Published in final edited form as:

Methods. 2018 July 01; 143: 121–133. doi:10.1016/j.ymeth.2018.02.025.

Methods for Construction and Characterization of Simple or Special Multifunctional RNA Nanoparticles Based on the 3WJ of Phi29 DNA Packaging Motor

Sijin Guo^{1,2}, Xijun Piao^{1,2}, Hui Li^{1,2}, and Peixuan Guo^{1,2,3,4,*}

¹Center for RNA Nanobiotechnology and Nanomedicine, The Ohio State University, Columbus, OH 43210, USA

²College of Pharmacy, Division of Pharmaceutics and Pharmaceutical Chemistry, The Ohio State University, Columbus, OH 43210, USA

³College of Medicine, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH 43210, USA

⁴James Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA

Abstract

The field of RNA nanotechnology has developed rapidly over the last decade, as more elaborate RNA nanoarchitectures and therapeutic RNA nanoparticles have been constructed, and their applications have been extensively explored. Now it is time to offer different levels of RNA construction methods for both the beginners and the experienced researchers or enterprisers. The first and second parts of this article will provide instructions on basic and simple methods for the assembly and characterization of RNA nanoparticles, mainly based on the pRNA three-way junction (pRNA-3WJ) of phi29 DNA packaging motor. The third part of this article will focus on specific methods for the construction of more sophisticated multivalent RNA nanoparticles for therapeutic applications. In these parts, some simple protocols are provided to facilitate the initiation of the RNA nanoparticle construction in labs new to the field of RNA nanotechnology. This article is intended to serve as a general reference aimed at both apprentices and senior scientists for their future design, construction and characterization of RNA nanoparticles based on the pRNA-3WJ of phi29 DNA packaging motor.

1. Introduction

Nanotechnology involves the study, engineering, and application of nanoscale materials across a wide range of scientific fields that include chemistry, biology, biophysics, materials sciences, and biomedical sciences. With defined structures and features, biomolecules such

*Correspondence should be addressed to: Peixuan Guo, PhD, guo.1091@osu.edu, Sylvan G. Frank Endowed Chair in Pharmaceutics and Drug Delivery System, The Ohio State University, 912 Biomedical Research Tower (BRT), 460 W 12th Ave., Columbus, OH 43210, USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

as proteins, peptides, lipids, DNA, and RNA can serve as natural building blocks for constructing nanomaterials from the bottom-up [1]. Among these molecules, RNA is an attractive candidate, since it can be manipulated at a similar level of simplicity as DNA, while possessing the versatility of different structures, functions, and biological activities like proteins [2]. RNA nanotechnology is conceptualized as the bottom-up self-assembly of nanometer scale RNA architectures, with their major frame composed of RNA [2,3]. The scaffolds, ligands, therapeutics, and regulators of RNA nanoparticles can be made up of only RNA or a hybrid of RNA with other chemicals. The field of RNA nanotechnology can be traced back to the first construction of RNA nanoparticles from the re-engineered phi29 packaging RNA (pRNA) molecules in 1998 [4]. The pRNA molecules were originally derived from bacteriophage phi29 DNA packaging motor [5,6]. They contain two interlocking loops that enable the formation of dimers, trimers, hexamers, and patterned superstructures via intermolecular interaction [7,8]. Unlike conventional RNA biology, which mainly studies RNA structures and functions, RNA nanotechnology is a unique field that focuses on the construction and application of RNA nanomaterials.

Two major approaches have been used for the construction of RNA nanoparticles. The first approach is the sequence-dependent self-folding of RNA nanoarchitectures, based on computational algorithms and prediction of secondary or tertiary structures [9,10]. The second approach employs the naturally-occurring RNA motifs as core building blocks, such as three-way junction, four-way junction, and kissing loops, to assemble RNA nanoparticles [11–22]. Particularly, the pRNA three-way junction (pRNA-3WJ) has been extensively used as a central core for the construction of multifunctional RNA nanoparticles [12,16,23–28]. The branched feature of the 3WJ motif allows different functional modules to be conveniently incorporated to the three helical regions, making it an ideal scaffold for targeted drug delivery. This multivalent property suggests that pRNA-3WJ nanoparticles have enormous potential for therapeutic, imaging and diagnostic applications.

Over the past decade, the hurdle of enzymatic instability of native RNA has been overcome by various chemical modifications such as 2'-fluoro (2'-F) [29–34], and the concern of thermodynamic instability has been addressed by the utilization of naturally-occurring highly stable RNA motifs such as pRNA-3WJ [12]. As a result, the field of RNA nanotechnology has seen significant advances in biomedical applications, as evidenced by a substantial increase of publications on RNA nanostructures. Compared to many other well-developed nano-delivery systems, such as liposomes and polymers, RNA nanotechnology is relatively new, with unique features and various advantages [2]. For example, the fundamental rule for RNA nanoparticle self-assembly relies on base-pairing, while many other synthetic nanoparticles are formed by covalent polymerization and hydrophobic interactions [35,36]. In addition, RNA nanoparticles are assembled spontaneously using highly programmable and predictable building blocks in a predefined manner that is very different from traditional routes. The construction of functional RNA nanoparticles integrates the knowledge of RNA chemistry, RNA biology, and computational approaches. Although the history and progress of RNA nanotechnology have been well-reviewed [2,3,11,37,38], only few literatures summarized the methods for constructing and characterizing RNA nanoparticles [39,40]. Here, by using pRNA-3WJ platform as a model, we seek to summarize the most general methods for constructing and characterizing both

simple, and relatively sophisticated multifunctional RNA nanoparticles for therapeutic, imaging, and diagnostic applications. For other types of RNA nanoparticles using different nanoscaffold designs, reader can refer to a previous protocol [40]. The goal of this article is to foster the interdisciplinary research, and to encourage greater participation of new investigators into the field of RNA nanotechnology.

2. Methods for *in vitro* synthesis of RNA

RNA nanoparticles are typically constructed from several single-stranded RNA (ssRNA) fragments shorter than 100-nucleotides (nt). These RNA strands can be synthesized either enzymatically or chemically, depending on the length of the RNA strands and the types of chemical modifications or labeling. This section mainly introduces the most common methods for the *in vitro* synthesis of RNA.

2.1 Production and purification of DNA templates

The common enzymatic approaches of *in vitro* RNA synthesis include SP6, T7, and T3 RNA transcription that require highly purified DNA templates with distinct promoter sequences. This article will mainly focus on the T7 RNA transcription. Prior to transcription, DNA template containing the T7 promoter (5'-TAATACGACTCACTATA-3') at the 5'-end can be obtained via standard polymerase chain reaction (PCR) using a pair of DNA primers, or directly annealed from two complementary oligonucleotides. The first two nucleotides after the T7 promoter should be GG, as these are preferred by T7 RNA polymerase. To ensure the high quality of RNA product, the DNA template must be of high purity. Therefore, the DNA template should be carefully purified to remove free nucleotides, salts, and any byproducts by commercially available gel extraction kits or spin columns (e.g. QIAEX II Gel Extraction Kit; Ambion NucAway spin column), or by electro-separation system. Particularly, the electro-separation system consists of an electrophoresis chamber and sample traps, driving the DNA samples to migrate out of the gel slices and retain in membrane traps under the driving force of electric field (Fig. 1).

- i. Firstly, run DNA template products in 2% (wt/vol) synergel-agarose gel in 1×TAE buffer (40 mM Tris-acetate and 1 mM EDTA) at 120 V for 40 min at room temperature (RT).
- ii. Place the gel on a TLC plate, excise the gel slices containing DNA bands of interest under UV light (254 nm) on a TLC (Thin Layer Chromatography) plate.
- iii. Place the BT1 membranes (a dense matrix which buffer ions and molecules less than 3–5 kD can pass through; available from GE Whatman) at points A and C to form an electrophoresis chamber, and a BT2 membrane (a microporous prefilter that prevents gel pieces or other particulates from passing through) at point B to form a trap.
- iv. Place the gel slices in the chamber. Add 1×TAE running buffer, and apply 100 V for 1 hour (h).
- v. The elution can be monitored by using UV light (254 nm) to visualize the DNA samples. Purified DNA samples in the trap are collected, and 2.5 volumes of

100% ethanol and one-tenth volume of 3 M sodium acetate are added to precipitate the DNA samples overnight at -20°C .

- vi. Centrifuge the samples solution at $16,500\times g$ for 30 min at 4°C and discard the supernatant. Wash the pellet with 70% (vol/vol) ethanol and dry the pellet for 10 min with a vacuum concentrator.
- vii. Dissolve the pellet in $1\times\text{TE}$ buffer (10 mM Tris-HCl and 1 mM EDTA) and store at -20°C until use. The suggested DNA template concentration is $0.5\text{ }\mu\text{g}/\mu\text{L}$.

2.2 In vitro RNA transcription with T7 RNA polymerase

In vitro T7 transcription is a standard method of synthesizing RNA molecules longer than 20-nt using a purified T7 RNA polymerase. This method can produce up to several milligrams of RNA with high quality. The purified DNA templates can be used in the commercially available or homemade T7 transcription kit to synthesize RNA [39,41–43]. A minimum of $0.5\text{ }\mu\text{g}$ of DNA template is required per $50\text{ }\mu\text{L}$ transcription. A typical T7 RNA transcription setup can be found in Table 1, and a specific procedure is described below.

- i. Mix the samples well in a 1.5 ml RNase-free Eppendorf tube. The transcription reaction can be scaled up or down maintaining the same reagent concentration ratios.
- ii. Incubate the reactions at 37°C for approximately 4 h.
- iii. Terminate the reaction by adding $0.5\text{ }\mu\text{L}$ RNase-free DNase I (1 mg/ml, Fermentas) and incubate at 37°C for another 15 min.
- iv. The transcription products are then prepared for purification by adding equal volume of $2\times$ denaturing loading dye. Run the samples on an 8% (wt/vol) polyacrylamide gel with 8 M urea in $1\times\text{TBE}$ buffer (89 mM Tris base, 200 mM boric acid and 2 mM EDTA) at 100 V at RT for 1–1.5 h.
- v. Place the gel on a TLC plate, excise the bands of interest under UV light (254 nm), and cut the gel slices to small pieces.
- vi. Elute the RNA from gel slices in $400\text{ }\mu\text{L}$ RNA elution buffer (0.5 M ammonium acetate, 10 mM EDTA and 0.1% (wt/vol) SDS in 0.05% (vol/vol) diethyl pyrocarbonate (DEPC)-treated water) at 37°C for 2 h. Collect the supernatant and add another $400\text{ }\mu\text{L}$ RNA elution buffer for a second elution for 1 h. Alternatively, the elution step can be replaced by the electro-separation system, as described in section 2.1 (Fig. 1).
- vii. Add 2.5 volumes of 100% ethanol and a one-tenth volume of 3 M sodium acetate to the supernatant, and precipitate RNA samples overnight at -20°C .
- viii. Centrifuge the samples solution at $16,500\times g$ for 30 min at 4°C and discard the supernatant. Wash the pellet with 70% (vol/vol) ethanol and dry the pellet for 10 min with a vacuum concentrator.
- ix. Dissolve the pellet in 0.05% (vol/vol) DEPC-treated water and store at -20°C until use.

2.3 In vitro 2'-F RNA transcription with Y639F mutant T7 RNA polymerase

2'-F modified RNA can be synthesized by *in vitro* transcription using 2'-F CTP, 2'-F UTP and Y639F mutant T7 RNA polymerase [44]. Compared to the normal transcription in section 2.2, the *in vitro* 2'-F RNA transcription requires a higher input of purified DNA template, an optimized 2'-F transcription buffer, and an extended reaction time (6~12 hours) to ensure sufficient yield (Table 2), because the Y639F mutant T7 RNA polymerase has a lower efficiency of incorporating ribonucleotides. It is generally recommended that over 2.5 µg of purified DNA templates are used for a 50 µl transcription reaction. After treatment with RNase-free DNase I, the 2'-F RNA transcripts must be purified by polyacrylamide gel electrophoresis (PAGE) or electro-separation system using the same procedure described in section 2.2.

2.4 Solid-phase chemical synthesis of short RNA by phosphoramidite chemistry

Solid-phase synthesis via standard phosphoramidite chemistry is a universal method for large scale production of short RNA oligonucleotides (<80-nt). The phosphoramidites are commercially available nucleoside derivatives with protecting groups. Notably, this technique allows the incorporation of various chemical modifications or labels to RNA oligonucleotides for different applications [45]. Opposite to enzymatic RNA synthesis, the solid-phase chemical synthesis proceeds in the 3' → 5' direction. It begins with the first phosphoramidite linked to controlled pore glass (CPG) beads as the 3'-end, followed by a series of successive reactions (deprotection, coupling, oxidation, and capping) to elongate the RNA strand by adding phosphoramidites based on the input sequence [46]. These reactions will continuously run in cycles until the last 5'-end nucleotide is attached. Next, the protecting groups at each base are cleaved by treatment with ammonia, and the synthesized RNA strand will be cleaved from the CPG simultaneously. The 2'-protecting group will then be removed by treatment with triethylamine trihydrofluoride (TEA.3HF) to yield a final RNA product. During synthesis, chemically-modified phosphoramidites such as 2'-F modified nucleotides or locked nucleic acid (LNA) can be incorporated into the RNA strand, and functional groups such as amine (-NH₂), alkyne (-C≡CH), thiol (-SH), biotin, or fluorophores can be incorporated at the 5'-, 3'-end, or other specific site within the sequence. After synthesis, the RNA products should be purified by a desalting column to remove free nucleotides and salts. High-performance liquid chromatography (HPLC) or denaturing PAGE is recommended to yield RNA products with higher purity.

3. Fundamental methods for the construction and characterization of simple pRNA-3WJ nanoparticles

3.1 Construction of pRNA-3WJ with high efficiency by bottom-up self-assembly

The pRNA-3WJ motif was derived from the central domain of the natural pRNA of bacteriophage phi29 (Fig. 2A) [12,47]. It can be constructed from three short ssRNA fragments (3WJ-a, 3WJ-b, and 3WJ-c) with unusually high efficiency by a bottom-up self-assembly approach (Fig. 2B). Briefly, these three fragments are mixed together at equal molar concentration in TMS buffer (50 mM Tris pH 8.0, 100 mM NaCl, 10 mM MgCl₂), followed by heating to 85 °C for 5 minutes and slowly cooled over 40 minutes to 4 °C on a

Thermal Cycler. It has been reported that the three separate strands co-assemble into pRNA-3WJ via a two-step mechanism, $3WJ\text{-b} + 3WJ\text{-c} \leftrightarrow 3WJ\text{-bc} + 3WJ\text{-a} \leftrightarrow 3WJ\text{-abc}$ [48]. The first step between 3WJ-b and 3WJ-c is highly dynamic since these two fragments only contain 8 complementary base pairs. Upon the second association with 3WJ-a, which contains 17 complementary base pairs to the 3WJ-bc dimer complex, the unstable dimer was locked into a highly stable 3WJ. Generally, this assembly process is highly efficient even in the absence of Mg^{2+} , as long as the three RNA fragments are mixed at equal molar ratio.

3.2 Purification and gel assay of pRNA-3WJ nanoparticles

The assembled pRNA-3WJ nanoparticles are generally purified on 12% native PAGE at 120V for 2 h in TBM running buffer (89 mM Tris, 200 mM borate acid, and 5 mM $MgCl_2$) at 4 °C. The major band of pRNA-3WJ samples is then excised from the gel under UV light (254 nm), and two elution approaches (see section 2.2) can be applied to recover the pRNA-3WJ samples. However, the RNA elution buffer should be supplied with 10 mM Mg^{2+} , if the assembly of RNA nanoparticles requires Mg^{2+} . After precipitation, the pellet should be dried and dissolved in 0.05% DEPC-treated water or TMS buffer. The purity of pRNA-3WJ can be confirmed by performing a step-wise assembly of pRNA-3WJ monomers (3WJ-a, 3WJ-b, or 3WJ-c), dimers (3WJ-a + 3WJ-b, 3WJ-b + 3WJ-c, or 3WJ-a + 3WJ-c), and pRNA-3WJ trimer (3WJ-a + 3WJ-b + 3WJ-c) on a native PAGE, while the unpurified pRNA-3WJ nanoparticle can be used as control for comparison. Approximate 200 ng RNA nanoparticles in gel can be greatly stained by ethidium bromide (EtBr), and visualized on a common gel imaging system.

3.3 Evaluation of the thermodynamic stability of the resulting RNA nanoparticles

The pRNA-3WJ scaffold was demonstrated to be highly thermodynamically stable, as evidenced by its high melting temperatures (T_m), resistance to denaturation at high urea concentration, and its ability to remain intact at ultra-low concentrations (picomolar) [12]. The thermodynamic parameters of pRNA-3WJs composed of different types of oligonucleotides (DNA, RNA, and 2'-F RNA) have been studied previously [49]. It was found that the formation of $3WJ_{RNA}$ displays the least favorable enthalpy change (ΔH) but the most favorable entropy change (ΔS), as compared to $3WJ_{DNA}$ and $3WJ_{2'-F}$. The Gibbs free energy (ΔG) of $3WJ_{RNA}$ formation, however, was higher than $3WJ_{DNA}$ but lower than $3WJ_{2'-F}$. This comparison indicated a mechanism of entropy-driven assembly of pRNA-3WJ. The thermodynamic parameters for pRNA-3WJs were typically obtained from a Van't Hoff curve by plotting the T_m versus the concentrations of the assembled pRNA-3WJs. There are two independent methods for the accurate measurements of T_m values of pRNA-3WJ, namely, temperature-gradient gel electrophoresis (TGGE) and quantitative real-time PCR (rtPCR).

3.3.1 Temperature-Gradient Gel Electrophoresis (TGGE) for T_m measurement

—TGGE (Biometra GmbH) applies a linearly increasing temperature gradient perpendicular to the electrical current on a polyacrylamide gel. As a result, each separate lane of RNA sample will be subjected to heating at a gradually-increased temperature across the gel, driving the nanoparticles to undergo a transition from the assembled to denatured conformation [50] (Fig. 2C). Sample in each lane is then quantitatively analyzed to generate

a melting curve, and the T_m is defined as the specific temperature when 50% of the RNA samples are dissociated [49]. Normally, the increasing temperature gradient will result in the swelling of gel pores, leading to the gradual increasing migration rate of the RNA nanoparticles. The dissociation of RNA nanoparticles can be visualized by a relatively sharp transition of the band migration. A specific procedure is described below.

- i. Load approximate 200 ng assembled pRNA-3WJ samples in each well of a 12% native PAGE, and run under 100 V in TBM buffer for 10 minutes to allow the samples to enter the gel.
- ii. Disassemble gel sandwich, clean and dry the backside of the gel support film.
- iii. Apply 150 μ L of thermal coupling solution (0.01% Triton) on the thermal block to clean the surface, and place the gel on the thermal block after drying the surface. Avoid leaving any bubbles trapped under the gel support film.
- iv. Cover the gel with a cover film. Attach pre-soaked buffer wicks on the top and bottom of the gel.
- v. Attach gel cover plate, and set up the temperature gradient program. Run the gel at 100 V for 1 h.

TGGE possesses some advantages over other common methods for T_m measurements, such as UV melting. It's especially useful for nucleic acid samples composed of multiple oligonucleotide strands, as each strand can be conveniently detected with different labeled fluorophores. Consequently, the dissociation of each strand from the complex can be monitored simultaneously. Another notable advantage of TGGE is that it's suitable for a wide range of concentrations of nucleic acid samples. By applying radiolabeling, fluorophores, and Ethidium Bromide (EtBr) staining, TGGE can detect the process of melting from low nanomolar to micromolar concentrations [49–51].

3.3.2 Real-time PCR for T_m measurement—Real-time PCR provides another technique for T_m measurement of RNA nanoparticles (Fig. 2D). It is especially favorable for high-throughput T_m screening of nucleic acid samples. Up to 96 samples can be measured simultaneously within 1 h. A specific procedure is described below.

- i. 18 μ L of purified pRNA-3WJ samples are mixed well with 2 μ L 10 $\times$ SYBR Green II. Generally, the final concentration of pRNA-3WJ can be 1–10 μ M.
- ii. Load the samples into an optical 96-well plate and seal the plate with a sealing film.
- iii. Samples are heated at 95 $^{\circ}$ C for 5 minutes in the Roche LightCycler 480 real-time PCR system, followed by slowly cooling at a rate of 0.11 $^{\circ}$ C/s to 20 $^{\circ}$ C for several cycles. The heating process generates an initial denaturing period for the nanoparticles, while the cooling process generates an assembling period.
- iv. The melting curves are obtained by real-time monitoring the fluorescence levels of SYBR Green II. The T_m is determined as the specific temperature when 50% of the fluorescence intensity is observed.

It has been well-confirmed that the two methods discussed above have a high degree of consistency for T_m measurements of 3WJ_{DNA}, 3WJ_{RNA}, and 3WJ_{2'-F} [49]. However, the real-time PCR method relies heavily on dye intercalation into base pairs, and may not be suitable for other highly chemically-modified oligonucleotide samples due to the decreased intercalation rate and efficiency. It's always recommended to use more than one method to confirm the T_m measurements.

3.4 Enhanced enzymatic stability of pRNA-3WJ by chemical modifications

Natural RNA molecules are susceptible to RNase-mediated degradation, extremely limiting their usefulness for *in vivo* applications. Over the past years, many chemical modification studies aiming to improve the enzymatic stability and the *in vivo* properties of RNA have been reported [29–34,52]. The common modifications include the modification of phosphodiester backbone, substitution of 2'-OH group, base modification, and ribose modification [38,52–54]. Particularly, 2'-F modification has been widely used to construct RNase-resistant pRNA-3WJ nanoparticles [12,16,23–28,54]. This modification typically replaces the 2'-OH group of cytidines (C) and uridines (U) with a 2'-fluoro, without affecting the authentic folding and assembly of the pRNA-3WJ [12]. The enhanced enzymatic stability of 2'-F RNA nanoparticles can be greatly evaluated by a serum stability assay [12,51,55], where 2'-F RNA nanoparticles are incubated with 10–50% fetal bovine serum at 37°C to mimic an *in vivo* environment for different time points (Fig. 2E). The resulting nanoparticles are run in native PAGE, and the percentage of intact nanoparticles can be quantitatively analyzed to generate a degradation curve. It was found that 2'-F modification further enhanced the thermodynamic stability of RNA nanoparticles [49,51]. Another advantage of 2'-F RNA is that it can be readily created by either *in vitro* transcription or solid-phase chemical synthesis (see section 2). The *in vitro* 2'-F transcription enables the production of long strands of 2'-F RNA. In addition, LNA modification on C and U can dramatically improve the half-life of RNA molecules *in vivo* [51]. The recent progress of chemical modifications has considerably facilitated the *in vivo* applications of RNA nanotechnology.

3.5 High mechanical stability of pRNA-3WJ due to Mg clamps

The pRNA-3WJ structure has been recently reported to be a novel mechanically anisotropic structure and shows potential applications in biomaterials. Single-molecule atomic force microscopy (AFM) can be used to investigate the mechanical property of pRNA-3WJ [47,56,57]. To study this mechanical stability, pRNA-3WJ was modified with amine groups at 5'- and 3'-end of different helical arms, and linked to the cantilever tip and substrate via two bi-functionalized PEG linkers [N-hydroxyl succinimide (NHS)–PEG–NHS], respectively. AFM pulling experiment was performed by retracting the cantilever tip at a constant velocity that gradually unfolded the pRNA-3WJ structure at a defined pulling direction. In the same way, amine groups can be modified at other helical arms to establish different pulling directions. The force spectroscopy curves were recorded to reveal the mechanical property of pRNA-3WJ. It was found that pRNA-3WJ structure displays strong resistance to stretching along its coaxial helices. In the presence of Mg²⁺, pRNA-3WJ could resist the rupture force of about 219 pN along the H1–H3 coaxial direction. This robust resistance is comparable to some mechanically strong elastomeric proteins such as titin

immunoglobulin domain. In the absence of Mg^{2+} , pRNA-3WJ exhibited weaker resistance to rupture forces along all directions, demonstrating a mechanism of Mg^{2+} -dependent mechanical property. This Mg^{2+} -dependent mechanical anisotropy is attributed to two Mg clamps between the phosphates of G23 and A90 as well as C24 and A89 (Fig. 2A). These two Mg clamps resist coaxial stretching in a cooperative manner and contribute to the extraordinary mechanical stability of pRNA-3WJ, making it a robust scaffold for constructing more sophisticated RNA nanoparticles.

3.6 Evaluation of size and shape of pRNA-3WJ nanoparticles

Size and shape are important parameters to evaluate the structural property of RNA nanoparticles. AFM has been developed as a powerful tool for the structural characterization of nanoparticles. The high resolution and compatible sample examination conditions make it suitable for RNA nanostructures [12,17,58–60]. To clearly visualize the branched structure of the pRNA-3WJ, three helical arms were extended with a 60 or 58 base pairs RNA helix [23,24]. After purification by PAGE, the size and shape of the extended pRNA-3WJ can be observed by AFM (Fig. 2F). While AFM is more favorable for analyzing the surface morphology of RNA nanoparticles, cryo-electron microscopy (cryo-EM) is another powerful technique to characterize their three-dimensional features [60,61]. In addition, dynamic light scattering (DLS) is useful to evaluate the size of pRNA-3WJ nanoparticles through the assessment of average hydrodynamic diameter (Fig. 2G). As alternatives, gel electrophoresis, including agarose gel and polyacrylamide gel, can be conveniently used to characterize the step-wise formation of RNA nanoparticles [12,49]. The combination of gel electrophoresis, DLS, AFM, and cryo-EM creates a general characterization system to evaluate the size and shape properties of RNA nanoparticles.

4. Specific methods for the construction of sophisticated pRNA-3WJ nanoparticles with various functionalities

4.1 Incorporation of functional modules to pRNA-3WJ scaffold

Apart from the high enzymatic and thermal stability, a multivalent nature is another favorable attribute of pRNA-3WJ nanoparticles. The pRNA-3WJ can be functionalized simultaneously with different modules to achieve synergistic effects. Generally, in order to construct multifunctional pRNA-3WJ nanoparticles, the types of functional modules and functionalities need to be well defined, and the global structure needs to be established before the RNA synthesis and construction (Fig. 3). The common functional modules include targeting ligands (chemical ligands or aptamers), therapeutic molecules [short interfering RNA (siRNA) or anti-micro RNA (anti-miRNA)], catalytic ribozymes [e.g. anti-hepatitis B virus hammerhead (anti-HBV) ribozyme], chemotherapeutic drugs (e.g. doxorubicin), and imaging probes (fluorophores or radionuclide). The general strategies for the multifunctionalization of RNA nanoparticles include both covalent and non-covalent methods, such as 5' - or 3' -end extensions, hybridization, and chemical conjugation or labeling. The rigidity of the pRNA-3WJ scaffold allows all incorporated modules to be spaced apart from each other, retaining their authentic folding and independent functionalities [62]. Recently, the integration of multiple functional modules into one

nanoparticle has been used for the treatments of various cancer models [16,23–28,63,64]. The following subsections will focus on the well-established methods for design, incorporation and construction of pRNA-3WJ nanoparticles with different functional modules.

4.2 pRNA-3WJ conjugated with chemical ligands for specific tumor targeting

Chemical ligands such as folate can be conjugated to pRNA-3WJ nanoparticles for specific cancer targeting [16,23,27,64]. Folate receptors are highly overexpressed on ~ 40% of tumor types, but show only limited expression in healthy organs and tissues [65]. This makes the folate ligand-receptor pair an efficient strategy for targeted drug delivery. Recent studies have shown that the folate-conjugated pRNA-3WJ nanoparticle can specifically bind to cancer cells *in vivo*, including glioblastoma [27], gastric cancer [23] and colorectal cancer metastasis in the liver, lungs and lymph node [64], with little or no accumulation in vital organs and tissues. The most common chemical strategies for the conjugation of folate to synthetic RNA can be summarized into two following categories.

4.2.1 Labeling RNA with folate using AMP or GMP derivatives during *in vitro* transcription—AMP and GMP derivatives refer to the chemically-modified adenosine monophosphate and guanosine monophosphate with reactive groups, respectively. A variety of AMP and GMP derivatives have been synthesized by using linker molecules through well-established chemistry [66–68]. These AMP or GMP derivatives can be used for single labeling of chemical ligands at the 5'-end of RNA because they are efficient initiators of T7 RNA transcription but cannot be used in the chain elongation step. By utilizing the AMP or GMP derivatives, RNA strands can be efficiently labeled with folate by a co-transcription procedure. A well-studied folate labeling procedure was performed by adding a AMP-hexanediamine (HDA)-folate derivative together with normal ribonucleoside triphosphates (rNTPs) during the *in vitro* RNA transcription [66]. Specifically, an AMP-HDA derivative is firstly synthesized through the direct coupling of HDA to AMP in the presence of condensing reagent N-ethyl-N''-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). On the other hand, folate molecules can be modified into folate-NHS ester derivative in the presence of N,N''-dicyclohexylcarbodiimide (DCC), NHS, and triethylamine (TEA) in dimethylsulfoxide (DMSO). Next, coupling folate-NHS ester with the free amino group of AMP-HDA results in the AMP-HDA-folate derivative product (Fig. 4A). To ensure a high yield of RNA production as well as folate labeling efficiency, an excess amount of AMP-HDA-folate should be used, compared to normal ATP. A 16:1 ratio of AMP-HDA-folate to ATP was reported to be the best ratio for this co-transcriptional folate labeling method [66].

4.2.2 Labeling RNA with folate by chemical conjugations of folate derivatives—Besides the co-transcriptional method, RNA can be labeled with folate via a wide range of chemical conjugations. These chemical conjugation strategies require that both the RNA and folate molecule are modified with a pair of reactive groups. For RNA, the reactive groups are more favorably attached at the 5'- or 3'-ends to avoid interfering the base pairing. The well-established reaction chemistry includes (Fig. 4B): 1) Reaction of a NHS ester, an activated carboxylic group, on the folate derivative with a free -NH₂ on the RNA strand. This reaction can yield a stable amide linkage under slightly alkaline conditions (pH 7.2 to

9); 2) Click reaction of an alkyne- or azide-modified folate derivative with an azide- or alkyne-labeled RNA strand, respectively. The copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction is an outstanding reaction for folate labeling of RNA due to its high reaction efficiency, mild reaction conditions, and high compatibility with other functional groups; 3) Reaction of a maleimide group on the folate derivative with a free -SH group labeled on the RNA strand. This reaction occurs under the pH 6.5–7.5, and results in a non-reversible stable thioether linkage. It should be noticed that all the folate derivatives above are commercially available. The RNA strands labeled with functional groups at the 5′- or 3′-end can be either chemically synthesized using the modified phosphoramidite derivatives, or directly purchased commercially (see section 2.4). After the chemical conjugations, the RNA-folate product should be purified by reverse phase HPLC or PAGE to remove unreacted molecules prior to the nanoparticle assembly.

4.3 pRNA-3WJ incorporated with aptamers for specific tumor targeting

RNA aptamers are RNA oligonucleotides selected via the systematic evolution of ligands by exponential enrichment (SELEX) to bind targets with high affinity and selectivity, similar to an antibody binding to an antigen. Many RNA aptamers have been extensively applied to cancer targeting, diagnosis, and therapeutics [69,70]. Here, this section will not focus on the generation of aptamers by SELEX technology, but the methods for incorporating well-developed aptamers to pRNA-3WJ scaffold. The first step is to determine a suitable aptamer for the specific objective. The sequence of aptamers can be rationally linked to the 5′- or 3′-end of one helical arm of 3WJ. For example, a 39-nt epidermal growth factor receptor aptamer (EGFR_{apt}) was incorporated to the pRNA-3WJ scaffold for efficient tumor targeting in the triple negative breast cancer model (Fig. 5A) [24]. The EGFR aptamer was directly extended from the 3′-end of 2′-F 3WJ-b, resulting in a 59-nt 2′-F EGFR_{apt}-displaying 3WJ-b strand. This strand can be synthesized by either *in vitro* 2′-F RNA transcription, or solid-phase chemical synthesis (see section 2), and mixed with 2′-F 3WJ-a and 2′-F 3WJ-c at equal molar concentration to assemble the 2′-F 3WJ-EGFR_{apt} nanoparticle. Instead of being extended from one strand of 3WJ, RNA aptamers can also be inserted between two strands of 3WJ to obtain a higher cell binding efficiency. For example, a 43-nt anti-prostate-specific membrane antigen A9g aptamer (PSMA_{apt}) was used to connect 3WJ-a and 3WJ-c, and specifically targeted prostate cancer after the assembly of 2′-F 3WJ-PSMA_{apt} (Fig. 5B) [25]. These aptamers retained their authentic folding and specific cell binding activities after incorporation to 3WJ. Typically, their *in vitro* and *in vivo* specific cancer cell binding capacity can be evaluated by flow cytometry analysis, confocal microscopy imaging, and *in vivo* biodistribution study.

4.3.1 In vitro evaluation of cell binding function of pRNA-3WJ incorporated with targeting ligands—After the construction of pRNA-3WJ with targeting ligands (folate or aptamers), their specific cell binding function should be evaluated *in vitro* prior to animal trials. The most common approaches include flow cytometry analysis and confocal microscopy imaging. In these cases, the RNA nanoparticles must be additionally labeled with fluorophores for detection and imaging (see section 4.8). The types of cell line vary according to the specific incorporated ligand or aptamer that recognizes the specific cell-

surface marker. To evaluate the cell binding efficiency by flow cytometry, a standard procedure will be:

- i. Trypsinize the cells and wash them with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 2 mM KH₂PO₄ pH 7.4).
- ii. Resuspend the cells to a concentration of 2×10^5 cells in 50 μ l ice-cold PBS buffer.
- iii. Add fluorophore-labeled RNA nanoparticles incorporated with targeting ligand to cell suspension at a final concentration of 50–100 nM, and incubate at 37 °C for 1–2 h. A typical control group will be a fluorophore-labeled RNA nanoparticle without targeting ligand.
- iv. Rinse the cells twice with PBS buffer, resuspend the cells and assay them by flow cytometer.

As an alternative, a standard confocal microscopy experiment for RNA nanoparticles will be:

- i. Seed 5×10^4 cells on glass coverslips in cell culture medium overnight.
- ii. Incubate the cells with either fluorophore-labeled RNA nanoparticles incorporated with targeting ligand or control groups at 37 °C for 2 h.
- iii. Gently wash the cells with PBS buffer twice, fix the cells with 4% (vol/vol) paraformaldehyde at RT for 30 min, and wash cells three times with PBS buffer.
- iv. Stain the cells cytoskeleton using Alexa Fluor 488 phalloidin for 30 min at RT, and rinse them with PBS buffer for three times.
- v. Mount the cells with Prolong Gold antifade reagent to stain cell nucleus.
- vi. Assess cell binding and entry event with a Zeiss LSM 510 laser-scanning confocal microscope.

4.3.2 In vivo biodistribution study of pRNA-3WJ incorporated with targeting ligands—Apart from *in vitro* cell binding experiments, the tumor targeting of pRNA-3WJ incorporated with folate or aptamers should be further evaluated by *in vivo* biodistribution. It should be noted that mice must be on a folate-deficient diet to avoid the competitive binding from free folate molecules, if folate is used as the targeting ligand. All RNA nanoparticles are recommended to be 2'-F modified to increase their enzymatic stability *in vivo*. A far-red or near-IR dye, such as Alexa 647, will be preferred for biodistribution imaging of RNA nanoparticles due to their better fluorescent signal penetration and low background noise. Tumor model varies depending on the types of incorporated targeting ligand to RNA nanoparticles. A detailed procedure is described below.

- i. Inject 4–6 weeks-old nude mice (nu/nu) with approximate 1×10^6 cancer cells in PBS buffer to generate tumor model (if using folate as the ligand, keep mice on a folate-deficient diet for 2 weeks before cancer cells injection).

- ii. Once the tumors grow to approximate 500 mm³, IV inject fluorophore-labeled RNA nanoparticles at a single dose of 20 µM in 100 µl PBS, or PBS buffer as a negative control through the tail vein.
- iii. Anesthetize the mice and perform whole-body imaging with an IVIS Spectrum Station at different time points (e.g. 1, 4, 8, 12, 24 h) after injection.
- iv. To further evaluate the biodistribution in organs and tumors, sacrifice the mice by CO₂ asphyxiation followed by cervical dislocation. Major organs including heart, lung, spleen, liver, and kidney as well as tumor are collected and subjected for fluorescence imaging using the IVIS Spectrum Station.

4.4 pRNA-3WJ harboring siRNA for tumor regression and gene knockdown

RNA interference (RNAi) is an important post-transcriptional method of gene regulation in many living organisms [71,72]. A previous protocol offers a well-established method for the design and assembly of siRNA-functionalized RNA nanoring and RNA nanocube [40]. Here, we mainly introduce the fast and simple method to construct siRNA-functionalized pRNA-3WJ nanoparticles for specific targeted delivery and tumor regression. Due to the same chemical nature, a typical 21–25 bp siRNA can be readily incorporated into pRNA-3WJ nanoparticles. The primary siRNA sense sequence can be extended from the 5′- or 3′-end of 3WJ, while the antisense strand is fused to sense strand by complementary base pairing. To ensure the subsequent Dicer processing, the siRNA can be spaced apart from the helical region of 3WJ by the introduction of a -UU- or -AA- bulge between these two modules, and the 2-nt overhang at 3′-end of the siRNA must be retained for Dicer recognition. It is important to note that the antisense strands are generally retained unmodified to avoid compromising the active silencing potency. It has been demonstrated that the incorporation of siRNA to pRNA-3WJ nanoparticles didn't affect the authentic folding and activities of the individual units [12,62]. A typical example is the incorporation of BRCA1 siRNA to pRNA-3WJ for gastric cancer regression (Fig. 5C) [23]. The BRCA1 sense strand was linked to the 5′-end of 2′-F 3WJ-b. The entire nanoparticle was designed to be constructed from four ssRNA fragments, including folate-displaying 2′-F 3WJ-a, BRCA1-harboring 2′-F 3WJ-b sense strand, 2′-F 3WJ-c, and BRCA1 antisense strand. This nanoparticle actively targeted gastric cancer and showed a significant inhibitory effect on gastric tumor growth *in vivo*. Another example is the incorporation of luciferase siRNA to pRNA-3WJ (Fig. 5D) [27]. Different from the previous design, the luciferase siRNA sense strand was linked at the 3′-end of 2′-F 3WJ-a. After the construction, 2′-F 3WJ nanoparticles harboring luciferase siRNA efficiently targeted human patient-derived glioblastoma stem cells and knocked down luciferase reporter gene expression. By incorporating siRNA to pRNA-3WJ platform, the specific tumor targeting strategy can potentially overcome the obstacles for siRNA therapeutics, such as improve the *in vivo* stability and half-life of the siRNA, prevent undesirable side effects, and increase the silencing potency of a given dose.

4.5 pRNA-3WJ harboring anti-miRNA for tumor regression

MiRNAs are another important part of RNAi therapeutics [73]. Typically, mature miRNAs are naturally-occurring small noncoding RNA molecules composed of around 22

nucleotides. They can regulate target genes by inhibiting the translation of specific mRNA or degrading the mRNA. MiRNAs play important roles in the regulation of cell cycle, differentiation, metabolism, apoptosis, oncogenesis, and tumor growth. In recent years, anti-miRNAs have been developed as therapeutics for tumor regression [74]. Similar to siRNA, small miRNA or anti-miRNA can be incorporated to the 5'- or 3'-end of pRNA-3WJ for targeted delivery. For example, a recent study showed that the specific delivery of anti-miRNA-21 by pRNA-3WJ to triple negative breast cancer can successfully achieve tumor regression (Fig. 5A) [24]. The anti-miRNA-21 used in this study was an 8-nt LNA sequence (5'-GATAAGCT-3') that is complementary to the oncogenic miRNA-21 seed region. The LNA modification significantly improves the binding affinity and specificity. To incorporate anti-miRNA-21 to the pRNA-3WJ, the anti-miRNA-21 was linked to a 25-nt DNA sequence which can be hybridized to 2'-F 3WJ-a with a 25-nt extended complementary sequence, leaving the 8-nt LNA as an overhang for miRNA targeting. The synthesis of this anti-miRNA-21 LNA strand can be readily accomplished by solid-phase chemical synthesis using LNA phosphoramidites (see section 2.4), or directly purchased commercially. Next, the 2'-F 3WJ harboring anti-miRNA-21 can be assembled from four fragments, using the same bottom-up self-assembly procedure as described above. Besides breast cancer, 3WJ nanoparticles harboring anti-miRNA-21 were also used for the treatment of glioblastoma and prostate cancer in animal models, and showed enhanced anti-tumor effect (Fig. 5B) [25,75].

4.6 pRNA-3WJ incorporated with ribozyme for catalytic functioning

Ribozymes are a class of RNA molecules with enzymatic activity similar to proteins [76,77]. These catalytic RNAs possess significant therapeutic potential because they can regulate protein translation, RNA splicing process, and viral replication. Like RNA aptamers, siRNA, and miRNA, ribozymes are another important functional module that can be readily incorporated to pRNA-3WJ nanoparticles. For example, the anti-HBV ribozyme is a RNA enzyme that cleaves the genomic RNA of HBV genome [12]. It was incorporated to a pRNA-3WJ scaffold by joining it to the helical region of 3WJ-a/3WJ-b branch (Fig. 6A). The 3WJ-a/b harboring anti-HBV ribozyme sequence can be synthesized by *in vitro* RNA transcription (see section 2), followed by mixing with 3WJ-c to form the 3WJ nanoparticle harboring anti-HBV ribozyme. After the construction, the original catalytic activity of the anti-HBV ribozyme was confirmed by its ability to cleave its 135-nt RNA genome substrates into two small fragments of 60-nt and 75-nt, respectively [12].

4.7 pRNA-3WJ intercalated with doxorubicin

Traditional chemotherapy still plays an essential role in cancer treatment. However, routine systemic administration of small chemotherapeutic drugs always requires a high dose to achieve therapeutic effects, leading to non-desirable side effects and non-specific toxicity. RNA nanotechnology shows great potential in overcoming the challenges of traditional chemotherapy, by lowering off-target effects, enhancing circulation time, lowering injection dose, and improving drug efficacy. Anti-cancer drugs such as doxorubicin (Dox) have been incorporated to pRNA-3WJ nanoparticles for targeted delivery to ovarian cancer [28]. To intercalate Dox within pRNA-3WJ, a 26-bp GC rich sequence was extended from the 3WJ-b/3WJ-c helical branch to serve as a drug-loading region (Fig. 6B). By gently mixing Dox

with pRNA-3WJ in intercalation buffer (0.1 M sodium acetate, 0.05 M NaCl, 0.01 M MgCl₂) at RT for 1 h, the planar Dox will preferentially intercalate between neighboring GC base pairs within the RNA helix. Excess Dox can be readily removed from the reaction mixture by passing it through a sephadex G50 spin column. After the conjugation, the drug loading efficiency can be determined by measuring the fluorescence intensity of dox with a fluorescence spectrophotometer. The non-covalent intercalation allows Dox to be released over time during cancer treatment. The incorporation of Dox to pRNA-3WJ has the potential to enhance its therapeutic efficiency at low doses through targeted drug delivery for cancer treatment.

4.8 pRNA-3WJ labeled with fluorophores for imaging

Fluorophores are useful for the *in vivo* localization of pRNA-3WJ nanoparticles. Covalent labeling of RNA with fluorophores can be accomplished or obtained in several ways, including common chemical conjugations (see section 4.2.2 and Fig. 4B), solid-phase chemical synthesis using fluorophores-modified phosphoramidites (see section 2.4), and directly purchase from commercial vendors. It is strongly recommended to purify the fluorescently labeled RNA strand from unlabeled RNA and excess fluorophores by reverse phase HPLC. After the pRNA-3WJ assembly, the fluorescent pRNA-3WJ nanoparticles, functionalized with targeting groups, can be used for *in vivo* imaging and detection (Fig. 5) [16,64,78].

5. Challenges and perspectives

RNA nanotechnology is an innovative and novel platform and its rapid progress in recent years has rendered tremendous potential for the treatment of various diseases, especially cancer treatments. The construction of functional RNA nanoparticles has been well-established, but the following challenges need to be addressed to facilitate the field toward clinic and industry:

1. Accurate prediction of the global folding of RNA structures and assembly of RNA nanoparticles continues to be challenging. The computational modeling of RNA nanoparticle construction requires the understanding of both thermodynamic and kinetic aspects of RNA folding. Although some user friendly RNA computation resources are available online, such as Mfold [79], RNA designer [80], Sfold [81], NUPACK [82], Nanofolder [20], and Hyperfold [83], the development of RNA structural computation is still at the early stage.
2. The drug loading capacity and controlled drug release of pRNA-3WJ needs to be improved. The branched feature enables the pRNA-3WJ scaffold to carry multiple functional modules or small therapeutic agents; however, the amount of payload is still limited. Different solutions have been developed, such as the construction of RNA dendrimers and RNA nanocages [59–61]. Nevertheless, some important issues, such as the capacity of drug loading, effective strategies for controlled drug release, await improvement.
3. The knowledge of intracellular processing and endosomal escape of RNA nanoparticles is still restricted. After incorporation of targeting ligands,

pRNA-3WJ nanoparticles can enter cells by receptor-mediated endocytosis. However, it is possible that the RNA nanoparticles are transferred to and trapped in the late endosomes, causing them to fail to reach their targets, especially for siRNA delivery. Some methods have been reported to assist endosome escape. For example, the use of pH-responsive chemical groups, such as acetal, hydrazone, and maleic acid amides, or acid protonating groups like imidazole, sulfonamide can help disrupt the endosome by proton sponge effects [84,85]. More studies are needed to apply these methods to the RNA nanoparticle platform.

4. The large-scale production and high cost issue of RNA nanoparticles remain one of the bottlenecks for their usefulness in clinical and industrial applications. Nevertheless, significant progress has been made to improve the chemical synthesis efficiency of RNA and the availability of chemical modifications. Furthermore, the rapid development of RNA production facilities and technologies are expected to decrease the cost of RNA nanoparticle production in the future.

6. Concluding remarks

The field of RNA nanotechnology has achieved many exciting developments in recent years, especially its application in targeted cancer therapy. The 3WJ motif derived from phi29 pRNA provides an ideal platform for constructing multifunctional RNA nanoparticles. By incorporating different functional modules to the branched region of 3WJ, RNA nanoparticles can be constructed via a bottom-up self-assembly approach with controllable structures, defined sizes, precise stoichiometries, and polyvalent functionalities. These RNA nanoparticles will play a significant role in biomedical sciences. This article covers the fundamental methods for the design, construction and characterization of both simple and sophisticated pRNA-3WJ nanoparticles. The methods discussed above are expected to help readers understand this field better and be used in their own research.

Acknowledgments

This research was supported by NIH grants R01EB019036, U01CA151648 and U01CA207946. P.G.'s Sylvan G. Frank Endowed Chair position in Pharmaceutics and Drug Delivery is funded by the CM Chen Foundation. P.G. is the consultant of Oxford Nanopore Technologies and Nanobio Delivery Pharmaceutical Co. Ltd; he is the cofounder of Shenzhen P&Z Bio-medical Co. Ltd and its subsidiary US P&Z Biological Technology LLC, as well as ExonanoRNA LLC.

Reference List

1. B Rehm. Bionanotechnology: Biological Self-assembly and its Applications Horizon Scientific Press; 2013
2. Guo P. The emerging field of RNA nanotechnology. *Nature Nanotechnology*. 2010; 5:833–842.
3. Jasinski D, Haque F, Binzel DW, Guo P. Advancement of the Emerging Field of RNA Nanotechnology. *ACS Nano*. 2017; 11:1142–1164. [PubMed: 28045501]
4. Guo P, Zhang C, Chen C, Trottier M, Garver K. Inter-RNA interaction of phage phi29 pRNA to form a hexameric complex for viral DNA transportation. *Mol Cell*. 1998; 2:149–155. [PubMed: 9702202]

5. Guo P, Erickson S, Anderson D. A small viral RNA is required for *in vitro* packaging of bacteriophage phi29 DNA. *Science*. 1987; 236:690–694. [PubMed: 3107124]
6. Guo P, Bailey S, Bodley JW, Anderson D. Characterization of the small RNA of the bacteriophage phi29 DNA packaging machine. *Nucleic Acids Res*. 1987; 15:7081–7090. [PubMed: 3116499]
7. Chen C, Zhang C, Guo P. Sequence requirement for hand-in-hand interaction in formation of pRNA dimers and hexamers to gear phi29 DNA translocation motor. *RNA*. 1999; 5:805–818. [PubMed: 10376879]
8. Shu Y, Haque F, Shu D, Li W, Zhu Z, Kotb M, Lyubchenko Y, Guo P. Fabrication of 14 Different RNA Nanoparticles for Specific Tumor Targeting without Accumulation in Normal Organs. *RNA*. 2013; 19:766–777.
9. Yingling YG, Shapiro BA. Computational design of an RNA hexagonal nanoring and an RNA nanotube. *Nano Letters*. 2007; 7:2328–2334. [PubMed: 17616164]
10. Afonin KA, Bindewald E, Yaghoubian AJ, Voss N, Jacovetty E, Shapiro BA, Jaeger L. In vitro assembly of cubic RNA-based scaffolds designed in silico. *Nat Nanotechnol*. 2010; 5:676–682. [PubMed: 20802494]
11. Westhof E, Masquida B, Jaeger L. RNA tectonics: Towards RNA design. *Folding & Design*. 1996; 1:R78–R88. [PubMed: 9079386]
12. Shu D, Shu Y, Haque F, Abdelmawla S, Guo P. Thermodynamically stable RNA three-way junctions for constructing multifunctional nanoparticles for delivery of therapeutics. *Nature Nanotechnology*. 2011; 6:658–667.
13. Severcan I, Geary C, Chworos A, Voss N, Jacovetty E, Jaeger L. A polyhedron made of tRNAs. *Nat Chem*. 2010; 2:772–779. [PubMed: 20729899]
14. Dibrov SM, McLean J, Parsons J, Hermann T. Self-assembling RNA square. *Proc Natl Acad Sci USA*. 2011; 108:6405–6408. [PubMed: 21464284]
15. Grabow WW, Zakrevsky P, Afonin KA, Chworos A, Shapiro BA, Jaeger L. Self-Assembling RNA Nanorings Based on RNAI/II Inverse Kissing Complexes. *Nano Lett*. 2011; 11:878–887. [PubMed: 21229999]
16. Haque F, Shu D, Shu Y, Shlyakhtenko L, Rychahou P, Evers M, Guo P. Ultrastable synergistic tetravalent RNA nanoparticles for targeting to cancers. *Nano Today*. 2012; 7:245–257. [PubMed: 23024702]
17. Geary C, Chworos A, Verzemnieks E, Voss NR, Jaeger L. Composing RNA Nanostructures from a Syntax of RNA Structural Modules. *Nano Lett*. 2017; 17:7095–7101. [PubMed: 29039189]
18. Leontis NB, Lescoute A, Westhof E. The building blocks and motifs of RNA architecture. *Curr Opin Struct Biol*. 2006; 16:279–287. [PubMed: 16713707]
19. Jaeger L, Westhof E, Leontis NB. TectoRNA: modular assembly units for the construction of RNA nano-objects. *Nucleic Acids Res*. 2001; 29:455–463. [PubMed: 11139616]
20. Bindewald E, Afonin K, Jaeger L, Shapiro BA. Multistrand RNA secondary structure prediction and nanostructure design including pseudoknots. *ACS Nano*. 2011; 5:9542–9551. [PubMed: 22067111]
21. Severcan I, Geary CVE, CA, Jaeger L. Square-shaped RNA particles from different RNA folds. *Nano Lett*. 2009; 9:1270–1277. [PubMed: 19239258]
22. Boerneke MA, Dibrov SM, Hermann T. Crystal-Structure-Guided Design of Self-Assembling RNA Nanotriangles. *Angew Chem Int Ed Engl*. 2016; 55:4097–4100. [PubMed: 26914842]
23. Cui D, Zhang C, Liu B, Shu Y, Du T, Shu D, Wang K, Dai F, Liu Y, Li C, Pan F, Yang Y, Ni J, Li H, Brand-Saberi B, Guo P. Regression of gastric cancer by systemic injection of RNA nanoparticles carrying both ligand and siRNA. *Scientific reports*. 2015; 5:10726. [PubMed: 26137913]
24. Shu D, Li H, Shu Y, Xiong G, Carson WE, Haque F, Xu R, Guo P. Systemic delivery of anti-miRNA for suppression of triple negative breast cancer utilizing RNA nanotechnology. *ACS Nano*. 2015; 9:9731–9740. [PubMed: 26387848]
25. Binzel D, Shu Y, Li H, Sun M, Zhang Q, Shu D, Guo B, Guo P. Specific Delivery of MiRNA for High Efficient Inhibition of Prostate Cancer by RNA Nanotechnology. *Molecular Therapy*. 2016; 24:1267–1277. [PubMed: 27125502]

26. Zhang Y, Leonard M, Shu Y, Yang Y, Shu D, Guo P, Zhang X. Overcoming Tamoxifen Resistance of Human Breast Cancer by Targeted Gene Silencing Using Multifunctional pRNA Nanoparticles. *ACS Nano*. 2017; 11:335–346. [PubMed: 27966906]
27. Lee TJ, Haque F, Shu D, Yoo JY, Li H, Yokel RA, Horbinski C, Kim TH, Kim SH, Nakano I, Kaur B, Croce CM, Guo P. RNA nanoparticles as a vector for targeted siRNA delivery into glioblastoma mouse model. *Oncotarget*. 2015; 6:14766–14776. [PubMed: 25885522]
28. Pi F, Zhang H, Li H, Thiviyanathan V, Gorenstein DG, Sood AK, Guo P. RNA nanoparticles harboring annexin A2 aptamer can target ovarian cancer for tumor-specific doxorubicin delivery. *Nanomedicine*. 2016; 13:1183–1193. [PubMed: 27890659]
29. Layzer JM, McCaffrey AP, Tanner AK, Huang Z, Kay MA, Sullenger BA. In vivo activity of nuclease-resistant siRNAs. *RNA*. 2004; 10:766–771. [PubMed: 15100431]
30. Shaw JP, Kent K, Bird J, Fishback J, Froehler B. Modified deoxyoligonucleotides stable to exonuclease degradation in serum. *Nucleic Acids Res*. 1991; 19:747–750. [PubMed: 1850122]
31. Kawasaki AM, Casper MD, Freier SM, Lesnik EA, Zounes MC, Cummins LL, Gonzalez C, Cook PD. Uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets. *J Med Chem*. 1993; 36:831–841. [PubMed: 8464037]
32. Elmen J, Thonberg H, Ljungberg K, Frieden M, Westergaard M, Xu Y, Wahren B, Liang Z, Orum H, Koch T, Wahlestedt C. Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res*. 2005; 33:439–447. [PubMed: 15653644]
33. Heidenreich O, Benseler F, Fahrenholz A, Eckstein F. High activity and stability of hammerhead ribozymes containing 2'-modified pyrimidine nucleosides and phosphorothioates. *J Biol Chem*. 1994; 269:2131–2138. [PubMed: 8294467]
34. Allerson CR, Sioufi N, Jarres R, Prakash TP, Naik N, Berdeja A, Wanders L, Griffey RH, Swayze EE, Bhat B. Fully 2'-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. *J Med Chem*. 2005; 48:901–904. [PubMed: 15715458]
35. Liechty WB, Kryscio DR, Slaughter BV, Peppas NA. Polymers for drug delivery systems. *Annu Rev Chem Biomol Eng*. 2010; 1:149–173. [PubMed: 22432577]
36. Shrestha H, Bala R, Arora S. Lipid-Based Drug Delivery Systems. *J Pharm (Cairo)*. 2014; 2014:801820. [PubMed: 26556202]
37. Guo P, , Haque F. *RNA Nanotechnology and Therapeutics* CRC Press; Boca Raton, FL: 2013
38. Li H, Lee T, Dziubla T, Pi F, Guo S, Xu J, Li C, Haque F, Liang X, Guo P. RNA as a stable polymer to build controllable and defined nanostructures for material and biomedical applications. *Nano Today*. 2015; 10:631–655. [PubMed: 26770259]
39. Shu Y, Shu D, Haque F, Guo P. Fabrication of pRNA nanoparticles to deliver therapeutic RNAs and bioactive compounds into tumor cells. *Nat Protoc*. 2013; 8:1635–1659. [PubMed: 23928498]
40. Afonin KA, Grabow WW, Walker FM, Bindewald E, Dobrovolskaia MA, Shapiro BA, Jaeger L. Design and self-assembly of siRNA-functionalized RNA nanoparticles for use in automated nanomedicine. *Nat Protoc*. 2011; 6:2022–2034. [PubMed: 22134126]
41. Milligan JF, Uhlenbeck OC. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol*. 1989; 180:51–62. [PubMed: 2482430]
42. Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acid Research*. 1987; 15(21):8783–8798.
43. Beckert B, Masquida B. Synthesis of RNA by in vitro transcription. *Methods Mol Biol*. 2011; 703:29–41. [PubMed: 21125481]
44. Padilla R, Sousa R. Efficient synthesis of nucleic acids heavily modified with non-canonical ribose 2'-groups using a mutant T7 RNA polymerase (RNAP). *Nucleic Acids Res*. 1999; 27:1561–1563. [PubMed: 10037823]
45. Beaucage SL, Reese CB. Recent advances in the chemical synthesis of RNA. *Curr Protoc Nucleic Acid Chem*. 2009; Chapter 2(Unit 2.16)(Unit–31):1–31.
46. Marshall WS, Kaiser RJ. Recent advances in the high-speed solid phase synthesis of RNA. *Curr Opin Chem Biol*. 2004; 8:222–229. [PubMed: 15183319]

47. Zhang H, Endrizzi JA, Shu Y, Haque F, Sauter C, Shlyakhtenko LS, Lyubchenko Y, Guo P, Chi YI. Crystal Structure of 3WJ Core Revealing Divalent Ion-promoted Thermostability and Assembly of the Phi29 Hexameric Motor pRNA. *RNA*. 2013; 19:1226–1237. [PubMed: 23884902]
48. Binzel DW, Khisamutdinov E, Vieweger M, Ortega J, Li J, Guo P. Mechanism of three-component collision to produce ultrastable pRNA three-way junction of Phi29 DNA-packaging motor by kinetic assessment. *RNA*. 2016; 22:1710–1718. [PubMed: 27672132]
49. Binzel DW, Khisamutdinov EF, Guo P. Entropy-driven one-step formation of Phi29 pRNA 3WJ from three RNA fragments. *Biochemistry*. 2014; 53:2221–2231. [PubMed: 24694349]
50. Benkato K, O'Brien B, Bui MN, Jasinski DL, Guo P, Khisamutdinov EF. Evaluation of Thermal Stability of RNA Nanoparticles by Temperature Gradient Gel Electrophoresis (TGGE) in Native Condition. *Methods Mol Biol*. 2017; 1632:123–133. [PubMed: 28730436]
51. Piao X, Wang H, Binzel D, Guo P. Assessment and Comparison of Thermal Stability of Phosphorothioate DNA, DNA, RNA, 2'-F RNA and LNA in the Context of Phi29 pRNA 3WJ. *RNA*. 2017; 24:67–76. [PubMed: 29051199]
52. Corey DR. Chemical modification: the key to clinical application of RNA interference? *J Clin Invest*. 2007; 117:3615–3622. [PubMed: 18060019]
53. Piao X, Xia X, Bong D. Bifacial peptide nucleic acid directs cooperative folding and assembly of binary, ternary, and quaternary DNA complexes. *Biochemistry*. 2013; 52:6313–6323. [PubMed: 23964711]
54. Liu J, Guo S, Cinier M, Shlyakhtenko LS, Shu Y, Chen C, Shen G, Guo P. Fabrication of stable and RNase-resistant RNA nanoparticles active in gearing the nanomotors for viral DNA packaging. *ACS Nano*. 2011; 5:237–246. [PubMed: 21155596]
55. Guo S, Li H, Ma M, Fu J, Dong Y, Guo P. Size, Shape and Sequence-dependent Immunogenicity of RNA Nanoparticles. *Molecular Therapy-Nucleic Acids*. 2017; 9:399–408. [PubMed: 29246318]
56. Xu Z, Sun Y, Weber JK, Cao Y, Wang W, Jasinski D, Guo P, Zhou R, Li J. Directional mechanical stability of Bacteriophage phi29 motor's 3WJ-pRNA: Extraordinary robustness along portal axis. *Sci Adv*. 2017; 3:e1601684. [PubMed: 28560321]
57. Sun Y, Di W, Li Y, Huang W, Wang X, Qin M, Wang W, Cao Y. Mg(2+) -Dependent High Mechanical Anisotropy of Three-Way-Junction pRNA as Revealed by Single-Molecule Force Spectroscopy. *Angew Chem Int Ed Engl*. 2017; 56:9376–9380. [PubMed: 28631866]
58. Khisamutdinov EF, Jasinski DL, Guo P. RNA as a boiling-resistant anionic polymer material to build robust structures with defined shape and stoichiometry. *ACS Nano*. 2014; 8:4771–4781. [PubMed: 24694194]
59. Sharma A, Haque F, Pi F, Shlyakhtenko L, Evers BM, Guo P. Controllable Self-assembly of RNA Dendrimers. *Nanomedicine-Nanotechnology Biology and Medicine*. 2015; 12:835–844.
60. Li H, Zhang K, Pi F, Guo S, Shlyakhtenko L, Chiu W, Shu D, Guo P. Controllable Self-Assembly of RNA Tetrahedrons with Precise Shape and Size for Cancer Targeting. *Adv Mater*. 2016; 28:7501–7507. [PubMed: 27322097]
61. Khisamutdinov EF, Jasinski DL, Li H, Zhang K, Chiu W, Guo P. Fabrication of RNA 3D Nanoprism for Loading and Protection of Small RNAs and Model Drugs. *Advanced Materials*. 2016; 28:100079–100087.
62. Shu D, Khisamutdinov E, Zhang L, Guo P. Programmable folding of fusion RNA complex driven by the 3WJ motif of phi29 motor pRNA. *Nucleic Acids Res*. 2013; 42:e10. [PubMed: 24084081]
63. Lee TJ, Haque F, Vieweger M, Yoo JY, Kaur B, Guo P, Croce CM. Functional assays for specific targeting and delivery of RNA nanoparticles to brain tumor. *Methods Mol Biol*. 2015; 1297:137–152. [PubMed: 25896001]
64. Rychahou P, Haque F, Shu Y, Zaytseva Y, Weiss HL, Lee EY, Mustain W, Valentino J, Guo P, Evers BM. Delivery of RNA nanoparticles into colorectal cancer metastases following systemic administration. *ACS Nano*. 2015; 9:1108–1116. [PubMed: 25652125]
65. Low PS, Kularatne SA. Folate-targeted therapeutic and imaging agents for cancer. *Curr Opin Chem Biol*. 2009; 13:256–262. [PubMed: 19419901]
66. Guo S, Huang F, Guo P. Construction of folate-conjugated pRNA of bacteriophage phi29 DNA packaging motor for delivery of chimeric siRNA to nasopharyngeal carcinoma cells. *Gene Ther*. 2006; 13:814–820. [PubMed: 16482206]

67. Huang F, Wang G, Coleman T, Li N. Synthesis of adenosine derivatives as transcription initiators and preparation of 5' fluorescein- and biotin-labeled RNA through one-step *in vitro* transcription. *RNA*. 2003; 9:1562–1570. [PubMed: 14624011]
68. Zhang L, Sun L, Cui Z, Gottlieb RL, Zhang B. 5'-sulfhydryl-modified RNA: initiator synthesis, *in vitro* transcription, and enzymatic incorporation. *Bioconjug Chem*. 2001; 12:939–948. [PubMed: 11716685]
69. Sun H, Zhu X, Lu PY, Rosato RR, Tan W, Zu Y. Oligonucleotide aptamers: new tools for targeted cancer therapy. *Mol Ther Nucleic Acids*. 2014; 3:e182. [PubMed: 25093706]
70. Zhou G, Wilson G, Hebbard L, Duan W, Liddle C, George J, Qiao L. Aptamers: A promising chemical antibody for cancer therapy. *Oncotarget*. 2016; 7:13446–13463. [PubMed: 26863567]
71. Aagaard L, Rossi JJ. RNAi therapeutics: Principles, prospects and challenges. *Advanced Drug Delivery Reviews*. 2007; 59:75–86. [PubMed: 17449137]
72. Bass BL. RNA interference. The short answer. *Nature*. 2001; 411:428–429. [PubMed: 11373658]
73. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nature Reviews Cancer*. 2006; 6:857–866. [PubMed: 17060945]
74. Garzon R, Calin GA, Croce CM. MicroRNAs in Cancer. *Annu Rev Med*. 2009; 60:167–179. [PubMed: 19630570]
75. Lee TJ, Yoo JY, Shu D, Li H, Zhang J, Yu JG, Jaime-Ramirez AC, Acunzo M, Romano G, Cui R, Sun HL, Luo Z, Old M, Kaur B, Guo P, Croce CM. RNA Nanoparticle-Based Targeted Therapy for Glioblastoma through Inhibition of Oncogenic miR-21. *Mol Ther*. 2017
76. Blount KF, Uhlenbeck OC. The hammerhead ribozyme. *Biochem Soc Trans*. 2002; 30:1119–1122. [PubMed: 12440986]
77. James HA, Gibson I. The therapeutic potential of ribozymes. *Blood*. 1998; 91:371–382. [PubMed: 9427689]
78. Jasinski DL, Yin H, Li Z, Guo P. The Hydrophobic Effect from Conjugated Chemicals or Drugs on *in Vivo* Biodistribution of RNA Nanoparticles. *Hum Gene Ther*. 2017
79. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*. 2003; 31:3406–3415. [PubMed: 12824337]
80. Andronescu M, Fejes AP, Hutter F, Hoos HH, Condon A. A new algorithm for RNA secondary structure design. *J Mol Biol*. 2004; 336:607–624. [PubMed: 15095976]
81. Ding Y, Chan CY, Lawrence CE. Sfold web server for statistical folding and rational design of nucleic acids. *Nucleic Acids Res*. 2004; 32:W135–W141. [PubMed: 15215366]
82. Zadeh JN, Steenberg CD, Bois JS, Wolfe BR, Pierce MB, Khan AR, Dirks RM, Pierce NA. NUPACK: Analysis and design of nucleic acid systems. *J Comput Chem*. 2011; 32:170–173. [PubMed: 20645303]
83. Bindewald E, Afonin KA, Viard M, Zakrevsky P, Kim T, Shapiro BA. Multistrand Structure Prediction of Nucleic Acid Assemblies and Design of RNA Switches. *Nano Lett*. 2016; 16:1726–1735. [PubMed: 26926528]
84. Pack DW, Putnam D, Langer R. Design of imidazole-containing endosomolytic biopolymers for gene delivery. *Biotechnology and Bioengineering*. 2000; 67:217–223. [PubMed: 10592519]
85. Shete HK, Prabhu RH, Patravale VB. Endosomal escape: a bottleneck in intracellular delivery. *J Nanosci Nanotechnol*. 2014; 14:460–474. [PubMed: 24730275]

Research highlights

1. The common methods and protocols for *in vitro* synthesis of RNA are summarized.
2. The instructions on basic and simple methods for the construction and characterization of RNA nanoparticles are introduced.
3. The specific methods for the construction of sophisticated multifunctional RNA nanoparticles for therapeutic, imaging, and diagnostic applications are introduced.
4. The current challenges and perspectives of construction and applications of RNA nanoparticles are discussed.

Figure 1.

Purification of DNA or RNA by electro-separation system. Negatively charged DNA or RNA migrate into and are retained in a membrane trap, while salt ions pass through.

Figure 2. Structure, assembly and characterization of pRNA-3WJ. (A) Secondary sequence of pRNA-3WJ (H1, H2, and H3 represent helical regions). (B) 3D computational modeling of the bottom-up self-assembly of pRNA-3WJ. (C) T_m measurement of 2'-F pRNA-3WJ by TGGE (M means monomer). (D) T_m measurement of 10 μM 2'-F pRNA-3WJ by real-time PCR. (E) Enzymatic stability assay of pRNA-3WJ with 2'-F modification on different strands in 10% FBS supplemented cell culture medium. (F) AFM image of the extended pRNA-3WJ. (G) Size distribution of pRNA-3WJ measured by DLS (n=3).

Figure 3. Strategies for RNA nanotechnology. The construction of RNA nanoparticles includes several distinct steps: conception, computation, synthesis, assembly, assessment and characterization, and applications.

Figure 4. Chemical conjugations and structures for RNA nanotechnology. (A) The chemical structure of synthesized AMP-HDA-folate derivative. (B) Chemical conjugation methods for labeling RNA with chemical ligands.

Figure 5. Multifunctional RNA nanoparticles based on pRNA-3WJ. (A, B) pRNA-3WJ incorporated with anti-miRNA and aptamers. (C, D) pRNA-3WJ incorporated with siRNA and folate.

Figure 6.
Illustration of functional pRNA-3WJ nanoparticles incorporated with (A) anti-HBV ribozyme, and (B) intercalated with doxorubicin.

Table 1

A T7 RNA transcription setup

Components	Amount	Note
5 × Transcription Buffer	10 ul	400 mM HEPES-KOH pH 7.5, 120 mM MgCl ₂ , 10 mM spermidine, and 200 mM DTT.
0.1 M DTT	5 ul	
25 mM rNTPs	10 ul	Added second to last; Made by mixing equal amounts of 100mM ATP, GTP, CTP, and UTP.
0.5 ug/ul DNA Template	1 ul	Purified prior to transcription.
DEPC-treated H ₂ O	14 ul	
T7 RNA Polymerase	10 ul	Added last.
Total	50 ul	

Table 2**A 2'-F T7 RNA transcription setup**

Components	Amount	Note
10 × Transcription Buffer	5 ul	400 mM Tris-acetate pH8.0, 10mM EDTA, 100 mM Mg-acetate, 5 mM MnCl ₂ , 80 mM spermidine, and 50 mM DTT.
0.1 M DTT	5 ul	
50 mM ATP, GTP, 2'-F CTP, and 2'-F UTP	5 ul for each (5 ul × 4 = 20 ul)	Added second to last.
0.5 ug/ul DNA Template	5 ul	Purified prior to transcription.
DEPC-treated H ₂ O	10 ul	
Y639F T7 RNA Polymerase	5 ul	Added last.
Total	50 ul	