Assembly of RNA Nanostructures from Double-Crossover Tiles

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Abstract

Artificial self-assembling RNA scaffolds can be produced from many types of RNA motifs that are rationally designed. These scaffolds are of interest as nanoscale organizers, with applications in drug delivery and synthetic cells. Here we describe design strategies, production methods, and imaging of micrometer-sized RNA nanotubes and lattices that assemble from RNA tiles comprising multiple distinct strands.

Key words RNA, RNA assembly, RNA nanotubes, RNA lattices

1 Introduction

RNA molecules spontaneously fold into a variety of structural motifs due to their base-pairing interactions. In living cells, natural motifs include aptamers, siRNA, miRNA, and ribozymes that can have functional properties such as catalysis and gene regulation. In nanotechnology, folding of artificial RNA molecules has been exploited to design a multitude of artificial scaffolds with demonstrated functionality in biomedical applications such as gene silencing and targeted therapeutic delivery [1, 2].

The synthesis of increasingly complex and large artificial RNA scaffolds has been sought after with the goal of building programmable molecular machines and organelles. While the first efforts in this direction take advantage of conserved tertiary motifs [3], recent work has taken inspiration from the success of design methods developed in DNA nanotechnology [4–6]. The tiling approach originally developed for DNA components can be adapted to RNA yielding structures that reach micrometers in size like their DNA counterparts [7–9]. RNA structures demonstrated with this approach include hollow nanotubes, filaments, and lattices, which can be obtained by specifying design parameters of double-crossover (DX) tiles (Fig. 1) [8, 9]. These tiles include three to

five short, distinct RNA strands that can be assembled either by gel-purification of individual strands followed by annealing or transcription of all strands in one-pot and annealing [8]. While RNA tile components can be expressed and interact inside cells, the formation of lattices and nanotubes in vivo remains challenging to demonstrate [10]. These RNA assemblies show promise as a vehicle for therapeutic delivery [7]. Understanding RNA design principles to build large assemblies will make it possible to build customizable scaffolds for drug delivery and for the spatial arrangement of components in synthetic cells.

Here we provide protocols for the design, production, and imaging of self-assembled RNA structures from RNA DX tile motifs.

2 Materials

Nuclease-free water and RNase removal agent (e.g., RNase Zap) are used throughout the Methods.

2.1 RNA Transcription

1. Synthetic PAGE purified template DNA strands from Integrated DNA Technologies (Coralville, IA, USA).
2. 10× TE buffer: 100 mM, 10 mM EDTA, pH 8.0 at 25 °C.
3. AmpliScribe™ T7-Flash™ Transcription Kit (Lucigen): T7 RNA Polymerase, 10× Transcription Reaction Buffer, ATP (100 mM), GTP (100 mM), CTP (100 mM), UTP (100 mM), DTT (100 mM), RiboGuard RNase Inhibitor (40 U/μL), nuclease-free water, control template DNA (0.5 μg/μL), RNase-free DNase I (1 U/μL), store at −20 °C.

2.2 Polyacrylamide Gel Components

1. 10× TBE buffer: 890 mM Tris–borate, 20 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0–8.5 at 25 °C.
2. Acrylamide/Bis 19:1, 40% (w/v) solution, store at 5 °C.
3. Disposable gel cassettes.
4. Urea.
5. Ammonium persulfate (APS): 10% (w/v) solution in water.
6. *N,N,N',N'*-Tetramethylethylenediamine (TEMED), store at 5 °C.

2.3 RNA Extraction, Elution, and Precipitation

1. Razor blades.
2. Plastic wrap.
3. Aluminum TLC plate, silica gel coated with fluorescent indicator F₂₅₄.
4. Sodium acetate.
5. Glycogen, store at −20 °C.

2.4 Assembly Buffer Components

1. Magnesium chloride.
2. 10× TAE: 400 mM Tris–acetate, and 10 mM EDTA, pH 8.5, store at 25 °C.

3 Methods

3.1 RNA Tile Design

1. Choose one of the tile designs in Fig. 1 (*see Note 1*), and choose a set of parameters from Table 1 (*see Notes 2 and 3*). Using the parameters of choice, the sequence of the RNA strands required for assembly can be designed using the DNA Design Toolbox [11]. While this toolbox is primarily for DNA nanostructure design, it can be used as a first-pass filter to design sequences that do not have bad secondary structures (both intra and inter-strand) at a specified temperature.
2. Confirm the absence of unwanted secondary structures in the designed RNA strands using NUPACK [12].
3. The number of sticky ends can be changed if one needs to tune the melting temperature of the nanotube assembly. Changing

Fig. 1 RNA-based tilings considered in this work. The “up-up” tiling has all the tiles showing the same side up and the “up-down” tiling has every other column of tiles in the assembly showing the same side up. The top row of the figure shows the 3D model of monomer tiles for each tiling. The second row shows an abstraction of the monomer tile that identifies tile domains that influence the assembly outcome. The third row illustrates how the monomers assemble to form an array and the bottom row shows the arrangement of faces of the monomers in the array in each tile type

Table 1
Parameters for RNA tile designs

Tiling type	Inter-tile crossover distance	Sticky end	Nick distance	Arm distance	Predominant structure formed	Avg. nanotube length
Up-up	22	6	8	8	2D arrays	NA
Up-up	23	7	8	8	Nanotubes	1.5 μm
Up-up	24	8	8	8	Nanotubes	1.5 μm
Up-up	25	9	8	8	2D filaments	NA
Up-up	26	10	8	8	2D filaments	NA
Up-down	28	5	9	11	Nanotubes	0.5 μm
Up-down	28	5	13	11	Nanotubes	0.5 μm
Up-down	28	5	11	11	No structures	NA

sticky-end length without adjusting any other design parameter will change the inter-tile crossover distance and result in different types of structures formed as shown in Table 1.

4. If parameters not listed in Table 1 are going to be tried, it is important to build 3D computer model of the design to ensure that the crossovers positions provided work with the A-form geometry of the RNA-RNA duplex (*see* **Note 4**). The angle with which the bases tilt with respect to the central helical axis can make crossovers at some pairs of positions impossible [4]. A 2D line drawing as shown in Fig. 1 does not capture this detail so a 3D computer model is needed.
5. RNA needed for the assembly can either be purchased directly from a vendor or instead the DNA needed for in vitro transcription of RNA strands can be purchased from a vendor. RNA synthesis by in vitro transcription can be done as described in Subheadings 3.5 and 3.6. The DNA templates will be ordered with a T7 RNA polymerase promoter [7].

3.2 DNA and RNA Quantitation

UV absorbance quantifies nucleic acids by measuring the absorbance of light through a DNA or RNA liquid solution to determine the concentration of molecules.

1. DNA or RNA purchased from the vendor is resuspended in nuclease-free water and vortexed until completely dissolved (*see* **Note 5**).
2. Determine the concentration of DNA and RNA by measuring the absorption at 260 nm according to Beer Lambert’s law using sequence-specific extinction coefficients.

3.3 Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) provides useful information by separating DNA and RNA molecules by their electrophoretic mobility.

3.3.1 Denaturing

Denaturing PAGE separates DNA and RNA molecules based on size and is used to determine sequence length of strands.

1. Prepare a 100 mL 8% (w/v) 19:1 acrylamide:bis and 7 M urea premix solution. Add 42 g of urea to 25 mL of nanopure water. Heat the solution until the urea is completely dissolved (*see Note 6*). Then add 10 mL of 10× TBE, and the appropriate volume for the desired polyacrylamide percentage. Typically, we prepare 10% polyacrylamide gel by adding 25 mL of the 40% acrylamide/bis 19:1, and then fill the remaining volume to 100 mL.
2. Cast the gel by adding in a 50-mL falcon tube 8 mL of the premix solution, 48 μL 10% APS, and 3.2 μL TEMED. Gently rock the tube avoiding the creation of bubbles.
3. Slowly pour the solution in an upright empty 8 cm × 8 cm with 1 mm width cassette.
4. Carefully add the gel comb, avoiding the creation of bubbles.
5. Allow the gel to polymerize for 2 h (*see Note 8*).
6. After the gel has polymerized, carefully remove the comb as not to disturb the wells.
7. Rinse the wells with running buffer, 1× TBE, to remove any residual unpolymerized remains.
8. Place the gel in the electrophoresis chamber and add 1× TBE buffer.
9. Prerun the gel at 10 V/cm at room temperature or 45 °C in 1× TBE for 10 min.
10. Carefully pipette the sample and loading buffer into the wells.
11. Run the gel for 50–60 min at room temperature or 45 °C.

3.4 Annealing dsDNA Templates

1. Dilute PAGE-purified DNA strands in nuclease-free water.
2. Mix the template (t) and non-template (nt) strands at a target concentration of 20 μM in a buffer of 10 mM Tris, 20 mM NaCl, and 1 mM EDTA.
3. Apply a heat ramp on a thermocycler or PCR machine. Heat the sample up to 95 °C, holding the maximum temperature for 5 min, and cooling to 20 °C at a rate of −1 °C/min.

3.5 RNA Transcription

1. In a 0.5-mL reaction tube, mix:
 - (a) 1× T7-Flash reaction buffer.
 - (b) 9 mM each NTPs.
 - (c) 10 mM DTT.

- (d) 1 μ g DNA template.
 - (e) 20 units of RiboGuard RNase Inhibitor.
 - (f) 1 unit of AmpliScribe T7-Flash enzyme solution.
2. Incubate the transcription mixture at 37 °C for 4 h (*see Note 10*).
 3. After the transcription reaction, to remove the DNA template, add 1 unit of RNase-free DNase I and incubate at 37 °C for 10 min.

3.6 RNA Purification

Purified RNA can be chemically synthesized and ordered directly from a vendor. Alternatively, one can produce RNA via an enzymatic reaction and purify the RNA.

1. Assemble a transcription mix (typically 20 μ L) as described in AmpliScribe™ T7-Flash™ Transcription Kit instructions.
2. Incubate the reaction mix at 37 °C for 4 h.
3. Add 1 μ L DNase I and incubate at 37 °C for 10 min to remove DNA templates.
4. Add equal volume of 2 $\times$ denaturing loading buffer, then load it on a 10–15% denaturing gel.
5. After the run, place an aluminum TLC plate, and silica gel coated with fluorescent indicator F254 and cover with plastic wrap.
6. Crack open the gel cassette and place the gel on the plastic wrap on the aluminum TLC plate.
7. Use a short-wave UV (~254 nm) light source to observe RNA transcripts in the gel.
8. Excise the RNA bands of interest, and chop them into small gel pieces. Collect the pieces in a 0.5-mL tube, filling about half the tube.
9. Add 350 μ L of 0.3 M sodium acetate (pH: 5.2–5.3) to completely submerge the pieces and incubate at 42 °C overnight.
10. Transfer the supernatant to a fresh tube and add 100 μ L of the 0.3 M sodium acetate (pH: 5.2–5.3) to the gel pieces. Vortex the content and transfer the 100 μ L of buffer to the other 350 μ L in the fresh 1.5-mL tube.
11. Add 1 mL of freezer cold ethanol (–20 °C) to the supernatant and 1 μ L of glycogen (20 μ g/ μ L) and vortex.
12. Incubate the sample at –20 °C overnight.
13. Spin the tube at >13,500 rpm at 4 °C for 15 min in a centrifuge.

14. Decant the supernatant with a pipette, then add 500 μL of 70% freezer cold ethanol, and spin (19,400 rcf) at 4 °C for 5 min.
15. Repeat **step 14**.
16. Remove as much supernatant as you can without disturbing the pellet.
17. Open tubes and place in vacufuge and allow to spin at room temperature for 10 min.
18. Resuspend the pellet in nuclease-free water and determine the concentration.
19. Store extracted and purified RNA at -20 °C.

3.7 Assembling RNA Nanotubes by Standard Anneal

1. In a 0.5-mL reaction tube, mix:
 - (a) $1\times$ TAE.
 - (b) 12.5 mM MgCl_2 .
 - (c) 1 μM of each strand.
2. Apply a heat ramp on a thermocycler or PCR machine. Heating the sample up to 70 °C, holding the maximum temperature for 5 min, and cooling to room temperature at a constant rate over 24 h.

3.8 Assembling RNA Nanotubes by One-Pot Transcription and Anneal

1. In a 0.5-mL reaction tube, mix:
 - (a) $1\times$ TAE.
 - (b) 22.5 mM MgCl_2 .
 - (c) 2.25 mM of each NTP.
 - (d) 10 mM DTT.
 - (e) 1/6 volume dilution of AmpliScribe T7-Flash Enzyme Solution.
 - (f) 0.5–1 μg of each DNA template.
2. Apply a heat ramp on a thermocycler or PCR machine. Incubate the sample at 37 °C for 15 min, then heat the sample up to 70 °C, holding the maximum temperature for 5 min, and cooling to room temperature at a constant rate over 24 h.

3.9 Atomic Force Microscopy

RNA nanotubes can be imaged in buffer with nanometer resolution using atomic force microscopy.

1. Place metal specimen disc on a hot plate and melt a small amount of a hot glue stick.
2. Place a mica disc that has been cut into a square and firmly secure it on the metal specimen disc.
3. Cleave the mica with a piece of tape.
4. Place 5 μL of sample on the freshly cleaved mica and allow it to incubate on the surface for 30 s.

5. Add 25 μL of the sample buffer on top of the sample on the mica.
6. Add 25 μL of the sample buffer to the AFM tip.
7. Sharp Nitride Lever (SNL) tips from Bruker with a nominal spring constant of 0.24 N/m are used for imaging, with a drive frequency of 9–10 kHz (*see* **Note 12**). The AFM data are collected with a Digital Instruments Multimode AFM, equipped with a Nanoscope III controller and quantitated using ImageJ.

3.10 Fluorescence Microscopy

1. Add 1–2 μL of a fluorescent labeled sample (*see* **Note 13**) on a precleaned coverslip.
2. Carefully place a clean glass slide on the sample on the coverslip.
3. Place one drop of immersion oil on a 60 $\times$ oil immersion objective and position the assembled slide on the stage.
4. Select the correct filter and focus on the sample.
5. Images are obtained with a NIKON TI-E inverted fluorescence microscope. Cy3 filter was used to image all tiles with Cy3 labeled S3 strand. A 60 $\times$ oil immersion objective is used to collect all images, with a standard exposure time of 600 ms.

4 Notes

1. There are two DX tile designs that have been shown to produce nanostructures. Figure 1 shows their 3D structure, strand composition, and tiling pattern. The “up-up” tiling system self-assembles with all the monomers having the same side up. The “up-down” tiling system self-assembles with tiles alternatingly facing up and down as shown in Fig. 1. For “up-up” tiling systems, monomer tiles with five unique strands have been shown to work [9]. For “up-down” tiling systems, monomer tiles with as low as three unique strands have been shown to work [8]. While the “up-down” design is more parsimonious with only three distinct strands, the “up-down” tiling designs are more versatile both in terms of the number of uniquely modifiable strands and resultant morphologies.
2. The important parameters to determine before the start of the design are: (a) inter-tile crossover distance, (b) sticky-end length, (c) nick distance, and (d) arm distance. These parameters are shown in Fig. 2.
3. Table 1 shows the resulting nanostructures and their characteristics based on the choice of the parameters listed above. Choose the tiling type and parameter values depending on the type and characteristics of nanostructures desired. The parameter combinations not listed in this table have not yet been tested.

Fig. 2 Example AFM and fluorescence microscopy images of RNA tile-based assemblies. (a) AFM image of up-down tile assembly. (b) AFM image of up-up tile assembly. (c) Representative fluorescence microscopy image of up-up tile assembly

4. Multiple tools are available to build 3D models: (a) PDB models can be built using NAMOT software [13] and edited and rendered using Chimera software [14]. (b) You can also build PDB models using simRNA [15] and export the PDB file to oxDNA [16, 17] if you want to run simulations with the model. PDB files can be converted to oxDNA using the TacoxDNA server [17]. Additionally, caution should be taken when using the TacoxDNA server for manipulating RNA as it currently does not support the import of uracil bases
5. Lyophilized DNA or RNA can also be resuspended in tris-buffer for longer storage time; however, the downstream application should be taken into account when selecting the storage buffer.
6. To quickly dissolve urea in water, microwave the solution in 10 s increments, careful not to let the solution boil.
7. Denaturing polyacrylamide premix can be stored at 4 °C for up to 4 weeks [18].
8. Gels can be cast and stored for later use in buffer wrapped with plastic wrap 4 °C for up to a week.
9. We recommend using a 10-basepair dsDNA ladder (Invitrogen) as a size marker and to stain gels with staining solution, SYBR gold (Molecular Probes) for quantitation of DNA and RNA molecules [19].
10. To increase the amount of transcripts produced, the incubation temperature can be changed from 37 °C to 42 °C.
11. According to the manufacturer, transcription reactions can be scaled linearly to increase yield of RNA products.
12. When imaging via AFM, sometimes samples will aggregate on the mica surface, so you may have to search the surface for the sample.

13. When preparing fluorescently labeled RNA strands, adding more than 25% will result in a lack of assemblies or in malformed assemblies. Adding 10% of fluorescently labeled strands is optimal.

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