Stable RNA nanoparticles as potential new generation drugs for cancer therapy

Yi Shu a,b, Fengmei Pi a,b, Ashwani Sharma a,b, Mehdi Rajabi a,b, Farzin Haque a,b, Dan Shu a,b, Markos Leggas a,b, B. Mark Evers a,b, Peixuan Guo a,b,*

a Markey Cancer Center, University of Kentucky, Lexington, KY 40536, USA
b Department of Pharmaceutical Sciences, University of Kentucky, Lexington, KY 40536, USA

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Human genome sequencing revealed that only ~1.5% of the DNA sequence coded for proteins. More and more evidence has uncovered that a substantial part of the 98.5% so-called “junk” DNAs actually code for noncoding RNAs. Two milestones, chemical drugs and protein drugs, have already appeared in the history of drug development, and it is expected that the third milestone in drug development will be RNA drugs or drugs that target RNA. This review focuses on the development of RNA therapeutics for potential cancer treatment by applying RNA nanotechnology. A therapeutic RNA nanoparticle is unique in that its scaffold, ligand, and therapeutic component can all be composed of RNA. The special physicochemical properties lend to the delivery of siRNA, miRNA, ribozymes, or riboswitches; imaging using fluorogenic RNA; and targeting using RNA aptamers. With recent advances in solving the chemical, enzymatic, and thermodynamic stability issues, RNA nanoparticles have been found to be advantageous for in vivo applications due to their uniform nano-scale size, precise stoichiometry, polyvalent nature, low immunogenicity, low toxicity, and target specificity. In vivo animal studies have revealed that RNA nanoparticles can specifically target tumors with favorable pharmacokinetic and pharmacodynamic parameters without unwanted accumulation in normal organs. This review summarizes the key studies that have led to the detailed understanding of RNA nanoparticle formation as well as chemical and thermodynamic stability issue. The methods for RNA nanoparticle construction, and the current challenges in the clinical application of RNA nanotechnology, such as endosome trapping and production costs, are also discussed.

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1. Introduction

Nanotechnology refers to the creation and application of materials using either a top-down approach or bottom-up assembly at the nanometer scale. In nature, a wide variety of macromolecules that form patterned arrays and highly-ordered structures in nano-scale have inspired several biomimetic strategies. Macromolecules, such as DNA, RNA, and proteins have intrinsically defined features with the potential to serve as versatile building blocks for bottom-up assembly of nanostructures and nano-devices.

More and more evidence has revealed that a substantial part of ~98.5% of the human genome, so-called “junk” DNA [1], codes for noncoding RNAs. These noncoding RNAs play major roles in gene regulation[5,6], cellular catalytic reaction [7], and so on [8]. The malfunction of some noncoding RNAs will end up as abnormal cellular activity closely related to cancers, for example, microRNAs (miRNAs) have been shown to function as oncosenes or tumor suppressors [9–13]; and snoRNAs (SNORD33, SNORD66, and SNORD76) were identified as biomarkers for non-small cell lung cancer [14]. Many other diseases, such as dilated cardiomyopathy and heart failure [15], were found to be related to RNA functionality. This has led to treatment strategies that use RNA as therapeutic targets [16,17]. In other aspects, the discoveries of small interfering RNAs (siRNAs) [18,19], ribozymes [20,21], riboswitches [22,23], and miRNAs [24,25] have induced a heightened interest in using RNAs as therapeutics for disease treatment.

Natural RNA possesses versatile sequences, secondary structures, and tertiary/quaternary interactions [26–28]. Several assembly mechanisms of naturally occurring RNA complexes have been applied to construct RNA nanoparticles with defined structure and stoichiometry via intra- and/or inter-molecular interactions. Through this innovative approach based on RNA nanotechnology [29,30], varieties of therapeutic RNA nanoparticles harboring multiple therapeutic modules, such as siRNA, aptamer, or miRNA, have been constructed. Each incorporated siRNA, aptamer, miRNA, or other functionalities within the nanoparticle fold into its respective, authentic structure and retain its independent function for specific cell binding, cell entry, gene silencing, catalytic function, in both in vitro and animal trials [31–33]. Following the two milestones of chemical and protein drugs, respectively, in medical history, we speculate that the third milestone in drug development will be RNA drugs or drugs that target RNA, thus, RNA nanoparticles have the potential to be a new generation of drugs. This review will discuss the application of the achievements in modern RNA nanotechnology for cancer therapy, especially focusing on well-constructed RNA-based nano-delivery systems.

2. Definition of RNA nanotechnology

RNA nanotechnology is a unique field that studies the design, fabrication, and application of RNA nanoparticles with architectures primarily made up of RNA via bottom-up self-assembly [29,30,34,35] (Fig. 1). This concept contrasts with other widely studied drug delivery nano-systems that conjugate functional RNA modules to polymers, lipids, dendrimers, gold, or other nanomaterial-based particles.


Compared to classical RNA studies, RNA nanotechnology is a relatively new field [36–41]. The first evidence showing that RNA
nanoparticles can be constructed by bottom-up self-assembly using reengineered RNA molecules was reported in 1998 [36] (Fig. 2A). The study, led by Peixuan Guo, demonstrated that dimeric, trimeric, and hexameric RNA nanoparticles can be constructed via bottom-up assembly using re-engineered RNA fragments derived from a viral RNA (pRNA) that functions as the gear component in the bacteriophage phi29 DNA packaging motor. This finding, published in *Molecular Cell* [36] and featured in *Cell* [42], is the proof-of-concept for RNA nanotechnology. The finding demonstrated that RNA nanoparticles can be fabricated with a level of simplicity as DNA. In addition, RNAs possess versatile tertiary structures and catalytic functions that mimic some proteins [29,30]. There are certain aspects which make RNA unique: 1) it contains varieties of RNA motifs such as helices, loops, bulges, stems, hairpins, and pseudo-knots to specify rich 3D architectures; 2) it possesses both canonical and noncanonical base pairings [43–45], as well as base stacking capabilities for inter- or intra-molecular interaction [46,47]; 3) it displays unique features in transcription, termination, splicing, and self-processing to produce RNA fragments which are able to self-assemble into nanoparticles *in vitro* and *in vivo* [36,48–52]; and 4) it exhibits special functional entities such as siRNAs [18,19], ribozymes [20,21], riboswitches [22,23], and miRNAs [24,25]. However, the extreme sensitivity of RNA to RNase degradation has made many scientists reluctant to embrace RNA nanotechnology. A major concern is that formation of RNA nanoparticles via self-assembly without a covalent linkage could lead to thermodynamic instability and dissociation in vivo. This concern has long hindered the application of RNA as a construction material. However, recent discoveries that chemically modified RNAs are resistant to RNase degradation have increased interest in RNA nanotechnology [29,30,53]. This advancement is essential for progress in the field, given that modified RNA nanoparticles fold correctly during assembly and retain biological functions similar to unmodified RNA [31,53,54]. In addition, the discovery of the thermodynamically stable three-way junction (3WJ) motif from phi29 motor pRNA [31–33,55] provides strong evidence for the potential application of RNA nanoparticles as therapeutics *in vivo*. Advancements in RNA nanotechnology should also be attributed to the National Cancer Institute (NCI) Nanotechnology group, which has shown great insight and promoted RNA nanotechnology for many years before its wide recognition by the scientific community.

Over the past two decades, significant advances have been made in understanding of the structure and structure-determined functions of naturally occurring, self-folding RNAs and these studies have laid a solid foundation for further development of RNA nanotechnology [38,43,44,56–63]. The accumulated information regarding the modularity of RNA architectures also allows use of small RNA modules for bottom-up design and assembly of artificial RNA structures (RNA tectonics) [38], RNA motifs [43,44,56] and RNA inter-/intra-molecular interactions [33,55,64] have been used to build up diverse RNA nanoparticles, such as squares [62], jigsaw puzzles [40], filaments [65], cubic scaffolds [61], and polyhedrons [63]. RNA folding and structural computation is essential for directing RNA motifs in RNA nanoparticle assembly. The computational design of nanostructures is relatively inexpensive and provides a faster examination of new structures. The major advances in RNA 3D computation, from traditional intra-molecular interactions to inter-molecular interactions, have been promoted by Bruce Shapiro and co-workers [61,66–69].

In 2005, Guo’s group reported that self-assembled dimeric pRNA nanoparticles could be used for specific delivery of therapeutics to cancer cells, and showed promising inhibition of tumor cell growth *in vitro* and in animal models [41,70,71]. This finding attracted the attention of NCI Nanotechnology program leaders, which initiated the NCI’s promotion of RNA nanotechnology for cancer therapy. Since then, pRNA molecules have been conjugated with varieties of therapeutic functionalities, including RNA aptamers, siRNAs, ribozymes, and miRNAs [31–33,41,49,55,70–77]. These findings paved the way for translating RNA nanotechnology into its clinical application for the treatment of various diseases such as cancer, viral infections, and potentially many diseases with underlying genetic causes.

The concept of RNA nanotechnology has been around for more than 15 years. However, the rapid development of the RNA nanotechnology has been hindered by several road-blocks for clinical applications. Great progress has been made to overcome those barricades, as discussed in the following sections.

4. Overcome the first barricade: chemical instability of RNAs

Natural RNA is extremely sensitive to RNase degradation and is especially unstable in blood plasma. Over the last few years, rapid progress has been made to improve the stability of RNA for *in vivo* application by chemical modification of RNA. These include modifications on the bases (e.g., 5-BrU and 5-IU) [78], modifications of the phosphate linkage (e.g., phosphothioate and boranophosphate) [79], alteration of the ribose 2′ hydroxyl group (e.g., 2′-F, 2′-OMe, or 2′-NH2) [53,80–82], synthesis of peptide nucleic acids (PNAs) [83], polycarboxylate nucleic acids (PCNAs) [84], locked nucleic acids (LNAs) and their respective derivatives with a bridge at different positions (2′-4′, 1′-3′) [85], and capping of the 3′-end [86]. For therapeutic purpose, these modifications have also been applied to siRNAs. The 5′-end of the antisense strand in siRNA is important for its activity and cannot be modified; however modification of 3′ ends is well-tolerated [87]. It has also been shown that 2′-OH in RNA is not an absolute requirement for siRNA activity, and thus can be modified to increase chemical stability of RNA in serum (and the modification should not disrupt RNAi potency) [88]. This further depends on size of the modification group, its position in the sequence and the total number of modified units in each strand, suggesting the concept of targeted RNA modification. Hong et al. [89] have shown that the 5′-UA/3′UA and 5′CA/3′UC pairings are the two most vulnerable sites to serum ribonuclease. With 5′-UA/3′UA being the most sensitive, modification at this particular site improves the stability of dsRNA in serum. The serum stability of siRNA can also be increased by 2′-F modifications of pyrimidine in the sense strand, keeping the antisense unaltered [90]. All these chemical modifications are very efficient in rendering RNA resistant to RNase in *vitro* and *in vivo*. However, the challenge still remains that the RNA often changes its folding properties and biological functioning. Therefore, development of a suitable method conferring RNase resistance without affecting its folding, nanoparticle assembly, and biological functioning is critical. It was recently found that among the aforementioned methods, 2′-F modifications have the least detrimental effects on RNA folding, nanoparticle assembly, and biological function [53]. In some special cases, fine-tuning may still be necessary to find an appropriate location that can be modified with minimal detrimental effects. By applying chemical modification to RNA, the instability of RNA *in vivo* is no longer an issue [31,32,54,55].

5. Overcome the second barricade: thermodynamic instability of RNA via self-assembly without covalent linkage

The thermodynamic stability of RNA nanoparticles with regard to the use of RNA nanoparticles as therapeutics is of paramount

![Fig. 2](https://example.com/fig2.png) The bacteriophage phi29 DNA packaging RNA and three “toolkits” for construction of pRNA nanoparticles based on pRNA structural features. (A) pRNA hexamer ring showing the proof-of-concept of RNA nanotechnology in 1998 [36,212]. (B) The primary sequence and secondary structure of pRNA. The 3WJ scaffold domain connects the helical foot domain and central R- and L-hand domain [55]. (C) Three “toolkits” for pRNA nanoparticle construction. Left panel: Toolkit I: extended pRNA hand-in-hand interaction. Middle panel [39,55]: Toolkit II: foot-to-foot interaction. Right panel: Toolkit III: branch extension [31,32,55]. In this figure, (A) was adapted from ref. [36,212], (B) was adapted from ref. [55], (C) was adapted from ref. [31,32,39,55] with permission.
importance. Systemic injection of several micro-liters of RNA solution into the body will result in a hundred-thousand fold dilution. Dissociation of assembled RNA nanoparticles at extremely low concentrations is a serious concern. Crosslinking agents, such as psoralen [91] and transition metal compounds [92] can promote the formation of stable RNA complexes. In a recent paper, a thermodynamically stable pRNA-3WJ core was assembled from 3 pieces of RNA in the absence of metal ions [31,32]. The 3WJ RNA nanoparticles are resistant to denaturation in urea, stable in serum (after 2'-F modifications), and remain intact at ultra-low concentrations. The results indicate that the pRNA-3WJ has an unusually low dissociation constant that is typically not found in other biological RNA structures [31,32]. Therefore, thermodynamic instability and in vivo dissociation are no longer a concern, especially for pRNA-based nanoparticles.

6. Combating the third barricade: low yield and high production costs

The most challenging aspect of applying RNA nanoparticles for clinical applications is the yield and cost of RNA production. RNA strands can be synthesized both chemically and enzymatically. Commercial RNA chemical synthesis can only offer up to 40 nucleotide (nt) (conservative) to up to 80 nt (with low yield and high cost). Several methods have been explored for synthesizing longer RNA strands. We found that up to 117 nt of phi29 pRNA can be synthesized using bipartite approach, and this pRNA displayed biological activity in viral DNA packaging [72]. New 2'-protecting groups such as ether, acetal, orthoester, ester, and O-acetaester, pivaloyloxymethyl (PivOM) have been utilized to improve the RNA chemical synthesis efficiency [93,94]. RNA ligase II has been shown to be a good alternative over the traditional T4 DNA ligase to generate longer RNA by ligation of two shorter synthetic RNA fragments [95]. In addition, chemically synthesized RNA strands can be installed with clickable groups and rapidly click conjugated together to generate longer RNAs through the copper(I)-promoted azide-alkyne cycloaddition reaction (click chemistry) [96,97]. Enzymatic RNA synthesis methods allow production of longer RNAs. However, in enzymatic synthesis, heterogeneity of the 3' end has been an issue; this problem can be addressed by extending the transcribed sequence beyond the intended end and then cleaving the RNA at the desired site using ribozymes, DNAzymes, or RNase H [49,95,98]. Large-scale RNA production from bacteria has been reported [99,100], but it is limited by using T7 promoter and producing only highly folded RNA motifs (such as RNA aptamer or phi29 motor pRNA) [75] flanked and escorted by the tRNA scaffold [99]. During the last five years, collaboration between academic institutes and commercial vendors, supported by one of the NCI Alliance Cancer Nanotechnology Platform Partnership Programs directed by Peixuan Guo, has allowed the cost of RNA synthesis and reagents for RNA production to decrease over time.

7. Advantages of using RNA nanoparticles for pharmaceutical applications

RNA nanotechnology recently has received more and more attention from scientists around the world due to its high potential regarding therapeutics, especially after overcoming aforementioned several major hurdles in the field. While the use of RNA for therapeutics is still in its infancy, it is already clear that RNA nanotechnology provides several advantages: 1) The nano-scale size and branched, ratchet shape of RNA nanoparticles facilitates passive targeting through tumors and narrow cavities penetration, enables enhanced permeability and retention (EPR) effects [29,30], and possesses favorable biodistribution [31,32,54,55]. 2) RNA nanoparticles can be synthesized with defined size, structure, and stoichiometry. 3) The polyamionic nature of RNA prevents it from nonspecifically crossing the negatively charged cell membranes [101–104]. 4) RNA nanoparticles are highly water-soluble and not prone to aggregation under normal physiological conditions. 5) RNA nanoparticles have less immunogenicity compared to other heterogeneous nanoparticles because they are composed of “polynucleic acid”, a biocompatible material. Unpredictable side effects arising from heterogeneous particles can thus be avoided [29–32]. Minimal induction of antibody production allows for repeated treatment for chronic diseases. 6) The multivalent nature of RNA nanoparticles allows conjugation of targeting, therapeutic and imaging modules, all in one nano-construct for achieving synergistic or enhanced effects without cross-linking [31,32]. 7) The RNA nano-scaffold displays favorable pharmacokinetic and pharmacodynamic profiles in vivo and is non-toxic in mice [54]. 8) The specific delivery and longer retention time reduce the dose required and associated side effects. The specific delivery can be achieved through both EPR effect and active targeting by conjugating with cancer marker specific ligands. Systemic injection of the thermodynamically and chemically stable RNA nanoparticles in mice revealed that the RNA nanoparticles strongly and specifically bound to tumor with minimum accumulation in liver, lungs, or any other vital organs or tissues [31,32,54]. 9) RNA is a chemical reagent. Therefore, the regulatory processes are expected to be more favorable compared to protein-based clinical reagents [29,30].

8. Comparing RNA nanoparticles with other nano-delivery systems

Several nano-delivery systems of different materials and physiochemical properties for the treatment of cancer and viral infections have been pursued (Fig. 3). They include lipid-based nanoparticles [105], polymer-based nanoparticles [106], viral nanoparticles [107,108], inorganic nanoparticles [109,110], and DNA nanoparticles [111,112].

8.1. Lipid-based nanoparticles

Among lipid based nano-delivery systems, liposomes are the first reported system to be used in drug delivery and one of the most thoroughly studied and successfully developed delivery systems [105]. The advantages of using liposomes for gene or chemical drug delivery are ease of preparation, low cost, and relative non-toxicity [113]. However, liposomes are thermodynamically unstable [113]. Reconstituted liposomes usually have limited reproducibility in particle size distribution after freeze-thaw cycles. In addition, they have encapsulated drug leakage issues, especially with water soluble drugs, as well as non-specific distribution and systemic toxicity because they are prone to retention in the liver, lungs, spleen, and other organs [114]. Much effort has been devoted to optimization of the liposome delivery system. Chemical modification of the phospholipid structure of liposomes and the incorporation of polyethylene glycol can modulate stability and the in vivo circulation profile [115,116]. Recently developed solid lipid nanoparticles (SLN) demonstrated enhanced biocompatibility [117,118], longer blood circulation time, and improved encapsulation of water-soluble drugs, resulting in their controlled and extended release [119,120].

8.2. Polymer-based nanoparticles

Polymers, both naturally adapted and artificially synthesized, can be used to construct nanoparticles [106,121]. The advantages of polymer-based nanoparticles include feasible incorporation of both hydrophilic and hydrophobic drugs, high structural stability, high drug encapsulation efficiency, high cellular uptake, improved drug release rate, and ease of modification to achieve multifunctional and targeted delivery. In the past, the drawbacks of these types of polymers have been lack of biodegradability, insufficient biocompatibility, toxicity, and immunogenicity arising from activation of the mononuclear phagocyte system [122]. A notable advance is the “smart” delivery vehicles that include thermo-sensitive [123,124] and pH-sensitive [125] polymer nanoparticles especially designed to improve the biocompatibility, in vivo pharmacokinetics, and targeting
efficacy. However, the production of uniform size nanoparticles and cost-effectiveness are still challenging.

8.3. Virus-based nanoparticles (VNPs)

Viral particles are robust protein cages that exhibit well-defined geometry and homogeneity, making them well suited for nanostructure fabrication. Genetic manipulation can be performed on the viral genome easily. The viral capsid is versatile and relatively rigid, which can be modified and conjugated with active biomolecules or chemical groups [126,127]. Although most of the VNPs are not typically human pathogens, reliable methods used to inactivate replicating viruses or convert replicating viruses to virus-like particles (VLPs) need to be standardized to ensure the safety for in vivo applications. Further studies on the toxicity, immunogenicity, and biodistribution of virus particles in vivo are needed to systematically evaluate the efficacy of VLPs.

8.4. Inorganic nanoparticles

Inorganic nanoparticles include carbon-based, metal-based, and semiconductor-based nanoparticles [128]. Most inorganic nanoparticles have been developed for use in medical imaging because of their brightness and photostability [129]. Some have great potential for photothermal therapy because of their high photothermal conversion rate. However, the biological incompatibility, toxicity, non-degradable nature, and entrapment by the lungs, liver, and kidneys are major disadvantages for the clinical application of inorganic nanoparticles.

8.5. DNA nanoparticles

DNA nanotechnology uses the complementary nature of the four nucleotide bases in DNA to construct nanomaterials [112]. The development of DNA nanotechnology relies on the control of DNA hybridization, self-assembly of stable branched DNA, programmability of DNA, and convenient synthesis of designed DNA sequences. Constructed DNA nanoparticles include individual folded structures [130–133], tile-shaped structures [134–136], and dynamic assemblies [137–141]. DNA nanoparticles can be functionalized by quantum dots, DNA aptamers, or other functional molecules. One example of the medical application of DNA nanoparticles involves the use of a DNA nanostructure to cage an enzyme that can be released to induce cell apoptosis upon delivery [111,142]. However, in comparison to RNA nanoparticles, the use of pure DNA nanoparticles for clinical application is limited due to the simplicity of the DNA structure and function, as compared to RNA.

9. RNA modules applied for cancer therapy

Conventional cancer therapy, including chemotherapy and radiotherapy, cannot distinguish malignant from non-cancerous organs and tissues. Severe side effects and toxicity have occurred in patients during the treatment regime. As such, specific target delivery is highly desired for advanced cancer therapy in order to achieve higher treatment efficacy but lower toxicity than that for conventional therapy. The RNA nanoparticle is one of the candidates for targeting delivery of therapeutics to cancer cells. There are many advantages of using RNA
nanoparticles for cancer therapy (see Section 2). The multivalency of the RNA nanoparticles allows for combining therapeutic (e.g. siRNA/miRNA/drug), targeting (e.g. aptamer and chemical ligand) and detection (e.g. radionucleotide, fluorophore) modules, all in one nanoparticle. The potential of the pRNA platform [34,74] for specific delivery of siRNA to cancer receptor targets and the silencing of particular genes has been demonstrated in breast cancer [143], prostate cancer [70], cervical cancer [143], nasopharyngeal carcinoma [71], leukemia [41,70], and ovarian cancer [77], as well as viral infections [144].

9.1. Current advances using siRNA for cancer therapy

RNAi therapy uses RNA molecules to regulate post-transcriptional gene expression. siRNA was first discovered in plants in 1999 [145], and then found in mammalian cells [146]. siRNAs are short, double-stranded RNA sequences, typically 21 to 25 bp in length, that regulate gene expression through the degradation of mRNA with a complimentary sequence. This is accomplished through the association of siRNA with the RNA-induced silencing complex (RISC) presenting in the cytoplasm of cells. The siRNAs undergo processing through the RISC and then scan and bind to intracellular mRNAs. The siRNAs provide specific gene silencing by only targeting genes in which the complimentary sequence is a 100% match to the siRNA. The objective of siRNA therapy is to suppress tumor growth by down-regulating genes responsible for malignant transformation and angiogenesis. To date, a number of gene encoding transcription factors, anti-apoptotic proteins, GTPases, RTks, adhesion molecules, and others have been targeted by siRNAs for gene therapy. The RNA nanoparticles can be rationally designed to harbor: 1) identical siRNAs targeted to the same locus on one gene; 2) different siRNAs targeted to different loci on one gene; and 3) different siRNAs targeted to different genes, thereby modulating multiple pathways to generate synergistic or additive therapeutic effects [31,32].

9.2. Current advances using miRNA for cancer therapy

Similar to siRNAs, miRNAs are short RNA structures responsible for regulating gene expression within cells. However, miRNAs differ from siRNAs in a few aspects. First, miRNAs are typically found as short hairpin loops before processing, and miRNAs are part of the noncoding region of RNAs found in plants and animals. Second, miRNAs can regulate multiple genes and do not require absolute sequence matching with the genes being regulated. Same as for siRNA, a complex with RISC is required for miRNA processing. After pre-miRNAs are turned into mature miRNAs, they bind to the 3′-untranslated region of mRNA and block gene expression through mRNA degradation or blocking translation. Recently, miRNA has received attention for its role in disease progression, and it has been used as an early marker for cancers, where miRNA levels can either be repressed or up-regulated. miRNAs can act as either tumor suppressors or oncogenes [9–12]. In specific diseases where tumor suppressor miRNAs are down regulated, endogenous miRNAs can be increased by delivering miRNAs to the affected cells for self-correction. Alternatively, anti-miRNAs can be delivered to knockdown oncogenic genes.

9.3. Current advances using aptamers for cancer therapy

Nucleic acids (single-stranded DNA or RNA), with the propensity to take various shapes, can bind to diverse targets with high affinity and specificity. The target can be small molecules, peptides, and proteins, as well as viruses and bacteria. These nucleic acids, termed aptamers, are selected from a large random pool by an in vitro selection process called SELEX (Systematic Evolution of Ligands by Exponential Enrichment) [147,148]. Theoretically, it is possible to evolve aptamers virtually against any molecular target; this makes them an attractive tool for regulating cellular processes and drug delivery to specific cellular targets, especially in cancer targeting [149], biomarker discovery, diagnostics, and therapeutics [150,151].

There have been several SELEX procedure modifications in the last decade [152], which include capillary electrophoresis (CE) SELEX, Spiegelmer technology, Genomic SELEX, Photo-SELEX, and Cell-SELEX. More recently, SELEX has been performed to select high affinity aptamers using high-throughput sequencing, unlike the traditional method that involves several rounds of selection and optimization. This high-throughput sequencing provides a large number of sequences as readouts from selection. However, this method can only read fixed-length sequences [153]. The current method of Aptamer Selection by K-mer Analysis of Sequencing (ASKAS) requires a single round positive selection from a pool, followed by high-throughput sequencing [154]. High affinity aptamers of varied lengths are selected using variable K-mer length sequences enriched in the sequence library. Introduction of modified nucleotides such 2′-F, 2′-NH3, 2′-O-CH3 or LNA makes them nuclease resistant and also enhances thermal and chemical stability [155].

In the last few years, a major challenge has been specific cellular targeting. Aptamers have been utilized to import a variety of cargos into cancer cells, including drugs, siRNA, and miRNA. Aptamers can be generated for a variety of cancer biomarkers (e.g. prostate-specific membrane antigen (PSMA) [90,149], epithelial cell adhesion molecule (EpCAM) [156], carcinoembryonic antigen (CEA) [157], CD4 [158], and epithelial growth factor receptors (EGFR) [159] and can be used to deliver drugs to cancer cells specifically (Table 1).

In addition to the ability to bind specific targets, aptamers have also been shown to be used as a therapeutic drug, e.g., Pegaptanib, the first aptamer-based drug approved by FDA [160]. Pegaptanib specifically binds to VEGF 165, a protein that plays a critical role in angiogenesis. Another aptamer that is in clinical trials, AS1411, specifically targets the cancer cell surface protein nucleolin, a bcl-2 mRNA binding protein involved in cell proliferation, and induces cell apoptosis [154].

| Table 1 |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|
| Reported aptamers against cancer cell markers. | | | | |
| Aptamer | Type | Cancer target | KD | Cancer |
| C2 | 2′ F RNA | Transferrin receptor | 17 nM | Leukemia, cancer, skin cancer, etc. [206] |
| EpDT3 | 2′ F RNA | EPICAM | 12 nM | Colon cancer, breast cancer [156] |
| xPSM-A10 | 2′ F RNA | PSMA | 1.19 nM | Prostate cancer [149] |
| S6 | 2′ F RNA | HER2/ErbB2/Neu | 94.6 nM | Breast cancer [207] |
| C1 | 2′ F RNA | HER2 | 45.8 nM | Breast cancer [159] |
| CL4 | 2′ F RNA | EGFR | 46 nM | Breast cancer [208] |
| Y1-1 | 2′ F RNA | CEA | < 1 nM | Metastasis of colon cancer [157] |
| Aptamer 14 | 2′ F RNA | CD4 | Unknown | Leukemia [158] |
| C10 | DNA | CD19. B cell specific cell surface marker protein | 45.6 nM | Burkitt like lymphoma [209] |
| Sge8 | DNA | Tyrosine-protein kinase-like 7 (PTK7) | 0.8 nM | Acute lymphoblastic leukemia cells [210] |
| TA6 | DNA thioaptamer | CD44, receptor for hyaluronic acid | 187 nM | Breast cancer, lymphoma, melanoma, etc. [211] |
RNA oligonucleotides can be chemically conjugated with many biological or chemical molecules [31,32]. A well-labeled construct consists of three components: a small molecule moiety, a spacer, and a reactive group (Fig. 4A). The small molecule moiety includes a reporter molecule (fluorophore), any kind of bioactive drug, or ligand. The spacer separates the small molecule moiety from the oligonucleotide and can be used to change the hydrophobicity or hydrophilicity of the molecule and alter the flexibility and spacing of the label relative to the oligonucleotide.

Examples of some common oligonucleotide labeling reactions are:

1) Reaction of a free amino group on an oligonucleotide with isothiocyanate derivative of a dye. The reaction involves an attack of the nucleophile on the central, electrophilic carbon of isothiocyanate group. The resulting electron shift creates a thiourea linkage between the nucleophile and oligo, with no leaving group; this formed thiourea linkage is more prone to degradation during long-term storage [161,162] (Fig. 4B1).

2) Reaction of a free amino group on an oligonucleotide with an activated carboxylic group (NHS-ester), which produces amide linkage. The use of amino groups for conjugation has certain limitations; for example, the nucleophilicity of amino groups is pH dependent, which means that these groups are protonated at lower pH, which reduces their reactivity towards the electrophiles [163,164] (Fig. 4B2).

3) Click reaction of an azide-modified or alkyne-modified oligonucleotide with an alkyne-modified label and azide-modified label, respectively. The copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction is an outstanding representative of the click reactions, which are characterized by high yield, mild reaction conditions, and an extremely high compatibility with functional groups [165,166] (Fig. 4B3).

4) Nucleophilic addition of a thiol-modified oligonucleotide to an α,β-unsaturated ketone like maleimide as an electron deficient carbon which produces thioether linkages [167] (Fig. 4B4).

5) Reaction of a phosphoramidite derivative of the labeling group with the oligonucleotide during solid phase synthesis [168] (Fig. 4B5).

11. Methods and current achievements for constructing RNA nanoparticles

The general principles of constructing RNA nanoparticles follow three steps: RNA building block extraction, rational computational design/modeling, and RNA nanoparticle fabrication. There are a lot of naturally occurring or artificial RNA motifs that can serve as the building blocks for the design and construction of a variety of RNA nanoparticles.

11.1. RNA nanoparticles derived from phi29 DNA packaging RNA (pRNA)

11.1.1. Structure and folding of pRNA into a hexameric ring in phi29 DNA packaging motor

The pRNA is a 117 nt RNA molecule that is an essential component of the phi29 DNA packaging motor [169]. The pRNA folds into a complex structure consisting of two major functional domains: 1) a helical domain containing a double-stranded RNA (dsRNA) region with an open 5’/3’ end, and 2) an interlocking domain consisting of two looped regions. These two looped regions are noted as the right- (R-) and left- (L-) hand loops and are connected by a 3WJ motif (Fig. 2B). The loops allow for intermolecular interactions between pRNA monomers. Each loop contains a 4-nt sequence that forms base pairs with complementary sequences of other loops [27,36,72]. The interactions between these molecules have been deemed “hand-in-hand” interactions, as they almost always allow pRNAs to “hold hands,” creating a series of multimers. It is believed that pRNA dimers serve as building blocks for the hexameric ring by intermolecular interactions [170], matching the same number of units as ATPase gp16 [171]. The formation of the hexameric ring within the motor has been revealed through a series of experiments [170–177].

11.1.2. Current achievements and advances in creating pRNA nanoparticles

Compared to many other RNA motifs in nature, pRNA exhibits extraordinary stability and can be used in a variety of applications through the creation of nanoparticles. A series of “toolkits” have been developed for constructing multifunctional RNA nanoparticles using special structural features derived from pRNA.

11.1.2.1. Toolkit I: Hand-in-hand interaction using phi29 pRNA interlocking loops

The interlocking loop regions of pRNA (R- and L-loops) allow for inter- molecular interactions between pRNA monomers via hand-in-hand interactions. A homo-dimer can be created using complimentary sequences within the same pRNA molecule, or a hetero-complex can be made by combining a number of pRNAs with the R-loop, matching the sequence of the L-loop of another pRNA. Through these interactions between building blocks, dimer, trimer, and hexamer rings have been created [27,36,39,41,70,73,178]. However, the resulting particles were not thermodynamically stable enough for nanotechnology applications. To increase the stability of the interlocking loops, approaches to extend the loop sequences were investigated [55]. Random extension of the loop sequences failed due to interferences in RNA folding and 3D conformation. Extensive investigations have resulted in a toolkit with a set of hand-in-hand loop sequences that can be used for constructing stable RNA polygonal nanoparticles. The loop-extended pRNAs were...
constructed by replacing the 4-nt loop complementary region in wild-type pRNA R- and L-loops with a 7-nt sequence [55]. Since altering the nucleic acid sequences might affect the global folding of pRNA molecules, pRNA with re-engineered loop sequences was thoroughly analyzed using the RNA folding program ‘Mfold’ [179]. Pairs of extended loop-loop sequences that exhibited dimer formation were used as potential candidates for building pRNA nano-scale assemblies. Using reengineered 7-nt loop-loop interactions, pRNA dimers, trimers, tetramers, pentamers, hexamers, and heptamers were assembled with high efficiency in the presence of Mg2+, as revealed by AFM imaging (Fig. 2C, left panel).

11.1.2.2. Toolkit II: Foot-to-foot interactions based on palindrome sequences for assembly of higher ordered pRNA nanoparticles. The 5′/3′ open region is located in the helical domain of the pRNA. This section allows the extension of sequences without compromising the folding of the entire pRNA molecule. A palindrome sequence reads the same way, from the 5′ to 3′ direction on one strand or from the 5′ to 3′ direction on the complementary strand (Fig. 2C, middle panel). We have shown that a palindrome sequence can be added to serve as a sticky end for linking two pRNA monomers via intermolecular interactions, denoted by foot-to-foot interactions [39]. In a similar fashion, palindrome sequences were utilized 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 assembled into foot-to-foot structures, as revealed by AFM imaging [39,55] (Fig. 2C, middle panel).

11.1.2.3. Toolkit III: Branch-extension for RNA nanoparticle production based on 3WJ motif. Within the pRNA interlocking domain is a 3WJ motif [31,32]. This 3WJ core was extracted from the pRNA and assembled from three individual RNA fragments with high efficiency in the absence of metal ions. The 3WJ creates a branched structure allowing the conjugation of various RNA functional moieties at each branch end. After incorporation of functional modules, the 3WJ core retains its original folding, and each module within the nanoparticle can fold into its respective authentic structure and retain its independent functionality [31].

Similar to the 3WJ, the X-shaped motif stems from the interlocking domain of the pRNA. The X-shaped motif extends the 3WJ motif and replaces the right-hand loop on the pRNA with a short, double-stranded sequence with an overall structure consisting of four RNA oligos [32]. The resulting self-assembled structure creates a scaffold that allows for the conjugation of four RNA functional motifs. The addition of RNA modules on each branch preserves folding and functionality, as well as the central folding of the X-shaped motif. Hexavalent RNA nanoparticles can be further constructed from 3WJ cores (denoted as “arm-on-arm” interactions) or by palindrome sequence-mediated linking of two pRNA-X cores [55] (Fig. 2C, right panel).

11.1.3. Functionalization of the robust pRNA platform

Given its strong natural folding, uniform nanometer-scale particle size distribution, and in vivo stability, pRNA-based nanoparticles and their derivatives have evolved into a versatile RNA nano-delivery platform. One of the aims of RNA nanotechnology is clinical application. Most notably, the phi29 pRNA has become a highly promising candidate for third generation of intracellular targeted drug delivery platform to treat cancers and viral infections. The system is highly stable and allows the conjugation of a variety of RNA functional groups for in vivo applications. These include siRNAs (for gene silencing), miRNAs (to down-regulate oncogenic genes or up-regulate tumor suppressor genes), RNA aptamers or chemical ligands (for recognizing and internalizing RNA nanoparticles into target cells via receptor-mediated endocytosis), ribozymes (for the catalytic function), and riboswitches (for modulating gene expression in response to stimuli).

11.1.3.1. Specific delivery of siRNA. siRNAs are double-stranded, and they can be easily incorporated at the 5′/3′ open end of a pRNA. The inclusion of an RNA aptamer on a second pRNA, and the subsequent assembly of pRNA/siRNA and the pRNA/aptamer dimer through hand-in-hand interactions, allow the specific targeting, delivery, and silencing of a gene in the cell type of interest. Alternatively, siRNA can also be fused to any branch of the pRNA-3WJ and pRNA-X motifs through bottom-up self-assembly and an RNA aptamer can be fused within the same nanoparticle as well. Due to the multivalent nature of pRNA nanoparticles, multiple copies of siRNA that target the same loci within one gene, or different siRNA that target different loci within one gene, can be incorporated into one RNA nanoparticle for enhanced or synergistic gene knock-down effects [32,72] (Fig. 5).

11.1.3.2. Specific delivery of miRNA. Much like siRNA conjugation with the pRNA system, miRNAs can be easily fused onto the pRNA 5′/3′ open region or to one of the branches on the pRNA-3WJ or pRNA-X motifs. The pRNA was shown to be an effective carrier of miRNA to silence vital gene by targeting the 3′-untranslated region of coxsackievirus genome [180,181]. A similar approach can be applied to deliver anti-miRNA to knock down oncogenic genes of miRNAs to increase endogenous tumor suppressor miRNAs.

11.1.3.3. Specific delivery of ribozymes. Ribozymes are a class of RNAs that have the kind of enzymatic activity similar to proteins. These catalytic RNAs play essential roles in amino acid linking during protein translation, RNA processing through splicing, and viral replication. The specific action and activity of ribozymes have made them a valuable target in the therapeutical field because of their ability to control viral replication [49] and gene expression [216]. Through bottom-up self-assembly, ribozymes can be easily incorporated into a monomer pRNA unit, the pRNA-3WJ, or the pRNA-X motif. Previously, the hepatitis B virus hammerhead ribozyme has been conjugated to these systems and shown to retain its enzymatic activity after delivery to infected cells through an RNA aptamer or other chemical targeting modules [31,32,49,55] (Fig. 6).

11.1.3.4. Delivering RNA nanoparticles to target cells. Delivery of RNA nanoparticles to target cells is achieved via the incorporation of ligands onto the polyvalent RNA nanoparticles. Three classes of ligands have been used for targeting: chemical ligands such as folate (Fig. 9A), RNA ligands such as aptamer, and peptide ligands such as RGD. Aptamers, chemical ligands, or peptides that specifically recognize cancer receptors can be incorporated into RNA nanoparticles as part of the functionality for polyvalent therapeutics. Chemical ligands can be incorporated at the terminal ends of the RNA oligos [31,32,55], while RNA aptamer sequences

**Fig. 5.** Enhanced gene knock-down effects of pRNA-X nanoparticle carrying multiple copies of firefly luciferase siRNAs [32]. RLU: relative luciferase unit. This figure was adapted from ref. [32] with permission.
can be rationally designed to link to the 5′/3′ end of any helical region of the pRNA-3WJ [31], pRNA-X [32], or monomeric pRNA [41,70]. Using the phi29 RNA system, various aptamers have been shown to fold correctly and retain their high binding affinity to the targets. These include several chimeric pRNA-containing aptamers used for binding to CD4 [41], gp120 of HIV [144,182], and PSMA [90,183] (Fig. 7).

11.1.3.5. pRNA nanoparticles carrying fluorogenic RNA functionality for in vitro monitoring of RNA folding and half-life in the cells or in vivo in real time. Finding the numerous new roles of cellular noncoding RNAs has made it possible to introduce RNA into cells for regulating the cell life cycle and for the treatment of diseases. The understanding of RNA folding and degradation, its intracellular half-life, and trafficking after it enters the cell are intriguing questions. Current methods to detect these processes in the cell are extremely challenging. Common assays that measure RNA half-life and degradation in vivo use radioactive or fluorescence indicators; however, if labeled RNA is degraded or misfolded, the isotope or fluorescence remains in the cell, and the signals are not a true indication of the presence of the original RNA induced into the cell. Another common method to measure RNA half-life is to isolate RNA from cells and detect the degradation by gel, capillary electrophoresis, or chromatography. However, RNases are released from cellular compartments when a cell is breaking down, and degradation of RNA in lysates occurs immediately after cell lysis. Recently, novel methods that monitor RNA degradation in living cells in real time have been reported; these methods use fluorogenic RNAs through the application of RNA nanotechnology [75,76,184,185].

The RNA aptamer that binds malachite green (MG) or 3,5-difluoro-4-hydroxybenzyldiene imidazolinone (DFHBI) was fused to the 3WJ motif to generate RNA nanoparticles, and the aptamer retained its function independently after fusion into the complex [75,76] (Fig. 8A). When the resulting RNA nanoparticle is misfolded, degraded, or denatured, the fluorescence disappears. Free MG is not fluorescent and only emits fluorescence after binding to its aptamer. Thus, fluorescence is emitted only if the RNA remains folded in the correct conformation. Therefore, using epifluorescence microscopy and fluorescence spectroscopy and without lysing the cells, the 3WJ-MG aptamer system can be used as a real-time indicator of the RNA’s degradation and folding in the cells [76] (Fig. 8B).

11.1.3.6. RNA nanoparticles fused with aptamer that bind to streptavidin or sephadex for purification. Aptamers reported to bind to streptavidin or sephadex [186,187] have been fused with pRNA nanoparticles for purifying RNA nanoparticles. The pRNA nanoparticles harboring streptavidin aptamers can bind to the streptavidin conjugated agarose bead and then eluted out by excess amount of biotin, while preserving correct structures and functions [33,55,75]. This paves the way for simple and rapid purification of RNA nanoparticles.

11.1.4. In vivo biodistribution and pharmacokinetics profile of pRNA nanoparticles

An ideal in vivo delivery system is one that is able to target cancer cells specifically by overcoming several biological barriers and deliver high doses of therapeutics. The realization of such a system faces challenges that include the construction of a suitable delivery system with sufficient ability to administer therapeutic moieties to desired targets with marginal or no collateral damage to the normal tissue [188,189]. Upon systemic injection in mice, the thermodynamically and chemically stable pRNA nanoparticles strongly and specifically bound to cancers with little accumulation in the liver, lungs, or any other vital organs or normal tissues [31,32,54,55] (Fig. 9B). Furthermore, pRNA nanoparticles displayed favorable pharmacological profiles: 10-fold (5–10 h) enhancement of half-life compared to regular siRNA counterparts (0.25–0.75 h); clearance (CL) of <0.13 L/kg/h; volume of distribution (Vd) of 1.2 L/kg; and non-induction of interferon-1 responses or cytokine production in mice (Fig. 10). Repeated intravenous administrations of pRNA nanoparticles, up to 30 mg/kg, into mice did not result in any toxicity [54].

A recent study revealed that pRNA nanoparticles can be delivered to the posterior of the eye and internalized into cornea and retina cells after subconjunctival injection [190] (Fig. 11). The targeted biodistribution and metabolic profiles of pRNA nanoparticles indicate the high potential of pRNA nanoparticles as carriers for treatment of ocular diseases.

11.2. Other RNA nanoparticles with therapeutic potential

11.2.1. TectoRNAs based on natural or artificial RNA motifs

Since Westhof et al. proposed the concept of RNA tectonics [191], a variety of RNA 2D and 3D nano-constructs (TectoRNAs) have been constructed (for review, see reference [64]). These constructs follow the principle that natural or artificially designed RNA motifs can serve as building blocks for assembly of RNA architectures by coordinating RNA motifs with certain lengths of base pairing, specific angles, and tertiary interactions, such as tetraloop/receptor interactions and kissing loop interactions. Examples of constructing TectoRNAs include: 1) RNA squares. Three natural RNA motifs (a five-way tRNA junction, a three-way junction, and a two-helix bend), known to direct helical topology into approximately 90° bends, were connected by kissing loop interactions to generate square-shaped tetrameric RNA nanoparticles [62]. 2) Jigsaw puzzles. The RNA tectosquares with sticky, interacting tails were self-assembled from the right angle motif (which specifies 90° angle corners) and two interacting hairpins (adopted from dimerization at the initiation site of human immunodeficiency virus (HIV) RNA) to form specific, non-covalent kissing loop interactions [40].
RNA I/II inverse kissing complexes [68]. 

RNA filaments. RNA filaments can be assembled from RNA motifs bearing tetraloop receptor interactions [37,39,65,192]. 

RNA cubic scaffolds. By computation-facilitated methods, the rationally designed RNA cubic scaffolds were assembled from RNA strands through the one-pot self-assembly process [61]. 

RNA polyhedrons. The stable RNA nanostructures with an anti-prism polyhedral shape can be efficiently synthesized with versatile tRNA fold as a structural building block [63]. 

The RNA nanostructure can be also formed with the facilitation of protein counterparts. Ohno H et al. constructed an equilateral RNA triangle using a kink-turn (K-turn) motif to induce a 120° bend at the corner. The K-turn motif can be further recognized by the large ribosomal protein L7Ae, used to stabilize the triangle RNA nano-structures [193]. 

Most TectoRNAs have been structure-based until recently. Jaeger's group and Shapiro's group started to conjugate siRNA and RNA aptamers onto their RNA nanoparticles [61,63,68,194,195]. SirNA can be attached to the dangling ends of the hexagonal RNA nanorings and released out by Dicer cleavage [68]. RNA nanocubes and RNA polyhedrons are also good candidates for introducing functional RNA modules such as aptamers and siRNAs [61,63].
This had a modiﬁcation to siRNA only after cellular uptake. This conversion provides microsponges showed the generation of ~21 nt small RNA fragments further self-assembled into nano-scale pleated sheets of hairpin RNA, predetermined sequences for RNAi [196]. The long RNA strands can be used an enzymatic RNA polymerization method called rolling circle 11.2.2. Polymer-like RNAi microsponge

11.2.2. Polymer-like RNAi microsponge

RNA itself is a high-molecular-weight polymer. Hammond’s group used an enzymatic RNA polymerization method called rolling circle transcription (RCT) to generate elongated RNA strands containing predetermined sequences for RNAi [196]. The long RNA strands can be further self-assembled into nano-scale pleated sheets of hairpin RNA, which in turn forms sponge-like microspheres. The polymer-like RNAi microsponges showed the generation of ~21 nt small RNA fragments after incubation with Dicer, indicating the promising in vitro processing of siRNA by the RNA machinery in cells, that converts the stable hairpin RNA to siRNA only after cellular uptake. This conversion provides protection for siRNA during delivery and transport to the cytoplasm. Using the uptake of a single RNAi-microsponge, greater than half a million siRNA copies can be delivered to a cell. The authors further introduced a synthetic polycation, polyethylenimine (PEI), into the RNAi-microsponges to serve two purposes: 1) condense the RNAi-microsponge from 2 μm to 200 nm, and 2) alter the net charge of the particle from negative to positive. Presumably, PEI condenses RNAi-microsponges into particles with a positively charged outer layer, which can bind to an anionic cell surface and internalize into the cells via endocytosis. Due to lower pH within the endosome, the protonation of PEI amine residues lowers the osmotic potential and causes osmotic swelling, which results in bursting of the endosome and the release of the PEI/RNAi microsponges into the cytoplasm. The siRNA can then be processed by RISC and trigger knock down of the target genes as shown in both in vitro and in vivo studies. This approach offers several benefits: low off-target effects, low toxicity, efﬁcient delivery, and high cargo capacity, and therefore could lead to novel therapeutic routes for siRNA delivery [196].

12. Challenges and perspectives

The utilization of RNA nanotechnology for medical and nanotechnological applications requires addressing the following issues.

The first challenge is the understanding of correct global folding of RNA constructs to ensure the function of the resulting RNA nanoparticles. RNA folding and structural computation is essential for the examination of new structural designs [61,66–69,197]. Several online resources have been developed, including Mfold [179], RNA designer [198], Sfold [199], and NUPACK [200]. However, prediction of RNA structure or folding for nanoparticle assembly remains a great challenge. Due to the tertiary/quaternary interactions, non-canonical base pairing, and inter-molecular contacts in assembly, more sophisticated softwares for RNA folding are required. Additional rules that govern RNA nanoparticle assembly have yet to be elucidated. Historically, computational method for RNA folding mainly focused on 2D structure. Using current RNA 2D prediction programs, typically only ~70% of folding is accurately predicted [179,201]. RNA nanotechnology necessitates the development of prediction software for 3D and 4D structures, the knowledge of which is still elusive. Optimistic progress in this area has been made [179,198–200], but the field is still in its infancy, and additional collective effort is still needed.

The second challenge is the stability of the therapeutic RNA nanoparticles. As aforementioned, RNA stability in serum and in vivo is no longer a concern. But due to metabolism and clearance issues, the most stable RNA might not necessarily be the most desirable therapeutics in vivo. Optimal retention time is suitable as far as pharmaceutical aspects are concerned. This challenge can be addressed by manipulating the concentration and location for incorporating chemically modiﬁed stable nucleotides.

The third challenge in ligand-mediated endocytosis for speciﬁc delivery of siRNA is endosome escape. By taking advantage of the multivalent properties of RNA nanoparticles, therapeutic molecules can be delivered in a complex with agents that facilitate endosome disruption and trigger the release of the delivered therapeutic molecules from the endosome. Methods for assisting endosome escape include the use of acid-responsive chemical functional groups, such as acid-cleavable linkers like acetal, hydrazone, and maleic amides, or acid protonating groups such as amino esters, amidazole, and sulfonamide [202]. Another approach is using cell-penetrating peptides (CPPs), or amphipathic peptides that lead to protonation of histidine residues to disrupt the endosome via proton sponge effects [203]. Finally, although great effort has been made to combat the low yield and high cost issue of large-scale production of RNAs, there are still problems remaining to be overcome. The bacterial fermentation method for industrial scale RNA synthesis is promising (see Section 5) [89,204,205], but it is limited by the use of the tRNA scaffold (including its promoter)
for expressing the tightly folded RNA motifs. Additional effort is desired to improve the in vivo RNA expression vector, as well as engineering host cells to be exonuclease and endonuclease free strains or to be able to up- 

take modified nucleotides to generate RNase resistant RNAs. Based on the 

rapid reduction of cost over the history of DNA synthesis, it is expected that the cost of RNA synthesis will gradually decrease with the development of industrial-scale RNA production technologies.

13. Conclusion

Natural or chemical synthetic RNA molecules can fold into pre- 

defined structures that can spontaneously assemble into nanoparticles with multiple functionalities. The field of RNA nanotechnology is still emerging, but will play an increasingly important role in medicine, bio- 

otechnology, synthetic biology, and nanotechnology. RNA nanoparticles are promising as a new generation of drug for cancer therapy.

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