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Innovations

Isolation of short RNAs with homogeneous 3′-ends using quaternary-amine anion exchange chromatography

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Abstract

Visualizing RNA–protein interactions through structural approaches requires the use of RNA molecules purified to homogeneity. We describe here a simple and effective method, free of acrylamide contamination and without using UV radiation, to separate *in vitro* synthesized, heterogeneous RNA transcripts (up to ∼15 nucleotides) at single-nucleotide resolution by quaternary-amine anion exchange chromatography. The quality of short RNAs isolated through this method is validated by gel electrophoresis, mass spectrometry, and crystallization with a protein-binding partner.

Keywords: short RNA; in vitro transcription; capping; chromatography; structural biology

Introduction

RNA plays a central role in biology, from fundamental processes like gene transcription and protein synthesis [1], to more specialized roles in catalysis [2], gene regulation [3], cell differentiation, as well as tissue and organ development [4]. These roles are mediated by a diverse array of RNA types within the cell, including rRNA, tRNA, mRNA, as well as a group of non-coding, generally shorter length, RNAs such as siRNA, miRNA, snRNA, lncRNA, and others [5]. Additionally, a variety of foreign RNAs from viruses play important roles in activating the host innate immune response, including 5′-triphosphate (5′-PPP)-RNA, capped RNA, and double-stranded RNA [6]. In almost all these functions, RNA is intimately associated with protein-binding partners. Our understanding of the intricate mechanisms that underlie the functions of ribonucleoprotein machineries has been gleaned by numerous structural studies of these complexes using techniques, such as X-ray crystallography and cryo-electron microscopy [7], which require highly purified and homogeneous samples for success.

For the most part, the majority of cellular RNAs are 5′-end monophosphorylated owing to the nature of their maturation processes from larger pre-RNAs [8]. Moreover, in many structural roles, the RNA component is relatively small in length or only small fragments of the larger physiological RNA are used, since RNA (particularly single-stranded RNA) is less structured than protein [9, 10]. In these cases, it is generally most efficient to chemically synthesize RNAs because of the high purity and cost–effectiveness for short monophosphate RNAs. However, a subset of physiological RNAs have structurally different 5′-ends, most notably mRNA, with the m7GTP cap structure that protects it from nuclease degradation in the cytoplasm [11]. Additionally, viral RNA genomes can also be capped, and their replication intermediates can exist as an uncapped 5′-PPP RNA form [12]. These viral signatures are important for recognition by host proteins such as retinoic acid-inducible gene I (RIG-I) or interferon-induced proteins with tetratricopeptide repeats (IFITs) during the innate immune response [13, 14]. To study these types of RNAs, chemical synthesis is difficult even for short-length 5′-PPP RNAs due to poor yields, the hazardous chemicals required for synthesis, and the degradation of the 5′-PPP group under the synthetic reaction conditions [15, 16]. Moreover, chemical synthesis of isotopically labeled RNA for NMR applications is costly [17]. Therefore, to produce RNA with an intact 5′-PPP structure or with isotope labeling, it is more appropriate and affordable to opt for *in vitro* synthesis with T7 RNA polymerase (RNAP) followed by purification of the RNA.

Whereas the purification of proteins using engineered fusion tags and well-established chromatographic methods has a rich history in biochemistry and structural biology, the techniques for RNA purification are not as well-developed. For RNA purification, denaturing polyacrylamide gel electrophoresis (PAGE) is the most widely used method to isolate RNA transcripts [18]. However, gel extraction for RNA purification has some limitations: for instance, the inevitable contamination with acrylamide; the challenge of separating full-length transcripts from aborted or run-off transcripts that differ in length by only one or a few nucleotides; the loss of RNA in the gel matrix; and the undesirability of working with UV radiation. Nevertheless, gel extraction is a robust method to obtain relatively homogeneous RNA. Additionally, because RNA is a charged molecule, anion exchange chromatography is a commonly used method to purify RNA as evidenced by several previous studies. Easton *et al.* reported the purification of relatively long RNAs [∼30–500 nucleotides (nt)] by diethylaminoethyl (DEAE) anion exchange chromatography [19]. Koubek *et al.* used a Mono-Q anion exchange column for purifying tRNAs, and found that it outperformed DEAE [20]. Karlsson *et al.* combined reverse-phase ion-

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pairing and denaturing ion-exchange chromatography to purify RNA samples ranging from 22 to 82 nt [21]. Separation based on size is not as effective as charge-based methods for RNA purification–size exclusion chromatography can, at best, separate transcripts with different oligomerization states, but cannot separate transcripts that are heterogeneous at their 3′-ends [19].

The RNAs purified by ion exchange in the above studies lacked homogeneity with respect to their 3′-ends, due to aborted or run-off transcripts. To obtain the homogeneity required at the 3′-end for structural studies, one must resort to encoding self-cleaving ribozymes into the transcript [22], which may be suitable for larger RNAs, but is an unnecessary complication for shorter RNAs (less than ∼15 nt). Here, we describe a method to purify short, *in vitro* transcribed RNAs using quaternary (Q) anion exchange chromatography with single-nucleotide resolution at the 3′-end and with milligram quantities of RNA suitable for structural studies.

Materials and methods

Purification of bacteriophage T7 RNA polymerase

The plasmid pAR1219 containing the cDNA for His_6-tagged bacteriophage T7 RNAP was transformed into *Escherichia coli* BL21 cells and plated onto 100 μg/ml ampicillin containing Luria broth (LB)-agar [25 g pre-mixed Luria broth powder (10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract), and 20 g/l agar]. A single colony was transferred into an LB medium containing 100 μg/ml ampicillin and grown overnight at 37°C. Twenty-five milliliters of the overnight starter culture was inoculated into 1 L LB containing 100 μg/ml ampicillin, and grown at 37°C until the OD_{600} was ∼0.6, followed by induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and growth for 3 h. The following purification procedures were adapted from a previous study on T7 RNAP [23]. Cells were harvested and resuspended in Ni-binding buffer [50 mM Tris–HCl pH 8.0, 500 mM NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol (βME)] supplemented with a Roche protease inhibitor cocktail tablet (Sigma-Aldrich) and 0.1% Triton. Cells were lysed using a French press and centrifuged at 50 000*g* for 30 min. The supernatant was loaded onto a 5 ml Ni-NTA column (HisTrap HP, Cytiva) equilibrated in Ni-binding buffer. The column was washed with 10% Ni-elution buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 200 mM imidazole, 2 mM βME). His-tagged T7 RNAP was eluted with a linear gradient of Ni-elution buffer. The eluted protein was dialyzed overnight against buffer containing 50 mM Tris–HCl pH 8.0, 100 mM NaCl, and 2 mM βME. The sample was then passed over a 5 ml HiTrap SP HP column (GE Healthcare) equilibrated with SP buffer A [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5 and 2 mM dithiothreitol (DTT)], and T7 RNAP was eluted using a linear gradient of SP buffer B (50 mM HEPES pH 7.5, 1 M NaCl, and 2 mM DTT). Fractions were pooled, concentrated and loaded onto a Superdex 75 Increase 10/300 column (GE Healthcare) equilibrated in gel filtration buffer containing 50 mM Tris–HCl pH 8.0, 200 mM NaCl, and 2 mM DTT. Fractions corresponding to T7 RNAP were pooled and concentrated to aliquots of ∼2–4 mg/mL in 50% glycerol, kept at −20°C for short-term use, and stored at −80°C (Supplementary Fig. S1). The final yield of T7 RNAP was ∼2 mg/l of culture.

In vitro transcription

Synthetic DNA oligonucleotides were used as templates for *in vitro* transcription (IVT). One strand contained a consensus 17 nt T7 promoter sequence plus an initializing G nucleotide; the other strand contained the complementary region to the T7 promoter, followed by a region that encodes the desired RNA sequence. To hinder the sliding of RNA polymerase, a stalling nucleotide was added since the target RNA sequence does not contain all four types of nucleotides. For instance, if C is absent from the RNA sequence, then the stalling nucleotide in the coding DNA template is G, which can be followed by an additional random nucleotide. In our case, rCTP is omitted when setting up the IVT reaction (Supplementary Table S1). The two DNA oligonucleotides were separately resuspended at a concentration of 100 μM in ribonuclease (RNase)-free water, after which an equal volume of each strand was transferred to a clean tube, annealed at 95°C for ∼3 min, and gradually cooled to room temperature. The concentration of annealed double-stranded (ds) DNA template was determined on a NanoVue (GE Healthcare) and diluted to a concentration of 500–1000 ng/μl. The IVT reaction consisted of the following ingredients: buffer, either 50 mM Tris–HCl, pH 8.1 or 50 mM HEPES-KOH, pH 7.5; 2 mM Spermidine; 20 mM DTT; 2–10 mM of each ribonucleoside triphosphates (rNTP) depending on the desired RNA sequence; 10–100 ng/μl dsDNA template; 50–200 μg/ml T7 RNAP; 20–50 mM $MgCl_2$; RNase-free water. T7 RNAP and $MgCl_2$ were added last. The reaction was carried out at 37°C for 3–4 h. Longer incubation times tended to cause RNA degradation. A series of small-scale (10 μl) reactions were initially carried out to optimize the yield for product RNA based on the type of buffer as well as concentrations of rNTP, DNA template, T7 RNAP, and $MgCl_2$ (Supplementary Fig. S2). Following optimization, the reactions were scaled up to 100–500 μl depending on the yield of the target RNA sequence. Pyrophosphate magnesium precipitate ($PPi{\cdot}Mg^{2+}$) was pelleted by centrifugation at 13 000 (15 700 *g*) rpm for 7 min and the supernatant was injected onto a Q anion exchange column. Optionally, $PPi{\cdot}Mg^{2+}$ can be clarified by quenching the reaction with 50 mM EDTA, pH 8.0 and centrifuged before loading onto a Q column (Note: EDTA can associate with the column, potentially complicating the elution profile of the RNA).

Q column purification of IVT reaction

A 5 ml HiTrap Q HP column was cleaned with buffer B (20 mM Tris–HCl pH 7.8 at 4°C, 2 M NaCl) and equilibrated in buffer A (20 mM Tris–HCl pH 7.8 at 4°C). The IVT reaction was injected onto the Q column in buffer A and initially washed with 11% buffer B, followed by gradient elution from 11% B to 16% B over 20 column volumes and was finally washed with 100% B. Pooled fractions were subject to ethanol precipitation in anhydrous alcohol with 0.3 M NaOAc pH 5.2 at −20°C overnight. The RNA was pelleted by centrifugation, washed with 70% ethanol, air-dried, and resuspended in RNase-free water for subsequent applications.

Vaccinia capping enzyme purification

The plasmid for expressing vaccinia capping enzyme was a kind gift from Dr Remco Sprangers (Universität Regensburg). The capping enzyme expression and purification were followed according to Fuchs *et al.* [24].

RNA capping reaction and purification of capped RNA

10× capping buffer (500 mM Tris pH 8.0, 50 mM KCl, 10 mM $MgCl_2$) was prepared. The capping reaction was set up in 1× capping buffer, ∼0.2–0.5 mM S-adenosyl-methionine (New England Biolabs), and ∼0.5 mM GTP, 5′-PPP GUAUA, capping enzyme, 1 mM DTT, and RNase-free water. Small-scale 10 μl capping reactions were performed with varied enzyme-to-RNA ratios to optimize capping efficiency. A large-scale capping reaction was set up at the optimized capping enzyme-to-RNA molar ratio. The reaction was incubated at 37°C for 2 h. The reaction was

centrifuged at 13 000 rpm (15 700 *g*) for 7 min and the supernatant was injected into a Q column and purified as above.

Mass spectrometry

The mass of RNA was determined by Liquid chromatography–mass spectrometry (LC–MS) using a Dionex Ultimate 3000 coupled to a Bruker Maxis Impact QTOF in negative ESI mode. Around 10–20 µl of 20 µM sample was separated on an AgilentAdvanceBio C18 column (particle size: 2.7 µM; pore size: 120 Å; diameter × length: 2.1 × 50 mm^2), followed by a programmed run with a gradient of 98% mobile phase A [100 mM hexafluoroisopropanol (HFIP) and 5 mM triethylamine (TEA) in H_2O] and 2% mobile phase B (MeOH) to 40% mobile phase A and 60% mobile phase B in 10 min. The data were processed using the Bruker DataAnalysis software v4.2.

Crystallization of IFIT1–RNA complex

The expression and purification of IFIT1 protein (L457E/L464E mutant) were carried out as previously described [25]. The protein–RNA complex was mixed at a molar ratio of 1.5:1 and incubated on ice for ∼30 min after which hanging crystallization drops were set up using the following conditions: 23–27% PEG 200, 100 mM Tris pH 8.1, 200 mM $CaCl_2$, ∼5 mg/ml protein complex at 4°C. Diffraction data were collected on a Bruker D8 Venture X-ray source. The data were processed using Bruker Proteum3 software and the structure was solved by difference Fourier calculation using the published structure (PDB 5udi) [24]. The structure was built and refined with Coot [26] and Refmac [27], respectively (Supplementary Table S2).

Results

Separation of *in vitro* transcribed 5′-PPP RNAs by anion-exchange chromatography

Our goal was to generate highly pure and homogeneous short 5′-PPP-RNAs (<15 nt in length) for the purpose of co-crystallization with IFIT proteins to understand their mechanism of binding RNA. These RNAs were generated using a standard protocol of IVT with T7 RNAP [28]. Initially, we performed a small-scale 10 µl IVT reaction of a 5 nt RNA (5′-PPP-GUAUA). Gel analysis of the products revealed the presence of abortive transcripts that differed in size by only one nucleotide at the 3′-end from the target product (Supplementary Fig. S2). Unfortunately, this mixture could not be separated using gel purification due to its inability to completely resolve bands.

We therefore wondered whether the mixture could instead be separated into homogeneous species by anion-exchange chromatography. For this purpose, we ran a large-scale 500 µl IVT reaction, which was then passed over a standard anion exchange 5 ml HiTrap Q HP column (GE Healthcare). A shallow gradient from 220 to 320 mM NaCl was used over 20 column volumes to elute the sample. We observed on the resulting chromatogram a series of peaks during the wash and elution from ∼50 to ∼115 ml retention volume. The initial three peaks from ∼50 to ∼75 ml were likely either unincorporated ribonucleoside triphosphates (rNTPs) or 2 to 3 nt abortive transcripts (Fig. 1A). The peaks from ∼75 to ∼115 ml, corresponding to larger RNA products, were divided into three pools (Fig. 1B), and run on a high percentage denaturing PAGE gel (20% polyacrylamide, 7 M urea) to ascertain their composition. Pools 1 and 2 corresponded to 4 nt abortive

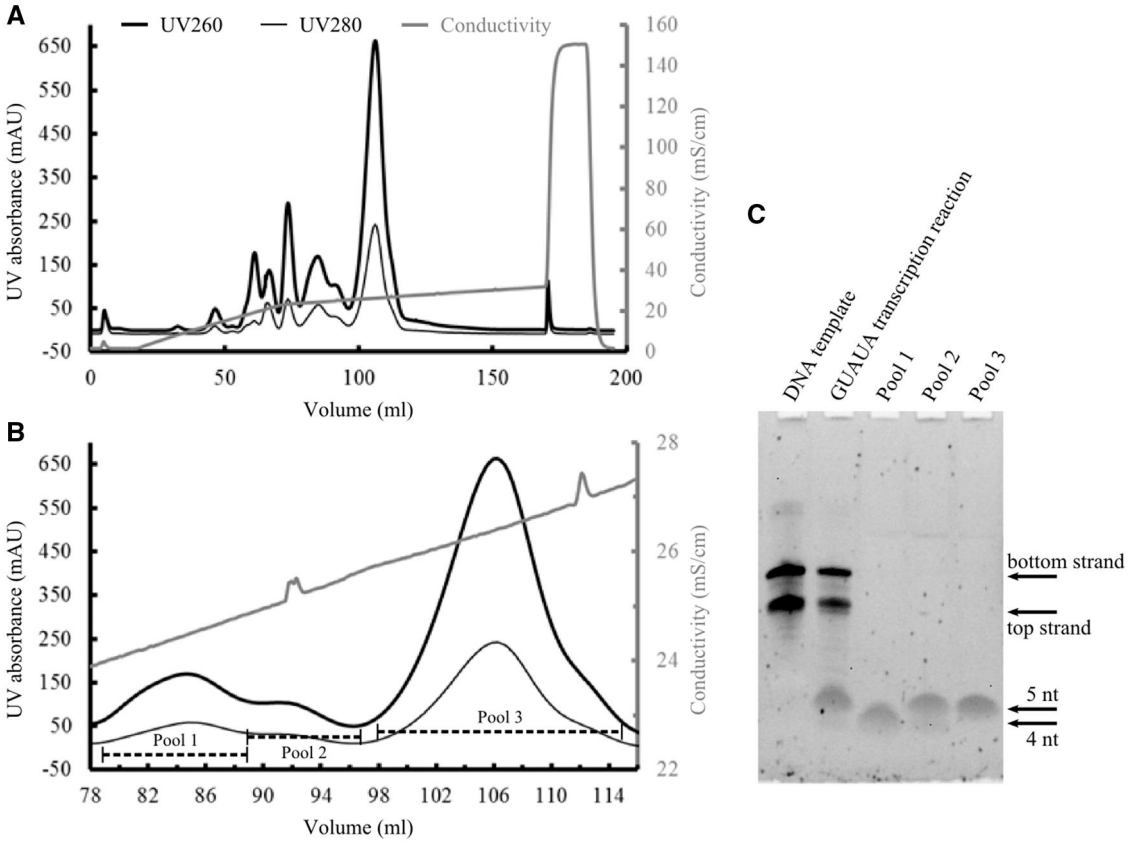

Figure 1. Purification of 5′-PPP GUAUA IVT reaction. (A) Q column chromatogram. (B) Enlarged view of chromatogram peaks containing RNA products. (C) Gel analysis of chromatogram peaks, 7 M urea, 20% denaturing PAGE at 200 V for 80 min, SyBr Gold staining. The top and bottom strand of DNA template are indicated along with RNA products.

transcripts and 4/5 nt transcripts, respectively, whereas Pool 3, the main peak, corresponded to the desired 5 nt-only transcripts (Fig. 1C). The peak of the desired product was completely resolved from the others in the chromatogram. Mass spectrometry of Pool 3 confirmed it as mainly containing 5′-PPP GUAUA, as well as very minor fractions of 5′-diphosphate- and monophosphate-terminated species (Supplementary Fig. S3).

With the successful purification of a 5 nt transcript, we wondered whether the technique could be extended to a longer 10 nt transcript, where we might expect it to become more difficult to discern single-nucleotide differences from run-off transcripts. The above experiment was repeated, this time loading a 200 µl IVT reaction of a 10 nt RNA (5′-PPP GGUAGAAUAU) onto the Q column. Once again, we observed a similar distribution of peaks from the elution profile, but this time only two peaks contained larger RNA products (Pool 1 and Pool 2) (Fig. 2A and B). Interestingly, gel analysis revealed that the desired 10 nt-product was found in Pool 1 and a run-off transcript containing a longer product was the main peak (Fig. 2C). Mass spectrometry analysis indicated that the larger product is an 11 nt run-off transcript with an extra U nucleotide added at the 3′-end (Supplementary Fig. S4).

Separation of capped RNA from the uncapped

In addition to the utility of short 5′-PPP RNAs for structural studies with IFITs, we were also interested in studying capped versions of these RNAs since m7GTP is a modification found on host mRNAs and some members of the IFIT family can accommodate viral capped RNAs [11, 25, 29]. RNA capping typically involves incubation of the purified 5′-PPP RNA from the IVT reaction with the Vaccinia capping enzyme, GTP and S-adenosyl methionine (SAM). Depending on the purity of the capping enzyme, the capping efficiency may not be optimal and separation of the final capped RNA product from uncapped RNA is generally required. As above, gel purification is inadequate to separate the RNAs due to overlap of bands. Previously, His-tagged eIF4E (cap-binding protein) coated Ni-NTA beads have been used to isolate capped RNA from uncapped transcripts [24]. Using eIF4E is perhaps the only effective technique for purifying long-capped RNAs.

However, for short-capped RNAs, given the above results on short 5′-PPP RNAs, we wondered whether a simple Q column could replace the otherwise more complex method of using eIF4E-based purification. We prepared a 1 ml capping reaction using ~0.7 mg of the 5′-PPP GUAUA purified from Pool 3 (shown in Fig. 1) and the His_6-tagged Vaccinia capping enzyme [24] incubated at a molar ratio of 1 to 20 (enzyme-to-RNA). After incubation, the reaction was centrifuged and injected into an equilibrated Q column. We observed two main peaks present at ~80–90 ml (Pool 1) and ~98–110 ml (Pool 2) on the chromatogram (Fig. 3A and B). Gel analysis and mass spectrometry indicated Pool 1 consisted of the capped 5 nt RNA and Pool 2 was composed of the uncapped RNA (Fig. 3C and Supplementary Fig. S5). Therefore, a Q column is capable of separating capped from uncapped RNA, at least for short RNAs.

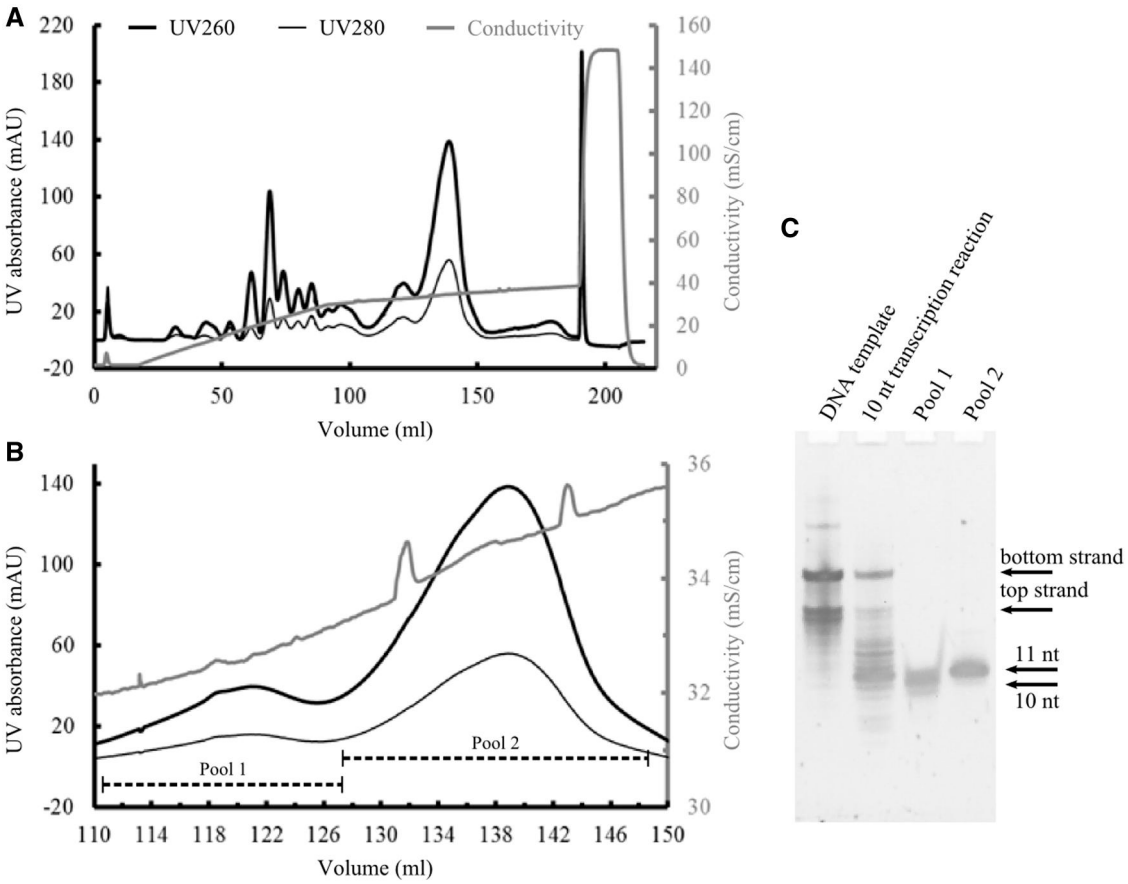

Figure 2. Purification of 5′-PPP GGUAGAAUAU IVT reaction. (A) Q column chromatogram. (B) Enlarged view of chromatogram peaks containing RNA products. (C) Gel analysis of chromatogram peaks, 7 M urea, 20% denaturing PAGE at 200 V for 80 min, SyBr Gold staining. The top and bottom strand of the DNA template are indicated, along with RNA products.

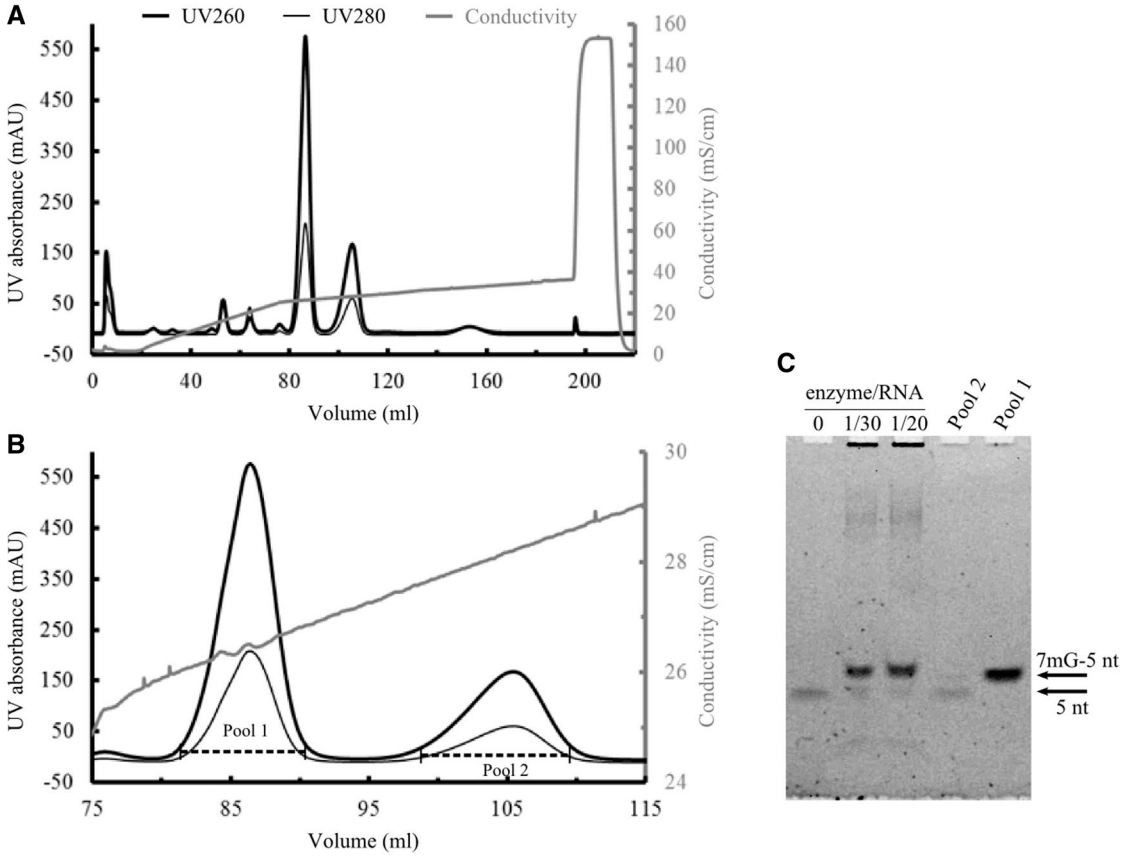

Figure 3. Purification of 5′-PPP GUAUA capping reaction using the (His)6-tagged capping enzyme. (A) Q column chromatogram. (B) Enlarged view of chromatogram peaks containing RNA products. (C) Gel analysis of chromatogram peaks, 7 M urea, 20% denaturing PAGE at 200 V for 50 min, SyBr Gold staining.

Crystallization of purified capped RNA with a protein complex

To confirm the quality of the capped RNA produced above, we crystallized and solved its structure bound to human IFIT1 protein, for which a crystal structure was previously determined [25]. The purified capped RNA was mixed with IFIT1 in a ratio of 1.5:1 (RNA: protein). Crystallization using the published conditions resulted in a crystal that diffracted to ∼2 Å resolution. A difference Fourier (Fo–Fc) map without inclusion of an RNA model revealed clear difference electron density for the m7GTP moiety and the first 4 nt of the 5 nt RNA, consistent with its sequence (Fig. 4). The presence of the fifth nucleotide was confirmed by mass spectrometry (Supplementary Fig. S5) but was not visible in the density, presumably due to flexibility, as reported in the initial structural study [25].

Discussion

The requirement of homogeneous RNA samples for structural studies has led to the development of a variety of new methodological advancements. While chemical synthesis can satisfy the RNA needs for many structural endeavors, when very long RNAs or 5′-PPP RNAs are required, *in vitro* transcription using bacteriophage T7 RNAP is currently indispensable. However, T7 RNAP has long been known to introduce heterogeneous transcripts at either the 5′-end near its initializing domain, or the 3′-end near the end of the template [30]. For example, early termination of transcription generates abortive transcripts, whereas sliding the polymerase off the DNA template generates run-off transcripts.

Various strategies have therefore been introduced to address the heterogeneity issue from the perspectives of sequence design and purification. Self-cleaving ribozymes, such as a 5′-hammerhead or 3′-hepatitis delta virus ribozymes, can be integrated into the termini of desired RNA sequence to flank the target transcript [31]. This approach is, however, not always ideal, since ribozyme cleavage can be incomplete, and the length of target transcript must be quite different from the ribozyme to achieve adequate separation in gel-extraction purification. Moreover, for short RNAs, it may become uneconomical to use this approach since most of the rNTP substrate will be consumed synthesizing the ribozyme, a factor that is especially relevant when using costly isotope-labeled rNTPs to synthesize short RNAs for NMR experiments. While the traditional gel purification method for RNAs is still the workhorse in these applications, it also has issues such as the inability to resolve heterogeneous transcripts that are close in length, even using very high percentage gels. Additionally, the neurotoxicity of acrylamide, the usage of UV radiation, and phenol/chloroform make the traditional approach less user- and environmentally friendly.

Our method of using standard Q column purification is well-suited both in terms of ease of use and minimizing the costs and hazards for applications that require pure and homogeneous 5′-PPP or capped RNAs. We found that single-nucleotide resolution purification at the 3′-end could be achieved with this approach where the target transcript was resolved from abortive/run-off products in separate chromatogram peaks. An important

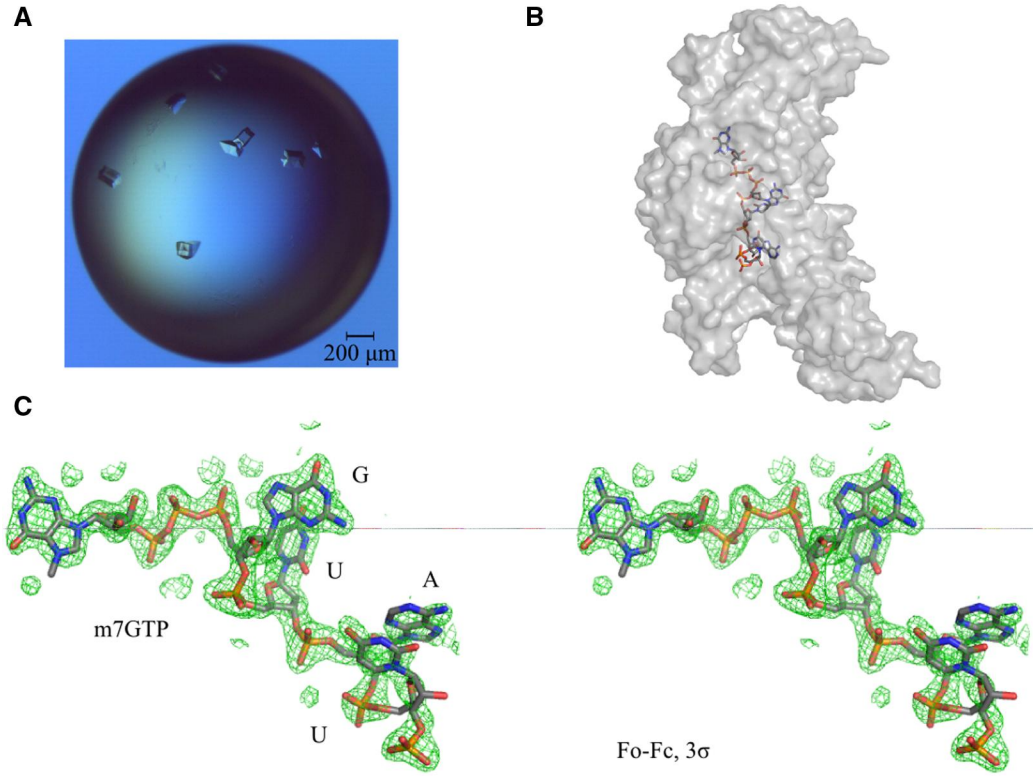

Figure 4. Crystal structure of m7GTP-GUAUA with IFIT1. (A) Crystals of IFIT1–RNA complex. (B) Overall structure of IFIT1 bound to the RNA. The protein is shown as a gray surface and the RNA is shown as sticks. (C) Stereo view of the Fo–Fc difference map before inclusion of RNA in the model, contoured at 3σ. Overlayed is the refined RNA model.

aspect of this approach is fine tuning the slope of the elution gradient. If the gradient is too steep, it will prevent resolution of the peaks leading to a mixture of species. On the other hand, too shallow of a gradient will result in much broader chromatogram peaks and dilution of the sample.

The main limitation of this method is its restriction of RNA length to probably at most ~15 nt. The likely reason for this is that, as the RNA length increases, the relative-charge difference between single-nucleotide variants decreases below the resolving power of the column. While adjusting the elution gradient, using a finer bead Q column (e.g. mono Q), or using a lower volume Q column may help overcome this, beyond a length of 15 nt, one will most likely have to resort to using gel purification in combination with a self-cleaving ribozyme to achieve 3′-end homogeneity. In summary, we describe here a simple and effective method for the purification of short 5′-PPP or capped RNAs, suitable for structural and other biochemical applications that require highly pure and homogeneous RNA.

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Author contributions

Zixian Li (Conceptualization [lead], Formal analysis [lead], Funding acquisition [supporting], Investigation [lead], Validation [lead], Writing—original draft [lead], Writing—review & editing [supporting]), Mia Bilic (Investigation [supporting]), and Bhushan Nagar (Funding acquisition [lead], Supervision [lead], Writing—review & editing [lead])

Supplementary data

Supplementary data is available at *Biology Methods and Protocols* online.

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Data availability

The data underlying this article are available in the article and in its online supplementary material.

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Innovations