The field of RNA nanotechnology is rapidly emerging. RNA can be manipulated with the simplicity characteristic of DNA to produce nanoparticles with a diversity of quaternary structures by self-assembly. Additionally RNA is tremendously versatile in its function and some RNA molecules display catalytic activities much like proteins. Thus, RNA has the advantage of both worlds. However, the instability of RNA has made many scientists flinch away from RNA nanotechnology. Other concerns that have deterred the progress of RNA therapeutics include the induction of interferons, stimulation of cytokines, and activation of other immune systems, as well as short pharmacokinetic profiles in vivo. This review will provide some solutions and perspectives on the chemical and thermodynamic stability, in vivo half-life and biodistribution, yield and production cost, in vivo toxicity and side effect, specific delivery and targeting, as well as endosomal trapping and escape.
work in Peixuan Guo’s lab (Fig. 1) demonstrated that RNA dimer, trimer, and hexamer nanoparticles can be assembled using reengineered RNA fragments derived from the pRNA (packaging RNA), a vital component to gear the DNA packaging motor of the bacteriophage phi29. This finding was published in Molecular Cell (Guo et al., 1998), and was featured in Cell (Hendrix, 1998), proving the concept of RNA nanotechnology.

In 2004, Guo’s group reported the systematic formation of pRNA nanoparticles using 2 technologies: hand-in-hand interactions and palindrome sequence-mediated self-annealing (Figs. 1A–C, 2B–C, 3) (Shu et al., 2004). In the succeeding years, through a series of papers, they showed that pRNA molecules could be conjugated with various therapeutic functionalities including aptamers, small interfering RNA (siRNA), ribozymes, and microRNA (miRNA) (Hoeprich et al., 2003; Guo et al., 2005; Khaled et al., 2005; Guo et al., 2006; Shu et al., 2009; Abdelmawla et al., 2011; Ye et al., 2011; Shu et al., 2011a; Shu et al., 2011b; Shu et al., 2011c; Zhang et al., 2009) (Figs. 2A–D, 4). These findings have paved the way for RNA nanotechnology to develop into a novel area of therapeutics for the treatment of various diseases such as cancer, viral infections, and genetic diseases.

The development of multivalent pRNA nanoparticles in the Guo lab is just one facet of the rapidly emerging field of RNA nanotechnology and therapeutics. Investigations of the folding and structure of RNA motifs and junctions have laid a foundation for the further development of RNA nanotechnology.
nanotechnology. Significant contributions on the fundamental studies of RNA structural motifs were made by Eric Westhof (Leontis and Westhof, 2003; Lescoute and Westhof, 2006; Jossinet et al., 2007), Neocles Leontis (Jaeger et al., 2001; Leontis and Westhof, 2003; Leontis et al., 2006), David Lilley (Lilley, 1999; McKinney et al., 2003; Schroeder et al., 2010), and Luc Jaeger (Jaeger et al., 2001; Severcan et al., 2009; Afonin et al., 2010; Severcan et al., 2010). Their fundamental work on RNA junctions (Leontis et al., 2006; Schroeder et al., 2010) and RNA tectonics (Jaeger et al., 2001) have been used to construct diverse RNA nanoparticles, such as squares (Severcan et al., 2009), jigsaw puzzles (Chworos et al., 2004), filaments (Jaeger and Leontis, 2000; Nasalean et al., 2006; Geary et al., 2010), cubic scaffolds (Afonin et al., 2010), and polyhedrons (Severcan et al., 2010). Advances in RNA 3-dimensional computation expanding from the traditional intramolecular interactions to intermolecular interactions promoted by Bruce Sharpiro and others.
has brought new energy into the RNA nanotechnology field (Mathews and Turner, 2006; Shapiro et al., 2007; Yingling and Shapiro, 2007; Bindewald et al., 2008a; Shapiro et al., 2008; Afonin et al., 2010; Kasprzak et al., 2010; Laing and Schlick, 2010; Bindewald et al., 2011; Grabow et al., 2011). These newly developed inter-RNA computational programs will greatly facilitate RNA nanoparticle design and construction.

RNA nanotechnology is a vigorous and rapidly emerging new field of science, as evidenced by the burst of publications on RNA nanostructures over the last 5 years, indicating strong interest in RNA nanotechnologies in diverse fields such as chemistry, biophysics, biochemistry, structural biology, microbiology, cancer biology, pharmacy, cell biology, and nanomedicine. Currently, PubMed shows that 92% (1,002 of the total 1,090) of publications with the key words “RNA nanostructure” were published after 2005. With the continued development of RNA nanotechnology, many well-respected and prestigious journals have begun to include articles focused on RNA nanotechnology in their journals, including Science (Delebecque et al., 2011), Nature Nanotechnology (Afonin et al., 2010; Editorial comment, 2011; Guo, 2010; Ohno et al., 2011; Shu et al., 2011a), PNAS (Dibrov et al., 2011), Nano Letters (Shu et al., 2004; Yingling and Shapiro, 2007; Grabow et al., 2011) Nano Today (Haque et al., 2012), and Nature Protocols (Afonin et al., 2011). In addition, new journals have been founded to cover topics on RNA nanotechnology, such as Nucleic Acid Therapeutics, WIREs RNA, and Molecular Therapy–Nucleic Acids. In 2009, the National Institutes of Health (NIH) launched the National Cancer Institute Alliance for Nanotechnology in Cancer to create and foster a community of scientists using novel nanotechnology approaches to diagnose, treat, and prevent cancers. As a result, a Cancer Nanotechnology Platform Partnership program entitled RNA Nanotechnology in Cancer Therapy directed by Dr. Peixuan Guo was established (http://nano.cancer.gov/action/programs/platforms/uc.asp). In 2010, the first International Conference of RNA Nanotechnology and Therapeutics (http://www.eng.uc.edu/nanomedicine/RNA2010) was held (Shukla et al., 2011) and a second conference is planned in April 3–5, 2013 at the University of Kentucky.

Uniqueness of RNA Nanotechnology

RNA has several unique attributes that make it a powerful biomaterial compared to DNA, such as high thermodynamic stability (Searle and Williams, 1993; Sugimoto et al., 1995; Freier et al., 1986), formation of canonical and non-canonical base pairs (Ikawa et al., 2004; Leontis et al., 2006; Li et al., 2006; Matsumura et al., 2009; Schroeder et al., 2010), base stacking properties (Searle and Williams, 1993; Sugimoto et al., 1995), and various in vivo attributes (Chang and Tinoco, 1994; Guo et al., 1998; Zhang et al., 1998; Chen et al., 2000; Hoeprich et al., 2003; Wagner et al., 2003; Bindewald et al., 2008b; Laurenti et al., 2010). RNA molecules can fold into unique structural motifs mediated by canonical and non-canonical base pairings and further stabilized by tertiary interactions and complex 3-dimensional architectures exhibiting pseudoknots, single stranded loops, bulges, hairpins, and base stacking. Currently, an RNA polymer up to 80 nt can be efficiently and commercially synthesized non-enzymatically. An 80-nt RNA can have up to 10^68 (4^88) unique sequences with the sequence variation specifying for many individual possible structures. Such a huge pool is a great resource to identify diverse building blocks of RNA nanoparticles for the design, assembly, and manufacturing of therapeutic nanoparticles via intra- and intermolecular interactions. RNA–RNA interaction is the most stable with lowest free energy among the RNA–RNA, DNA–RNA, and DNA–DNA interactions (Lesnik and Freier, 1995; Gyi et al., 1996; Shu et al., 2011a; Binzel and Guo, unpublished results).

Techniques for the Construction of Therapeutic RNA Nanoparticles

RNA can fold into well-defined tertiary structures with specialized functionalities. The structural motifs and tertiary interactions has been examined in many RNA molecules and the information gleaned has been used to rationally design the building blocks that self-assemble into RNA nanoparticles (Figs. 2–4), as discussed below.

Hand-in-hand (loop–loop) interactions

Bacteriophage phi29 pRNA has 2 defined domains (Fig. 1): a 5′/3′-end helical domain (Zhang et al., 1994) and an interlocking loop region, which is located at the central part of the pRNA sequence (Reid et al., 1994; Zhang et al., 1994 and 1995a; Chen et al., 2000). The central domain of each pRNA subunit contains 2 interlocking loops, known as the right- and left-hand loops, that can be re-engineered to form dimers, trimers, or hexamers via hand-in-hand interactions (Fig. 2A–C) (Guo et al., 1987; Guo et al., 1998; Chen et al., 2000; Shu et al., 2003; Shu et al., 2004; Zhang et al., 1998). The 2 domains fold separately, and replacement of the helical domain with a siRNA does not affect pRNA structure, folding, or intermolecular interactions (Zhang et al., 1994; Trottier et al., 2000). This hand-in-hand interaction approach has recently been used by Bruce Shapiro and Luc Jaeger for construction of RNA nanoparticles with different shapes (Fig. 2E-F) (Yingling and Shapiro, 2007; Afonin et al., 2011; Grabow et al., 2011). The kissing loop of human immunodeficiency virus (HIV) RNA (Chang and Tinoco, 1994; Bindewald et al., 2008b) and the hand-in-arm interaction of Drosophila bicoid mRNA (Wagner et al., 2004) can be constructed utilizing a similar approach.

Robust RNA motif as a scaffold to build multivalent nanoparticles

Mechanically constructing fusion complexes of DNA, RNA, or protein can be easily accomplished, but it is difficult to ensure that the individual modules within the complex will appropriately fold and function after fusion. Recently, it was reported that the 3-way junction (3WJ) is a motif of the phi29 pRNA that can be assembled from 3 small RNA oligos with unusually high affinity in absence of metal salts. The resulting complex displays thermodynamically stable properties, resistant to denaturation even in presence of 8 M urea, and remains intact without dissociating at ultra-low concentrations. RNA nanoparticles harboring a variety of functionalities (siRNA, ribozyme, aptamer, riboswitch, miRNA, or folate) were constructed using the pRNA (Zhang et al., 1995b; Hoeprich et al., 2003), or its 3WJ core as a scaffold with perfect folding and function (Shu et al., 2011a; Haque et al., 2012) (Fig. 4). The 3WJ-pRNA is tightly folded and serves as a driving
force for the folding of other modules. As a result, individual functionalities can be placed at each branch without affecting the folding of other branches. The sequences for therapeutic and reporter moieties can be rationally designed to fuse with the sequences of the 3WJ strands a3WJ, b3WJ, and c3WJ, respectively. The 3 RNA fragments can then be assembled into RNA nanoparticles and their folding evaluated by in vitro and in vivo functional assays (Figs. 4–6).

**Palindrome sequence mediated formation of RNA dimers**

Palindrome sequences can promote the self-formation of pRNA dimers, tetramers, and arrays with high efficiency (Shu et al., 2004). In a similar manner, addition of self-complementary palindrome sequences to either the 5' or 3' end of one of the strands of the 3WJ-pRNA core results in the bridging of two 3WJs that harbor multiple functionalities via intermolecular interactions thereby generating a tetramer with 4 therapeutic and reporter moieties (Fig. 3).

**RNA junctions as LEGO® pieces to build quaternary structures**

Large RNA constructs can be fabricated by non-templated assembly via modular design, such that the complex can be self assembled from the basic building blocks without any external influence, assembled based on a modular design without any external template required (Chworos et al., 2004; Severcan et al., 2009; Severcan et al., 2010). Examples include Tecto-RNA, 2-, 3-, and 4-way junctions (2-/3-/4WJ) (Fig. 4), and self-assembly by coliE1 kissing loop interactions or kissing loops engineered that mimic this type of interaction (Fig. 2) and phi29 pRNA multimerization and quaternary architectures (Prats et al., 1990; Clever et al., 1996; Mujeeb A et al., 1998; Jaeger and Leontis, 2000; Shu et al., 2003; Shu et al., 2004; Guo et al., 2005; Khaled et al., 2005; Grabow et al., 2011).  

**RNA binding proteins to serve as junctions for the formation of arrays**

Ribosomal proteins have been shown to interact with RNA to form a nanostructure with a shape similar to an equilateral triangle (Ohno et al., 2011). In each triangle, three proteins are bound to an RNA scaffold-containing kink-turn motifs (Schroeder et al., 2010) for protein binding. The kink-turn allows the RNA to bend by ~60° at three positions, thus forming a triangle. The resulting protein–RNA complex could have potential applications in medicine, biotechnology, and nanotechnology.

**Combination of rolling circle transcription of RNA and self-assembly to produce giant spherical RNA particles**

A method has been developed by using rolling circle transcription to form siRNA concatemers that self-assemble into sponge-like microspheres (Fig. 7). The RNA interference (RNAi)-microsponges consist of cleavable RNA strands can be processed by the cellular machinery to convert the stable hairpin RNA to siRNA after cellular uptake. This finding reveals that RNA, which is a special class of polymer, displays the intrinsic property of other chemical polymers that form lamellar spherulites, such as that from polyethylene when nucleated in the bulk state or in solution (Lee et al., 2012). Generally, pure RNA is negatively charged, and direct cellular uptake remains insignificant due to electrostatic repulsion from the negatively charged cell membrane. Hammond and colleagues (Lee et al., 2012) used synthetic...
poly(ethyleneimine) (PEI) to condense the RNAi-microsponge from 2 μm to 200 nm. By this approach, the net charge of microspheres were shifted from negative to positive and subsequently internalized into cells. It is commonly believed that at the lower pH environment within the endosome, protonation of amine residues of PEI can lower the osmotic potential and cause osmotic swelling, which can result in bursting of the endosome to release the siRNA. It would be interesting to investigate whether the feasibility of PEI/RNAi-microsponges reported by Lee et al., as therapeutic agents can be improved by including ligands for specific targeting. Variable mechanisms and routes such as phagocytosis, macropinocytosis, and clathrin- or caveolae-mediated endocytosis all can lead to the internalization of nanoparticles. It would be worthy to evaluate which route is involved in the cellular uptake of such large PEI/RNAi-microsponges and to include targeting moieties to achieve specific delivery in vivo. In addition, extensive studies in xenograft models have revealed that delivery of nanomaterials requires a delicate balance between extravasation

FIG. 4. Construction of thermodynamically stable trivalent pRNA-based 3-way junction (3WJ) nanoparticles. (A) Sequence of pRNA monomer Ab′ (Guo et al., 1998). Green box: central 3WJ domain. In pRNA Ab′, A and b′ represent right- and left-hand loops respectively. (B) 3WJ domain composed of 3 RNA oligomers in black, red, and blue. Helical segments are represented as H1, H2, and H3. (C) Three pRNA molecules bound at the 3WJ-pRNA core sequence (black, red, and blue), and (D) its accompanying AFM images; scale bar = 30 nm. (E) Multi-module RNA nanoparticles harboring siRNA, ribozyme, and aptamer, and (F) its accompanying AFM images; scale bar = 20 nm. Figures reproduced with permission from Shu et al., 2011a, © 2011 Nature Publishing Group (NPG).
from the porous tumor vasculature and particles trapped by the monocyte phagocytic system or Kupffer cells in lung, spleen, and liver. It would be interesting to determine whether the PEI/RNAi-microsponge can be formulated in a way to evade the monocyte phagocytic system and organ accumulation.

Construction of RNA-Based Nanoparticles for Therapeutic Applications

There has been a heightened interest in RNA therapeutics since the discovery of siRNA (Fire et al., 1998; Hamilton and Baulcombe, 1999; Brummelkamp et al., 2002; Carmichael, 2002; Jacque et al., 2002; Li et al., 2002; Varambally et al., 2002), ribozymes (Guerrier-Takada et al., 1983; Zaug et al., 1983; Forster and Symons, 1987; Nava Sarver et al., 1990; Sarver et al., 1990; Chowrrira et al., 1991) and anti-sense RNA (Coleman et al., 1985; Knecht and Loomis, 1987), since they have been shown to down-regulate the expression of specific genes in viral-infected or cancerous cells. However, the use of siRNA in gene therapy has been significantly limited due to the difficulty of targeting the siRNA to specific cells. The advantage of using phi29 pRNA as a delivery medium is based on its ability to form stable multimers, which can be manipulated and sequence-controlled (Guo et al., 1998; Chen et al., 2000; Shu et al., 2003). This particular system, by applying the hand-in-hand approach and the robust pRNA 3WJ motif, provides superior pliancy for constructing polyvalent delivery vehicles containing multiple components (Figs. 2–4). For instance, one subunit of a dimeric, trimeric, or tetrameric RNA nanoparticle can be modified to contain a RNA aptamer that binds to a specific cell-surface receptor, thereby acting as a ligand for receptor-mediated endocytosis. A second subunit of the multimer can contain a reporter moiety such as a gold particle (Moll and Guo, 2007) or fluorescent dye for evaluating cell binding and entry. A third subunit can be designed to contain a component that enhances endosome disruption so the therapeutic molecules are released. A fourth (or fifth or sixth, if necessary) subunit of the RNA nanoparticle can carry a therapeutic siRNA.

FIG. 5. Assembly and stability studies of 3WJ-pRNA. In the tables, “+” indicates the presence of the strand in samples of the corresponding lanes. (A) 15% native polyacrylamide gel electrophoresis (PAGE) showing the assembly of the 3WJ core, stained by ethidium bromide (upper) and SYBR green 2 (lower). (B) Melting temperature curves for the assembly of the 3WJ core. Melting curves for the individual strands (brown, green, silver), the 2-strand combinations (blue, cyan, pink) and the 3-strand combination (red) are shown. (C) Melting curves for 11 different RNA 3WJ core motifs assembled from 3 oligos for each 3WJ motif under physiological buffer. (D–F) Competition and dissociation assays of 3WJ-pRNA. (D) Temperature effects on the stability of the 3WJ-pRNA core, denoted as [ab*c]3WJ, evaluated by 16% native gel. A fixed concentration of Cy3-labelled [ab*c]3WJ was incubated with varying concentrations of unlabelled b3WJ at 37°C. (E) Urea denaturing effects on the stability of [ab*c]3WJ evaluated by 16% native gel. A fixed concentration of labeled [ab*c]3WJ was incubated with unlabelled b3WJ at 1:1 ratio in the presence of 0–6 M urea at 25°C. (F) Dissociation assay for the [32P]-3WJ-pRNA complex harboring 3 monomeric pRNAs by 2-fold serial dilution (lanes 1–9). The monomer unit is shown on the left. Figures reproduced with permission from Shu et al., 2011a, © 2011 NPG.
FIG. 6. Apoptosis and binding assays of chimeric therapeutic pRNA. (A) Apoptosis induced by transfection of chimeric pRNA harboring siRNA targeting survivin using Lipofectamine 2000. Breast cancer MCF-7 cells were transfected with pRNA/siRNA (survivin) and apoptosis was monitored by propidium iodide– annexin A5 double labeling followed by flow cytometry. Cells in the bottom right quadrant represent apoptotic cells. The mutant pRNA/siRNA was transfected in parallel as a negative control. (B) Specific delivery of chimeric pRNA/siRNA by folate-pRNA. Flow cytometry analyses of the binding of fluorescein isothiocyanate (FITC)-labeled folate-pRNA to nasopharyngeal carcinoma (KB) cells. Left: Cells were incubated with folate-pRNA labeled with FITC. Middle: Cells were preincubated with free folate, which served as a blocking agent to compete with folate-pRNA for binding to the receptor. Right: Binding was also tested using folate-free pRNA labeled with FITC as a negative control. The percentages of FITC-positive cells are shown in the top right quadrants. (C) Confocal images showed targeting of folate receptor positive (FR+) KB cells by co-localization (overlap, 4) of cytoplasm (green, 1) and RNA nanoparticles (red, 2). (D) 3WJ-pRNA nanoparticles target folate receptor positive (FR+) tumor xenografts on systemic administration in nude mice. Upper panel: whole body; lower panel: organ imaging (Lv, liver; K, kidney; H, heart; L, lung; S, spleen; I, intestine; M, muscle; T, tumor). Figures reproduced with permission: A–B, Guo et al., 2005, © 2005 Mary Ann Liebert, Inc.; C–D, Shu et al., 2011a, © 2011 NPG.
FIG. 7. Self-assembled RNA nanoparticles as potential therapeutic agents. AFM images of (A–B) rationally designed RNA 1-dimensional and 2-dimensional arrays in vivo (Delebecque et al., 2011); (C) RNA bundles (scale bar = 50 nm) (Cayrol et al., 2009); (D) AFM images of pRNA arrays (Shu et al., 2004). (E) Transmission electron microscopy (TEM) images of RNA microsponges (Lee et al., 2012). Figures reproduced with permission: A–B, Delebecque et al., 2011, © 2011 AAAS; C, Cayrol et al., 2009, © 2009 ACS; D, Shu et al., 2004, © 2004 ACS; E, Lee et al., 2012, © 2012 NPG.

FIG. 8. Chimeric pRNA-aptamer-siRNA nanoparticles for human immunodeficiency virus (HIV) therapy. (A) The pRNA-aptamer mediated targeted delivery of siRNA using chimeric pRNA–anti-gp120 aptamer. The anti-gp120 aptamer is responsible for binding to HIV-1 gp120 protein. (B) Cell-type specific binding studies of pRNA aptamer chimeras. Cy3-labeled pRNA aptamers were incubated with Chinese hamster ovary (CHO)-gp160 cells and CHO-EE control cells. Cell surface binding of Cy3-labeled chimeras was assessed by confocal imaging. (C) The inhibition of HIV-1 infection mediated by pRNA-aptamer chimeras. Both antigp120 aptamer and pRNA-aptamer chimera neutralized HIV-1 infection in HIV-infected human peripheral blood mononuclear cells (PBMCs) (NL4-3 strain) culture. Data represent the average of triplicate measurements (Zhou et al., 2008; Zhou et al., 2011). Figure A courtesy of Dr. Jiehua Zhou and Dr. John Rossi. Figures B–C reproduced with permission from Zhou et al., 2011, © 2011 Elsevier.
ribozyme, riboswitch, miRNA, or another complementary drug. The incorporation of each of the functional modules to pRNA scaffold are discussed below.

**siRNA**

RNAi is a key post-transcriptional gene silencing mechanism that has evolved in plants and some animals. The siRNAs are typically 21–25-bp dsRNA strands with 2-nt overhangs at the 3’ ends. The siRNAs bind to a protein complex in the cytoplasm called the RNA induced silencing complex (RISC). The siRNA/RISC complex then scans and intercepts intracellular mRNA containing a complementary sequence to the bound siRNA. The intercepted mRNA is cleaved and degraded, thereby silencing the expression of that gene (Fire et al., 1998; Brummelkamp et al., 2002; Carmichael, 2002; Jacque et al., 2002; Li et al., 2002; Var-ambally et al., 2002).

Since the siRNA is double stranded, the incorporation of siRNA into RNA nanoparticles is readily accomplished by simply fusing the siRNA sequences at one of the helical stems of the 3WJ (Fig. 4) (Shu et al., 2011a), by replacing the end helical segment of the monomeric pRNA with siRNA sequences (Fig. 2) (Liu et al., 2007), or by attaching the siRNA to the RNA assemblies, whereby the 5’ or 3’ ends of the sequences that constitute the assemblies are extended and either the sense or the antisense sequences are then hybridized to these extended sequences to form the siRNAs (Fig. 2) (Afonin et al., 2011; Grabow et al., 2011). For increased stability, typically chemically modified 2’F nucleotides are used to modify the sense strand. Since chemical modifications might compromise the silencing potency of RNA nanoparticles containing siRNA components, the region of the Dicer processing site needs to be rationally designed and use of C or U nucleotides should be avoided if 2’-F C/U modifications were introduced.

**miRNA**

The miRNAs are typically short (~23 nt) RNA strands that are naturally found in plants and animals. They are part of noncoding RNA sequences and play an important role in gene regulation by binding onto specific messenger RNA sites responsible for protein coding (Bartel, 2009). Recently, it has been found that miRNA plays an important role in the control or development of cancers (He and Hannon, 2004), cardiac diseases (Chen et al., 2008), and regulation of the nervous system (Maes et al., 2009). Within each of the diseases, miRNA levels are either up- or down-regulated.

Recent work and discoveries have led the idea of using miRNAs in therapy for gene regulation in cell mutations (Fig. 9) (Bader et al., 2010). In diseases where miRNAs are seen to be down-regulated, levels can be synthetically increased through the delivery via the approach of the pRNA or its 3WJ core (Ye et al., 2011; Shu et al., 2011a; Ye et al., 2012). Similar to siRNA, the miRNA sequences can be conjugated onto each branch of the 3WJ and delivered to the diseased cells, which would then undergo normal Dicer processing in vivo through the RISC complex, returning normal gene regulation in the diseased cells. Different from siRNA, miRNA has a much broader target by regulating several genetic pathways (Kaminski and Slack, 2011).

**Ribozymes**

Ribozymes are RNA molecules that can catalyze chemical reactions (Kruger et al., 1982; Guerrier-Takada et al., 1983). Ribozymes have compact and specific structures that enable them to catalyze trans-esterification and hydrolysis reactions. They can intercept and cleave mRNA or the genome of RNA viruses and thus have a significant therapeutic impact.

A chimeric pRNA monomer containing a hammerhead ribozyme was designed to cleave the poly(A) signal on hepatitis B virus (HBV) mRNA. Cleavage of the HBV mRNA was nearly complete in vitro and HBV replication was inhibited in vivo by this chimeric pRNA (Hoeprich et al., 2003). The anti-apoptosis factor, survivin, regulates tumor development and progression. A chimeric pRNA containing a hammerhead ribozyme designed to target survivin mRNA was shown to suppress survivin gene expression and initiate apoptosis in cell cultures (Liu et al., 2007). It was shown that the HBV ribozyme can also cleave the poly(A) signal from HBV mRNA after being incorporated into the 3WJ nanoparticles (Fig. 4) (Liu et al., 2007; Shu et al., 2011a).

**Riboswitches**

A riboswitch (Tucker and Breaker, 2005; Barrick and Breaker, 2007; Cheah et al., 2007; Breaker, 2008; Breaker, 2012) is a component of some specific miRNAs that binds a small molecule and controls the expression of that mRNA in response to the concentration of the small molecule. Riboswitches fold into intricate structures that typically recognize metabolites and have evolved as metabolic control mechanisms in bacteria. Riboswitches can regulate gene expression by several means, including premature termination of mRNA transcription, ribosome binding, and inhibition of mRNA translation, mRNA cleavage, and even mRNA degradation. There is substantial interest in engineering artificial riboswitches to create a new generation of regulators to control the expression level of targeted genes in response to interactions with small drug-like molecules.

Such RNA-based gene-control machines have the potential to supply nanoscale, cis-acting, modular systems, incapable of inducing antibody production, for use in future gene therapies (Henkin, 2008; Ogawa and Maeda, 2008; Shahbabian et al., 2009). If RNA nanoparticles with riboswitch modules can be constructed in vivo, it would be possible to regulate biological functions in vivo.

**Aptamers**

A RNA aptamer is a RNA molecule that binds a specific ligand through the formation of a recognition structure (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Mi et al., 2010). RNA aptamers with the ability to bind specific targets with high affinity can be extracted from a pool of random RNA oligonucleotides by in vitro SELEX (systematic evolution of ligands by exponential enrichment) (Ellington and Szostak, 1990; Tuerk and Gold, 1990).

Aptamers that specifically bind to target cancer receptors can be incorporated into the RNA nanoparticles as part of the functionality of the polyvalent therapeutics. The selected receptor-binding RNA aptamers can be rationally designed to link to the 5'/3' end of any helical region of the 3WJ. It is important to ensure that the aptamer folds correctly and that
its binding affinity to the target cell surface marker is maintained. Several chimeric pRNA containing aptamers have already been successfully used for binding to CD4 (Khaled et al., 2005), gp120 (Zhou et al., 2008; Zhou et al., 2011) of HIV (Fig. 8), or prostate cancer prostate-specific membrane antigen (McNamara et al., 2006; Dassie et al., 2009).

**Advantages of RNA Nanotechnology for In Vivo Applications**

Various types of therapeutic RNA have been developed, and their applications for the treatment of diseases are just beginning to be fully realized. Although gene silencing with high efficacy and specificity by a variety of interference strategies and RNA molecules has been achieved in vitro, the effective delivery of therapeutic RNAs to specific cells in vivo remains challenging. Development of an efficient, specific, and nonpathogenic nanodevice for delivering multiple therapeutics in vivo is highly desirable. Application of RNA nanotechnology has significant advantages in this endeavor, as outlined below.

**Polyvalent delivery for generating synergistic effects**

The polyvalent RNA nanoparticles can deliver up to 6 kinds of molecules to specific cells including therapeutics, detection modules, drugs, or other functionalities (Guo, 2005; Guo et al., 2005; Nakashima et al., 2011; Chang et al., 2012a; Chang et al., 2012b). This particular system provides remarkable flexibility for the construction of polyvalent delivery vehicles since it is based on a modular design. Hence, individual RNA subunits with various cargos can be constructed separately and assembled into the final quaternary complex by mixing them together in any desired combination (Shu et al., 2011a; Haque et al., 2012). For example, the deliverable RNA nanoparticle can be engineered to carry therapeutic siRNAs, ribozymes, or antisense RNAs against multiple genes or different regions of one target gene, and RNA aptamers or folic acid for targeted delivery (Fig. 6). The other subunits of the RNA nanoparticle may carry anti-cancer drugs to enhance the therapeutic effect or to overcome the drug resistance by combination therapy. The therapeutics, detection molecules, or drug may also be combined into one nanoparticle, making the concomitant therapy and detection of the therapeutics possible with a single administration.

**Defined size, structure, and stoichiometry**

Currently, the use of polymer for siRNA or drug delivery have been reported extensively (Nimesh et al., 2011; Singha et al., 2011; Troiber and Wagner, 2011; Duncan, 2011). RNA is a polymer (polynucleic acid). Different from other polymers
such as polyethylene glycol, 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 and increased safety. The clearly defined structure and stoichiometry might facilitate FDA approval of RNA nanoparticles as therapeutic agents.

Nanoscale size for enhanced permeability and retention effects

The size of a nanoparticle is commonly thought to be the fundamental factor for effective delivery to diseased tissues. Many studies suggest that nanoparticles ranging from 10–100 nm (Gao et al., 2005; Jain, 2005; Li and Szoka, 2007) are the optimal size because they are large enough to avoid excretion through the urine, yet small enough to bind to cell surface receptors and enter the cells via receptor-mediated endocytosis (Li and Szoka, 2007). During the development of solid tumors, angiogenesis occurs to supply enough oxygen and nutrients to the fast-growing tumor cells. These newly formed blood vessels, unlike the tight blood vessels in most normal tissues, are leaky because of gaps between them and adjacent endothelial cells. This allows the particles that are usually excluded from the normal tissue to navigate 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 sizes ranging between 20 and 40 nm (Liu et al., 2010; Shu et al., 2004; Abdelmawla et al., 2011; Shu et al., 2011a), 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 cross cell membranes, and non-formulated siRNAs have been reported to be easily excreted by the body (de Fougerolles et al., 2007; Kim and Rossi, 2007; Rozema et al., 2007). Nanoparticle delivery of siRNAs or other therapeutics has the potential to improve the pharmacokinetics (PK), pharmacodynamics (PD), and biodistribution, as well as reduce potential toxicity (Shu et al., 2004; Guo et al., 2005; Khaled et al., 2005; Abdelmawla et al., 2011). Furthermore, the PK and PD of pRNA nanoparticles can be improved by introducing chemical modifications to the RNA backbone. Chemically modified RNA is resistant to RNase degradation, which makes RNA nanoparticles more stable and increases their retention time during blood circulation. Specific delivery and longer retention time including the enhanced permeability and retention effect also reduces the dosage necessary for effective therapy.

Targeted delivery to cancer cells

The pRNA nanoparticles can carry both a therapeutic agent and a ligand for the targeted delivery of the nanoparticles to specific tissues and cell types. 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 pRNA system provides for both higher delivery efficiency and reduced off-target toxicity (Abdelmawla et al., 2011).

Non-induction of an antibody response to ensure repeated treatments

Protein-free RNA nanoparticles, such as the pRNA system, contain RNA aptamers designed to act as receptor antagonists with similar binding specificities as protein antagonists. However, RNA nanoparticles have a much lower antibody-inducing activity (Abdelmawla et al., 2011). Thus, the repeated administration of RNA nanoparticles during the treatment of chronic diseases is less likely to result in complications as a result of immune responses.

Challenges, Solutions, and Perspectives in RNA Nanotechnology

Although great progress has been achieved by applying RNA nanotechnology in medical applications, many challenges still remain. Herein we provide some solutions and perspectives on the chemical and thermodynamic instability, short in vivo half-life and biodistribution, low yield and high production cost, in vivo toxicity and side effects, and specific delivery and targeting, as well as endosomal escape.

Chemical instability

One of the major concerns on the use of RNA nanoparticles in therapeutics is the chemical stability of RNA itself. Natural RNA is extremely sensitive to degradation by RNases and is especially unstable in the body or serum. The stability of RNA has long been an obstacle to its application as a construction material. Over the last few years, rapid progress has been made in improving the stability of RNA, which include chemical modifications of the bases (e.g., 5-Br-Ura and 5-I-Ura); modifications of the phosphate linkage (e.g., phosphothioate, boranophosphate); alteration of the 2’ carbon (e.g., 2’-F, 2’-OMe or 2’-NH2) (Watts et al., 2008; Singh et al., 2010); synthesis of peptide nucleic acids, locked nucleic acids, and their respective derivatives; polycarbamate nucleic acids (Madhuri and Kumar, 2010) or locked nucleic acids with a bridge at different positions (2’-4’, 1’-3’) (Mathe and Perigaud, 2008); and capping of the 3’-end (Patra and Richert, 2009). All these methods are very efficient in increasing RNase resistance in vitro and in vivo. However, the challenge is that after chemical modification, the folding properties, and biological function of a RNA molecule change. The development of a method that confers resistance to RNase degradation while not changing the characteristic structure, self-assembly, and biological function of a RNA nanoparticle is critical. It was recently found that for all the aforementioned methods, the 2’-F have minimal detrimental effect on folding, assembly and function (Liu et al., 2010). While in some special cases, fine-tuning is necessary to find a location that can be modified with minimal detrimental effect, RNase degradation in vivo is no longer a concern. It has been shown that RNA degradation in serum occurred more frequently at vulnerable sites. Fine-tuning of these sites by mutation or alteration protect siRNAs from degradation in serum (Hong et al., 2010).

Thermodynamic instability

The thermodynamic stability of the RNA nanoparticles is of paramount importance with regard to the use of RNA nanoparticles as therapeutics. Injection of several microliters or milliliters of RNA solution into the body will result in several
hundred thousand–fold dilutions. Dissociation of bottom-up assembled RNA nanoparticles at extremely low concentrations after in vivo dilution will be a serious concern. In a recent paper, thermodynamically stable pRNA 3WJ core scaffold was assembled from 3 to 6 pieces of RNA in the absence of metal salts. The 3WJ complex was stable in serum, remained intact at ultra low concentrations and was even resistant to denaturation in 8M urea, (Fig. 5) (Shu et al., 2011a; Haque et al., 2012). More importantly, various functionalities such as siRNA, ribozyme, or receptor-binding aptamer incorporated into the 3WJ core resulted in the formation of polyvalent particles displaying all the authentic functionalities in vitro and in vivo (Fig. 6). Therefore, the thermodynamic stability and in vivo dissociation are no longer a concern for pRNA-based nanoparticles.

**Short in vivo half-life**

The other important factor in therapeutics is the PK of the drug. In order to improve the stability of RNA in vivo, a variety of chemical modifications have been introduced into RNA, as discussed previously. Chemically modified siRNA are RNase resistant, while retaining their biological activity (Liu et al., 2010). However, the half-life of modified siRNA in vivo is only 15–45 min (Morrissey et al., 2005; Behlke, 2006).

Another critical factor that determines the in vivo retention time is the size of the RNA nanoparticles. Many studies suggest that particles ranging from 10 to 100 nm are the optimal size for a non-viral vector—large enough to be retained by the body, yet small enough to bind to cell surface receptors and pass through cell membranes (Prabha et al., 2002). RNA nanoparticles designed in the range between 20 and 40 nm are usually excluded from the normal tissue that has tighter blood vessels, but enter into the tumor interstitial space and concentrate in the tumor via enhanced permeability and retention effects, since the angiogenic blood vessels have larger gaps. This optimal size range for RNA nanoparticles also improves the biodistribution and ensures the longer retention time for in vivo delivery. It has been reported that the half-life of chemically modified pRNA nanoparticles was 5–10 hours, in comparison to 0.8 hours for the siRNA counterparts (Abdelmawla et al., 2011). So the concerns about the in vivo retention and half-life of RNA nanoparticles have been significantly reduced by the application of chemical modifications.

**Low yield and high production costs**

A major limiting factor of RNA nanotechnology in therapeutic applications is the cost of the nanoparticle construction, especially for RNA nanoparticles that require larger RNAs. RNA oligonucleotides can be prepared by enzymatic transcription or automated solid-phase synthesis. Enzymatic synthesis can produce relatively long transcripts in significant quantities, while commercial non-enzymatic RNA chemical synthesis can only produce RNAs that are 40–80 nt long. The longest chemically synthetic RNA with biological activity is 117-nt long (Guo et al., unpublished data). When it comes to the synthesis of relatively long RNA oligonucleotides, the yield of a RNA oligo decreases greatly as the length of the oligo increases (Reese, 2002; Marshall and Kaiser, 2004).

Classical approaches based on the t-Butyldimethylsiloxy protecting group for the 2’-hydroxy are limited to short sequences, whereas more recent approaches based on 5’-O-DMT-2’-O-[(trisisopropylsilyl)-oxy]methyl (2’-O-TOM) protecting scheme (Pitsch et al., 2001), and 5’-O-Silyl-2’-O-orthoester (2’-ACE) protecting group combination (Scaringe et al., 1998) have provided a more effective tool for the chemical synthesis of longer RNAs. The cost of RNA synthesis is expected to gradually drop with the development of industrial-scale RNA production techniques. For example, the cost of synthesizing DNA oligos was 100-fold higher 20 years ago compared to the cost today.

RNA can also be produced by enzymatic synthesis with in vitro transcription, but the heterogeneity of the 3’-end of the RNA products presents a problem. To work around the heterogeneity issue, the transcribed sequence can be extended beyond the intended end and then cleaved at the desired site using small ribozymes, DNAzyme, RNase H, or a cis-cleaving hammerhead or ribozyme (Feng et al., 2001; Hoeprich et al., 2003). RNase ligase 2 has also been shown to be a good alternative over the traditional T4 DNA ligase to obtain longer RNAs by the ligation of 2 shorter synthetic RNA fragments.

To circumvent this issue of yield and cost, a clever approach is to employ the bottom-up assembly (1 of the 2 basic approaches of nanotechnology) of the RNA nanoparticles. The production of oligonucleotides with functional moieties then becomes a scalable process (chemically) and with a modular design the complex can be self-assembled from the basic building blocks. Using this methodology, thermodynamically and chemically stable pRNA-based nanoparticles with functional modules were successfully fabricated using a bipartite, tripartite, and tetrapartite approach with various modifications (Shu et al., 2011a; Shu et al., 2011b).

The most economic way for industrial scale production of RNA is by fermentation in bacteria. Cloning and production of RNA in bacteria with high yield has been reported (Wichtweckarn et al., 1992; Ponchon and Dardel, 2007; Ponchon et al., 2009; Delebecque et al., 2011; Ponchon and Dardel, 2011). Bacteria fermentation is the direction for industry production, but currently, the bacteria high yield production of RNA nanoparticles with therapeutic functionality has not been reported.

**Toxicity, in vivo safety, and side effects**

From an in vivo delivery and therapeutic point of view, it is essential that the nanoparticles have favorable pharmacological profiles concerning biodistribution, pharmacokinetics (stability, half-life, and clearance rate), immune response (antibody induction, α and β interferon, toll-like and innate immunity, PKR effect, and cytokine induction), specific targeting, and efficiency of gene silencing. The induction of innate immunity and certain organ toxicity has been a major concern in using RNA nanoparticles for therapeutic applications. If the RNA is single stranded then type-II interferons (IFN-α) should be used as a marker for toxicity assay; if the RNA is double stranded it is type-I interferons (INF-α/IFN-β) that are important. Other immunotoxicity issues, such as hypersensitivity, complement activation, and fever-like reactions can all be dose-limiting factors. In addition, it has been reported that the immunotoxicity of siRNA is sequence specific. The potential toxic effects of delivery vehicles should also be explored.

Some of the favorable pharmacological profiles of the pRNA-based nanoparticles have been reported recently (Abdelmawla et al., 2011; Shu et al., 2011b). It is exciting to find
that the half-life of the pRNA nanoparticles has been extended 10-fold (5–10 hours) in comparison with their regular siRNA counterparts (15–45 minutes) with a clearance rate of <0.13 L/kg/hour and a volume of distribution of 1.2 L/kg. The pRNA nanoparticles induced neither an interferon response (OAS1, MX1, or IFITM1) nor cytokine production in mice, even after repeated intravenous administrations in mice, up to 30 mg/kg. Fluorescent folate-pRNA nanoparticles efficiently and specifically bound to and were internalized by folate receptor-bearing cancer cells in vitro. Systemic injection of pRNA nanoparticles specifically and dose-dependently targeted folate receptor [FR(+)] xenograft tumor in mice with minimal or no accumulation in normal tissues (Fig. 6) (Abdelmawla et al., 2011; Shu et al., 2011b). However, rodents might not necessarily present the same effects of nucleic acid toxicity as humans. This type of toxicity should be further tested in preclinical studies using non-human primates.

**Specific delivery and targeting problems**

In order for RNA nanoparticles to be useful as therapeutic agents, they must be capable of targeting specific cells. The pRNA nanoparticles in the 15–50 nm size range will not enter cells randomly or nonspecifically. Hence, a nanotechnological approach was adopted to construct pRNA nanoparticles harboring various targeting molecules, such as RNA aptamers or folate. One example is the specific delivery of pRNA nanoparticles to the folate receptor, overexpressed on the surface of nasopharyngeal carcinoma cells (KB cells). The folate moiety readily binds to the folate receptor and the nanoparticles rapidly enter the cells via receptor-mediated endocytosis (Fig. 6) (Guo et al., 2005; Khaled et al., 2005; Guo et al., 2006; Shu et al., 2011a).

Although ligand-mediated specific delivery is an exciting approach, RNA aptamers and ligands currently available are limited. Each cancer type requires a specific ligand. As a result, development of more RNA aptamers for specific targeting is imperative. Screening methods derived from SELEX have shown considerable promise and is compatible with RNA nanotechnology approaches. Once an aptamer has been selected, it could easily be incorporated into RNA nanoparticles and retain their binding function in vivo. The development of such a screening system is being actively pursued.

**Endosome trapping**

One of the major problems encountered in DNA or protein delivery is degradation of the therapeutic molecules in the endocytic pathway. Similarly, the greatest challenge in ligand-mediated endocytosis for specific delivery of siRNA or therapeutic RNA nanoparticles is endosomal escape. After receptor-mediated endocytosis, the nanoparticles are trapped in the endosomes within the cells. This keeps the siRNA from being able to be processed by the Dicer machinery, and therefore the siRNA is not able to knockdown a specifically targeted gene.

By taking advantage of the oligomerization properties of pRNA, we can deliver therapeutic molecules in a complex with endosome-disrupting agents. A number of substances that disrupt endosomes and mediate endosome escape of therapeutic molecules have been described in the literature. Defective or psoralen-inactivated adenovirus particles have shown promise since they have considerable endosomolytic activity (Cotten et al., 1992). Synthetic peptides that mimic the membrane-fusing region of the hemaglutinin of influenza virus have also been successfully used in gene delivery systems to facilitate endosomal escape (Plank et al., 1994; Mastrobattista et al., 2002; Van Rossenberg et al., 2002). Polymeric endosome-disrupting gene delivery vectors, such as poly(ε-amino ester) (Lim et al., 2002) or poly(DL-lactide-co-glycolide) (Panyam et al., 2002) have also been reported. Polymers harboring varieties of chemical moieties have been reported to enhance disruption of the endosome. Using the multivalent property of RNA nanoparticles, the endosome-escaping reagents can be incorporated into the RNA nanoparticles. One subunit of the deliverable RNA complex (dimer, trimer, or hexamer) can be altered to contain an RNA aptamer that acts as a ligand for a cell-surface receptor and induces uptake by receptor-mediated endocytosis upon binding. Another 1 or 2 subunits of the RNA complex can be altered to contain components that facilitate endosome disruption for the release of the delivered therapeutic molecules from the endosome. The other subunits of the RNA complex can be used to carry a therapeutic siRNA, a ribozyme, miRNA, a riboswitch, or a chemical drug.

Methods for assisting endosome escape include the use of synthetic polymers to form siRNA/polymer polyplexes, the complexation of siRNA with lipids to form lipid nanoparticles, or the siRNA association with cell-penetrating peptides (CPPs) or endosome-disrupting chemicals. Design considerations for the formation of siRNA/polymer polyplexes for endosomal release and gene silencing efficiency have been recently reviewed (Kwon, 2011). For increased endosomal escape, a common technique is to create polymers with various types of acid-responsive chemical functional groups such as acid-cleavable linkers including acetal, hydrazone, and maleic amides or acid protonating groups such as β-amino esters, imidazole, and sulfonamide (Kwon, 2011). In the case of acid-cleavable linkers, the cationic termini of the polymer complexes become protonated within the acidic pH of the endosome, which can destabilize the endosome via the proton sponge effect. In addition, the loss of the cationic branches after acid hydrolysis reduces the interactions between the siRNA and polymer, resulting in release of the siRNA for Dicer processing (Kwon, 2011).

Another approach to siRNA delivery and endosomal disruption is to use CPPs. In most ways, CPPs deliver siRNA into cells in the same way as siRNA/polymer polyplexes and lipid nanoparticles. The anionic siRNA interact with cationic peptides, which enter the cell via endocytosis and carry the siRNA in along with them. Recently, amphipathic peptides have received a great deal of attention for nucleic acid delivery. These peptides are usually short and contain a high number of histidine and leucine residues. In a recent paper (Langlet-Bertin et al., 2010), the siRNA delivery efficiency of a cationic amphipathic peptide, LAH4, and several of its derivatives were compared. Each of these peptides was able to deliver siRNA for the luciferase gene to mammalian cells and was shown to be at least as effective as common lipid based transfection reagents such as lipofectamine. The exact mechanism of endosomal disruption by amphipathic peptides is still unknown, however it is likely that that acidic pH of the endosome leads to protonation of the histidine residues, which frees the peptides from the siRNA they were carrying and allows them to disrupt the endosome via the proton
sponge effect (Langlet-Bertin et al., 2010). The proton sponge effect occurs after accumulation of a weak base in the endosome, which neutralizes the lumen of the endosome and results in an increase in the endosome’s osmolarity (Midoux et al., 2009). As a result, the endosome swells and is no longer able to hold its contents leading to siRNA being released into the cytosol.

The pH dependence of endosome escape was investigated in a recent report using a commercially available amphipathic peptide called Endo-Porter that has been used to deliver various nucleic acid cargos to mammalian cells (Bartz et al., 2011). It was shown that Endo-Porter requires the acidification of the endosome since the activity of the peptide was blocked by bafilomycin A. At physiological pH, Endo-Porter does not appear to form secondary structures such as the α-helix that it forms at pH between 5.0 and 6.0. The exact mechanism for how the α-helical structure is able to disrupt the endosome is unknown, but it may be a result of interaction between the endosome membrane and the α-helix resulting in the formation of a large pore in the endosome or disruption of its membrane (Bartz et al., 2011).

Although there are numerous types of nanoparticles capable of endosomal disruption, there is still a major challenge associated with their future use for widespread therapeutic use: specific targeting. Currently, synthetic polymer nanoparticles, lipid nanoparticles, and amphipathic peptides are all non-specific, which greatly limits their usefulness in vivo. One of the advantages of RNA nanoparticles is the use of targeting moieties such as RNA aptamers or receptor-targeting ligands, such as folate, but endosome escape is still an issue. Both types of delivery have their associated challenges to overcome. If combined into one particle, it may be possible to create a specific targeting RNA nanoparticle capable of escaping the endosome and paving the way toward a new and powerful form of RNA therapeutics.

Conclusions

The self-assembly property of RNA can be utilized as a powerful bottom-up approach to rationally design and create nanostructures through the integration of biological, chemical, physical, and computational techniques. This approach relies upon the cooperative interactions of individual RNA subunits to spontaneously self-assemble into larger multimeric structures in a predefined manner. The feasibility and utility of RNA nanotechnology in therapeutics is beginning to be realized. Currently, the RNA therapeutics industry faces the following challenges: (1) chemical instability, (2) thermodynamic instability, (3) short in vivo half-life, (4) low yield and high production costs, (5) in vivo safety and side effect issues, (6) difficulty in specific delivery and targeting in vivo, and (7) endosome trapping after delivery into the cell. The first 5 challenges have been overcome to a significant extent. Specific targeting and endosome escape are still a major issue that remains hurdles and make many companies cautious about aggressively pursuing the use of RNA as therapeutics. Cancer targeting has progressed with the approach of RNA nanotechnology, but greater efforts are needed to tailor the delivery efficiency and specificity to individual cancers. Combination of RNA with other chemical polymers to improve endosome escape has faced the challenges of undefined structure and stoichiometry as well as nanoparticle accumulation in normal organs. Many research groups, including our own, are working to solve the endosome escape issues. Once these challenges are overcome, the field of RNA nanotechnology and therapeutics will surely become a clinical reality.

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