306812) (*see* **Note 9**).

7. Ultracentrifugation Vacuum Grease: Vacuum seal grease (Beckman #335148) (*see* **Note 10**).

2.3 For Fractionation and Sample Analysis

1. Tubes: Fisherbrand Premium Microcentrifuge tubes, 1.5 mL. Catalog No: 05-408-120. Autoclaved.

2. Pipette: 200 μL pipette, Denville Scientific Inc. and sterile pipette 200 μL pipette tips.

3. Fraction Collector (Optional): Beckman Coulter Fraction Recovery System, No: 343890 (*see* **Note 11**).

4. Agarose: Low-melting, molecular biology grade. Fisher BioReagents. Catalog No: BP165-25.

5. Agarose Gel Buffer/Running Buffer: 1× TAE (40 mM Tris–acetate, 1 mM EDTA) with 10 mM $MgCl_2$. Add about 100 mL water to a 1-L glass beaker with a magnetic stir bar. While stirring, add 0.00725 g Tris–acetate, 0.000292 g EDTA, and 2.03 g $MgCl_2$ hexahydrate and transfer to the glass beaker. Bring the volume to 1 L.

6. Microwave.

7. Imaging System: Typhoon FLA 7000 imaging system, GE Healthcare.

8. Agarose Gel Electrophoresis System: Mini-sub cell GT system, Bio-Rad.

9. Ethidium Bromide: 1 % Solution, Molecular Grade. Fisher BioReagents Catalog No: BP1302-10.

10. Sample Concentration: Amicon Ultra 0.5 mL Centrifugal Filters, Millipore.

3 Methods

3.1 CsCl Equilibrium Density method for RNA Purification

Carry out all procedures at RT unless otherwise specified.

This method details the purification of RNA prepared by in vitro transcription using T7 RNA polymerase as described in detail previously [8]. CsCl centrifugation will remove unincorporated nucleotides as well as proteins and other impurities from the transcription reaction mixture based on differences in density. The sample being used for centrifugation in the following procedure can be used directly after in vitro transcription without further modification (Fig. 1).

3.1.1 Preparation of d = 1.65–1.95 CsCl Gradient

1. Prepare two CsCl solutions with densities of 1.65 (0.790 g/mL) and 1.95 (1.23 g/mL) as detailed previously (*see* **Note 5**).

2. Prepare CsCl sample solution with density equal to 1.75 (1.164 g/mL). Dilute the sample from RNA transcription to 1.0 mL (maximum sample volume for 5 mL centrifuge tube is 1.0 mL) with 1× TE buffer. To the diluted sample add 1.7452 g CsCl and mix thoroughly with a vortexer (*see* **Note 5**). Adjust the final volume to 1.5 mL.

3. Pipette the 1.5 mL sample in CsCl solution, $d = 1.75$, into a 5.0 mL ultracentrifuge tube (Beckman #326819).

4. Using a syringe and long needle, pipette the $d = 1.95$ CsCl solution to the bottom of the ultracentrifuge tube, below the sample CsCl solution

5. On top of the sample solution, pipette 1.5 mL of $d = 1.65$ CsCl solution.

6. Cap the centrifuge tube (*see* **Note 12**).

3.1.2 Ultracentrifugation Procedure

At all times be gentle when handling the tubes with the prepared CsCl gradients. Too rough of handling will disturb the gradient and affect the sample purification.

1. Place the ultracentrifuge tube into the centrifugation buckets.

2. Using an analytical balance, measure and record the weight of each bucket and respective bucket top with the tube inside. It is extremely important to equilibrate weights of buckets that will be across from each other when attached to the rotor (*see* **Note 13**). To make the weights equal, use water to match weights between the pairs of buckets. For each 0.01 g of difference, pipette 10 μL of water into the bucket with less weight (*see* **Note 14**).

3. Once the buckets are balanced, tightly secure the tops of the buckets to prevent leaking. Finally, secure the buckets onto the swinging-bucket rotor (Beckman SW Ti 55) (*see* **Note 15**).

4. Holding onto the base and the top of the rotor, carefully place the rotor into the ultracentrifuge (Beckman L-80 ultracentrifuge) (*see* **Note 16**).

5. Bring the temperature down to 4.0 °C and turn on the vacuum to equilibrate the rotor and sample. Wait until the final temperature and lowest pressure (<20 μm) are reached before commencing rotation (*see* **Note 17**).

6. Spin the rotor (Beckman SW 55 Ti) at 45,000 (246,078 × *g*) RPM for 16 h. (*see* **Note 18**).

7. After centrifugation, wait for the rotor to come to a complete stop and the RPM reading on the ultracentrifuge reaches "0." Once the rotor has stopped, release the vacuum and open the lid. Carefully grabbing the base and the top of the rotor, remove the rotor form the ultracentrifuge and place it back on the rotor stand.

8. Remove the rotor buckets and place them back in the bucket holder rack. Remove the tubes and place them in a sample rack. It is important to now thoroughly clean and dry the buckets as excess moisture can cause damage to the buckets.

9. Proceed to Subheading 3.3 for fractionation and sample analysis.

3.2 Cushioned Rate-Zonal Sucrose Gradient for Nanoparticle Separation

This method details the separation of the final nanoparticle assembly from incorrect structures such as misformed aggregates and unincorporated strands. The RNA assemblies should be annealed prior to purification by ultracentrifugation in 1× TMS buffer. Maximum sample volume for ultracentrifugation is 500 μL.

3.2.1 Preparation of 5–20 % (v/v) Sucrose Density Gradient Preparation

Gradient Preparation by Gradient Maker

1. Prepare two sucrose solutions, 5 % and 20 % (w/v), in 1× TMS buffer by diluting 5 and 20 g, respectively, of sucrose to 100 mL volume in a graduated cylinder. Transfer to a glass beaker and use a magnetic stir bar to thoroughly mix the solutions.

2. Into a 5.0 mL polypropylene (Beckman #326819) ultracentrifuge tube, cleaned with a mild detergent and thoroughly dried, pipette 2.4 mL of 5 % sucrose solution. In a 5-mL syringe with a long tip needle, carefully syringe 2.4 mL of 20 % (w/v) sucrose solution to the bottom of the ultracentrifuge tube. Insert the cap of the centrifuge tube into the top of the tube (*see* **Note 12**).

3. In this method, the gradient was made using the BioComp Gradient Master Model 106 (BioComp Instruments) gradient maker (*see* **Note 19**). For the specified gradient, 5–20 % sucrose (w/v), set the gradient maker to spin for 1 min and 13 s at an angle of 86° at 16 RPM. After the gradient maker is done, remove the ultracentrifuge tube and place in a sample holder rack (*see* **Note 20**).

Gradient Preparation by Manual Layering (if Gradient Maker Not Available)

This method details the procedure to make the 5–20 % (w/v) sucrose gradient if there is no automated gradient maker available.

1. By the same procedure previously detailed, make 5, 10, 15, and 20 % (w/v) sucrose solutions.

2. Using the prepared solutions, layer from most dense (20 %) to least dense (5 %) with 1.2 mL of each solution. Carefully layer the solutions into a 5.0-mL (Beckman #326819) ultracentrifuge tube using a long-tip hypodermic needle (*see* **Note 21**).

3. Incubate the tube overnight at 4 °C to form a continuous gradient, ideally in a cold room (*see* **Note 22**).

3.2.2 Sample Loading and Ultracentrifugation Procedure

At all times be gentle when handling the tubes with the prepared sucrose gradients. Too rough of handling will disturb the gradient and affect the sample purification.

1. Before being loaded, ensure the sample is in its native annealing buffer (*see* **Note 23**). To load the sample, slowly pipette a maximum volume of 100 µL on top of the previously prepared sucrose gradient (*see* **Note 24**).

2. Using an analytical balance, measure and record the weight of each bucket and respective cap with the tube inside. It is extremely important to equilibrate weights of buckets that will be across from each other when attached to the rotor (*see* **Note 13**). Use water to equal bucket weights. For each 0.01 g of difference, pipette 10 µL of water into the bucket with less weight (*see* **Note 14**).

3. Once the buckets are balanced, tightly secure the tops of the buckets to prevent leaking. Finally, secure the buckets onto the swinging-bucket rotor (Beckman SW Ti 55) (*see* **Note 15**).

4. Holding onto the base and the top of the rotor, carefully place the rotor into the ultracentrifuge (Beckman L-80 ultracentrifuge) (*see* **Note 16**).

5. Bring the temperature down to 4.0 °C and turn on the vacuum to equilibrate the rotor and sample. Wait until the final temperature and lowest pressure (<20 µm) are reached before commencing rotation (*see* **Note 17**).

6. Spin the rotor (Beckman SW 55 Ti) at 50,000 (303,800 × g) RPM for 7 h (*see* **Note 18**).

7. After centrifugation, wait for the rotor to come to a complete stop and the RPM reading on the ultracentrifuge has reached "0." Once the rotor has stopped, release the vacuum and open the lid. Carefully grabbing the base and the top of the rotor, remove the rotor from the ultracentrifuge and place it back on the stand.

8. Remove the rotor buckets and place them back in the bucket holder rack. Remove the tubes and place them in a sample rack. It is important to now thoroughly clean and dry the buckets as excess moisture can cause damage to the buckets.

3.2.3 Preparation of Cushioned Sucrose Gradient for Rate-Zonal Purification of RNA Nanoparticles

This method details the use of a high density sucrose "cushion" at the bottom of the gradient during the rate-zonal purification of RNA nanoparticles (Fig. 3d). Utilizing a cushion has multiple advantages. For low amounts of sample, the sample can be run to the bottom of the gradient and then stopped at the cushion. This allows the samples to be collected at a higher concentration (fewer fractions) while avoiding pelleting. Pelleting can sometimes lead to damage of sensitive samples during resuspension. Additionally, using a cushion enhances the collection of nanoparticles that display multiple conformations, which would otherwise spread to multiple fractions. Importantly, when purifying nanoparticles that contain small molecule therapeutics or reporter molecules, a cushion will help to purify the nanoparticle complex from free small molecules by allowing large separation between the two species. The size of the complex can easily separate from free drug due to the diffusion of the small molecule, which will prevent the small molecule from co-localizing in the denser (lower) portion of the gradient.

1. First prepare the 5–20 % sucrose gradient as described earlier in section 3.2.1. However, when using the BioComp gradient maker reduce the volume of each solution to 2.3 mL and when manually layering each solution decrease the volume of each solution to 1.15 mL. This will account for the additional volume needed for the high-density sucrose cushion.

2. After preparation of the 5–20 % sucrose gradient, with decreased volume, use a pipette to load 2 mL of 60 % sucrose solution to the bottom of the gradient. Slowly pipette the 60 % sucrose solution down the side of the ultracentrifuge tube, from the top of the gradient. Because of the high density of the 60 % sucrose solution, it will quickly migrate to the bottom of the tube forming a sucrose cushion.

3. Let the cushion settle for 5 min being careful not to disturb the solution in the tube.

4. For sample loading and fractionation follow the same procedures detailed for sucrose gradient rate-zonal ultracentrifugation without use of the cushion.

3.3 Fractionation and Sample Analysis/Recovery (Same for CsCl and Sucrose)

During this step it is important to keep track of each fraction when removing it from the tubes. Pre-label autoclaved tubes to ensure accuracy of fractions.

1. To remove fractions, use a pipette to remove 200 μL from the very top of the CsCl or sucrose gradient and place in a pre-labeled tube to ensure accuracy of fraction numbers (*see* **Note 25**).

2. Continue to remove 200 μL fractions from the very top of the tube until the tubes are completely empty (*see* **Note 26**).

3. Prepare a 2.0 % non-denaturing agarose gel containing 1× EtBr gel stain (2.0 g agarose, diluted to 100 mL in 1× TAE Buffer containing 10 mM-Mg) (*see* **Note 27**).

4. Into each well of the agarose gel load 10 μL aliquots of each fraction. Run for 30 min at 120 V at RT in 1× TAE buffer with 10 mM $MgCl_2$.

5. After the gel has finished running, scan on a Typhoon FLA 7000 laser scanner for EtBr signal. Similar gel scanning devices can also be used (*see* **Note 28**).

6. Combine the fractions containing the desired products and reconstitute into the native folding buffer, 1× TMS, using Amicon Ultra 0.5 mL centrifugal filters (*see* **Note 29**).

3.4 **Expected Results**   Synthesis and purification of RNA nanoparticles utilizing the pRNA from the phi29 bacteriophage was performed. In nature, the pRNA forms a hexameric ring that is used in gearing the phi29 DNA packaging motor [1, 25]. Previously, the native pRNA structure was engineered to assemble into diverse RNA nanoparticles including dimers, trimers, tetramers, hexamers, and larger supramolecular complexes utilizing hand-in-hand and foot-to-foot interactions [1, 7, 8, 26, 27]. As demonstration of expected results, we show here the purification of pRNA monomer and dimer constructs from two different pRNA monomers, Ab′ and Ba′ [28]. Monomers were purified by CsCl density gradient ultracentrifugation and dimer constructs by 5–20 % sucrose gradient rate-zonal ultracentrifugation. The Ab′ and Ba′ pRNA monomers assemble via hand-in-hand kissing loops to form dimer constructs (Fig. 4a). CsCl density gradient ultracentrifugation was used to purify RNA directly after in vitro transcription from the proteins and DNA template using the procedures detailed here. Post in vitro RNA transcription, the reaction mixture contains proteins and DNA template, which are undesired products. The DNA template is degraded by DNase enzyme and the proteins are easily separated from the RNA product by the difference in their densities. The high density of RNA results in the products migrating to the densest portion of the gradient. Despite a small size difference between monomers and dimer, ~120 nucleotide monomer and ~240 nucleotide dimer, the monomer and dimer can be separated by sucrose density gradient ultracentrifugation following the procedures detailed here (Fig. 4c). The recovered fractions demonstrated comparable dimer assembly to RNA nanoparticles purified by PAGE (Fig. 4d).

4 Notes

1. Adding reagents to already circulating water helps to dissolve them easily. If there is trouble dissolving the reagents, the glass beaker can be heated gently to 37 °C. However, pH must be adjusted at room temperature.

2. Use caution when handling EDTA. EDTA is a skin, eye, and respiratory tract irritant

3. $MgCl_2$ will absorb moisture from the air. Be sure to handle as quickly as possible and then recap the reagent bottle tightly to prevent moisture absorption [29].

4. Use caution when handling concentrated HCl. HCl is a strong acid and is very corrosive. HCl is a strong eye, skin, and respiratory tract irritant. HCl can cause severe burns, so be sure to handle with gloves and wear splash goggles to prevent it from getting into eyes.

5. Be sure to mix vigorously enough to have a homogeneous mixture of CsCl or sucrose and 1× TE buffer.

6. Be sure regular maintenance has been carried out on the ultracentrifuge to ensure proper operation and safety for the user.

7. Before use, inspect rotor to make sure there are no cracks or disfigurements; use of damaged rotors could result in danger to the user and damage to the ultracentrifuge.

8. Before use, inspect buckets for cracks and/or disfigurements; use of damaged buckets could result in danger to the user and damage to the ultracentrifuge.

9. Before using the ultracentrifuge, be sure the rotation bearings are properly lubricated.

10. Prior to ultracentrifugation ensure the vacuum seal is properly greased. A poorly greased vacuum seal will not allow the proper low-pressure environment to be reached and ultracentrifugation will not be able to commence.

11. The Fraction Recovery System works by puncturing a whole in the bottom of the ultracentrifugation tube and collection occurs from the bottom of the tube. This system can be a viable alternative to the method described here; however, this equipment is optional.

12. When capping the ultracentrifuge tube make sure to cap at an angle to prevent air bubbles from forming in the gradient. Keep air bubbles out while preparing the gradient. When syringing higher density CsCl or sucrose do so slowly and with extreme care to avoid mixing the high density with the low density solutions. The gradient used will not be identical for all complexes. In some cases the ideal gradient to use must be experimentally determined.

13. Each bucket is labeled with a number 1–6, 1 is across from 4, 2 from 5, 3 from 6. It is important that each pair be identical in weight and are placed across from each other on the rotor. Also, even if six samples are not being run, it is important that all buckets be in place during centrifugation and run with equal weight.

14. Do not pipette into the ultracentrifuge tube; pipette into the space surrounding the tube inside the bucket.

15. Before beginning ultracentrifugation, make sure each bucket is securely fixed to the rotor. Do this by gently but firmly tugging down on each bucket once it is attached to the rotor. However, while doing this do not disturb the gradient and sample in the tube.

16. The rotor must rotate freely to ensure proper function of the ultracentrifuge. Ensure that the connection is sufficiently greased (Beckman #306812) and test by rotating the rotor clockwise by hand. If it moves freely, it is sufficiently lubricated.

17. A proper vacuum seal is required to hold the vacuum constant. Make sure the seal is sufficient, if not apply grease to ensure a proper seal (Beckman #335148).

18. The optimal centrifugation time depends on the exact mass and shape of the RNA complex. This should be experimentally determined. Avoid excessive centrifugation as this may lead to sample pelleting at the bottom of the tube, which will negatively affect the purification of the RNA assembly. Pelleting time, or k-factor, can be calculated quickly by equation $t = k/s$, where t is run time in hours required to pellet a particle of known sedimentation coefficient s (in Svedberg units, S). The *k-factor* is a function of the maximum and minimum radius of a rotor and the run speed in RPM. The *k-factor* for the SW-55 Ti rotor at 45,000 RPM is 72.4. After determining the sedimentation coefficient of the particle of interest, the pelleting time can be calculated and a shorter time should be used. For details, please refer to Beckman Coulter Application note: *Using k-Factor to Compare Rotor Efficiency (CENT-66APP)*.

19. This exact model of gradient maker is not required. Follow the gradient maker protocol for correct angle, speed, and duration.

20. Always prepare gradients with location in mind. Try to move the formed gradients as little as possible as moving the gradients can disturb the final density gradient.

21. Pipette as carefully as possible when stacking the different densities of CsCl or sucrose. Taking care not to mix the different gradients will lead to a better quality gradient.

22. A cold room is preferred over a refrigerator. Repeated opening and closing of a refrigerator door will disturb the gradient during its equilibration.

23. Sample should be in its native annealing buffer, 1× TMS.

24. When pipetting the sample onto the top of the gradient take great care to pipette slowly and get as narrow a band as possible of the sample. This will lead to higher resolution during ultracentrifugation.

25. 200 μL may not be an ideal volume for every purification situation. In a case where the RNA complex is not as well separated a lower fraction volume may be ideal, in cases where resolution is high larger fraction volumes may be ideal. This should be experimentally determined.

26. Be sure to use a new pipette tip with each fraction to avoid contamination.

27. Be careful handling EtBr, it is thought to act as a mutagen by intercalating with dsDNA and dsRNA.

28. Be sure to include a molecular weight marker in the agarose gel to determine which fractions are the correctly formed complexes.

29. Follow the manufacturer's protocol. The size of the RNA complex being purified will determine the specific filter to be used. Centrifugal force less than $4,500 \times g$ should be used in order to minimize sample damage and loss. Typically this will result in 50–100 μL of purified RNA complex.

Acknowledgements

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