Published in final edited form as:

Nat Protoc. 2013 September ; 8(9): . doi:10.1038/nprot.2013.097.

Fabrication of pRNA nanoparticles to deliver therapeutic RNAs and bioactive compounds into tumor cells

Yi Shu, Dan Shu, Farzin Haque, and Peixuan Guo*

Nanobiotechnology Center, Markey Cancer Center, and Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536, USA

Abstract

RNA nanotechnology is a term that refers to the design, fabrication, and utilization of nanoparticles mainly composed of ribonucleic acids via bottom-up self-assembly. The packaging RNA (pRNA) of the bacteriophage phi29 DNA packaging motor has been developed into a nano-delivery platform. This protocol describes the synthesis, assembly, and functionalization of pRNA nanoparticles based on three 'toolkits' derived from pRNA structural features: interlocking loops for hand-in-hand interactions, palindrome sequences for foot-to-foot interactions, and an RNA three-way junction for branch-extension. siRNAs, ribozymes, aptamers, chemical ligands, fluorophores, and other functionalities can also be fused to the pRNA prior to the assembly of the nanoparticles, so as to ensure the production of homogeneous nanoparticles and the retention of appropriate folding and function of the incorporated modules. The resulting self-assembled multivalent pRNA nanoparticles are thermodynamically and chemically stable, and they remain intact at ultra-low concentrations. Gene silencing effects are progressively enhanced with increasing number of siRNA in each pRNA nanoparticle. Systemic injection of the pRNA nanoparticles into xenograft-bearing mice has revealed strong binding to tumors without accumulation in vital organs or tissues. The pRNA-based nano-delivery scaffold paves a new way towards nanotechnological application of pRNA-based nanoparticles for disease detection and treatment. The time required for completing one round of this protocol is 3–4 weeks, including *in vitro* functional assays, or 2–3 months including *in vivo* studies.

Keywords

Nanotechnology; Nanobiotechnology; Bionanotechnology; Nanomotor; DNA packaging motor; Bacteriophage; RNA Nanotechnology; RNA therapeutics; Bottom-up assembly; Virus assembly; Nanomotor

INTRODUCTION

RNA molecules can be manipulated as easily as DNA, with the difference that RNA has functions and structural versatility similar to those of proteins. These properties make RNA a suitable candidate to serve as building block for applications in nanotechnology and nanomedicine^{1–4}. The first proof-of-concept in the field of RNA nanotechnology was published in 1998⁵, and this research field has since developed rapidly during the last

*Address correspondence to: Peixuan Guo, Department of Pharmaceutical Sciences, Biopharm Complex Building, Room 565, 789 S. Limestone Street Lexington, KY, USA 40536-0596, Phone: 859-218-0128, Fax: 859-257-1307, peixuan.guo@uky.edu.

AUTHOR CONTRIBUTIONS

P.G. conceived and led the project. Y.S., D.S., and F.H. designed and conducted the experiments and co-wrote the manuscript with P.G.

COMPETING FINANCIAL INTERESTS

P.G. is a co-founder of Kylin Therapeutics, Inc. and Biomotor and Nucleic Acid Nanotechnology Development Corp., Ltd.

several years^{1,3,5–11}. The rationale for using RNA as a nanomaterial is based on its unique features: 1) RNA is made up of a sugar-phosphate backbone chain with four ribonucleobases, adenine (A), guanine (G), cytosine (C), and uracil (U). Various combinations of the four nucleotides can result in different nucleotide sequences with unique secondary structural viability^{12–14}. 2) The structural and folding properties of RNA structural motifs have been elucidated, which has laid the foundation of RNA nanoparticle construction^{8,14–24}. The inter-molecular (between RNA molecules) and intra-molecular (within a RNA molecule) interactions of RNA tertiary motifs via both canonical and non-canonical pairing, as well as base stacking, ensure the formation of strong RNA tertiary structures. 3) RNA structural motifs and tertiary interactions can be isolated from known RNA structures available in public databases (e.g. PDB) and can be used as the building blocks in the rational design and grafting of RNA nanoparticles^{17,19,25,26}. 4) Small RNA molecules can be incorporated in RNA nanoparticles to add functionalities that regulate cell behavior. The finding that 98.5% of the human genome codes for non-coding RNA²⁷ has revolutionized the view of the role that RNA plays in cells. RNA molecules are actively involved in catalyzing biological reactions²⁸, regulating gene expression^{29–32}, and sensing or responding to cellular signals^{33–35}. In recent years, a plethora of natural or artificial RNA molecules have been discovered or synthesized, including siRNA^{36–43}, ribozymes^{44–48}, anti-sense RNAs^{49,50}, miRNAs^{29,51–53}, riboswitches^{54,55} and RNA aptamers^{56–60}.

RNA nanoparticle construction, as described in this protocol, involves building block extraction, rational nanoparticle design, RNA nanoparticle fabrication, functionalization; the latter is performed concomitantly to nanoparticle assembly, functional assessment, characterization, and utilization (Fig. 1). RNA motifs extracted from known RNA structures can serve as the building block for RNA nano-scaffold assembly. RNA tertiary structure interactions, including inter-molecular and intra-molecular interactions, as well as ion effects are key considerations for assembly. Functional RNA molecules, such as siRNAs, miRNAs, ribozymes, riboswitches, and RNA aptamers, can then be incorporated into the scaffold to functionalize RNA nanoparticles. Computational prediction of RNA folding and structure is an essential step for producing new structural designs^{61–66}. Available online resources include *Mfold*⁶⁷, RNA designer⁶⁸, *Sfold*⁶⁹, NUPACK⁷⁰, and others⁷¹.

Because of its structural features, bacteriophage phi29 packaging RNA (pRNA) has been extensively utilized for the assembly of RNA nanoparticles (Fig. 2a). The pRNA forms a hexameric ring on the connector, and along with the viral protein gp16, which serves as ATPase to hydrolyze ATP, gears the phi29 DNA packaging motor to package the viral genome into the preformed procapsid^{5,72,73}. Each pRNA contains a helical domain (5'/3' paired ends denoted as the foot, Fig. 2b)^{74–79}, a central domain (bases 23–97) containing the right- and left-hand loops for intermolecular interactions^{75,76,80,81} (Fig. 2b), and a three-way junction (3WJ) motif (Fig. 2b). An array of techniques or 'toolkits' (Fig. 3) that are based on pRNA structure (modular interaction and branch extension) has been developed to construct diverse RNA nanostructures with multiple functionalities as polyvalent delivery systems for nanotechnological and nanomedical applications^{82–92}.

Comparison with other methods

Many types of nano-delivery systems with different materials and physiochemical properties have been pursued over the years, the merits of a few such systems are discussed below.

Lipid-based nanoparticles—The advantages of using liposomes for gene or drug delivery are ease of preparation, low cost, and efficient cell entry⁹³. However, liposomes are thermodynamically unstable⁹³, their particle size is only limitedly reproducible, and they can incur in nonspecific cell fusion, entry and distribution⁹⁴. Nevertheless, it has been

demonstrated that chemical modifications in the phospholipid structure of liposomes can modulate their stability and *in vivo* circulation^{95,96,97}. Recently developed solid lipid nanoparticles have been shown to decrease the mobility of encapsulated drugs, resulting in their controlled and extended release, as well as an increased blood circulation time^{98,99}.

Polymer-based nanoparticles—The advantages of using both natural and synthetic polymers include ease of construction, high structural stability, high drug encapsulation efficiency, high cellular uptake, improved drug releasing rate, their ability to escape endosomes by way of protonation, and ease of modification to achieve multifunctional and targeted delivery^{100,101}. Major concerns for their use have been nonbiodegradability, insufficient biocompatibility, toxicity, and the triggering of immunogenic reactions by the mononuclear phagocyte system¹⁰². Nevertheless, the development of biodegradable and biocompatible polymers has rendered possible a progress towards the clinical application of polymer based nano-delivery systems¹⁰³. Recently, ‘smart’ delivery vehicles, including thermosensitive^{104,105} and pH-sensitive¹⁰⁶ polymer nanoparticles, have been designed that improve the biocompatibility, *in vivo* pharmacokinetics, and targeting efficacy of polymer nanocarriers. However, synthesizing uniform nanoparticles with defined structure and stoichiometry has still to be fully achieved as an objective.

Virus-based nanoparticles (VNPs)—Viral particles are robust protein cages with homogeneous and well-defined geometry that effect active or passive cell entry, which makes them attractive for nanostructure fabrication. The viral genome can be relatively easily manipulated and the viral particles can be modified and conjugated with active biomolecules or chemical groups^{107,108}. Although most of the VNPs are not typically human pathogens, reliable methods to inactivate replicating viruses or convert replicating viruses to virus-like particles need to be standardized to ensure the safety of the *in vivo* application of VNPs. Substantial concerns exist regarding the possible incorporation of the viral genome into host chromosomes as well as their possible toxicity, immunogenicity, and biodistribution.

Inorganic nanoparticles—Inorganic nanoparticles can be carbon-based, metal-based, and semiconductor-based¹⁰⁹. Most inorganic nanoparticles have been developed for use in medical imaging because of their brightness, high contrast, and photostability¹¹⁰. Some of them have great potential for photothermal therapy. However, biological incompatibility, toxicity, non-degradability, and entrapment of the inorganic nanoparticles by the lung, liver, and kidney are major disadvantages for their clinical application.

DNA-based nanoparticles—DNA nanotechnology has a great potential for diverse applications. This technology uses the complementarity patterns of the four bases in DNA to construct variety of nanostructures and nanomachines¹¹¹. Constructed DNA nanoparticles include individual folding structures^{112–115}, tile-shaped structures^{116–118}, and dynamic assemblies^{119–123}. A large number of reports on DNA nanotechnology has been published over the years^{112,124–133}. Most DNA nanostructures have been used as templates to direct and support the assembly of other nanoarchitectures^{134–140}. DNA nanoparticles can be functionalized by quantum dots¹⁴¹, DNA aptamers¹⁴², or other functional molecules. One example of medical application of DNA nanoparticles involves the use of a DNA nanostructure to cage an enzyme that could be released to induce cell apoptosis¹⁴³. However, in comparison to RNA nanotechnology (see next section), the use of pure DNA nanoparticles for clinical application is limited by their relative structural and functional simplicity, see Table 1.

RNA-based nanoparticles—The RNA nanoparticles described in this protocol have many favorable attributes: 1) They have defined size, structure, and stoichiometry. The size of pRNA nanoparticles can be easily controlled by the primary RNA sequences in which the length and sequence of RNA determines the global folding and size of each RNA subunit or by making use of tertiary interactions that are designed to control the assembling stoichiometry of the RNA nanoparticles. The hand-in-hand interactions via interlocking loops (Toolkit I, Fig. 3a), foot-to-foot interactions via palindrome sequences (Toolkit II, Fig. 3b), and branch extension (Toolkit III, Fig. 3c,d) can help the pRNA nanoparticles to grow into different oligomers in a controllable manner. Thus, unpredictable side effects such as off-target effect and *in vivo* toxicity arising from heterogeneous particles can be avoided^{1,3,90,91,144}. 2) The nanoscale size and branched ratchet shape of our RNA nanoparticles facilitate tumor penetration and enable them to take advantage of the enhanced permeability and retention (EPR) effect, via which some nanoparticles tend to accumulate preferentially in cancerous tissues rather than healthy tissues. 3) Nonspecific cell entry can be avoided since it is unfavorable for the polyanionic RNA to cross the negatively charged cell membranes^{145–148}. 4) RNA nanoparticles are highly soluble, homogeneous, and are not prone to aggregation. They do not need to be linked to PEG (polyethylene glycol) or serum albumins in order to increase their aqueous solubility or stability^{1,144}. 5) The multivalent nature of RNA nanoparticles enables their facile functionalization for simultaneous targeting and/or delivery of additional therapeutic and targeting ligands for achieving synergistic effects⁹¹. 6) Chemically modified RNA is resistant to RNase degradation and retains correct folding and biological functions^{90,91,149}. 7) RNA nanoparticles can be thermodynamically stable; therefore, the entire construct can remain intact in the body at ultra-low concentrations^{90,91}. 8) RNA-based nanoparticles have favorable pharmacokinetic and pharmacodynamic profiles *in vivo*¹⁵⁰. 9) Systemic injection of thermodynamically and chemically stable RNA nanoparticles into mice has revealed that the RNA nanoparticles strongly and specifically bind to cancer cells without accumulating in vital organs or tissues^{90,91,150}. 10) This protein-free system induces minimum host-immune responses that will allow multi dose treatment of cancer and other chronic diseases. This approach is particularly applicable to patients who produce neutralizing antibodies in response to protein-based reagents. 11) Economic industrial-scale production by enzymatic transcription is possible in a cell-free system. Modular design allows self-assembly of engineered RNA fragments^{1,144}. 12) RNA is classified as a chemical reagent by the FDA; therefore, the regulatory approval process in the USA is expected to be easier for RNA-based nanoparticles than for protein-based clinical reagents^{1,144}.

Potential applications

Due to their multivalency, RNA nanoparticles can harbor a variety of functionalities in different combinations to achieve the desired purpose. The size of pRNA-based nanoparticles is ideal for passive delivery into tumors via the EPR effect, which will minimize the off-target effects or toxicity *in vivo*. Previous studies have demonstrated that the size of the nanoparticles is crucial for their *in vivo* behavior. If the size is smaller than 10nm, the nanoparticles are likely to diffuse nonspecifically into normal tissue and organs and cause off-target effects; if the size is larger than 100nm, the nanoparticles are less likely to enter the cells by receptor-mediated endocytosis and more likely to be trapped into liver and lung, stimulate microphage activity, and generate organ toxicity^{151–153}. Active delivery can be achieved by introducing targeting moieties into the RNA nanoparticles. These functionalized RNA nanoparticles, with the combination of detection molecules, targeting moieties, and therapeutics, can prospectively be applied to the diagnosis and therapy of cancer or viral disease. pRNA nanoparticles have been used to escort siRNA to silence genes and destroy cancer cells in leukemia, lung, breast, ovarian, prostate, and many other cancers^{82–89}. Furthermore, multiple therapeutics, reporters, and/or targeting payloads can

be incorporated into the same RNA nanoparticle for achieving synergistic or enhanced therapeutic effects^{83,89,91}. In addition, the assembled RNA nano-scaffold can be potentially utilized to achieve the spatial organization of enzymes and/or proteins to reconstitute metabolic pathways²⁵, as well as the construction of biosensors for various applications^{154,155}.

Limitations

The sensitivity of RNA to RNase degradation and its potential dissociation due to the noncovalent link of self-assembled RNA quaternary structure has made many scientists shy away from RNA nanotechnology. However, chemical modification of the RNA ribose ring, such as 2'-fluorine (2'-F) modification, has been introduced as a successful strategy to increase RNA resistance to RNase degradation^{156–161}. Furthermore, the finding that certain chemically modified RNAs preserve their folding property and original biological function implies the feasibility of RNA nanotechnology with diverse applications¹⁴⁹. Another key challenge for *in vivo* application of RNA nanoparticles is the dissociation that occurs in very dilute *in vivo* conditions after systemic injection. This tendency is no longer a concern, as demonstrated in the recent finding that the pRNA 3WJ core and its derivative are thermodynamically stable, resistant to denaturation, and remain intact at ultra-low concentrations after systemic injection^{90,91}.

Some other limitations include, size limitations (typically less than 80 nt) of industrial scale synthesis of chemically modified RNA; cost of chemically synthesized RNA nanoparticles; and endosome escape of RNA nanoparticles after cell entry. The cost and size limitations can be overcome with time as the technology develops further. Incorporation of endosome-disrupting reagents^{162–165} might improve endosome escape of RNA nanoparticles.

Experimental design

In Figure 1, the workflow of this protocol is shown. When implementing the Procedure, please be aware that, as mentioned above, the functionalization of the RNA nanoparticles, **Steps 11–20**, is achieved concomitantly with RNA nanoparticle assembly. Therefore, the types, function, and number of functionalities to be fused to the RNA nanoparticles need to be established before-hand, prior to nanoparticle and DNA primer design (see Reagent setup).

This protocol is modular in nature, and, depending on specific experimental requirements, any number (from zero to five) of the five functionalizations covered in the Procedure — incorporation of siRNAs, RNA aptamers, RNA ribozymes, targeting ligands, and fluorophores — and described in sequential **Steps 11–20** can be implemented in no pre-specified order. Similarly, the RNA nanoparticle characterization approaches described (**Steps 21–31**) are mutually independent procedural 'modules', some or all of which can be implemented, keeping in mind that the order in which they are performed is unimportant. In general, for a good level of RNA nanoparticle characterization, we recommend implementing at least one mutually alternative experiment in each step (**Steps 21–31**).

MATERIALS

REAGENTS

CRITICAL: Unless specified, all the reagents can be replaced with other brands, if available.

- Diethyl pyrocarbonate (DEPC, Sigma, cat no. D-5758)
- Milli-Q water, 18.2 MΩ cm⁻¹ resistivity

- Urea, for molecular biology, DNase-free, RNase-free, and protease-free (Acros, cat no. 327380050)
- Tris base (Fisher, cat no. BP-152-5)
- Acetic acid, glacial (HAc, Fisher, cat no. A38C-212)
!CAUTION: HAc is a weak acid; it is corrosive and is a skin, eye, and respiratory tract irritant. Wear gloves and goggles, and dispense it in a vented hood.
- Hydrochloric acid (HCl; Fisher, cat. no. A144-212)
!CAUTION: HCl is a strong acid; it is very corrosive and is a skin, eye, and respiratory tract irritant. HCl can cause severe burns. Wear gloves and goggles, and dispense it in a vented hood.
- EDTA disodium salt, dihydrate (Sigma, cat no. E-5134)
!CAUTION: EDTA is a skin, eye, and respiratory tract irritant.
- Sodium hydroxide (NaOH, Fisher, cat no. S318-3)
!CAUTION: NaOH is corrosive and can cause chemical burns. Wear gloves, lab coat, and eye protection when handling the material or its solutions. Dissolution of NaOH is highly exothermic, and the resulting heat may cause heat burns or ignite flammables. It also produces heat when reacted with acids.
- Potassium hydroxide (KOH, Fisher, cat no. P250-500)
!CAUTION: KOH is corrosive and water reactive. Cause severe eye and skin burns. Wear gloves and goggles.
- HEPES, for molecular biology (Fisher, cat no. BP310-500)
- Glycine (Fisher, cat no. BP381-500)
- Sucrose (Fisher, cat no. S5-3)
- Formamide (Fisher, cat no. F84-1)
!CAUTION: Formamide is harmful if swallowed, inhaled or absorbed through the skin. Cause eye and skin irritation. May cause respiratory tract irritation, central nervous system effect, and liver damage. Wear gloves.
- Glycerol (Fisher, cat no. G33-4)
- Potassium chloride (KCl, Mallinckrodt, cat no. 6858)
- Sodium chloride (NaCl, Fisher, cat no. S271-500)
- Magnesium chloride hexahydrate (MgCl_2 , Fisher, cat no. M33-500)
- Magnesium acetate (MgOAc_2 , Sigma, cat no. M-0631)
- Manganese chloride (MnCl_2 , Sigma, cat no. M3634)
- Sodium phosphate dibasic heptahydrate (Na_2HPO_4 , Fisher, cat no. S373-500)
!CAUTION: May cause irritation to eye, skin, and respiratory tract. Wear gloves, mask, and goggles.
- Potassium phosphate monobasic (KH_2PO_4 , Mallinckrodt, cat no. 7100)
!CAUTION: May cause irritation to eye, skin, and respiratory tract. Wear gloves, mask, and goggles.

- Ethylenediamine dihydrochloride (Thermo Scientific, cat no. 23031)
!CAUTION: Ethylenediamine may cause eye, skin and respiratory tract irritation. May be harmful if swallowed, inhaled, or absorbed through the skin. Wear gloves, mask, and goggles.
- Cystamine dihydrochloride, 97% (Acros Organics, cat no.111770250)
!CAUTION: Cystamine causes eye, skin, and respiratory tract irritation. May be harmful if swallowed, inhaled, or absorbed through the skin. Wear gloves, mask and goggles.
- Sodium tetraborate ($\text{Na}_2\text{B}_4\text{O}_7$, Fisher, cat no. S252-10)
- Tween 20 (Anatrace, T1003)
- 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Thermo Scientific, cat no. 22980)
!CAUTION: EDC is moisture sensitive. May cause eye and skin irritation. Wear gloves and goggles.
- Imidazole, ACS reagent (Sigma, cat. no. I2399)
- Malachite green oxalate (Acros Organics, cat no. 229780250)
!CAUTION: Malachite green is light sensitive. Harmful and dangerous to the environment.
- Spermidine trihydrochloride (Sigma, cat no. S2501)
!CAUTION: Spermidine causes skin, eye, and respiratory tract irritation. Wear gloves, mask and goggles.
- Dithiothreitol (DTT, Acros, cat no. 16568-0050)
!CAUTION: DTT is an eye irritant.
- Ethanol, 100% (Pharmco-AAPER, cat no. 111000200)
!CAUTION: Ethanol is a flammable liquid.
- Methanol (Fisher, A412-4)
!CAUTION: Methanol is a dangerous, poisonous, and flammable liquid and vapor. Harmful if inhaled. May be fatal or cause blindness if swallowed. May cause eye and skin irritation. May cause central nervous system depress, kidney damage, reproductive and fetal effects. Wear gloves and goggles, and dispense it in a vented hood.
- Sodium acetate trihydrate (NaOAc , Fisher, cat no. S209-3)
- Boric acid (Fisher, cat no. A74-500)
- Acrylamide, 99+%, electrophoresis grade (Acros Organics, cat no. 164850025)
!CAUTION: Acrylamide may cause cancer and heritable genetic damages. Harmful by inhalation and in contact with skin. Toxic if swallowed. Wear gloves and mask.
- Bis-Acrylamide (Fisher, BP171-100)

!CAUTION: Bis-Acrylamide causes eye, skin and respiratory tract irritation. Harmful if inhaled, swallowed, or absorbed through skin. May cause central nervous system effects. Wear gloves, goggles, and mask.

- Acrylamide/Bis 40% (wt/vol) solution, acrylamide/bis-acrylamide ratio 29:1 (Fisher, cat no. BP1408-1)
- TEMED, electrophoresis grade (Fisher, cat no. BP150-20)

!CAUTION: TEMED is a skin and respiratory tract irritant.

- Ammonium persulfate (APS, Fisher, cat no. BP179-100)
- Ammonium acetate (NH₄OAc, J.T. Baker, cat no. 0596-01)
- Sodium dodecyl sulfate (SDS, Fisher, cat no. S529-500)

!CAUTION: SDS is an eye and respiratory tract irritant.

- Agarose, molecular biology grade (Thermo scientific, cat no. 17852)
- Synergel (Diversified Biotech, cat no. SYN-100)
- Clorox regular bleach (VWR, cat. no. 37001-058)

!CAUTION: Bleach is corrosive, and can cause severe irritation or damage to eyes and skin. It is harmful if swallowed.

- Ethidium bromide (EtBr, Fisher, cat. no. P1302-10)

!CAUTION: EtBr is a strong carcinogen; it is a skin, eye, and respiratory tract irritant. Wear gloves, and dispense in a hood.

- Xylene cyanol (Sigma, cat. no. X-4126)

!CAUTION: Xylene cyanol is a skin and respiratory tract irritant.

- Bromophenol blue (Sigma, cat. no. B-8026)

!CAUTION: Bromophenol blue is a skin and respiratory tract irritant.

- QIAEX® II gel extraction kit (Qiagen, cat no. 20021)
- Illustra™ RNAspin Mini kits (GE Healthcare, cat no. 25-0500-72)
- TRIZOL® reagent (Life Technologies, cat no. 15596-018)

!CAUTION: TRIZOL® reagent is toxic in contact with skin and if swallowed. Causes burns. Wear gloves.

- GoTaq Flexi DNA polymerase Kit: including GoTaq Flexi DNA polymerase (5 U/μl, Promega, cat no. M8295), 5X colorless GoTaq Flexi reaction buffer (Promega, cat no. M8901), 25 mM MgCl₂ solution (Promega, cat no. A3511)
- 100 mM dNTP set, PCR grade (Life Technologies, cat no. 10297-117)
- T7 RNA polymerase (homemade, comparable purity and activity as commercial counterparts)
- Adenosine 5'-triphosphate (ATP, Sigma, cat no. A-7699)
- Guanosine 5'-triphosphate (GTP, Sigma, cat no. G-8877)
- Cytidine 5'-triphosphate (CTP, Sigma cat no. C-1631)
- Uridine 5'-triphosphate (UTP, Sigma, U-6750)

- Y639F mutant T7 RNA polymerase (homemade, comparable purity and activity as commercial counterparts)
- 2'-deoxy, 2'-fluoro cytosine 5'-triphosphate (2'-F CTP, Trilink, 50 mM solution)
- 2'-deoxy, 2'-fluoro uridine 5'-triphosphate (2'-F UTP, Trilink, 50 mM solution)
- RNase free DNase I (1 mg/ml, Fermentas, cat. no. FEREN0521)
- RNase A (DNase and protease-free; Thermo Scientific, cat. no. EN0531)
- Superscript® III first strand synthesis system for RT-PCR (Life Technologies, cat no. 18080-051)
- Dual-luciferase reporter assay system (Promega, cat no. E1960)
- Folic acid (Fisher, cat no. BP25195)
- RPMI medium 1640 (1×), folic acid free (Gibco by Life Technologies, cat no. 27016-021)
- RPMI-1640 medium (Hyclone, cat no. SH30255.01)
- Fetal bovine serum, heat inactivated (FBS, Sigma, cat no. F4135)
- Penicillin–streptomycin (PS, Gibco by Life Technologies, cat no. 15070-063)
- 0.25% (wt/vol) Trypsin–EDTA (1×) (Gibco by Life Technologies, cat no. 15140-122)
- Label IT® nucleic acid labeling kits: Label IT® CyTM 3 labeling kit (Mirus, cat no. MIR3600); Label IT® CyTM 5 labeling kit (Mirus, cat no. MIR3700); Label IT® fluorescein labeling kit (Mirus, cat no. MIR3200)
- SYBR® green I nucleic acid gel stain (Life Technologies, cat no. S-7567)
- Prolong® gold antifade reagent with DAPI (Life Technologies, cat no. P36935)
- TO-PRO®-3 iodide (642/661) (Life Technologies, cat no. T3605)
- Alexa Fluor® 488 phalloidin (Life technologies, cat no. A12379)
- Fluoromount-G (Southern Biotechnology Associates, cat no.0100-01)
- exACT gene 50bp mini DNA ladder (Fisher, cat no. BP2570100)
- Spectro multicolor broad range protein ladder (Fisher, cat no. SM1841)
- Anti-human survivin antibody (R&D systems, cat no. AF886)
- Anti β -actin antibody (Sigma, cat no. A2103)
- Goat anti-rabbit IgG, HRP-conjugated (Millipore, cat no. 12-348)
- ImmobilonTM western chemiluminescent HRP substrate (Millipore, cat no. WBKL S0100)
- Lipofectamine® 2000 transfection reagent (Life Technologies, cat no. 11668-019)
- RIPA buffer (Sigma, cat no. R0278)
- pEGFP-N2 vector (BD Bioscience Clontech, cat no.6081-1)
- pGL3-control vector (Promega, cat no. E1741)
- pRL-TK vector (Promega, cat no. E2241)

- Folate-DNA (Homemade as described in **Step 19**, and sequence is shown in Table 3) Cy3/Cy5/FAM-DNA (synthesized from Integrated DNA Technology or other appropriate commercial supplier)
- UTP, [α - ^{32}P]-3000Ci/mmol 10mCi/mL (PerkinElmer, cat no. NEG007H250UC)
!CAUTION: This reagent is radioactive. Shield protection is required. Wear lab coat and gloves. Follow the regulation of radioactive materials handling.
- CyTM3 NHS ester (GE Healthcare, cat no. PA13101)
!CAUTION: CyTM3 NHS ester is light sensitive. Toxic if swallowed. Wear protective gloves.
- CyTM3 Maleimide mono-reactive dye (GE Healthcare, cat no. PA23031)
!CAUTION: CyTM3 Maleimide Mono-reactive Dye is light sensitive. Toxic if swallowed. Wear protective gloves.
- Dimethyl sulfoxide (DMSO, Acros Organics, cat no. 348445000)
!CAUTION: Avoid contact with skin and eyes.
- Cy3 (Cy5)-AMP or GMP (homemade)¹⁶⁶
- Streptavidin (STV) agarose resin (Thermo Scientific, cat no. 20353)
- 6-week-old male nude mice (nu/nu) (Taconic, model no. NCRNU-M)

EQUIPMENT

- Freezer (−80 °C and −20 °C) and 4 °C refrigerator
- Milli-Q water purification system
- PCR machine (Eppendorf Mastercycler Gradient, model 5331)
- Real-time PCR detection system (Roche LightCycler 480 real-time PCR System)
- NanoDrop 2000 spectrophotometer (Thermo Scientific)
- pH meter (Fisher, Dual channel pH/ion meter AR25)
- Eppendorf centrifuge 5424 (Eppendorf)
- Agarose gel electrophoresis system (Biorad, mini-sub cell GT system)
- PAGE gel electrophoresis system (Hoefer, SE250 mighty small II for 8 × 7 cm gel)
- TGGE system (Biometra, model 024-000)
- Eagle eye II imaging system (Stratagen)
- Fluorescence microscope (Olympus)
- Optical microscope (Motic company, cat. no. SFC-11)
- SynergyTM 4 microplate reader (BioTek)
- Flow cytometer (Beckman)
- Fluorolog fluorospectrometer (Horiba Jobin Yvon)
- Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss)
- Trans-Blot® SD semi-dry transfer cell (Bio-Rad)
- Biosafety cabinet (Thermo Scientific, model 1307)

- Forma series II water jacket CO₂ incubator (Thermo Scientific)
- Typhoon FLA 7000 imaging system (GE Healthcare)
- Hand hold Mineralight® lamp (UVP, model UVGL-25)
- Flexible TLC plates (Selecto Scientific, cat no. 11078)
- SAVANT DNA120 Speed Vac concentrator (Thermo Electron Corporation)
- NucAway™ spin columns (Ambion, cat no. AM10070)
- Microfluro 1 96-well white Microtiter plates (Thermo Scientific, cat no. 14-245-194A)
- Immun-Blot™ PVDF membrane for protein blotting (Bio-rad, cat no. 162-0177)
- Premium autoradiography film (Deville Scientific, cat no. E3012)
- Cyclone storage phosphor system (Packard)
- Elutrap electroelution system (Whatman)
- MultiMode AFM NanoScope IV system (Veeco/Digital Instruments) operating in tapping mode.
- Regular tapping Mode Silicon Probes (Olympus from Asylum Research)
- Non-contact NSG01_DLC probes (K-Tek Nanotechnology)
- Mica sheets (green or ruby mica) (Asheville-Schoonmaker Mica Company)
- DynaPro 99 Dynamic Light Scattering (Wyatt Technology Corporation)
- Surgery station
- Syringe (28G1/2), operation scissors and tweezers (Fisher Scientific)
- IVIS® *in vivo* imaging system (PerkinElmer Inc.)
- *Mfold* (<http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form>)
- siRNA Wizard (<http://www.sirnawizard.com/>)
- AFM image analysis software (Veeco)
- *ImageJ* (<http://rsbweb.nih.gov/ij/download.html>), free download and installation. User instruction is also available online (<http://rsbweb.nih.gov/ij/>).

REAGENT SETUP

▲ **CRITICAL STEP:** Prepare all reagents and perform all experiments using pure water (Milli-Q, 18.2 MΩ cm⁻¹ resistivity). Use DEPC-treated water for experiments involving RNA.

!CAUTION: Wear gloves at all times.

DNA primers for *in vitro* RNA transcription—Determine the required RNA sequences for assembling RNA nanoparticles and design DNA sequences accordingly. Computational prediction of RNA folding and structure is necessary for determine the RNA sequence for pRNA nanoparticle construction. Predict the individual folding of pRNA nano-scaffold and functional RNAs using online RNA folding program, such as *Mfold*⁶⁷. Then, fuse the RNA sequences of scaffold and functional modules and run the folding program again. Compare the fusion structure with individual structures. The optimal condition is the core scaffold and

functional modules retain their individual authentic folding after fusion. Otherwise, a linker sequence (such as poly A or poly U) can be introduced to separate the functional RNA from scaffold structures to avoid potential folding interference. The sequences of core scaffolds include loop-extended pRNA, pRNA-3WJ, pRNA-X motif, and functional modules, as listed in Tables 2 and 3. Introduce a T7 promoter sequence (5'-TAATACGACTCACTATA-3') at the 5'-end of the DNA sequence to initiate RNA transcription. Design then the necessary DNA primers and order them from an appropriate commercial supplier.

▲ **CRITICAL STEP:** Make sure the RNA sequence starts with G after the T7 promoter sequence. Consider adding an extra G to the RNA 5'-end to increase the transcription efficiency without interfering with the folding of the RNA strand.

0.05% (vol/vol) DEPC aqueous solution. Add 0.05 ml of DEPC to 99.5 ml of pure water and shake the solution vigorously. Let incubate at 37 °C overnight, and then autoclave the solution to remove DEPC. This reagent can be stored in room temperature (RT, 23 °C) for 1 year.

0.5 M EDTA, pH 8. Dissolve EDTA in pure water. Stir vigorously and adjust pH to 8 with NaOH. This reagent can be stored at RT for 1 year.

Tris-Acetate EDTA (TAE) buffer, 1×. TAE buffer (1×) contains 40 mM Tris-acetate, 1 mM EDTA. This reagent can be stored at RT for 1 year.

Tris-Borate EDTA (TBE) buffer, 1×. TBE buffer (1×) contains 89 mM Tris base, 200 mM boric acid, and 2 mM EDTA. This buffer can be stored at RT for 1 year.

Tris-Borate Magnesium (TBM) buffer, 1×. TBM buffer (1×) contains 89 mM Tris base, 200 mM boric acid, and 5 mM MgCl₂. This buffer can be stored at RT for 1 year.

3 M NaOAc, pH 6.5. Dissolve NaOAc in pure water. Adjust pH to 6.5 with HAc. After autoclaving, this buffer can be stored at RT for 1 year.

2 M MgCl₂. This buffer can be stored at RT for 1 year.

Tris-Magnesium Saline (TMS) buffer, 1×. TMS buffer (1×) contains 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM MgCl₂. This buffer can be stored at RT for 1 year.

SDS running buffer, 1×. SDS running buffer (1×) contains 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 0.1% (wt/vol) SDS. This buffer can be stored at RT for 1 year.

6× loading buffer. 6× loading buffer contains 40% (wt/vol) sucrose, 0.1% (wt/vol) xylene cyanol FF, and 0.1% (wt/vol) bromophenol blue. This buffer can be stored at -20 °C for 1 year.

2× TBE loading buffer. 2× TBE loading buffer contains 95% (vol/vol) formamide, 18 mM EDTA, 0.025% (wt/vol) SDS, 0.025% (wt/vol) bromophenol blue, and 0.025% (wt/vol) xylene cyanol. This buffer can be stored at RT for 1 year.

2× SDS loading buffer. 2× SDS loading buffer contains 100 mM Tris-HCl (pH 6.8), 4% (wt/vol) SDS, 0.2% (wt/vol) bromophenol blue, 20% (vol/vol) glycerol, and 200 mM DTT. Store the SDS gel-loading buffer without DTT at RT. Add DTT from a 1 M stock just before using the buffer. After the addition of DTT, this buffer can be stored at 4 °C for 1 wk. 1 M DTT can be stored at -20 °C for 1 year.

10 % (wt/vol) APS. 10% (wt/vol) APS needs to be freshly made and can be stored at 4 °C for 1 wk.

SDS PAGE Transfer Buffer (pH 8.3), 1×. SDS PAGE transfer buffer (1×) contains 25 mM Tris base, 19 mM glycine, 20% (vol/vol) methanol. This buffer can be stored at RT for 1 year.

▲ **CRITICAL STEP:** Do not adjust pH.

Tris Buffered Saline (TBS) buffer, 1×. TBS buffer (1×) contains 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl. Tris concentration in TBS buffer varies from 20 mM to 100 mM from recipe to recipe. 50 mM is a commonly used concentration. This buffer can be stored at RT for up to 1 year. Add 0.1% (vol/vol) Tween-20 freshly to make TBS-T buffer for blotting membrane wash.

Agarose-synergel, 2% (wt/vol): Dissolve 0.64 g of synergel in 1 ml of 100% ethanol. Add 0.7 g of agarose and 100 ml of TAE buffer. Heat until agarose is dissolved completely and pour before the gel cools down. Add 1 µl of EtBr per 25 ml of gel. The gel needs to be freshly prepared.

!CAUTION: EtBr is a strong carcinogen; it is a skin, eye and respiratory tract irritant. Wear gloves and dispense it in the hood.

Urea denaturing PAGE gel, 10–15% (wt/vol). Combine 10–15% (wt/vol; 37.5:1) acrylamide, 8 M urea, 10% (wt/vol) APS and TEMED. The gel should be freshly prepared.

Native PAGE gel (TBM or TBE), 10–15% (wt/vol). Combine 10–15% (wt/vol; 37.5:1) acrylamide, 1× Tris-borate (TB) buffer (pH 7.8), 10 mM MgCl₂ (or 2 mM EDTA), 10% (wt/vol) APS and TEMED. The gel should be freshly prepared.

SDS-PAGE gel, 15% (wt/vol). The 15% (wt/vol) separation gel contains 1.5 M Tris-HCl (pH 8.8), 20% (wt/vol) SDS, acrylamide/bisacrylamide (30%/0.8%, wt/vol), water, 10% (wt/vol) APS and TEMED; 5% (wt/vol). Stacking gel contains 0.5 M Tris-HCl (pH 6.8), 20% (wt/vol) SDS, acrylamide/bisacrylamide (30%/0.8%, wt/vol), water, 10% (wt/vol) APS and TEMED. The gels should be freshly prepared.

T7 RNA polymerase transcription buffer, 5×. T7 RNA polymerase transcription buffer (5×) contains 400 mM HEPES-KOH (pH 7.5), 120 mM MgCl₂, 10 mM spermidine, and 200 mM DTT. Filter the buffer using 0.22 µm filter and aliquot. This buffer can be stored at –20 °C for up to 1 year.

rNTPs mix, 25 mM each. Make stock solution of each rNTP at 100 mM and adjust pH to 7.4 using HCl. Mix equal amounts of 100 mM rATP, rGTP, rCTP and rUTP to make the rNTPs mix. Aliquot the solution. This buffer can be stored at –20 °C for up to 1 year.

dNTPs mix, 2.5 mM each. This buffer can be stored at –20 °C for 1 year.

100 mM DTT solution. This buffer can be stored at –20 °C for 1 year.

RNA elution buffer, 1×. RNA elution buffer (1×) contains 0.5 M NH₄OAc, 10 mM EDTA, 0.1% (wt/vol) SDS in 0.05% (vol/vol) DEPC treated water. After autoclave, this buffer can be stored at RT for 6 months.

2'-F Transcription Buffer, 10×. 2'-F transcription buffer (10×) contains 400 mM Tris-acetate pH 8.0, 50 mM DTT, 10 mM EDTA, 100 mM Mg-acetate, 5 mM MnCl₂, and 80 mM spermidine. Filter the buffer using 0.22 µm filter and aliquot. This buffer can be stored at –20 °C for 1 year.

Annealing buffer, 10×. Annealing buffer (10×) contains 500 mM Tris-HCl pH 7.5, 500 mM NaCl, and 10 mM EDTA. Filter the buffer using 0.22 μm filter and aliquot. This buffer can be stored at RT for 1 year.

MG binding buffer, 10×. MG binding buffer (10×) contains 1 M KCl, 50 mM MgCl₂, and 100 mM HEPES (pH 7.4). This buffer can be stored at RT for 1 year.

Ribozyme reaction buffer, 10×. Ribozyme reaction buffer (10×) contains 200 mM MgCl₂, 200 mM NaCl, and 500 mM Tris-HCl, pH 7.5. This buffer can be stored at RT for 1 year.

0.1 M imidazole, pH 6. This buffer can be stored at RT for 1 year.

0.25 M ethylenediamine in 0.1 M imidazole, pH 6. This buffer can be stored at −20 °C for 6 months.

0.25 M cystamine in 0.1 M imidazole, pH 6. This buffer can be stored at −20 °C for 6 months.

5'-end labeling reaction buffer, 1×. 5'-end labeling reaction buffer (1×) contains 7.5 mM sodium phosphate, 150 mM NaCl, and 10 mM EDTA; pH 7.2. This buffer can be stored at RT for 1 year.

0.1M sodium tetraborate labeling buffer, pH 8.5. This buffer can be stored at −20 °C for 1 year.

Phosphate Buffered Saline (PBS), 1×. PBS buffer (1×) contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄; pH 7.4. This buffer can be stored at RT for 1 year.

STV binding buffer, 1×. STV binding buffer (1×) contains PBS buffer with 10 mM Mg²⁺. This buffer can be stored at RT for 1 year.

PROCEDURE

Amplification of the DNA template via PCR TIMING: ~1d

- 1 Suspend the DNA primers using TE buffer to a final concentration of 100 μM.
 ■ **PAUSE POINT:** The DNA primer solutions thus obtained can be stored for a few years at −20 °C.
- 2 Mix together 20 μl of 5X GoTaq Flexi Buffer, 10 μl of 25 mM MgCl₂ solution, 8 μl of 2.5 mM dNTPs solution, 2 μl each of up-stream and down-stream primer, 1 μl of DNA template (10–20 ng/μl), 0.5 μl of GoTaq polymerase, and adjust the final volume to 100 μl with 0.05% (vol/vol) DEPC water. Please note that the PCR reaction can be scaled up or down using the same concentration ratios just detailed.
- 3 Set up a PCR program as follows: 95 °C 5 min, 22 cycles × (94 °C 1 min, 55 °C 1 min, 72 °C 1 min), 72 °C 5 min, then keep at 4 °C. Run the PCR program.
- 4 Mix 2–5 μl PCR products with 6x running buffer and run in a 2% Syner Agarose gel in 1x TAE buffer (120 V at RT for 35 min) to verify the PCR products by size. Use exACT gene 50 bp mini DNA ladder as the size control.

?TROUBLESHOOTING

- 5 Add 2.5 volumes of 100% ethanol and 1/10 volume of 3 M NaOAc (pH 6.5) to the PCR products and allow them to precipitate at −20 °C overnight.

- 6 Centrifuge the sample at 16,500 g for 30 min at 4 °C, remove the supernatant, wash the pellet with 70% (vol/vol) ethanol once, and then dry the pellet for 5 min using a speed vacuum.
- 7 Re-suspend the pellet in 20 µl of 0.05% (vol/vol) DEPC water (5x concentrated).
 - **PAUSE POINT:** The DNA template can be stored at –20 °C in the short term (6 months) or at –80 °C for the long term (1 year +).
- 8 (OPTIONAL) If you plan to synthesize 2'-F-modified RNA, the DNA template will have to be of high quality. In this case, therefore, run the DNA products from the PCR in a 2% Syner Agarose gel using the conditions detailed in **Step 4**. Cut the band of interest under UV light and extract the PCR DNA template from the gel using the QIAEX II gel extraction kit (follow the manufacturer's guidelines). Concentrate if necessary to reach at least 0.5 µg/ul of amplified DNA template. Please note that If the DNA PCR products show specific and clean bands in the agarose gel (see **Step 4**), then an alternative to additional gel purification steps is to pass DNA through NucAway™ spin columns to remove free nucleotides and salts following manufacturer's guidelines.

!CAUTION: UV light is harmful for eyes, wear protective goggles or use shield while handling the experiments.

 - **CRITICAL STEP:** The purity and quantity of DNA used for 2'-F transcription are critical. The amount of DNA template needed for a typical 20-µl transcription reaction should not be lower than 1 µg.
 - **PAUSE POINT:** The DNA template can be stored at –20 °C in the short term (6 month) or at –80 °C for the long term (1 year +).

***In vitro* RNA synthesis TIMING: 2–3 d**

- 9 Synthesize RNA strands according to option A, if the objective is long unmodified RNA, option B, if it is long, chemically 2'-F-modified RNA, or option C if it is short unmodified or chemically modified RNA.

A. *In vitro* RNA transcription by T7 RNA polymerase

- i. Mix 10 µl of DNA template from **Step 7** with 10 µl of 5× transcription buffer, 10 µl of 25 mM NTPs solution, 5 µl of 100 mM DTT, and 10 µl of T7 RNA polymerase. Adjust the final reaction volume to 50 µL adding 0.05% (vol/vol) DEPC water. Incubate at 37 °C for 4 h. Please note that the transcription reaction can be scaled up or down using the same concentration ratios just detailed.
- ii. Terminate the transcription process by adding 1 µl of RNase-free DNase I and incubating at 37 °C for another 30 min.
- iii. Add 2× loading buffer to the transcription products and run the samples in 8% polyacrylamide gel with 8 M urea in TBE buffer at 100 V at RT.
- iv. Place the gel on the TLC plate and excise the band corresponding to the RNA of the desired length under the UV light (254 nm). Elute the RNA from the gel at 37 °C using RNA elution buffer (2 h for the first elution and 2 h for the second elution). Please note that the passive elution just described can be replaced by an electro-elution step by using the elutrap electroelution system and following manufacturer's guidelines.

- v. Take the supernatant from the elution step and add to it 2.5 volumes of 100% ethanol and 1/10 volume of 3 M NaOAc. Allow RNA to precipitate overnight at -20°C .
- vi. Centrifuge at 16,500 g for 30 min at 4°C and remove the supernatant; wash the pellet with 70% (vol/vol) ethanol and speed vacuum-dry the pellet for 5 min.
- vii. Dissolve the pellet in 0.05% (vol/vol) DEPC water.
 - **PAUSE POINT:** Store the resulting RNA solution at -20°C for the short term (6 months) or at -80°C for the long term (1 year).

?TROUBLESHOOTING

B. *In vitro* RNA transcription by Y639F mutant T7 RNA polymerase

- i. Use purified DNA PCR products (from **Step 8**) as the template to synthesize the 2'-F modified RNA *in vitro*^{167,168}. For this purpose, mix $>2.5\ \mu\text{g}$ of DNA template with $5\ \mu\text{l}$ of 10X 2'-F transcription buffer, $5\ \mu\text{l}$ of 100 mM DTT, $5\ \mu\text{l}$ of 50 mM 2'-F CTP, $5\ \mu\text{l}$ of 50 mM 2'-F UTP, $5\ \mu\text{l}$ of 50 mM ATP, $5\ \mu\text{l}$ of 50 mM GTP, and $5\ \mu\text{l}$ of Y639F mutant T7 RNA polymerase. Adjust the final volume to $50\ \mu\text{l}$ by adding 0.05% (vol/vol) DEPC water. Incubate at 37°C overnight. Please note that the transcription reaction can be scaled up or down using the same concentration ratios just detailed.
- ii. Purify RNA by following **Step 9 A ii–vi**. Please note that the yield of *in vitro*-transcribed 2'-F-modified RNA is usually at least 20% lower than that of the corresponding non-modified RNA.
 - **PAUSE POINT:** Store the resulting RNA solution at -20°C for the short term (6 months) or at -80°C for the long term (1 year +).

?TROUBLESHOOTING

C. Synthesis of short unmodified or chemically modified RNA oligos (< 30 nt)

- i. Order desired RNA oligonucleotides from either Integrated DNA Technology or Trilink, Inc. (following manufacturer's instructions).
 - **PAUSE POINT:** Store the synthesized RNA oligos at -80°C for 1 year.

Assembly of thermodynamically stable RNA nanoparticles using 'toolkits' TIMING: ~1 wk

CRITICAL: Following pRNA nomenclature¹⁶⁹, please note that the right-hand (R-loop) sequence is assigned an upper case letter (i.e., A, B, ...), and the left-hand (L-loop) sequence a lower case letter with a prime (i.e., a', b', ...). A pair of the same letters, one upper and one lower case (e.g. Aa'), designates a complementary sequence in the R/L interlocking loop, while different letters indicate a lack of interlocking sequence complementarity. To distinguish from the wild-type pRNA, "Ex" is added before the letters (e.g. ExAa') to indicate that the interlocking loop-loop interaction is extended (Fig. 3a). The 3WJ domain of phi29 pRNA is constructed using three RNA oligos, denoted as a_{3WJ}, b_{3WJ}, and, c_{3WJ}. The three branches are named H3-1, H3-2, and H3-3, respectively (Fig. 3c) The 3WJ-derived X-shaped pRNA is constructed using four RNA oligos denoted a_X, b_X, c_X, and c_{3WJ}. The four branches are named H4-1, H4-2, H4-3, and H4-4, respectively (Fig. 3d).

- 10 Assemble the nanoparticles according to option A, if constructing RNA nanoparticles via hand-in-hand interactions (Fig. 4a–e), option B, to achieve modular assembly employing foot-to-foot interactions (Fig. 4 f–j), or option C, if assembling them by branch extension (Fig. 4k–n).

A. Assembly of RNA nanoparticles via hand-in-hand modular design

- i. Construct the loop-extended pRNAs by replacing the 4-nt loop complementary region of the wild-type pRNA R- and L-loops with a series of extended loop sequences (Table 4).
 - **CRITICAL STEP:** Since altering the nucleic acid sequences might affect the global folding of pRNA molecules, the pRNA with re-engineered loop sequences should be thoroughly analyzed with the online RNA folding program, such as *Mfold*⁶⁷.
- ii. Design RNA sequences using reengineered loop-loop interactions (Table 4): pRNA dimer (ExAb'-ExBa'), trimer (ExBa'-ExCb'-ExAc'), tetramer (ExBa'-ExCb'-ExDc'-ExAd'), pentamer (ExBa'-ExCb'-ExDc'-ExFd'-ExAf'), hexamer (ExBa'-ExCb'-ExDc'-ExEd'-ExFe'-ExAf'), and heptamer (ExBa'-ExCb'-ExDc'-ExEd'-ExFe'-ExGf'-ExAg'). Design primers according to the instructions in Reagents setup and synthesize RNA strands following **Steps 1–9** of the Procedure.
- iii. Mix required monomeric subunits to assemble hand-in-hand pRNA polyvalent nanoparticles in an equal molar ratio in TMS buffer. Incubate at 37 °C for 1 h and assay the formation of RNA complexes via 6% native PAGE gel run in TBM buffer under 80V for 3–4 h at 4 °C.
- iv. Purify the hand-in-hand assemblages by 6% native PAGE gel run in TBM buffer. Excise the band corresponding to each assembled multimer (dimer, trimer, tetramer, pentamer, hexamer, or heptamer, respectively) and elute the RNA by using RNA elution buffer in the presence of 10 mM Mg²⁺. Precipitate the RNA as in **Steps 9 A v–vi** and rehydrate the pellet in TMS buffer.
 - **PAUSE POINT:** The RNA nanoparticles obtained can be stored at –20 °C for the short term (1 month) or at –80 °C for the long term (6 months).

?TROUBLESHOOTING

B. Assembly of RNA nanoparticles via foot-to-foot modular design

- i. A palindrome sequence reads the same way in the 5' to 3' direction on one strand as in the 5' to 3' direction on a complementary strand. Design the 6-nt palindrome sequences following the chart in Fig. 3b, and introduce the palindrome sequence at the 3'-end of the RNA building blocks to bridge RNA nanostructures, motifs, or scaffolds for self-assembling RNA hexamers, octamers, decamers, and dodecamers or any other duplex with an even number of subunits.
- ii. Extend the 3'-end of ExAb' with a palindrome sequence (5' CGAUCG 3') to construct foot-to-foot RNA complexes. Design primers according to the instructions in Reagents setup and synthesize RNA strands by following **Steps 1–9** of the Procedure.
- iii. Introduce an ExAb' subunit with a 3'-over hanged palindrome sequence into trimer (ExBa'-ExCb'-ExAc'), tetramer (ExBa'-ExCb'-ExDc'-ExAd'), pentamer (ExBa'-ExCb'-ExDc'-ExFd'-ExAf'), hexamer (ExBa'-ExCb'-ExDc'-ExEd'-ExFe'-ExAf'), and heptamer (ExBa'-ExCb'-ExDc'-ExEd'-ExFe'-ExGf'-ExAg') at an equal molar ratio in TMS buffer. Incubate at 37 °C for 1 h and assay the formation of RNA complexes using 4% native PAGE gel run in TBM buffer under 80V for 4–5 h at 4 °C.
- iv. Purify the foot-to-foot assemblages by 4% native PAGE gel run in TBM buffer. Excise the band corresponding to each assembled foot-to-foot constructs (twin

dimer, twin trimer, twin tetramer, twin pentamer, twin hexamer, or twin heptamer, respectively) and elute the RNA using RNA elution buffer in the presence of 10 mM Mg^{2+} . Precipitate the RNA as in **Steps 9 A v–vi** and rehydrate the pellet in TMS buffer.

▲ **CRITICAL STEP:** The assemblage of the hand-in-hand and foot-to-foot nanostructures requires the presence of at least 5 mM Mg^{2+} . Add Mg^{2+} into the RNA elution buffer to ensure structure integrity during the purification process. Keep the purified products in TMS buffer when storing.

■ **PAUSE POINT:** The RNA nanoparticles obtained can be stored at $-20^{\circ}C$ for the short term (1 month) or at $-80^{\circ}C$ for the long term (6 months).

C. Assembly of RNA nanoparticles by branch extension

- i. Construct the 3WJ domain by mixing together the three RNA oligos denoted as a_{3WJ} , b_{3WJ} , and, c_{3WJ} at 1:1:1 molar ratio in 0.05% (vol/vol) DEPC-treated water or TMS buffer. Load the resulting solution onto a 15% native PAGE gel and run the gel in TBM buffer under 100V at $4^{\circ}C$ for 1–2 h to purify the complex. The assembly of 3WJ domain from three RNA oligos is highly efficient as long as the three RNA oligos were mixed in equal molar ratio. There will be only one major 3WJ band slightly lower than xylene cyanol dye position. Excise that band from the gel and elute the RNA in RNA elution buffer for ~4 h at $37^{\circ}C$, followed by ethanol precipitation overnight. Rehydrate the dried pellet in DEPC-treated water or TMS buffer.
- ii. Analyze the formation of the complexes using 15% native PAGE or 8 M urea PAGE gel in TBM running buffer. After running the gel under 100 V at $4^{\circ}C$ for 3 h, visualize the RNA by EtBr or SBYR Green II staining.
- iii. Construct the pRNA-X motif by opening the R-loop of the pRNA subunit and inserting a 9-bp sequence, thereby forming a double helical segment (H4-2), and extending the H4-3 helix by 4 bp. The X-shaped motif can then be assembled from four RNA oligos, denoted as a_X , b_X , c_X , and c_{3WJ} . Mix the four RNA oligos in a 1:1:1:1 ratio in the absence of metal salts. For the detailed assemblage procedure, please refer to **Steps 10 C i–ii**.

■ **PAUSE POINT:** The RNA nanoparticles obtained can be stored at $-20^{\circ}C$ for the short term (3 months) or at $-80^{\circ}C$ for the long term (1 year).

Construction and assay of RNA nanoparticles fused with siRNA. TIMING: ~1 wk

CRITICAL: Please note that, as mentioned in the Experimental design, the sub-sections that cover **Steps 11–20** are procedural ‘modules’, none, some, or all of which can be implemented, depending on the needs of the experimenter, in no pre-specified order.

- 11 Altering the primary sequences of the 5'/3'-end helical region of an RNA nanoparticle does not affect the structure and folding of the RNA, as long as the two strands are paired⁷⁷; furthermore, extensive studies have revealed that double-stranded siRNA are able to replace the helical region in RNA nanoparticles and retain their function^{82,83,88–91}. The siRNA sequences can be separated from the scaffold sequences by introducing an AA or UU bulge on the double helical strands. The 3' end 2-nt overhang of the siRNA should be kept for Dicer recognition, binding, and further processing. In order to assay the incorporated siRNA function, a negative control siRNA should be designed to compare with the active siRNA component. There are two kind of negative control siRNA design, one is a point mutation along the siRNA sequence and

the other is a scrambled design using siRNA Wizard (<http://www.sirnawizard.com/>). After design, synthesize the siRNA-fused RNA nanoparticles according to **Steps 1–10**.

- 12 Assay the efficacy of the RNA nanoparticles fused to siRNAs that has the purpose to knock down a fluorescent reporter (option A), a luminescent reporter (option B), or an anti-apoptotic factor (option C).

A. Assaying function of RNA nanoparticles fused with *GFP* siRNA

- i. Seed 10^5 KB cells (human nasopharyngeal epidermal carcinoma) in 24-well plates using RPMI-1640 with 10% FBS a day prior to transfection.
- ii. 24 h after seeding, check the cell confluence, and, once the cells are 70–80% confluent, co-transfect them with GFP-expressing plasmid pGFP-N2 and RNA nanoparticles harboring *GFP* siRNA (or RNA nanoparticles harboring GFP negative control siRNA (see step 11) using Lipofactamine® 2000 transfection reagent (follow manufacturer's guidelines).
- iii. 24 h after transfection, measure the effect at the level of GFP expression by fluorescence microscopy ^{82,83}.

B. Assaying function of RNA nanoparticles fused with *Luciferase* siRNA

- i. Seed 10^5 KB cells in 24-well plates using RPMI-1640 with 10% FBS a day prior to transfection.
- ii. 24 h after seeding, check cell confluence, and, once the cells are 70–80% confluent, co-transfect them with RNA nanoparticles harboring *firefly luciferase* siRNA (or RNA nanoparticles harboring negative control *firefly luciferase* siRNA) with both plasmid pGL3 coding for firefly luciferase and pRL-TK coding for renilla luciferase by Lipofactamine® 2000 transfection reagent (follow manufacturer's guidelines). pRL-TK coding for renilla luciferase serves as an internal control to normalize the luciferase data.
- iii. 24 h after transfection, use the Dual-luciferase reporter assay system to assay the *firefly luciferase* activity after fusion with the RNA nanoparticle (follow manufacturer's guidelines). Briefly, wash cells once with PBS and lyse with passive lysis buffer. Shake plates for 15 min at RT. Add 20 μ l of lysate to 100 μ l of luciferase assay reagent (LAR II) to a Microfluoro 1 96-well white microtiter plates and measure firefly luciferase activity by using Synergy™ 4 microplate reader. Upon addition of 100 μ l of Stop & Glo reagent, obtain control measurements of *renilla luciferase* activity. Normalize the previously obtained data with respect to the *renilla* activity to determine the average ratio of *firefly* to *renilla* activity over several trials (Fig. 5a).
- iv. Due to the multivalency of the RNA nanoparticles, multiple copies of siRNA targeting to the same loci within one gene, or different siRNAs targeting different loci within one gene, can be applied to one RNA nanoparticle to generate enhanced gene knock-down effects. The Dual-luciferase report assay system can also be used to assay the enhanced siRNAs targeting effects towards the *firefly luciferase* gene ^{91,169} (Fig. 6).

C. Assaying function of RNA nanoparticles fused with *Survivin* siRNA [Au; Subheading OK? Yes.]

- i. KB cells over-express survivin. Seed 10^5 KB cells in 24-well plates using RPMI-1640 with 10% FBS a day prior to transfection.
- ii. Transfect cells using Lipofactamine® 2000 transfection reagent (follow manufacturer's guidelines) with 25 nM of the RNA nanoparticle harboring *survivin* siRNA. Similarly do the same experiment with negative control RNA nanoparticles with scrambled *survivin* siRNA, and a positive control *survivin* siRNA (Ambion, Inc.) to evaluate the silencing efficiency of the RNA nanoparticles.
- iii. After 48 h of treatment, collect cells and assess target gene silencing effects by both RT-PCR/qRT-PCR assay on mRNA level and western blot to assay protein level (Fig. 5b).
- iv. To conduct the RT-PCR/qRT-PCR assay, extract total RNA using Illustra RNAspin Mini kits. Synthesize first DNA strand using SuperScript™ III first-strand synthesis system (follow manufacturer's guidelines), see below for primers.

Gene	Left primer	Right primer
<i>GAPDH</i> ¹⁷⁰	5'-GCCACATCGCTCAGACAC-3'	5'-GCCCAATACGACCAATCC-3'
<i>BIRC5</i> ¹⁷¹	5'-CACCGCATCTCTACATTCAAGA-3'	5'-CAAGTCTGGCTCGTTCTCAGT-3'

- v. Perform PCR using GoTaq Flexi DNA polymerase (Promega). For this purpose, prepare a reaction mixture to a final volume of 25 μ L that contains complementary DNA from first-strand synthesis, 1 $\times$ GoTaq Flexi colorless buffer, 2.5 mmol/l Mg^{2+} , 0.2 mmol/l dNTPs, 0.2 μ mol/l in each primer, and 0.02 U/ μ l GoTaq Flexi DNA polymerase. Use the following PCR program: 95 °C for 5 min then 25 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min. Assay PCR results using 2% Syner/Agarose gel electrophoresis and visualize by EtBr staining.
- vi. Perform real-time PCR using Roche universal probe library assay (follow manufacturer's guidelines). All reactions should be carried out in a final volume of 10 μ l and assayed in triplicate. Performing qRT-PCR on Lightcycler 480 for 45 cycles. Analyze the data by the comparative Ct Method ($\Delta\Delta$ Ct Method) following published procedures¹⁷². Briefly, assay each sample in triplicates, obtain cycle time (Ct) and calculate the average Ct. Obtain Δ Ct by normalizing the average Ct of the gene of interest (*BIRC5*) to the average Ct of the reference gene (*GADPH*). Choose the negative control group as the calibrator and the $\Delta\Delta$ CT, or calibrated value, for each sample = Δ Ct_{sample} - Δ Ct_{calibrator}. The fold-induction for each sample relative to the calibrator = $2(-\Delta\Delta$ Ct). Plot the resulting induction values as a bar graph for comparison.
- vii. To perform western blot assays, lyse cells by RIPA lysis buffer and extract the cell total protein. Load equal amounts of protein with 2 $\times$ SDS loading buffer onto 15% SDS-PAGE and run the gel at RT at 100 V for 2~3 h. Electrophoretically transfer the protein to Immobilon-PVDF membranes using Trans-Blot® SD semi-dry transfer cell. Probe the membrane with anti-human survivin antibody (1:4000 dilution) and anti β -actin antibody (1:5000 dilution) overnight, followed by 1:10000 goat anti-rabbit IgG, HRP-conjugated secondary antibody for 1 h. Blot

membrane using Immobilon™ western chemiluminescent HRP substrate and expose to film for autoradiography.

Construction and *in vitro* assay of RNA nanoparticles fused to RNA aptamers TIMING: 3–4 d

- 13 Like antibodies, RNA aptamers selected from systematic evolution of ligands by exponential enrichment (SELEX)^{56–60} are able to bind to specific targets including proteins, organic compounds, and nucleic acids^{173–175}. Design suitable aptamers for your own objectives keeping in mind that aptamers can be linked to the 3' and 5' end of the RNA scaffold and that, to facilitate independent folding, poly U or poly A linkers can be placed between the RNA scaffold and the aptamer. After design, synthesize the aptamer-fused RNA nanoparticles according to **Steps 1–10**.
- 14 Assay function of aptamer-fused RNA nanoparticles by fluorescence emission (option A), a property which can be used for *in vivo* tracking, (see Fig. 5c); cell binding (option B), which can be used as a way to define targets for delivery; and ligand binding (option C), which can be exploited for the purification of RNA nanoparticles (Fig. 5d).

A. Assaying function of RNA nanoparticles fused with aptamers that fluoresce upon dye binding

- i. Analyze the malachite green (MG)–binding aptamer in monomeric form or in RNA nanoparticle assemblies by native PAGE run in TBM running buffer at 4 °C, 90V for 3–4 h. The RNA nanoparticle without MG-binding aptamer serves as negative control. Stain the gel with 5–10 μM MG in MG binding buffer and image with Typhoon FLA 7000 under Cy5 channel. MG is not fluorescent by itself, but emits fluorescent light only after binding to the RNA aptamer.
- ii. Mix the MG-binding aptamer in monomeric form (without fusion to RNA nanoparticles), RNA nanoparticles harboring the MG-binding aptamer, and RNA nanoparticle without MG-binding aptamer (at a concentration of 100 nM) with MG dye (2 μM) in MG binding buffer. Incubate the reaction at RT for 30 min and measure the fluorescence using Fluorolog fluorospectrometer (excitation wavelength = 615 nm and emission spectrum = 630–800 nm or excitation wavelength = 425 nm and emission spectrum = 575–800 nm) (Fig. 5c).^{90,176} The RNA nanoparticles without the MG-binding aptamer serve as a negative control, while the MG-binding aptamer by itself serves as a positive control.

B. Assaying function of RNA nanoparticles fused with aptamers recognizing a specific cell-surface marker

- i. Maintain cells [American Type Culture Collection (ATCC)] in complete culture medium. Trypsinize cells and rinse them with PBS once. Incubate 2×10^5 cells with either Cy3-labeled RNA nanoparticles harboring an aptamer or with control RNA nanoparticles not harboring the aptamer at 37 °C for 1–4h. After washing with PBS, resuspend cells in PBS buffer and assay them by flow cytometry.
- ii. (OPTIONAL) This assay can be performed via confocal microscopy instead of the flow cytometry approach described in step 14B i. For this purpose, grow cells on glass coverslides in complete culture medium overnight. Incubate cells with either Cy3-labeled RNA nanoparticles harboring an aptamer or with control RNA not harboring the aptamer at 37 °C for 2 h. After washing with PBS, fix cells with 4% (vol/vol) paraformaldehyde at RT for 30 min. Wash cells with PBS. Stain cells

using Alexa Fluor® 488 phalloidin (follow manufacturer's guidelines) for the cytoskeleton and TO-PRO®-3 iodide (642/661) (follow manufacture's guidelines) for the nucleus. Mount the cells using Fluoromount-G. Assay for binding and cell entry with a Zeiss LSM 510 laser scanning confocal microscope.

C. Assaying function of RNA nanoparticles fused with aptamers that bind to streptavidin or sephadex

- i. Premix and preassemble RNA nanoparticles harboring streptavidin (STV) - binding aptamer ¹⁷⁷ in STV binding buffer before incubation with STV agarose resin.
- ii. Equilibrate 50 µl of STV resin in a test tube at RT and wash with STV binding buffer three times.
- iii. Incubate RNA nanoparticles harboring STV binding aptamer with STV resin at RT for 1 h. RNA nanoparticles without the STV binding aptamer serve as a negative control. After incubation, spin the resin at 500 g for 1 min and remove the supernatant (pass through). Add STV binding buffer and incubate with resin for 15 min to wash. Spin the resin at 500 g for 1 min and remove the supernatant. Repeat washing step seven times (washes 1–7).
- iv. Elute out RNA with 50 µL of 5 mM biotin in STV binding buffer; the elution step can be repeated twice (elutions 1–2). Analyze the sample fractions (RNA before loading, pass through, washes 1–7, and elutions 1–2) by native PAGE run in TBM buffer and visualized with EtBr staining (Fig. 5d).

Construction and assay of RNA nanoparticles harboring ribozymes **TIMING: 1 d**

- 15 RNA ribozymes (RNA enzymes) have the ability to catalyze chemical reactions ^{44,48}, cleave mRNA, and regulate gene expression at post-transcriptional level. In particular, an anti-HBV ribozyme cleaves the genomic RNA of the Hepatitis B Virus genome ⁸⁴. An RNA nanoparticle fused with this ribozyme is able to cleave the 135-nt HBV RNA genome substrate into two fragments, of 60-nt and 75-nt, respectively ^{84,90} (Fig. 5e). RNA nanoparticles that harbor anti-*survivin* ribozymes, on the other hand, target the anti-apoptosis factor *survivin* and down-regulate genes involved in tumor development and progression ⁸⁶. Design suitable ribozymes to be fused to your RNA nanoparticle keeping in mind that ribozymes can be linked to the 3' and 5' end of RNA scaffold. To facilitate independent folding, poly U or poly A linkers can be placed between the RNA scaffold and the ribozyme. Synthesize ribozyme-fused RNA nanoparticles following **Step 1–10**. Please note that two 'anti-HBV ribozyme' nanoparticles should be synthesized, one that contains the active ribozyme — the positive control ⁸⁴ — and one in which the fused ribozyme has one point mutation that abolishes its function, which serves as a negative control, see Table 3 for the sequences of the active and disabled anti-HBV ribozyme.
- 16 To assay ribozyme-fused RNA nanoparticles for their function, radiolabel the RNA substrate by incorporating [α -³²P] UTP during transcription. For this purpose, SpeedVac dry 10 µl of [α -³²P] UTP. Add to the dried [α -³²P] UTP 10 µl of DNA template from Step 7 with 10 µl of 5× transcription buffer, 10 µl of 25 mM NTPs solution, 5 µl of 100 mM DTT, and 10 µl of T7 RNA polymerase. Adjust the final reaction volume to 50 µl by adding 0.05% (vol/vol) DEPC water. Incubate the resulting solution at 37 °C for 4 h. This transcription reaction can be scaled up or down maintaining the reagents' proportions just detailed. Purify RNA according to **Step 9A**. Alternatively, fluorescent label the HBV

genomic RNA substrate using Label IT® nucleic acid labeling kits (following manufacturer's guidelines).

!CAUTION: This reagent is radioactive. Shield protection is required. Wear lab coat and gloves. Follow the regulation of radioactive materials handling.

- 17 Incubate the RNA nanoparticles harboring anti-HBV ribozyme at 37 °C for 60 min in ribozyme reaction buffer.
- 18 After incubation, load samples with 2× loading buffer on 8 M urea/10% (wt/vol) PAGE gel at RT for 1–2 h for autoradiograph or fluorescent imaging.

Construction of RNA nanoparticles harboring targeting ligands **TIMING: 3–4 d**

- 19 Conjugate the 5'-end-NH₂ of the DNA oligo (ordered from IDT, please also see Table 3) with folate-N-hydroxysuccinimide ester (folate-NHS ester) according to the published procedure¹⁷⁸. Purify the folate-DNA conjugates with HPLC. Please note that folate-labeled RNA oligos with the same sequence as the DNA oligo can also be obtained from Trilink. Design the RNA scaffold with a sequence complementary to the folate-DNA/RNA sequences for annealing and docking the folate into the RNA nanoparticles. Mix equal molar amounts of folate-DNA/RNA conjugate and RNA scaffolds in annealing buffer. Incubate at 80 °C for 5 min and slowly cool down to room temperature. Purify the RNA nanoparticle harboring folate by either 8% denaturing PAGE or 10% native PAGE gel in TBE buffer. Assay the folate-mediated binding of RNA nanoparticles to the folate receptor and subsequent cell internalization according to **Step 14 B** (see also Fig. 5f).

?TROUBLESHOOTING

▲ **CRITICAL STEP:** To ensure sufficient binding, the cells need be kept in a folate-free medium for at least 12 h prior to the binding assay.

Construction of RNA nanoparticles harboring imaging molecules **TIMING: 3–4 d**

- 20 Many different strategies have been developed to achieve co-transcriptional (option A) or post-transcriptional (option B, C, and D) labeling of RNA molecules. These labeling strategies have been demonstrated to be highly efficient for the preparation of long-chain RNAs^{169,179,180} and can be distinguished according to whether they implement single-molecule labeling (option A, B, and C) or random labeling of the whole RNA chain (option D). Co-transcriptional labeling method is easy to handle but the yield of labeling products cannot be guaranteed. Use this approach for experiments only requiring small amount of labeled RNA. Post-transcriptional single-stranded RNA can be used for large scale RNA labeling; however, it is multiple-step process, reaction conditions are harsh, and may cause damage to RNA strands. Use this method for chemically modified stable RNA strands. Post-transcriptional whole chain labeling method is suitable for RNA without folding dependent function.

A. Single-molecule labeling of RNA by incorporating fluorescent AMP or GMP during *in vitro* transcription—In this approach, the labeling is performed at the 5'-end of the RNA, and methods to accomplish this goal with a single chemical group have been reported^{179–181}. Perform labeling during *in vitro* transcription using T7 RNA polymerase. Monophosphate AMP or GMP can be used with the polymerase only for initiation, but not for chain extension which requires a triphosphate.

▲ **CRITICAL STEP:** GMP initiates transcription using dsDNA template containing the T7 promoter sequence: 5'-TAATACGACTCACTATATA-3'. AMP initiates transcription using dsDNA templates containing the T7 class II promoter (Ø2.5): 5'-TAATACGACTCACTATTA-3'.

- i. Set up the *in vitro* transcription reaction by mixing the dsDNA template with 10 µl of 5× transcription buffer, 10 µl of rNTPs solution (25 mM GTP, 25 mM CTP, 25 mM UTP, and 2.5 mM ATP), 5 µL 100 mM Cy3-AMP, 5 µl of 100 mM DTT, and 10 µl of T7 RNA polymerase. Adjust the final reaction volume to 50 µl by 0.05% (vol/vol) DEPC water. Incubate at 37 °C overnight. The transcription reaction can be scaled up or down maintaining the reagents' proportions just detailed.

▲ **CRITICAL STEP:** In order to increase the incorporation efficacy of the Cy3-AMP, the ATP concentration is decreased 1/10 to reduce the chance of competition with the Cy3-AMP for transcription initiation. However, reducing the ATP can compromise RNA yield. Other alternative methods are recommended if large-scale labeling of the RNA is required for the experiments.

- ii. Purify the labeled RNA strand from the unlabeled RNA by electrophoresis under denaturing condition. After cutting the band out of the gel and eluting, homogeneous pRNA with one Cy3 (or Cy5) is obtained, as determined by gel electrophoresis of the purified products.
- iii. Determine the RNA concentration by UV/Vis spectrophotometry or Nanodrop 2000 at OD260 and Cy3 concentration by measuring the absorbance at 550 nm ($\epsilon_{550\text{ nm}} = 150,000$). Calculate labeling efficiency as the molar concentration of Cy3 divided by the molar concentration of RNA.

B. Single-molecule labeling of RNA by annealing with fluorescently labeled DNA oligos

- i. Order single fluorophore-labeled DNA or RNA oligonucleotides from Integrated DNA Technology using solid phase synthesis.
- ii. Anneal the fluorescent labeled DNA or RNA oligo onto the RNA nanoparticle according to **Steps 19**.

C. Single-molecule labeling of RNA with fluorescent molecules using chemical strategies—!CAUTION: Since RNA is exposed to harsh experimental condition for long periods, wear gloves for all of the experimental procedures to protect your samples from possible RNase contamination.

- i. Transcribe the RNA with 5'-monophosphate: use standard T7 RNA transcription condition, except add an extra 10 mM GMP. Most RNA will be produced with 5'-monophosphate¹⁸². Purify the RNA with 8% Urea PAGE for future modifications.
- ii. Modify the 5'-end of RNA with amine (-NH₂) group: Weigh 1.25 mg EDC in a test tube. Dissolve dry RNA (up to 15 nmol) in 7.5 µL 5'-end labeling reaction buffer, add all of it to the EDC tube, quickly add 5 µl of 0.25 M ethylenediamine solution, and mix to dissolve the EDC. Add 20 µl of imidazole solution, mix and incubate at 37 °C for 2 h. Use NucAway™ spin columns to remove EDC and ethylenediamine from the reaction.

■ **PAUSE POINT:** 5'-NH₂-labeled RNA can be stored at -20 °C for up to 1 year without losing the reactivity of the amine group.

- iii. To label 2'-F-modified NH₂-RNA with CyTM3 NHS ester, speed-vacuum dry –NH₂ labeled RNA from the previous step. Dissolve 1 mg of CyTM3 NHS ester in 100 µl DMSO as a dye stock solution. Please note that this dye stock solution is only stable with good reactivity for up to two weeks at –20 °C. Add 3.5 µl of dye stock solution, 1.8 µl of DEPC treated water, and 18.8 µl of 5'-end labeling reaction buffer into the testing tube to dissolve the dried RNA. Mix and react overnight at RT. Ethanol precipitate the RNA and purify the final Cy3 labeled RNA product by 8% Urea PAGE.
- ▲ **CRITICAL STEP:** –NH₂ and –NHS reaction takes an incubation time (overnight) that is relatively long. This labeling approach is not suitable for RNA without chemical modifications, as this RNA will be degraded in such harsh reaction conditions.
- iv. (OPTIONAL) To label non-chemically modified RNA, modify 5'-end of RNA with -thiol (–SH) group: Weigh 1.25 mg EDC in a test tube. Dissolve dried RNA (up to 15 nmol) in 7.5 µl of 5'-end labeling reaction buffer. Add dissolved RNA into the EDC tube, quickly add 5 µl of 0.25 M cystamine in 0.1 M imidazole solution, and mix to dissolve the EDC. Add 20 µl of imidazole, mix and incubate at 37 °C for 2 h. Use NucAwayTM spin columns to remove EDC and cystamine from the reaction. The labeled RNA, which has a 5'-disulfide bond, can be stored frozen (see Pause Point below). To create a free –SH group for subsequent utilization of the modified RNA's 5'-thiol reactivity, add 10 mM DTT for 30 min.
- **PAUSE POINT:** The labeled RNA can be stored frozen for up to 1 year at –20 °C.
- v. (OPTIONAL) Label SH-RNA with CyTM3 Maleimide mono-reactive dye: Dissolve 1 mg of CyTM3 maleimide mono-reactive dye in 100 µl DMSO as a dye stock solution. Please note that this dye stock solution is only stable with good reactivity for up to two weeks at –20 °C. To instate –SH reactivity in the modified RNA (see previous step), add 10 mM DTT to the labeled RNA from the previous step to open the disulfide bond. Incubate at RT for 30 min. Use desalting column to remove the DTT and adjust the RNA with PBS buffer. Add 100 nmol CyTM3 Maleimide mono-reactive dye into the RNA, react for 2 h at RT. Ethanol-precipitate and purify the final labeling products via 8% Urea PAGE.

D. Whole-chain labeling of RNA nanoparticles

- i. Achieve post-transcriptional fluorescent labeling of the RNA molecule using functionalized fluorophores harboring a mono-alkylating reactive group developed by Mirus. Label the RNA strand using Label IT® nucleic acid labeling kits following manufacturer's guidelines.

?TROUBLESHOOTING

▲ **CRITICAL STEP:** Whole-chain RNA labeling may cause misfolding and loss of function of the RNA strands. The region of the RNA strand that is to be labeled should, therefore, be carefully considered. It is better to introduce whole-chain labels in the RNA strands that are responsible for assembling RNA scaffolds but have no functional role.

▲ **CRITICAL STEP:** During Cy3 labeling, light should be avoided during all of the experimental procedures. 3 M NaOAc should be prepared at pH 6.5 as the Cy3 rapidly degrades in acidic pHs.

Assessment of the successful assembly of RNA nanoparticles **TIMING: 3–4 d**

▲ **CRITICAL STEP:** Please note that, as mentioned in the Experimental design, the sub-sections that cover steps 21–31 are procedural ‘modules’, some, or all of which can be implemented, in no particular order.

- 21** Assay the assembly of RNA nanoparticles by native PAGE (option A) for quick validation of desired products or atomic force microscopy (option B) for detailed structural characterization of RNA nanoparticles (Fig. 4).

A. Assay the assembly of RNA nanoparticles by native PAGE

- i.** A simple and easy way to assay the assembly of RNA nanoparticles is by gel shift assays. Visualize the corresponding bands, as described in previous **Step 10A iii**.

B. Assaying the assembly of RNA nanoparticles by atomic force microscopy (AFM)

- i.** Synthesize 1-(3-aminopropyl) silatrane (APS) following the procedures in reference ^{183,184}. Incubate freshly cleaved mica with 167 nM 1-(3-aminopropyl) silatrane to generate APS mica ^{183,184}.
- ii.** Dilute the RNA samples with TMS buffer to a final concentration of 3–5 nM. Deposit a droplet of the sample (5–10 μ l) immediately on the APS mica. After 2 min incubation on the surface, wash off the excess sample using DEPC-treated water and dry with a flow of argon gas.
- iii.** AFM images in air can be acquired using MultiMode AFM NanoScope IV system operating in tapping mode. Two types of AFM probes are used for tapping mode imaging in air: 1) regular tapping Mode Silicon Probes with a spring constant of about 42 N/m and 300–320 kHz resonant frequency and 2) non-contact NSG01_DLC probes with a spring constant of ~5.5 N/m and 120–150 kHz resonance frequency.
- iv.** Analyze the AFM images with image analysis software provided with the AFM (following manufacturer instructions).

Determination of RNA nanoparticle hydrodynamic radius **TIMING: 1d**

- 22** Dissolve RNA nanoparticles in DEPC-treated water to a final concentration of 1–2 μ g/ μ l. Measure and analyze the hydrodynamic radius of RNA nanoparticles using DynaPro 99 dynamic light scattering with the temperature set at 25 °C (follow manufacturer’s guideline).

Determination of RNA nanoparticle melting temperature **TIMING: 1–2 d**

- 23** Assess the RNA melting temperature (T_m) to evaluate its thermostability implementing one of two alternative approaches: SYBR green I fluorescence emission (option A), a high throughput approach for analyzing multiple samples at the same time; or temperature gradient gel electrophoresis (option B), which can only analyze one sample at a time within a limited temperature range.

A. T_m measurement based on SYBR green I fluorescence emission

- i.** Conduct the melting experiments by monitoring the fluorescence of the RNA nanoparticles using the LightCycler® 480 real-time PCR system.

- ii. Mix 1× SYBR green I dye (emission 465–510 nm), which binds double-stranded nucleic acids, but not to single-stranded, with the RNA strands required for nanoparticle assembly, at RT in physiological TMS buffer.
- iii. Slowly cool down the RNA samples from 95 °C to 20 °C at the ramping rate of 0.11 °C/sec. Analyze data by LightCycler® 480 analysis software (following manufacturer instructions) using the first derivative of the melting profile (follow manufacturer's guidelines) (Fig. 7e). Represent the T_m value using the mean and standard deviation from at least three independent experiments.

B. T_m measurement based on temperature gradient gel electrophoresis (TGGE)

- i. Adjust the experimental setup to have a linear temperature gradient perpendicular to the electric field.
- ii. Set up temperature gradient in the appropriate range.
- iii. Load 10 μ l of the RNA sample combined with 2 μ l of 6× loading buffer and run on 8% or 10% native PAGE at 20 W for 1 h. 10 mM $MgCl_2$ is present in both the gel and the electrophoresis buffer.
- iv. Stain gels with EtBr and image by Typhoon FLA 7000 (GE Healthcare).
- v. Analyze the fraction of RNA assembled within the total RNA using *ImageJ*, and fit the melting curve of each construct using nonlinear sigmoidal fitting.

Assessment of the thermodynamic stability of RNA nanoparticles TIMING: 1–2d

- 24** Assess the thermodynamic stability of assembled RNA nanoparticles by approaches: dissociation conditions at different temperature by option A, resistance to chemical denaturation by option B, and dissociation at ultra-low concentrations by option C.

A. Assessment of dissociation conditions of RNA nanoparticles by competition assays and radiolabel chasing at different temperature

- i. Construct Cy3-labeled 3WJ-pRNA core $[a\ b^*\ c]_{3WJ}$ using three RNA oligos, a_{3WJ} , Cy3- b_{3WJ} , and c_{3WJ} mixed at 1:1:1 molar ratio in DEPC-treated water or TMS buffer.
- ii. While keeping the concentration of the labeled $[a\ b^*\ c]_{3WJ}$ fixed, incubate 6 solutions with labeled $[a\ b^*\ c]_{3WJ}$: varying concentration ratios of the unlabeled b_{3WJ} (1:0, 1:0.1, 1:1, 1:10, 1:100, 1:1000) for 30 min at 25 °C, 37 °C, and 55 °C, respectively. Load the samples onto 16% native gel for visualization by Typhoon FLA 7000 under Cy3 channel (Fig. 7a). The labeled RNA fragment b^* that gets dissociated or exchanged from the intact RNA nanoparticle $[a\ b^*\ c]_{3WJ}$ at different temperature will appear as a band migrating faster than the intact RNA complex.

B. Assessing RNA nanoparticle dissociation in the presence of different concentrations of urea

- i. Prepare two solutions containing unlabeled b_{3WJ} and Cy3-labeled $[a\ b^*\ c]_{3WJ}$, in which the concentration of $[a\ b^*\ c]_{3WJ}$ is the same but the concentration of b_{3WJ} is such that the mole ratios between the two species ($[a\ b^*\ c]_{3WJ}$:unlabeled b_{3WJ}) is 1:1 and 1:5, respectively. Incubate four aliquots of each of the two solutions at RT for 30 min with different concentrations of urea (0, 2, 4, and 6 M). Load the samples onto 16% native for visualization by Typhoon FLA 7000 under Cy3

channel (Fig. 7b). The labeled RNA fragment b* that gets dissociated or exchanged from the intact RNA nanoparticle [a b* c]_{3WJ} upon treatment with different concentrations of urea will appear as a band migrating faster than the intact RNA complex.

C. Assay to test RNA nanoparticle dissociation at extremely low concentrations

- i. Label RNA nanoparticles using radiolabel assays according to **Step 16**. Purify [³²P]-labeled RNA nanoparticles by denaturing PAGE according to **Steps 9A iii–vii**.
- ii. Dilute the RNA nanoparticles solution serially from 40 nM to 160 pM in TMS buffer, and then load each solution onto 8% native PAGE gel for autoradiography (see Fig. 7c).

!CAUTION: This reagent is radioactive. Shield protection is required. Wear lab coat and gloves. Follow the regulation of radioactive materials handling.

Assay to determine chemical stability of RNA nanoparticle **TIMING: 2–3 d**

- 25 Assemble RNA nanoparticles with or without chemical modification, for instance with or without 2'-F-modification.
- 26 Incubate the RNA nanoparticles with RPMI-1640 medium containing 10% fetal bovine serum or with RNase A (2-fold serial diluted RNase A starting from 1 mg/ml) at 37 °C. Collect solution aliquots containing 200 ng of RNA at each of the following time points: 10 min, 1 h, 12 h, 24 h, and 36 h for RPMI-1640 medium containing 10% fetal bovine serum treated samples. Collect solution aliquots containing 200 ng of RNA at 36 h for RNase A treated samples. Analyze these test solutions by 8% native PAGE gel (see Fig. 7d).

Assessing *in vivo* tumor targeting of pRNA nanoparticles **TIMING**

1–2 months, depending on how long it takes to establish tumor xenografts.

!CAUTION: The *in vivo* studies can be only performed following appropriate institutional regulatory board guidelines and the permission of the animal protocol must be obtained before performing any experiment.

- 27 Feed 6-week-old male nude mice (nu/nu) with a folate-free diet for a total of 2 wk before the experiment.
 ▲ **CRITICAL STEP:** If using folate as the targeting molecules for delivery, keeping mice on a folate-free diet will free the folate receptor from folate and avoid the competitive binding of free folate to the folate receptor, which will ensure the success of the *in vivo* targeting assay.
- 28 Inject mice with cancer cells (taking folate receptor positive KB cells as example) ~1×10⁶ cells per mouse in folate-free RPMI-1640 medium.
- 29 Measure tumor diameters with calipers and calculate the tumor volume in mm³ by the formula:

$$volume = \frac{(width)^2 \times length}{2}$$

- 30** Once the tumors grow to $\sim 500 \text{ mm}^3$ (for KB cell based xenografts, it usually takes about 10 d), inject mice intravenously through the tail vein with a single dose of $600 \mu\text{g}$ of 2'-F U/C modified Folate-AlexaFluor647-labeled pRNA nanoparticles (about 15 nmol in PBS buffer, equal to 24 mg/kg).
- ▲ **CRITICAL STEP:** All the RNA nanoparticles utilized for *in vivo* studies must be chemical modified (2'-F) in order to increase the chemical stability *in vivo*. Otherwise, unmodified RNA will be degraded by ribonuclease in plasma immediately upon injection. To evaluate the targeting, the RNA nanoparticles should be labeled with far-red or near infrared dye such as Alexa 647 for better signal penetration and low background noise.
- 31** 24 h after injection, carry out whole-body imaging was with an IVIS® Lumina Station with the body of the mouse lying sideways in the imaging chamber (see Fig. 8).
- 32** After whole-body imaging, euthanize the mouse by CO_2 asphyxiation and follow with cervical dislocation. Dissect and image the tumors, liver, spleen, heart, lung, intestine, kidney, and skeletal muscles of the mice individually.

TIMING

- Step 1–8, Amplification of the DNA template via PCR: $\sim 1 \text{ d}$
- Step 9, *In vitro* RNA synthesis: 2–3 d
- Step 10, Assembly of thermodynamically stable RNA nanoparticles using 'toolkits': $\sim 1 \text{ wk}$
- Step 11–12, Construction and assay of RNA nanoparticles fused with siRNA: $\sim 1 \text{ wk}$
- Step 13–14, Construction and *in vitro* assay of RNA nanoparticles fused to RNA aptamers: 3–4 d
- Step 15–18, Construction and assay of RNA nanoparticles harboring ribozymes: 1 d
- Step 19, Construction of RNA nanoparticles harboring targeting ligands: 3–4 d
- Step 20, Construction of RNA nanoparticles harboring imaging molecules: 3–4 d
- Step 21, Assessment of the successful assembly of RNA nanoparticles: 3–4 d
- Step 22, Determination of RNA nanoparticle hydrodynamic radius: 1 d
- Step 23, Determination of RNA nanoparticle melting temperature: 1–2 d
- Step 24, Assessment of the thermodynamic stability of RNA nanoparticles: 1–2 d
- Step 25–26, Assay to determine chemical stability of RNA nanoparticle: 2–3 d
- Step 27–32, Assessing *in vivo* tumor targeting of pRNA nanoparticles: 1–2 months

TROUBLESHOOTING

Table 5

Troubleshooting

Step	Problem	Possible Reason	Solution
4	Incorrect size of PCR product	Incorrect PCR primers design	Re-design the primers

Step	Problem	Possible Reason	Solution
	Non-specific PCR product	PCR condition is not optimized or Incorrect primer design	Optimizing the PCR condition or applying the DNA purification procedure in Step 8 to obtain expected DNA products
9A	No RNA products	No T7 promoter sequence	Add T7 promoter sequence at the 5'-end of dsDNA
	Low yield of RNA production	The transcription starting sequence is not strong enough	Strong transcription initiation with GG after T7 promoter sequence. Adding an extra G to 5'-end to increase the transcription efficacy without interfering with the folding of the RNA
9B	No or low yield of transcription of 2'-F modified RNA	Impure dsDNA template	Purify dsDNA template through gel extraction
		Inappropriate salt condition	Pass the dsDNA template through desalting column.
10A	No formation of RNA nanoparticles	The extension of the loop sequences affect the global folding of RNA	Thoroughly analyze the folding of re-engineered RNA sequence with the online RNA folding program such as <i>Mfold</i> ⁶⁷ , etc.
		No Mg ²⁺ present	Make sure to include Mg ²⁺ at least at a 5 mM concentration
19	No folate-mediated binding observed	Folate receptors are occupied by free folic acid	Cells need be kept in a folate-free medium for at least 12 h prior to the binding assay
20D	Loss of function of RNA after labeling	Heavy labeling can cause misfolding of RNA strand	Introduce whole-chain labeling to the RNA strands only for assembling RNA scaffolds. Otherwise, end labeling methods should be considered.

ANTICIPATED RESULTS

This protocol provides detailed procedures for constructing RNA nanoparticles using the unique structural features of bacteriophage phi29 pRNA. Series of assembly 'toolkits' can be used to assemble a variety of multivalent nanoparticles. We have also provided the comprehensive methods to functionalize pRNA nanoparticles with targeting, detection, and therapeutic compartments (Fig. 1). Conjugation of functionalities is achieved prior, not subsequently, to the assembly of the RNA nanoparticles, thus ensuring the production of homogeneous nanoparticles with appropriate folding and function after fusion. Anticipated results of this protocol are as follows:

1. The resulting RNA nanoparticles will be homogeneous and can be manufactured with high reproducibility and known stoichiometry^{90,91}.
2. The resulting RNA nanoparticles will be chemically and thermodynamically stable and will remain intact at ultra-low concentrations (160 nM as shown in Fig. 7c)^{90,91}.
3. The diameter of resulting RNA nanoparticles typically will range from 10–50 nm, which is an optimal size to greatly improve the *in vivo* pharmacokinetics, pharmacodynamics, biodistribution, and toxicology profiles^{90,91,150}.
4. Cell type-specific gene targeting can be achieved via target delivery modules, which reduce off-target toxicity and lower the concentration of the drug administered; thus, reducing the side effects of the therapeutics¹⁵⁰.
5. Protein-free RNA nanoparticles that contain RNA aptamers as anti-receptors can be have higher specificity than protein anti-receptors, while displaying the lowest

antibody-inducing activity, which provides an opportunity for repeated administration and treatment of chronic diseases¹⁵⁰.

6. RNA nanoparticles have a modular design, so they can self-assemble via a bottom-up approach to harbor multiple therapeutic reporters and/or targeting payloads to achieve enhanced or synergetic effects^{82–89,89–91}.
7. We have demonstrated that the RNA nanoparticles harboring different functional modules retain their folding and independent functionalities for specific cell binding, cell entry, gene silencing, catalytic function, and cancer targeting, both *in vitro* and in animal trials^{90,91}.
8. Systemic injection of the thermodynamically and chemically stable RNA nanoparticles into mice revealed that the RNA nanoparticles strongly and specifically bind to cancers without accumulating in the liver, lungs, or any other vital organs or tissues (Fig. 8)^{90,91,150}.

Acknowledgments

The research was supported by National Institutes of Health grants EB003730 and CA151648, and the Arnold and Mabel Beckman Initiative for Macular Research External Grant 1108 to P. Guo. AFM images were obtained by L. Shlyakhtenko and Y. Lyubchenko at the Nanoimaging Core Facility, University of Nebraska Medical Center supported by the NIH SIG Program and the UNMC Program of ENRI. We thank D. Rodger and M. Chow at University of Kentucky for the help with Dynamic Light Scattering; F. Huang at The University of Southern Mississippi for providing Cy3 and Cy5 labeled AMP and GMP; J. Haak, E. Khisamutdinov, E. Beabout, and F. Pi for help with the manuscript preparation. P. Guo is a cofounder of Kylin Therapeutics, Inc., and Biomotor and Nucleic Acid Nanotechnology Development Corp. Ltd.

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Figure 1. General workflow of the present protocol for the design, construction, functionalization, characterization and use of pRNA nanoparticles.

Figure 2. Structure of phi29 DNA packaging motor and packaging RNA

(a) Bacteriophage phi29 DNA packaging motor. Six copies of pRNAs assemble into a hexamer ring to gear the viral DNA packaging motor. (b) The primary sequence and secondary structure of wild-type pRNA. The two domains (helical foot and central R- and L-hand) are connected by a 3WJ core (Reproduced with permission from ref ⁹²).

Figure 3. Three ‘toolkits’ for constructing pRNA nanoparticles

(a) Toolkit I: Extended pRNA interlocking loop sequences. (b) Toolkit II: Principle for the design of foot-to-foot 6-nt palindrome sequences. (c) Toolkit III: pRNA 3WJ motif for the construction of three-branch RNA nanoparticles. (d) pRNA 3WJ derived X-shaped pRNA motif for the construction of four-branch RNA nanoparticles. (Reproduced with permission from refs ^{90–92}).

Figure 4. AFM images of diverse pRNA nanoparticles constructed using the Toolkits I, II and III (a) Loop extended pRNA trimer. (b) Loop extended pRNA tetramer. (c) Loop extended pRNA pentamer. (d) Loop extended pRNA hexamer. (e) Loop extended pRNA heptamer. (f) Foot-to-foot trimer. (g) Foot-to-foot tetramer. (h) Foot-to-foot pentamer. (i) Foot-to-foot hexamer. (j) Foot-to-foot heptamer. (k) 3WJ-pRNA. (l) X-pRNA. (m) Foot-to-foot branched hexamer. (n) Arm-on-arm branched hexamer. The second column is the magnified images of individual nanoparticles. Scale bar: 10 nm.. (Reproduced with permission from ref ⁹²).

Figure 5. Functional assay of moieties incorporated in pRNA nanoparticles

(a) Dual-luciferase assay for target gene knock-down of luciferase gene. The relative firefly luciferase activity reflects the level of luciferase gene expression and is obtained by normalizing firefly luciferase activity using the internal control renilla luciferase activity. Error bars represent standard deviations (N = 3). (b) Target gene knock-down effects of BIRC5 siRNA showed by RT-PCR (GADPH is the endogenous control) on mRNA level and western blot assay (β -actin bands served as loading control) on protein level. (c) Fluorogenic assay demonstrating fluorescence emission in the presence of the chemical MG binding to the MG aptamer incorporated in the pRNA vector using 6% native PAGE (lane 1 and 2 are pRNA monomer and dimer control; lanes 3–6 are loop-extended pRNA monomer,

dimer, trimer and tetramer, respectively, without the MG aptamer; lanes 7–8 are loop-extended monomer and tetramers, respectively, harboring the MG binding aptamer), visualized by MG fluorescent imaging (left panel), ethidium bromide staining (center panel), and MG fluorescence emission (right panel). **(d)** STV (streptavidin)-binding assay using STV affinity column to demonstrate the correct folding of the STV-aptamer incorporated into the RNA nanoparticles visualized by both MG staining in native PAGE gel. **(e)** Assessing the catalytic activity of the HBV ribozyme incorporated into the 3WJ-pRNA. **(f)** Flow cytometry and confocal images revealed the binding and specific entry of fluorescent-[3WJ-pRNA/FA] nanoparticles into folate-receptor-positive (FR+) cells. Negative control is Cy3-[3WJ-pRNA/NH₂] (without FA). Co-localization (overlap, 4); cytoplasm (green, 1); RNA nanoparticles (red, 2) (magnified, right panel); nuclei (blue, 3). **(a, b)** reproduced with permission from ref ⁹¹; **c,d** reproduced with permission from ref ⁹²; **e,f** reproduced with permission from ref ⁹⁰).

Figure 6. Construction of tetravalent pRNA-X nanoparticles harboring multiple siRNA for enhanced gene silencing effects

Sequences and notations of siRNA used in tetravalent constructs: Blue: siLuci-1; red: siLuci-2; green: siLuci-3; orange: siLuci-4; black: control siRNA. (a) Quantification of luciferase gene expression: Effects of increasing number of different Luciferase siRNAs (siLuci-1, 2, 3 and 4) incorporated in the pRNA-X motif. RLU, relative luciferase units. Error bars represent standard deviations ($N = 3$). (b) siRNA and pRNA-X nanoparticles were transfected into HT29 GFP-Luc cells (siRNA 1 and 2: 100 nM; pRNA nanoparticle: 1 nM). Cells were seeded in 96-well plate and images were taken by IVIS® *in vivo* imaging system. Image shows overlay of luminescence and white light channels. RLU, relative luciferase units. Error bars represent standard deviations ($N = 3$). (Reproduced with permission from ref ⁹¹).

Figure 7. Stability assays for pRNA nanoparticle characterization

(a) Temperature effects on the stability of the 3WJ-pRNA core, denoted as [ab*c]3WJ, evaluated by 16% native gel. A fixed concentration of Cy3-labelled [ab*c]3WJ was incubated with varying concentrations of unlabelled b3WJ at 25 °C, 37 °C, and 55 °C. (b) Urea denaturing effects on the stability of [ab*c]3WJ evaluated by 16% native gel. A fixed concentration of labelled [ab*c]3WJ was incubated with unlabelled b3WJ at ratios of 1:1 and 1:5 in the presence of 0–6 M urea at 25 °C. (c) Dissociation assay for the [³²P]-3WJ-pRNA complex harboring three monomeric pRNAs by twofold serial dilution (lanes 1–9). The monomer unit is shown on the left. (d) Stability assay for RNA nanoparticles (unmodified and 2'-fluorine (2'-F)-modified RNA nanoparticles) in 10% (vol/vol) serum at different time points and digestion by RNase A with 2-fold serial dilution starting with an RNA nanoparticle concentration of 1 mg/ml. (e) Melting curves for each of the 11 RNA 3WJ core motifs assembled from three oligos for each 3WJ motif under physiological buffer TMS. (a, b, c, f reproduced with permission from ref ⁹⁰; d reproduced with permission from ref ⁹²).

Figure 8. *In vivo* binding and targeting of pRNA-X nanoparticles

(a) The pRNA-X nanoparticles (harboring folate and Alexa-647) specifically targeted folate-receptor-positive tumor xenografts upon systemic administration in nude mice, as revealed by (a) whole body imaging and (b) internal organ imaging (After 8 h) (Lv=liver, S=spleen, K=kidney, H/L=heart/liver). Control: PBS treated mice. Scale bar: fluorescent intensity. (Reproduced with permission from ref ⁹¹).

Table 1Difference between RNA and DNA (adapted and modified from Ref ¹).

		DNA	RNA
Elements
Structure and folding		Simple canonical Watson-Crick (W-C) base pairing, rare tertiary structure formation	Folding through canonical and non-canonical base pairing as well as base stacking. Versatile tertiary structures and rich structural elements
Acidic effect		Depurination: Apurine DNA is sensitive to cleavage	Stable
Alkaline effect		Stable up to pH 12	Sensitive to alkaline hydrolysis
Configuration		Predominantly B form:	A form:
		<ul style="list-style-type: none"> • Base pairs/turn of the helix: 10.5; • Pitch: 3.5 nm; • Helix rise/bp: 0.314 nm; • Humidity: Nucleotide:H₂O = 1:1 	<ul style="list-style-type: none"> • Base pairs/turn of the helix: 10.9; • Pitch: 2.5 nm; • Helix rise/bp: 0.275 nm; • Humidity: Nucleotide:H₂O = 1:0.7
Chemical stability		Relatively stable but sensitive to DNase	Unstable, sensitive to RNase
Stability after chemical modification		Not applicable	Highly stable after modification ^{90,91}
Thermal stability		G:C pairs more stable than A:T	Thermally more stable than DNA ^{1,90,91} , especially for RNA motifs and modules with particular bends or stacks
Free energy, ΔG°		-1.4 KJ.mol ⁻¹ per bp stack ¹⁸⁵	-3.6 to -8.5 KJ.mol ⁻¹ per bp stack ¹⁸⁵
Helix formation		A minimum of 4 nucleotides needed	A minimum of 2 nucleotides needed ^{81,186}
Intermolecular interactions		Cohesive ends, crossover motifs	Cohesive ends, crossover motifs, kissing loops, interlocking loops
Intracellular processing		Rare	Editing, splicing, capping, polyadenylation, Dicer processing, ribozyme cleavage, ribonuclease processing, on/off switching by metabolites, etc.
Intracellular production		Duplication of original copies	Controllable transcription defined by promoter and terminator
<i>In vivo</i>	Initiation	Origin of replication with primer	Promoter, exact nucleotide to start without primer
	Termination	No nature sequence for replication termination	Specific transcription terminators
<i>In vitro</i>	Enzymatic synthesis	DNA polymerase, PCR	T7/SP6 Transcription
	Chemical synthesis	Up to 160 nucleotides; low cost	Up to 117 nucleotide; high cost and low yields

Table 2Sequence of RNA nanoparticle scaffold (adapted from Ref ⁹²).

Name	RNA sequence (5'→3')
loop extended pRNA	GGAAUGGUACGUACUCCAUGUCAUGUGUAUGU UGGGGAUUAxxxxxxCUGAUUGAGUUCAGCCCACAU ACUUUGUUGAUUxxxxxxGUCAAUCAUGGCAAAAGU GCACGCUACUUUCC (refer to Table 4 for detailed x sequence)
a_{3WJ}	UUGCCAUGUGUAUGUGGG
3WJ-pRNA	b_{3WJ} CCCACAUACUUUGUUGAUCC
c_{3WJ}	GGAUCAAUCAUGGCAA
pRNA-X	a_X UUGCCAUGUGUAUGUGGGUCCAGCAC
b_X	GUGCUGGAACUGACUGC
c_X	GCAGUCAGCCCACAUACUUUGUUGAUCC
c_{3WJ}	GGAUCAAUCAUGGCAA

Table 3Sequence of inserted functional moieties (adapted from Ref ⁹²).

Name	RNA sequence (5'→3')
Malachite Green - binding aptamer	5'-AUGGUAACGAAUGA-3' 5'-CAAUCCGACAU-3'
STV-binding aptamer	5'-CGACCAGAAUCAUGCAAGUGCGUAAGAUAGUCGCGGGUCG-3'
NH₂-DNA	5'-NH ₂ -CTCCCGGCCGCCATGGCCGCGGGAT-3'
Folate- DNA	5'-Folate-CTCCCGGCCGCCATGGCCGCGGGAT-3'
Anti-HBV ribozyme	Active ribozyme: 5'-CAAAUUCUUUACUGAUGAGUCCGUGAGGACGAAACGGGUC-3' disabled ribozyme: 5'-CAAAUUCUUUACUAAUGAGUCCGUGAGGACGAAACGGGUC-3'
Firefly luciferase siRNA	siLuci 1: sense: 5'-GUGCGCUGCUGGUGCCAAC-3' anti-sense: 3'-CACGCGACGACACGGUUG-5' siLuci 2: sense: 5'-CUUACGCUGAGUACUUCGA-3' anti-sense: 3'-GAAUGCGACUCAUGAAGCU-5' siLuci 3: sense: 5'-GCUAUGAAACGAUAUGGGC-3' anti-sense: 3'-CGAUACUUUGCUAUACCCG-5' siLuci 4: sense: 5'-UUCGUCACAUCUCAUCUAC-3' anti-sense: 3'-AAGCAGUGUAGAGUAGAUG-5' control: sense: 5'-UCUCCUUCACGAAACCGAC-3' anti-sense: 3'-AGAGGAAGUGCUUUGGCUG-5'

Table 4

Reengineered hand-in-hand loop interactions (adapted from Ref ⁹²).

Loop name	Right-hand loop (5'→3')	Left-hand loop (5'→3')
	ExX	Exx'
ExAa'	gauuaAGUGGAC	uGUCCACU
ExBb'	gauuaACAGGCA	uUGCCUGU
ExCc'	gauuaGCGUUCU	uAGAACGC
ExDd'	gauuaAGGCUAG	uCUAGCCU
ExEe'	gauuaAGCACCA	uUGGUGCU
ExFf'	gauuaAGACGUG	uCACGUCU
ExGg'	gauuaCACUAUC	uGAUAGUG