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Assembly of Multifunctional Phi29 pRNA Nanoparticles for Specific Delivery of SiRNA and other Therapeutics to Targeted Cells

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Abstract

Recent advances in RNA nanotechnology have led to the emergence of a new field and brought vitality to the area of therapeutics (Guo P, The Emerging Field of RNA Nanotechnology, *Nature Nanotechnology*, 2010). Due to the complementary nature of the four nucleotides and its special catalytic activity, RNA can be manipulated with simplicity characteristic of DNA, while possessing versatile structure and diverse function similar to proteins. Loops and tertiary architecture serve as mounting dovetails or wedges to eliminate external linking dowels. Unique features in transcription, termination, self-assembly, self-processing, and acid-resistance enable *in vivo* production of nanoparticles harboring aptamer, siRNA, ribozyme, riboswitch, or other regulators for therapy, detection, regulation, and intracellular computation. The unique property of noncanonical base-pairing and stacking enables RNA to fold into well-defined structures for constructing nanoparticles with special functionalities.

Bacteriophage phi29 DNA packaging motor is geared by a ring consisting of six packaging RNA (pRNA) molecules. pRNA is able to form a multimeric complex via the interaction of two reengineered interlocking loops. This unique feature makes it an ideal polyvalent vehicle for nanomachine fabrication, pathogen detection, and delivery of siRNA or other therapeutics. This review describes methods in using pRNA as a building block for the construction of RNA dimers, trimers and hexamers as nanoparticles in medical applications. Methods for industrial-scale production of large and stable RNA nanoparticles will be introduced. The unique favorable PK (pharmacokinetics) profile with a half life ($T_{1/2}$) of 5–10 hours comparing to 0.25 of conventional 2'-F siRNA, and advantageous *in vivo* features such as non-toxicity, non-induction of interferons or non-stimulating of cytokine response in animals will also be reviewed.

Keywords

bacteriophage phi29; nanomotors; nanotechnology; nanobiotechnology; bottom-up assembly; DNA packaging; pRNA nanoparticle; RNAi; Cell-type specific delivery; viral assembly

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1. Introduction

Nanotechnology involves modification, engineering, and/or assembly of organized materials at the nanometer scale[1–4]. RNA molecules can be designed and manipulated at a level of simplicity similar to DNA[5–11], while possessing the versatility in structure, function, and even enzymatic activity similar to that of proteins[5,12–18]. This property makes RNA an attractive candidate for nanotechnological applications[4]. Building-blocks are first synthesized after computing intra- and inter-molecular folding[19–24]. Nanoparticles are then built via spontaneous templated [5,14,15] or nontemplated self-assembly as planned[6,7].

In recent years, more and more functional RNA molecules, naturally or artificially engineered, have been discovered. Like antibodies, RNA aptamers selected from systematic evolution of ligands by exponential enrichment (SELEX)[25–29] are able to bind to specific targets, including proteins, organic compounds, and nucleic acids[30–32]. The ability to recognize specific cell surface markers through the formation of binding pockets and the capability of internalization by the targeted cells pave a new way for targeted delivery[33–37]. In the early 1980s, Thomas Cech[13] and Sydney Altman[38] found RNA molecules had the ability to catalyze chemical reactions. In 1998, Andrew Fire and Craig Mello discovered RNAi (RNA interference), which can regulate gene expression on a post-transcriptional level[39]. These RNA molecules have significant therapeutic potential and are capable of regulating gene function by intercepting and cleaving mRNA or the genome of RNA viruses. The discovery of RNAi has heightened interest in RNA therapeutics[39], since several RNA based therapeutic approaches using small interfering RNAs (siRNAs) [4,40–45], ribozymes[46–49], and anti-sense RNAs[50,51,51] have been shown to down-regulate specific gene expression in cancerous or viral-infected cells. The successful application of RNA-based therapeutics for the treatment of cancer and infectious diseases requires several features: 1) intact delivery to desired cells; 2) capability of entering cells; 3) surviving degradation by nucleases within the cell; 4) trafficking into the appropriate cell compartments; 5) correct folding of siRNA or ribozyme in the cell, if fused to a carrier; 6) specific delivery to cells; and 7) the release and incorporation of siRNA into RISC once siRNA is delivered into cells. In addition, the RNA particle should have low toxicity and high retention times in the body. Hence, the development of a safe, efficient, specific, and nonpathogenic system for the delivery of therapeutic RNA is highly desirable.

Bacterial virus phi29 DNA packaging RNA (pRNA) molecule contains an intermolecular interaction central domain and a helical domain as the 5'/3' paired region (Fig. 1A). Via the interaction of two interlocking loops, the pRNA molecules form dimers, trimers, hexamers, and patterned superstructures[7]. This property of forming self-assembled nanostructure makes pRNA ideal building blocks for bottom-up assembly. The pRNA interlocking central domain is a nucleation core with very low free energy, which folds independently of the newly incorporated moieties[52]. Thus, pRNA can be engineered and fabricated as a polyvalent and nanoscale delivery system capable of delivering multiple therapeutics into specific cells. Incubation of the pRNA containing receptor-binding moieties and gene-silencing subunits, resulted in cell binding and transportation of the chimeric pRNA/siRNA, pRNA/ribozyme into cells, subsequently silencing the targeted genes and modulating programmed cell death[53–55].

pRNA can be chemically synthesized and modified starting from small RNA fragments. The resulting nanoparticle can be assembled through the bottom-up assembly approach. The structural and molecular features of phi29 pRNA allow its easy manipulation, making it possible to redesign its components as gene targeting and delivery vehicles (Fig. 1D).

2. The Biological Function of pRNA on Phi29 DNA Packaging Motor

All linear double-stranded DNA viruses including bacteriophage phi29[56] possess a common feature that their genome is packaged into a preformed procapsid during the maturation of the virion. This process is accomplished by an ATP-driven packaging motor (Fig. 1C)[57]. However, the most exciting aspect of the phi29 DNA packaging process is the discovery of a small viral RNA, called pRNA (Fig. 1A)[12], which is 120 bases and transcribed from the left end of the phi29 genome. It has also been revealed that pRNA contains two functional domains[58,59], one facilitates the formation of pRNA hexameric ring (Fig. 1B) and binds to the connector while the other binds to the DNA-packaging enzyme gp16[60]. By using the energy from gp16 hydrolyzing ATP, this very powerful DNA packaging motor then gears the viral genome into the preformed procapsid. After the complete packaging, it is possible that pRNA leaves the connector before the collar protein gp11 and tail knob gp9 protein block the connector channel and assemble the intact viral particles[61].

3. Structure of Phi29 Motor pRNA

pRNA is a crucial component in the phi29 DNA packaging motor and contains two functional domains (Fig. 1A). The intermolecular interaction domain is located at the central region (bases 23–97) and within this domain there are two loops (right hand loop and left hand loop) which are responsible for the hand-in-hand interaction through the four complementary base sequences within these two loops. The other domain is a DNA translocation domain which is located at the 5'/3' paired ends. The right hand loop (bases 45–48) and the left hand loop (bases 82–85) allow for the formation of pRNA dimers, trimers and hexamer rings via intermolecular base-pairing [5,62].

Nucleotides 23–97 are the key components in the formation of pRNA multimers. This pRNA interlocking central domain constitutes a nucleation core with very low free energy. As mentioned before, pRNA is constituted with two domains and both of them can fold independently. The ability to form pRNA multimers is not affected by 5' or 3'-end truncation before residue #23 and after residue #97[52]. Thus, end conjugation of pRNA with chemical moiety of fusing pRNA with a receptor-binding RNA aptamer, siRNA, or ribozyme would not disturb pRNA dimer formation or interfere with the function of inserted moieties [53–55,63,64].

4. Nomenclature

To simplify the description of RNA construction and multimer assembly, uppercase letters will be used to represent the right hand loop of pRNA and lowercase letters to represent the left hand loop (Fig. 1A). The same letters in upper and lower cases indicate complementary sequences for loop/loop interaction, while different letters indicate non-complementary loops. For example, pRNA A–b' represents pRNA with a non-complementary right loop A (5'G₄₅G₄₆A₄₇C₄₈) and a left loop b' (3'U₈₅G₈₄C₈₃G₈₂). Dimer formation requires a right loop B (5'A₄₅C₄₆G₄₇C₄₈) and left loop a' (5'C₄₅C₄₆U₄₇G₄₈) of pRNA B–a'[52].

5. Bottom-up Assembly of pRNA Nanoparticles

Taking advantages of the folding independence of pRNA's two functional domains, end conjugation of pRNA with chemical moiety, fusing pRNA with a receptor-binding RNA aptamer, siRNA, ribozyme or other chemical groups generally do not disturb the interlocking interaction or interfere with the function of inserted moieties (Fig. 1D). Then, the engineered monomeric pRNA chimera (Fig. 2) can be further assembled as various nanoparticles including dimers, trimers and tetramers through different strategies.

5.1 Construction of Monomeric building block of pRNA Chimera with Biological and Pharmaceutical Functionalities

5.1.1 Monomeric pRNA chimera harboring therapeutic RNAs

pRNA chimera harboring siRNA (Fig. 2): The pRNA double-stranded 5'/3' end helical domain and intermolecular binding domain fold independently of each other. Complementary modification studies have revealed that altering the primary sequences of any nucleotide of the helical region does not affect pRNA structure and folding as long as the two strands are paired[65]. Extensive studies revealed that siRNA was a ~21–23nt RNA duplex [41,42,45,66]. Thus, it was possible to replace the helical region in pRNA with double-stranded siRNA. A variety of chimeric pRNAs with different targets were constructed to carry siRNA connected to nt# 29 and 91 of the pRNA (Fig. 1A). Such a pRNA/siRNA chimera was proven to be the building block which successfully inhibited targeting gene expression. A pRNA/siRNA chimera harboring siRNA targeting coxsackievirus B3 (CVB3) protease gene was constructed and was able to knock down gene expression specifically and inhibit viral replication *in vitro*[67]. We also selected a model involving the Metallothionein-IIA (MT-IIA) gene as a proof of concept. The specific knock-down of the MT-IIA gene by constructed chimera pRNA/siRNA (MT-IIA) can reduce ovarian cancer cells viability significantly[68]. Pre-clinical study also showed that pRNA/siRNA chimera targeting anti-apoptotic factor survivin gene can drive cancer cells undergo apoptosis and prevent tumorigenesis in xenograft mice models[53,54].

pRNA chimera harboring ribozyme (Fig. 2). Using the circular permutation approach[69–71], almost any nucleotide of the entire pRNA can serve as either the new 5' or 3'-end of the RNA monomer. Connecting the pRNA 5'/3' ends with variable sequences did not disturb its folding and function[53–55,63,64]. These unique features, which help prevent two common problems: exonuclease degradation and misfolding in the cell, make pRNA an ideal vector to carry therapeutic RNA such as ribozyme. A pRNA-based vector was designed to carry hammerhead ribozymes that cleave the hepatitis B virus (HBV) polyA signal[63]. The pRNA/ribozyme(survivin), which targeted the anti-apoptosis factor survivin to down-regulate genes involved in tumor development and progression, was also shown to suppress survivin expression and initiate apoptosis[55].

5.1.2 Construction of monomeric pRNA chimera for cell targeting

pRNA chimera harboring aptamer (Fig. 2): *In vitro* SELEX[31,32] of RNA aptamers which bind to specific targets has become a powerful tool for selecting RNA molecules that target specific cell surface receptor. Aptamers were linked to the 3' and 5' end of pRNA. To facilitate independent folding, poly U or poly A linkers were placed between the pRNA and the aptamer. The nascent 5'/3' end of the pRNA were relocated to nt 71 and 75 (Fig. 1A) using the circular permutation approach[69,72]. The tightly folded nt 71 and 75 region protected the 5'/3' end from exonuclease digestion. One of the pRNA/aptamer constructs harboring anti-HIV gp120 aptamer was proved to bind to and are internalized into cells expressing HIV gp120. Moreover, the pRNA-aptamer chimeras alone also provide HIV inhibitory function by blocking viral infectivity in an acute *in vitro* challenge assay[73].

pRNA chimera harboring chemical ligands (Fig. 2): Chemical ligands like folate which can recognize specific cell surface markers can also be conjugated to pRNA for specific targeting [64,67,68]. A complementary DNA oligo can be annealed to the end of the pRNA which has the 3'-end extension with 14–26 nucleotides. The assembled folate-pRNA nanoparticles are able to bind and internalize into cancer cells specifically and efficiently (Fig. 3A) and were applied for systematic target delivery of therapeutics *in vivo* (Fig. 3B). Synthetic DNA oligos functionalized with different chemical groups, such as folate, were

used for therapeutic pRNA construction (Fig. 2). Details of the pRNA labeling strategies are discussed below (section 5.1.3).

5.1.3. Chemical strategies for pRNA conjugation with ligands, fluorophores, and other chemical moieties—Unlike most of the other nanodelivery system, pRNA technology offers the possibility to form multivalent nanoparticles with accurate control of the stoichiometry of the different functional elements. Detection, ligand targeting, and drug or gene delivery elements can be conjugated to pRNA and then assembled into one nanoparticle through the bottom-up approach. Although, synthetic RNA can be modified with a wide variety of reactive moieties for chemical conjugation, they will be not discussed in this paper. Readers can refer to a previous review[74]. Alternatively, many different strategies have been developed for post-transcriptional or co-transcriptional functionalization of RNA molecules. These labeling strategies have been demonstrated to be highly efficient for long chain RNA, such as pRNA (120 bases), and can be distinguished following single molecule labeling or random labeling of the whole chain of the RNA[75].

5.1.3.1 Whole chain labeling of pRNA: Bifunctional alkylating agents are known to promote crosslinking of DNA or RNA molecules. Using a similar strategy, post-transcriptional fluorescent labeling of the pRNA molecule was readily achieved using functionalized fluorophores, harboring a mono-alkylating reactive group, developed by Mirus (Label IT labeling reagent, Mirus). More recently, it was demonstrated that T7 RNA polymerase can be used for *in vitro* enzymatic fluorescent labeling of the RNA molecule using the new reagent tCTP[76]. Different T7 RNA polymerase mutants were constructed to recognize and permit the incorporation of 2'-modified triphosphate ribonucleotide such as 2'-Ome, 2'-F, 2'-NH₂, 2'-N₃ into the RNA chain[77–80]. Reactive functions (amino or azido) can be imagined to further conjugate to the RNA molecules. Alternatively, 2'-F pRNA molecules were constructed and shown to present an improved resistance to RNase degradation compared to unmodified RNA, when both pyrimidines were substituted by their 2'-F counterparts[81].

5.1.3.2 Single labeling of pRNA with fluorescent molecules or other chemical reactive groups: Although it is not difficult to incorporate a single label during solid phase synthesis of short RNA, synthesis of long RNAs greater than about 80 nts only rely on the enzymatic methods and the single labeling of the RNA is hard to achieve with high labeling efficiency. To overcome this challenge and label the longer RNA with a single functional group, AMP and GMP derivatives which have been demonstrated to be efficient initiators in RNA transcription by T7 RNA polymerase but cannot be used in the elongation step, were designed (Fig. 4). Efficient labeling of RNA molecules at the specific 5' position can be readily achieved by either one-step transcription initiation or a two-step procedure of transcription and post-transcriptional modification. Various kinds of AMP and GMP derivatives were synthesized by conjugating different chemical groups with AMP or GMP through linker molecules through established chemistry[64,82–85]. The amino- and thiol-reactive derivatives, AMP-hexanediamine (HDA)[82,86] and 5'-deoxy-5'-thioguanosine-5'-monophosphorothioate (GSMP)[83] respectively, present both reactive moieties for further conjugation with ligands or detection markers for the production of deliverable polyvalent therapeutic particles. Whereas the synthesis of GSMP requires a more complicated chemical process[83], an AMP-HDA derivative was synthesized in one-step through the direct coupling of HDA to AMP at pH 6.5 in the presence of 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) [86]. AMP-HDA can then be purified either simply by removing the excess of HDA using ion exchange chromatography[86] or reverse-phase chromatography[82].

The reactive aliphatic amine of AMP-HDA can be used for further functionalization by common coupling reactions with *N*-hydroxysuccinimide (NHS) activated compounds. Fluorescent dyes (FITC, Cy5, Cy3), targeting moieties (folic acid)[87], or also biotin can be coupled to AMP-HDA and the resulting derivatives are shown to be efficient for the construction of single labeled pRNA molecule[88]. Alternatively, these NHS activated compounds can be directly coupled to the end NH₂ group of single labeled RNA fragments as a post-transcriptional labeling approach.

Unlike most of the common dyes and biotin, folic acid is not commercially available through its NHS ester activated form. Reaction of folic acid in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC), NHS, and triethylamine (TEA) in dimethylsulfoxide (DMSO) lead to its conversion into its NHS ester derivatives, which can be purified from the reaction mixture by precipitation[89]. Alternatively, folate labeling of pRNA was also achieved through a two-step procedure involving primarily the coupling of the folate-NHS ester with a 5'-NH₂-DNA oligo. HPLC purification of the coupling product, followed by the annealing with pRNA has led to pRNA-folate conjugates.

SH-groups, provided by GSMP, can be used to link either a NH₂-group via a hetero-bifunctional crosslinker such as *N*-[β -Maleimidopropoxy] succinimide ester (BMPS) or any common chemicals that contain maleimide, vinyl sulfone or pyridyl disulfide. Using similar approaches, it was possible to efficiently synthesize RNA directly and incorporate traditional coupling reactive groups, such as amino -NH₂, -COOH, Maleimide, or NHS for constructing polyvalent RNA nanoparticles. The NH₂- group was used to link any particles with a COOH-group with the help of EDC. NH₂/NH₂ interactions can be achieved via heterobifunctional crosslinkers.

5.1.4 Two-piece assembly and enzymatic ligation of pRNA nanoparticles—One problem in RNAi therapy is the requirement for the generation of relatively large quantities of RNA. Industrial scale of chemically synthesized RNA is one of the approaches to scale up the RNA synthesis. However, each pRNA nanoparticle is over the size limit of commercial RNA synthesis (~80 nucleotides). To produce polyvalent therapeutic pRNA nanoparticles in a large scale quantity, the phi29 pRNA can be assembled from two pieces of pRNA modules[90]. A similar principle in using two-piece modules can be applied to the assembly of therapeutic pRNA chimeras. Besides the two-piece modules reported recently[90], other modules from different locations and regions of the pRNA template were designed and tested.

Different kinds of two-piece modules were constructed by opening at the different positions of the pRNA. After chemical or enzymatic synthesis of each fragment, the two RNA fragments are annealed to form the intact particle, simply by mixing them together, heating up to 80°C and slowly cooling down to room temperature. The assembled two-piece pRNA modules maintain the folding characteristics of the intact pRNA and are able to form a dimer with its partner. Preliminary data also demonstrated that the chimeric pRNA two-piece modules can be processed and silence the target gene in the same manner as the intact particle[91].

Enzymatic ligation of the two-piece RNAs into one intact particle is found to be an efficient strategy to face the dissociation problem observed in some two-piece RNAs. Within the pRNA structure, we have already found one high-yielding ligation site in the pRNA interlocking domain[92]. After synthesizing the small RNA fragments separately, the additional RNA ligation used to assemble the longer RNA fragments is realized using T4 RNA ligase[93]. The ligation of the fragments will ensure the stability and the correct folding of the entire particle.

5.2. Construction of multimeric pRNA nanoparticles based on pRNA structure

The self-assembly of pRNA nanoparticles is a prominent bottom-up approach and represents an important idea that biomolecules can be successfully integrated into nanotechnology[7,53,54,94,95]. Such an approach relies on the cooperative interaction of individual RNA molecules that spontaneously assemble in a predefined manner and form larger 2D or 3D-structures.

There are two main categories of self-assembly: non-templated and templated nanoparticle assembly. Non-templated assembly involves the formation of a larger structure by individual components without influence from external forces. The formation of pRNA dimers, trimers and tetramers falls into this category. On the contrary, template nanoparticle assembly involves the interaction of RNAs under the influence of a specific external force, structure, or spatial constraint. In the phi29 bacteriophage, pRNA dimers serve as the building blocks to form a hexameric ring through the binding to the connector embedded in the viral procapsid which serves as the template for hexameric ring assembly[15].

5.2.1 Construction of dimeric pRNA nanoparticles—Hand-in-hand dimers are formed by the intermolecular interaction of interlocking right and left loops that are trans-complementary (Fig. 5A). For example, pRNA Ab' molecule interacts quantitatively with an equal mole ratio of pRNA Ba' molecule, forming a dimer in the presence of at least 5 mM Mg^{2+} [61]. Dimers of an extended configuration (twins) can also be efficiently self-assembled in solution by introducing a palindrome sequence (5'GCUAGC3') into the 3'-end of the RNA chimera (Fig. 5C). The assembled foot-to-foot dimer (twin) is formed by annealing such a 3'-end overhanged sequence by simply heating up to 80°C and slowly cooling down to room temperature.

5.2.2 Construction of trimeric pRNA nanoparticles—A similar mechanism is utilized to construct chimeric pRNA trimers. When molecules of pRNA Ab', Bc' and Ca' are mixed together at equal mole concentrations (Fig. 5B), stable pRNA trimers are formed with very high efficiency (up to 100%) via the interlocking loops of the three pRNAs[5–7] in the presence of 5 mM Mg^{2+} .

5.2.3 Construction of multivalent pRNA nanoparticles for detection and therapeutic application—The use of small RNA in gene therapy was significantly hampered due to the difficulties of producing a safe and efficient system which is able to specifically recognize and target specific cells. The strength of using phi29 pRNA as a delivery vehicle relies on its ability to easily form stable multimers which could be manipulated and sequence-controlled[5,6,61]. This particular system provides unprecedented versatility in constructing polyvalent delivery vehicles by separately constructing individual pRNA subunits with various cargos and mixing them together in any desired combination[3].

These nanoparticles can carry multiple components, including molecules for specific cell recognition, image detection, endosome disruption, and therapeutic treatment (Fig. 6). One subunit of the deliverable RNA nanoparticles (dimer, trimer or tetramer) can be modified to carry a RNA aptamer that binds to a specific cell-surface receptor, thereby inducing receptor-mediated endocytosis. The second subunit of the multimer can carry reporting molecules such as gold particles or fluorescent beads for the evaluation of cell binding and entry. The third subunit can be re-engineered to carry components that can be used to enhance endosome disruption so that the therapeutic molecules are released. The fourth (or fifth and sixth, if needed) subunit of the RNA nanoparticles can carry therapeutic siRNA, ribozyme RNAs, antisense RNA, or other drugs to be delivered.

6. The advantages of using pRNA nanoparticles for therapeutics

Phi29 pRNA-derived carriers act as an innovative and versatile targeted delivery system for cancer treatment. Therapeutic options for patients with cancer are extremely limited. A high percentage of late-stage cancers are also resistant toward current conventional chemo- or radio-therapies. Thus, there is an urgent need to develop a new treatment regime for patients suffering from cancers. Our work using pRNA as a vector to specifically deliver therapeutics to targeted tumor cells serves both as a model to prove the concept of using RNA nanotechnology for cancer treatment and will pave a way toward clinical trials for noninvasive treatment of cancer. One might ask why use phi29 pRNA nanoparticles over the numerous other anti-cancer delivery platforms under development. The phi29 pRNA system is unique and offers the following numerous advantages: 1) polyvalent delivery; 2) controllable structure and precise stoichiometry; 3) nanoscale size; 4) targeted delivery; and 5) non-induction of antibody to ensure repeated treatments.

6.1 Polyvalent delivery

The polyvalent pRNA nanoparticles can deliver up to six kinds of molecules to specific cells including therapeutics, detection molecules, drugs, or other functionalities[3]. This particular system provides unprecedented versatility in constructing polyvalent delivery vehicles by separately constructing individual pRNA subunits with various cargos and mixing them together in any desired combination. For example, the deliverable pRNA nanoparticles can be re-engineered to carry therapeutic siRNAs, ribozyme RNAs, antisense RNAs against multiple targets or different regions of one target gene, and RNA aptamers or folic acid for delivery. The other subunits of the pRNA nanoparticles may carry anti-cancer drugs to enhance the therapeutic effect or to overcome the drug resistance by combination therapy. The therapeutics or detection molecules may also be combined into one nanoparticle, making the concomitant therapy and detection of the therapeutics possible with only one administration.

6.2 Controllable structure and precise stoichiometry

The homogeneity in size of the pRNA nanoparticles is of extreme importance. Highly efficient and controlled bottom-up self-assembly yields nanoparticles with well defined structures and stoichiometry. This characteristic is highly valuable for the reproducible manufacturing of drugs for increased safety and will facilitate the U.S. Food and Drug Administration (FDA) approval of the therapeutic reagents.

6.3 Nanoscale size

It is commonly accepted that the size of a nanoparticles is paramount for effective delivery to diseased tissues. Many studies suggest that particles ranging from 10–100 nm[96–98] are the optimal size for a nonviral vector: large enough to be retained by the body, yet small enough to pass through the cell membrane[97] and access the cell surface receptors. During the development of solid tumors, angiogenesis occurs to supply enough oxygen and nutrients to the fast growing tumor cells. The angiogenic blood vessels, unlike the tight blood vessels in most normal tissues, have gaps between adjacent endothelial cells. This allows the particles that are usually excluded from the normal tissue to extravagate through these gaps into the tumor interstitial space and concentrate in the tumor, in a size-dependent manner. The pRNA nanoparticles (dimers, trimers or tetramers) have optimal sizes ranging between 20 and 40 nm which improves the biodistribution of the therapeutic pRNA nanoparticles in the blood circulation system while the average size of a normal single siRNA molecule is well below 10 nm, which represents a major challenge for the siRNA delivery *in vivo*. In addition, the polyanionic nature of RNA makes it difficult to penetrate the cell membrane, and non-formulated siRNAs have been reported to be easily excreted by

the body[99–102]. Nanoparticle delivery of siRNA or other therapeutics has the potential to improve the PK, pharmacodynamics, and biodistribution, as well as to provide a lower toxicity in treatment, as discussed in Section 8[92].

Furthermore, the PK and pharmacodynamics of the pRNA nanoparticles has been improved by introducing chemical modifications to the RNA backbone. The chemically modified RNA can be resistant to RNase, which makes RNA nanoparticles more stable and increases their retention time during blood circulation. The specific delivery and longer retention time of pRNA nanoparticles also assures the usage of a lower dose for the treatment.

6.4 Targeted delivery

pRNA nanoparticles can carry both a therapeutic agent and a recognition ligand for targeted delivery to specific cells. The incorporation of a receptor binding aptamer, folate, or other ligands to the pRNA complex with simple procedures ensures the specific binding and targeted delivery to cells. In combination with the advantage of nano-scale size, the system provides the both advantages of higher delivery efficiency and reduced off-target toxicity[92].

6.5 Non-induction of an antibody response to ensure repeated treatments

Using such protein-free RNA nanoparticles with RNA aptamers as anti-receptors will yield specificity as compared with protein anti-receptors and a lower antibody-inducing activity, thus providing an opportunity for repeated administration and treatment of chronic diseases.

7. The broad medical applications of the pRNA nanodelivery platform

The potential of the pRNA platform[3,103] for the construction of RNA nanoparticles to carry receptor-binding ligands for specific delivery of siRNA to target and silence particular genes have been explored for a variety of cancer and viral infected cells, including breast cancer[104], prostate cancer[53], cervical cancer[104], nasopharyngeal carcinoma[64], leukemia[53,54], and ovarian cancer[68], as well as coxsackievirus infected cells[67] (Table 1). The data demonstrated that the pRNA nanoparticle is a nanodelivery platform that can be applied broadly to diverse medical applications.

8. Pharmacokinetics and toxicity studies in animal trials

Many factors affect the utility and effectiveness of a nanodelivery system. These include metabolic stability, induction of both innate and adaptive immunity, pharmacokinetic (PK) behavior and toxicological properties. It is generally believed that the optimal particle size for nanodelivery is 10 to 100 nm. This size range is large enough to avoid kidney filtration of particles less than 10 nm, but small enough to penetrate tissues and enter cells via receptor-mediated endocytosis, and facilitate intracellular trafficking, while also minimizing reticuloendothelial system (RES) mediated clearance[4]. The commonly used lipid and polymer based nanoparticles with larger particle sizes and being hydrophobic cause accumulation in the liver kupffer cells and spleen macrophages, as well as lung macrophages [4]. In addition, the induction of innate immunity and certain organ toxicity has been a major concern in siRNA therapeutics[105]. Recently, it has been demonstrated that 2'-F-modified pRNA nanoparticles were chemically and metabolically stable in mice and demonstrated a favorable PK profile of plasma half-life ($T_{1/2}$) of 5 to 10 hours, in contrast to $T_{1/2}$ of 0.25 hour reported for siRNAs with similar chemical modifications (Fig. 7A), a clearance value (Cl) smaller than 0.13L/kg/hr, and a volume of distribution (V_d) of 1.2L/kg (Fig. 7B)[92]. With fluorescent and folate labeling, the pRNA nanoparticles were found to be specifically self-delivered to folate receptor positive xenograft tumor in mice upon systemic administration, with minimal accumulation in normal organs or tissues. These

particles, composed of pure RNA, did not provoke interferon response, nor did they stimulate cytokine production in mice[92]. Repeated intravenous administrations at doses up to 30 mg per kilogram did not cause toxicity in mice[92]. The result of this and other pharmacological studies suggest that pRNA nanoparticles carry all the preferred pharmacological features to serve as safe drugs with broad clinical applications.

9. Industry scale production of pRNA nanoparticles

RNA can be manipulated with simplicity characteristic of DNA and bears versatile structure and catalytic function similar to proteins. It is an ideal building block in nanotechnology and nanomedicine. However, standing in awe of its sensitivity to RNase degradation has made many scientists hesitant to apply RNA nanotechnology. Recently, it has been reported that 2'-F modified pRNA retained its natural property for correct folding in dimer formation, appropriate structure in binding to the phi29 procapsid, as well as authentic function in driving the DNA packaging nanomotor, and producing infectious viral particles (Fig. 8) [106]. The data reveals that it is possible to manufacture RNase-resistant, biologically active and chemically stable RNA building blocks for application in nanotechnology.

pRNA is a 120-nucleotide molecule. Currently, chemical synthesis of RNA with 120 nucleotides in large quantities is very challenging. In addition, labeling of specific locations of pRNA requires the understanding of its modular organization. For *in vivo* trials or clinical applications, one technical hurdle is the lack of a scalable industry process to produce sufficient quantities of RNA. Recently, a two-piece approach has been reported for the construction of a functional 117-nucleotide pRNA using two synthetic RNA fragments with modifications at different locations. The resulting bipartite pRNA was fully competent in pRNA dimer formation, in the packaging of DNA via the nanomotor and in the *in vitro* assembly of phi29 virions [91]. The pRNA subunit assembled from two-piece fragments harboring siRNAs or receptor-binding ligands were also able to assemble into dimers. Dimers carrying different functionalities were able to bind cancer cells specifically, enter the cells and silence specific genes of interest. The pRNA nanoparticles were processed by Dicer to release the siRNA embedded in the nanoparticles. This two piece approach has also been demonstrated to be adapted for total chemical synthesis[92], enabling industrial scale manufacturing. The results pave a way toward the treatment of diseases using synthetic pRNA/siRNA chimeric nanoparticles.

10. Concluding remarks

The phi29 pRNA system is a unique delivery system, which takes advantage of the structural features of pRNA including the presence of two domains which fold independently and the facility of re-engineering to form different kinds of desired particles through interlocking loop-loop interactions or end palindrome sequences. The assembled nano-scale particles harboring functional moieties offer many advantages over the numerous other anti-cancer delivery platforms under development such as polyvalent delivery, controllable structure and precise stoichiometry, nanoscale size, targeted delivery, long half life *in vivo*, no induction of interferon, and toll-like immunity, low or no toxicity, and non-induction of an antibody response to ensure repeated treatments. Consequently, pRNA nanoparticles delivery will potentially lead to an innovative therapeutic strategy for developing an effective and safe treatment for cancer and other diseases.

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Abbreviations

pRNA	Phi29 motor packaging RNA
RNAi	RNA interference
SELEX	systematic evolution of ligands by exponential enrichment
HBV	hepatitis B virus
CVB3	coxsackievirus B3
MT-IIA	Metallothionein-IIA
HDA	AMP-hexanediamine
GSMP	5'-deoxy-5'-thioguanosine-5'-monophosphorothioate
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
NHS	<i>N</i> -hydroxysuccinimide
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
TEA	triethylamine
DMSO	dimethylsulfoxide
BMPS	<i>N</i> -[β -Maleimidopropyl]succinimide ester
RES	reticuloendothelial system
Cl	clearance value
V_d	volume of distribution
PK	pharmacokinetics

Reference List

1. Niemeyer CM. The developments of semisynthetic DNA-protein conjugates. *Trends Biotechnol.* 2002; 20:395–401. [PubMed: 12175771]
2. Schmidt OG, Eberl K. Nanotechnology. Thin solid films roll up into nanotubes. *Nature.* 2001; 410:168. [PubMed: 11242068]
3. Guo P. RNA Nanotechnology: Engineering, Assembly and Applications in Detection, Gene Delivery and Therapy. *Journal of Nanoscience and Nanotechnology.* 2005; 5(12):1964–1982. [PubMed: 16430131]
4. Guo P, Coban O, Snead NM, Trebley J, Hoeprich S, Guo S, Shu Y. Engineering RNA for Targeted siRNA Delivery and Medical Application. *Advanced Drug Delivery Reviews.* 2010; 62:650–666. [PubMed: 20230868]
5. Guo P, Zhang C, Chen C, Trottier M, Garver K. Inter-RNA interaction of phage phi29 pRNA to form a hexameric complex for viral DNA transportation. *Mol Cell.* 1998; 2:149–155. [PubMed: 9702202]
6. Shu D, Huang L, Hoeprich S, Guo P. Construction of phi29 DNA-packaging RNA (pRNA) monomers, dimers and trimers with variable sizes and shapes as potential parts for nano-devices. *J Nanosci Nanotechnol.* 2003; 3:295–302. [PubMed: 14598442]
7. Shu D, Moll WD, Deng Z, Mao C, Guo P. Bottom-up assembly of RNA arrays and superstructures as potential parts in nanotechnology. *Nano Lett.* 2004; 4:1717–1723. [PubMed: 21171616]
8. Jaeger L, Westhof E, Leontis NB. TectoRNA: modular assembly units for the construction of RNA nano-objects. *Nucleic Acids Res.* 2001; 29:455–463. [PubMed: 11139616]
9. Jaeger L, Leontis NB. Tecto-RNA: One dimensional self-assembly through tertiary interactions. *Angew Chem Int Ed Engl.* 2000; 39:2521–2524. [PubMed: 10941124]

10. Hansma HG, Oroudjev E, Baudrey S, Jaeger L. TectoRNA and 'kissing-loop' RNA: atomic force microscopy of self-assembling RNA structures. *J Microsc.* 2003; 212:273–279. [PubMed: 14629553]
11. Afonin KA, Bindewald E, Yaghoubian AJ, Voss N, Jacovetty E, Shapiro BA, Jaeger L. In vitro assembly of cubic RNA-based scaffolds designed in silico. *Nat Nanotechnol.* 2010; 5:676–682. [PubMed: 20802494]
12. Guo P, Erickson S, Anderson D. A small viral RNA is required for *in vitro* packaging of bacteriophage phi29 DNA. *Science.* 1987; 236:690–694. [PubMed: 3107124]
13. Cech TR, Zaug AJ, Grabowski PJ. In vitro splicing of the ribosomal RNA precursor of Tetrahymena: involvement of a guanosine nucleotide in the excision of the intervening sequence. *Cell.* 1981; 27:487–496. [PubMed: 6101203]
14. Shu D, Zhang H, Jin J, Guo P. Counting of six pRNAs of phi29 DNA-packaging motor with customized single molecule dual-view system. *EMBO J.* 2007; 26:527–537. [PubMed: 17245435]
15. Xiao F, Zhang H, Guo P. Novel mechanism of hexamer ring assembly in protein/RNA interactions revealed by single molecule imaging. *Nucleic Acids Res.* 2008; 36(20):6620–6632. [PubMed: 18940870]
16. Kruger K, Grabowski PJ, Zaug AJ, Sands J, Gottschling DE, Cech TR. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. *Cell.* 1982; 31:147–157. [PubMed: 6297745]
17. Cech TR, Tanner NK, Tinoco I, Weir BR, Zuker M, Perlman PS. Secondary structure of the tetrahymena ribosomal RNA intervening sequence: structural homology with fungal mitochondrial intervening sequences. *Proc Natl Acad Sci USA.* 1983; 80:3903–3907. [PubMed: 6306649]
18. Zaug AJ, Grabowski PJ, Cech TR. Autocatalytic cyclization of an excised intervening sequence RNA is a cleavage-ligation reaction. *Nature.* 1983; 301:578–583. [PubMed: 6186917]
19. Hoepflich S, Guo P. Computer modeling of three-dimensional structure of DNA-packaging RNA(pRNA) monomer, dimer, and hexamer of phi29 DNA Packaging motor. *J BiolChem.* 2002; 277(23):20794–20803.
20. Leontis NB, Westhof E. Analysis of RNA motifs. *Curr Opin Struct Biol.* 2003; 13:300–308. [PubMed: 12831880]
21. Kasprzak W, Bindewald E, Kim TJ, Jaeger L, Shapiro BA. Use of RNA structure flexibility data in nanostructure modeling. *Methods.* 2010
22. Yingling YG, Shapiro BA. Computational design of an RNA hexagonal nanoring and an RNA nanotube. *Nano Lett.* 2007; 7:2328–2334. [PubMed: 17616164]
23. Bindewald E, Hayes R, Yingling YG, Kasprzak W, Shapiro BA. RNAJunction: a database of RNA junctions and kissing loops for three-dimensional structural analysis and nanodesign. *Nucleic Acids Res.* 2008; 36:D392–D397. [PubMed: 17947325]
24. Bindewald E, Grunewald C, Boyle B, O'Connor M, Shapiro BA. Computational strategies for the automated design of RNA nanoscale structures from building blocks using NanoTiler. *J Mol Graph Model.* 2008; 27:299–308. [PubMed: 18838281]
25. Bouvet P. Determination of nucleic acid recognition sequences by SELEX. *Methods Mol Biol.* 2001; 148:603–610. [PubMed: 11357617]
26. Ciesiolka J, Gorski J, Yarus M. Selection of an RNA domain that binds Zn²⁺ RNA. 1995; 1:538–550. [PubMed: 7489515]
27. Clark S, Remcho V. Aptamers as analytical reagents. *Electrophoresis.* 2002; 23:1335–1340. [PubMed: 12007135]
28. Kraus E, James W, Barclay AN. Cutting edge: novel RNA ligands able to bind CD4 antigen and inhibit CD4+ T lymphocyte function. *J Immunol.* 1998; 160:5209–5212. [PubMed: 9605115]
29. Shu D, Guo P. A Viral RNA that binds ATP and contains an motif similar to an ATP-binding aptamer from SELEX. *J Biol Chem.* 2003; 278(9):7119–7125. [PubMed: 12444088]
30. Gold L. The SELEX process: a surprising source of therapeutic and diagnostic compounds. *Harvey Lect.* 1995; 91:47–57. [PubMed: 9127985]
31. Ellington AD, Szostak JW. *In vitro* selection of RNA molecules that bind specific ligands. *Nature.* 1990; 346:818–822. [PubMed: 1697402]

32. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science*. 1990; 249:505–510. [PubMed: 2200121]
33. McNamara JO, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA, Giangrande PH. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat Biotech*. 2006; 24:1005–1015.
34. Dassie JP, Liu XY, Thomas GS, Whitaker RM, Thiel KW, Stockdale KR, Meyerholz DK, McCaffrey AP, McNamara JO, Giangrande PH. Systemic administration of optimized aptamer-siRNA chimeras promotes regression of PSMA-expressing tumors. *Nat Biotechnol*. 2009; 27:839–849. [PubMed: 19701187]
35. Zhou J, Swiderski P, Li H, Zhang J, Neff CP, Akkina R, Rossi JJ. Selection, characterization and application of new RNA HIV gp 120 aptamers for facile delivery of Dicer substrate siRNAs into HIV infected cells. *Nucleic Acids Res*. 2009; 37:3094–3109. [PubMed: 19304999]
36. Zhou J, Li H, Zaia J, Rossi JJ. Novel dual inhibitory function aptamer-siRNA delivery system for HIV-1 therapy. *Mol Ther*. 2008; 16:1481–1489. [PubMed: 18461053]
37. Cerchia L, deV F. Targeting cancer cells with nucleic acid aptamers. *Trends Biotechnol*. 2010; 28:517–525. [PubMed: 20719399]
38. Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S. The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. *Cell*. 1983; 35:849–857. [PubMed: 6197186]
39. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998; 391:806–811. [PubMed: 9486653]
40. Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. *Nat Rev Genet*. 2009; 10:94–108. [PubMed: 19148191]
41. Li H, Li WX, Ding SW. Induction and suppression of RNA silencing by an animal virus. *Science*. 2002; 296:1319–1321. [PubMed: 12016316]
42. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science*. 2002; 296:550–553. [PubMed: 11910072]
43. Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. *Nature*. 2002; 418:435–438. [PubMed: 12087358]
44. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP, Rubin MA, Chinnaiyan AM. The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature*. 2002; 419:624–629. [PubMed: 12374981]
45. Carmichael GG. Medicine: silencing viruses with RNA. *Nature*. 2002; 418:379–380. [PubMed: 12140542]
46. Sarver NA, Cantin EM, Chang PS, Zaia JA, Ladne PA, Stephens DA, Rossi JJ. Ribozymes as potential anti-HIV-1 therapeutic agents. *Science*. 1990; 247:1222–1225. [PubMed: 2107573]
47. Chowrira BM, Berzal-Herranz A, Burke JM. Novel guanosine requirement for catalysis by the hairpin ribozyme. *Nature*. 1991; 354:320–322. [PubMed: 1956383]
48. Forster AC, Symons RH. Self-cleavage of virusoid RNA is performed by the proposed 55-nucleotide active site. *Cell*. 1987; 50:9–16. [PubMed: 3594567]
49. Sarver N, Cantin EM, Chang PS, Zaia JA, Ladne PA, Stephens DA, Rossi JJ. Ribozymes as Potential Anti-HIV-1 Therapeutic Agents. *Science*. 1990; 24:1222–1225. [PubMed: 2107573]
50. Coleman J, Hirashima A, Inocuchi Y, Green PJ, Inouye M. A novel immune system against bacteriophage infection using complementary RNA (micRNA). *Nature*. 1985; 315:601–603. [PubMed: 2409446]
51. Knecht DA, Loomis WF. Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. *Science*. 1987; 236:1081–1086. [PubMed: 3576221]
52. Chen C, Zhang C, Guo P. Sequence requirement for hand-in-hand interaction in formation of pRNA dimers and hexamers to gear phi29 DNA translocation motor. *RNA*. 1999; 5:805–818. [PubMed: 10376879]
53. Guo S, Tschammer N, Mohammed S, Guo P. Specific delivery of therapeutic RNAs to cancer cells via the dimerization mechanism of phi29 motor pRNA. *Hum Gene Ther*. 2005; 16:1097–1109. [PubMed: 16149908]

54. Khaled A, Guo S, Li F, Guo P. Controllable Self-Assembly of Nanoparticles for Specific Delivery of Multiple Therapeutic Molecules to Cancer Cells Using RNA Nanotechnology. *Nano Letters*. 2005; 5:1797–1808. [PubMed: 16159227]
55. Liu H, Guo S, Roll R, Li J, Diao Z, Shao N, Riley MR, Cole AM, Robinson JP, Snead NM, Shen G, Guo P. Phi29 pRNA Vector for Efficient Escort of Hammerhead Ribozyme Targeting Survivin in Multiple Cancer Cells. *Cancer Biol Ther*. 2007; 6:697–704. [PubMed: 17426446]
56. Guo P, Trottier M. Biological and biochemical properties of the small viral RNA (pRNA) essential for the packaging of the double-stranded DNA of phage ϕ 29. *Seminars in Virology*. 1994; 5:27–37.
57. Earnshaw WC, Casjens SR. DNA packaging by the double-stranded DNA bacteriophages. *Cell*. 1980; 21:319–331. [PubMed: 6447542]
58. Reid RJD, Bodley JW, Anderson D. Identification of bacteriophage phi29 prohead RNA (pRNA) domains necessary for *in vitro* DNA-gp3 packaging. *J Biol Chem*. 1994; 269:9084–9089. [PubMed: 8132646]
59. Zhang CL, Tellinghuisen T, Guo P. Confirmation of the helical structure of the 5'/3' termini of the essential DNA packaging pRNA of phage ϕ 29. *RNA*. 1995; 1:1041–1050. [PubMed: 8595559]
60. Lee TJ, Guo P. Interaction of gp16 with pRNA and DNA for genome packaging by the motor of bacterial virus phi29. *J Mol Biol*. 2006; 356:589–599. [PubMed: 16376938]
61. Chen C, Sheng S, Shao Z, Guo P. A dimer as a building block in assembling RNA: A hexamer that gears bacterial virus phi29 DNA-translocating machinery. *J Biol Chem*. 2000; 275(23):17510–17516. [PubMed: 10748150]
62. Zhang F, Lemieux S, Wu X, St-Arnaud S, McMurray CT, Major F, Anderson D. Function of hexameric RNA in packaging of bacteriophage phi29 DNA *in vitro*. *Mol Cell*. 1998; 2:141–147. [PubMed: 9702201]
63. Hoepflich S, Zhou Q, Guo S, Qi G, Wang Y, Guo P. Bacterial virus phi29 pRNA as a hammerhead ribozyme escort to destroy hepatitis B virus. *Gene Ther*. 2003; 10:1258–1267. [PubMed: 12858191]
64. Guo S, Huang F, Guo P. Construction of folate-conjugated pRNA of bacteriophage phi29 DNA packaging motor for delivery of chimeric siRNA to nasopharyngeal carcinoma cells. *Gene Ther*. 2006; 13:814–820. [PubMed: 16482206]
65. Zhang CL, Lee C-S, Guo P. The proximate 5' and 3' ends of the 120-base viral RNA (pRNA) are crucial for the packaging of bacteriophage ϕ 29 DNA. *Virology*. 1994; 201:77–85. [PubMed: 8178491]
66. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*. 2001; 411:494–498. [PubMed: 11373684]
67. Zhang HM, Su Y, Guo S, Yuan J, Lim T, Liu J, Guo P, Yang D. Targeted delivery of anti-coxsackievirus siRNAs using ligand-conjugated packaging RNAs. *Antiviral Res*. 2009; 83:307–316. [PubMed: 19616030]
68. Tarapore P, Shu Y, Guo P, Ho SM. Application of Phi29 Motor pRNA for Targeted Therapeutic Delivery of siRNA Silencing Metallothionein-IIA and Survivin in Ovarian Cancers. *Mol Ther*. 2010 In Press.
69. Zhang CL, Tellinghuisen T, Guo P. Use of circular permutation to assess six bulges and four loops of DNA-Packaging pRNA of bacteriophage phi29. *RNA*. 1997; 3:315–322. [PubMed: 9056768]
70. Nolan JM, Burke DH, Pace NR. Circularly Permuted tRNAs as Specific Photoaffinity Probes of Ribonuclease P RNA Structure. *Science*. 1993; 261:762–765. [PubMed: 7688143]
71. Pan T, Uhlenbeck OC. Circularly permuted DNA, RNA and proteins-a review. *Gene*. 1993; 125:111–114. [PubMed: 7681803]
72. Zhang CL, Trottier M, Guo PX. Circularly permuted viral pRNA active and specific in the packaging of bacteriophage ϕ 29 DNA. *Virology*. 1995; 207:442–451. [PubMed: 7533964]
73. Zhou J, Shu Y, Guo P, Smith DD, Rossi JJ. Dual Functional RNA Nanoparticles Containing Phi29 Motor pRNA and Anti-gp120 Aptamer for Cell-type Specific Delivery and HIV-1 Inhibition. *Methods*. 2011

74. Singh Y, Murat P, Defrancq E. Recent developments in oligonucleotide conjugation. *Chem Soc Rev.* 2010; 39:2054–2070. [PubMed: 20393645]
75. Shu D, Zhang H, Petrenko R, Meller J, Guo P. Dual-channel single-molecule fluorescence resonance energy transfer to establish distance parameters for RNA nanoparticles. *Nucleic Acid Research.* 2010; 4:6843–6853.
76. Stengel G, Urban M, Purse BW, Kuchta RD. Incorporation of the fluorescent ribonucleotide analogue tCTP by T7 RNA polymerase. *Anal Chem.* 2010; 82:1082–1089. [PubMed: 20067253]
77. Huang Y, Eckstein F, Padilla R, Sousa R. Mechanism of ribose 2'-group discrimination by an RNA polymerase. *Biochemistry.* 1997; 36:8231–8242. [PubMed: 9204868]
78. Padilla R, Sousa R. Efficient synthesis of nucleic acids heavily modified with non-canonical ribose 2'-groups using a mutant T7 RNA polymerase (RNAP). *Nucleic Acids Res.* 1999; 27:1561–1563. [PubMed: 10037823]
79. Sousa R, Padilla R. A mutant T7 RNA polymerase as a DNA polymerase. *EMBO J.* 1995; 14:4609–4621. [PubMed: 7556104]
80. Padilla R, Sousa R. A Y639F/H784A T7 RNA polymerase double mutant displays superior properties for synthesizing RNAs with non-canonical NTPs. *Nucleic Acids Research.* 2002; 30:e138.
81. Kawasaki AM, Casper MD, Freier SM, Lesnik EA, Zounes MC, Cummins LL, Gonzalez C, Cook PD. Uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets. *J Med Chem.* 1993; 36:831–841. [PubMed: 8464037]
82. Huang F, Wang G, Coleman T, Li N. Synthesis of adenosine derivatives as transcription initiators and preparation of 5' fluorescein- and biotin-labeled RNA through one-step *in vitro* transcription. *RNA.* 2003; 9:1562–1570. [PubMed: 14624011]
83. Zhang L, Sun L, Cui Z, Gottlieb RL, Zhang B. 5'-sulfhydryl-modified RNA: initiator synthesis, *in vitro* transcription, and enzymatic incorporation. *Bioconjug Chem.* 2001; 12:939–948. [PubMed: 11716685]
84. Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res.* 1987; 15:8783–8798. [PubMed: 3684574]
85. Bruce AG, Uhlenbeck OC. Reactions at the termini of tRNA with T4 RNA ligase. *Nucleic Acids Res.* 1978; 5:3665–3677. [PubMed: 724498]
86. Huang F, He J, Zhang Y, Guo Y. Synthesis of biotin-AMP conjugate for 5' biotin labeling of RNA through one-step *in vitro* transcription. *Nat Protoc.* 2008; 3:1848–1861. [PubMed: 18989262]
87. Laing BM, Guo P, Bergstrom DE. Optimized method for the synthesis and purification of adenosine - Folic acid conjugates for use as transcription initiators in the preparation of modified RNA. *Methods.* 2010
88. Huang F. Efficient incorporation of CoA, NAD and FAD into RNA by *in vitro* transcription. *Nucleic Acids Res.* 2003; 31:e8. [PubMed: 12560511]
89. Zhang P, Zhang Z, Yang Y, Li Y. Folate-PEG modified poly(2-(2-aminoethoxy)ethoxy)phosphazene/DNA nanoparticles for gene delivery: synthesis, preparation and *in vitro* transfection efficiency. *Int J Pharm.* 2010; 392:241–248. [PubMed: 20298769]
90. Fang Y, Shu D, Xiao F, Guo P, Qin PZ. Modular assembly of chimeric phi29 packaging RNAs that support DNA packaging. *Biochemical and Biophysical Research Communications.* 2008; 372:589–594. [PubMed: 18514064]
91. Shu Y, Cinier M, Fox SR, Ben-Johnathan N, Guo P. Assembly of Therapeutic pRNA-siRNA Nanoparticles Using Bipartite Approach. *Molecular Therapy Accepted.* 2011
92. Abdelmawla S, Guo S, Zhang L, Pulkuri S, Patankar P, Conley P, Trebley J, Guo P, Li QX. Pharmacological Characterization of Chemically Synthesized Monomeric pRNA Nanoparticles for Systemic Delivery. *Molecular Therapy Accepted.* 2011
93. Uhlenbeck OC, England TE. Enzymatic oligoribonucleotide synthesis with T4 RNA ligase. *Biochemistry.* 1978; 17:2069–2076. [PubMed: 667012]
94. Glotzer SC. Materials science. Some Assembly Required. *Science.* 2004; 306:419–420. [PubMed: 15486279]

95. Gates BD, Xu Q, Stewart M, Ryan D, Willson CG, Whitesides GM. New approaches to nanofabrication: molding, printing, and other techniques. *Chem Rev.* 2005; 105:1171–1196. [PubMed: 15826012]
96. Jain KK. The role of nanobiotechnology in drug discovery. *Drug Discov Today.* 2005; 10:1435–1442. [PubMed: 16243263]
97. Li W, Szoka F. Lipid-based Nanoparticles for Nucleic Acid Delivery. *Pharm Res.* 2007; 24:438–449. [PubMed: 17252188]
98. Gao H, Shi W, Freund LB. Mechanics of receptor-mediated endocytosis. *Proc Natl Acad Sci USA.* 2005; 102:9469–9474. [PubMed: 15972807]
99. de Fougerolles A, Vornlocher HP, Maraganore J, Lieberman J. Interfering with disease: a progress report on siRNA-based therapeutics. *Nat Rev Drug Discov.* 2007; 6:443–453. [PubMed: 17541417]
100. Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. *Nat Rev Genet.* 2007; 8:173–184. [PubMed: 17304245]
101. Rozema DB, Lewis DL, Wakefield DH, Wong SC, Klein JJ, Roesch PL, Bertin SL, Reppen TW, Chu Q, Blokhin AV, Hagstrom JE, Wolff JA. Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes. *Proceedings of the National Academy of Sciences.* 2007; 104:12982–12987.
102. De Paula D, Bentley MV, Mahato RI. Hydrophobization and bioconjugation for enhanced siRNA delivery and targeting. *RNA.* 2007; 13:431–450. [PubMed: 17329355]
103. Shu Y, Shu D, Diao Z, Shen G, Guo P. Fabrication of Polyvalent Therapeutic RNA Nanoparticles for Specific Delivery of siRNA, Ribozyme and Drugs to Targeted Cells for Cancer Therapy. *IEEE/NIH Life Science Systems and Applications Workshop.* 2009:9–12. [PubMed: 21243099]
104. Li L, Liu J, Diao Z, Guo P, Shen G. Evaluation of specific delivery of chimeric phi29 pRNA/siRNA nanoparticles to multiple tumor cells. *Molecular BioSystems.* 2009; 5:1361–1368. [PubMed: 19823753]
105. Robbins M, Judge A, MacLachlan I. siRNA and Innate Immunity. *Oligonucleotides.* 2009; 19:89–101. [PubMed: 19441890]
106. Liu J, Guo S, Cinier M, Shlyakhtenko L, Shu Y, Chen C, Shen G, Guo P. Fabrication of Stable and RNase- Resistant RNA Nanoparticles Active in Gearing the Nanomotors for Viral DNA Packaging. *ACS Nano.* 2010 In Press.
107. Shu D, Huang L, Guo P. A simple mathematical formula for stoichiometry quantitation of viral and nanobiological assemblage using slopes of log/log plot curves. *J. Virol Meth.* 2003; 115(1): 19–30.

Fig. 1. The illustration of phi29 pRNA structure, function and application. A. Primary sequence and the folding structure of the pRNA. B. The assembled hexamer ring via hand-in-hand interaction (adapted from [15] with permission from the authors and the publisher). C. The phi29 pRNA packaging motor is geared by pRNA rings to package DNA into procapsid[12] (adapted from [107] with permission from the authors and the publisher). D. pRNA has the potential to be used as a building block for assembly of therapeutic RNA nanoparticles.

Fig. 2.

The construction of pRNA chimeras including therapeutic portion like pRNA/siRNA and pRNA/ribozyme as well as delivery portion such as pRNA/aptamer and pRNA conjugated with chemical ligands.

Fig. 3.

Target delivery of folate labeled pRNA nanoparticles *in vitro*[91] and *in vivo*[92]. A. a. Flow cytometry data showed the specific and efficient binding of the folate-pRNA nanoparticles to cancer cells. b. Confocal images showed the internalization of folate-pRNA nanoparticles after binding. B. a. Target delivery of pRNA nanoparticles *in vivo*. b. The pRNA nanoparticles were delivered and accumulated into FR positive tumor specifically compared to normal tissues and organs. Lv = liver; K = kidney; H = heart; L = lung; S = spleen; I = intestine; M = muscle; T = tumor. (Figures are adapted from [91] and [92] with permission from the authors and the publisher).

Fig. 4.
The chemical structure of the synthesized AMP and GMP derivatives.

Fig. 5. Methods for constructing pRNA nanoparticles including A. hand-in-hand dimer, B. trimer, C. foot-to-foot dimer and D. Tetramer (adapted from [6] with permission from the authors and the publisher).

Fig. 6.

Illustration of constructed therapeutic pRNA multimers. A. pRNA chimeric monomer with 3'-end annealed oligo which conjugates the detection molecule and chemical ligand at the same time. B. a. Twin dimer formed through palindrome sequence; b. Dimer formation through loop-loop interaction. C. Trimeric RNA chimera. D. Tetrameric pRNA chimera and E. Hexameric pRNA particle (adapted from [103] with permission from the authors and the publisher).

A. Secondary PK parameters of pRNA nanoparticles

Key parameters	2'-F-pRNA	siRNA
AUC_{last} (hr*ng/mL)	2×10^5	1.1×10^3
$T_{1/2}$ (hrs)	5~10	0.25
V_d (L/kg)	1.2	0.36
Cl (L/kg/hr)	0.13	1.0
Dose (mg/kg)	24	1.2

B. **Fig. 7.**

Pharmacological features of pRNA nanoparticles. (A) The PK profile of pRNA nanoparticles compared to siRNA *in vivo*. (B) The pRNA nanoparticle did not induce an interferon response *in vitro*. KB cells were transfected with 50 nM pRNA (2'-F modified vs. non-modified), 50 nM siRNA, and 1 µg/mL poly I:C using Fugene-HD. The cells were harvested 24 hours later and semi-quantitative RT-PCR was conducted to test for the expression of the IFN responsive genes (OAS1, OAS2, MX1 and IFITM1). (adapted from [92] with permission from the authors and the publisher).

Fig. 8. Atomic force microscopy images showing hand-in-hand and foot-to-foot dimer nanoparticles of non-modified pRNA and 2'-F C/U pRNA, respectively (adapted from [106] with permission from the authors and the publisher).

Table 1

Application of pRNA platform for the treatment of diseases

Name of the Disease	Receptors for delivery	Genes as target	References
ovarian cancer	folate receptor	Metallothionein-IIA and Survivin	[68]
breast cancer	folate receptor	Survivin	[55,104]
cervical cancer	folate receptor	Survivin	[104]
nasopharyngeal carcinoma	folate receptor	Survivin	[64]
Leukimia	CD4 receptor	Survivin, BAD and BIM	[53,54]
prostate cancer		Survivin	[53]
coxsackievirus infection	folate receptor	CVB3 protease 2A gene	[67]
HIV infection	gp120	Tat/Rev or TNPO3	[73]