RNA AS A UNIQUE POLYMER TO BUILD CONTROLLABLE NANOSTRUCTURES FOR NANOMEDICINE AND NANOTECHNOLOGY

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Hui Li, Student

Dr. Peixuan Guo, Major Professor

Dr. David Feola, Director of Graduate Studies
RNA AS A UNIQUE POLYMER TO BUILD CONTROLLABLE
NANOSTRUCTURES FOR NANOMEDICINE AND
NANOTECHNOLOGY

DISSertation

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By

Hui Li

Lexington, Kentucky

Director: Dr. Peixuan Guo, Professor of Pharmaceutical Sciences

Lexington, Kentucky

2016

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RNA nanotechnology is an emerging field that involves the design, construction and functionalization of nanostructures composed mainly of RNA for applications in biomedical and material sciences. RNA is a unique polymer with structural simplicity like DNA and functional diversity like proteins. A variety of RNA nanostructures have been reported with different geometrical structures and functionalities. This dissertation describes the design and construction of novel two-dimensional and three-dimensional self-assembled RNA nanostructures with applications in therapeutics delivery, cancer targeting and immunomodulation. Firstly, by using the ultra-stable pRNA three-way junction motif with controllable angles and arm lengths, tetrahedral architectures composed purely of RNA were successfully assembled via one-pot bottom-up assembly with high efficiency and thermal stability. By introducing arm sizes of 22 bp and 55 bp, two RNA tetrahedrons with similar global contour structure but with different sizes of 8 nm and 17 nm were successfully assembled. The RNA tetrahedrons were also highly amenable to functionalization. Fluorogenic RNA aptamers, ribozyme, siRNA, and protein-binding RNA aptamers were integrated into the tetrahedrons by simply fusing the respective sequences with the tetrahedral core modules. Secondly, I reported the design and construction of molecularly defined RNA cages with cube and dodecahedron shapes based on the stable pRNA 3WJ. The RNA cages can be easily self-assembled by single-step annealing. The RNA cages were further characterized by gel electrophoresis, cryo-electron microscopy and atomic force microscopy, confirming the spontaneous formation of the RNA cages. I also demonstrated that the constructed RNA cages could be used to deliver model drugs such as immunomodulatory CpG DNA into cells and elicit enhanced immune responses. Thirdly, by using the modular multi-domain strategy, molecular defined RNA nanowires can be successfully self-assembled via a bottom-up approach. Only four different 44-nucleotide single-stranded RNAs were used to assemble the RNA nanowire. The reported RNA nanowire has the potential to be explored in the future as the carrier for drug delivery or matrix for tissue engineering. Fourthly, the construction of RNA polygons for delivering immunoactive CpG oligonucleotides will be presented. When CpG oligonucleotides were incorporated into the RNA polygons, their immunomodulation effect for cytokine TNF-α and IL-6 induction was greatly enhanced, while RNA polygon controls induced unnoticeable cytokine induction. Moreover, the RNA polygons were delivered to macrophages specifically and the degree of immunostimulation greatly depended on the size, shape, and the number of payload per RNA polygon. Collectively, these findings demonstrated RNA nanotechnology can produce controllable nanostructures with different functionalities and result in potential applications in nanomedicine and nanotechnology.
KEYWORDS: RNA Nanotechnology, pRNA, Three-way Junction, Nanostructures, Nanomedicine

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11/14/2016
Date
RNA AS A UNIQUE POLYMER TO BUILD CONTROLLABLE
NANOSTRUCTURES FOR NANOMEDICINE AND NANOTECHNOLOGY

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11/15/2016
Date
To my parents and wife for their continued love and support
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<td>2’-Fluoro RNA Modification</td>
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<td>2’-O-Me</td>
<td>2’-O-Methyl RNA Modification</td>
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<tr>
<td>3WJ</td>
<td>Three Way Junction</td>
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<td>4WJ</td>
<td>Four Way Junction</td>
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<tr>
<td>ADP</td>
<td>Adenosine 5’-Diphosphate</td>
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<td>AFM</td>
<td>Atomic Force Microscopy</td>
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<td>AMP</td>
<td>Adenosine Monophosphate</td>
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<td>ANA</td>
<td>Altritol Nucleic Acid</td>
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<td>APS</td>
<td>Aminopropyl Silatran</td>
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<td>ASO</td>
<td>Antisense Oligonucleotides</td>
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<tr>
<td>CAM</td>
<td>Cell Adhesion Molecule</td>
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<tr>
<td>cas9</td>
<td>CRISPR associated protein 9</td>
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<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>c-di-GMP</td>
<td>Cyclic Dimeric Guanosine Monophosphate</td>
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<tr>
<td>cGMP</td>
<td>Cyclic Guanosine Monophosphate</td>
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<tr>
<td>CpG</td>
<td>Cytosine-phosphate-guanine</td>
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<tr>
<td>cRCT</td>
<td>Complementary Rolling Circle Transcription</td>
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<tr>
<td>CRISPR</td>
<td>Clustered Regularly Interspaced Short Palindromic Repeats</td>
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<td>CT</td>
<td>Computed Tomography</td>
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<td>CTF</td>
<td>Contrast Transfer Function</td>
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<td>CVB3</td>
<td>Coxsackievirus B3</td>
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<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<td>DFHBI</td>
<td>3,5-Difluoro-4-Hydroxybenzylidene Imidazolinone</td>
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<td>DIS</td>
<td>Dimerization Initiation Site</td>
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<td>DLS</td>
<td>Dynamic Light Scattering</td>
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<td>DME/F-12</td>
<td>Mixture of Dulbecco's Modified Eagle's Medium (DME) and Ham's F-12 Nutrient Mixture</td>
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<td>Deoxyribonucleic Acid</td>
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<td>Doxorubicin</td>
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<td>EB</td>
<td>Ethidium Bromide</td>
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<td>ECL</td>
<td>Electrochemiluminescence</td>
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<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
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<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>EISA</td>
<td>Evaporation-induced Self-assembly</td>
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<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<td>EM</td>
<td>Electron Microscopy</td>
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<td>Abbreviation</td>
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<tr>
<td>EMEM</td>
<td>Eagle's Minimum Essential Medium</td>
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<td>EpCAM</td>
<td>Epithelial Cell Adhesion Molecule</td>
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<td>FANA</td>
<td>Fluoro-β-d-Arabinonucleotide</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>Fc</td>
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<td>FMN</td>
<td>Flavin Mononucleotide</td>
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<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
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<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
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<tr>
<td>GEMM</td>
<td>Genes for the Environment, Membranes, and Motility</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GMP</td>
<td>Good Manufacturing Practice</td>
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<td>GNP</td>
<td>Gold Nanoparticle</td>
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<td>gRNA</td>
<td>Guide RNA</td>
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<td>HBV</td>
<td>Hepatitis B Virus</td>
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<td>HCV</td>
<td>Hepatitis C Virus</td>
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<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>hMMP-9</td>
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<td>MRI</td>
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<td>mRNA</td>
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<td>Term / Definition</td>
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<td>PCR</td>
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Chapter 1: Introduction and Literature Review

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1. Brief Summary

Chapter 1 begins this dissertation with an overview of the hypothesis and summarizes the research progress and current state of ribonucleic acid (RNA) nanotechnology and RNA nanomedicine. The unique properties of RNA as a polymer are introduced. The current methods for the construction of various RNA nanostructures and the applications of these RNA nanostructures in nanotechnology and nanomedicine are also reviewed.

Chapter 2 studies the design and self-assembly of multifunctional 3D RNA tetrahedrons based on the pRNA-Three Way Junction (3WJ) motif. The 3D structures of the assembled RNA tetrahedrons are examined by both atomic force microscopy (AFM) and cryo-electron microscopy (EM). The sizes of the RNA tetrahedrons are tuned by changing the number of RNA base pairs per edge. Several functional modules including aptamers, ribozyme and small interfering RNA (siRNA) are incorporated into the RNA tetrahedron and their biological functions are examined. In vitro cell binding and in vivo biodistribution studies are performed to show that the RNA tetrahedron functionalized with EGFR-aptamer can target to xenograft breast cancer. These results indicated that 3D RNA nanoparticles have the potential to deliver imaging modules and therapeutics for cancer diagnosis and therapy.

Chapter 3 reports the design and self-assembly of RNA cube and dodecahedron cages with defined shapes and sizes by using the pRNA 3WJ as the basic building block. The 3D structures of the assembled RNA cages are examined by cryo-EM and AFM. In addition, the designed RNA cube cages are applied to deliver immunoactive cytosine-phosphate-guanine (CpG) DNA to macrophage cells and induce immune responses.
Chapter 4 studies the design and bottom-up self-assembly of RNA nanowire with length in the micrometer scale. The constructed RNA nanowire is examined by cryo-EM, negative staining transmission electron microscopy (TEM), fluorescent microscopy and AFM. This study showcased an example of the expansion of RNA nanostructures to RNA microstructures.

Chapter 5 reports the design, construction and characterization of RNA polygons as multivalent nanocarriers of vaccine adjuvants such as CpG DNA for immune modulation. It was found that the sizes and shapes of the RNA nanostructures played important roles in the induction of immune responses. This study illustrated that RNA nanoparticles have the potential to be developed as potent immunomodulators or vaccine adjuvants.

Chapter 6 briefly summarizes the main conclusions of this dissertation and discusses the future directions and prospects in RNA nanotechnology and RNA nanomedicine.

2. Hypothesis

The central hypothesis of this dissertation is that RNA is a unique polymer that can be used to build controllable and defined nanostructures for applications in nanomedicine and nanotechnology.
3. Literature Review

Introduction

Polymers have been extensively used by humans since the earliest recorded history. Tree saps, tar and plant fibers, all represent the earliest natural polymeric materials used by humans [1]. Since the 1950s, through continued synthesis and manufacturing improvements, synthetic polymers have dramatically improved in quality and strength to the point where they, in many cases, can have mechanical properties that better match desired use, as compared to alternative natural counterparts. Synthetic polymers have the manufacturing advantages of rapid production rates and synthetic simplicity. However, owing to the inherently random nature of most polymerization mechanisms, chain polydispersity and controlled shaping remain two of the key limiting hurdles that have led to increased variable performance and overall reduced control of the final properties [2-4]. As such, there has been much effort towards developing synthetic building blocks and schemes that can create monodisperse polymer systems [5-7]. These, however, are typically very expensive, time consuming and difficult to control.

As a result of these developments, we are at another unique turning point in the evolution and design of polymers. We once again turn towards nature as our inspiration for the development of advanced biomaterials. By applying what we have learned about the complexity and versatility of nucleic acid structures, it is now possible to create materials with revolutionary properties previously considered unattainable through synthetic strategies. Recent advances in RNA chemistry, RNA biology and RNA nanotechnology have shown that RNA as a biopolymer not only shares the common characteristics of other polymers, but also possesses a range of unique properties
advantageous for applications in nanotechnology as well as biomedical and material sciences [8-14].

Herein, we will (1) delineate the physiochemical properties of polymers that can be applied to RNA, (2) introduce the unique properties of RNA as a polymer, (3) review the current methods for constructing RNA nanostructures for diverse applications, and (4) discuss the future prospects including challenges, solutions and new directions of this nascent field.

Physical and chemical properties of polymers in relationship to RNA nanotechnology

The word (poly)-(mer) means (many)-(parts) and refers to macromolecules consisting of a large number of repeating elementary units covalently joined together. These ‘mers’ control the inter-chain interactions and dictate the final polymer characteristics. Given the large variations in polymer morphologies, multiple ways are used to describe polymer structure. Polymers can be formed from one type of monomer (homopolymers) or of various monomers (heteropolymers). Heteropolymers can be further described by the arrangement of the monomers along the backbone, including alternating, random, and block formations. Heteropolymers also adopt more elaborate structures, including star, ring, branched, dendrimeric, crosslinked, ladder, and others [15-23]. These large arrays of potential structures and chemical diversity offer a rich repertoire of available properties from which one can custom-design a polymer for a desired application.
Biological macromolecules, as natural building blocks, are critical for the functioning of living organisms. RNA is one of the five most important biological macromolecules in addition to DNA, proteins, lipids and carbohydrates. With some aspects similar to DNA, RNA, which is composed of four nucleotides including adenosine (A), cytosine (C), guanosine (G) and uridine (U), is special in its homogeneity. RNA is a homopolymer of nucleotide, but is also a heteropolymer of A, U, G, and C. Each nucleotide contains a ribose sugar, a nucleobase, and a phosphate group. The nucleotides are covalently linked together through 3’ → 5’ phosphodiester bonds between adjacent ribose units, giving the directionality to the sugar-phosphate backbone that defines RNA as a polynucleic acid. The phosphate moieties in the backbone are negatively charged, making RNA a polyanionic macromolecule at physiological pH. RNA molecules are typically single-stranded. However, Watson-Crick (canonical) base-pair interactions (A:U and G:C), wobble base pairing (such as G:U) [24] or other non-canonical base pairing such as twelve basic geometric families of edge-to-edge interaction (Watson-Crick, Hoogsteen/CH or sugar edge) with the orientation of glycosidic bonds relative to the hydrogen bonds (cis or trans) [25-28], all together give rise to various structural conformations. Unique RNA structures include loops, hairpins, bulges, stems, pseudoknots, junctions, etc., which are essential elements to guide and drive RNA molecules to assemble into desired structures [12, 29-32]. On the contrary, synthetic polymers are usually formed via a polymerization reaction, a chemical process through which many monomers form covalent bonds between each other and finally form a long chain or network, imparting them with their desired structural properties.
Generally, the properties of polymers are strongly dependent on their monomer chemistry. Moreover, the molecular weight and 2D structure (e.g., linear chain-shaped structure or branch-shaped structure) also play important roles in determining the characteristics and properties of a specific polymer. Important properties of chemical polymers include [1]: (1) rheological properties, (2) solubility, (3) volumetric and viscometric properties, (4) stress-strain relationships, (5) electrical properties, (6) thermal properties, (7) optical properties, (8) stiffness, (9) flex life, (10) hardness, and (11) chemical resistance. Various physical or chemical tests can be readily performed to characterize these properties for RNA polymers.

Traditional synthetic polymers are heterogeneous and have typical polydispersity index greater than 1 [33, 34]. In contrast, RNA is synthesized by either in vitro transcription or solid phase chemistry. Both approaches are based on stepwise reactions and will generate RNA polymers with defined sequences, structures, and molecular weights. This leads to a polydispersity index of 1 of a specific RNA. Structurally, the average molecular weight determines the mechanical property of the polymer. However, as polydispersity increases, the increased lower molecular weight chains can act as a lubricating/solvating system, reducing its mechanical strength and integrity. As increases in temperature result in increased molecular motion, these weak associations begin to destabilize and reduce mechanical integrity. Such polymers are classically referred to as thermoplastics [35], as their mechanical strength weakens with increased temperature. However, this property also means they can be intentionally deformed at higher temperatures and then regain their structural properties once cooled. Thermodynamically, the RNA molecule is relatively more stable at lower temperature and intends to dissociate
or misfold at higher temperatures. Thus, RNA displays the typical thermoplasticity of polymers. Boiling-resistant RNA has been discovered and constructed [36], suggesting a potential application of RNA in molding technology.

Chemical polymers have a wide range of applications in biomedical and material sciences. Specific applications include, but are not limited to [1, 37-50]: (1) sutures for surgery, (2) dental devices, (3) orthopedic fixation devices, (4) scaffolds for tissue engineering, (5) drug formulation (e.g., controlled release of drugs, excipients for drug stabilization or solubilization), (6) biodegradable vascular stents, (7) biodegradable soft tissue anchors, (8) implantable biomedical devices, (9) flexible transparent displays, (10) field effect transistors, (11) solar cell panels, (12) printing electronic circuits, (13) organic light-emitting diodes, and (14) supercapacitors. Usually, an ideal chemical polymer for biomedical and industrial applications should exhibit the majority of the following properties: (1) non-toxic, (2) no or low immunogenicity (if applicable, for \textit{in vivo} use), (3) biodegradable and metabolizable, (4) mechanical features that fit the needs of the specific application, (5) easily sterilizable, (6) processability, (7) scalability, (8) electrical conductivity, and (9) thermostability. RNA as a polymer favorably displays many of these properties, as described below.

**Unique properties of RNA as a polymer to build nanostructures**

\textit{The 2'-hydroxyl group makes RNA dramatically different from DNA}

The characteristic of RNA that defines and differentiates it from DNA is the 2'-hydroxyl on each ribose sugar of the backbone. The 2'-OH group offers RNA a special property, which can be either an advantage or a disadvantage. From a structural point of
view, the advantage of this additional hydroxyl group is that it locks the ribose sugar into a 3’-endo chair conformation. As a result, it is structurally favorable for the RNA double helix to adopt the A-form which is ~20% shorter and wider rather than the B-form that is typically present in the DNA double helix. Moreover, the 2’-OH group in RNA is chemically active and is able to initiate a nucleophilic attack on the adjacent 3’ phosphodiester bond in an $S_N2$ reaction. This cleaves the RNA sugar-phosphate backbone and this chemical mechanism underlies the basis of catalytic self-cleavage observed in ribozymes [51]. The disadvantage is that the 2’-OH group makes the RNA susceptible to nuclease digestion since many ribonuclease (RNases) recognize the structure of RNAs including the 2’-OH group as specific binding sites. However, such enzymatic instability has been overcome by applying chemical modification to the 2’-OH group (see the sections Enzymatic stability of RNA and Methodology II: Chemical modification of RNA).

**Thermodynamic stability of RNA**

From a thermodynamic point of view, the stability of the double helix of RNA can be evaluated by measuring the Gibbs free energy ($\Delta G_0$) required for double helix formation, or conversely, double helix unwinding ($\Delta G_0 = -\Delta G_0$helix formation = $\Delta G_0$helix unwinding). Remarkably, the RNA double helix is more thermodynamically stable than the DNA double helix considering $\Delta G_0$ for RNA double helix formation is, on the average, $-3.6$ to $-8.5$ kJ/mol per base pair stacked and $\Delta G_0$ for DNA double helix formation is $-1.4$ kJ/mol per base pair stacked [52]. Moreover, the presence of special motifs such as bends, stacks, junctions and loops in the tertiary structure of RNA may
also further improve its stability [8]. In addition, various proteins, such as RNA chaperone proteins, and metal ions, such as Mg\textsuperscript{2+} may also interact or coordinate with RNA and significantly contribute to the stability of RNA [53, 54].

*Enzymatic stability of RNA*

In biological systems, the instability of RNA is mainly the result of enzymatic degradation by RNases. The presence of RNases in living organisms is universal, suggesting that RNase-mediated RNA degradation is a vital process for normal biological function. Several important roles of RNases have been revealed, including degrading surplus cellular RNA [55, 56], editing messenger RNA, processing microRNA and other non-coding RNA during their maturation [57, 58], defending against the invasion of viral RNA [59], and providing crucial machinery for RNA interference (RNAi) [60, 61]. RNases can be divided into two major categories: (1) endo-ribonucleases that cleave phosphodiester bonds within the RNA backbone [62]. Examples include RNase A, RNase H, RNase I, RNase III, RNase L, RNase P, RNase PhyM, RNase T1, RNase T2, RNase U2, RNase V; (2) exo-ribonucleases that cleave phosphodiester bonds at either the 5’ or the 3’ end of an RNA chain [63]. Examples include polynucleotide phosphorylase (PNPase), RNase PH, RNase II, RNase R, RNase D, RNase T, oligoribonuclease, exoribonuclease I, exoribonuclease II. RNA is indeed very sensitive to degradation by RNases, which confers a very short half-life and thus a poor pharmacokinetic profile to most RNA molecules. This degradation limits the *in vivo* application of RNA molecules as therapeutics. However, chemical modifications of RNA can overcome this shortcoming. For example, the substitution of the 2’ hydroxyl group with a Fluorine (2’-
F), O-methyl (2’-O-Me) or Amine (2’-NH$_2$) dramatically increases the stability of RNA \textit{in vivo} by preventing degradation by RNases [64-66]. Recent studies also showed that the stability of siRNA in serum is also highly depended on the specific RNA sequences and the degradation of both short and long RNA duplexes mostly occurred at UA/UA or CA/UG sites [67].

Synthetic polymers always show good stability against various enzymes. However, biodegradable polymers that can be degraded by enzymes such as proteases, oxidoreductases and phospholipases are more beneficial for therapeutic applications [68]. Nanocarriers composed of enzyme sensitive polymers can control the site of cellular uptake of carriers and drug release to improve drug efficacy [69-71]. Synthetic polymers can also be designed to be non-degradable or degradable under certain physiological conditions. For example, Poly(lactic-co-glycolic acid) (PLGA) (which is generally regarded as safe) is approved by the Food and Drug Administration (FDA), as it can be degraded in the body by hydrolysis and has now been widely used to fabricate nanoparticles for drug delivery. Poly (beta-amino ester) is another class of degradable biomaterial developed by the Langer group [72], which can bind negatively-charged RNA or DNA and facilitate gene transfections. Although biodegradable polymers are highly attractive for drug delivery purposes, their degradation rate is very hard to control and the pharmacokinetic and biodistribution profiles of the nanocarriers can be elusive.

Synthetic polymers usually are composed of repeated same subunit, while RNA is the repetition of four subunits A, U, G and C in a specific sequence order. Therefore, RNA polymers might be advantageous concerning controllable biodegradability [73, 74] simply by tuning the ratio and location of 2’-modified nucleotides in the RNA sequence.
The versatile and plastic properties of RNA

The versatility of RNA is highly evident given the diversity of structural repertoires available in nature, which include simple structures such as helical stems and single stranded hairpin loops to more complicated structures such as multi-way junctions and pseudoknots [75-84]. Persistence length is a basic mechanical property used in polymer science to measure the flexibility and stiffness of a polymer. If a polymer is shorter than the persistence length, the molecule will basically be a rigid rod. From a mechanical perspective, the persistence length of dsRNA (62–63 nm) is slightly longer than dsDNA (45–50 nm) in an aqueous solution [85, 86]. The stretch modulus of short dsRNA (<150 bp) is ~100 pN, which is 3 times lower than dsDNA of similar length, is based on AFM and tweezer studies [87]. Interestingly, RNA was observed to shorten upon twisting with two-orders of magnitude slower timescale compared to DNA, which in contrast lengthened with a faster timescale. More recently, single molecule force spectroscopy was used to study ~10 nm long dsDNA containing ribonucleoside monophosphates (rNMPs) located at specific positions within the DNA strands [88]. The results demonstrated that the perturbation of stiffness and the plastic properties by guanosine 5’-monophosphate (rG) intrusions is location and sequence dependent, and can either soften or stiffen the DNA complex. Molecular dynamics (MD) simulations indicated that the perturbations are from local structural distortions arising from hydrogen bonding between the OH group of rG and electronegative sites of either the phosphate backbone or vicinal base. However, many factors such as sequence, local structural environment, and metal ion concentrations [89, 90] can significantly influence RNA versatility, stiffness and plasticity at the molecular level [91]. The balance of the ion-
mediated electrostatic force and the non-electrostatic (chemical) forces determine the equilibrium structure of the RNA. It is common to find that one RNA molecule can exist in more than one conformation, and one kind of RNA molecule can display multiple bands in native gel electrophoresis due to the redistribution of modular motif and local thermal energy. The versatile and plastic properties of RNA as a polymer have been applied to the construction of RNA polygons by stretching the internal angle of the three-way junction (3WJ) of phi29 DNA packaging motor from 60° to 90° to 108° to transit from triangle, square, and pentagon structures, respectively [92].

**RNA can form complex 3D structures in nanoscale**

While most biologically active DNAs are double-stranded, in contrast, most RNAs in living organisms are single-stranded. However, RNA molecules contain self-complementary sequences which facilitate the self-folding of these RNAs. In addition to Watson-Crick base pairing, non-Watson–Crick base pairs and coaxial stacking of helices also play important roles in promoting RNAs folding into complex 3D structures [12, 53]. This remarkable folding capability not only provides the structural basis for the diverse biochemical functions of different RNA molecules, but also provides huge opportunities for designing novel RNA nanostructures. For example, the subdomain IIa-1 RNA of the internal ribosome entry site (IRES) from hepatitis C virus (HCV) has a unique 90° bend L-shaped structure. Molecular modeling using this L-shaped motif showed that a planar square shaped 3D nanoparticle can be formed with four repeating L-shaped motifs, as verified by gel shift assay and fluorescence resonance energy transfer (FRET) assay [93] (Figure 1.11). Moreover, it has been found that proteins, small molecule ligands (such as
thiamine pyrophosphate (TPP) and flavin mononucleotide (FMN)) as well as monovalent and/or divalent metal ions are also important mediators of RNA folding, which could add another layer of complexity to the assembly of RNA nanostructures [53, 94].

Toolkits and methodologies for the construction of RNA nanostructures

The diverse function of RNA molecules in cells such as ribozymes, riboswitches, microRNAs, long non-coding RNAs and aptamers had attracted increasing attention in the scientific community. All these properties of RNA originated from nature that RNA can adapt different structures and spatial conformations. The laws of how nature manipulates RNA structures can also be explored to construct artificial RNA nanostructures. Besides the template-guided folding of RNA nanostructures that is similar to the tactics in DNA nanotechnology, self-assembly of RNA building blocks is another leading approach for bottom-up RNA nanotechnology. A wide variety of RNA nanostructures has been successfully constructed based on different assembly principles and approaches (Figure 1.1). In this section, I will review several toolkits and methodologies that have shown great success for constructing multifunctional RNA nanostructures.

Toolkit I: Hand-in-hand interactions involving RNA loops

Taking the construction of a wooden table as an analogy, external dowels are used to link variety of wood blocks into a structure. One of the unique properties of RNA is its folding into loops and hairpin structures. RNA loops can serve as inter-RNA mounting dovetails, thus external dowels are not necessary for self-assembly into a unique structure.
The packaging RNA (pRNA) in the phi29 DNA packaging motor [95-103] forms a hexameric ring structure through interlocking of two looped regions in each pRNA molecule, named the right- and left-hand loops (Figure 1.1A). The loop close to the 5’-end of the pRNA is defined as the right-hand loop, while the loop close to the 3’-end of the pRNA is defined as the left-hand loop. The left-hand and right-hand were brought together from interlocking interaction among four nucleotide sequences in the loops. Single pRNA molecule with self-complimentary sequences in the loops can be used to construct homodimer nanoparticles, or several pRNAs with the right-hand loop matching the sequence of the left-hand loop in another pRNA can be used to construct heterodimer nanoparticles. Through the interlocking loop interactions between different pRNA, dimer, trimer, and hexamer RNA nanoparticles have been created [104-113]. The interlocking loop sequences were further extended to increase the thermodynamic stability of generated RNA nanoparticles [114]. A toolkit with a set of hand-in-hand loop sequences has been designed and tested for constructing stable RNA polygonal nanoparticles. Loop extended RNA pairs with confirmed dimer formation capability were used as building blocks for higher order RNA oligomer nanoparticles (Figure 1.1B). Using a reengineered loop extended pRNA toolkit, pRNA dimer, trimer, tetramer, pentamer, hexamer, and heptamer were constructed with highly efficient self-assembly, as shown by gel shift assays and AFM imaging [114].

Other loop–loop interactions have also been reported to build various RNA nanostructures. For example, noncovalent loop–loop contacts based on RNAI/IIi kissing-loop complex have been used to build RNA nanorings which are thermostable, ribonuclease resistant and capable of delivering RNA interference modules [85, 115, 116]
Moreover, RNA kiss-loops were also used to build square-shaped tetrameric RNA nanoparticles and three-dimensional polyhedrons based on rationally designed RNAs [117-119] (Figure 1.1D and F). In addition, micrometer-scale RNA filaments have also been constructed by the rational design of tectoRNAs incorporating 4-way junction (4WJ) motifs, hairpin loops and their cognate loop–receptors [120, 121].

**Toolkit II: Foot-to-foot interactions involving palindrome sequence and sticky ends**

pRNA nanoparticles can also be constructed through the foot to foot interaction between pRNA monomers. While hand-in-hand interaction involves the “left” and “right” hand and the interaction is “multiple” and “intermolecular”, foot-to-foot interaction includes only one foot that is the one unique palindrome sequence by self-dimerization interaction of the identical RNA molecule. The foot domain is located in the helical 5’/3’ open region of pRNA. Extending this region with helical sequences does not compromise the entire pRNA molecule folding. A palindrome sequence reads the same whether from 5’ → 3’ direction on one strand or from 5’ → 3’ direction on the complementary strand. Extending the 3’ end of pRNA monomer with a palindrome sequence can serve as a sticky end for linking two pRNA monomers, and is denoted as a foot-to-foot interaction [113]. The foot-to-foot interaction by palindrome sequences can also be utilized to bridge RNA nanostructures, motifs, or scaffolds for constructing RNA hexamers, octamers, decamers, and dodecamers (Figure 1.1K). A pRNA array was constructed by combining hand in hand-loop-interactions and foot-to-foot palindrome sequence interactions [113] (Figure 1.1C). Recently, strategies involving both loop–loop interaction and sticky-end
cohesion were also reported to assemble three-dimensional and structurally well-defined RNA nanoprisms by re-engineering pRNA [122].

*Toolkit III: Grafts of motifs from naturally-occurring RNA molecules*

Synthetic RNA nanoarchitectures can be designed with the known naturally-occurring three-dimensional RNA motifs. Particularly, some RNA motifs in nature exhibit extraordinary stability, which could provide advantages in the assembly of RNA nanostructures. For example, the phi29 motor pRNA [54, 111, 123], tRNA [119], 5S RNA [124], and RNAI/II inverse kissing complexes [116] have been used to create synthetic RNA nanostructures [36, 93, 119, 125, 126]. The central domain of the pRNA molecule contains a 3WJ core structure [54]. This 3WJ can be assembled from three individual RNA fragments with high efficiency in the absence of metal ions [124]. The 3WJ motif itself creates a branched structure that allows for constructing multifunctional RNA nanoparticles with different functional moieties at each end of the branches. Since the 3WJ motif has three branches, RNA nanoparticles can also be designed and fabricated via branch extension [114] of the 3WJ motif ([Figure 1.1L](#)). The 3WJ core retained its original folding and the conjugated RNA module on the branches can fold into their authentic structure and remain functional [124, 127]. For example, after incorporation into the 3WJ motif, hepatitis B Virus (HBV) ribozyme retain its capacity to cleave its RNA substrate and generate smaller cleavage products, and malachite green (MG) binding aptamer was also able to bind MG to emit fluorescence as shown in the fluorescent spectra ([Figure 1.2A and B](#)). This property makes the pRNA 3WJ structure an extraordinary nanocarrier for targeted gene delivery [124]. Moreover, an X-shaped...
motif from the central domain of the pRNA was discovered through extending the right-hand loop with a double-stranded sequence [73]. The X-shaped pRNA motif can be self-assembled from four RNA oligos and it creates a scaffold that allows for the conjugation of four RNA functional moieties at the same time. Functional RNA modules such as survivin siRNA preserved their target gene knock-down effect after being fused to the X-shaped structure (Figure 1.2C). Branched hexavalent RNA nanoparticles were further constructed from three 3WJ motifs, integrated by one 3WJ core motif structure or by palindrome sequence mediated linking of two pRNA-X cores [114].

Recently, Khisamutdinov et al. reported using RNA as an anionic polymer to build programmable self-assembling boiling-resistant RNA nanostructures based on pRNA-3WJ [36] (Figure 1.1N). A triangular shaped RNA nanoparticle was designed and prepared by carefully joining extended helices to the thermodynamically stable pRNA-3WJ motif of the bacteriophage phi29 DNA packaging motor. The step-wise self-assembly of the triangle RNA nanoparticles was confirmed by native polyacrylamide gel electrophoresis (PAGE) analysis, and AFM imaging revealed the triangular shape of the nanoparticles as expected. The constructed nanoparticles are thermodynamically ultrastable and robust. Functional motifs including siRNA, ribozyme, folate and fluorogenic RNA aptamers retained their activities after conjugation to the RNA nanoparticles. Moreover, these RNA triangles could be used as building blocks to construct supramolecular complexes, such as RNA hexagons and patterned hexagonal arrays. The pRNA-3WJ is also highly tunable [92]; the naturally preserved angle between the helices H1 and H2 of pRNA-3WJ is \( \sim 60^\circ \) and this angle can be stretched to form square-shaped (90° angle) and pentagon-shaped (108° angle) nanoparticles with pre-
designed sequences (Figure 1.3A). The polygons were formed in an one-step self-assembly manner with efficiency >90%, as demonstrated by gel shift assays. The different sizes and shapes of the polygons were also confirmed by dynamic light scattering (DLS) and AFM. The equilibrium dissociation constants (K_D) for triangle, square and pentagon are 18.8 nM, 20.3 nM and 22.5 nM, and the melting temperature (T_m) values are 56 °C, 53 °C and 50 °C, respectively, revealing structure and shape-dependent thermodynamic features. In a recent paper by Jasinski et al, the authors showed that square-shaped RNA nanoparticles with fluorogenic and ribozymatic properties as well as different sizes can be successfully constructed by utilizing the pRNA-3WJ at each corner and different length RNA duplexes at each edge of the square RNA nanoparticles [126] (Figure 1.3B). DLS and AFM determined the sizes of the small, medium, and large square RNA nanoparticles are 4.0, 11.2, and 24.9 nm, respectively. The physiochemical properties of the nanoparticles can be easily tuned, as the utilization of 2′-F RNA as the core strand significantly increased the melting temperature as well as the nanoparticle's resistance to serum-mediated degradation.

Naturally occurring stable RNA-protein complexes were also explored as building blocks for nanostructures. For example, the assembly of archaeal box C/D small ribonucleoproteins (sRNPs) comprising the L7Ae protein from Archaeoglobus fulgidus and a box C/D RNA has been utilized to build an equilateral triangle shaped synthetic RNA protein complex [128] (Figure 1.1H). The construction relies on the interaction between ribosomal protein L7Ae and the Kink-turn motif in box C/D RNA. L7Ae can bind to the box C/D K-turn motif through hydrogen bonding with high specificity and affinity [129]. It was reported that using RNA-protein complex as building block for
nanostructures enhanced its stability in serum comparing to pure unmodified RNA based nanoparticles [130]. The ribonucleoprotein (RNP) nanostructure could serve as a scaffold for protein or RNA functionalities. For example, an affibody peptide, which can recognize human epidermal growth factor receptor 2 (HER2), was connected to a RNP triangle through fusing to a L7Ae protein, and the functionality of the affibody peptide was still retained. siRNA targeting to GFP gene can be conjugated to the RNP triangle through extending the RNA strand and the GFP siRNA on RNP nanoparticles still can be processed by dicer and knock down GFP expression in cells.

Toolkit IV: RNA origami

DNA origami, which involves the folding of a long template single stranded DNA aided by multiple short staple DNA strands, was reported in 2006 [131]. A wide range of 2D and 3D nanostructures have been successfully constructed using the DNA origami technique [132-136]. In 2014, a similar approach for RNA origami was reported to fold single stranded RNA into RNA tiles and further assembled into hexagonal lattices [137] (Figure 1.1M). A variety of RNA tertiary motifs, including the 180° kissing loop motif of the HIV-1 DIS [138], the 120° loop–loop complex of RNA i/ii inverse loop [115], and the tetraloops GNRA and UNCG [139] were used to mediate internal RNA tertiary interactions, resulting in organized and scalable RNA crossover tiles. The designed RNA origami nanostructures are also robust, which could form by either annealing or co-transcription on mica. In contrast to DNA origami, this RNA origami approach does not need short staple strands to facilitate the folding of the origami structure, but using RNA modules such as kissing loops to replace the role of staple strands. Interestingly, a
different RNA origami approach which is a direct extension of the DNA origami to RNA was also reported in 2014 [140] (Figure 1.1P). In this study, a single-stranded RNA scaffold and multiple staple RNA strands were used to assemble defined RNA nanostructures including a 7-helix bundled RNA tile and a 6-helix bundled RNA tube. The authors also showed that functional modules such as biotin could be introduced into the RNA origami structures by chemical modifications of the scaffold strands.

**Toolkit V: RNA/DNA hybrid nanostructures**

RNA/DNA hybrids can also be used to construct functional nanoarchitectures. Similar to split-protein systems, RNA/DNA hybrids can be computationally designed for activating different split functionalities in the presence of respective equivalent strands [141-144] (Figure 1.4A). Toehold interactions were used to trigger disassociation of RNA/DNA hybrids and re-association of the double-stranded RNA and DNA. The thermodynamics and kinetics of the toehold interactions can be tuned to control the dissociation and re-association processes. RNA interference modules as well as other functionalities such as fluorophores and RNA aptamers can be successfully triggered inside mammalian cells. This RNA/DNA hybrids concept has also been applied to other nanostructures such as nanorings and cubes [115, 116, 145-147], and the spilt functionalities still retain their authentic functionalities. Because the RNA/DNA hybrid is more enzymatically stable than RNA, this approach has the potential to improve the pharmacological profiles of RNA-based nanoparticles. Moreover, controlled release of the split functionalities opens a new avenue for the development of nucleic acid-based switches to modulate cellular functions in vivo.
Methodology I: Rolling circle transcription

Typically, the design and production of RNA nanoparticles have relied on the production of discrete RNA strands that can be assembled in a controlled and predictive fashion to generate nanoparticles with a defined structure and stoichiometry. Hammond and colleagues have departed from this approach with an innovative strategy that generates monodisperse spherulitic RNA particles from extremely high molecular weight RNA strands by using a rolling circle transcription (RCT) approach [148, 149] (Figure 1.1J). They created a circular DNA construct containing a siRNA gene without any terminator sequences and proceeded by a T7 promoter. As a result, T7 RNA polymerase can continuously transcribe the circular DNA hundreds of times to generate a tremendous number of copies of the tandem RNA unit. As the continuous RNA strand is transcribed, magnesium pyrophosphate crystals are simultaneously generated, and the RNA strands attach to the crystallite surfaces to form composite structures; each structure grows in length to become fiber-like, then, forms sheet-like lamellae. The lamellae, typically ~10 nm thick, finally condense into spherulites with diameters varying from a few micrometers to hundreds of micrometers, referred by the author as RNAi microsponges. To achieve efficient cellular uptake, Polyethylenimine (PEI) was introduced to condense the RNAi-microsponge from 2 µm to 200 nm, and to protect the RNA from degradation by RNase. The PEI introduced a positively charged outer layer to the microsponges to facilitate cell binding and entry. It was also demonstrated that the spherulitic RNAi-microsponges produced ~21 nucleotides (nt) siRNA fragments after incubation with Dicer and could transfect a cancer cell line and silence firefly luciferase expression. Gene expression knockdown in vivo by intratumoral injection of the PEI-condensed RNAi-
microsponges was also reported. This innovative approach opens new directions to RNA nanoparticle self-assembly and siRNA delivery. However, the therapeutic potential and in vivo safety of this approach requires further evaluation, as PEI has been shown to cause well-known adverse side effects, such as high cytotoxicity [150, 151]. In addition, further reduction of the size of the particles is recommended to avoid non-specific healthy organ accumulation.

In material sciences, polymeric membranes are at the forefront in the chemical and biotechnology industry because of their versatile applications such as water purification, dehydrogenation of natural gas, dialysis of blood, and removal of cell particles [152]. Recently, Lee's group demonstrated rolling circle transcription can also be applied to the synthesis of macroscopic RNA membranes that has the potential as a controlled drug-release system [153] (Figure 1.1O). The RNA membrane was fabricated by T7 RNA polymerase transcription system with a combination of complementary rolling circle transcription (cRCT) and evaporation-induced self-assembly (EISA). The circular DNA template and complementary circular DNA template were prepared with long linear DNA and the promoter DNA for starting the rolling circle transcription (RCT). Upon addition of T7 polymerase to these two circular DNA templates, thousands of copies of single stranded RNA and single stranded complementary RNA were continuously generated. The two complementary RNA strands hybridized to each other thereby forming the double stranded RNA for the large-area of RNA membrane. After the cRCT process, the water in the RNA membrane was evaporated and the dried RNA membrane was concentrated by the EISA process. The self-assembled RNA membrane was fabricated densely on the tube wall during the evaporation process. The durability of the fabricated
RNA membrane was tested under various harsh conditions, including RNase and DNase-rich environments. In addition, they confirmed the application of the RNA membrane as an enzyme-responsive drug-release system with doxorubicin and siRNA. The results showed that the RNA membrane can provide a high drug-loading efficiency and can be a great candidate for membrane-based drug delivery.

**Methodology II: Chemical modification of RNA**

A great deal of work on chemical modifications aiming to improve the chemical stability and in vivo properties of RNA has been reported [154-157]. Common chemical modifications of RNA can be categorized into five classes: (1) Modification of internucleotide phosphodiester backbone. This type of modification is the most classic and simplest method to improve the performance of RNA in the biological environment. For example, creation of a phosphorothioate (PS) linkage by replacing one non-bridging oxygen atom on the phosphate backbone with a sulfur atom substantially increases the stability of RNA in vitro and in vivo [158]. Other backbone modifications such as boranophosphate, phosphoramidate and methylphosphonate have also been explored to enhance the resistance to nuclease-mediated degradation [159-161]. However, cytotoxic side-effects were also observed if extensive modifications were applied [162]. (2) Substitution of 2′-OH group. This is the most widely used approach since it is well-tolerated and can enhance nuclease resistance as well as reduce immunogenicity. For example, the naturally occurring 2′-O-methyl (OMe) modification is nontoxic and is able to prevent immune activation while conferring biological stability simultaneously [162] and [163]. Guo's lab also reported that incorporation of 2′-fluoro nucleotides into the
pRNA scaffold allows the creation of stable and RNase-resistant RNA nanoparticles with correct folding and authentic biological activities. The melting temperature of 2′-F RNA was further enhanced compared to unmodified RNA [164]. The chemically modified pRNA-based nanoparticles have shown promising applications in cancer therapy [36, 66, 165-168]. Recent study also showed that 2′-F modified pRNA nanoparticles are resistant to 125I and 131Cs radiation with clinically relevant doses, which is a required property for applying these RNA nanoparticles as delivery vehicles for targeted radiation therapy [169]. Other options for 2′-modification like 2′-fluoro-β-d-arabinonucleotide (FANA) have also shown promise in gene silencing applications [170]. (3) Locked nucleic acids (LNA) and unlocked nucleic acid (UNA). LNA is another class of 2′-modification in which 2′-O and 4′-C is linked via a methylene bridge. This locked linkage constrains the ribose ring into the C3′-endo conformation, which confers both significant increase in thermostability and enhancement in nuclease resistance [171]. Successful applications of LNA such as 2′-amino-LNA have been studied by Astakhova et al. to show great promise in the development of biosensor, aptamer and other nanomaterials [172]. Antisense oligonucleotides (ASO) containing LNA also has been reported to have higher potency and specificity [173] and [174], but would also cause liver toxicity in mouse models if LNAs were extensively incorporated into the sequence [175]. In the other hand, UNA is an acyclic and structurally flexible-RNA analogue in which the C2′–C3′ bond is absent. As a result, the binding affinity of UNA towards its complementary strand is decreased [176]. (4) Modification of ribonucleotide base. This approach is less commonly used than other modification approaches described thus far. However, recently RNA tube nanostructures have been reported to be constructed successfully by using RNA scaffolds
with 5-biotinylated modified and 5-aminoallyl modified uracil [140]. Other common modified bases such as 2-thio-, 4-thio, 5-iodo-, 5-bromo-, dihydro-, pseudo-uracil and diamino-purine also have been demonstrated to confer enhancement in stability as well as specificity of base-pairing interactions [156, 177, 178]. (5) Modification of ribose moiety. This modification strategy, for example, altritol nucleic acid (ANA) and hexitol nucleic acid (HNA), is mainly applied in siRNA design to enhance potency and nuclease resistance [179]. Besides these classes, other novel methods such as photocaging modification have been used to control RNAi induction [180]. The particular effect of various chemical modifications on RNA are summarized in Table 1.1. It is expected that the rational choice of precise chemical modifications will greatly contribute to the development of RNA nanostructures for applications in biomedical sciences and material sciences. Moreover, the strategy of combining different modification approaches also has the potential to improve the properties of RNA more dramatically.

Methodology III: Computational approach

The first step in RNA nanoparticle construction is the consideration of the blueprint [181]. This requires an understanding of the assembly and folding mechanism in the bottom-up assembly. Designing the sequence of the building block is critical for successful RNA nanostructure assembly, which can be achieved by experience and brainstorming taking into consideration RNA folding, complementation, hand-in-hand interaction, foot-to-foot interaction, and the use of thermostable motifs, kissing loops, sticky ends, helices and stem loops. All RNA nanoparticles constructed based on phi29 motor pRNA were achieved via experience and brainstorming without computer
algorithm other than the traditional RNA folding program developed by Zuker 30 years ago [182]. It is expected that computer algorithms will facilitate RNA nanoparticle construction. A variety of computer programs such as NanoFolder, NanoTiler and RNA2D3D are available to facilitate the in silico design of RNA sequences (these sequences may contain inter-strand and intra-strand pseudoknot-like interactions) capable of self-assembly into multi-sequence RNA nanostructures and the 3D modeling of such structures [183-185]. For example, by utilizing computational modeling and sequence optimization, three-dimensional cubic RNA-based scaffolds can be successfully designed and engineered with precise control over their shape, size and composition [145]. Moreover, online RNA structure databases such as RNAJunction database also provide useful RNA structures for designing RNA nanostructures [186].

**Application of RNA as a polymer in biomedical sciences**

RNA as a natural and biocompatible polymer has many advantages for biomedical applications. It carries a negative charge at physiological conditions, which disallows nonspecific passing through negatively charged cell membrane. With the conjugation of chemical ligands and/or RNA aptamers, RNA nanoparticles can be designed for specific cell targeting. It is less toxic compared with protein-based nanoparticles since it can avoid antibody induction (protein-free nanoparticle), allowing repeated treatment of chronic diseases. It also does not induce an interferon response nor cytokine production [92, 187]. RNA nanoparticles designed with a size range 10–40 nm display favorable pharmacokinetic properties [187], such as extended half-life *in vivo* (5–12 h compared to 0.25–0.75 h for siRNA), clearance: < 0.13 L/kg/h, volume of distribution: 1.2 L/kg.
As a building block for nanoparticles, RNA can be synthesized with defined structure and stoichiometry. Multivalent RNA nanoparticles can be constructed using special RNA motifs as building blocks that combine therapy, targeting, and detection, all functionalities in one particle.

RNA as a biocompatible nanomaterial for tissue engineering

Tissue engineering is a new area with large amounts of active research and some recent success. Biocompatible nanomaterials are needed for various applications in tissue engineering, such as scaffolds or arrays that can function as temporary matrices and/or niches for the controlled deposition, infiltration, proliferation, and differentiation of cells. It would also be advantageous if these nanomaterials could be highly biocompatible and mimic the natural tissue microenvironment in vivo.

Shu et al. reported that nanometer scale 3D RNA arrays can be assembled by using pRNA as building blocks [113] (Figure 1.1C). By rational design, the authors incorporated palindrome sequences (nucleotide sequences that read the same $5' \rightarrow 3'$ on one strand and $3' \rightarrow 5'$ on the complementary strand) into the $3'$-end of the pRNA. The palindrome sequences served as links for bridging two pRNAs via foot-on-foot interactions. Loop–loop interactions were further used to link pRNA molecules into a chain. The formation of pRNA arrays was analyzed by PAGE and it was found that the arrays were too large to run into the gel and got trapped in the gel wells. The shape of the pRNA arrays was further characterized by AFM. Under AFM imaging, the pRNA arrays presented as bundles with micron scale. These RNA arrays are unusually stable and resistant to a wide range of temperatures, salt concentrations, and pH environments. The
microstructures of the RNA assays are also tailorable by changing the RNA nucleotide sequences. Since RNA molecules are highly biocompatible and not toxic, these RNA arrays have the potential to be good tissue engineering scaffolds.

**RNA nanoparticles for targeted therapeutic delivery**

Targeted delivery is a major challenge that nucleic acid therapeutics is facing. RNA nanoparticles with chemical ligand and nucleic acid aptamers have shown great promise in this regard. The utilization of RNA nanostructures as a platform for targeted therapeutics delivery has been successfully demonstrated by a series of studies using the phi29 pRNA system. In 2003, Hoeprich et al. constructed a pRNA-based carrier to deliver hammerhead ribozymes [188]. Upon conjugation to the pRNA 5′/3′ ends, the HBV ribozymes were able to fold correctly and almost completely cleaved the polyA signal of HBV mRNA in vitro. Targeted therapeutics delivery to specific cancer cells can be achieved by using ligand-conjugated RNA nanoparticles as carriers. Furthermore, the cargo in vivo release profile from RNA nanoparticles can also be controlled through the rational design of RNA nanoparticles. In 2005, Guo et al. reported the construction of chimeric pRNA dimer with one subunit harboring folate and the other subunit harboring a gene silencing siRNA targeting survivin [109]. Incubation of these pRNA dimers with cancer cells resulted in receptor-mediated binding and entry to cells and induced efficient gene silencing. Animal trials showed that ex vivo delivery of the pRNA dimer harboring both folate and survivin siRNA could suppress tumor development. Khaled et al. studied the fabrication of a protein-free 20–40 nm pRNA trimer which could harbor three functional modules including siRNA, CD4 aptamer or folate ligands and a fluorescent
dye per nanoparticle [112]. They showed that the pRNA trimer could also bind and enter into cells and modulate gene expression and apoptosis both in vitro and in vivo. In 2006, Guo et al., reported incorporating a folate-AMP into the 5′-end of phi29 pRNA and showed that folate conjugated pRNA dimer nanoparticles were able to deliver survivin siRNA into nasopharyngeal epidermal carcinoma cells and silence the target gene [189].

The pRNA system can also be used to deliver anti-virus siRNAs. Zhang et al., developed a folate-linked pRNA conjugated with the siRNA targeting the Coxsackievirus B3 (CVB3) protease 2A (siRNA/2A) [190]. They observed that the modified pRNA could achieve a similar antiviral effect to that of siRNA/2A alone and also strongly inhibited CVB3 replication.

The latest advance in the utilization of phi29 pRNA for therapeutics delivery is the discovery of a phi29 pRNA 3WJ motif with unusual thermodynamic stability. The slope of the melting temperature curve of the three-fragment RNA complex is close to 90°, indicating extremely low ΔG of the phi29 pRNA-3WJ complex [124]. The pRNA 3WJ motif was used as a RNA scaffold to construct bi-, tri-, and tetra-valent RNA nanoparticles with very high chemical and thermodynamic stability [73, 124, 181]. The resulting RNA nanoparticles are resistant to denaturation in 8 M urea and do not dissociate at ultra-low concentrations. Each arm of the 3WJ or X-motif can harbor one siRNA, ribozyme, miRNA, or aptamer without affecting the folding of the central core, and each daughter RNA molecule within the nanoparticle folds into respective correct structure with authentic biological function. The effects of gene silencing progressively increased with increasing number of siRNA modules in the RNA nanoparticle [73]. These RNA nanoparticles can specifically target subcutaneous tumor xenografts [73, 124,
168], as well as orthotopic breast cancer [174] and intracranial glioma tumors [165] without detectable accumulation in liver, lung or other healthy organs or tissues (Figure 1.5A–C). A recent study also showed that the pRNA-3WJ nanoparticle conjugated with folate can specifically target colorectal cancer metastasis in the liver, lungs and lymph node simultaneously in vivo, without accumulation in normal liver or lung parenchyma [166] (Figure 1.5D). Pharmacological analysis in mice indicated that the pRNA nanoparticles display favorable pharmacokinetic (PK) and pharmacodynamic (PD) profiles, with in vivo half-life extended 10-fold compared to siRNA alone and did not induce adverse immune response including cytokine, interferon, antibody, and other toxic effects [187]. The regression of gastric cancer and breast cancer by RNA nanoparticles harboring siRNA [73, 124, 168] or anti-miRNA [174] has also been reported recently.

A recent paper also reported the development of novel immunomodulators by engineering rationally designed RNA polygonal nanoparticles [92]. When immunological adjuvants CpG DNA were incorporated into the RNA polygons, potent immunostimulation (cytokine TNF-α and IL-6 induction) was observed both in vitro and in vivo, compared to controls. Moreover, the RNA nanoparticles could deliver CpG DNA to macrophages specifically and the degree of immunostimulation significantly depended on the size, shape, and the number of payload per RNA nanoparticle. This finding demonstrates that RNA nanotechnology, such as developing RNA nanoparticles based on pRNA, has great potential to develop novel immunomodulators.
RNA nanoparticles for controlled drug delivery

Many pre-clinical studies are evaluating RNAi as novel therapeutics for different diseases. However, one concern is that single gene targeted therapy might eventually fail due to mutations over time or development of alternative signaling cascades or escape pathways. Multivalent RNA nanoparticles can be a very promising vehicle for delivery of multiple siRNAs to suppress multiple genes simultaneously. A recent publication by Afonin et al. showed that several RNA and RNA/DNA nanocubes could be functionalized with multiple double stranded RNAs and the siRNAs can be conditionally released through ssDNA toehold-mediated interactions. Furthermore the RNA nanocubes can be tracked intracellularly through FRET (fluorescence resonance energy transfer) studies using fluorophores [191]. In addition, spherical nucleic acid (SNA) nanoparticle conjugates developed by the Mirkin group have also shown promise for systemic siRNA delivery for treating diseases such as glioblastoma [192, 193] (Figure 1.4D). Spherical nucleic acids are densely packed and highly oriented nucleic acids covalently conjugated to the surface of a spherical nanoparticle core. In vivo studies on glioma-bearing mice showed that SNA nanoparticle conjugates targeting the oncoprotein Bcl2Like12 effectively increased intratumoral apoptosis, and reduced tumor burden and progression without adverse side effects [193].

DNA origamis have been exploited as a carrier for the chemical drug doxorubicin through noncovalent intercalation interactions. The DNA origami/doxorubicin nanoparticles were able to enter into doxorubicin-resistant cancer cells and circumvent their drug resistance [194]. Furthermore, the DNA origami/doxorubicin nanoparticles showed a slow drug release profile at pH 7.4 under physiological conditions but showed
enhanced release capability in pH 5.5 which is similar to the pH of acidic subcellular organelles in tumors [195]. As we discussed in previous section (Toolkit IV: RNA origami), RNA origami structures have been reported [137, 140]. With favorable thermodynamic stability and excellent serum stability after chemical modifications, RNA origami is expected to be more favorable than its counterpart DNA origami as a drug carrier for achieving controlled drug release.

**RNA nanoparticles for vascular targeting**

A critical feature to drug delivery is the transportation of therapeutics to their intended target sites. As almost all cells in the body are within ~100 µm of the vasculature, the vascular delivery route represents a promising mean for site specific therapeutics delivery [196]. Owing to the significant heterogeneity in endothelial cells, both temporally and regionally, it has been observed that the vascular endothelium has unique expression of surface markers that are highly regionally variant. In other words, it is potentially possible to target unique endothelial zip codes to locally accumulate nanocarriers [197].

Current strategies for vascular targeting of nanocarriers have primarily focused on antibody and peptide based strategies, targeting to surface markers CD31 (PECAM-1) [198] and [199], inflammatory markers (ICAM-1, VCAM-1) [200-205], and thrombomodulin [206]. Targeting of CD31, ICAM-1 and VCAM represents a unique opportunity as the binding of nanoparticles to their sites highlights a novel endocytic mechanism termed as CAM mediated endocytosis [199, 207]. The use of antibodies as targeting agents is technically challenging owing to their size, cost of production and
potential immune responses. RNA aptamers have already shown exciting advantages in vasculature targeting. High affinity aptamers (56 pM) against P-selectin have been developed [208]. Microbubbles coated with a low density (1000 copies/µm²) of P-selectin aptamers demonstrated high adhesion even under shear rates as high as 1700 L/s. The real promise of vascular-targeted RNA nanoparticles comes from the combination of the high binding affinity to carrier sizes below 70 nm. The capillary bed represents the greatest promise of vascular targeting, where there already exists a highly active transcytosis mechanism, with approximately ten thousand times more caveolae than other endothelial cells [209]. These 70 nm endocytic vesicles are an exciting target for drug delivery [210] yet require a carrier system like RNA nanoparticles that are simultaneously small and have high affinity to permit transcytosis and tissue bed delivery.

**RNA nanoparticles as non-invasive medical detection reagents**

Functional RNA structures such as aptamers can be used for developing non-invasive molecular and cellular imaging reagents which may have applications in the diagnosis of various diseases including cancer, cardiovascular diseases, and infectious diseases. Considering it is relatively easy to conjugate a fluorophore, radionuclide, quantum dot (QD), gold nanoparticle, or other imaging functional groups to RNA, RNA molecules with high binding affinity to their targets hold great potential as a platform to construct imaging probes which can specifically detect their targets in vitro and in vivo.

Fluorescent RNA nanoparticles conjugated with aptamer have been widely explored for cancer detection. For example, Bagalkot et al. [211] developed a QD-aptamer-doxorubicin conjugate nanoparticle as a cancer-targeted imaging, sensing and
A10 RNA aptamer, which specifically binds to the extracellular domain of the prostate specific membrane antigen (PSMA), was conjugated to the surface of fluorescent quantum dots and the anticancer drug doxorubicin was intercalated into the double-stranded stem of the A10 aptamer. In the conjugate nanoparticle, fluorescence of doxorubicin was quenched by the aptamer through donor-quencher FRET, and the fluorescence of QD was quenched by doxorubicin through donor-acceptor FRET mechanism. When the nanoparticle enters the prostate cancer cell and doxorubicin is released, the fluorescence of QD and doxorubicin is re-activated. Other RNA aptamers conjugated with fluorescent dye chemicals have also been utilized for breast cancer diagnosis, such as EpCAM aptamer labeled with DY647, TYE665 or FITC [212].

Conjugating RNA aptamer with radioactive material can also be applied as diagnostic radiopharmaceuticals to detect cancer cell markers. In a recent study reported by Gomes et al. [213] a 36 nucleotide long truncated RNA aptamer with 2′F pyrimidine and 2′-O-methyl purine modification was constructed for targeting to the human matrix metalloprotease 9 (hMMP-9), which promotes tumor metastasis and is an important marker of malignant tumors. The 5′-end amine modified RNA aptamer was conjugated to N-hydroxysuccinimidyl mercaptoacetyltriglycinate (MAG3-NHS), which served as a chelator for Technetium-99m ($^{99m}$Tc). The $^{99m}$Tc labeled modified RNA aptamer was able to specifically detect its target in human brain tumors with autoradiography.

With the conjugation of the PSMA RNA aptamer to the surface of gold nanoparticles (GNPs) [214], a targeted computed tomography (CT) imaging and therapy system for prostate cancer can also be established (Figure 1.4C). The CT imaging study
revealed that the PSMA aptamer-conjugated GNPs could generate more than four-fold
greater CT intensity for a PSMA-expressing cell line than a non-PSMA-expressing cell
line, suggesting good specificity in detection of the targeted cancer cells.

RNA nanoparticles for intracellular imaging and detection

An interesting example of applying RNA in intracellular imaging is the Spinach
RNA aptamer, which is the RNA mimic of Green fluorescent protein (GFP). GFP has
been extensively utilized as a reporter protein in cell and molecular biology. Similarly,
the RNA mimics of GFP should also have remarkable applications in biomedical research,
especially for intracellular imaging of RNA. By using the method of systematic evolution
of ligands by exponential enrichment (SELEX), Paige et al. developed a novel class of
RNA aptamers, termed Spinach that bind to fluorophores resembling the fluorophore in
GFP [215] (Figure 1.6A). Upon binding of the targeted fluorophores, these RNA
aptamers were capable of emitting tunable fluorescence with comparable brightness to
enhanced GFP (EGFP) and many other fluorescent proteins. Moreover, the RNA-
fluorophore complex is resistant to photobleaching. Interestingly, the Spinach RNA
aptamer can be expressed in vivo by fusing to other cellular RNAs, for example, 5S RNA,
to enable live-cell imaging and monitoring of these cellular RNAs. Furthermore,
fluorescent sensors for detecting a variety of small molecules and cellular metabolites,
including adenosine 5‘-diphosphate (ADP) and S-adenosylmethionine (SAM), in vitro
and in living cells could also be generated by combining the Spinach aptamer, a
transducer, and a target-binding aptamer [216] (Figure 1.6B).

Reif et al. [217] recently reported another novel method to enable real-time monitoring of RNA folding and degradation in living cells based on the pRNA-3WJ. The authors designed and constructed RNA nanoparticles by incorporating an RNA aptamer capable of binding to malachite green (MG), the hepatitis B virus ribozyme and the luciferase siRNA into the pRNA 3WJ. When MG aptamer binds to MG dye, it will emit fluorescent light only if the aptamer folds correctly. The MG aptamer system can then be used to monitor the degradation of the constructed RNA nanoparticles by fluorescent microscopy and fluorescence spectroscopy. By using this novel design, the authors determined the half-life ($t_{1/2}$) of the MG aptamer containing pRNA-3WJ inside living cells to be $\sim 4.3$ h.

RNA as a polymer for biosensor systems

The biomolecular-based detection system has been widely investigated in many applications including clinical diagnosis, food industry, environmental monitoring industry, and security industry [218, 219]. Conventionally, biosensors can detect proteins, nucleic acids such as DNA or RNA sequences, and small organic molecules [219-221]. RNA molecules are well suited to serve as detection tools for small molecules, antibiotics, peptides, metal ions, and ligands [222, 223] because of their unique folding structures, functional conformation, predictable base pairing, and high fidelity. These characteristics of RNA allow for the development of ribozyme or aptamer, and aptazyme-based biosensors [224-226]. Also, as the target, RNA can be used to develop the biosensor for the detection of messenger RNA or micro RNA [227-230]. Various detection methods of RNA-based biosensors were proposed such as electrochemical type, fluorescence type,
optical type, and electrical type. Also, there are various detection platforms for RNA-based biosensors (Figure 1.7) [231-236]. In this section, we briefly introduce RNA-based biosensors for biomedical and environmental monitoring applications.

The first proof of concept of using RNA in a biosensor was developed by Breaker Group [222]. Self-cleaving hammerhead-ribozymes were created with seven different RNA switches and these immobilized each pixel that responded allosterically to six types of analytes (Co$^{2+}$, cGMP, cCMP, cAMP, FMN and theophylline). The platform of biosensor array was prepared on a polystyrene cell culture plate which was coated with gold by physical vapor deposition. The ribozyme was immobilized onto gold surface via a 5′-thiotriphosphate-terminated RNA moiety. When individual analyte was added, the corresponding RNA biosensor captured the analyte and induced the self-cleavage and release of the 3’ fragment of the ribozyme. They also showed the quantitative and qualitative measurement of cAMP in culture media from E. coli strand.

RNA can also serve as the detection analyte to detect viable E. coli as an indicator organism in drinking water, which was proposed by the Baeumner group [229, 230]. This RNA-based biosensor was generated with the extraction and amplification of mRNA molecules from E. coli in 20 min. Viable E. coli in the water were identified and quantified using a 200 nt target sequence from mRNA. The detection limit of the biosensor system could detect around 40 viable E. coli in water (5 fmol per sample) using the electrochemiluminescence (ECL) detection method. They introduced the isothermal amplification technique, a nucleic acid sequence-based amplification (NASBA) for mRNA amplification.
RNA was also applied to develop medical diagnosis biosensors. Theophylline (1, 3-dimethylxanthine) is a common agent in the bronchodilators and used for acute and chronic asthma. Gothelf group developed a RNA-based electrochemical biosensor for theophylline in serum with a ferrocence (Fc) redox probe [226, 234]. In this study, the thiol group-modified and amino group-modified RNA aptamer were immobilized onto gold substrate via covalent bonding between thiol group and gold substrate. Then, the Fc redox probe was covalently attached to the 3′-amino group of RNA aptamer through Fc-carboxylic acid NHS reaction. Usually, the RNA aptamer stays on the open conformation in absence of theophylline. However, when the theophylline added to RNA aptamer-modified electrode, the aptamer folds into the conformationally restricted hairpin structure. As a result, this phenomenon changed the electron transfer that was monitored by cyclic and differential pulse voltammetry. Moreover, specific recognition of dopamine by the RNA aptamer allows selective amperometric detection of dopamine from 100 nM to 5 μM range in the presence of competitive neurotransmitters such as catechol, epinephrine, norepinephrine, and others [233].

In an alternative approach, the Hammond group used a RNA-based fluorescent biosensor for detecting cyclic di-GMP and cyclic AMP-GMP [227]. The c-di-GMP was generated by a GEMM (Genes for the Environment, Membranes, and Motility)-I riboswitch aptamer and the riboswitch ligand mutation can recognize the c-AMP-GMP, c-di-GMP. The fluorescence turn-on was activated by the 3, 5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) fluorescent molecule for cell imaging. This system provided selective and sensitive detection for each cyclic dinucleotide. Thus,
there are many examples demonstrating that RNA molecules are promising candidates for biosensor applications.

**Application of RNA as a polymer in material sciences**

*RNA as a polymer for biocomputation systems*

Silicon-based computer systems are ubiquitous [237] but new processors to implement computation, communication, and controls are needed to meet the demands of various applications. Gordon Moore's Law suggested that due to the increasing demand of the memory, computer electronic devices on the microprocessor will be doubled every 18 months. Scientists and engineers are wondering whether current computer technology growth can be continued in the next ten years. They raised the concern that silicon microprocessor speed and miniaturization will eventually reach limits by current technology. Thus, molecular-scale computing has been explored since 1994 due to the predictable ending of Moore's Law for silicon-based computer devices.

In the last few decades, biotechnology has been integrated with nanotechnology and electrical engineering [238-240]. As a result, the field of bioelectronics has been created [241] and [242]. Bioelectronics has led to the development of the nanoscale biochip, biosensor, and biocomputation devices such as information storage devices, logic gates, field-effect transistors and computation systems (*Table 1.2*) [243-246]. Typically, bioelectronic devices are composed of a biological actuation portion, and an electronic signal transduction portion. So far, the biological component is usually composed of biomolecules, such as protein and DNA [240], [243] and [245]. Recently, some pioneering groups have suggested that RNA-based bioelectronic devices also
exhibited the logic gate behavior, processing functions, and biocomputational functions [181, 247-251]. The advantages of RNA-based computation are summarized in Figure 1.8. Biocomputation approaches for information control, storage, and processing using RNA-based devices brings new possibilities that embody multiple functions in biological systems. The development of biological computers composed of artificial RNA molecules to operate inside living cells or tissues would provide a new avenue. A combination of these results will pave the way to develop the new-concept of biocomputer development in the future such as tissue-mimicked computations.

RNA as a polymer for logic gates

In computer science, the ‘logic’ term is defined as circuits which gives an output corresponding to a set of two input values (True ‘1’, High voltage and False ‘0’, Low voltage). The logic gate is required to have two input values for performing Boolean function [242]. Thereby, it gives the logical output to perform the next function. Usually, man-made computers consist of millions of combinational logic gates. Thus, logic gates are essential components of the computer. As mentioned in the last section (RNA as a polymer for biocomputation systems), in the last few decades, the concept of a biomolecule-based computation system has been proposed or explored to solve the current limitation of silicon-based computations [247, 252-256]. The first attempt of molecular-scale computation was developed by Adleman's group in 1994 [252]. They introduced the encoded DNA molecule to solve the Hamiltonian path problem. This research established a milestone for the field of biomolecule-based computations. Since then, several groups proposed the DNA polymer-based logic systems [253, 255, 256].
The basic operation mechanism of DNA-based logic gate is combinational DNA hybridization that undergoes conformational change or chemical reaction and includes pH, temperature, light, electrical, or electrochemical signals [252-256]. The function of logic gate gives the discrete state change such as structure, fluorescence, absorbance, and electrochemical or electrical current as the output. Like this, the various types of DNA-based computations have been proposed. DNA, however, is hard to use to perform the complex functions because of its simple functionality. To develop the new-concept of biocomputation systems, RNA is a candidate owing to its intriguing characteristics. The folding and assembly of RNA molecules drive themselves into secondary and tertiary structures using the formation of hairpin loops, dovetails, bulges, and internal loops [10]. Thereby, RNA has functionality such as aptamer, ribozyme, siRNA, non-coding RNA, and circular RNA [181]. These various functional groups can be easily applied to the new concept of logic gate behavior, information storage, information processing, and computations as the novel elements [257-265].

An autonomous biomolecular-based computer has been proposed to regulate the gene expression “logically” [249]. The basic computation rule is governed by ‘diagnostic rule’ for prostate cancer stage detection. If the specific genes relating to prostate cancer are overexpressed, then ssDNAs will bind to their mRNA and inhibit the protein synthesis. The level of specific RNA and the concentration of specific molecule which regulates the point mutations were regarded as an input. The release of short ssDNA modulated the levels of gene expression for anticancer activity, as the output logically. The computation module has two states, “positive” and “negative”, in response to the level of specific gene expression. This biocomputation concept is related with the
identification of mRNAs of disease-related genes. Thereby, it can be applied to cancer diagnosis systems based on logic analysis.

A RNAi-based logic evaluator has also been reported to perform Boolean logic corresponding to input molecules in the human kidney cells [250]. The reported biological circuit is composed of several mRNA species that encode the same protein but have different non-coding regions. This protein served as the output. As an input, siRNAs were used to control the degradation of the target mRNA and the expression of the output protein. Expressions with up to five logic variables were directly evaluated by this system.

RNA as a polymer for information processor

In computer sciences, the information processor is a unit that receives the input information and processes it into another form based on programmed functions. RNA-based information processing devices that operate logic gates, signal filtering, and cooperativity functions have been reported [251, 260, 266]. RNA devices composed of ribozymes and RNA aptamers received, processed and transmitted the molecular inputs to express the green fluorescent protein as output. As a sensor part, the RNA aptamer was introduced and a hammerhead ribozyme was used to process the cleaving of the aptamer. Also, the transmitter part was composed of sequences that bind to the aptamer and ribozymes. It was suggested that the RNA aptamer and ribozyme combination can be used as actuating elements for multi-functional information processor development [251, 266].

An intracellular RNA computation device in a single mammalian cell has also been reported [260]. Trigger-controlled transcription factors were used to regulate the
gene expression individually and the RNA-binding protein was used to inhibit the translation of transcripts on target RNA motifs. This computation biosynthetic circuit can provide the NOT, AND, NAND, and N-IMPLY expression functions in individual cells. Moreover, two N-IMPLY expression functions can combine with other cells to operate XOR gate functions and three logic gates can perform the half-adder and half-subtractor functions for arithmetic calculation.

**RNA as a polymer for resistive biomolecular memory**

RNA can also provide several advantages such as ease of construction, well-defined structure and thermodynamic stability for the development of biomolecular memory devices. Based on the discovery of the ultra-high thermostable pRNA-3WJ, a pRNA-3WJ/QD hybrid nanoparticle for resistive biomolecular memory application has been constructed [267]. The QD was used as a semiconducting part for storage of the electrons and pRNA-3WJ was used as an insulating part and serves as the bridge between QD and metal substrate. The electrical measurement (I–V) was conducted to this hybrid structure for resistive memory performances. This study showed the RNA polymer conjugates can be used directly for molecular memory device fabrication.

**Hybrid RNA polymers as semiconductors**

Biomolecular hybrids of a conducting polymer [poly (O-methoxy aniline) (POMA)] and dsRNA have been investigated as semiconductors by Routh et al. in 2009 [268]. Fourier transform infrared (FTIR) spectroscopy indicated that three types of supramolecular interactions including electrostatic, H-bonding, and pi–pi interactions
were involved in these hybrid polymers. The circular dichroism spectra of the hybrid polymers indicated that the dsRNA underwent a small distortion in conformation from the canonical A-form RNA towards the B-form RNA during the formation of the hybrid polymers. Ultraviolet–visible spectroscopy revealed that the interaction of POMA on RNA occurred much faster than that on DNA. Morphological study by TEM further revealed that these POMA-RNA hybrids exhibited a fibrillar network structure in the nanometer scale. Conductivity studies revealed that the conductivity values of the POMA-RNA hybrids were three orders of magnitude higher than that of RNA alone. Moreover, when RNA concentrations increased in the hybrids, the conductivity values of the POMA-RNA hybrids also decreased. The I–V curves of the POMA-RNA hybrid polymers also clearly exhibited a hysteresis loop and demonstrated a semiconducting nature. Based on these interesting physical and electronic characteristics, the POMA-RNA hybrids have the potential to be candidates for fabricating biosensors for other applications.

**Conjugation of RNA to graphene**

Graphene is a one-atom thick, two-dimensional honeycomb lattice composed of sp2-bonded carbon atoms. It can be used as a fundamental building block for constructing other graphite-based structures such as fullerenes and nanotubes [269, 270]. This newly discovered carbon-based material has been a popular subject in material science and nanotechnology. It has been used for the sensing of a toxin Microcystin-LR in water [271-274]. RNA aptamer has been covalently immobilized on graphene oxide and a polydispersed stable RNA-graphene oxide nanosheet has been constructed [275]. The
RNA attached to these nanosheets was resistant to nuclease degradation and the nanosheets competitively absorbed trace amounts of the peptide toxin microcystin-LR from drinking water. PEI-grafted graphene oxide was also used to deliver siRNA as well as anticancer drug doxorubicin to cancer cells [276]. RNA has also been used as a surfactant to exfoliate flakes of graphene from nanocrystalline graphite to produce transparent and conducting RNA-graphene-based thin films [277], which has a potential for a variety of electronic applications. Further research in RNA-graphene nanocomposites may open a new avenue towards many applications of graphene-based conductive materials.

*Conjugation of RNA with other nanoparticles*

The conjugation of RNA with nanoparticles such as quantum dot, iron oxide nanoparticle or gold nanoparticle has been successfully demonstrated by a series of studies for nanosized imaging, therapy and diagnosis [278-283]. For example, siRNA and tumor-homing peptides (F3) were conjugated to the PEGylated QD core as a scaffold [279, 280]. The complex was efficiently delivered to HeLa cells, released from the endosomal compartment, and triggered knockdown of EGFP signal. This leads to dual purpose of treatment and imaging. In another case, siRNA was also conjugated to the iron oxide nanoparticle [281, 283] for dual purpose: (1) *in vivo* transfer of siRNA and (2) imaging the accumulation of siRNA in tumor through magnetic resonance imaging (MRI) and near-infrared *in vivo* optical imaging (NIRF) using near-infrared Cy5.5 dye. Another report showed that the conjugation of gold nanoparticle and RNA also increased the availability of the tethered RNA splicing enhancer [284].
In 2007, the pRNA of the phage phi29 DNA-packaging motor was conjugated to the gold nanoparticles via a thiol group for single particle quantification in bacterial virus assembly [282]. The pRNA-gold nanoparticle conjugates were bound to procapsid by in vitro phage assembly. The results demonstrated the feasibility in using RNA-gold nanoparticle for single molecule imaging and counting of biological machines.

**Prospects**

RNA is a polymer by nature. Recent technological advances to make RNA chemically and enzymatically stable [65, 66] and the discovery of unusual thermostability of some RNA motifs, as well as important biomedical applications [124, 125, 132, 133, 285] have propelled the concept of RNA as a polymeric building block [36] into a new horizon. The concern of yield and production costs has been and will be addressed continually by industry scale production and fermentation. It is expected that applications of RNA as a polymer and as building blocks will appear more and more in therapeutics, detection, sensing, nanoelectronic devices, and other polymer industries. The anion nature, the thermodynamic stability, the insulating property, the self-assembly capability and other novel features such as versatility, molecular-level plasticity, as well as the potential semiconductor behavior will make RNA unique for exploring new scientific territories. RNA has been shown to be major components of cells and leading functionality of life, and it is expected that RNA will also be the momentous material of daily life in the future.
**Acknowledgements**

This work was supported by grants EB019036, EB003730, EB012135 from NIH/NIBIB and CA151648 from NIH/NCI to P.G., Leading Foreign Research Institute Recruitment Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (MSIP) (2013K1A4A3055268) to T.L., National Distinguished Young Scholars grant (NSFC No: 31225009) and the “Strategic Priority Research Program” of the Chinese Academy of Sciences Grant no. XDA09030301 to X.L. The funding to P.G.’s Endowed Chair in Nanobiotechnology position is from the William Fairish Endowment Fund. P.G. is the cofounder of Biomotor and RNA Nanotechnology Development Corp. Ltd. We would like to thank Dr. Richard N. Greenberg, Dr. Daniel W. Pack, Dr. Yi Shu and Dr. Ashwani Sharma for their constructive comments.
Table 1.1. Summary of chemical modifications of RNA.

<table>
<thead>
<tr>
<th>Chemical Modifications</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate backbone</td>
<td>·Improve nuclease resistance. ·Combine with other modifications to dramatically improve RNA property</td>
<td>·Destabilize siRNA duplexes (e.g. decreases $T_m$ by 0.5 °C per PS linkage). ·Extensive modification causes cytotoxic effect.</td>
<td>[158-163]</td>
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<td>·Phosphorothioate (PS)</td>
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<tr>
<td>·Boranophosphate (BO)</td>
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<tr>
<td>·Phosphonoacetate (PACE)</td>
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<tr>
<td>·Phosphoramidate</td>
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<td>·Methylphosphonate</td>
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<tr>
<td>2′-OH group</td>
<td>·Significantly improve nuclease resistance. ·Greatly thermo-stabilizes dsRNA duplex (e.g. increase $T_m$ 0.5-0.7 °C per 2′-OMe and 1 °C per 2′-F). ·Particularly, 2′-OMe is nontoxic and prevents immune activation. ·Bulky 2′-modifications can modulate thermos-stability and duplex asymmetry, and also give higher binding affinity.</td>
<td>·Extensive or full modification will reduce or fully deactivate siRNA potency. ·Bulky 2′-modifications are only tolerated at limited position owing to their distortion of RNA helix structure.</td>
<td>[36, 66, 162-170, 174]</td>
</tr>
<tr>
<td>·Small 2′-substituents (e.g. 2′-O-methyl (2′-OMe), 2′-fluoro (2′-F), 2′-aminoethyl, 2′-deoxy-2′-fluorooarabinonucleic acid (2′-F-ANA))</td>
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<td></td>
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<tr>
<td>·Bulky 2′-modifications (e.g. 2′-O-MOE, 2′-O-allyl)</td>
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<tr>
<td>Locked nucleic acid (LNA)</td>
<td>·LNA enhances the complementary binding affinity and greatly improves thermostability by 2-10 °C per incorporation, as well as improves nuclease resistance and reduces RNA immunogenicity. ·Each UNA destabilizes duplex by 5.8 °C to improve local destabilization of siRNA duplex, and enhance biostability in vivo.</td>
<td>·LNA would probably cause liver toxicity. ·Extensive modification with LNA and UNA generally results in decreased activity of siRNA and failure in annealing of dsRNA, respectively.</td>
<td>[171-176]</td>
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<tr>
<td>Unlock nucleic acid (UNA)</td>
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<tr>
<td>Ribose moiety</td>
<td>·Enhance thermostability (e.g. 2′-F-ANA increases $T_m$ of RNA duplex by 0.5-0.8 °C per modification). ·Improve nuclease resistance.</td>
<td>·Modification at seed region would slightly reduce siRNA potency.</td>
<td>[179]</td>
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<tr>
<td>·Altritol nucleic acid (ANA)</td>
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<td>·Hexitol nucleic acid (HNA)</td>
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<td>·2′-deoxy-2′-fluorooarabinonucleic acid (2′-F-ANA)</td>
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<td>·Cyclohexenyl nucleic acid</td>
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<td>Ribonucleotide base (5-bromo-, 5-iodo-, 2-thio-, 4-thio-uracil, dihydro-, pseudo, 5-biotinylated, 5-aminooallyluracil, diamino-purine)</td>
<td>·Stabilize base-pairing and to enhance binding specificity. ·Particularly, 2-thio- and pseudo-uracil reduce cellular immune response.</td>
<td>·Some base modifications (e.g. 5-bromo- and 5-iodo-uracil) will affect siRNA potency.</td>
<td>[140, 178]</td>
</tr>
</tbody>
</table>
Table 1.2. Classification of RNA polymer-based bioelectronics.

<table>
<thead>
<tr>
<th>RNA-based bioelectronics</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Biocomputation devices</td>
<td></td>
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<tr>
<td>Biologic gate</td>
<td>[239, 244]</td>
</tr>
<tr>
<td>Biomemory</td>
<td>[239, 244, 267]</td>
</tr>
<tr>
<td>Bioinformation processor</td>
<td>[243, 244, 246, 248, 249, 251]</td>
</tr>
<tr>
<td>Biosensors</td>
<td></td>
</tr>
<tr>
<td>Biomedical sensor</td>
<td>[216, 218, 222, 223, 226-228]</td>
</tr>
<tr>
<td>Environmental sensor</td>
<td>[229, 230]</td>
</tr>
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</table>
A. HBV Ribozyme Assay
B. MG Amplimer Assay
C. Survivin Gene Knock-down
Figure 1.2. Construction and functional assays of thermodynamically stable pRNA 3WJ or pRNA-X nanoparticles. (A) HBV ribozyme cleavage assay showing the catalytic activity of the HBV ribozyme is retained after its incorporation into pRNA-3WJ nanoparticles. Cleave products are highlighted in the red box. Representative results from at least three independent experiments are shown. (B) MG aptamer fluorescence assay showing the function of the MG aptamer is retained after its incorporation into pRNA-3WJ nanoparticles [124]. Representative results from at least three independent experiments are shown. Adapted with permission from Ref. [124]. Copyright 2011 Nature Publishing Group. (C) Survivin gene knockdown assay showing the gene silencing activity of the surviving siRNA is retained after its incorporation into pRNA-X nanoparticles [73]. Representative results from at least three independent experiments are shown. Reprinted with permission from Ref. [73]. Copyright 2012 Elsevier.
Figure 1.3. Construction of RNA polygons based on pRNA-3WJ. (A) Construction of RNA triangle, square and pentagon by tuning the interior pRNA-3WJ angle [92]. Representative results from thirty-nine raw AFM images are shown. Reprinted with permission from Ref. [92]. Copyright 2014 Oxford University Press. (B) Construction of
RNA squares with tunable sizes [126]. Representative results from fifty-eight raw AFM images are shown. Reprinted with permission from Ref. [126]. Copyright 2014 American Chemical Society.
Figure 1.4. Functional RNA nanostructures for therapeutics delivery and medical detection. (A) Schematic illustration shows triggered siRNA delivery based on RNA/DNA hybrid nanostructures [141]. Control DNA duplexes fluorescently labeled with Alexa 488 and Alexa 546 were unable to recombine (upper part) and fluorescently labeled RNA/DNA hybrids were programmed for re-association and FRET occurred when the siRNA was released (lower part) [141]. Reprinted with permission from Ref. [141]. Copyright 2013 Nature Publishing Group. (B) QD-aptamer-doxorubicin conjugate nanoparticles as a cancer-targeted imaging, sensing and treatment platform [211]. Reprinted with permission from Ref. [211]. Copyright 2007 American Chemical Society. (C) PSMA aptamer-conjugated gold nanoparticles for targeted molecular CT imaging and therapy of prostate cancer [214]. Reprinted with permission from Ref. [214]. Copyright 2010 American Chemical Society. (D) Spherical nucleic acid scaffold for loading RNA therapeutics [192]. Spherical nucleic acids are densely packed and highly oriented nucleic acids covalently conjugated to the surface of a spherical nanoparticle core. Reprinted with permission from Ref. [192]. Copyright 2014 American Chemical Society.
Figure 1.5. pRNA nanoparticles for cancer targeting. (A) pRNA-3WJ nanoparticles target folate-receptor positive tumor xenografts [124]. Upper panel: whole body; lower panel: organ imaging (Lv, liver; K, kidney; H, heart; L, lung; S, spleen; I, intestine; M, muscle; T, tumor). Reprinted with permission from Ref. [124]. Copyright 2011 Nature Publishing Group. (B) pRNA-3WJ nanoparticles target glioma [165]. Folate conjugated-
pRNA nanoparticles with Alexa 647 labeling (FA-Alexa647-pRNA-3WJ) showed stronger binding to glioma compared with folate-free pRNA nanoparticles (Alexa647-pRNA-3WJ). Reprinted with permission from Ref. [165]. Copyright 2015 Impact Journals, LLC. (C) pRNA-X nanoparticles target folate-receptor positive tumor xenografts [73]. Reprinted with permission from Ref. [73]. Copyright 2012 Elsevier. (D) Folate conjugated-pRNA nanoparticles with Alexa 647 labeling (FA-pRNA-Alexa647) target colorectal cancer metastases [166]. pRNA nanoparticles with Alexa 647 labeling but without folate (pRNA-Alexa647) was used as a control. Reprinted with permission from Ref. [166]. Copyright 2015 American Chemical Society.
Figure 1.6. Spinach RNA aptamer for intracellular imaging and sensing. (A) Live-cell imaging of Spinach-tagged 5S RNA [215]. Fluorescence and phase images showed the comparison of HEK293T cells expressing 5S RNA tagged with either Spinach RNA (5S-Spinach) or negative control Lambda RNA (5S-Lambda). Fluorescence was detected in 5S-Spinach-expressing cells in the presence of 20 μM DFHBI (+DFHBI), and granule formation was detected in cells treated with 600 mM sucrose for 30 min (↑Suc). Nuclear borders assessed by Hoechst 33342 staining were highlighted by white dashed lines [215].
Representative results from at least three experimental replicates are shown. Reprinted with permission from Ref. [215]. Copyright 2011 The American Association for the Advancement of Science. (B) Imaging cellular metabolites in E. coli with sensor RNA [216]. Upper panel: the sensor RNA comprises Spinach (black), a transducer (orange), and a target-binding aptamer (blue). Target binding to the aptamer promotes stabilization of the transducer stem, enabling Spinach to fold and activate DFHBI fluorescence. Lower left panel: emission spectra of the SAM sensor in the presence or absence of SAM. Lower right panel: patterns of SAM accumulation after adding methionine to E. coli expressing the SAM sensor RNA were observed under a fluorescent microscope [216]. Scale bar, 5 µm. Representative results from at least three experimental replicates are shown. Reprinted with permission from Ref. [216]. Copyright 2012 The American Association for the Advancement of Science.
Figure 1.7. The basic constitution of RNA-based biosensor.
Figure 1.8. Advantages of RNA-based biocomputation.

- Capability of operating inside cells or tissue.
- Controllable and predictable self-assembly and hybridization.
- Well-defined structure, size, and stoichiometry.
- Long or short non-coding RNAs.
- Modularity by using aptamer, ribozyme, siRNA, miRNA, riboswitch, and other.
- Multi-functionality.
Chapter 2: Controllable Self-assembly of RNA Tetrahedrons with Precise Shape and Size for Cancer Targeting

This chapter was reproduced (with some modification) with permission from Hui Li†, Kaiming Zhang†, Fengmei Pi, Sijin Guo, Luda Shlyakhtenko, Wah Chiu, Dan Shu and Peixuan Guo. “Controllable Self-assembly of RNA Tetrahedrons with Precise Shape and Size for Cancer Targeting.” Advanced Materials. 2016. doi:10.1002/adma.201601976. †: Contributed equally. Special thanks to Dr. Kaiming Zhang for help in preparing Figure 2.1f, 2.2, 2.6e, 2.7 and Table 2.1, Fengmei Pi for help in preparing Figure 2.11b, Sijin Guo for help in preparing Figure 2.3a, 2.3b, 2.4 and 2.6c, Dr. Luda Shlyakhtenko for help in preparing Figure 2.1e and 2.6e. Author contribution: Dr. Kaiming Zhang performed the cryo-EM imaging and single particle reconstruction based on the cryo-EM raw data under the supervision of Dr. Wah Chiu. Fengmei Pi provided assistance for the luciferase gene silencing assay. Sijin Guo provided assistance for the dynamic light scattering experiments and nanoparticle stability studies. Dr. Luda Shlyakhtenko performed the AFM studies. Dr. Dan Shu provided assistance for the animal trial, data analysis and financial support. Hui Li conceived the idea of building RNA tetrahedron nanoparticles based on pRNA-3WJ, designed the 2D sequence and 3D computational model of RNA tetrahedron nanoparticles, performed all the other experiments, analyzed all the data and drafted the manuscript. Dr. Peixuan Guo led and supervised the whole study and provided financial support. All the authors revised and approved the manuscripts.

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Abstract

Since RNA shows an integer of 11 nucleotides per helix turn of $360^\circ$, in comparison to DNA that shows a non-integer of 10.5 nucleotides per helix turn, I propose that different sizes of RNA 3D structures can be constructed with precise control and the size of the particle will increase linearly by increasing the nucleotide number as multiples of eleven. I used the ultra-stable pRNA-3WJ motif with controllable angles and arm lengths to construct tetrahedral architectures composed purely of RNA via one-pot bottom-up assembly with high efficiency and thermal stability. By introducing arm sizes of 22 bp and 55 bp, respectively, I constructed two RNA tetrahedrons with similar global contour structure, but with different sizes of 8 nm and 17 nm, respectively. AFM and cryo-EM imaging clearly demonstrated the 3D shapes of RNA tetrahedrons that were consistent with the designs. The RNA tetrahedrons were also highly amenable to functionalization. Fluorogenic RNA aptamers, ribozyme, siRNA, and protein-binding RNA aptamers were integrated into the tetrahedrons by simply fusing the respective sequences with the tetrahedral core modules. Upon systemic injection, 2’-F modified RNase resistant RNA tetrahedrons harboring EGFR aptamers specifically targeted orthotopic breast tumors without getting trapped in healthy organs. The favorable biodistribution highlight their potentials as safe and specific drug delivery vectors. The reported design principles can be extended to construct higher order polyhedral RNA architectures for various applications.

Keywords: RNA nanotechnology; RNA nanoparticle; Phi29 packaging RNA; Three-way junction; Tetrahedron
Introduction

The Watson-Crick base pairing properties of DNA and RNA have led to their development as excellent building blocks for the construction of nanomaterials by bottom-up self-assembly [74, 133-135, 181, 286]. For constructing larger architectures by directional or angular extension, it is necessary to extend the building blocks with a defined angle or orientation. This has been challenging for DNA since one helix turn of 360° is 10.5 nucleotides for B-form DNA. A non-integer per helix turn will result in the twisting of the extension angle or the restriction in orientation control. In RNA, the number of nucleotides per helix turn is an integer of 11 for A-form RNA, which is a common structure for most RNA sequences (Appendix 1 Supplementary Figure 1). I propose that this unique property of RNA will enable RNA structural growth with precise one directional control, which will facilitate the construction of large size architectures for materials science, computer device, and biomedical applications.

RNA nanotechnology is an emerging field that involves the design, construction and functionalization of nanometer-scale particles composed mainly of RNA for applications in biomedical and material sciences [181]. Previous reports have shown that a variety of RNA nanostructures can be constructed with defined sizes, shapes and stoichiometry, including triangles [36], squares [93, 118, 126], bundles [113, 121], 2D arrays [36, 117], hexamers [54, 111, 116] and 3D cages [119, 122, 145] by bottom-up self-assembly based on intra- and inter-RNA interactions. We have extensively utilized the structural features of bacteriophage phi29 packaging RNA [123] to construct varieties of RNA nanoparticles via loop-loop interactions [111, 113, 114, 125], palindrome sequence mediated foot-to-foot interactions [113, 114], and three-way junction (3WJ)
motif[36,74,92,114,124]. More recently, we constructed 2D polygons such as triangle, square and pentagon[92] using the pRNA-3WJ as core scaffold at the vertices. Here I extended our previous 2D work to design and construct 3D RNA nanoparticles with controllable shape and size.

Tetrahedral geometry is attractive because of its intrinsic mechanical rigidity and structural stability. It is a pyramid-like structure with four triangular faces and six edges. Several methods have been used to construct DNA based tetrahedrons, such as an origami approach based on a long single DNA strand[287], complementary hybridization of strands using sticky ends[288,289], and hierarchical assembly of tiles[290]. RNA is an attractive alternative building block due to its high thermal stability[164,52] and versatility in structures[181,291], well beyond the simplistic canonical Watson-Crick base pairing in DNA nanostructures. Herein, the well-characterized ultrastable 3WJ motif[54,124] was used as the core scaffold to construct tetrahedral architectures. The RNA tetrahedrons have defined 3D structure as revealed by atomic force microscope (AFM), as well as by single particle cryo-electron microscopy (cryo-EM) that has long been found to be very challenging in imaging pure RNA structures without forming complex with proteins[292]. Moreover, for the functionalization of the RNA tetrahedrons, aptamers, ribozyme and siRNA were placed at the edges of the RNA tetrahedrons with high precision without disrupting the overall structure. Importantly, the functional modules were incorporated prior to the assembly of the RNA tetrahedrons to ensure the production of homogeneous nanoparticles with high yield. Biodistribution studies revealed that RNA tetrahedrons functionalized with EGFR targeting RNA aptamer specifically targeted orthotopic breast tumors without detectable accumulation in healthy
vital organs. The RNA tetrahedrons are envisioned to have a broad impact in nanotechnology arena, such as for organizing nanoscale materials with high precision, encapsulation of functional materials within its hollow cavity, targeted therapy to diseased cells and as image-guided delivery vectors.

Materials and Methods

RNA tetrahedron design, synthesis, and self-assembly

The crystal structure of the pRNA 3WJ motif (PDB ID: 4KZ2) was used for designing RNA tetrahedrons utilizing computational programs UCSF Chimera, Swiss PDB Viewer and the PyMOL Molecular Graphics System. RNA strands were synthesized by in vitro T7 transcription using corresponding DNA templates generated by polymerase chain reactions (PCR) following previously reported procedures [125]. DNA oligos for PCR and for the assembly of DNA tetrahedrons were ordered from Integrated DNA Technologies, Inc. (Coralville, IA). For a 50 µl transcription reaction, 10 µl of DNA template with 10 µl of 5× transcription buffer, 10 µl of 25 mM NTPs solution, 5 µl of 100 mM DTT, 10 µl of T7 RNA polymerase and 5 µl 0.05% (vol/vol) DEPC water were mixed together and incubated at 37 °C for 4 h. The transcription reaction was stopped by adding 1 µl of DNase I and incubating at 37 °C for another 30 min to digest the DNA template. For generating 2’-F modified RNA tetrahedrons, Y693F mutant T7 polymerase and 2’-F modified cytosine (C) and uracil (U) nucleotides were used to synthesize the corresponding RNA strands. For a 50 µl 2’F transcription reaction, 2.5 µg of DNA template diluted in 15 µl 0.05% (vol/vol) DEPC water, 5 µl of 10× 2’-F transcription buffer, 5 µl of 100 mM DTT, 5 µl of 50 mM 2’-F CTP, 5 µl of 50 mM 2’-F
UTP, 5 µl of 50 mM ATP, 5 µl of 50 mM GTP, and 5 µl of Y639F mutant T7 RNA polymerase were mixed together and incubated at 37 °C overnight. All synthesized RNA strands were purified by 8 M urea, 8% denaturing PAGE. RNA tetrahedrons were assembled in a one-pot manner by mixing the four RNA strands in equimolar concentrations (100 nM) in 1× Tris buffer (100 mM NaCl, 50 mM Tris, pH 8.0), and heated to 95 °C for 5 minutes and slowly cooled to 4 °C over 45 minutes using a PCR machine. The step-wise assembly of the RNA tetrahedrons were verified by native PAGE gels run in 1× TBM (89 mM Tris, 200 mM boric acid, and 2.5 mM MgCl₂) buffer at 90 V, 4 °C. The gels were stained with ethidium bromide (EB) and imaged by Typhoon FLA 7000 (GE Healthcare, Marlborough, MA). For AFM imaging and cryo-EM imaging, the assembled RNA tetrahedrons were further purified by native PAGE.

The detailed sequences for assembling the designed RNA tetrahedrons are listed below:

a. RNA sequences of the 8 nm RNA tetrahedron.

<table>
<thead>
<tr>
<th>Strand</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strand 1</td>
<td>5’- GGACUGAUACGAAUCAUCGUGUAGCACCAGCUGUAAUCG AUGUGUACGGAAGAGCCUAUGCCCAUCCAUACUUUGUU CUACUAUGGCG -3’</td>
</tr>
<tr>
<td>Strand 2</td>
<td>5’- GGUGCUACACGAUGUGUAAGCCGACCACGUUAGCGGAAUGUU CGUACUUUGUUCAUGCGAGGGCCUCAGAUAUACCGGAAUCAU CGAUUACAGCU -3’</td>
</tr>
<tr>
<td>Strand 3</td>
<td>5’- GGGCAGUUGAGAUGUGUACGAACAUUCCGCUAAGUCUG GCUACUUUGUUCGUACAGUCCCGCCAUAGUAGAAUCAU</td>
</tr>
<tr>
<td>Strand</td>
<td>Sequence</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Strand 4</td>
<td>CGUAUCACCAU -3'</td>
</tr>
<tr>
<td></td>
<td>5’- GGCCUCGCAUGAAUAUCUAACUGCCCAUGGUGAUACG</td>
</tr>
<tr>
<td></td>
<td>AUGUGUAGGAUGGGCAUAGGCUCUCCCGUACUUUGUU</td>
</tr>
<tr>
<td></td>
<td>CGGUAAUUGGAC -3’</td>
</tr>
</tbody>
</table>

b. RNA sequences of the 17 nm RNA tetrahedron.

<table>
<thead>
<tr>
<th>Strand 1</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5’- GGAUGACAGGGUGUCAAUGCACUGAUACGAAUCAUCGUGUAGCCCA</td>
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<td></td>
<td>UAGCAGAUUCCGAUUGUACUGACCAGCUCCAGGUUCUACAGACGA</td>
</tr>
<tr>
<td></td>
<td>GCUGUAAUCGGAUGGUACGGGAAGAGAGACCUACUUGCGUGG</td>
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<tr>
<td></td>
<td>CUCCCCUCUACUGCAUGUGUAUGCCCAUCCUACUUUGUU</td>
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<tr>
<td></td>
<td>CUACUAAUGGCGGAAGUAUGCAUGCAUGCAUG -3’</td>
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<table>
<thead>
<tr>
<th>Strand 2</th>
<th>Sequence</th>
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<tr>
<td></td>
<td>5’- GGACGAGUACAAUCGAAUCUGCUACACGAGAUGUGUAGCCCA</td>
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<td></td>
<td>GACUUAGUGUCUGACCGACCGACACUCGAGUCGAGCGAC</td>
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<tr>
<td></td>
<td>GGAAUGUUCGUACUUUGUUCUAGCGAGGGCGAUAGCGCCU</td>
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<tr>
<td></td>
<td>AGCUGCCUCUGACUGGACCUAGUGUCCAAUACCGAAUCAUC</td>
</tr>
<tr>
<td></td>
<td>GAUUACAGCUCUGUCUGUAGAACCUGA -3’</td>
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<table>
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<tr>
<th>Strand 3</th>
<th>Sequence</th>
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<tr>
<td></td>
<td>5’- GGAGAGCGUAGGUCCGUACGCAAGUAGGAUGUGUAGCGA</td>
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<tr>
<td></td>
<td>ACAUUCGUCGUCGACUGACGAGUCGUCGUCGACGACA</td>
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<tr>
<td></td>
<td>GUAUCAACCAUGAAACAUCAUCACGAACAG -3’</td>
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<table>
<thead>
<tr>
<th>Strand 4</th>
<th>Sequence</th>
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</thead>
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<tr>
<td></td>
<td>5’- GGCAGCUAGGCGCUAUCGCCCUCGCAUGGAUAUCUCUCAA</td>
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</table>
c. RNA sequences of the different functional modules.

<table>
<thead>
<tr>
<th>Module</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG Aptamer</td>
<td>5’- GGAUCCCGACUGGCGAGGCCAGGAACGAAUGGAGUC -3’</td>
</tr>
<tr>
<td>Spinach Aptamer</td>
<td>5’- GGACGCAACUGAAUGAAAAUGGUGAAGGACGGGUCCAGGCUUUGGUAGUGUAGA -3’</td>
</tr>
<tr>
<td>HBV Ribozyme</td>
<td>5’- GGGACGAAAAAAAAACAAAUUCUUUACUGAUGAGUCCGUGAGCC -3’</td>
</tr>
<tr>
<td>Streptavidin Aptamer</td>
<td>5’- GGAUGCGGCGCGCCGACCAGAUAUCAUGCAAGUGCAAGUCCGGA -3’</td>
</tr>
<tr>
<td>EGFR Aptamer</td>
<td>5’- GCCUUAGUAACGUGCUUUGAUGUACGUAUCGACAGGA -3’</td>
</tr>
<tr>
<td>Luciferase siRNA Sense Strand</td>
<td>5’- AACUUACGUGAUCUUCGAUU -3’</td>
</tr>
<tr>
<td>Luciferase siRNA Antisense Strand</td>
<td>5’- UCGAAGUACUCAGCGUAAGU -3’</td>
</tr>
</tbody>
</table>
**Atomic force microscopy**

Both 8 nm and 17 nm RNA tetrahedrons were imaged by AFM, following previously reported procedures [293]. Briefly, RNA tetrahedrons (5-10 µL at 3-5 nM) were deposited on the aminopropyl silatrane (APS)-modified mica surface and excess samples were washed with diethylpyrocarbonate (DEPC)-treated water and dried before imaging. AFM imaging was conducted using the MultiMode AFM NanoScope IV system (Veeco, Plainview, NY) operated in tapping mode.

**Dynamic light scattering measurements**

The apparent hydrodynamic sizes and zeta potential of the assembled RNA tetrahedrons were measured by Zetasizer nano-ZS (Malvern Instruments, Ltd, Malvern, United Kingdom) at 25 °C, respectively. For size measurements, 500 µl 100 nM RNA tetrahedron in 1× Tris buffer was used. For zeta potential measurements, 800 µl 100 nM RNA tetrahedron in 1× Tris buffer was used. The laser wavelength used for the experiments was 633 nm. The data were obtained from at least three independent measurements. The intensity distribution was used to calculate the sizes of the nanoparticles.

**Melting temperature measurements**

The melting temperature of RNA, 2’-F RNA and DNA tetrahedrons were measured by the Roche Lightcycler 480 real-time PCR machine, following previously reported procedures [124, 125]. Briefly, all tetrahedrons were assembled in 20 µl 1× Tris buffer in the presence of 1× SYBR Green II dye (Invitrogen, Carlsbad, CA) with final
nanoparticle concentration of 250 nM. The samples were heated to 95 °C for 5 min and then slowly cooled down to room temperature at a rate of 0.11 °C/s using the Roche Lightcycler 480 real-time PCR machine. The melting temperature was obtained from at least three independent measurements.

**Cryo-electron microscopy (cryo-EM)**

RNA tetrahedron nanoparticles solution (2 µl at 0.3 mg/ml) was applied onto a glow-discharged 200-mesh R1.2/1.3 Quantifoil grid. The grids were blotted for 3 s and rapidly frozen in liquid ethane using a Vitrobot Mark IV (FEI, Hillsboro, OR). Then the grids were transferred to JEM2200FS cryo-electron microscope (JEOL, Tokyo, Japan) operated at 200 kV with in-column energy filter with a slit of 20 eV for screening. Micrographs of 17 nm tetrahedron were recorded with a direct detection device (DDD) (DE-12 3k × 4k camera, Direct Electron, LP, San Diego, CA) operating in movie mode at a recording rate of 25 raw frames per second at 20,000× microscope magnification (corresponding to a calibrated sampling of 2.5 Å per pixel) and a dose rate of ~25 electrons/sec/Å² with a total exposure time of 3 s. Micrographs of 8 nm tetrahedron were recorded on a 4k × 4k CCD camera (Gatan, Pleasanton, CA) at 60,000× microscope magnification (corresponding to a calibrated sampling of 1.8 Å per pixel) and a dose rate of ~30 electrons/sec/Å² with a total exposure time of 1.5 s. A total 131 images of 17 nm tetrahedron and 52 images of 8 nm tetrahedron were collected with a defocus range of 2−4 µm.
Single particle image processing and 3D reconstruction

The image processing software package EMAN2.1, was used for the micrograph evaluation, particle picking, contrast transfer function correction, 2-D reference-free class averaging, initial model building and 3-D refinement of the cryo-EM data. Particle images recorded on DE-20 detector used the averaged sum of 72 raw frames per specimen area. The final frame average was computed from averages of every three consecutive frames to correct for the specimen movement (Courtesy of B. Bammes). For the 17 nm tetrahedron, a total of 1662 particles were boxed to generate the 2D class averages for building the initial models. At last, 1582 particles were used for final refinement, applying the tetrahedral symmetry. For the 8 nm tetrahedron, a total of 1346 particles were boxed to generate the 2D class averages for building the initial models. In total, 1254 particles were used for final refinement, applying the tetrahedral symmetry. The resolution for the final maps were estimated by the 0.143 criterion of FSC curve without any mask. 23 Å and 19 Å Gauss low-pass filter were applied to the final 3D maps displayed in the Chimera software package.

Tilt-pair validation for cryo-EM map

Tilt-pair validation data were collected at two goniometer angles, 0 ° and –10 °, for each region of the grid. A particle set of 50 particles from two tilt pairs of 17 nm RNA tetrahedron was used for the validation test. The test was performed using e2tiltvalidate.py program in EMAN2. More details about the tilt-pair validation are listed in Table 2.1.
**HBV ribozyme activity assay**

HBV ribozyme activity assay was performed according to previously reported procedures [124, 188]. Briefly, HBV RNA substrate was labeled by Cy5 using Cy5 Label IT® Kit (Mirus Bio LLC, Madison, WI) and incubated with 50 µL 200 nM RNA tetrahedrons harboring HBV ribozyme at 37 °C for 1 hour at 4:1 molar ratio. pRNA conjugated with HBV ribozyme was used as the positive control, while RNA tetrahedron only and pRNA conjugated with disabled HBV ribozyme (G→A mutation) were used as negative controls. The cleavage products of the HBV RNA substrate were further analyzed by 8% denaturing PAGE and imaged by Typhoon FLA 7000 (GE Healthcare, Marlborough, MA) under the Cy5 channel.

**Malachite green (MG) and Spinach aptamer fluorescence assays**

Measurements of MG and Spinach aptamer fluorescence emissions in solution were performed according to previously reported methods [36, 124]. Briefly, 100 µl at 100 nM multifunctional RNA tetrahedrons harboring MG and Spinach aptamers were incubated with 2 µM MG dye and 2 µM DFHBI (Lucerna, Inc., New York, NY) at room temperature. pRNA-3WJ harboring MG and Spinach aptamers were used as the positive control. RNA tetrahedron only and dye only were used as negative controls. Fluorescence emission spectrum was recorded using a fluorospectrophotometer (Horiba Jobin Yvon, Edison, NJ), excited at 450 nm (465-750 nm scanning for emission) for Spinach aptamer and 615 nm (625-750 nm scanning for emission) for MG aptamer.
Streptavidin (STV) aptamer binding assay

Multifunctional RNA tetrahedrons harboring STV aptamer (50 µL at 400 nM) were incubated with STV agarose resin (Thermo Scientific, Waltham, MA) in binding buffer (1× PBS with 10 mM Mg²⁺) at room temperature for 1 hr. Then the resin was spun at 500× g for 1 min and the supernatant was collected. Binding buffer (50 µL) was added to the resin to wash the resin for three times. The wash fractions were also collected. After washing, biotin (50 µL at 5 mM in 1× PBS with 10 mM Mg²⁺) was added to the resin three times to elute the RNA. All the collected supernatant, washing, and elution fractions were measured by Nanodrop (Thermo Scientific, Waltham, MA) for absorbance at 260 nm and further analyzed by PAGE. RNA tetrahedrons without STV aptamer were used as negative control.

Transfection assay for tetrahedron harboring siRNAs

5×10⁴ MCF-7 cells (human breast adenocarcinoma cell line) were grown on 24 well plate (TPP tech, Switzerland) with Eagle's Minimum Essential Medium (EMEM) (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and 0.01 mg/ml of bovine insulin (Cell Applications, San Diego, CA) at 37 °C in humidified air containing 5% CO₂ overnight. The cells were co-transfected with 1 nM RNA tetrahedrons and both plasmid DNA pGL3 encoding firefly luciferase and pRT-LK (Promega, Madison, WI) encoding renilla luciferase by lipofectamine2000 (Life technologies, Carlsbad, CA) following manufacturer’s instructions. Luciferase siRNA (4 nM) was used as the positive control, while 5 nM scramble siRNA was used as the negative control. Dual luciferase assay (Promega, Madison, WI) was performed to
evaluate the siRNA silencing effects 48 h post transfection. Briefly, cells were washed with PBS once, then lysed with 100 µl of passive lysis buffer per well. Luciferase assay reagent (LARII) (50 µl) was added to 20 µl of cell lysate in a 96 well plate, and the firefly luciferase activity was measured. Then 50 µl of Stop and Glow buffer was added to measure the control renilla luciferase activity on a microplate reader (Synergy 4, Bio Tek Instruments, Inc, Winooski, VT). The firefly luciferase activity was then normalized with renilla luciferase activity to determine whether luciferase siRNA are still functional after incorporation into RNA tetrahedrons. At least four independent wells were tested for each sample.

**In vitro binding of RNA tetrahedrons harboring EGFR aptamers**

$5 \times 10^4$ EGFR-positive MDA-MB-231 cells (human breast adenocarcinoma cell line) were grown on glass cover slides in 24-well plates in a 1:1 mixture of Dulbecco's modified Eagle's medium (DME) and Ham's F-12 nutrient mixture (DME/F-12 (1:1 medium) with 10% FBS at 37 °C in humidified air containing 5% CO$_2$ overnight. RNA tetrahedrons harboring EGFR aptamers and labeled by Alexa 647 were diluted in 300 µl Opti-MEM medium to final concentration at 100 nM and incubated with the cells for 1 h at 37 °C. RNA tetrahedrons without EGFR aptamer at 100 nM in 300 µl Opti-MEM medium were used as the negative control. After the incubation, the cells were washed with PBS and then fixed by 4% paraformaldehyde. Cellular actin were stained by Alexa 488 Phalloidin (Life Technologies, Carlsbad, CA) and the Prolong Gold antifade reagent with DAPI (Life Technologies, Carlsbad, CA) were used to stain the nuclei and mount
the cells. The images were obtained by the FluoView FV1000-Filter Confocal Microscope System (Olympus, Tokyo, Japan).

**In vivo targeting of RNA tetrahedrons to tumor-bearing mice**

All protocols and procedures involved in the animal experiments were approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC). Female athymic nu/nu mice (4-8 weeks old) were purchased from Taconic Biosciences, Inc. To generate the orthotopic triple-negative breast cancer (TNBC) xenograft tumors, $2 \times 10^6$ MDA-MB-231 cells were re-suspended in PBS and injected into the mammary fat pad of the mice. To generate the KB cells (human cervix carcinoma cell line) xenograft, $2 \times 10^6$ KB cells were re-suspended in PBS and injected subcutaneously to nude mice. The xenograft tumors were apparent within 2 weeks after the implantation. RNA tetrahedrons harboring EGFR aptamers and Alexa 647 in $1 \times$ PBS (100 µL at 2.4 µM) were then injected into the tumor bearing mice. PBS injected mice were used as negative control. For the animal trial on the mice bearing KB cells xenograft, RNA tetrahedrons without EGFR aptamer were also included as control. The mice were sacrificed and dissected 8 hours after injection. The major organs including heart, lung, liver, spleen, kidney as well as tumors were collected and subjected to biodistribution analysis via fluorescence imaging using IVIS Spectrum station (Caliper Life Sciences, Waltham, MA) with excitation at 640 nm and emission at 680 nm.
Statistical analysis

The results were presented as mean ± standard deviation. Statistical differences were evaluated using unpaired Student’s t-test, and p<0.05 was considered significant.

Results and Discussion

Design and self-assembly of RNA tetrahedrons

In this study, the pRNA-3WJ motif [54, 124] (Figure 2.1a) was used as a core module and placed at each of the four vertices to build RNA tetrahedrons (Figure 2.1b). A total of four RNA strands were designed consisting of four pRNA 3WJ core sequences and six linking RNA sequences. A 3D model of the RNA tetrahedron (Figure 2.1c) was then generated using computational modeling software UCSF Chimera [294], Swiss PDB Viewer (http://www.expasy.org/spdbv/) and PyMOL Molecular Graphics System (https://www.pymol.org/). The resulting computational model exhibited authentic tetrahedral conformation. For assembling the RNA tetrahedrons, the four RNA strands were synthesized by in vitro transcription and then mixed in stoichiometric ratio and annealed in 1×Tris buffer in a one-pot manner. Step-wise assembly of the complex was observed by native polyacrylamide gel electrophoresis (PAGE) (Figure 2.1d).

Physicochemical characterization of RNA tetrahedrons

For 3D RNA tetrahedrons, the most convincing structural characterization in their native state comes from single particle cryo-EM studies. We first analyzed the 3D structure of the 8 nm RNA tetrahedrons with 2-helix turn per edge (Figure 2.1f). Single particle 3D reconstruction was applied by analyzing a total of 1254 particles collected
from cryo-EM images and achieved a final resolution of 19 Å (gold standard criterion, 0.143 Fourier shell correlation, Figure 2.2). The 3D reconstruction data revealed that the RNA tetrahedron has a clear overall shape consistent with the computational 3D model (Figure 2.1c), which agrees with the predicted size of 8 nm. The 2D computed projections of the reconstructed RNA tetrahedron 3D model showed clear match to the 2D class averages of the raw particles, suggesting the reconstructed 3D model truly represented the native structure and conformation of the designed RNA tetrahedron. The images from cryo-EM accord with the global images obtained by AFM imaging (Figure 2.1e). However, the central cavity of each tetrahedron was too small to be resolved by AFM. The observed size discrepancy is due to limitations in the diameter of the AFM tip. To further characterize the size of the RNA tetrahedrons, dynamic light scattering (DLS) was performed. DLS assumes that the particles have an average globular geometry in solution. The apparent hydrodynamic size of the RNA tetrahedron was determined to be 8.5 ± 2.4 nm (Figure 2.3a).

The surface charge of RNA tetrahedrons, measured as zeta potential, was also evaluated by DLS. As expected, due to the phosphate backbone of nucleotides, RNA tetrahedrons have a negative surface charge with a single peak at −14.9 ± 1.0 mV (Figure 2.3b). This negative surface charge is advantageous for the overall colloid stability of RNA nanoparticles and prevents forming aggregation in solution. Moreover, negative surface charge could also reduce the non-specific interaction of the nanoparticles with the reticuloendothelial system (RES) and minimize non-specific cell entry, which is attractive for in vivo targeted drug delivery and theranostic applications [295].
To assess the thermodynamic stability of RNA tetrahedrons, I investigated their melting temperatures ($T_m$) by measuring their fluorescence intensities in the presence of SYBR Green II dye with the change of temperature on a real-time PCR machine. Melting experiments revealed that the assembled RNA tetrahedron had a very smooth, high-slope temperature dependent melting curve with a $T_m$ of 71.3 ± 1.8 °C (Figure 2.3c). The high slope indicates cooperative assembly of the tetrahedron from its four component strands. Moreover, I compared the melting curve of RNA, 2'-F RNA and DNA tetrahedrons. The results showed that 2'-F RNA tetrahedrons was the most stable with a $T_m$ of 77.7 ± 2.4 °C, followed by RNA tetrahedron with a $T_m$ of 71.3 ± 1.8 °C, and finally DNA tetrahedron with a much lower $T_m$ of 58.3 ± 0.5 °C (Figure 2.3d). These results were in agreement with previously reported thermodynamic stability of nucleic acids with the order of stability: 2’-F RNA > RNA > DNA [52, 164].

To investigate their enzymatic stability, unmodified and 2’-F modified (U and C nucleotides) RNA tetrahedrons were incubated in cell culture medium with 10% fetal bovine serum (FBS) (Figure 2.4). At specific time points, aliquots were extracted and evaluated by native PAGE. The unmodified RNA tetrahedrons were degraded within 15 minutes, while 2’-F counterparts were stable over an extended period of time, well beyond 24 hrs. The resistance to serum-mediated degradation combined with the high thermodynamic stability is particularly attractive for the in vivo application of these RNA nanoparticles.

To demonstrate the precise tunable sizes of the RNA tetrahedrons, I designed a larger 17 nm RNA tetrahedron in which every edge of the tetrahedron was extended to 55 bp equal to 5 helix turns (Figure 2.5). This larger nanoparticle has the same overall
tetrahedral geometry in our design (Figure 2.6a). Upon annealing the four component strands in one pot self-assembly, the larger RNA tetrahedron assembled with high efficiency, as revealed by native PAGE analysis (Figure 2.6b). DLS experiments revealed that the hydrodynamic diameter of the larger RNA tetrahedron was 16.9 ± 1.6 nm, which is in agreement with the designed dimensions (Figure 2.6c). Moreover, AFM imaging was clearly able to resolve the tetrahedral morphology along with the inner cavities (Figure 2.6d). Since the RNA tetrahedrons were dried on the APS-modified mica surface before imaging in air, flattened tetrahedral shapes were observed. The RNA nanoparticles were also highly homogenous in shape and structure, demonstrating the robustness of the self-assembly of RNA tetrahedrons. Cryo-EM image further showed the very clear RNA tetrahedron nanoparticles (indicated by red circles) with the expected sizes and geometries (Figure 2.6e). Single particle 3D reconstruction of a total of 1582 particles collected from 131 cryo-EM images achieved a resolution of 23 Å (gold standard criterion, 0.143 Fourier shell correlation, Figure 2.7).

**In vitro and in vivo functional assessments of multifunctional RNA tetrahedrons**

To evaluate the application of tetrahedrons, four different functional modules including a hepatitis B virus (HBV) ribozyme, fluorogenic aptamers for Malachite Green (MG) or Spinach, and a streptavidin-binding aptamer (Figure 2.8a) were incorporated into the tetrahedron structure. The sequences of the functional modules were simply fused with the sequences of the 3WJ core, and then synthesized by *in vitro* transcription. After annealing the strands, the step-wise self-assembly was evaluated by native PAGE analysis to confirm the successful assembly of the RNA tetrahedrons (Figure 2.8b).
Functional assays were then conducted to determine whether the modules retained their authentic folding and functionalities upon incorporation into the RNA tetrahedrons.

Retention of the HBV ribozyme catalytic activity: The HBV ribozyme is a hammerhead ribozyme that can target and cleavage the 135-nt HBV genomic RNA substrate [188]. The HBV ribozyme was fused to the RNA tetrahedron by extending one of the strands of the nanoparticle. After incubation with the RNA tetrahedron harboring the HBV ribozyme, the HBV substrate was cleaved into fragments with smaller molecular weights, as revealed by PAGE analysis (Figure 2.8c). The yield of the cleavage reaction was comparable with the positive control (pRNA harboring HBV ribozyme). In contrast, RNA tetrahedron by itself or harboring disabled HBV ribozyme (G→A mutation in catalytic site) had no catalytic effects. The result confirmed that the designed multifunctional RNA tetrahedron successfully escorted the HBV ribozyme, and the catalytic activity was retained after ribozyme sequence was fused to the tetrahedron.

Assessment of the fluorogenic properties of MG and Spinach aptamers: Both MG [296, 297] and Spinach [215, 216] aptamers are well-characterized for their fluorogenic properties, which emit fluorescence upon binding of their respective dye targets, triphenylmethane and DFHBI. To verify that the MG and spinach aptamers incorporated into the multifunctional RNA tetrahedron are still functional and folded correctly, fluorescence studies were performed using a fluorospectrophotometer. The fluorescence emission spectrums showed that both aptamers retained their ability to bind their respective dyes and emitted strong fluorescence at a similar level to the positive control, indicating the retention of the correct folding and functionalities of these fluorogenic
aptamers (Figure 2.8d-e). The fluorogenic modules fused with tetrahedron nanoparticles can have potential applications for imaging these nanoparticles in cells.

Assay for streptavidin (STV) binding: Upon incubation with STV agarose resins, the multifunctional RNA tetrahedrons harboring STV aptamer [298] successfully bound to the resin with high affinity and were eluted by biotin (Figure 2.8f). In contrast, the negative control tetrahedron did not bind to the resin and also did not show up in the elution fractions. The results indicated that the fusion with RNA tetrahedron did not interfere with the native structure and function of the STV aptamer. Moreover, in PAGE analysis of the eluted multifunctional nanoparticles harboring MG and Spinach aptamer, they still retained their fluorogenic properties, indicating that the RNA tetrahedron structure promoted the correct folding of the two aptamers after fusion into the RNA nanoparticle (Figure 2.9). The results demonstrated that the fused STV aptamer could be potentially employed as a handle to specifically purify the assembled multifunctional RNA tetrahedrons by using STV agarose resins.

Assay for luciferase gene silencing: Dual-luciferase reporter assay was utilized to study the gene silencing effects of the RNA tetrahedron harboring four siRNAs targeting the same region of the Firefly luciferase gene (Figure 2.10). The Renilla luciferase, which was not the target of the luciferase siRNA, served as an internal control. The relative ratio of the expression level of the firefly luciferase to the Renilla luciferase was used to evaluate the targeted gene silencing effect in MCF-7 cells upon transfection. The results revealed that the tetrahedron harboring four luciferase siRNAs with 1 nM concentration achieved ~90% gene silencing, which is similar to the positive control of luciferase siRNA only at 4 nM concentration. In contrast, the tetrahedron only and
scrambled control did not show any noticeable gene silencing effects (Figure 2.11b). These results demonstrated that luciferase siRNA still retained their gene silencing ability after incorporation into the RNA tetrahedrons, suggesting that RNA tetrahedrons could serve as a vehicle for intracellular siRNA delivery.

Cellular binding and entry of RNA tetrahedrons harboring EGFR aptamers: For effective cancer therapy, it is critical to guide therapeutics to specific cancer cells. The Epidermal Growth Factor Receptor (EGFR) family of Receptor Tyrosine Kinases (RTK) are highly prevalent in both primary tumors and metastatic breast cancer cells, making them an ideal candidate for targeted therapies [299]. RNA aptamers have been developed to bind to EGFR receptors [300-303] with high selectivity and sensitivity. Here I incorporated EGFR targeting RNA aptamers into RNA tetrahedrons (Figure 2.12) and evaluated their cellular binding by confocal microscopy (Figure 2.11a). Alexa 647 (red) labeled RNA strand was incorporated into tetrahedrons for fluorescence imaging. Tetrahedrons without EGFR aptamer were used as negative control. Confocal imaging showed that tetrahedron nanoparticles with EGFR aptamers strongly bound to EGFR (+) MDA-MB-231 cells (Figure 2.11a), as revealed by the co-localization of the Alexa 647-labeled RNA (red) and cellular actin (green) (Figure 2.11a). In contrast, negative control “naked” tetrahedrons without the EFGR aptamer showed negligible cellular binding. The results suggest that the EFGR aptamer could facilitate the binding of RNA tetrahedrons to EGFR-expressing cancer cells.

*In vivo* targeting in xenograft cancer mouse models: For studying the *in vivo* cancer-targeting properties of the RNA tetrahedrons, we generated orthotopic breast cancer mouse models by injecting MDA-MB-231 cells directly into the mammary fat pad
of athymic nude mice [174] to generate xenografts. Nuclease-resistant 2’-F RNA tetrahedrons harboring EGFR aptamers and Alexa 647 were systemically injected into mice and biodistribution monitored by whole body imaging. The mice were sacrificed 8 hours post injection and their organs were collected for ex vivo imaging (Figure 2.11c). RNA tetrahedrons were not detected in any other organs except the breast tumor, indicating that the nanoparticles were cleared from normal organs quickly and did not accumulate in the liver, lung, spleen or kidney after systemic injection. In another confirmatory animal trial by using EGFR-expressing KB cells xenograft mouse model, the similar cancer-targeting ability of the RNA tetrahedrons was also observed (Figure 2.13). Previous biodistribution experiment of pRNA 3WJ RNA nanoparticles harboring EGFR aptamer also showed similar results [174]. The cancer-targeting ability of the RNA tetrahedrons would make this delivery system an attractive candidate for future targeted cancer imaging studies and/or cancer therapy.

**Conclusion**

In conclusion, here I report the design and self-assembly of multifunctional 3D RNA tetrahedrons based on the ultrastable pRNA-3WJ motif. The constructed RNA tetrahedrons have defined 3D structures as revealed by both AFM and single particle cryo-EM. The size of the RNA tetrahedrons can be easily tuned by changing the number of RNA base pairs per edge. Melting experiments revealed its high thermodynamic stability. Aptamers, ribozyme and siRNA were successfully incorporated into the RNA tetrahedron with their correct folding and optimal functionality. Cellular binding and biodistribution study showed that the RNA tetrahedron functionalized with EGFR-
aptamer targeted orthotopic breast tumors without detectable accumulation in other healthy organs. These results suggest that 3D RNA tetrahedron nanoparticles have the potential to escort imaging modules and therapeutics for \textit{in vivo} cancer diagnosis and therapy. Moreover, since the current study only used \textit{in vivo} fluorescent imaging to study the biodistribution, it should be note that it is difficult to accurately quantify the exact percentage of nanoparticles distributed into the tumor. In the future, radiolabeled RNA nanoparticles should be used to accurately quantify the biodistribution profile and also get a clear picture regarding how the RNA nanoparticles was metabolized and cleared from the body.

\textbf{Acknowledgements}

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Figure 2.1. Design and assembly of 8 nm RNA tetrahedrons. (a) 2D sequence of pRNA monomer showing the central pRNA-3WJ motif. The 22-nucleotide core sequence (with red color) of pRNA-3WJ are used to construct RNA tetrahedrons. (b) 2D sequences and (c) 3D computational model of RNA tetrahedrons. (d) 7% native PAGE gel showing step-wise assembly of RNA tetrahedrons. '+' indicates the presence of the strands. M: ultra low range DNA Ladder. Representative results from at least three independent experiments are shown. (e) AFM images of 8 nm RNA tetrahedrons. Representative
results from twenty-one raw AFM images are shown. (f) Single particle cryo-EM 3D reconstruction of 8 nm RNA tetrahedrons. A total of 1346 particles were used to generate the 2D class averages for building the initial models. A total of 1254 particles were used for the final refinement of the model by applying the tetrahedral symmetry.
Figure 2.2. (a) Cryo-EM raw images of the 8 nm RNA tetrahedrons. A representative image from 52 raw images of 8 nm RNA tetrahedron was shown. (b) Reference free 2D class averages of the 8 nm RNA tetrahedrons. (c) 0.143 Fourier shell correlation. A total of 1346 particles were used to generate the 2D class averages for building the initial models. A total of 1254 particles were used for the final refinement of the model by applying the tetrahedral symmetry.
**Figure 2.3.** Physicochemical characterization of RNA tetrahedrons. Dynamic Light Scattering (DLS) assay showing (a) the hydrodynamic size and (b) the zeta potential of RNA tetrahedrons. Representative results from three independent measurements are shown. (c) Melting curve of RNA tetrahedron complex and each of the four component strands. Representative results from three independent measurements are shown. (d) Comparison of melting curves for RNA, 2'-F and DNA tetrahedrons. Representative results from three independent measurements are shown.
**Figure 2.4.** Serum stability assay of 2’-F RNA tetrahedron and unmodified RNA tetrahedron. (a) 2’-F RNA tetrahedrons and (b) unmodified RNA tetrahedrons were incubated in 10% FBS for different time points. (c) Quantification of intact particles in panels A and B using Image J. Data was from one experiment. Similar serum stability assays have been performed for other 2’F-modified RNA nanoparticles for numerous times, and similar results were observed and published previously [124, 125, 174].
**Figure 2.5.** 2D sequence of the larger RNA tetrahedron with 55 bp per edge. The 22-nucleotide core sequence of pRNA-3WJ was highlighted in red color.
Figure 2.6. Design, assembly and characterization of 17 nm RNA tetrahedrons. (a) Schematic showing tunable size conversion (from 22 bp per edge to 55 bp per edge) of RNA tetrahedrons. (b) 6% native PAGE gel showing step-wise assembly of larger RNA tetrahedrons. '+' indicates the presence of the strands. M: 100 bp DNA ladder. Representative results from at least three independent experiments are shown. (c) DLS
assay showing the hydrodynamic size of larger RNA tetrahedrons. Representative results from three independent measurements are shown. (d) AFM images of 17 nm RNA tetrahedrons. Representative results from sixteen raw AFM images are shown. (e) Cryo-EM images and 3D reconstruction of 17 nm RNA tetrahedrons. A representative image (left) from 131 raw images of 17 nm RNA tetrahedron was shown. A total of 1662 particles were used to generate the 2D class averages for building the initial models. A total of 1582 particles were used for final refinement by applying the tetrahedral symmetry.
Figure 2.7. (a) Reference free 2D class averages of the raw 17 nm RNA tetrahedrons. (b) 0.143 Fourier shell correlation of the 17 nm RNA tetrahedron. (c) Tilt-pair validation of 3D structure of the 17 nm RNA tetrahedron. A total of 1662 particles were used to generate the 2D class averages for building the initial models. A total of 1582 particles were used for final refinement by applying the tetrahedral symmetry. A particle set of 50 particles from two tilt pairs of 17 nm RNA tetrahedron was used for the validation test. The test was performed using e2tiltvalidate.py program in EMAN2.
**Figure 2.8.** Functional characterization of multifunctional RNA tetrahedrons. (a) Schematic showing multifunctional RNA tetrahedrons harboring HBV ribozyme, MG aptamer, Spinach aptamer and STV aptamer. (b) 7% native PAGE gel showing step-wise assembly of multifunctional RNA tetrahedrons. '+' indicates the presence of the strands. Representative results from at least two independent experiments are shown. (c) Ribozyme activity assay showing cleavage of 135 nt substrate. Representative results from at least three independent experiments are shown. Fluorogenic assay demonstrating fluorescence emission of (d) MG aptamer and (e) Spinach aptamer. Data was from one experiment, and the fluorogenic properties of MG aptamer and Spinach aptamer were further confirmed by PAGE (Figure 2.9). (f) Streptavidin (STV) aptamer binding assay using STV affinity column. The error bars represent standard deviation from three independent measurements. The binding property of STV aptamer was further confirmed by PAGE (Figure 2.9). ***: p < 0.001 versus corresponding control value, unpaired Student's t-test.
**Figure 2.9.** 7% native PAGE assay of multifunctional RNA tetrahedrons with (top) and without (bottom) Streptavidin aptamer after binding, washing and elution from the STV column. The gels were visualized by ethidium bromide staining (left), Spinach aptamer fluorescence (middle) and MG aptamer fluorescence (right). Data was from one experiment, and the purpose of this experiment is to confirm the previous studies of the fluorogenic properties of MG aptamer (Figure 2.8D) and Spinach aptamer (Figure 2.8E) as well as the binding properties of Streptavidin aptamer (Figure 2.8F).
Figure 2.10. 2D sequence of the RNA tetrahedron harboring luciferase siRNAs. Total four luciferase siRNAs were incorporated into one RNA tetrahedron.
Figure 2.11. *In vitro* and *in vivo* evaluation of RNA tetrahedrons harboring siRNA and cancer-targeting aptamers. (a) Confocal images showing RNA tetrahedron (with and without EGFR aptamers) binding to MDA-MB-231 cells. Representative results from at least three technical repeats are shown. Similar cell binding experiment of pRNA 3WJ RNA nanoparticles harboring EGFR aptamer was conducted by Dr. Dan Shu, and similar results were observed and published previously [174]. (b) Luciferase siRNA silencing effects assayed by dual luciferase assay. The error bars represent standard deviations from at least four independent wells. Representative results from at least two independent experiments are shown. ****: p < 0.0001 versus plasmid only control, unpaired Student's *t*-test. (c) Biodistribution assay in orthotopic MDA-MB-231 tumor-bearing mice after systemic tail vein injection of RNA tetrahedrons harboring EGFR aptamers. Data was
from one experiment, and the biodistribution experiment was repeated and further confirmed in KB xenograft mouse model (Figure 2.13). Similar biodistribution experiment of pRNA 3WJ RNA nanoparticles harboring EGFR aptamer was also conducted by Dr. Dan Shu, and similar results were observed and published previously [174].
Figure 2.12. 2D sequence of the RNA tetrahedron harboring EGFR aptamers (highlighted in the purple boxes) and Alexa 647 fluorescent probe (highlighted in the red box).
Figure 2.13. Biodistribution of tetrahedron nanoparticles with EGFR aptamer in KB xenograft mouse model. PBS saline and tetrahedron without EGFR aptamer treated mice were used as control. Data was from one experiment, and the purpose of this experiment is to confirm the previous biodistribution study on MDA-MB-231 xenograft mouse model (Figure 2.11c).
Table 2.1. (Related to Figure 2.7) Tilt geometry computed for tilt pair of images, using the final 3D model of the 17 nm RNA Tetrahedron.

<table>
<thead>
<tr>
<th></th>
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<tr>
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<tr>
<td>RMSD Tilt Axis</td>
<td>50.33</td>
</tr>
<tr>
<td>Tilt Angle via Microscope</td>
<td>15</td>
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Chapter 3: Controllable Self-assembly of Molecularly Defined RNA Cages with Cube and Dodecahedron Shapes based on Stable pRNA Three-way Junction for Therapeutics Delivery

This chapter is adapted from a manuscript entitled “Controllable Self-assembly of Molecularly Defined RNA Cages based on Stable pRNA Three-way Junction for Therapeutics Delivery” by Hui Li, Sijin Guo, Kaiming Zhang, Luda Shlyakhtenko, Wah Chiu and Peixuan Guo. The manuscript is in preparation for submission to Advanced Materials. Special thanks to Dr. Kaiming Zhang for help in preparing Figure 3.1, 3.6 and 3.11, Sijin Guo for help in preparing Figure 3.12, Dr. Luda Shlyakhtenko for help in preparing Figure 3.5 and 3.9. Author contribution: Sijin Guo performed the ELISA experiments. Dr. Kaiming Zhang performed the cryo-EM imaging and single particle reconstruction based on the cryo-EM raw data under the supervision of Dr. Wah Chiu. Dr. Luda Shlyakhtenko performed the AFM studies. Hui Li conceived the idea of building RNA cube and dodecahedron nanoparticles based on pRNA-3WJ, designed the 2D sequence and 3D computational model of RNA cube and dodecahedron nanoparticles, performed all the other experiments, analyzed all the data and drafted the manuscript. Dr. Peixuan Guo led and supervised the whole study and provided financial support.

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Abstract

Over the past decade, RNA has been shown to be a unique and programmable biopolymer that can be exploited to self-assemble nanoscale objects with sophisticated structures and rich functionalities. Herein, I report the design and construction of molecularly defined RNA cages with cube and dodecahedron shapes based on the stable pRNA 3WJ. The RNA cages can be easily self-assembled by single-step annealing. The RNA cages were further characterized by gel electrophoresis, cryo-electron microscopy and atomic force microscopy, confirming the spontaneous formation of the RNA cages. I also demonstrated that the constructed RNA cages could be used to deliver model drugs such as immunomodulatory CpG DNA into cells and elicit enhanced immune responses. The RNA cages can be further explored to be a new generation of molecularly defined nanocarriers for drug delivery.

Keywords: RNA nanotechnology; RNA nanostructures; Cage; Cube; Dodecahedron
Introduction

RNA nanotechnology is an emerging research field that focuses on utilizing RNA as a unique and programmable biopolymer to self-assemble nanoscale objects with sophisticated structures and rich functionalities [181]. Similar to DNA, most of RNA-RNA interactions are based on Watson-Crick base pairings and these base pairings are largely predictable. However, in contrast to DNA, RNA-RNA interactions also involves many non-canonical base pairings, and RNA can form complicated secondary structures such as single-stranded loops, bugles, hairpins, pseudoknots and junctions [12, 29-31]. In addition, RNA is one of the most important biomolecules in a cell and plays a critical role in normal physiology as well as various diseases. Previous research has shown that a variety of 2D and 3D RNA nanostructures including triangles [36], squares [93, 118, 126], pentagons [92], tetrahedrons [304], polyhedrons [119] and prisms [122] can be successfully constructed based on utilizing naturally-occurring RNA structures and motifs as basic building blocks. These RNA nanostructures can also be easily functionalized by incorporating functional groups such as aptamers, siRNAs and ribozymes [36, 126, 304]. These functionalized RNA nanostructures have the potential for many applications in biomedical and material sciences such as drug delivery, disease diagnosis and tissue engineering [181, 286]. Here I report the design and construction of molecularly defined RNA cages with cube and dodecahedron shapes based on the stable pRNA 3WJ [54]. To demonstrate the versatility of the stable pRNA 3WJ as a basic building block for RNA nanostructures, I designed and constructed an extended pRNA 3WJ and then cryo-EM was used to characterize the extended pRNA 3WJ. The core motif of the pRNA 3WJ was then used to construct RNA cages with cube and
dodecahedron shapes. The assembled RNA cages have defined 3D structures as revealed by AFM and cryo-EM. I also demonstrated that the constructed RNA cages could be used to deliver model drugs such as immunomodulatory CpG DNA [305] into cells and elicit enhanced immune responses.

**Materials and Methods**

**RNA design, synthesis, and self-assembly**

The extended pRNA 3WJ was designed by extending the three branches of the pRNA 3WJ by sixty perfectly base-paired nucleotides. The NanoEngineer program [306] was used to facilitate the design of the RNA cages. Six RNA strands were designed for assembling the RNA cube, while twelve RNA strands were designed for assembling the RNA dodecahedron. Each RNA strand represents one face of the RNA cube or dodecahedron. The nucleotide sequence of the individual RNA strand was designed based on the pRNA 3WJ sequence and perfectly base-paired double-stranded RNA sequences. RNA strands were synthesized by *in vitro* T7 transcription following previously reported procedures [125]. Briefly, DNA templates for generating the RNA strands were made by polymerase chain reactions (PCR). DNA oligo for PCR were directly ordered from Integrated DNA Technologies, Inc. (Coralville, IA). For a 50 µl transcription reaction, 10 µl of DNA template with 10 µl of 5× transcription buffer, 10 µl of 25 mM NTPs solution, 5 µl of 100 mM DTT, 10 µl of T7 RNA polymerase and 5 µl 0.05% (vol/vol) DEPC water were mixed together and incubated at 37 °C for 4 h. The transcription reaction was stopped by adding 1 µl of DNase I and incubating at 37 °C for another 30 min to digest the DNA template. For generating 2′-F modified RNA
tetrahedrons, Y693F mutant T7 polymerase and 2’-F modified cytosine (C) and uracil (U) nucleotides were used to synthesize the corresponding RNA strands. For a 50 µl 2’F transcription reaction, 2.5 µg of DNA template diluted in 15 µl 0.05% (vol/vol) DEPC water, 5 µl of 10× 2’-F transcription buffer, 5 µl of 100 mM DTT, 5 µl of 50 mM 2’-F CTP, 5 µl of 50 mM 2’-F UTP, 5 µl of 50 mM ATP, 5 µl of 50 mM GTP, and 5 µl of Y639F mutant T7 RNA polymerase were mixed together and incubated at 37 °C overnight. After transcription, the RNA strands were purified by 8 M urea, 8% denaturing PAGE, respectively.

The detailed sequences for assembling the designed structures are listed below:

a. RNA sequences for assembling the extended pRNA 3WJ:

Strand 1: Extended 3WJ-a

5’-GGGACAGCACACAGAGCAGCAGCUUGAGACUCAGCGUACUUCUGGCAA
GGUACGUACUUUGUCAUGUGUAUGUUGUUGGCGAGACGCGGAUACCAG
UAGUCACCUCUAGUGCUCUAUCGUAGAAGUGUAUGGACUGAUGACGCC -3’

Strand 2: Extended 3WJ-b

5’-GGCGUCAUGCUACACUUCUACGGAUAGACUAGCGACUCUAGGACUACUAGGUA
UCGCCGUCUGCGCCCAUACUUUGUUGAUGGGAUGGAUCAUCUUGGAA
UCUGGGUGAUCAGUGGCGCCACGCGACGUAUGGAUGGACGCC -3’

Strand 3: Extended 3WJ-c

5’-GGCUCACUAUACGUGCACGCUGGCACUGGGAUCAUCGAGAUCACGAGAUACAG
UGAUCUCAUCUAGUCAAUCUGCGAAAGUGCCGUACUUUGCCAGAAGUA
CGCUGAGUCUCAGCUGCUCUGUGUGUGGCCC -3’
The extended pRNA 3WJ was self-assembled in a one-pot manner by mixing the corresponding RNA strands in equimolar concentrations (1 µM) in 1× annealing buffer (10mM Tris-HCl, 50mM NaCl, 1mM EDTA, pH=8.0) and heated to 95 °C and slowly cool down to room temperature. For cryo-EM imaging, the assembled extended pRNA 3WJ were further purified by native PAGE.

b. RNA sequences for assembling the RNA cube:

Strand 1:

5’- GGUGCUACACGAGUGUGUAGCCAGACUUAGCGAAUGUUCGAUGUGUAC GGGAAGAGCUCUAUGCCCAUCCAUGGUGUACUGCAUGGCGAGGCGCCUCCAUAACCCG AUGUGUACGAAUACAGCU -3’

Strand 2:

5’- GGCUCUUCCCGUACUUUGUUCGUGUGUCGUCCAGACUGUCGAAAUCAU GUUACAGUGCCCAUACUGCAUACUAACUCUACUCUGCCCAUUGGGAUACG UUGUUCAGGGAUGGCCUA -3’

Strand 3:

5’- GGGCAGUUGAGUUGUUCUAUGGAGUAUACCACGAGUCUCGGAUAUUUG GUUGCAGCCCUCUCCUACCCUAUCAUCAUGCUGUAGGACGCGUCCUCCGA UGUAUCUACGUAUCACCAU -3’

Strand 4:

5’- GGAUCAGCUCUAACUAAGACUCGAGUACACUUUGCAACUUUUUGUUCAU CGUGUAGCACCACGUGUACUUUGUUGGUAGGGAAGACGAGGU ACGAAUAUCAGUGGAGGC -3’

Strand 5:
5’- GGACGACACAGAAUCAUCGAACAUUCCCGCUAGUCUGGCUACUAAAGU
UGCAAGUGUACACGUAGUCUUGUUUCAUUACCAGACGUAUGCGUGAUGCUA
UACUUUGUUUUCGACAGUCU -3’

Strand 6:
5’- GGCACUGUAACAUGUGUAUAGCAUCACGCAUCGUGCUGUAUGUGUAU
GAGCUGAUCGCACUGCAUACUGUAUCGAGCGAGAUCUGUGUAUACUC
AUGUGUAUGUGUGAUG -3’

c. RNA sequences for assembling the RNA dodecahedron

Strand 1:
5’- GGUGCUACACGAUGUGUAAGUCGCCACGUGAGACACGUGCAUGUGUAC
UGUGUCGUUCAAACUGUGUGUAUGUGUAUGGGAAGAGCGCCACGCCAUCC
AUGUGUAAGCCAGACUUAGCGGAAUGGUUCGAUGUGUAUGGUACAUACGCU -3’

Strand 2:
5’- GGACGACACAGUACUUUUUGUUUACCGACGUAUGCGUGAUGCUGUAACAU
CGUACUCCGGGUUCCUCAAACCGGUAUCAUGACUACGUACACUUACCAGCUU
ACUUGUGUAGGCUCUGUACGCACCAUAUGGAAUCAUUCGACAGUCU -3’

Strand 3:
5’- GGUAUGAGCGUUGUACUUUUUGUCAUGCGAGGCCGUCUUCAAUCCGGCUACUU
UGUUAGCAUCACGCAUCGUCGUGUAAAUCUAUGGAGACUGCCAGGGCCUACGUCGACG
GACUUACUUUUGGUGAGACCGACACGUGCUGCUAGAAUCAUCAUGGAC
G -3’

Strand 4:
5’- GGUACGCAUGCUACUUUUUGUUGCCCUAGACAAUGAGACUGACCUCUU
GUUUCGAGACGUGUCCGUCUCACGAAUAUCUGUGUAGCACCAGCUGUAA
UCGUACUUUGUUGCACUAACAUCGCAGGAUCAACAAUAUGAGACAGAC
AUGA -3’
Strand 5:
5’- GGGCAGUUGAGUACUUUGUUGAGACGCGACCUAACAUGAGACACUACUUU
GUUUGUCGAUCCCGGAGUUGUAGUGCAAUAUCGAACAUUCCCUGUAAGUCU
GGCUACUUUGUUGUCUCUGGAAGAGUGCAAGAAUAUAUCUGUAUCA
CCAU -3’
Strand 6:
5’- GGAUCAGCUCAAAUAUCUGUGAUCUGUAAACGCAGCCAGUACUUUGUU
AUCUUGGACUCUUCGAGAGCAAAUAUCUGGAUGGGCAUAGGCUCUUCCCG
UACUUGUUCUCAUAUGGUGCUAGACGAGCUAAUAUCACAGUACGGC -3’
Strand 7:
5’- GGAUGUACAGAUACUUUGUUGGUUGAGGAAGACGGAUGAUCUGUGU
ACGGUAUUUGGACGCGCCUCGCAUGAAUAUCACCCCGGACUGUCUGAGAUGACG
UAAUCAUCAGUGACUACCCGCAUGCGUCAGUACUUUGUUCACGUCCAGAU -3’
Strand 8:
5’- GGACUUGCUCUACUUUGUUGUACGCUACUCAGACAGUGCGGUGAUGUGU
AACAGCUCAUACCCGUGACAUUGAUGUGUAAGGCUCAGUCUCAUGUCUAG
GCAAUCAUGACAGCGAUCUGUGGACAGUAGAAUAUCGCAGGUGAGA -3’
Strand 9:
5’- GGUGAGUACUGUACUUUGUUCUACUGUCCACAGUUCGCUGUAUCAUGUGU
AGCAUGCUACCUCUAUGUCUGUCAUGUGUAUGCGUCAUGGUUAGGGUCGCU
AAAUCUAUGCUAGUUCACCACGCACUCGAGUCUAAUCAUGACCAGCUGA

Strand 10:
5’- GGUGAACUAGCAUGGUACUCAACUGCCCAUGGUGAUACGGAUGUGUAA
CUCGGUGCGUUACACGAUCACAUGUGUAUAUGUUCAUGCUUGACGAGC
AAUCAUCGUUACUAAACUGAUGCGAGACUACUUUGUUAGACUCGAGUC
-3’

Strand 11:
5’- GGCUGUCCUUCUACUUUGUUGGCUCGCUCAUCAGCAUGGAUCAUUACU
UGUUGAGCUAGUCCGCCCUGCUACUGAUGUGUAAGUUUGCAAGUGUACACG
AGUCAUGUGUAUCUGUACAUCUCUGGAGCUAAUCAUCAUCAACAGACA
-3’

Strand 12:
5’- GGUAGUCACUGAUGUGUAAGGCAGGAAGCUCUCAGCGAUGUGUAC
AGUACUCACCAUCUGAGCGUCAUGUGUAGUCUCGCAUCAGCUUAGUAACG
AUGUGUAGAAGGACAGCUGGCUUGUGUAGUGUACUGACGCAUGC
-3’

**d. DNA sequences for assembling RNA cube harboring CpG DNA**

CpG DNA strand 1:
5’- TCCATGACGTTCCTGACGTTTTTTTTTGTTGCTACACGATGTTAGCCAGACT
TAGCGGAATGTTCGATGTTACGGGAAGAGCC

CpG DNA strand 2:
5’- TCCATGACGTTCCTGACGTTTTTTTTATGCGCCATCCATGTTACATGCGAG
GCCGTTCAATACCGATGTTACGATTACAGCT

CpG DNA strand 3:
5'-TCCATGACGTTCCTGACGTTTTTTTGGCUCTGTAACATGTGTATAGCATCA
CGCATACGTCGTAATGTGTATGAGCTGATCC -3’

CpG DNA strand 4:
5'-TCCATGACGTTCCTGACGTTTTTTTGGCCTGCTACTGATGTGTATCGAGCAG
ATCGTGGTATACTCATGTGTATGTCGAGTATG -3’

The RNA cube or dodecahedron was self-assembled in a one-pot manner by mixing the corresponding RNA strands in equimolar concentrations (0.3 µM for RNA cube and 0.5 µM for RNA dodecahedron) in 1× Tris buffer and heated to 95 °C and slowly cool down to room temperature. The RNA cube harboring CpG DNA was self-assembled in a one-pot manner by mixing the corresponding RNA and DNA strands in equimolar concentrations (5 µM) in 1× annealing buffer (10mM Tris-HCl, 50mM NaCl, 1mM EDTA, pH=8.0) and heated to 95 °C and slowly cool down to room temperature. The step-wise self-assembly of the RNA cages was verified by PAGE or agarose gels. The gels were stained with ethidium bromide (EB) and imaged by Typhoon FLA 7000 (GE Healthcare, Marlborough, MA). For AFM imaging, the assembled RNA cube or dodecahedron was further purified by native PAGE. For cryo-EM imaging, the assembled RNA cube was further purified by native PAGE, while the RNA dodecahedron was further purified by an Amicon Ultra-0.5 mL centrifugal filter (100K molecular weight cut-off).

**Cryo-electron microscopy of the extended pRNA 3WJ**

2 µl of extended pRNA 3WJ solution (0.3 mg/ml) was applied onto a glow-discharged 200-mesh R1.2/1.3 Quantifoil grid. The grids were blotted for 1.5 s and
rapidly frozen in liquid ethane using a Vitrobot Mark IV (FEI, Hillsboro, OR). Then the grid was transferred to JEM2200FS cryo-electron microscope (JEOL, Tokyo, Japan) operated at 200 kV with in-column filter for screening. Micrographs were recorded on a 4k×4k CCD camera (Gatan, Pleasanton, CA) at 50,000× microscope magnification (calibrated sampling of 2.16 Å per pixel) and a dose rate of ~13 electrons/sec/Å² with a total exposure time of 4 s. Ninety-eight images were collected with a defocus range of 2~4 μm.

**Single particle image processing and 3D reconstruction of the extended pRNA 3WJ**

The image processing software package EMAN2 was used for the micrograph evaluation, particle picking, contrast transfer function (CTF) correction, 2D reference-free class averaging, initial model building and 3D refinement of the cryo-electron microscopy data. A total of 4702 particles were boxed to generate the 2D class averages for building the initial models. In total, 4340 particles were used for final refinement, applying the icosahedral symmetry. The resolution for the final maps was estimated by the 0.143 criterion of FSC curve without any mask. A 30 Å Gauss low-pass filter was applied to the final 3D map displayed in the Chimera software package.

**Cryo-electron microscopy of the RNA cube**

For cryo-electron microscopy, 2 μl of RNA cube cage sample (0.45 mg/ml) was applied onto a glow-discharged 200-mesh R1.2/1.3 Quantifoil grid. The grid was blotted and rapidly frozen in liquid ethane using a Vitrobot IV (FEI, Hillsboro, OR). Micrographs were recorded with a direct detection device (DDD) (DE-20 4k×5k camera,
Direct Electron, LP, San Diego, CA) operating in movie mode at a recording rate of 25 raw frames per second at 40,000× microscope magnification (corresponding to a calibrated sampling of 1.64 Å per pixel) and a dose rate of ~20 electrons per second per Å² with a total exposure time of 3 s. A total of 149 images of RNA cube were collected with a defocus range of 2~4 µm.

**Single particle image processing and 3D reconstruction of the RNA cube**

The image processing software package EMAN2 was used for the micrograph evaluation, particle picking, CTF correction, 2-D reference-free class averaging, initial model building and 3-D refinement of the cryo-electron microscopy data. A total of 5106 particles were boxed to generate the 2D class averages for building the initial model. Finally, 4371 particles were used for final refinement, applying the octahedron symmetry. The resolution for the final map was estimated by the 0.143 criterion of FSC curve without any mask. Sixteen Å Gauss low-pass filter was applied to the final 3D map displayed in the Chimera software package.

**Cryo-electron microscopy of the RNA dodecahedron**

RNA dodecahedron nanoparticles solution (2 µl at 1 mg/ml) was applied onto a glow-discharged 200-mesh R1.2/1.3 Quantifoil grid. The grids were blotted for 1.5 s and rapidly frozen in liquid ethane using a Vitrobot Mark IV (FEI, Hillsboro, OR). Then the grid was transferred to JEM2200FS cryo-electron microscope (JEOL, Tokyo, Japan) operated at 200 kV with in-column filter for screening. Micrographs were recorded on a 4k × 4k CCD camera (Gatan, Pleasanton, CA) at 40,000× microscope magnification
(calibrated sampling of 2.83 Å per pixel) and a dose rate of ~20 electrons electrons/sec/Å² with a total exposure time of 3 s. Fifty-five images were collected with a defocus range of 2~5 μm.

**Single particle image processing and 3D reconstruction of the RNA dodecahedron**

The image processing software package EMAN2 was used for the micrograph evaluation, particle picking, CTF correction, 2-D reference-free class averaging, initial model building and 3-D refinement of the cryo-electron microscopy data. A total of 1598 particles were boxed to generate the 2D class averages for building the initial models. At last, 991 particles were used for final refinement, applying the icosahedral symmetry. The resolution for the final maps was estimated by the 0.143 criterion of FSC curve without any mask. A 27 Å Gauss low-pass filter was applied to the final 3D map displayed in the Chimera software package.

**Atomic force microscopy**

The RNA cube or dodecahedron samples (5-10 μL at 3-5 nM) were deposited on the aminopropyl silatrane (APS)-modified mica surface and excess samples were washed with DEPC-treated water and dried before imaging. AFM images were obtained by using the MultiMode AFM NanoScope IV system (Veeco, Plainview, NY) operated in tapping mode following previously published procedures [293].
Determine the level of immunomodulation by RNA cube nanoparticles harboring CpG DNA via enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells were plated into 24-well plates with the density of $2.5 \times 10^5$ cells per well and cultured overnight. RNA cube nanoparticles harboring different numbers of CpG ODNs ($10 \mu l$ at $5 \mu M$ in $1\times$ annealing buffer ($10mM$ Tris-HCl, $50mM$ NaCl, $1mM$ EDTA, pH=8.0)) were diluted in $300 \mu l$ Opti-MEM medium (Life Technologies, Carlsbad, CA) and then incubated with the RAW 264.7 cells for 8 hours at $37 ^\circ C$ in humidified air containing $5\%$ CO$_2$. Then the cell culture supernatant of each well was collected and stored at -80 $^\circ C$ freezer until use. The concentration of TNF-$\alpha$ and IL-6 in the supernatant was determined by enzyme-linked immunosorbent assay (ELISA) by using Mouse ELISA MAX™ Deluxe sets (BioLegend, Inc., San Diego, CA), following protocols provided by the manufacturer. At least three independent wells were tested for each nanoparticle and control samples.

Statistical analysis

The results were presented as mean ± standard deviation. Statistical differences were evaluated using unpaired Student’s $t$-test, and $p<0.05$ was considered significant.

Results and Discussion

Design, construction and characterization of extended pRNA three-way junction as a flexible building block for RNA nanostructures

The pRNA 3WJ has been demonstrated by previous studies as a thermodynamically stable RNA motif and has been widely used in constructing RNA
nanostructures as a fundamental building block [36, 73, 92, 114, 124, 304]. However, because the size of the pRNA 3WJ is relative small (~ 4 nm), it is difficult to directly and clearly observe the pRNA 3WJ under electron microscope. In order to better characterize the pRNA 3WJ, I extended the three branches of the pRNA 3WJ by sixty perfectly base-paired nucleotides, respectively, to make a larger RNA 3WJ that is large enough to be observed by electron microscope. The designed extended pRNA 3WJ is still composed of three RNA strands with length of 139 nucleotides, 138 nucleotides and 134 nucleotides. To characterize the extended pRNA 3WJ in detail, cryo-electron microscope was applied to directly observe its structure (Figure 3.1). Cryo-EM is a powerful technique for imagining three-dimensional (3D) structure of biological macromolecules under their native states [292]. Cryo-EM images 2D class averages and single particle reconstruction showed very clear extended pRNA 3WJ with expected three-branched shape and dimension. Moreover, in the raw images of the cryo-EM results, different conformations of the extended pRNA 3WJ with different angles among the three RNA branches were observed, suggesting conformational flexibility of the pRNA 3WJ. Importantly, as demonstrated in the following sections of this chapter, this conformational flexibility can be exploited to build novel 3D RNA nanostructures such as cubes and dodecahedrons by adjusting the angles among the three RNA branches.

**Design and self-assembly of the RNA cube cages**

A typical cube structure has eight vertexes, six square faces and twelve edges. A 3D computational model of the RNA cube was first generated using the software NanoEngineer (Figure 3.2a). The resulting computational model exhibited authentic
cubic conformation and the eight RNA 3WJs can be clearly observed in the model. For designing the RNA cages with cube shape, eight pRNA 3WJs (four of them are reverse pRNA 3WJs) are positioned into the vertexes and twelve double-stranded RNAs are positioned into the edges of the cube to link the individual pRNA 3WJs. A total of six RNA strands were designed to construct the RNA cube, while each RNA strand represents one square face of the RNA cube (Figure 3.3). For assembling the RNA cube, the designed six RNA strands were synthesized by *in vitro* transcription and then mixed in stoichiometric ratio and annealed in 1×TMS buffer in a one-pot manner. Step-wise assembly of the RNA cubic cages was observed by a PAGE gel, confirming the formation of the high molecular weight RNA cubes (Figure 3.4).

**Design and self-assembly of the RNA dodecahedron cages**

A typical dodecahedron structure has twenty vertexes, twelve faces and thirty edges. Similar to the RNA cube, a 3D computational model of the RNA dodecahedron was generated using the same software NanoEngineer (Figure 3.2b). The resulting computational model exhibited authentic dodecahedron conformation and the twenty RNA 3WJs can be clearly observed in the model. For designing the RNA cages with dodecahedron shape, twenty pRNA 3WJs are positioned into the vertexes and thirty double-stranded RNAs are positioned into the edges of the dodecahedron to link the individual pRNA 3WJs. A total of twelve RNA strands were designed to construct the RNA dodecahedron, while each RNA strand represents one face of the RNA dodecahedron (Figure 3.5). For assembling the RNA dodecahedron, the designed twelve RNA strands were synthesized by *in vitro* transcription and then mixed in stoichiometric
ratio and annealed in 1×TMS buffer in a one-pot manner. Step-wise self-assembly of the RNA dodecahedron cages was clearly observed by running an agarose gel, suggesting the formation of the high molecular weight RNA dodecahedron (Figure 3.6).

**Imaging of the RNA cube cages by atomic force microscopy**

Atomic force microscopy (AFM) has been extensively used to characterize a variety of nucleic acid nanostructures [293]. AFM was also applied to characterize the RNA cube cages. Before AFM imaging, the self-assembled RNA cube was deposited on an APS-modified mica surface, washed with water and dried in room temperature. AFM studies of the RNA cube clearly revealed the shape of the cube, which was similar to its theoretical prediction (Figure 3.7). The height of the RNA cube was found to be around 7-8 nm, which was consistent to the height of the RNA cube after drying (the RNA cube was collapsed on the mica surface after drying, which gave a distorted dimension).

**Imaging of the RNA cube cages by cryo-electron microscopy**

To further confirm the formation of the RNA cube cages, cryo-electron microscopy (EM) was applied to direct image the RNA cube cages. Cryo-EM images showed very clear RNA cubes with expected dimensions. The single particle 3D reconstruction data revealed that the RNA cube has a clear overall shape and conformation in agreement with the computational 3D model and the predicted size of 10 nm (Figure 3.8). The six faces of the cube cage can be clearly observed in the reconstructed 3D model. The 2D computer projections of the reconstructed RNA cube
model showed good match to the 2D class averages of the raw images, suggesting the reconstructed 3D model correctly represented the native structure of the RNA cube.

**Imaging of the RNA dodecahedron cages by atomic force microscopy**

Similar to the RNA cube cages, AFM was also applied to characterize the RNA dodecahedron cages. Before AFM imaging, the self-assembled RNA dodecahedron was deposited on an APS-modified mica surface, washed with water and dried in room temperature. AFM studies of the RNA dodecahedron clearly revealed the shape of the cube, which was in agreement to its theoretical prediction ([Figure 3.9](#)). The height of the RNA dodecahedron was found to be around 8 nm, which was consistent to the distorted dimension of the RNA dodecahedron after drying. Moreover, the nuclease-resistant 2’F-modified RNA dodecahedrons were also imaged by AFM and similar images were observed under AFM ([Figure 3.10](#)). It should be noted that a sharper AFM tip was used for imaging the 2’F-modified RNA dodecahedron, so the resolution of the image of the 2’F-modified RNA dodecahedron was higher and more details of the inner cavities were observed. Overall, the AFM result suggests that 2’F-modification did not significantly change the conformation of the RNA dodecahedron.

**Imaging of the RNA dodecahedron cages by cryo-electron microscopy**

To further confirm the successful self-assembly of the RNA dodecahedron cages, cryo-electron microscopy (EM) was also applied to direct image the RNA dodecahedron cages. Cryo-EM images showed very clear RNA dodecahedrons with expected shapes and sizes. The single particle 3D reconstruction data revealed that the assembled RNA
dodecahedron has a clear overall 3D conformation in agreement with the computational 3D model and the predicted size of 20 nm (Figure 3.11). The twelve faces of the dodecahedron cage can be clearly observed in the reconstructed 3D model. The 2D computer projections of the reconstructed RNA dodecahedron model showed good match to the 2D class averages of the raw images, confirming the reconstructed 3D model truly represented the native structure of the RNA dodecahedron.

**Immunomodulation in macrophage cells by CpG DNA escorted by RNA cages**

The synthetic CpG ODN are a type of DNA molecule that could induce potent immune response both *in vitro* and *in vivo* [305, 307]. Herein, based on the hypothesis that 3D display of CpG DNA by nanoparticles will create synergistic effect and enhance the immune response, I utilized the constructed RNA cube to deliver CpG DNA by incorporating 4 CpG DNA oligos to the 4 edges of each RNA cube. RNA cubes harboring CpG DNA were then incubated with macrophage-like RAW 264.7 cells, and the cell culture supernatant was collected to determine the TNF-alpha and IL-6 cytokine level by ELISA. The ELISA assay showed that CpG DNA delivered by the RNA cube induced significantly enhanced immune response, and the induced immune response is strongly dependent on the number of immunostimulatory molecules per RNA cube (Figure 3.12). More CpG DNA molecules incorporated into the RNA cube, stronger immune responses were observed, while the RNA cube without CpG DNA induced negligible immune response. These results suggested the designed RNA cube has the potential to be employed as a novel vehicle for immunoactive CpG DNA delivery.
Conclusion

In this study, I reported the successful bottom-up self-assembly of molecular defined RNA cages with cube and dodecahedron shapes by using the stable pRNA 3WJ as the basic building block. The constructed RNA cages have defined 3D structures as revealed by both cryo-EM and AFM. Comparing with previously reported RNA cages [119, 122, 145], the RNA cages reported here has more regular and defined shapes. Moreover, since the thermodynamic stable pRNA 3WJ was used as the building block to build the RNA cages, it is expected that the constructed cages will also have remarkable thermodynamic stability. Furthermore, I also found that the designed RNA cube cages could be used to deliver immunostimulatory CpG DNA to macrophage-like RAW 264.7 cells and elicit enhanced immune response. The extent of the induced immune response is also strongly dependent on the number of CpG DNA molecules loaded into the RNA cube. This study showcased that the reported RNA cages have the potential to be explored in the future as molecularly defined nanocarriers for therapeutics delivery. Another appealing future research direction is to develop RNA cages with tunable 3D conformation (for example, 3D RNA cube with different length, width and height) and investigate the effect of different 3D shapes on the intracellular fate and in vivo behaviors of these RNA cages.

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Figure 3.1. Cryo-EM characterization of the extended pRNA 3WJ nanoparticles. (a) A representative raw image showing a typical microscopic view of the extended pRNA 3WJ nanoparticles under cryo-EM. Scale bar: 50 Å. A total of ninety-eight raw images were collected with a defocus range of 2–4 µm. (b) Forty representative raw particles extracted from ninety-eight raw cryo-EM images of the extended pRNA 3WJ nanoparticles. (c) 2D class averages of the extended pRNA 3WJ nanoparticles based on the cryo-EM data. (d) Reconstructed 3D map of the extended pRNA 3WJ nanoparticles based on the cryo-EM data. Four views of the 3D map at four different angles (90°
rotation) are shown in the figure. Scale bar: 260 Å. A total of 4702 particles were used to generate the 2D class averages for building the initial models, and 4340 particles were used for final refinement by applying the icosahedral symmetry.
Figure 3.2. Computation models of (a) RNA cube and (b) RNA dodecahedron designed by NanoEngineer program. Two views from two different angles are shown for the RNA cube model and RNA dodecahedron model, respectively. The RNA cube was composed of six different RNA strands and contains eight 3WJs in its vertexes, while the RNA dodecahedron was composed of twelve different RNA strands and contains twenty 3WJs in its vertexes.
Figure 3.3. 2D sequence design of the RNA cube. The RNA cube was composed of six different RNA strands (1-6) highlighted with six different colors. The 5' and 3' end of the individual RNA strand is labeled in the figure. The pRNA 3WJ motif is highlighted in orange color (Rev denotes the reverse pRNA 3WJ sequence).
Figure 3.4. 6% native TBM PAGE showing the step-wise self-assembly of the designed RNA cube. 100 bp DNA ladder is used as the molecular weight marker. The final product (cube 1+2+3+4+5+6) showed a distinct band (highlighted in the red square), demonstrating correct assembly of the designed RNA cube. Representative results from three independent experiments are shown.
Figure 3.5. 2D sequence design of the RNA dodecahedron. The RNA dodecahedron was composed of twelve different RNA strands (1-12) highlighted with twelve different colors. The 5’ and 3’ end of the individual RNA strand is labeled in the figure. The pRNA 3WJ motif is highlighted in orange color.
Figure 3.6. 2.5% agarose gel showing the step-wise self-assembly of the RNA dodecahedron. 100bp DNA ladder is used as the molecular weight marker. The final product (dodecahedron 1-12) showed a distinct band (highlighted in the red square), demonstrating correct assembly of the designed RNA dodecahedron. Representative results from three independent experiments are shown.
Figure 3.7. AFM characterization of the RNA cube. Left panel shows a typical AFM view of the RNA cube. Right panel shows the magnified view of individual RNA cube. Note that the RNA cube was collapsed on the mica surface after drying, which gave a distorted dimension. Representative results from eight raw AFM images are shown.
Figure 3.8. Cryo-EM characterization of the RNA cube with single particle reconstruction. (a) A representative raw cryo-EM image with box highlighting individual RNA cube. Scale bar: 100 Å. A total of 149 raw images of RNA cube were collected with a defocus range of 2~4 µm. (b) Classes of the RNA cube as observed by cryo-EM with corresponding projections of the reconstructed 3D RNA cube model. (c) 2D class averages (top), starting 3D model (middle) and refined 3D model of the RNA cube (bottom). Two views of the refined 3D model at two different angles (30 ° rotation) are shown in the figure. Scale bar: 100 Å. A total of 5106 particles were used to generate the 2D class averages for building the initial model, and 4371 particles were used for final refinement by applying the octahedron symmetry.
Figure 3.9. AFM characterization of the RNA dodecahedron. Left panel shows a typical AFM view of the RNA dodecahedron. Right panel shows the magnified view of individual RNA dodecahedron. Note that the RNA dodecahedron was collapsed on the mica surface after drying, which gave a distorted dimension. Representative results from thirty-six raw AFM images are shown.
Figure 3.10. AFM characterization of the 2’F-modified RNA dodecahedron. Left panel shows a typical AFM view of the 2’F-modified RNA dodecahedron. Right panel shows the magnified view of individual 2’F-modified RNA dodecahedron. Note that the 2’F-modified RNA dodecahedron was collapsed on the mica surface after drying, which gave a distorted dimension. It should also be noted that a sharper AFM tip was used for imaging the 2’F-modified RNA dodecahedron, so the resolution of the image of the 2’F-modified RNA dodecahedron was higher and more details of the inner cavities were observed. Representative results from fifteen raw AFM images are shown.
Figure 3.11. Cryo-EM characterization of the RNA dodecahedron with single particle reconstruction. (a) A representative raw cryo-EM image with box highlighting individual RNA dodecahedron. Scale bar: 500 Å. A total of fifty-five raw images were collected with a defocus range of 2~5 µm. (b) Reconstructed 3D RNA dodecahedron model. Two views of the reconstructed 3D model at two different angles (90 ° rotation) are shown in the figure. Scale bar: 200 Å. (c) Classes (denoted as C) of the RNA dodecahedron as observed by cryo-EM with corresponding projections (denoted as P) of the reconstructed 3D RNA dodecahedron model. A total of 1598 particles were used to generate the 2D class averages for building the initial models and 991 particles were used for final refinement by applying the icosahedral symmetry.
Figure 3.12. Determination of the level of immunomodulation by RNA cube nanoparticles harboring CpG DNA via ELISA. (a) Determination of the level of TNF-α after incubating RNA cube nanoparticles harboring CpG DNA with macrophage RAW 264.7 cells. The error bars represent standard deviations from three independent wells. ***: p < 0.001 versus 200 nM DNA CpG only control, unpaired Student's t-test. ****: p < 0.0001 versus 200 nM DNA CpG only control, unpaired Student's t-test. The induction of TNF-α in RAW 264.7 cells by 50 nM RNA cube harboring 4 CpGs, 50 nM RNA cube
only control and 200 nM DNA CpG only control was independently repeated one more time with triplicate independent wells for each sample and similar results were observed. (b) Determination of the level of IL-6 after incubating RNA cube nanoparticles harboring CpG DNA with macrophage RAW 264.7 cells. The error bars represent standard deviations from three independent wells. **: p < 0.01 versus 200 nM DNA CpG only control, unpaired Student's t-test. ***: p < 0.001 versus 200 nM DNA CpG only control, unpaired Student's t-test. ****: p < 0.0001 versus 200 nM DNA CpG only control, unpaired Student's t-test. The induction of IL-6 in RAW 264.7 cells by 50 nM RNA cube harboring 4 CpGs, 50 nM RNA cube only control and 200 nM DNA CpG only control was independently repeated one more time with triplicate independent wells for each sample and similar results were observed.
Chapter 4: Bottom-up Self-assembly of Molecularly Defined RNA Nanowire

This chapter is adapted from a manuscript entitled “Bottom-up Self-assembly of Molecularly Defined RNA Nanowire” by Hui Li, Kaiming Zhang, Wah Chiu and Peixuan Guo. The manuscript is in preparation for submission to Small. Special thanks to Dr. Kaiming Zhang for help in preparing Figure 4.2 and 4.3. Author contribution: Dr. Kaiming Zhang performed the TEM and cryo-EM imaging under the supervision of Dr. Wah Chiu. Hui Li conceived the idea of building RNA nanowire via the modular multi-domain strategy, designed the 2D sequence and 3D computational model of RNA nanowire, performed all the other experiments, analyzed all the data and drafted the manuscript. Dr. Peixuan Guo led and supervised the whole study and provided financial support.
Abstract

Since the proof-of-concept of RNA nanotechnology in 1998, RNA has been shown to be an exceptional and smart biopolymer that can be employed to create nanostructures with various conformations and diverse functionalities. In this study, I report the design and construction of a molecularly defined RNA nanowire that was only assembled from four different 44-nucleotide long single-stranded RNAs by using the modular multi-domain strategy. The RNA nanowire can be easily constructed by single-step annealing. The RNA nanowire was further characterized by cryo-electron microscopy, negative staining transmission electron microscopy, atomic force microscopy and fluorescent microscopy, confirming the spontaneous self-assembly of the RNA nanowire. Interestingly, I also found that the RNA nanowire can self-grow to micrometer-scale wire-shaped structure, expanding the horizon of RNA nanostructures to RNA microstructures. The reported RNA nanowire can be further explored as the carrier for drug delivery or the extracellular matrix for tissue engineering or the conductive nanowire after coating with conductive metals.

Keywords: RNA nanotechnology; RNA nanostructures; Nanowire
**Introduction**

RNA nanotechnology is an emerging research field that utilizes RNA as a unique and smart biomaterial to create nanostructures with various conformations and diverse functionalities [181]. Previous research has shown that a variety of 2D and 3D RNA nanostructures with defined shapes and sizes can be successfully constructed and functionalized [36, 54, 93, 111, 113, 116-119, 121, 122, 126, 147]. These RNA nanostructures have the potential for many useful applications in biomedical and material sciences such as drug delivery and tissue engineering [286]. Here I report the design and construction of molecularly defined RNA nanowire with micrometer length by using the modular multi-domain strategy [308]. The modular multi-domain strategy has been used to assemble DNA tubes and DNA ribbons with tunable width [308]. In my study, I applied this modular multi-domain strategy to RNA nanotechnology. Only four different 44-nucleotide long single-stranded RNAs were designed and used to assemble the RNA nanowire. The assembled RNA nanowire has defined 3D structure as revealed by cryo-EM, negative staining TEM, AFM and fluorescent microscopy. Interestingly, I also found that the RNA nanowire can self-grow to micrometer-scale wire-shaped structure based on non-covalent RNA base pairing interactions, expanding the horizon of RNA nanostructures to RNA microstructures. The reported RNA nanowire can be further explored as the carrier for drug delivery, the extracellular matrix for tissue engineering or the conductive nanowire after coating with conductive metals.
Materials and Methods

RNA nanowire design, synthesis, and self-assembly

The NanoEngineer program [306] was used to facilitate the design of the molecularly defined RNA nanowire. The modular multi-domain strategy previously reported for assembling DNA tubes and DNA ribbons was employed to design the RNA nanowire. In addition, different from the DNA double helix, 11 nucleotides per helix turn for RNA double helix were used in the design. RNA strands were synthesized by *in vitro* T7 transcription following previously reported procedures. DNA templates for generating the RNA strands were made by polymerase chain reactions (PCR). DNA oligo for PCR were directly purchased from Integrated DNA Technologies, Inc (Coralville, Iowa, USA). For a 50 µl transcription reaction, 10 µl of DNA template with 10 µl of 5× transcription buffer, 10 µl of 25 mM NTPs solution, 5 µl of 100 mM DTT, 10 µl of T7 RNA polymerase and 5 µl 0.05% (vol/vol) DEPC water were mixed together and incubated at 37 °C for 4 h. The transcription reaction was stopped by adding 1 µl of DNase I and incubating at 37 °C for another 30 min to digest the DNA template. After transcription, the RNA strands were purified by 8 M urea and 8% denaturing PAGE. The RNA nanowire was self-assembled in a one-pot manner by mixing the four different RNA strands in equimolar concentrations (1.4 µM) in 1× TMS buffer and heated to 95 °C and slowly cooled down to room temperature. The step-wise self-assembly of the RNA nanowire was further verified by a 3% agarose gel. The gel was stained with ethidium bromide (EB) and imaged by Typhoon FLA 7000 (GE Healthcare, Marlborough, MA). The detailed sequence for assembling the RNA nanowire are listed below:
Nanowire Strand 1:
5’- GGUGCUACACG CGAUUACAGCU UGUCGAGUAC CGGGCAGUUG AG -3’
Nanowire Strand 2:
5’- GGUUCAGACCG CGGGAAGAGCC CGUGUAGCAC CAGCUGUAAC CG -3’
Nanowire Strand 3:
5’- GGUGCUGUGUC AGCUGUCAGAA CGGUCUGAAC CGGCUCUUCC CG -3’
Nanowire Strand 4:
5’- GGUACUCGACA CUCAACUGCCC GACACAGCAC CUUCUGACAG CU -3’

**Atomic force microscopy**

The RNA nanowire (10 µl at 0.02 mg/ml) was first deposited on a mica surface and excess samples were washed with water and dried on room temperature before imaging. AFM images were obtained by using a Bruker AXS Dimension Icon Atomic/Magnetic Force Microscope with ScanAsyst (Bruker, Billerica, MA) operated in tapping mode.

**Fluorescent microscopy**

One RNA strand for assembling the RNA nanowire was labeled by Cy3 by using the Label IT® Nucleic Acid Labeling Reagents kit (Mirus Bio LLC, Madison, WI). Fluorescent RNA nanowire was assembled by mixing three unlabeled RNA strands with one Cy3-labeled RNA strand in equimolar concentrations (8 µM) in 1× TMS buffer. The mixture was heated to 95 °C and cooled to room temperature. The assembled RNA nanowire (1 µL at 8 µM) was further diluted in 1× TMS buffer by 10 times and deposited
on glass slide and observed under 40 × or 20 × objective lens using a fluorescent microscope (Olympus Corporation, Tokyo, Japan).

**Electron microscopy**

RNA nanowire sample (6 µl at 0.09 mg/ml) was transferred onto the EM grid and then the grid was negatively stained with 2% (w/v) uranyl acetate for 10 s. Electron micrographs were recorded with a JEOL 2100 transmission electron microscope (JEOL, Tokyo, Japan). For cryo-electron microscopy, 2 µl of nanowire sample (0.45 mg/ml) was applied onto a glow-discharged 200-mesh R3.5/1 Quantifoil grid. The grid was blotted and rapidly frozen in liquid ethane using a Vitrobot IV (FEI, Hillsboro, OR). Micrographs were recorded on a 4k × 4k CCD camera (Gatan, Pleasanton, CA) at 40,000× microscope magnification (corresponding to a calibrated sampling of 2.83 Å per pixel) and a dose rate of ~20 e⁻/s/Å² with a total exposure time of 3 s.

**Results**

**Design and self-assembly of the RNA nanowire**

RNA nanowire was designed by adopting the modular multi-domain strategy previously reported for assembling DNA tubes and DNA ribbons [308]. However, in contrast with DNA that has 10.5 nucleotides per helix turn, the number of nucleotides per helix turn is an integer of 11 for A-form RNA. As a result, I have used 11-nucleotide sequence as a single domain in our RNA nanowire design. By using the NanoEngineer program, I first built the computational model of the RNA nanowire (Figure 4.1a). A total of four single-stranded RNA strands were designed consisting of four single
domains per RNA strand. The detailed 2D sequence of the RNA nanowire is shown in Figure 4.1b. It is expected that these designed RNA domains will base pair with their corresponding domains and spontaneously grow in size and finally form RNA nanowire with micrometer-scale length. For assembling the RNA nanowire, the four RNA strands were synthesized by \textit{in vitro} transcription and then mixed in stoichiometric ratio and annealed in 1×TMS buffer in a one-pot manner. Step-wise assembly of the RNA nanowire was observed by a 3\% agarose gel, confirming the formation of the high molecular weight RNA nanowire (Figure 4.1c).

**Imaging of the RNA nanowire by cryo-electron microscopy and negative staining transmission electron microscopy**

To further confirm the formation of the RNA nanowire, cryo-electron microscopy (EM) was applied to direct image the RNA nanowire. Cryo-EM is a useful technique for characterizing the native structure of biological macromolecules including RNA molecules [292]. I expected that cryo-EM imaging would provide meaningful data to show the native structure of the constructed RNA nanowire. Cryo-EM images indeed showed very clear long RNA nanowires with expected width and dimensions (Figure 4.2). From the cryo-EM images, it could also be observed that two or more RNA nanowires could intertwine to from higher order structures. This is plausible since the assembly buffer contains magnesium ions, which could shield part of the negative charge of the RNA phosphate backbone and induce intertwining of RNA nanowires.

Moreover, transmission electron microscopy (TEM) was also used to characterize the RNA nanowire under negative staining by 2\% (w/v) uranyl acetate. Similar to the
results obtained by cryo-EM, long RNA nanowires were also observed under negative staining TEM as expected (Figure 4.3).

**Imaging of the RNA nanowire by fluorescent microscopy**

Because it was found that the length of the RNA nanowire was in the micrometer range as revealed by EM and AFM, I speculated that RNA nanowire could also be imaged by fluorescent microscopy. For preparing the fluorescently labeled RNA nanowire, one RNA strand was labeled with Cy3 and subsequently mixed with the other three non-labeled RNA strands. Under the fluorescent microscope, the RNA nanowire can be clearly observed with expected micrometer length, further confirming its successful self-assembly (Figure 4.4a).

**Imaging of the RNA nanowire by atomic force microscopy**

Atomic force microscopy (AFM) has been widely used to characterize nucleic acid nanostructures [293]. I also applied AFM to characterize the self-assembled RNA nanowire. Before AFM imaging, the self-assembled RNA nanowire was deposited on a mica surface, washed with water and dried in room temperature. AFM studies of the RNA nanowire clearly revealed the shape of the nanowire, which was similar to its theoretical prediction (Figure 4.4b). The height of the RNA nanowire was found to be around 4 nm, which was consistent with the height of the RNA nanowire under dried condition.
Discussion and Conclusion

As a central molecule in molecular biology, RNA not only plays an indispensable role in modulating gene expression and maintain the normal flow of genetic information, but also can form delicate 3D structures with defined shape and atomic level precision. Previous work in RNA nanotechnology has demonstrated that a range of 2D and 3D nanostructures can be successfully constructed based on utilizing naturally occurring RNA motifs and/or computational design [36, 54, 93, 111, 113, 116-119, 121, 122, 126, 147]. Specifically, there are some notable previous studies that applied RNA to build array- or filament-like structures based on non-covalent RNA-RNA interactions [113, 121]. For example, Shu et al. reported in 2004 that nanometer scale 3D RNA arrays could be constructed by linking pRNA molecules into a chain-like structure via foot-on-foot interactions based on palindrome sequences as well as loop–loop interactions [113]. The formation of the array was analyzed by PAGE and the structure of the array was further confirmed by AFM imaging. Moreover, Nasalean et al. reported in 2006 that micrometer-scale RNA filaments could be successfully constructed in a controlled manner by using H-shaped RNA molecules involving RNA 4WJ, specific hairpin loops and loop–receptors [121]. Under TEM, the RNA filaments were clearly visualized, confirming the successful self-assembly of the designed structure. In this current study, I reported the bottom-up self-assembly of molecular defined RNA nanowire by applying the modular multi-domain strategy [308] in RNA nanotechnology. The modular multi-domain strategy was firstly reported by Yin et al. in DNA nanotechnology to synthesize DNA tubes with monodisperse and programmable circumferences [308]. However, comparing with DNA, RNA is more structurally diverse and has important biological functionalities.
that cannot be replaced by DNA. In this regard, I set out to explore the feasibility to design and assemble RNA nanowires via the modular multi-domain strategy [308]. Notably, since the number of nucleotides per helix turn is 11 for A-form RNA (DNA has 10.5 nucleotides per helix turn), I designed multiple 11-nucleotide sequences as the modular domains for the self-assembly of the RNA nanowire. As revealed in a series of characterization experiments including cryo-EM, negative staining TEM, fluorescent microscopy and AFM, the constructed RNA nanowire has a defined wire-like 3D structure that is in agreement with its theoretical predication. Comparing with previously reported RNA array and RNA filaments, this newly designed RNA nanowire was self-assembled purely based on RNA base pairing and did not involve any naturally occurring RNA motifs or junctions. Notably, only four artificially designed short RNA strands were used to assembly the RNA nanowire. In summary, this study showcases the expanding of the horizon of RNA nanostructures to RNA microstructures by incorporating the modular multi-domain strategy previously used in DNA nanotechnology [308]. How to control the width and length of the assembled RNA nanowire will be an interesting direction for future studies. Moreover, the reported RNA nanowire can be further explored for a variety of applications such as the carrier for drug delivery, the extracellular matrix for tissue engineering or the conductive nanowire after coating with conductive metals.
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Figure 4.1. The design and self-assembly of the molecularly defined RNA nanowire. (a) The computational model of the RNA nanowire designed by NanoEngineer program. (b) 2D sequence of the RNA nanowire. The RNA nanowire was assembled from four different 44-nucleotide long single-stranded RNAs. The 5’ and 3’ end of the individual single-stranded RNA is labeled in the figure. (c) 3% agarose gel examine the self-assembly of the RNA nanowire. 100 bp DNA ladder is used as the molecular weight marker. The final product (nanowire 1+2+3+4) showed bands with high molecular weight (highlighted in the red square), demonstrating successful assembly of the designed RNA nanowire. Representative results from three independent experiments are shown.
Figure 4.2. Cryo-EM imaging of the RNA nanowire. Left panel shows a typical cryo-EM view of the RNA nanowire (scale bar: 100 nm). Right panel shows the magnified view of the RNA nanowire (scale bar: 20 nm). Nanowire structures are clearly visible in the images. Representative results from a total of seven raw cryo-EM images are shown.
**Figure 4.3.** TEM imaging of the negatively stained RNA nanowire. Two representative TEM images from a total of seven raw TEM images are shown. Nanowire structures are clearly visible in the images. It is also notable that two or more RNA nanowires could intertwine to form higher order structures. Scale bar: 200 nm.
Figure 4.4. Fluorescent microscopy and AFM imaging of the RNA nanowire. (a) Fluorescent microscopy imaging of the RNA nanowire deposited on a glass slide. Scale bar: 50 µm. A representative image from a total of eleven raw fluorescent microscopy images is shown. (b) AFM imaging of the RNA nanowire deposited on mica surface. Scale bar: 910 nm. A representative image from a total of sixteen raw AFM images is shown. Nanowire structures are clearly visible in both fluorescent microscopy and AFM images.
Chapter 5: Engineering RNA Nanoparticles for Potent Immunomodulation

Part of this chapter was reproduced (with some modification) with permission from Emil F. Khisamutdinov†, Hui Li†, Daniel L. Jasinski†, Jiao Chen, Jian Fu and Peixuan Guo. “Enhancing immunomodulation on innate immunity by shape transition among RNA triangle, square and pentagon nanovehicles.” Nucleic Acids Res. 2014 Nov 1; 42(15):9996-10004. †: Joint First Authors. Special thanks to Dr. Emil F. Khisamutdinov for help in preparing Figure 5.1 and 5.7, Daniel L. Jasinski in Figure 5.2d, 5.4 and 5.6a, Dr. Jiao Chen in Figure 5.3b, 5.3c and 5.4, and Dr. Luda Shlyakhtenko in Figure 5.2c. Author contribution: Dr. Emil F. Khisamutdinov and Daniel L. Jasinski designed and constructed RNA polygon nanoparticles based on the tunable angle of pRNA-3WJ. Hui Li conceived the idea of using RNA polygon nanoparticles to deliver CpG oligos to immune cells, designed and constructed RNA triangles harboring CpG oligos, performed cell and animal testing of RNA polygon nanoparticles harboring CpG oligos. Dr. Jiao Chen provided assistance to the ELISA experiments and animal experiments under the supervision of Dr. Jian Fu. Dr. Emil F. Khisamutdinov, Hui Li, Daniel L. Jasinski and Dr. Peixuan Guo analyzed all the data and drafted the manuscript. Dr. Peixuan Guo led and supervised the whole study and provided financial support.
Abstract

Modulation of immune response through cytokine induction is a vital process in vaccine development and immune therapy. It is desirable to develop potent and non-toxic immunomodulators to promote successful cancer immunotherapy. In this study, I report the development of novel immunomodulators by engineering rationally designed RNA nanoparticles based on the pRNA of the bacteriophage phi29 DNA packaging motor. RNA nanoparticles with triangular, square and pentagonal shape could be successfully constructed by stretching the angle of the pRNA three-way junction from 60° to 90° or 108°. When immunoactive CpG oligodeoxynucleotides (ODNs) were incorporated into the RNA nanoparticles by DNA-RNA hybridization, their immunomodulation effect for cytokine TNF-α and IL-6 induction was greatly enhanced both in vitro and in vivo, while RNA nanoparticle controls induced unnoticeable cytokine induction. Moreover, the RNA nanoparticles were delivered to macrophages specifically and the degree of immunostimulation greatly depended on the size, shape, and the number of payload per RNA nanoparticles. Stronger immune response was observed when the number of adjuvants per nanoparticle was increased, demonstrating the advantage of shape transition of the nanoparticles from triangle into pentagon, which can carry five adjuvants. This finding demonstrates that RNA nanotechnology such as developing RNA nanoparticles based on pRNA has great potential to develop novel immunomodulators for cancer immunotherapy.

Keywords: RNA nanotechnology; pRNA; CpG; Immunomodulator; Immunotherapy
Introduction

Cancer and infectious diseases such as influenza and human immunodeficiency virus infection / acquired immunodeficiency syndrome (HIV/AIDS) are still huge threats to public health worldwide [309, 310]. Prophylactic and treatment vaccines could offer promising opportunities for controlling these diseases [311, 312]. However, despite tens of years of extensive research, such vaccines are still far away from completely controlling these diseases due to either low effectiveness or safety issues. New strategies and approaches in designing and developing new vaccines and adjuvants targeting these diseases are urgently needed. CpG oligodeoxynucleotides (ODNs) are a type of immunostimulatory DNA molecules containing the CpG motif which is unmethylated cytosine-phosphate-guanine dinucleotide with optimized flanking sequence [305]. Previous reports have shown that CpG DNA strongly activates mammalian innate immune system by interacting with various immune cells via endosomal Toll-like receptor 9 (TLR9) [305, 313]. The therapeutic potential of CpG DNA has also been explored in a number of clinical applications, including vaccine adjuvants, anticancer agents, immunoprotective agents and anti-allergic agents [307, 314]. Recently, several groups have reported the utilization of nucleic acid nanostructures to deliver immunostimulatory CpG DNA. However, most of the published reports were using DNA nanostructures such as DNA tetrahedron [315], DNA polypod-like structures [316] or DNA origami structure [317].

In this study, I demonstrated that rationally designed RNA nanoparticles, based on the pRNA of the bacteriophage phi29 DNA packaging motor [123], have the potential to serve as a new generation of delivery system for immunomodulators such as CpG
DNA. By stretching the angle of the pRNA three-way junction from 60° to 90° or 108°, RNA nanoparticles with triangular, square and pentagonal shapes could be successfully constructed. When immunoactive CpG ODNs were further incorporated into the RNA triangular, square and pentagonal nanoparticles by DNA-RNA hybridization, their immunomodulation effect for cytokine TNF-α and IL-6 induction was greatly enhanced both in vitro and in vivo, while RNA nanoparticle controls induced unnoticeable cytokine induction. Moreover, the RNA nanoparticles were delivered to macrophages specifically and the degree of immunostimulation greatly depended on the size, shape, and the number of payload per RNA nanoparticles. Stronger immune response was observed when the number of adjuvants per nanoparticle was increased, demonstrating the advantage of shape transition of the nanoparticles from triangle into pentagon which can carry five adjuvants. This finding suggested that RNA nanotechnology such as developing RNA nanoparticles based on pRNA has great potential to develop novel immunomodulators. To the best of my knowledge, I reported the first time the use of RNA nanostructures derived from bacterial phage Phi29 pRNA to deliver CpG DNA.

Materials and Methods

RNA nanoparticles design, synthesis, and self-assembly

The 3WJ crystal structure of the pRNA molecule (PDB ID: 4KZ2) [54] was primarily used for designing polygon models using Swiss PDB viewer, as previously described [36]. RNA strands for corresponding nanoparticles were synthesized by in vitro T7 transcription using polymerase chain reaction (PCR) generated DNA templates. DNA oligo for PCR were directly ordered from Integrated DNA Technologies, Inc. (Coralville,
IA). For generating 2’-F modified RNA polygons, Y693F mutant T7 polymerase and 2’-F modified cytosine (C) and uracil (U) nucleotides were used to synthesize the corresponding RNA strands. RNAs were further purified by denaturing 8M urea 8% PAGE. Cy5 whole chain labeled RNA was prepared by using Cy5 Label IT® Kit (Mirus Bio LLC, Madison, WI). Cy3-labeled CpG DNA was ordered from Integrated DNA Technologies, Inc. (Coralville, IA). In this Cy3-labeled CpG DNA, Cy3 was conjugated to the 5’ end of the DNA through a phosphate bond. RNA nanoparticles self-assembly was examined by running a 2% agarose gel. The gel was stained with ethidium bromide (EB) and imaged by Typhoon FLA 7000 (GE Healthcare, Marlborough, MA).

The detailed sequence to construct the RNA triangle harboring three CpG ODNs are listed below:

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triangle-A RNA RNA</td>
<td>5’- GGGCCGUCAAUCAUGGCAAGUGUCCGCCAUACUUUGUUGCACGCCUCGGAAUGCAUACGACUGUA -3’</td>
</tr>
<tr>
<td>Triangle-B RNA RNA</td>
<td>5’ - GGGCGUGCAAUCAUGGCAACGGAUGAGCAUACUUUGUUGGCUGGCCUAUUGGAAAUGGCGGUAUG G -3’</td>
</tr>
<tr>
<td>Triangle-C RNA RNA</td>
<td>5’ - GGCCAGCCAUCAUGGCAAUACACGCAUACUUUGUUGACGCGCCAGUAAACACUCGCAUGGCUU -3’</td>
</tr>
<tr>
<td>Triangle-D</td>
<td>5’- GGACACCUUGUCAUGUGUAUGCGGUGUAUAUGUGUCAUG</td>
</tr>
</tbody>
</table>
RNA
UGUAUGCUCAUUCGUUGCAUGUGUAUGGC -3’

CpG-A DNA
5’- TCCATGACGTTCTGACGTTTTTTTTCTACAGTCGTATT
GCATTTCCGA -3’

CpG-B DNA
5’- TCCATGACGTTCTGACGTTTTTTTTCATAACGCGCATT
TC CAACTA -3’

CpG-C DNA
5’- TCCATGACGTTCTGACGTTTTTTTTAAGCACATGCGA
TGTTTAAC -3’

The detailed sequences to construct the shape transition (triangle to square to pentagon) nanoparticles harboring CpG ODNs are listed below:

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPG-TRI-A</td>
<td>5’- GGGAGCCGUCAAUCAUGGCAAGUGUCCGCCAUA</td>
</tr>
<tr>
<td>(for triangle)</td>
<td>CUUUGUUGCAGCUCUCUCGGAAUGCAAACGACUGUA -3’</td>
</tr>
</tbody>
</table>

| CPG-TRI-B     | 5’- GGGAGCGUGCAUCAUGGCAACGAUAGAGCAUACUU |
| (for triangle)| GUUUGCUGGCUCUAGUUGGAAAUGGCGGUAGGA -3’ |

| CPG-TRI-C     | 5’- GGGACCAGCCAAUCAUGGCAAUAACGCAUCUU |
| (for triangle)| GUUGACGCGGCUCAGUUAAACAUACGCAUGGCUU -3’ |

<p>| TRI-D         | 5’- GGACACUUUGCAUGUGUAUGCGUGUAUAAUGUC |</p>
<table>
<thead>
<tr>
<th>(for triangle)</th>
<th>AUGUGUAUGCUCUAUCGUUGUAUGUGUAUGGC -3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPG-SQR-A (for square)</td>
<td>5’- GGGAGCCGCUAAUCAUGGCAAGGUCGCCAUA</td>
</tr>
<tr>
<td>(for square)</td>
<td>CUUUGUUGCACGCUCUCUCGGAAUGGAAUACGUUGUA -3’</td>
</tr>
<tr>
<td>CPG-SQR-B (for square)</td>
<td>5’- GGGAGCGUGCAAUCAUGGCAAGGCAUGCACAUCUU</td>
</tr>
<tr>
<td>(for square)</td>
<td>GUUGCGACCUCUCUUGAUCUUGAUGCUGAU -3’</td>
</tr>
<tr>
<td>CPG-SQR-C (for square)</td>
<td>5’- GGGAGGUGCAGCAUCAUGGCAAGGCAAGCAUCUU</td>
</tr>
<tr>
<td>(for square)</td>
<td>GUUGGCUGGUCUCUAGUGAAUGGCGGUAUGG -3’</td>
</tr>
<tr>
<td>CPG-SQR-D (for square)</td>
<td>5’- GGGACCAGGCAAUCAUGGCAAAUACACGCAUACUU</td>
</tr>
<tr>
<td>(for square)</td>
<td>GUUAGCUGGCCUCUCAGUAAACAUACGCAUGUGCUU -3’</td>
</tr>
<tr>
<td>SQR-F (for square)</td>
<td>5’- GGACACUUGUCAUGUGUAUGCGUGUAUAUGGC</td>
</tr>
<tr>
<td>(for square)</td>
<td>AUGUGUAUGCUCUAUCGUUGUAUGUGUAUGCGAUGGCG</td>
</tr>
<tr>
<td></td>
<td>UUGUCAUGUGUAUGGC -3’</td>
</tr>
<tr>
<td>CPG-PENT-A (for pentagon)</td>
<td>5’- GGGAGCGCUAAUCAUGGCAAGGUCGCCAUA</td>
</tr>
<tr>
<td>(for pentagon)</td>
<td>GUUGUAGGGUCUCAUGUUAAGUAACGUCUAGAAU -3’</td>
</tr>
<tr>
<td>CPG-PENT-B (for pentagon)</td>
<td>5’- GGGACCCUAAUCAUGGCAAAUAGUAGCGCCAUA</td>
</tr>
<tr>
<td>(for pentagon)</td>
<td>GUUGCAGCGUCGAAAGCUUGUGUCUAUGGU -3’</td>
</tr>
<tr>
<td>CPG-PENT-C (for pentagon)</td>
<td>5’- GGGAGCGUGCAUCAUGGCAAGCGCAUCGCAUACUUU</td>
</tr>
<tr>
<td>--------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>GUUGCGACCUCUCUAUUGAUCUAUGAUCGUACGAU-3’</td>
</tr>
<tr>
<td>CPG-PENT-D (for pentagon)</td>
<td>5’- GGGAGGUCGCAAUAUGGCAACGUAAGGCAUACUUU</td>
</tr>
<tr>
<td></td>
<td>GUUGGCUGGUCUCUAUUGGAAAAUGGCGGUAGG -3’</td>
</tr>
<tr>
<td>CPG-PENT-E (for pentagon)</td>
<td>5’- GGGACCAGCAUAUGGCAUAUACGCAUACUUU</td>
</tr>
<tr>
<td></td>
<td>GUUGACGGUCUCAGUAAACACGCAUGUGCUU -3’</td>
</tr>
<tr>
<td>PENT-F (for pentagon)</td>
<td>5’- GGACACUUGUCAUGUGUAUGCGUGUACAUUGUCAUGU</td>
</tr>
<tr>
<td></td>
<td>GUAUGCUCAUCGUUGUCAUGUGUAUGCGAUGCUGUUGU</td>
</tr>
<tr>
<td></td>
<td>CAUGUGUAUGGCGCAUAUUUGUCAUGUGUAUGGC -3’</td>
</tr>
<tr>
<td>CPG-A</td>
<td>5’- TCCATGACGTTTCTGAGTTTTTTTTACAGTCGTAT</td>
</tr>
<tr>
<td></td>
<td>TGCATTCCGA -3’</td>
</tr>
<tr>
<td>CPG-B</td>
<td>5’- TCCATGACGTTTCTGACGTTTTTTTCCATACCGCCA</td>
</tr>
<tr>
<td></td>
<td>TTTCCAACTA -3’</td>
</tr>
<tr>
<td>CPG-C</td>
<td>5’- TCCATGACGTTTCTGACGTTTTTTTAAGCACATGCG</td>
</tr>
<tr>
<td></td>
<td>ATGTTTAACT -3’</td>
</tr>
<tr>
<td>CPG-D</td>
<td>5’- TCCATGACGTTTCTGACGTTTTTTTATCGTACGATC</td>
</tr>
<tr>
<td></td>
<td>ATAGATCAAT -3’</td>
</tr>
</tbody>
</table>
RNA polygons were assembled in one pot by mixing equimolar concentrations (final concentration of 1 µM) of the corresponding RNA strands in 1× TMS buffer (50 mM TRIS pH = 8.0, 100 mM NaCl, and 10 mM MgCl₂). Samples were annealed for 1 h by controlled slow cooling from 80 °C to 4 °C.

**Dynamic light scattering**

Apparent hydrodynamic sizes of RNA nanoparticles (10 µM) in 50 µL 1× TMS buffer were measured by Zetasizer nano-ZS (Malvern Instruments, Ltd, Malvern, United Kingdom) at 25 °C. The laser wavelength was 633 nm. The intensity distribution was used to calculate the sizes of the nanoparticles.

**Atomic force microscopy imaging**

RNA triangles harboring CpG DNAs were imaged by AFM following previously reported methods [293]. Briefly, the RNA samples were diluted with 1 × TMS buffer to a final concentration of 3-5 nM. Then, the droplet of samples (5-10 µL) was immediately deposited on APS-modified mica surface. After 2 min incubation on the surface, excess samples were washed with DEPC-treated water and dried under a flow of Argon gas.
AFM images in air were acquired using MultiMode AFM NanoScope IV system (Veeco/Digital Instruments, Santa Barbara, CA) operating in tapping mode.

**Cell cultures**

Mouse macrophage-like RAW 264.7 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin at 37 °C in humidified air containing 5% CO₂. Cells were then seeded on 24-well plates or 96-well plates at the density of $5 \times 10^5$ cells/mL and cultured overnight before use.

**Cytokine secretion from RAW 264.7 cells**

RAW 264.7 cells were plated into 24-well plates with the density of $2.5 \times 10^5$ cells per well and cultured overnight. Then RNA nanoparticles harboring different numbers of CpG ODNs were diluted in Opti-MEM medium (Life Technologies Corporation, Carlsbad, CA) and then added to the cells. The cells were continually cultured for 8 hours at 37 °C in humidified air containing 5% CO₂, and the cell culture supernatant were collected and stored at -80 °C until use. The concentration of TNF-α and IL-6 in the supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using Mouse ELISA MAX™ Deluxe sets (BioLegend, Inc., San Diego, CA), following protocols provided by the manufacturer. At least three independent wells were tested for each nanoparticle and control samples.
Confocal microscopy imaging

RAW 264.7 cells were seeded on glass coverslips in 24-well plates and cultured at 37 °C in humidified air containing 5% CO₂ overnight. The culture medium was removed and the cells were washed with Opti-MEM medium twice to remove dead cells. RNA nanoparticles harboring Cy3-labeled CpG DNA or Cy3-labeled CpG DNA only were diluted in Opti-MEM medium and added to the cells. After 4 hour incubation at 37 °C in humidified air containing 5% CO₂, the cells were washed twice with PBS and fixed with 4% formaldehyde. ProLong® Gold Antifade Reagent with DAPI (Life Technologies Corporation, Carlsbad, CA) was used to stain the cell nucleus and mount the samples. Alexa Fluor® 488 phalloidin (Life Technologies Corporation, Carlsbad, CA) was used to stain actin. The images were obtained by using Olympus FV1000 confocal microscope or Olympus fluorescent microscope (Olympus Corporation, Tokyo, Japan).

Flow cytometry assay

RAW 264.7 cells were detached from the cell culture flask by using a cell scraper. The cells were washed with Opti-MEM medium and aliquot in 1.5 mL eppendorf tubes at the density of $5 \times 10^5$ cells per tube. RNA nanoparticles harboring Cy3-labeled CpG DNA or Cy3-labeled CpG DNA only were diluted in Opti-MEM medium and incubated with the cells at 37 °C for 1.5 hour. The cells were vertexed every 30 minutes during the incubation. After washing with PBS, the cells were resuspended in PBS and the intensity of fluorescence was determined by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).
**Serum stability assay of RNA polygons harboring CpG DNA**

Preassembled complexes (1 µM) of RNA triangle, square and pentagon (2’-F modified) harboring DNA CpG were incubated in RPMI-1640 medium containing 10% fetal bovine serum. Aliquots (10 µL) were taken at 0 hr, 1 hr, 3 hr, 6 hr, 8hr and 16 hr time points after incubation at 37 °C, followed by analysis using 6% native PAGE gels. The gel was further stained with ethidium bromide (EB) and imaged by Typhoon FLA 7000 (GE Healthcare, Marlborough, MA).

**Cytotoxicity assay**

The cytotoxicity of RNA nanoparticles harboring CpG ODNs was evaluated by the MTT assay kit (Promega, Madison, WI), according to the protocol provided by the manufacturer. Briefly, RAW 264.7 cells were seeded at 96 well plates and cultured at 37 °C in humidified air containing 5% CO₂ overnight. RNA nanoparticles harboring CpG ODNs and controls were dissolved in fresh cell culture medium at the indicated concentrations and added to the cells for incubation at 37 °C for 24 hours. Then 15 µL of the dye solution was added to each well, followed by 4 hour incubation at 37 °C, Next, 100µl of the solubilization solution was added to each well and the plate was future incubated at room temperature on a plate shaker until the formazan crystals were completely solubilized. The absorbance was measured at 570 nm using a microplate reader. The cell viability was calculated relative to the absorbance of the cell only control (viability of cell only control = 1). At least three independent wells were tested for each sample.
For cytotoxicity assay on KB cells, RNA nanoparticles harboring CpG ODNs and control samples were incubated with KB cells for 16 hours (two independent wells for each sample). After 16 hours, cell viability was examined by the MTT assay kit (Promega, Madison, WI), following the previously described procedure.

Cytokine secretion from mice

Male CD-1 mice (4-5 week old) were purchased from Charles River Laboratories. All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Kentucky and were performed in accordance with guidelines issued by the National Institutes of Health for the care of laboratory animals. For in vivo immunostimulation, RNA triangular nanoparticles harboring CpG ODN, RNA triangular nanoparticles, or CpG ODN were dissolved in PBS and administrated to the mice via tail vein injection at 2 mg/kg (CpG ODN per body weight). The same volume of PBS was injected as a control. Blood samples were collected 3 hours post-injection by cardiac puncture. Serum was prepared by centrifugation at 12800 g for 10 min. Serum TNF-α and IL-6 levels were determined by enzyme-linked immunosorbent assay (ELISA) using Mouse ELISA MAX™ Deluxe sets (BioLegend, Inc., San Diego, CA), following protocols provided by the manufacturer. Two independent measurements of the cytokine levels from serum aliquots of the tested mouse were performed.

Statistical analysis

The results were presented as mean ± standard deviation. Statistical differences were evaluated using unpaired Student’s t-test, and p<0.05 was considered significant.
Results

Design of RNA triangles harboring CpG DNA

The structural features of the recently discovered ultrastable pRNA 3WJ module from the bacteriophage Phi 29 DNA packaging motor [54] were utilized for in silico design of the RNA triangles (Figure 5.1a). The particular angle of the 3WJ formed by H1 and H2 is 60 °, which can be used to build a triangular shape nanoparticle [36] as the normal triangle also has the internal angle of 60 ° (Figure 5.1b). Each RNA triangle contained a pRNA 3WJ motif at each vertex. Each triangle was composed of three external short RNA strands and one internal long RNA strand. For the construction of RNA triangles harboring CpG DNA, I designed extended external short strands with sticky ends for RNA-DNA hybridization. I hypothesized that the RNA sticky ends will base pair with the CpG DNA with DNA sticky ends and form hybrid RNA-DNA nanoparticles. A schematic design of the RNA triangle nanoparticle harboring 3 CpG DNAs is shown in Figure 5.2a. In Figure 5.2a, the RNA sequence for the assembly of the RNA triangle is marked in blue color, and RNA sticky ends for DNA-RNA hybridization is marked in green color, and CpG DNA is marked in red color. I hypothesized that when these designed RNA and DNA strands mixed together, a triangular RNA nanoparticle with CpG DNA at each end will be successfully self-assembled.
Construction and physicochemical characterization of RNA triangles harboring CpG DNA

RNA triangles harboring CpG DNA were assembled in one pot by mixing equimolar concentrations (final concentration of 1 µM) of four RNA strands and three DNA strands in 1X TMS buffer. To evaluate the step-wise self-assembly of the designed RNA nanoparticles, Cy5-labeled RNA triangle D strand and a 2% agarose gel was used (Figure 5.2b). Specifically, the image under Cy5 channel demonstrated the participation of triangle D strand in all the assembly intermediates as well as the final products. The image under EB channel showed the successful assembly of the designed RNA nanoparticle.

To further evaluate the size and shape of the resulting RNA assemblies, structural characterization of RNA triangles harboring CpG DNA was conducted by atomic force microscopy (AFM). AFM images revealed that the shapes of resulting RNA nanoparticles were similar to their predicted 3D models (Figure 5.2c). In addition, the elongated arms of each RNA nanoparticle are clearly visible, demonstrating the successful incorporation of the designed DNA strands into the RNA triangles. The estimated average size was found to be around 20 nm. However, this value does not reflect the true size of the designed RNA nanoparticles due to the AFM tip convolution.

DLS was performed to determine the apparent hydrodynamic size for RNA triangles harboring three CpG ODNs (Figure 5.2d). The diameter was found to be 14.2 ± 0.8 nm based on the intensity distribution, which agreed with its corresponding 3D model. However, there was a difference between the sizes determined by AFM and DLS. This could be attributed to the different mechanism in determining nanoparticle size as DLS
calculates the average size of nanoparticles in solution assuming that the nanoparticles have globular shapes, while AFM imaging can produce larger images of the nanoparticles because of the size of the used AFM tip. However, the sizes determined by the two techniques were in agreement with our designed dimension of the nanoparticles.

**Evaluation of the interaction between RNA triangles harboring CpG DNA and macrophage cells**

CpG oligonucleotide is a ligand for TLR-9 [305, 313], and previous studies have found that it can be used as vaccine adjuvant or immunotherapy reagent for disease control and treatment [307, 314, 318]. To evaluate whether the RNA triangles can enhance the efficacy of CpG DNA, I further studied the interaction of the designed RNA nanoparticles with macrophage cells. Using the mice microphage-like RAW 264.7 cells as the model, I evaluated the cell binding and entry by using flow cytometry (**Figure 5.3a**). The flow cytometry experiment revealed that RNA triangle nanoparticles significantly enhance cellular binding and entry of CpG DNA. Moreover, the level of inflammatory cytokine TNF-α excretion by the cells when they were incubated with the RNA triangles and CpG DNA was measured by using the enzyme-linked immunosorbent assay (ELISA) (**Figure 5.3b**). As shown in Figure 5.3b, compared with cells treated with triangle only and CpG DNA only, cells treated with CpG DNA loaded triangle showed significantly increased TNF-α level. And this activity also shows good dosage-response relationship (**Figure 5.3c**), as higher concentration of nanoparticles induced higher level of TNF-α.
Comparison of the modulation effect of RNA triangle, square, and pentagon nanoparticles harboring CpG DNA

By increasing the number of external strands and the propagation of the central or internal strand, the tension on the inter-helical angle of the pRNA three-way junction could be increased to 90 ° and 108 °, allowing for the structural transition of RNA triangles to squares and pentagons. To evaluate whether the different shape of RNA polygons could affect the immunomodulation effect, we designed and constructed new RNA polygons harboring different numbers of CpG DNAs. Similar to the experiments described above, the immunostimulatory efficacy of different RNA polygons was evaluated by measuring the release of cytokines after addition to mouse macrophage-like RAW 264.7. The triangular RNA nanoparticle coupled with only one CpG exhibited the highest level of cytokine induction for both TNF-α and IL-6 comparing to square and pentagonal RNA nanoparticles (Figure 5.4a). Upon increasing the number of CpG per nanoparticle there is opposite effect, as pentagonal RNA nanocarrier showed the highest level of the induction of both TNF-α and IL-6 (Figure 5.4b). The results suggest that the cytokine release by CpG coupled to RNA polygons with different shape remarkably increases the immunostimulatory activity compared to CpG alone. RNA nanoparticles with the size of about 10-nm such as the triangle with a single CpG induced the greatest amount of TNF-α and IL-6. In addition, the induction of cytokines is highly dependent on the number of CpG per polygon. With the increasing number of CpG per polygon, stronger immune response is observed, demonstrating the advantage of the transition of triangle into pentagon which can carry five CpG (Figure 5.4c). Moreover, I also evaluated the toxicity of the tested RNA nanoparticles by MTT assay. The toxicity assay
revealed that no significant cytotoxicity was induced on RAW 264.7 cells upon the incubation with the tested RNA nanoparticles (Figure 5.5). Furthermore, a similar cytotoxicity assay was performed one more time by using KB cells, and similar results were observed (Appendix 3, Supplementary Figure 3).

Comparison of cellular uptake by different RNA polygons harboring CpG DNA

Previously, it has been demonstrated that the CpG oligonucleotide can be readily recognized by TLR9 on the endosomal membrane of macrophages, resulting in cellular uptake of the CpG adjuvants [319, 320]. To investigate whether there is a difference between the efficiency of RNA polygons binding to the cells, I quantified the cellular uptake of polygons-CpG by using the flow cytometric assay [124, 125]. Figure 5.6a demonstrates the binding of different RNA polygons-CpG to the RAW 264.7 cells in a dose-dependent manner. Results demonstrate that there is an increase in binding efficiency from triangle to pentagon with more CpGs. Overall, RNA polygons-CpG complexes have significantly more binding efficiency to cells compared to CpG only.

This observation was further confirmed by confocal microscope images (Figure 5.6b) revealing that RNA nanoparticle harboring CpGs are localized in the cytoplasm exclusively and there are much higher amounts of triangular nanoparticles inside the cell compared to free CpG, suggesting that RNA nanoparticles could efficiently enhance the cellular uptake of the CpG adjuvants.

Collectively, both flow cytometry and confocal microscope imaging results demonstrate that all RNA nanoparticles with different shape and harboring CpGs have much stronger binding and cellular entry to the macrophage-like RAW 264.7 cells
compared to free CpGs. In addition, cellular uptake is highly dependent on the number of CpG per polygon. With the increasing number of CpG per polygon, more efficient cell entry is observed (Figure 5.6a), demonstrating the advantage of the transition of triangle into pentagon which can carry five CpG. Notably, all RNA polygons-CpG complexes still can be detected in the gel after 16 h incubation in 10% fetal bovine serum (FBS) indicating robustness of the assembled complex in extracellular environment (Figure 5.7).

Enhancement of modulation effect in vivo by immunological CpG DNA adjuvant incorporated into RNA nanoparticles

To examine whether RNA nanoparticles retain their immunostimulatory activity in vivo, CpG DNA containing triangle, free triangle and free CpG DNA were administered to CD-1 mice by injection into the tail vein at 2 mg/kg (CpG oligonucleotide per body weight), following determination of cytokine TNF-α and IL-6 levels after 3 hour post administration in collected blood serum. Figure 5.4d shows that free triangle nanoparticle and free CpG did not induce any cytokine production, whereas the complex triangle-CpG resulted in elevated levels of both cytokines. The difference in immunostimulatory activity of triangle-CpG was estimated to be 10-fold comparing to free CpG in vivo. These data are in agreement with the in vitro stimulation of murine RAW 264.7 cells. Furthermore, a confirmatory follow-up animal trial was also performed by using square-CpG nanoparticles, and similar immunostimulatory activity were observed (Appendix 2, Supplementary Figure 2).
Discussion

Previous studies have demonstrated that rationally designed RNA nanoparticles can be used as potent multifunctional nanocarriers for delivery of therapeutics [73, 124, 125]. In this study, I demonstrated that rationally designed RNA polygons have the potential to serve as multivalent nanocarriers of vaccine adjuvants and particularly of CpG ODNs [305]. The designed RNAs self-assemble into distinct non-toxic homogeneous nanoparticles. I also found that size and shape of the RNA nanostructures are important factors for the induction of immune responses. There is a correlation between the RNA size and the number of CpGs that can be incorporated. The highest level of secretion of pro-inflammatory cytokines TNF-α, IL-6 was obtained with the smallest triangle nanoparticle harboring one CpG compared to square and pentagon nanoparticles. However, upon increasing the numbers of CpG per RNA nanoparticle the cytokines induction was affected more by pentagon. This study illustrates the importance of the size and shape of RNA nanoparticles for improvement of activity of CpG based vaccines targeting infectious diseases and cancer cells as well as for an increase immune-response by the innate and adaptive immune systems. The RNA nanoparticles harboring CpGs are effective, versatile and easy to manufacture, offering new solutions to address the unmet needs in current vaccines and adjuvants development.

In summary, the development of RNA nanoparticles for delivering CpG DNA as novel vaccine adjuvants can provide several advantages: (1) The RNA nanoparticle-based adjuvants have defined size, structure and stoichiometry. (2) Due to the multivalent nature of RNA nanoparticles, multiple antigen and adjuvants could be incorporated into one nanoparticle for achieving synergistic or enhanced immune repose such as cytokine
induction and antibody production. (3) Nanosize of the particles will facilitate tissue penetration and target to important immune tissues or organs such as lymph nodes for achieving targeted and enhanced immune stimulation. (4) Multiple targeting ligands could be incorporated into one particle for achieving better targeting to immune cells such as B cells, T cells, dendritic cells and macrophages. (5) RNA nanoparticles are also thermodynamically stable. For example, the three-way junction RNA nanoparticles are resistant to denaturation in 8 M urea and do not dissociate at ultra-low concentrations [124]. (6) The cellular uptake of the RNA nanoparticle-based adjuvants is greatly enhanced compared to CpG DNA only. The immune response induced by the RNA nanoparticle-based adjuvants is also greatly enhanced compared to CpG DNA only.

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Figure 5.1. Structural features of the pRNA 3WJ motif. (a) Secondary structure of 3WJ motif with base pairs annotated using Leontis-Westhof nomenclature [321]. (b) Tertiary structure of 3WJ motif with indication of $\angle AOB \sim 60^\circ$ angle formed between H1 and H2. Angle corresponds to inner angles of polygons.
Figure 5.2. Design, construction and characterization of RNA triangles harboring CpG DNA. (a) Schematic illustration of the design and self-assembly of RNA triangles harboring CpG DNA. (b) 2% agarose gel showing the step-wise self-assembly of the designed RNA nanoparticles. Cy5 channel showing the incorporation of Cy5 Triangle D strands into the RNA nanoparticles. EB channel showing the step-wise self-assembly of the total RNA and DNA strands. Representative results from at least two independent
experiments are shown. (c) AFM images of RNA triangles harboring CpG DNA. Representative results from a total of ten raw AFM images are shown. (d) The size of RNA triangles harboring CpG DNA determined by DLS. Representative results from three independent measurements are shown.
Figure 5.3. Evaluation of the interaction between RNA triangles harboring CpG DNA and macrophage-like RAW 264.7 cells. (a) Evaluation of the cell binding and entry by flow cytometry. Representative results from at least two independent experiments are shown. (b) Determination of TNF-α level in cell culture supernatant by ELISA. The error bars represent standard deviations from three independent wells. ****: p < 0.0001 versus 150 nM DNA CpG only control, unpaired Student’s t-test. The induction of TNF-α by triangle-CpG, triangle only control and CpG DNA only control was independently repeated two additional times with triplicate independent wells for each sample and similar results were observed. (c) Concentration study of RNA triangles harboring CpG
DNA. The error bars represent standard deviations from three independent wells. *: $p < 0.05$ versus 12.5 nM Triangle-CpG control, unpaired Student's t-test. ***: $p < 0.001$ versus 12.5 nM Triangle-CpG control, unpaired Student's t-test.
Figure 5.4. Effect of cytokine induction in macrophage-like RAW 264.7 cells and mice by RNA polygons harboring CpG DNA and RNA polygons control. (a) Induction of
TNF-α in RAW 264.7 cells by 50 nM RNA polygons harboring one CpG or multiple CpGs. The error bars represent standard deviations of measurements from three independent wells. **: p < 0.01 versus 250 nM DNA CpG only control, unpaired Student's t-test. ****: p < 0.0001 versus 250 nM DNA CpG only control, unpaired Student's t-test. The induction of TNF-α in RAW 264.7 cells by 50 nM RNA square harboring 4 CpGs, 50 nM RNA square only control and 200 nM DNA CpG only control was independently repeated at least three additional times with triplicate independent wells for each sample and similar results were observed. (b) Induction of IL-6 in RAW264.7 cells by 50 nM RNA polygons harboring one CpG or multiple CpGs. The error bars represent standard deviations from three independent wells. **: p < 0.01 versus 250 nM DNA CpG only control, unpaired Student's t-test. ***: p < 0.001 versus 250 nM DNA CpG only control, unpaired Student's t-test. ****: p < 0.0001 versus 250 nM DNA CpG only control, unpaired Student's t-test. The induction of IL-6 in RAW 264.7 cells by 50 nM RNA square harboring 4 CpGs, 50 nM RNA square only control and 200 nM DNA CpG only control was independently repeated at least three additional times with triplicate independent wells for each sample and similar results were observed. (c) Increase of TNF-α induction in RAW 264.7 cells with increasing number of CpGs per RNA square or pentagon. The error bars represent standard deviations from three independent wells. For RNA square samples: ***: p < 0.001 versus 200 nM DNA CpG only control, unpaired Student's t-test. ****: p < 0.0001 versus 200 nM DNA CpG only control, unpaired Student's t-test. For RNA pentagon samples: **: p < 0.01 versus 250 nM DNA CpG only control, unpaired Student's t-test. ****: p < 0.0001 versus 250 nM DNA CpG only control, unpaired Student's t-test. (d) Immunostimulatory activity by
triangle-CpG nanoparticles in animal model. The error bars represent the range from two independent measurements of the cytokine levels from serum aliquots of the tested mouse. **: p < 0.01 versus 2 mg/kg DNA CpG only control, unpaired Student's t-test. ***: p < 0.001 versus 2 mg/kg DNA CpG only control, unpaired Student's t-test. A confirmatory follow-up animal trial was performed by using square-CpG nanoparticles, and similar results were observed (Appendix 2, Supplementary Figure 2).
**Figure 5.5.** Effect of RNA triangle harboring CpG DNA, triangle control and CpG DNA only control on RAW 264.7 cell viability. The cells were incubated with different concentrations (200 nM, 100 nM, 50 nM and 25 nM) of RNA triangles only, RNA triangles harboring 3 CpGs and CpG only. The error bars represent standard deviations from three independent wells. *: p < 0.05 versus cell only control, unpaired Student's t-test. ns: non-significant, unpaired Student's t-test. A similar cytotoxicity assay was performed one more time by using KB cells, and similar results were observed (Appendix 3, Supplementary Figure 3).
Figure 5.6. Comparison of RNA polygon-CpG complexes binding to the RAW 264.7 cells. (a) The plot represents the summary of the flow cytometry data showing RNA nanoparticles binding to the cell in a dose dependent manner. Data was from one experiment, and four different concentrations of each sample and CpG DNA only control were tested in this experiment. The cell binding of triangle-3CpGs and CpG DNA only control was assayed by flow cytometry for two additional times and similar results were observed. (b) Confocal images showing the binding comparison of the triangle-CpG and
CpG only control to the RAW 264.7 cells by co-localization of nucleus (blue), actin (green), and CpGs (red) signals. Representative results from two independent experiments are shown.
Figure 5.7. Serum stability assay of RNA polygons harboring CpG DNA. Preassembled complexes (1 μM) of 2’-F modified RNA triangle, square and pentagon harboring CpG DNA were incubated in RPMI-1640 medium containing 10% fetal bovine serum. Aliquots (10 μL) were taken at 0 hr, 1 hr, 3 hr, 6 hr, 8hr and 16 hr time points after incubation at 37 °C, followed by analysis using 6% native PAGE gels. Data was from one experiment. A similar serum stability assay was performed one more time for RNA triangle harboring CpG DNA, and similar results were observed. Moreover, similar serum stability assays have also been performed for other 2’F-modified RNA nanoparticles for numerous times, and similar results were also observed and published previously [124, 125, 174].
Chapter 6: Conclusions and Perspectives
In the final chapter of this dissertation, I review the main conclusions of this dissertation and discuss the directions of future work in RNA nanotechnology and RNA nanomedicine.

**Conclusions**

The emerging field of RNA nanotechnology, which is the study of RNA nanostructures constructed by utilizing inter-RNA interactions, has opened a new arena in nanotechnology and nanomedicine. Indeed, RNA can offer several advantages for building controllable nanostructures with diverse applications. On one hand, RNA is almost as simplistic as DNA in terms of base pairing. Like DNA, RNA also has four different bases A, C, U and G, and most interactions between these bases can be predicted. On the other hand, RNA also has diverse folding and functions like protein. For example, many bulges, loops, pseudoknots and non-canonical base pairing exist in RNA structures that are critical to their biological activities [12, 29-32]. Moreover, many new biological functions have been discovered recently with RNA especially non-coding RNA [322]. For example, siRNA [323] and miRNA [324, 325] can be used to silence specific genes. Ribozymes can catalyze specific biochemical reactions [326, 327]. Riboswitches [328] and long non-coding RNAs [329] play important roles in gene regulation. Guide RNAs (gRNAs) can direct Cas9 (CRISPR associated protein 9) nuclease to edit genes at specific genomic loci [330]. All of these advantages make RNA nanotechnology a very intriguing and promising research field. In this dissertation, I have presented the design and construction of several RNA nanostructures with defined shapes and sizes, and studied their potential applications in nanotechnology and nanomedicine.
• First, I reported the design and self-assembly of multifunctional 3D RNA tetrahedrons based on the pRNA-3WJ motif [304]. The assembled RNA tetrahedrons have defined 3D structures as revealed by both AFM and cryo-EM. The size of the RNA tetrahedrons can be easily tuned by changing the number of RNA base pairs per edge. Several functional modules including aptamers, ribozyme and siRNA were successfully incorporated into the RNA tetrahedron. *In vitro* cell binding showed that the RNA tetrahedron functionalized with EGFR-aptamer has strong binding to EGFR-positive cells. *In vivo* biodistribution study showed that the RNA tetrahedron functionalized with EGFR-aptamer targeted to cancer xenografts in mouse models. These results indicated that 3D RNA tetrahedron nanoparticles have the potential to deliver imaging modules and therapeutics for cancer diagnosis and therapy.

• Secondly, I reported the design and self-assembly of RNA cube and dodecahedron cages with defined shapes and sizes by using the pRNA 3WJ as the basic building block. The assembled RNA cages have distinct 3D structures as revealed by cryo-EM and AFM. In addition, the designed RNA cube cages can be applied to deliver immunoactive CpG DNAs to macrophage cells and elicit enhanced immune responses.

• Thirdly, I reported the design and bottom-up self-assembly of the RNA nanowire with the length of several micrometers by using the modular multi-domain strategy [308]. The constructed RNA nanowire has been characterized by cryo-EM, negative staining TEM, fluorescent microscopy and AFM. This study demonstrated the expanding of the toolboxes of RNA nanostructures to RNA
microstructures. Comparing with previously reported RNA array and RNA filaments, this newly designed RNA nanowire was self-assembled purely based on RNA base pairing and only four artificially-designed short RNA strands were needed to assemble the RNA nanowire.

- Fourthly, I reported the design and construction of RNA polygons to serve as multivalent nanocarriers of vaccine adjuvants and particularly of CpG DNA [92]. The designed RNA and DNA strands self-assembled into distinct homogeneous nanoparticles with triangular, square or pentagonal shapes. I also found that size and shape of the RNA nanostructures played important roles in the induction of immune responses. This finding demonstrated that rationally designed RNA nanoparticles have great potential to be developed as novel immunomodulators or vaccine adjuvants.

**Perspectives**

RNA is not only an informational molecule that can transfer genetic information, but also a structural molecule that can be utilized for programmable self-assembly by engineering its sequence. In addition, RNA molecules can be designed to form artificial nanostructures with defined chemistry, structure, size and stoichiometry for applications in nanotechnology and nanomedicine [286]. In the future, one new direction of research is to expand the structural and functional diversity of 3D RNA nanostructures by exploring novel RNA motifs and RNA folding pathways. In addition, it is also possible to employ RNA-binding proteins to build artificial RNA-protein chimeric nanostructures that equipped with both RNA-based functionality and protein-based functionality [128].
It will be also interesting to explore the possibility to incorporate classic methodologies in DNA nanotechnology (such as the DNA origami technique [331] and computer-aided design of DNA nanostructures by utilizing specialized software [332]) into RNA nanotechnology. It is expected that RNA mimics of DNA motifs widely used in DNA nanotechnology such as the DNA 4WJ [333] and DNA double-crossover molecules [334] will also play important roles in the construction of artificial RNA nanostructures. To obtain a more thorough understanding of the 3D conformation of the designed RNA nanostructures, high-resolution AFM, cryo-EM, X-ray diffraction and nuclear magnetic resonance (NMR) spectroscopy can be applied to characterize these RNA nanostructures with the aim to acquire atomic-level details. From an application perspective, one intriguing research direction is to test drug loading to these artificial RNA nanostructures. Particularly, 3D RNA nanoparticles may have the potential to encapsulate and protect a range of different therapeutics including nucleic acid therapeutics, small molecule therapeutics and protein therapeutics. For example, nucleic acid therapeutics such as siRNA and miRNA can be loaded into RNA nanoparticles via sticky end-mediated cohesion, while small molecule therapeutics such as paclitaxel and doxorubicin can be loaded via covalent conjugation or non-covalent binding to the RNA double helix. In addition, it will also be appealing to combine RNA nanoparticles with other nanotechnology approaches and investigate whether other nanotechnology approaches can improve the pharmacological properties of RNA nanoparticles. For instance, it will be interesting to encapsulate RNA nanoparticles into liposomes [335] or exosomes [336] and study the intracellular traffic and fate of liposome/exosome-encapsulated RNA nanoparticles. Furthermore, for the successful clinical application of RNA nanoparticles,
the production of clinical-grade RNA nanoparticles in large scale under Good Manufacturing Practice (GMP) regulation [337] will also be an important prerequisite. Currently, there are two mainstream approaches to produce RNA molecules: 1) in vitro transcription based on corresponding DNA templates [338]; 2) solid-phase synthesis based on phosphoramidite chemistry [339]. It is expected that automation of these synthesis procedures will greatly reduce the cost and improve the efficiency for large-scale production of RNA molecules. Another alternative approach for RNA production is the expression of recombinant RNA molecules in bacterial cells such as Escherichia coli [331, 340]. Since recombinant protein technology has been widely used in the modern biotechnology industry for manufacturing protein therapeutics [341], the future improvements of recombinant RNA technology also has the potential to pave the way for producing highly pure RNA molecules for clinical applications.
Appendix 1

Supplementary Figure 1: Schematic of the 3D structure of an A-form RNA helix [137]. Reprinted with permission from Ref. [137]. Copyright 2014 The American Association for the Advancement of Science. A-form RNA helix has 11 base pairs per helix turn, 32.7° helix twist per base pair and 2.9 Å rise per nucleotide. The minor groove is also named as shallow groove since it is wide and shallow compared to the major groove. Moreover, from the top view the RNA helix has a hollow central cavity, which is significantly different from DNA.
Supplementary Figure 2: Immunostimulation by square-CpG nanoparticles in mice. CpG DNA containing square nanoparticles, free square nanoparticles and free CpG DNA were dissolved in PBS and administered to CD-1 mice by injection into the tail vein at 2 mg/kg (CpG oligonucleotide per body weight). Blood samples were collected 3 hours post-injection by cardiac puncture. Serum was prepared by centrifugation at 12800 g for 10 min. The levels of both TNF-α and IL-6 in serum were determined by ELISA. The error bars represent the standard error of the mean (SEM) from the measurements of six mice per treatment group. **: p < 0.01 versus 2 mg/kg DNA CpG only control, unpaired Student's t-test. Data was collected in collaboration with Sijin Guo and Mengshi Ma.
Appendix 3

Supplementary Figure 3: Effect of RNA triangle harboring CpG DNA, triangle control and CpG DNA only control on KB cell viability. KB cells were incubated with 100 nM or 300 nM triangle, triangle-3CpGs, and CpG DNA only respectively for 16 hours. After 16 hours, cell viability was examined by MTT assay. The error bars represent the range of measurement from two independent wells. ns: non-significant compared with cell only control, unpaired Student's t-test.
RNA as a stable polymer to build controllable and defined nanostructures for material and biomedical applications

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Summary The value of polymers is manifested in their vital use as building blocks in material and life sciences. Ribonucleic acid (RNA) is a polynucleic acid, but its polymeric nature in materials and technological applications is often overlooked due to an impression that RNA is seemingly unstable. Recent findings that certain modifications can make RNA resistant to RNase degradation while retaining its authentic folding property and biological function, and the discovery of ultra-thermostable RNA motifs have adequately addressed the concerns of RNA instability. RNA can serve as a unique polymeric material to build varieties of nanostructures including nanoparticles, polyrons, arrays, bundles, membrane, and microsponges that have potential applications in biomedical and material sciences. Since 2005, more than a thousand publications on RNA nanostructures have been published in diverse fields, indicating a remarkable increase of interest in the emerging field of RNA nanotechnology. In this review, we aim to: delineate the physical and chemical properties of polymers that can be applied to RNA; introduce the unique properties of RNA as a polymer; review the current methods for the construction of RNA nanostructures; describe its applications in material, biomedical and computer sciences; and, discuss the challenges and future prospects in this field.

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Introduction

Polymers have been extensively used by humans since the earliest recorded history. Tree saps, tar and plant fibers, all represent the earliest natural polymeric materials used by humans [1].

Since the 1950s, through continued synthesis and manufacturing improvements, synthetic polymers have dramatically improved in quality and strength to the point where they, in many cases, can have mechanical properties that better match desired use, as compared to alternative natural counterparts. Synthetic polymers have the manufacturing advantages of rapid production rates and synthetic simplicity. However, owing to the inherently random nature of most polymerization mechanisms, chain polydispersity and controlled shaping remain two of the key limiting hurdles that have led to increased variable performance and overall reduced control of the final properties [2–4]. As such, there has been much effort towards developing synthetic building blocks and schemes that can create monodisperse polymer systems [5–7]. These, however, are typically very expensive, time consuming and difficult to control.

As a result of these developments, we are at another unique turning point in the evolution and design of polymers. We once again turn towards nature as our inspiration for the development of advanced biomaterials. By applying what we have learned about the complexity and versatility of nucleic acid structures, it is now possible to create materials with revolutionary properties previously considered unattainable through synthetic strategies. Recent advances in RNA chemistry, RNA biology and RNA nanotechnology have shown that RNA as a biopolymer not only shares the common characteristics of other polymers, but also possesses a range of unique properties advantageous for applications in nanotechnology as well as biomedical and material sciences [8–14].

In this review, we will (1) delineate the physicochemical properties of polymers that can be applied to RNA, (2) introduce the unique properties of RNA as a polymer, (3) review the current methods for constructing RNA nanostructures for diverse applications, and (4) discuss the future prospects including challenges, solutions and new directions of this nascent field.

Physical and chemical properties of polymers in relationship to RNA nanotechnology

The word (poly)-(mer) means (many)-(parts) and refers to macromolecules consisting of a large number of repeating elementary units covalently joined together. These ‘mers’ control the inter-chain interactions, and dictate the final polymer characteristics. Given the large variations in polymer morphologies, multiple ways are used to describe polymer structure. Polymers can be formed from one type of monomer (homopolymers) or of various monomers (heteropolymers). Heteropolymers can be further described by the arrangement of the monomers along the backbone, including alternating, random, and block formations and also adopt more elaborate structures, including star, ring, branched, dendrimeric, crosslinked, ladder, and others [15–23]. These large arrays of potential structures and chemical diversity offer a rich repertoire of available properties from which one can custom-design a polymer for a desired application.

Biological macromolecules, as natural building blocks, are critical for the functioning of living organisms. RNA is one of the five most important biological macromolecules in addition to DNA, proteins, lipids and carbohydrates. With some aspects similar to DNA, RNA, composed of four nucleotides including adenosine (A), cytosine (C), guanosine (G) and uridine (U), is special in its homogeneity. RNA is a homopolymer of nucleotide, but is also a heteropolymer of A, U, G, and C. Each nucleotide contains a ribose sugar, a nucleoside, and a phosphate group. The nucleotides are covalently linked together through 3′ → 5′ phosphodiester bonds between adjacent ribose units, giving the directionality to the sugar-phosphate backbone that defines RNA as a polynucleic acid. The phosphate moieties in the backbone are negatively charged, making RNA a polyanionic macromolecule at physiological pH. RNA molecules are typically single-stranded; however, Watson-Crick (canonical) base-pair interactions (A:U and G:C), wobble base pairing (such as G:U [24] or other non-canonical base pairing such as twelve basic geometric families of edge-to-edge interaction (Watson-Crick, Hoogsteen/CH or sugar edge) with the orientation of glycosidic bonds relative to the hydrogen bonds (cis or trans) [25–28], all together give rise to various structural conformations exhibiting loops, hairpins, bulges, stems, pseudoknots, junctions, etc., which are essential elements to guide and drive RNA molecules to assemble into desired structures [12,29–31].

On the contrary, synthetic polymers are usually formed via polymerization reaction, a chemical process through which many monomers form covalent bonds between each other and finally form a long chain or network, imparting them with their desired structural properties.

Generally, the properties of polymers are strongly dependent on their monomer chemistry. Moreover, the molecular weight and 2D structure (e.g., linear chain-shaped structure or branch-shaped structure) also play important roles in determining the characteristics and properties of a specific polymer. Important properties of chemical polymers include [32]: (1) rheological properties, (2) solubility, (3) volumetric and viscometric properties, (4) stress-strain relationships, (5) electrical properties, (6) thermal properties, (7) optical properties, (8) stiffness, (9) flex life, (10) hardness, (11) chemical resistance, etc. Various physical or chemical tests can be readily performed to characterize these properties for RNA polymers.

Traditional synthetic polymers are heterogeneous and have typical polydispersity index greater than 1 [33,34]. In contrast, RNA is synthesized by either in vitro transcription or solid phase chemistry. Both approaches are based on stepwise reactions and will generate RNA polymers with defined sequences, structures, and molecular weights. This leads to a polydispersity index of 1 of a specific RNA. Structurally, the average molecular weight determines the mechanical property of the polymer. However, as polydispersity increases, the increased lower molecular weight chains can act as a lubricating/solvating system, reducing its mechanical strength and integrity. As increases in temperature result in increased molecular motion, these weak associations begin to destabilize and reduce
mechanical integrity. Such polymers are classically referred to as thermoplastics [35], as their mechanical strength weakens with increased temperature. However, this property also means they can be intentionally deformed at higher temperatures and then regain their structural properties once cooled. Thermodynamically, RNA molecule is relatively more stable at lower temperature and tends to dissociate or misfold at higher temperatures. Thus, RNA displays the typical thermoplasticity of polymers. Boiling-resistant RNA has been discovered and constructed [36], suggesting a potential application of RNA in molding technology.

Chemical polymers have a wide range of applications in biomedical and material sciences. Specific applications include, but not limited to [1, 37–50]: (1) sutures for surgery, (2) dental devices, (3) orthopedic fixation devices, (4) scaffolds for tissue engineering, (5) drug formulation (e.g., controlled release of drugs, excipients for drug stabilization or solubilization), (6) biodegradable vascular stents, (7) biodegradable soft tissue anchors, (8) implantable biomedical devices, (9) flexible transparent displays, (10) field effect transistors, (11) solar cell panels, (12) printing electronic circuits, (13) organic light-emitting diodes, (14) supercapacitors, etc. Usually, an ideal chemical polymer for biomedical and industrial applications should exhibit the majority of the following properties: (1) non-toxic, (2) no or low immunogenicity (if applicable, for in vivo use), (3) biodegradable and metabolizable, (4) mechanical features that fit the needs of the specific application, (5) easily sterilizable, (6) processability, (7) scalability, (8) electrical conductivity, and (9) thermostability. RNA as a polymer favorably displays many of these properties, as described below.

Unique properties of RNA as a polymer to build nanostructures

The 2'-hydroxyl group makes RNA dramatically different from DNA

The characteristic of RNA that defines and differentiates it from DNA is the 2'-hydroxyl on each ribose sugar of the backbone. The 2'-OH group offers RNA a special property, which can be either an advantage or a disadvantage. From a structural point of view, the advantage of this additional hydroxyl group is that it locks the ribose sugar into a 3'-endo chair conformation. As a result, it is structurally favorable for the RNA double helix to adopt the A-form which is ~20% shorter and wider rather than the B-form that is typically present in the DNA double helix. Moreover, the 2'-OH group in RNA is chemically active and is able to initiate a nucleophilic attack on the adjacent 3' phosphodiester bond in an S<sub>2</sub>2 reaction. This cleaves the RNA sugar-phosphate backbone and this chemical mechanism underlies the basis of catalytic self-cleavage observed in ribozymes [51]. The disadvantage is that the 2'-OH group makes the RNA susceptible to nuclease digestion since many RNases recognize the structure of RNAs including the 2'-OH group as specific binding sites. However, such enzymatic instability has been overcome by applying chemical modification of the 2'-OH group (see the sections Enzymatic stability of RNA and Methodology II: Chemical modification of RNA).

Thermodynamic stability of RNA

From a thermodynamic point of view, the stability of the double helix of RNA can be evaluated by measuring the Gibbs free energy (ΔG<sub>th</sub>) required for double helix formation, or conversely, double helix unwinding (ΔG<sub>th</sub> = −ΔG<sub>th</sub> formation + ΔG<sub>th</sub> unwinding). Remarkably, the RNA double helix is more thermodynamically stable than the DNA double helix considering ΔG<sub>th</sub> for RNA double helix formation is, on the average, −3.6 to −8.5 kJ/mol per base pair stacked and ΔG<sub>th</sub> for DNA double helix formation is −1.4 kJ/mol per base pair stacked [52]. Moreover, the presence of special motifs such as bends, stacks, junctions and loops in the tertiary structure of RNA may also further improve its stability [8]. In addition, various proteins, such as RNA chaperone proteins, and metal ions, such as Mg<sup>2+</sup> may also interact or coordinate with RNA and significantly contribute to the stability of RNA [53,54].

Enzymatic stability of RNA

In biological systems, the instability of RNA is mainly the result of enzymatic degradation by ribonucleases (RNases). The presence of RNases in living organisms is universal, suggesting that RNA-mediated RNA degradation is a vital process for normal biological function. Several important roles of RNases have been revealed, including degrading surplus cellular RNA [55,56], editing messenger RNA, processing microRNA and other non-coding RNA during their maturation [57,58], defending against the invasion of viral RNA [59], and providing crucial machinery for RNA interference (RNAi) [60,61]. RNases can be divided into two major categories: (1) endo-ribonucleases that cleave phosphodiester bonds within the RNA backbone [62]. Examples include RNase A, RNase H, RNase I, RNase II, RNase L, RNase P, RNase PH, RNase T1, RNase T2, RNase U2, RNase V, etc.; (2) exo-ribonucleases that cleave phosphodiester bonds at either the 5' or the 3' end of an RNA chain [63]. Examples include polynucleotide phosphorylase (PNPase), RNase PH, RNase II, RNase R, RNase D, RNase T, oligoribonuclease, exoribonuclease I, exoribonuclease II, etc. RNA is indeed very sensitive to degradation by RNases, which confers a very short half-life and thus a poor pharmacokinetic profile to most RNA molecules. This degradation limits the in vivo application of RNA molecules as therapeutics. However, chemical modifications of RNA can overcome this shortcoming. For example, the substitution of the 2'-hydroxyl group with a Fluorine (2'-F), O-methyl (2'-O-Me) or Amine (2'-NH<sub>2</sub>) dramatically increases the stability of RNA in vivo by preventing degradation by RNases [64–66]. Recent studies also showed that the stability of siRNA in serum is also highly dependent on the specific RNA sequences and the degradation of both short and long RNA duplexes mostly occurred at UA/UA or CA/UG sites [67].

Synthetic polymers always show good stability against various enzymes. However, biodegradable polymers that can be degraded by enzymes such as proteases, oxidoreductases and phospholipases are more beneficial for therapeutic applications [68]. The nanocarriers composed of enzyme-sensitive polymers can control the site of cellular uptake of carriers and drug release to improve the drug efficacy [69–71]. Synthetic polymers can also be designed to be
non-degradable or degradable under certain physiological conditions. For example, Poly(lactide-co-glycolide acid) (PLGA) (which is generally regarded as safe) is approved by FDA, as it can be degraded in the body by hydrolysis and has now been widely used to fabricate nanoparticles for drug delivery. Poly (beta-amino ester) is another class of degradable biomaterial developed by Langer group [72], which can bind negatively-charged RNA or DNA and facilitate gene transfections. Although the biodegradable polymers are highly attractive for drug delivery purposes, their degradation rate is very hard to control and the pharmacokinetics and biodistribution profiles of the nanocarriers can be elusive. Synthetic polymers usually are composed of repeated same subunit, while RNA is the repetition of four subunits A, U, G and C in a specific sequence order. Therefore, RNA polymers might be advantageous concerning controllable biodegradability [73, 74] simply by tuning the ratio and location of 2'-modified nucleotides in the RNA sequence.

The versatile and plastic properties of RNA

The versatility of RNA is highly evident given the diversity of structural repertoires available in nature, which include simple structures such as helical stems and single stranded hairpin loops to more complicated structures such as multi-way junctions and pseudoknots [75–84]. Persistence length is a basic mechanical property used in polymer science to measure the flexibility and stiffness of a polymer. If a polymer is shorter than the persistence length, the molecule will basically be a rigid rod. From a mechanical perspective, the persistence length of dsRNA (62–63 nm) is slightly longer than dsDNA (45–50 nm) in an aqueous solution [85, 86]. The stretch modulus of short dsRNA (<150 bp) is ~100 pN, which is 3 times lower than dsDNA of similar length, is based on AFM and tweezer studies [87]. Interestingly, RNA was observed to shorten upon twisting with two-orders of magnitude slower timescale compared to DNA, which in contrast lengthened with a faster timescale. More recently, single molecule force spectroscopy were used to study ~10 nm long dsDNA containing ribonucleoside monophosphates (rNMPs) located at specific positions within the DNA strands [88]. The results demonstrated that the perturbation of stiffness and the plastic properties by Rg intrusions is location and sequence dependent, and can either soften or stiffen the DNA complex. MD simulations indicated that the perturbations are from local structural distortions arising from hydrogen bonding between the O1 group of Rg and electronegative sites of either the phosphate backbone or vicinal base. However, many factors such as sequence, local structural environment, and metal ion concentrations [89, 90] can significantly influence RNA versatility, stiffness and plasticity at the molecular level [91]. The balance of the ion-mediated electrostatic force and the non-electrostatic (chemical) forces determine the equilibrium structure of the RNA. It is common to find that one RNA molecule can exist in more than one conformation, and one kind of RNA molecule can display multiple bands in native gel electrophoresis due to the redistribution of modular motif and local thermal energy. The versatile and plastic properties of RNA as polymer have been applied to the construction of RNA polymers by stretching the internal angle of the three-way junction (3WJ) of phi29 DNA packaging motor from 60° to 90° to 108° to transit from triangle, square, and pentagon structures, respectively [92].

RNA can form complex 3D structures in nanoscale

While most biologically active DNAs are double-stranded, in contrast, most RNAs in living organisms are single-stranded. However, RNA molecules contain self-complementary sequences which facilitate the self-folding of these RNAs. In addition to Watson-Crick base paring, non-Watson-Crick base pairs and coaxial stacking of helices also play important roles in promoting RNAs folding into complex 3D structures [12, 53]. This remarkable folding capability not only provides the structural basis for the diverse biochemical functions of different RNA molecules, but also provides huge opportunities for designing novel RNA nanostructures. For example, the subdomain Ila-1 RNA of the IRES from HCV has a unique 90° bend L-shaped structure. Molecular modeling using this L-shaped motif showed that a square shaped nanoparticle can be formed with four repeating L-shaped motifs, as verified by gel shift assay and FRET assay [93] (Fig. 1H). Moreover, it has been found that proteins, small molecule ligands as well as monovalent and/or divalent metal ions are also important mediators of RNA folding, which could add another layer of complexity to the assembly of RNA nanostructures [53].

Toolkits and methodologies for the construction of RNA nanostructures

The diverse function of RNA molecules in cells such as ribozymes, riboswitches, microRNAs, long non-coding RNAs and aptamers had attracted increasing attention in the scientific community. All these properties of RNA originated from nature that RNA can adapt different structures and spatial conformations. The laws of how nature manipulates RNA structures can also be explored to construct artificial RNA nanostructures. Besides the template-guided folding of RNA nanostructures that is similar to the tactics in DNA nanotechnology, self-assembly of RNA building blocks is another leading approach for bottom-up RNA nanotechnology. A wide variety of RNA nanostructures have been successfully constructed based on different assembly principles and approaches (Fig. 1). In this section, we will review several toolkits and methodologies that have shown great success for constructing multifunctional RNA nanostructures.

Toolkit I: Hand-in-hand interactions involving RNA loops

Taking the construction of a wooden table as an analogy, external dowels are used to link variety of wood blocks into a structure. One of the unique properties of RNA is its folding into loops and hairpin structures. RNA loops can serve as inter-RNA mounting dovetails, thus external-dowels are not necessary for self-assembly into unique structure. The packaging RNA (pRNA) in phi29 DNA packaging motor [94–102] forms a hexameric ring structure through interlocking of two looped regions in each pRNA molecule, named
the right- and left-hand loops (Fig. 1A). The left-hand and right-hand were brought together from interlocking interaction among four nucleotide sequences in the loops. Single pRNA molecule with self-complementary sequences in the loops can be used to construct homodimer nanoparticles, or several pRNAs with the right-hand loop matching the sequence of the left-hand loop in another pRNA can be used to construct heterodimer nanoparticles. Through the interlocking loop interactions between different pRNA, dimer, trimer, and hexamer RNA nanoparticles have been created [103–112]. The interlocking loop sequences were further extended to increase the thermodynamic stability of generated RNA nanoparticles [113]. A toolkit with a set of hand-in-hand loop sequences has been designed and tested for constructing stable RNA polygonal nanoparticles. Loop extended RNA pairs with confirmed dimer formation capability were used as building blocks for higher order RNA oligomer nanoparticles (Fig. 1B). Using a reengineered loop extended pRNA toolkit, pRNA dimer, trimer, tetramer, pentamer, hexamer, and heptamer were constructed with highly efficient self-assembly, as shown by gel shift assays and AFM imaging [113].

Other loop-loop interactions have also been reported to build various RNA nanostructures. For example, noncovalent loop-loop interactions based on RNA/III kissing-loop complex have been used to build RNA nanorings which are thermostable, ribonuclease resistant and capable of delivering RNA interference modules [85,114,115] (Fig. 1E).
Moreover, RNA kiss-loops were also used to build square-shaped tetrameric RNA nanoparticles and three-dimensional polyhedrons based on rationally designed RNAs [116–118] (Fig. 1D and F). In addition, micrometer-scale RNA filaments have also been constructed by the rational design of tecto-RNAs incorporating 4-way junction (4WJ) motifs, hairpin loops and their cognate loop–receptors [119,120].

**Toolkit II: Foot-to-foot interactions involving palindrome sequence and sticky ends**

pRNA nanoparticles can also be constructed through the foot to foot interaction between pRNA monomers. While hand-in-hand interaction involve “left” and “right” hand and the interaction is “multiple” and “inter” heterosexual, foot-to-foot interaction includes only one foot that is the one unique palindrome sequence by self-homosexual interaction of the identical RNA molecule. The foot domain is located in the helical 5’/3’ open region of pRNA. Extending this region with helical sequences does not compromise the entire pRNA molecular folding. A palindrome sequence reads the same whether from 5’ → 3’ direction on one strand or from 5’ → 3’ direction on the complementary strand. Extending the 3’ end of pRNA monomer with a palindrome sequence can serve as a sticky end for linking two pRNA monomers, and is denoted as a foot-to-foot interaction [112]. The foot-to-foot interaction by palindromic sequences can also be utilized to bridge RNA nanostructures, motifs, or scaffolds for constructing RNA hexamers, octamers, decamers, and dodecamers (Fig. 1K). A pRNA array was constructed by combining hand in hand-loop interactions and foot-to-foot palindrome sequence interactions [112] (Fig. 1C). Recently, strategies involving both loop–loop interaction and sticky-end cohesion were also reported to assemble three-dimensional and structurally well-defined DNA nanostructures by re-engineering pRNA [121].

**Toolkit III: Grafts of motifs from naturally-occurring RNA molecules**

Synthetic RNA nanoarchitectures can be designed with the known naturally-occurring three-dimensional RNA motifs. Particularly, some RNA motifs in nature exhibit extraordinary stability, for example, phi29 motor pRNA [54,110,122], tRNA [118], 5S RNA [123], and others [115]. These motifs have been used to create synthetic RNA nanostructures [36,93,118,124,125]. The central domain of the pRNA molecule contains a three way junction (3WJ) core structure [54]. This 3WJ can be assembled from three individual RNA fragments with high efficiency in the absence of metal ions [123]. The 3WJ motif itself creates a branched structure that allows for constructing multifunctional RNA nanoparticles with different functional moieties at each end of the branches. RNA nanoparticles can also be designed and fabricated via branch extension tool kit [113] based on the 3WJ motif (Fig. 1L). 3WJ core retained its original folding and the conjugated RNA module on the branches can fold into their authentic structure and remain functional [123,126]. For example, after incorporated into 3WJ motif, HBV ribozyme retains its capacity to cleave its RNA substrate and generate smaller cleavage products, and MG binding aptamer was also able to bind MG to emit fluorescence as shown in the fluorescent spectra (Fig. 2A and B). This property makes pRNA 3WJ structure an extraordinary nanocarrier for targeted gene delivery [123]. Moreover, an X-shaped motif from the central domain of the pRNA was discovered through extending the right-hand loop with a double-stranded sequence [73]. The X-shaped pRNA motif can be self-assembled from four RNA oligos and it creates a scaffold that allows for the conjugation of four RNA functional moieties at the same time. Functional RNA modules such as survivin siRNA preserved their target gene knockdown effect after being fused to the X-shaped structure (Fig. 2C). Branched hexavalent RNA nanoparticles were further constructed from three 3WJ motifs, integrated by one 3WJ core motif structure or by palindrome sequence mediated linking of two pRNA-X cores [113].

Recently, Khisamatulin et al. reported using RNA as an anionic polymer to build programmable self-assembling boiling-resistant RNA nanostructures based on pRNA-3WJ [36] (Fig. 1N). A triangular shaped RNA nanoparticle was designed and prepared by carefully joining extended helices to the thermodynamically stable pRNA-3WJ motif of the bacteriophage phi29 DNA packaging motor. The step-wise self-assembly of the triangle RNA nanoparticles was confirmed by native PAGE analysis, and AFM imaging revealed the triangular shape of the nanoparticles as expected. The constructed nanoparticles are thermodynamically ultra-stable and robust. Functional motifs including siRNA, ribozyme, folate and fluorogenic RNA aptamers retained their activities after conjugation to the RNA nanoparticles. Moreover, these RNA triangles could be used as building blocks to construct supramolecular complexes such as RNA hexagons and patterned hexagonal arrays. The pRNA-3WJ is also highly tunable [92], the naturally preserved angle between the helices H1 and H2 of pRNA-3WJ is ~60° and this angle can be stretched to form square-shaped (90°) and pentagonal shape (108°) nanoparticles with pre-designed sequences (Fig. 3A). The polygons formed by one-step self-assembly manner with efficiency ~90%, as demonstrated by gel shift assays. The different sizes and shapes of the polygons were also confirmed by dynamic light scattering (DLS) and AFM. The equilibrium dissociation constants (Kd) for triangle, square and pentagon are 18.8 nM, 20.3 nM and 22.5 nM, and the Tm values are 56 °C, 53 °C and 50 °C, respectively, revealing structure and shape-dependent thermodynamic features. In a recent paper by Jasinski et al., the authors showed that square-shaped RNA nanoparticles with fluorogenic and ribozymatic properties as well as different sizes can be successfully constructed by utilizing the pRNA-3WJ at each corner and different length RNA duplexes at each edge of the square RNA nanoparticles [125] (Fig. 3B). DLS and AFM determined the sizes of the small, medium, and large square RNA nanoparticles are 4.0, 11.2, and 24.9 nm, respectively. The physiochemical properties of the nanoparticles can be easily tuned, as the utilization of 2’-F RNA as the core strand significantly increased the melting temperature as well as the nanoparticle’s resistance to serum-mediated degradation.

Naturally occurring stable RNA-protein complexes were also explored as building blocks for nanostructures. For example, the assembly of archaeal box C/D SRNPs comprising the L7Ae protein from Archaeoglobus fulgidus and
a box C/D RNA has been utilized to build an equilateral triangle shaped synthetic RNA protein complex [127] (Fig. 1H). The construction relies on the interaction between ribosomal protein L7Ae and the Kink-turn motif in box C/D RNA. L7Ae can bind to the box C/D K-turn motif through hydrogen bonding with high specificity and affinity [128]. It was reported that using RNA-protein complex (RNP) as building block for nanostructures enhanced its stability in serum comparing to pure unmodified RNA based nanoparticles [129]. The RNP nanostructure could be served as a scaffold for protein or RNA functionalities. For example, an affibody peptide, which can recognize HER2 receptor, was connected to RNP triangle through fusing to L7Ae protein, and the functionality of affibody peptide was still retained. siRNA targeting to GFP gene can be conjugated to the RNP triangle through extending the RNA strand and the GFP siRNA on RNP nanoparticles still can be processed by dicer and knock down GFP expression in cells.

**Toolkit IV: RNA origami**

DNA origami, which involves the folding of a long template single stranded DNA aided by multiple short staple DNA strands, was reported in 2006 [130]. A wide range of 2D and 3D nanostructures have been successfully constructed using the DNA origami technique [131–135]. In 2014, a similar approach for RNA origami was reported to fold single stranded RNA into RNA tiles and further assembled into hexagonal lattices [136] (Fig. 1M). A variety of RNA tertiary motifs, including the 180° kissing loop motif of the HIV-1 DIS [137], the 120° loop–loop complex of RNA i/ii inverse loop [114], and the tetraloops GNRA and UNCG [138] were used to mediate internal RNA tertiary interactions, resulting in organized and scalable RNA crossover tiles. The designed RNA origami nanostructures are also robust, which could form by either annealing or co-transcription on mice. In contrast to DNA origami, this RNA origami approach does not need short staple strands to facilitate the folding of the origami structure, but using RNA modules such as kissing loops to replace the role of staple strands. Interestingly, a different RNA origami approach which is a direct extension of the DNA origami to RNA was also reported in 2014 [139] (Fig. 1P). In this study, a single-stranded RNA scaffold and multiple staple RNA strands were used to assemble defined RNA nanostructures including a 7-helix bundled RNA tile and a 6-helix bundled RNA tube. The authors also showed that functional modules such as biotin could be introduced into the RNA origami structures by chemical modifications of the scaffold strands.

**Toolkit V: RNA/DNA hybrid nanostructures**

RNA/DNA hybrids can also be used to construct functional nanoarchitectures. Similar to split-protein systems, RNA/DNA hybrids can be computationally designed for activating different split functionalities in the presence of respective equivalent strands [140–143] (Fig. 5A). Toehold interactions were used to trigger dissociation of RNA/DNA hybrids and re-association of the double-stranded RNA and DNA. The thermodynamics and kinetics of the toehold interactions can be tuned to control the dissociation and re-association processes. RNA interference modules
as well as other functionalities such as fluorophores and RNA aptamers can be successfully triggered inside mammalian cells. This RNA/DNA hybrids concept has also been applied to other nanostructures such as nanorings and cubes [114,115,144–146], and the split functionalities still retain their authentic functionalities. Because RNA/DNA hybrid is more enzymatically stable than RNA, this approach has the potential to improve the pharmacological profiles of RNA-based nanoparticles. Moreover, controlled release of the split functionalities opens a new avenue for the development of nucleic acid-based switches to modulate cellular functions in vivo.
Methodology I: Rolling circle transcription

Typically, the design and production of RNA nanoparticles have relied on the production of discrete RNA strands that can be assembled in a controlled and predictive fashion to generate nanoparticles with a defined structure and stoichiometry. Hammond and colleagues have departed from this approach with an innovative strategy that generates monodisperse spherical RNA particles from extremely high molecular weight RNA strands by using a rolling circle transcription (RCT) approach [147,148] (Fig. 1J). They created a circular DNA construct containing a siRNA gene without any terminator sequences and proceeded by a T7 promoter. As a result, T7 RNA polymerase can continuously transcribe the circular DNA hundreds of times to generate a tremendous number of copies of the tandem RNA unit. As the continuous RNA strand is transcribed, magnesium pyrophosphate crystals are simultaneously generated, and the RNA strands attach to the crystallite surfaces to form composite structures; each structure grows in length to become fiber-like, then, forms sheet-like lamellae. The lamellae, typically ~10 nm thick, finally condense into spherulites with diameters varying from a few micrometers to hundreds of micrometers, referred to by the author as RNAi microsponges. To achieve efficient cellular uptake, PEI was introduced to condense the RNAi-microsponge from 2 μm to 200 nm, and to protect the RNA from degradation by RNase. The PEI introduced a positively charged outer layer to the microsponges to facilitate cell binding and entry. It was also demonstrated that the spherical RNAi-microsponges produced ~21 nt siRNA fragments after incubation with Dicer and could transfect a cancer cell line and silence firefly luciferase expression. Gene expression knockdown in vivo by intratumoral injection of the PEI-condensed RNAi-microsponges was also reported. This innovative approach opens new directions to RNA nanoparticle self-assembly and siRNA delivery. However, the therapeutic potential and in vivo safety of this approach requires further evaluation, as PEI has been shown to cause well-known adverse side effects, such as high cytotoxicity [149,150]. In addition, further reduction of the size of the particles is recommended to avoid non-specific healthy organ accumulation.

In material sciences, polymeric membranes are at the forefront in the chemical and biotechnology industry because of their versatile applications such as water purification, dehydrogenation of natural gas, dialysis of blood, removal of cell particles, and others [151]. Recently, Lee's group demonstrated rolling circle transcription can also be applied to the synthesis of macroscopic RNA membranes that has the potential as a controlled drug-release system [152]. (Fig. 1C). The RNA membrane was fabricated by T7 RNA polymerase transcription system with a combination of complementary rolling circle transcription (cRCT) and evaporation-induced self-assembly (EISA). The circular DNA template and complementary circular DNA template were prepared with long linear DNA and the promoter DNA for starting the RCT, respectively. Upon addition of T7 polymerase to these two circular DNA templates, thousands of copies of single stranded RNA and single stranded complementary RNA were continuously generated. The two complementary RNA strands hybridized to each other thereby forming the double stranded RNA for the large-area of RNA membrane. After the cRCT process, the water in the RNA membrane was evaporated and the dried RNA membrane was concentrated by EISA process. The self-assembled RNA membrane was fabricated densely on the tube wall during the evaporation process. The durability of the fabricated RNA membrane was tested under various harsh conditions, including RNase and DNase-rich environments. In addition, they confirmed the application of the RNA membrane as an enzyme-responsive drug-release system with doxorubicin and siRNA. The results showed that the RNA membrane can provide a high drug-loading efficiency and can be a great candidate for future membrane industry.

Methodology II: Chemical modification of RNA

A great deal of work on chemical modifications aiming to improve the chemical stability and in vivo properties of RNA have been reported [153–156]. Common chemical modifications of RNA can be categorized into five classes: (1) Modification of inter-nucleotide phosphodiester backbone. This type of modification is the most classic and simplest method to improve the performance of RNA in the biological environment. For example, creation of a phosphorothioate (PS) linkage by replacing one non-bridging oxygen atom on the phosphate backbone with a sulfur atom substantially increases the stability of RNA in vitro and in vivo [157]. Other backbone modifications such as boronophosphate, phosphoramide and methylphosphonate have also been explored to enhance the resistance to nuclease-mediated degradation [158–160]. However, cytotoxic side-effects were also observed if extensive modifications were applied [161]. (2) Substitution of 2'-OH group. This is the most widely used approach since it is well-tolerated, and can enhance nuclease resistance as well as reduce immunogenicity. For example, the naturally occurring 2'-O-methyl (OMe) modification is nontoxic and able to prevent immune activation while conferring biological stability simultaneously [161,162]. Guo’s lab also reported that incorporation of 2'-Fluoro nucleotides to 3RNA scaffold allows the creation of stable and RNase-resistant RNA nanoparticles with correct folding and authentic biological activities. The melting temperature of 2'-F RNA was further enhanced compared to unmodified RNA [163]. The chemically modified 3RNA-based nanoparticles have shown promising applications in cancer therapy [36,66,164–167]. Recent study also showed that 2'-F modified 3RNA nanoparticles are resistant to 1-I25 and Cs-131 radiation with clinically relevant doses, which is a required property for applying these RNA nanoparticles as delivery vehicles for targeted radiation therapy [168]. Other options for 2'-modification like 2'-fluoro-β-D-arabinonucleotide (FANA) have also shown promise in gene silencing applications [169]. (3) Locked nucleic acids (LNA) and unlocked nucleic acid (UNA). LNA is another class of 2'-modification in which 2'-O and 4'-C is linked via a methylene bridge. This locked linkage constrains the ribose ring to a C3'-endo conformation which confers both significant increase in thermostability and enhancement in nuclease resistance [170]. Successful applications of LNA such as 2'-amino-LNA have been studied by Astakhova et al. to show great promise in the development of biosensor, aptamer
and other nanomaterials [171]. Antisense oligonucleotides (ASOs) containing LNA also has been reported to have higher potency and specificity [172,173], but would also cause liver toxicity in mouse models if LNAs were extensively incorporated into the sequence [174]. In the other hand, UNA is an acyclic and structurally flexible-RNA analogue in which the C2′–C3′ bond is absent. As a result, the binding affinity of UNA towards its complementary strand is decreased [175].


This approach is less commonly used than other modification approaches described thus far. However, recently RNA tube nanostructures have been report to be constructed successfully by using RNA scaffolds with 5-biotinylated modified and 5-aminooxyl modified uracil [139]. Other common modified bases such as 2-thio-, 4-thio, 5-ido-, 5-bromo-, dihydro-, pseudo-uracil and diamino-purine also have been demonstrated to confer enhancement in stability as well as specificity of base-pairing interactions [155,176,177].

5. Modification of ribose molety.

This modification strategy, for example, altritol nucleic acid (ANA) and hexitol nucleic acid (HNA), is mainly applied in siRNA design to enhance potency and nuclease resistance [178]. Besides these classes, other novel methods such as photocaging modification have been used to control RNAi induction [179]. The particular effect of various chemical modifications on RNA are summarized in Table 1. It is expected that the rational choice of precise chemical modifications will greatly contribute to the development of RNA nanostructures for applications in biomedical sciences and materials sciences.

Moreover, the strategy of combining different modification approaches also has the potential to improve the properties of RNA more dramatically.

Methodology III: Computational approach

The first step in RNA nanoparticle construction is the consideration of the blueprint [180]. This requires an understanding of the assembly and folding mechanism in the bottom-up assembly. Designing the sequence of the building block is critical for successful RNA nanostructure assembly, which can be achieved by experience and brainstorming taking into consideration of RNA folding, complementation, hand-in-hand interaction, foot-to-foot interaction, and the use of thermostable motifs, kissing loops, sticky ends, helices, stem loops, etc. All RNA nanoparticles constructed based on pH29 motor pRNA was achieved via experience and brainstorming without computer algorithms besides the traditional RNA folding program developed by Zuker 30 years ago [181]. It is expected that computer algorithms will facilitate RNA nanoparticle construction. A variety of computer programs such as NanoFolder, NanoTiler and RNA2D3D are available to facilitate the in silico design of RNA sequences (these sequences may contain inter-strand and intra-strand pseudoknot-like interactions) capable of self-assembly into multi-sequence RNA nanostructures and the 3D modeling of such structures [162–184]. For example, by utilizing computational modeling and sequence optimization, three-dimensional cubic RNA-based scaffolds can be successfully designed and engineered with precise control over their shape, size and composition [144]. Moreover, online RNA structure databases such as RNAJunction database also provides useful RNA structures for designing RNA nanostructures [185].

Application of RNA as a polymer in biomedical sciences

RNA as a natural and biocompatible polymer has many advantages for biomedical applications. It carries a negative charge at physiological conditions, which disallows nonspecific passing through negatively charged cell membranes. With the conjugation of chemical ligands and/or RNA aptamers, RNA nanoparticles can be designed for specific cell targeting. It is less toxic compared with protein based nanoparticles since it can avoid antibody induction (protein-free nanoparticle), allowing repeated treatment of chronic diseases. It also does not induce an interferon response nor cytokine production [92–186]. RNA nanoparticles designed with a size range 10–40 nm display favorable pharmacokinetic properties [186], such as extended half-life in vivo (5–12 h compared to 0.25–0.75 h for siRNA), clearance: <0.13 L/kg, volume of distribution: 1.2 L/kg. Furthermore, RNA nanoparticles are classified as chemical drugs rather than biological entities. This classification will facilitate drug approval.

As a building block for nanoparticles, RNA can be synthesized with defined structure and stoichiometry. Multivalent RNA nanoparticles can be constructed using special RNA motifs as building blocks that combine therapy, targeting, and detection, all functionalities in one particle.

RNA as a biocompatible nanomaterial for tissue engineering

Tissue engineering is a new area with lots of active research and some recent success. Biocompatible nanomaterials are needed for various applications in tissue engineering, such as scaffolds or arrays that can function as temporary matrices and/or niches for the controlled deposition, infiltration, proliferation, and differentiation of cells. It would also be advantageous if these nanomaterials could be highly biocompatible and mimic the natural tissue microenvironment in vivo.

Shu et al. reported that nanometer scale 3D RNA arrays can be assembled by using pRNA as building blocks [112] (Fig. 1C). By rational design, the authors incorporated palindromic sequences (nucleotide sequences that read the same 5′ → 3′ on one strand and 3′ → 5′ on the complementary strand) into the 3′-end of the pRNA. The palindromic sequences served as links for bridging two pRNAs via foot-on-foot interactions. Loop–loop interactions were further used to link pRNA molecules into a chain. The formation of pRNA arrays was confirmed with both polycrylamide gel electrophoresis (PAGE) and AFM. These RNA arrays are unusually stable and resistant to a wide range of temperatures, salt concentrations, and pH environments. The microstructures of the RNA assays are also tailor-made by changing the RNA nucleotide sequences. Since RNA molecules are highly biocompatible and not toxic, these RNA arrays have the potential to be good tissue engineering scaffolds.
### Table 1  Summary of chemical modifications of RNA.

<table>
<thead>
<tr>
<th>Chemical modifications</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate backbone</td>
<td>• Improve nuclease resistance</td>
<td>• Destabilize siRNA duplexes (e.g. decreases Tm by 0.5°C per PS linkage)</td>
<td>[157–162]</td>
</tr>
<tr>
<td>• Phosphorothioate (PS)</td>
<td>• Combine with other modifications to dramatically improve RNA property</td>
<td>• Extensive modification causes cytotoxic effect</td>
<td></td>
</tr>
<tr>
<td>• Boranophosphate (BO)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Phosphonacetate (RACE)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>• Phosphoramidate</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>• Methylphosphonate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Z′-OH group</td>
<td>• Significantly improve nuclease resistance</td>
<td>• Extensive or full modification will reduce or fully deactivate siRNA potency</td>
<td>[36,66,161–169,173]</td>
</tr>
<tr>
<td>• Small Z′-substituents (e.g., Z′-O-methyl (Z′-OMe), 0.5°C per Z′-F)</td>
<td>• Greatly thermo-stabilizes dsRNA duplex (e.g. increase Tm of 2°-OMe and 1°C per Z′-F)</td>
<td>• Bulky Z′-modifications are only tolerated at limited position owing to their distortion of RNA helix structure</td>
<td></td>
</tr>
<tr>
<td>• Z′-aminoethyl, 2′-deoxy-Z′-fluoroarabinonucleic acid (Z′-F-ANA)</td>
<td>• Particularly, Z′-OMe is nontoxic and prevents immune activation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Bulky Z′-modifications (e.g., Z′-O-MOE, Z′-O-allyl)</td>
<td>• Bulky Z′-modifications can modulate thermo-stability and duplex asymmetry, and also give higher binding affinity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locked nucleic acid (LNA)</td>
<td>• LNA enhances the complementary binding affinity and greatly improves thermostability by 2–10°C per incorporation, as well as improves nuclease resistance and reduces RNA immunogenicity</td>
<td>• LNA would probably cause liver toxicity</td>
<td>[170–175]</td>
</tr>
<tr>
<td>Unlock nucleic acid (UNA)</td>
<td>• Each LNA destabilizes duplex by 5–8°C to improve local destabilization of siRNA duplex, and enhance biostability in vivo</td>
<td>• Extensive modification with LNA and UNA generally results in decreased activity of siRNA and failure in annealing of dsRNA, respectively</td>
<td></td>
</tr>
<tr>
<td>Ribose moeity</td>
<td>• Attritol nucleic acid (ANA)</td>
<td>• Modification at seed region would slightly reduce siRNA potency</td>
<td>[178]</td>
</tr>
<tr>
<td>• Hexitol nucleic acid (HNA)</td>
<td>• Enhance thermostability (e.g. 2°-F-ANA increases Tm of RNA duplex by 0.5–0.8°C per modification)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 2′-deoxy-2′-fluoroarabinonucleic acid (2′-F-ANA)</td>
<td>• Improve nuclease resistance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Cyclohexenyl nucleic acid</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ribonucleotide base</td>
<td>• Stabilize base-pairing and to enhance binding specificity</td>
<td>• Some base modifications (e.g. 5-bromouracil and 5-iodo-uracil) will affect siRNA potency</td>
<td>[139,177]</td>
</tr>
<tr>
<td>5-bromo-, 5-iodo-, 2-thio-, 4-thio-uracil, dihydro-pseudo, 5-biotinylated, 5-aminoallyl-uracil, diamino-purine)</td>
<td>• Particularly, 2-thio- and pseudo-uracil reduce cellular immune response</td>
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</table>

### RNA nanoparticles for targeted therapeutic delivery

Targeted delivery is a major challenge that nucleic acid therapeutics is facing. RNA nanoparticles with chemical ligand and nucleic acid aptamers have shown great promise in this regard. The utilization of RNA nanostructures as a platform for targeted therapeutics delivery has been successfully demonstrated by a series of studies using the phi29 pRNA system. In 2003, Hoeprich et al. constructed a pRNA-based carrier to deliver hammerhead ribozymes [187]. Upon conjugation to the pRNA 5′/3′ ends, the HBV ribozymes were able to fold correctly and almost completely cleaved the polyA signal of HBV mRNA in vitro. Targeted therapeutics delivery to specific cancer cells can be achieved by using ligand-conjugated RNA nanoparticles as carriers. Furthermore, the cargo in vivo release profile from RNA nanoparticles can also be controlled through the rational design of RNA
nanoparticles. In 2005, Guo et al. reported the construction of chimeric pRNA dimer with one subunit harboring folate and the other subunit harboring a gene silencing sRNA targeting surviving [100]. Incubation of these pRNA dimers with cancer cells resulted in receptor-mediated binding and entry to cells and induced efficient gene silencing. Animal trials showed that ex vivo delivery of the pRNA dimer harboring both folate and survivin sRNA could suppress tumor development. Khaled et al. studied the fabrication of a protein-free 20–40 nm pRNA trimar which could harbor three functional modules including sRNA, CD4 aptamer or folate ligands and a fluorescent dye per nanoparticle [111]. They showed that the pRNA trimar could also bind and enter into cells and modulate gene expression and apoptosis both in vitro and in vivo. In 2006, Guo et al., reported incorporating a folate-AMP into the 5’-end of phi29 pRNA and showed that folate conjugated pRNA dimers nanoparticles were able to deliver survivin sRNA into nasopharyngeal epidermal carcinoma cells and silence the target gene [188]. The pRNA system can also be used to deliver anti-virus sRNAs. Zhang et al., developed a folate-linked pRNA conjugated with the sRNA targeting the Coxackievirus B3 (CVB3) protease 2A (sRNA/2A) [189]. They observed that the modified pRNA could achieve a similar antiviral effect to that of sRNA/2A alone and also strongly inhibited CVB3 replication.

The latest advance in the utilization of phi29 pRNA for therapeutics delivery is the discovery of a phi29 pRNA three-way junction (3WJ) motif with unusual thermodynamic stability. The slope of the melting temperature curve of the three-fragment RNA complex is close to 90°, indicating extremely low G′ of the phi29 pRNA-3WJ complex [123]. The pRNA 3WJ motif was used as a RNA scaffold to construct bi-, tri-, and tetra-valent RNA nanoparticles with very high chemical and thermodynamic stability [73,123,180]. The resulting RNA nanoparticles are resistant to denaturation in 8 M urea and do not dissociate at ultra-low concentrations both in vitro and in vivo. Each arm of the 3WJ or X-motif can harbor one sRNA, ribozyme, miRNA, or aptamer without affecting the folding of the central core, and each daughter RNA molecule within the nanoparticle folds into respective correct structure with authentic biological function. The effects of gene silencing progressively increased with increasing number of sRNAs modules in the RNA nanoparticle. More importantly, systemic delivery of targeting ligand containing RNA nanoparticles into tumor-bearing mice revealed that the RNA nanoparticles remained intact in vivo without showing any sign of dissociation or degradation. These RNA nanoparticles can specifically target subcutaneous tumor xenografts [73,123,167], as well as orthotopic breast cancer [173] and intracranial glioma tumors [164] without detectable accumulation in liver, lung or other healthy organs or tissues (Fig. 4A–C). A recent study also showed that the pRNA-3WJ nanoparticle conjugated with folate can specifically target colorectal cancer metastasis in the liver, lungs and lymph node simultaneously in vivo, without accumulation in normal liver or lung parenchyma [165] (Fig. 4D). Pharmacological analysis in mice indicated that the pRNA nanoparticles display favorable pharmacokinetic (PK) and pharmacodynamic (PD) profiles, with in vivo half-life extended 10-fold compared to sRNA alone and did not induce adverse immune response including cytokine, interferon, antibody, and other toxic effects [186]. The regression of gastric cancer and breast cancer by RNA nanoparticles harboring siRNA [73,123,167] or anti-miRNA [173] has also been reported recently.

A recent paper also reported the development of novel immunomodulators by engineering rationally designed RNA polygonal nanoparticles [92]. When immunological adjuvants CpG DNA were incorporated into the RNA polygons, potent immunostimulation (cytokine TNF-α and IL-6 induction) was observed both in vitro and in vivo, compared to controls. Moreover, the RNA nanoparticles could deliver CpG DNA to macrophages specifically and the degree of immunostimulation significantly depended on the size, shape, and the number of payload per RNA nanoparticle. This finding demonstrates that RNA nanotechnology, such as developing RNA nanoparticles based on pRNA, has great potential to develop novel immunomodulators.

RNA nanoparticles for controlled drug delivery

Many pre-clinical studies are evaluating RNAs as novel therapeutics for different diseases. However, one concern is that single gene targeted therapy might eventually fail due to mutations over time or development of alternative signaling cascades or escape pathways. Multivalent RNA nanoparticles can be a very promising vehicle for delivery of multiple siRNAs to suppress multiple genes simultaneously. A recent publication by Afonin et al. showed that several RNA and RNA/DNA nanocubes could be functionalized with multiple double stranded RNAs and the siRNAs can be conditionally released through ssDNA toehold-mediated interactions. Furthermore the RNA nanocubes can be tracked intracellularly through FRET ( Förster resonance energy transfer) studies using fluorophores [190]. In addition, spherical nucleic acid (SNA) nanoparticle conjugates developed by Mirkin group have also shown promise for systemic sRNA delivery for treating diseases such as glioblastoma [191,192] (Fig. 5D). In vivo studies on glioma-bearing mice showed that SNA nanoparticle conjugates targeting the oncoprotein Bcl2L1e12 effectively increased intratumoral apoptosis, and reduced tumor burden and progression without adverse side effects [192].

DNA origamis have been exploited as a carrier for chemical drug doxorubicin through noncovalent intercalation interactions. The DNA origami/doxorubicin nanoparticles were able to enter into doxorubicin-resistant cancer cells and circumvent their drug resistance [193]. Furthermore, the DNA origami/doxorubicin nanoparticles showed a slow drug release profile at pH 7.4 under physiological conditions but showed enhanced release capability in pH 5.5 corresponding to tumor subcellular organelles [194]. As we discussed in previous section, RNA origami structures have been reported [136]. With favorable thermodynamic stability and excellent serum stability after chemical modifications, RNA origami is expected to be more favorable than its counterpart DNA origami as a drug carrier for achieving controlled drug release.

RNA nanoparticles for vascular targeting

A critical feature to drug delivery is the transportation of therapeutics to their intended target sites. Arguably, as
almost all cells in the body are within ~100 μm of the vasculature, and by way a vascular endothelial cell, it stands to reason that the vascular delivery route represents the most promising mean of any site specific delivery strategy [195]. Owing to the significant heterogeneity in endothelial cells, both temporally and regionally, it has been observed that the vascular endothelium has unique expression of surface markers that are highly regionally variant. In other words, it is potentially possible to target unique endothelial zip codes to locally accumulate nanocarriers [196].

Current strategies for vascular targeting of nanocarriers have primarily focused on antibody and peptide based strategies, targeting to surface markers CD31 (PECAM-1) [197,198], inflammatory markers (ICAM-1, VCAM-1) [199–204], and thrombomodulin [205]. Targeting of CD31, ICAM1 and VCAM represents a unique opportunity as their binding of nanoparticles to their sites highlights a novel endocytic mechanism, CAM mediated endocytosis [198,206]. The use of antibodies as targeting agents is technically challenging owing to their size, cost of production and potential immune responses. RNA aptamers have already shown exciting advantages in vasculature targeting. High affinity aptamers (56 pm) against P-selectin have been developed [207]. Microbubbles coated with a low density (1000 copie/μm²) of P-selectin aptamers demonstrated high adhesion even under shear rates as high as 1700 L/s. The
real promise of vascular-targeted RNA nanoparticles comes from the combination of the high binding affinity to carrier sizes below 70 nm. The capillary bed represents the greatest promise of vascular targeting, where there already exists a highly active transcytosis mechanism, with approximately ten thousand times more caveolae than other endothelial cells [208]. These 70 nm endocytic vesicles are an exciting target for drug delivery [209] yet require a carrier system like RNA nanoparticles that are simultaneously small and have high affinity to permit transcytosis and tissue bed delivery.

RNA nanoparticles as non-invasive medical detection reagents

Functional RNA structures such as aptamers can be used for developing non-invasive molecular and cellular imaging reagents which may have applications in the diagnosis of various diseases including cancer, cardiovascular diseases, and infectious diseases. Considering it is relatively easy to conjugate a fluorophore, radionuclide, quantum dot (QD), gold nanoparticle, or other imaging functional groups to RNA, RNA molecules with high binding affinity to their targets hold great potential as a platform to construct imaging probes which can specifically detect their targets in vitro and in vivo.

Fluorescent RNA nanoparticles conjugated with aptamer have been widely explored for cancer detection. For example, Bagalkot et al. [210] developed a QD-aptamer-doxorubicin conjugate nanoparticle as a cancer-targeted imaging, sensing and treatment platform (Fig. 5B). A10 RNA aptamer, which specifically binds to the extracellular domain of the prostate specific membrane antigen (PSMA), was conjugated to the surface of fluorescent quantum dots and the anticancer drug doxorubicin was intercalated into the double-stranded stem of the A10 aptamer. In the conjugate nanoparticle, fluorescence of doxorubicin was quenched by the aptamer through donor-quencher FRET, and the fluorescence of QD was quenched by DOX through donor-acceptor FRET mechanism. When the nanoparticle enters the prostate cancer cell and doxorubicin is released, the fluorescence of QD and DOX is re-activated. Other RNA aptamers conjugated with fluorescent dye chemicals have also been utilized for breast cancer diagnosis, such as EpCAM aptamer labeled with DY647, TYE665 or FITC [211].
Conjugating RNA aptamer with radioactive material can also be applied as diagnostic radiopharmaceuticals to detect cancer cell markers. In a recent study reported by Gomes et al. [212] a 36 nucleotide long truncated RNA aptamer with 2F pyrimidine and 2'-O-methyl purine modification was constructed for targeting to the human matrix metalloprotease 9 (MMP-9), which promotes tumor metastasis and is an important marker of malignant tumors. The 5'-end amine modified RNA aptamer was conjugated to MAG3-NHS, which served as a chelator for Technetium-99m (99mTc). The 99mTc labeled modified RNA aptamer was able to specifically detect its target in human brain tumors with autoradiography.

With the conjugation of the PSMA RNA aptamer to the surface of gold nanoparticles (GNPs) [213], a targeted computed tomography (CT) imaging and therapy system for prostate cancer can also be established (Fig. 5C). The CT imaging study revealed that the PSMA aptamer-conjugated GNP could generate more than four-fold greater CT intensity for a PSMA-expressing cell line than a non-PSMA-expressing cell line, suggesting good specificity in detection of the targeted cancer cells.

RNA nanoparticles for intracellular imaging and detection

An interesting example of applying RNA in intracellular imaging is the Spinach RNA aptamer, which is the RNA mimic of Green fluorescent protein (GFP). GFP has been extensively utilized as a reporter protein in cell and molecular biology. Similarly, the RNA mimics of GFP should also have remarkable applications in biomedical research, especially for intracellular imaging of RNA. By using the method of systematic evolution of ligands by exponential enrichment (SELEX), Paige et al. developed a novel class of RNA aptamers, termed Spinach that bind to fluorophores resembling the fluorophore in GFP [214] (Fig. 6A). Upon binding of the targeted fluorophores, these RNA aptamers were capable of emitting tunable fluorescence with comparable brightness to enhanced GFP (EGFP) and many other fluorescent proteins. Moreover, the RNA-fluorophore complex is resistant to photobleaching. Interestingly, the Spinach RNA aptamer can be expressed in vivo by fusing to other cellular RNAs, for example, 5S RNA, to enable live-cell imaging and monitoring of these cellular RNAs. Furthermore, fluorescent sensors for detecting a variety of small molecules and cellular metabolites, including adenosine 5'-diphosphate (ADP) and 5-adenosylmethionine (SAM), in vitro and in living cells could also be generated by combining the Spinach aptamer, a transducer, and a target-binding aptamer [215] (Fig. 6B).

Reif et al. [216] recently reported another novel method to enable real-time monitoring of RNA folding and degradation in living cells based on the pRNA-3WJ. The authors designed and constructed RNA nanoparticles by incorporating an RNA aptamer capable of binding to malachite green (MG), the hepatitis B virus ribozyme and the luciferase siRNA into the pRNA 3WJ. When MG aptamer binds to MG dye, it will emit fluorescent light only if the aptamer folds correctly. The MG aptamer system can then be used to monitor the degradation of the constructed RNA nanoparticles by fluorescent microscopy and fluorescence spectroscopy. By using this novel design, the authors determined the half-life (t1/2) of the MG aptamer containing pRNA-3WJ inside living cells to be ~4.3 h.

RNA as a polymer for biosensor systems

The biomolecular-based detection system has been widely investigated in many applications including clinical diagnosis, food industry, environmental monitoring industry, and security industry [217,218]. Conventionally, biosensors can detect proteins, nucleic acids such as DNA or RNA sequences, small organic molecules, and others [218–220]. RNA molecules are well suited to serve as detection tools for small molecules, for example, antibiotics, peptides, metal ions, ligands, etc. [221,222] because of their unique folding structures, functional conformation, predictable base-pairing, and high fidelity. These characteristics of RNA allow for the development of ribozyme or aptamer, and aptazyme-based biosensors [223–225]. Also, as the target, RNA can be used to develop the biosensor for the detection of messenger RNA or micro RNA [226–229]. Various detection methods of RNA-based biosensors were proposed such as electrochemical type, fluorescence type, optical type, and electrical type. Also, there are various detection platforms for RNA-based biosensors (Fig. 7) [230–235]. In this section, we briefly introduce RNA-based biosensors for biomedical and environmental monitoring applications.

The first proof of concept of using RNA in a biosensor was developed by Breaker Group [221]. Self-cleaving hammerhead-ribozymes were created with seven different RNA switches and these immobilized each pixel that responded allosterically to six types of analytes (Co2+, cGMP, cAMP, FAM, FMN and theophylline). The platform of biosensor array was prepared on a poly styrene cell culture plate which was coated with gold by physical vapor deposition. The ribozyme was immobilized onto gold surface via a 5'-thiotriphosphate-terminated RNA moiety. Then, each hammerhead ribozymes cleaved off their 3' fragment according to the addition of individual analytes. They also showed the quantitative and qualitative measurement of cAMP in culture media from E. coli strain.

RNA can also serve as the detection analyte to detect viable E. coli as an indicator organism in drinking water, which was proposed by Baumer group [228,229]. This RNA-based biosensor was generated with the extraction and amplification of mRNA molecules from E. coli in 20 min. Viable E. coli in the water were identified and quantified using a 200 nt target sequence from mRNA. The detection limit of the biosensor system could detect around 40 viable E. coli in water (5 fmol per sample) using the electrochemiluminescence (ECL) detection method. They introduced the isothermal amplification technique, a nucleic acid sequence-based amplification (NASBA) for mRNA amplification.

RNA was also applied to develop medical diagnosis biosensors. Theophylline (1,3-dimethylxanthine) is a common agent in the bronchodilators and used for acute and chronic asthma. Gottelf group developed a RNA-based electrochemical biosensor for theophylline in serum with a ferrocene (Fc) redox probe [225,233]. In this study, the thiol group-modified and amino group-modified RNA aptamer was immobilized onto gold substrate via
Figure 6  Spinach RNA aptamer for intracellular imaging and sensing. (A) Live-cell imaging of Spinach-tagged 5S RNA[214]. Reprinted with permission from Ref. [214]. Copyright 2011 The American Association for the Advancement of Science. (B) Imaging cellular metabolites in E. coli with sensor RNA[215]. The sensor RNA comprises Spinach (black), a transducer (orange), and a target-binding aptamer (blue). Reprinted with permission from Ref. [215]. Copyright 2012 The American Association for the Advancement of Science.

Figure 7  The basic constitution of RNA-based biosensor.

cova lent bonding between thiol group and gold substrate. Then, the Fe redox probe was covalently attached to the 3'-amino group of RNA aptamer through Fe-carboxylic acid NHS reaction. Usually, the RNA aptamer stays on the open conformation in absence of theophylline. However, when

the theophylline added to RNA aptamer-modified electrode, the aptamer folds into the conformationally restricted hairpin structure. As a result, this phenomenon changed the electron transfer that was monitored by cyclic and differential pulse voltammetry. Moreover, specific recognition of
Table 2  Classification of RNA polymer-based bioelectronics.

<table>
<thead>
<tr>
<th>RNA-based bioelectronics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocomputation devices</td>
<td>[238,243]</td>
</tr>
<tr>
<td>Biologic gate</td>
<td></td>
</tr>
<tr>
<td>Biomemory</td>
<td>[238,243,266]</td>
</tr>
<tr>
<td>Bioinformation processor</td>
<td>[242,243,245,247,248,250]</td>
</tr>
<tr>
<td>Biosensors</td>
<td></td>
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<tr>
<td>Biomedical sensor</td>
<td>[215,217,221,222,225–227]</td>
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<tr>
<td>Environmental sensor</td>
<td>[228,229]</td>
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Dopamine by the RNA aptamer allows selective amperometric detection of dopamine from 100 nM to 5 μM range in the presence of competitive neurotransmitters such as catechol, epinephrine, norepinephrine, and others [232].

In an alternative approach, Hammonds group used a RNA-based fluorescent biosensor for detecting the cyclic di-GMP and cyclic AMP-GMP [226]. The c-di-GMP was generated by a GEMM-I riboswitch aptamer and the riboswitch ligand mutation can recognize the c-AMP-GMP, c-di-GMP. The fluorescence turn-on was activated by the DPhH fluorescent molecule for cell imaging. This system provided selective and sensitive detection for each cyclic dinucleotide. Thus, there are many examples demonstrating that RNA molecules are promising candidates for biosensor applications.

Application of RNA as a polymer in material sciences

RNA as a polymer for biocomputation systems

Silicon-based computer systems are ubiquitous [236] but new processors to implement computation, communication, and controls are needed to meet the demands of various applications. Gordon Moore’s Law suggested that due to the increasing demand of the memory, computer electronic devices on the microprocessor will be doubled every 18 months. Scientists and engineers are wondering whether current computer technology growth can be continued in the next ten years. They raised the concern that silicon microprocessor speed and miniaturization will eventually reach limits by current technology. Thus, molecular-scale computing has been explored since 1994 due to the predictable ending of Moore’s Law for silicon-based computer devices.

In the last few decades, biotechnology has been integrated with nanotechnology and electrical engineering [237–239]. As a result, the field of bioelectronics has been created [240,241]. Bioelectronics has led to the development of the nanoscale biochip, biosensor, and biocomputation devices such as information storage devices, logic gates, field-effect transistors and computation systems (Table 2) [242–245]. Typically, bioelectronic devices are composed of a biological actuation portion, and an electronic signal transduction portion. So far, the biological component is usually composed of biomolecules, such as protein and RNA [239,242,244]. Recently, some pioneering groups have suggested that the RNA-based bioelectronic devices also exhibited the logic gate behavior, processing functions, and biocomputations [180,246–250]. The advantages of RNA-based computation are summarized in Fig. 8. Bio-computing approaches for information control, storage, and processing using RNA-based devices brings new possibilities that embody multiple functions in biological systems. The development of biological computers composed of artificial RNA molecules to operate inside living cells or tissues would provide a new avenue. A combination of these results will pave the way to develop the new concept of biocomputer development in the future such as tissue-mimicked computations.

RNA as a polymer for logic gates

In computer science, the ‘logic’ term is defined as circuits which give an output corresponding to a set of two input values (True ‘1’, High voltage and False ‘0’, Low voltage). The logic gate is required to have two input values for performing Boolean function [241]. Thereby, it gives the logical output to perform the next function. Usually, man-made computers consist of millions of combinational logic gates. Thus, logic gates are essential components of the computer. As aforementioned, in the last few decades,
the concept of a biomolecule-based computation system has been proposed or explored to solve the current limitation of silicon-based computations [246, 251–255]. The first attempt of molecular-scale computation was developed by Adleman’s group in 1994 [251]. They introduced the encoded DNA molecule to solve the Hamiltonian path problem. This research established a milestone for the field of biomolecule-based computations. Since then, several groups proposed the DNA polymer-based logic systems [252, 254, 255]. The basic operation mechanism of DNA-based logic gate is combinational DNA hybridization that undergoes conformational change or chemical reaction and includes pH, temperature, light, electrical, or electrochemical signals [251–255]. The function of logic gate gives the discrete state change such as structure, fluorescence, absorbance, and electrochemical or electrical current as the output. Like this, various types of DNA-based computations have been proposed. DNA, however, is hard to use to perform the complex functions because of its simple functionality. To develop the new-concept of biocomputations systems, RNA is a candidate owing to its intriguing characteristics. The folding and assembly of RNA molecules drive themselves into secondary and tertiary structures using the formation of hairpin loops, dovetails, bulges, and internal loops, etc [10]. Thereby, RNA has functionality such as aptamer, ribozyme, siRNA, non-coding RNA, circular RNA, etc. [180] These various functional groups can be easily applied to the new concept of logic gate behavior, information storage, information processing, and computations as the novel elements [256–264].

An autonomous biomolecular-based computer has been proposed to regulate the gene expression "logically" [248]. The basic computation rule is governed by ‘diagnostic rule’ for prostate cancer state detection. If the specific gene relating to prostate cancer are overexpressed, then ssDNA will bind to their mRNA and inhibits the protein synthesis. The level of specific RNA and the concentration of specific molecule which regulates the point mutations were regarded as an input. The release of short ssDNA modulated the levels of gene expression for anticancer activity, as the output logically. The computation module has two states, “positive” and “negative”, in response to the level of specific gene expression. This bio-computation concept is related with the identification of mRNAs of disease-related genes. Thereby, it can be applied to cancer diagnosis systems based on logic analysis.

A RNA-based logic evaluator has also been reported to perform Boolean logic corresponding to input molecules in the human kidney cells [249]. The reported biological circuit is composed of several mRNA species that encode the same protein but have different non-coding regions. This protein served as the output. As an input, ssiRNAs were used to control the degradation of the target mRNA and the expression of the output protein. Expressions with up to five logic variables were directly evaluated by this system.

RNA as a polymer for information processor

In computer sciences, the information processor is a unit that receives the input information and processes it into another form based on programmed functions. RNA-based information processing devices that operate logic gates, signal filtering, and cooperativity functions have been reported [250, 259, 265]. RNA devices composed of ribozymes and RNA aptamers received, processed and transmitted the molecular inputs to express the green fluorescent protein as output. As a sensor part, the RNA aptamer was introduced and a hammerhead ribozyme was used to process the cleaving of the aptamer. Also, the transmitter part was composed of sequences that bind to the aptamer and ribozymes. It was suggested that the RNA aptamer and ribozyme combination can be used as actuating elements for multi-functional information processor development [250, 265].

An intracellular RNA computation device in a single mammalian cell has also been reported [259]. Trigger-controlled transcription factors were used to regulate the gene expression individually and the RNA-binding protein was used to inhibit the translation of transcripts on target RNA motifs. This computation biosynthetic circuit can provide the N OT, AND, NAND, and N-IMPLY expression functions in individual cells. Moreover, two N-IMPLY expression functions can combine with other cells to operate XOR gate functions and three logic gates can perform the half-adder and half-subtractor functions for arithmetic calculation.

RNA as a polymer for resistive biomolecular memory

RNA can also provide several advantages such as ease of construction, well defined structure and thermodynamic stability for the development of biomolecular memory devices. Based on the discovery of the ultra-high thermostable pRNA-3WJ, a pRNA-3WJ/QD hybrid nanoparticle for resistive biomolecular memory application has been constructed [266]. The QD was used as a semiconducting part for storage of the electrons and pRNA-3WJ was used as an insulating part and serves as the bridge between QD and metal substrate. The electrical measurement (I–V) was conducted to this hybrid structure for resistive memory performances. This study showed the RNA polymer conjugates can be used directly for molecular memory device fabrication.

Hybrid RNA polymers as semiconductors

Biomolecular hybrids of a conducting polymer [poly(3-methoxy aniline) (POMA)] and dsRNA have been reported as semiconductors [267]. Fourier transform infrared spectroscopy (FTIR) indicated that these hybrid polymers are held together by electrostatic, H-bonding, and π–π Interactions. The circular dichroism spectra of the hybrid polymers indicated that the dsRNA underwent a small distortion in conformation from the canonical A-form towards the B-form during the formation of the hybrid polymers. TEM micrographs revealed that these polymers create a fibrillar network structure. The conductivity values of the POMA-RNA hybrids were three orders of magnitude higher than that of RNA alone. The I–V curves of the POMA-RNA hybrid polymers also demonstrated a semiconducting nature. Based on these physical characteristics, the POMA-RNA hybrids have the potential to be candidates for fabricating biosensors for other applications.
Conjugation of RNA to graphene

Graphene is a one-atom thick, two-dimensional honeycomb lattice composed of sp²-bonded carbon atoms. It can be used as a fundamental building block for constructing other graphite-based structures such as fullerences and nanotubes [268,269]. This newly discovered carbon-based material has been a popular subject in material science and nanotechnology. It has been used for the sensing of a toxin Microcystin-LR in water [270–273]. RNA aptamer have been covalently immobilized on graphene oxide and a polydispersed stable RNA-graphene oxide nanosheet have been constructed [274]. The RNA attached to these nanosheets was resistant to nuclease degradation and the nanosheets competitively absorbed trace amounts of the peptide toxin microcystin-LR from drinking water. PEI-grafted graphene oxide was also used to deliver short interfering RNA (siRNA) as well as anti-cancer drug doxorubicin to cancer cells [275]. RNA has also been used as a surfactant to exfoliate flakes of graphene from nanocrystalline graphite to produce transparent and conducting RNA-graphene-based thin films [276], which has a potential for a variety of electronic applications. Further research in RNA-graphene nanocomposites may open a new avenue towards many applications of graphene-based conductive materials.

Conjugation of RNA with other nanoparticles

The conjugation of a RNA with nanoparticles such as quantum dot, iron oxide nanoparticle or gold particle has been successfully demonstrated by a series of studies for nanized imaging, therapy and diagnosis [277–282]. For example, siRNA and tumor-homing peptides (F3) were conjugated to the PEGylated QD core as a scaffold [278,279]. The complex was efficiently delivered to HeLa cells, released from the endosomal compartment, and triggered knockdown of EGFP signal. This leads to dual purpose of treatment and imaging. In another case, siRNA was also conjugated to the iron oxide nanoparticle [280,282] for dual purpose: (1) in vivo transfer of siRNA and (2) imaging the accumulation of siRNA in tumor through magnetic resonance imaging (MRI) and near-infrared in vivo optical imaging (NIRF) using near-infrared Cy5.5 dye. Another report showed that the conjugation of gold nanoparticle and RNA also increased the availability of the tethered RNA splicing enhancer [283].

In 2007, the pRNA of the phage phi29 DNA-packaging motor was conjugated to the gold nanoparticles via a thiol group for single particle quantification in bacterial virus assembly [281]. The pRNA-gold nanoparticle conjugates were used for procapsid by in vitro phage assembly. The results demonstrated the feasibility in using RNA-gold nanoparticle for single molecule imaging and counting of biological machines.

Prospects

RNA is a polymer by nature. Recent technological advances to make RNA chemically and enzymatically stable [55,66] and the discovery of unusual thermostability of some RNA motifs, as well as important biomedical applications [123–126,284] have propelled the concept of RNA as a polymeric building block [36] into a new horizon. The concern of yield and production cost has been addressed continually by industry scale production and fermentation. It is expected that applications of RNA as a polymer and as building blocks will appear more and more in therapeutics, detection, sensing, nanoelectronic devices, and other polymer industries. The anion nature, the thermodynamic stability, the Insulating property, the self-assembly capability and other novel features such as versatility, molecular-level plasticity, as well as the potential semiconductor behavior will make RNA unique for exploring new scientific territories. RNA has been shown to be major components of cells and leading functionality of life, and it is expected that RNA will also be the momentous material of daily life in the future.

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References

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Controllable Self-Assembly of RNA Tetrahedrons with Precise Shape and Size for Cancer Targeting

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The Watson-Crick base pairing properties of DNA and RNA have led to their development as excellent building blocks for the construction of nanomaterials by bottom-up self-assembly.[1-4] For constructing larger architectures by directional or angular extension, it is necessary to extend the building blocks with a defined angle or orientation. This has been challenging for DNA since one helix turn of 360° is 10.5 nucleotides for the regular B-DNA. A non-integer per helix turn will result in the twisting of the extension angle or the restriction in orientation control. In RNA, the number of nucleotides per helix turn is an integer of 11 for A-form RNA, which is a common structure for most RNA sequences. We propose that this unique property of RNA will enable RNA structural growth with precise one directional control, which will facilitate the construction of large size architectures for materials science, computer device, and biomedical applications.

RNA nanotechnology is an emerging field that involves the design, construction, and functionalization of nanometer-scale particles composed mainly of RNA for applications in biomedical and material sciences.[2] Previous reports have shown that a variety of RNA nanostructures can be constructed with defined sizes, shapes, and stoichiometry, including triangles,[7] squares,[8-10] bundles,[11,12] 2D arrays,[7,11] hexamers,[14-16] and 3D cages[17-19] by bottom-up self-assembly based on intra- and inter-RNA interactions. We have extensively utilized the structural features of bacteriophage phi29 packaging RNA[20] to construct varieties of RNA nanoparticles via loop-loop interactions.[11,21,22] palindrome sequence mediated foot-to-foot interactions,[11,22] and three-way junction (3JW) motif.[21,22] More recently, we constructed 2D polygons such as triangle, square, and pentagon[22]

using the pRNA-3JW as core scaffold at the vertices. Here we extended our previous 2D work to design and construct 3D RNA nanoparticles with controllable shape and size.

Tetrahedral geometry is attractive because of their intrinsic mechanical rigidity and structural stability. It is a pyramid-like structure with four triangular faces and six edges. Several methods have been used to construct DNA-based tetrahedrons, such as an origami approach based on a long single DNA strand,[20,31] complementary hybridization of strands using sticky ends,[12,21,22] and hierarchical assembly of tiles.[20] RNA is an attractive alternative building block due to its high thermal stability[20,30] and versatility in structures,[12,22] well beyond the simplistic canonical Watson-Crick base pairing in DNA nanostructures. Herein, we used our well-characterized ultrastable 3JW motif[12,22] as core scaffold to construct tetrahedral architectures. The RNA tetrahedrons have defined 3D structure as revealed by atomic force microscope (AFM), as well as by single-particle cryo-electron microscopy (cryo-EM) that has long been found to be very challenging in imaging pure RNA structures without forming complex with proteins.[33] Moreover, for the functionalization of the RNA tetrahedrons, aptamers, ribozyme, and siRNA were placed at the edges of the RNA tetrahedrons with high precision without disrupting the overall structure. Importantly, the functional modules were incorporated prior to the assembly of the RNA tetrahedrons to ensure the production of homogeneous nanoparticles with high yield. Biodistribution studies revealed that RNA tetrahedrons functionalized with EGFR-targeting RNA aptamer specifically targeted orthotopic breast tumors without detectable accumulation in healthy vital organs. The RNA tetrahedrons are envisioned to have a broad impact in nanotechnology arena, such as for organizing nanoscale materials with high precision, encapsulation of functional materials within its hollow cavity, targeted therapy to diseased cells, and as image-guided delivery vectors.

In this study, the pRNA-3JW motif[12,22] (Figure 1a) was used as a core module and placed at each of the four vertices to build RNA tetrahedrons (Figure 1b). A total of four RNA strands were designed consisting of four pRNA 3JW core sequences and six linking RNA sequences. A 3D model of the RNA tetrahedron (Figure 1c) was then generated using computational modeling software UCSF Chimera,[34] Swiss PDB Viewer (http://www.expasy.org/spdbv/), and PyMOL Molecular Graphics System (https://www.pymol.org/). The resulting computational model exhibited authentic tetrahedral conformation. For assembling the RNA tetrahedrons, the four RNA strands were synthesized by in vitro transcription and then mixed in stoichiometric ratio and annealed in 1x Tris buffer in a one-pot manner. Step-wise assembly of the complex was observed by native polyacrylamide gel electrophoresis (PAGE) (Figure 1d).
Figure 1. Design and assembly of 8 nm RNA tetrahedrons. a) 2D sequence of pRNA monomer showing the central pRNA-[W] motif. The 22 nucleotide core sequence (with red color) of pRNA-[W] is used to construct RNA tetrahedrons. b-c) 2D sequences (b) and 3D computational model (c) of RNA tetrahedrons. d) 7% native PAGE gel showing step-wise assembly of RNA tetrahedrons. ‘+’ indicates the presence of the strands. M: Ultralow range DNA Ladder. e) AFM images and f) single-particle cryo-EM 3D reconstruction of 8 nm RNA tetrahedrons.

For 3D RNA tetrahedrons, the most convincing structural characterization in their native state comes from single-particle cryo-EM studies. We first analyzed the 3D structure of the 8 nm RNA tetrahedrons with two-helix turn per edge (Figure 1f). Single-particle 3D reconstruction was applied by analyzing a total of 1254 particles collected from cryo-EM images and achieved a final resolution of 19 Å (gold standard criterion, 0.143 Fourier shell correlation, Figure S1, Supporting Information). The 3D reconstruction data revealed that the RNA tetrahedron has a clear overall shape consistent with the computational 3D model (Figure 1c) which agrees with the predicted size of 8 nm. The 2D computed projections of the reconstructed RNA tetrahedron 3D model showed clear match to the 2D class averages of the raw particles, suggesting the reconstructed 3D model truly represented the native structure and conformation of the designed RNA tetrahedron. The images from cryo-EM accord with the global images obtained by AFM imaging (Figure 1f). However, the central cavities of each tetrahedron were too small to be resolved by AFM and gave an apparent size of 12.5 ± 0.6 nm. The observed size discrepancy is due to limitations in the diameter of the AFM tip. To further characterize the size of the RNA tetrahedrons, dynamic light scattering (DLS) was performed. DLS assumes that the particles have an average globular geometry in solution. The apparent hydrodynamic size of the RNA tetrahedron was determined to be 8.3 ± 2.4 nm (Figure 2a).

The surface charge of RNA tetrahedrons, measured as zeta potential, was also evaluated by DLS. As expected, due to the phosphate backbone of nucleotides, RNA tetrahedrons have a negative surface charge with a single peak at −14.9 ± 1.0 mV. This negative surface charge is advantageous for the overall colloidal stability of RNA nanoparticles and prevents forming aggregation in solution. Moreover, negative surface charge could also reduce the non-specific interaction of the nanoparticles with the reticuloendothelial system (RES) and minimize non-specific cell entry, which is attractive for in vivo targeted drug delivery and theranostic applications.[35]

To assess the thermodynamic stability of RNA tetrahedrons, we investigated their melting temperatures ($T_m$) by measuring their fluorescence intensities in the presence of SYBR Green II dye with the charge of temperature on a real-time PCR machine. Melting experiments revealed that the assembled RNA tetrahedrons had a very smooth, high-slope temperature dependent melting curve with a $T_m$ of 71.3 ± 1.8 °C. The high slope indicates cooperative assembly of the tetrahedron from its four component strands. Moreover, we compared the melting curve of RNA, 2’-F RNA and DNA tetrahedrons. The results showed that 2’-F RNA tetrahedrons was the most stable with a
$T_m$ of 77.7 ± 2.4 °C, followed by RNA tetrahedron with a $T_m$ of 71.3 ± 1.8 °C, and finally DNA tetrahedron with a much lower $T_m$ of 58.3 ± 0.5 °C. These results were in agreement with previously reported thermodynamic stability of nucleic acids with the order of stability: Z'-F RNA > RNA > DNA.\textsuperscript{30,31}

To investigate their enzymatic stability, we incubated unmodified and Z'-F modified (U and C nucleotides) RNA tetrahedrons in cell culture medium with 10% FBS (fetal bovine serum) (Figure S2, Supporting Information). At specific time points, aliquots were extracted and evaluated by native PAGE. The unmodified RNA tetrahedrons were degraded within 15 min, while Z'-F counterparts were stable over an extended period of time, well beyond 24 h. The resistance to serum-mediated degradation combined with the high thermodynamic stability is particularly attractive for the in vivo application of these RNA nanoparticles.

To demonstrate the precise tunable sizes of the RNA tetrahedrons, we designed a larger 17 nm RNA tetrahedron in which every edge of the tetrahedron was extended to 55 bp equal to five helix turns (Figure S3, Supporting Information). This larger nanoparticle has the same overall tetrahedral geometry in our design (Figure 3a). Upon annealing the four-component strands in one-pot self-assembly, the larger RNA tetrahedron assembled with high efficiency, as revealed by native PAGE analysis (Figure 3b). DLS experiments revealed that the hydrodynamic diameter of the larger RNA tetrahedron was 16.9 ± 1.6 nm, which is in agreement with the designed dimensions (Figure 3c). Moreover, AFM imaging was clearly able to resolve the tetrahedral morphology along with the inner cavities (Figure 3d). Since the RNA tetrahedrons were dried on the APS-modified mica surface before imaging in air, flattened tetrahedral shapes were observed. The RNA nanoparticles were also highly homogenous in shape and structure, demonstrating the robustness of the self-assembly of RNA tetrahedrons. Cryo-EM image further showed the very clear RNA tetrahedron nanoparticles (indicated by red circles) with the expected sizes and geometries (Figure 3e). Single-particle 3D reconstruction of a total of 1582 particles collected from 131 cryo-EM images achieved a resolution of 23 Å (gold standard criterion, 0.143 Fourier shell correlation, Figure S4, Supporting Information).

To evaluate the application of tetrahedrons, four different functional modules including a hepatitis B virus (HBV) ribozyme, fluorogenic aptamers for malachite green (MG) or Spinach, and a streptavidin-binding aptamer (Figure 4a) were incorporated into the tetrahedron structure. The sequences of the functional modules were simply fused with the sequences of the 3W1 core, and then synthesized by in vitro transcription. After annealing the strands, the step-wise self-assembly was evaluated by native PAGE analysis to confirm the successful assembly of the RNA tetrahedrons (Figure 4b). Functional assays were then conducted to determine whether the modules retained their authentic folding and functionalities upon incorporation into the RNA tetrahedrons.
Figure 3. Design, assembly, and characterization of 17 nm RNA tetrahedrons. a) Schematic showing tunable size conversion (from 22 bp per edge to 55 bp per edge) of RNA tetrahedrons. b) 6% native PAGE gel showing step-wise assembly of larger RNA tetrahedrons. "$\cdot$" indicates the presence of the strands. M: 100 bp DNA ladder. c) DLS assay showing the hydrodynamic size of larger RNA tetrahedrons. d) AFM images and e) cryo-EM images and 3D reconstruction of RNA tetrahedrons.

The HBV ribozyme is a hammerhead ribozyme that can target and cleave the 135 nt HBV genomic RNA substrate. The HBV ribozyme was fused to the RNA tetrahedron by extending one of the strands of the nanoparticle. After incubation with the RNA tetrahedron harboring the HBV ribozyme, the HBV substrate was cleaved into fragments with smaller molecular weights, as revealed by PAGE analysis (Figure 4c). The yield of the cleavage reaction was comparable with the positive control (pRNA harboring HBV ribozyme). In contrast, RNA tetrahedron by itself or harboring disabled HBV ribozyme (G→A mutation in catalytic site) had no catalytic effects. The result confirmed that the designed multifunctional RNA tetrahedron successfully escorted the HBV ribozyme, and the catalytic activity was retained after ribozyme sequence was fused to the tetrahedron.

Both MG[13-19] and Spinach[20-26] aptamers are well-characterized for their fluorogenic properties, which emit fluorescence upon binding of their respective dye targets, triphenylmethane and 3,5-difluoro-4-hydroxybenzilidene imidazole (DFBI). To verify that the MG and Spinach aptamers incorporated into the multifunctional RNA tetrahedron are still functional and folded correctly, fluorescence studies were performed using a fluorospectrophotometer. The fluorescence emission spectra showed that both the aptamers retained their ability to bind their respective dyes and emitted strong fluorescence at a similar level to the positive control, indicating the retention of the correct folding and functionalities of these fluorogenic aptamers (Figure 4d,e). The fluorogenic modules fused with tetrahedron nanoparticles can have potential applications for imaging these nanoparticles in cells.

Upon incubation with STV agarose resins, the multifunctional RNA tetrahedrons harboring STV aptamer[27] successfully bind to the resin with high affinity and were eluted by biotin (Figure 4f). In contrast, the negative control tetrahedron...
did not bind to the resin and also did not show up in the elution fractions. The results indicated that the fusion with RNA tetrahedron did not interfere with the native structure and function of the STV aptamer. Moreover, in PAGE analysis of the eluted multifunctional nanoparticles harboring MG and Spinach aptamers, they still retained their fluorogenic properties, indicating that the RNA tetrahedron structure promoted the correct folding of the two aptamers after fusion into the RNA nanoparticle (Figure S5, Supporting Information). The results demonstrated that the fused STV aptamer could be potentially employed as a handle to specifically purify the assembled multifunctional RNA tetrahedrons by using STV agarose resins.

Dual-luciferase reporter assay was utilized to study the gene silencing effects of the RNA tetrahedron harboring four siRNAs targeting the same region of the firefly luciferase gene (Figure S6, Supporting Information). The Renilla luciferase, which was not the target of the luciferase siRNA, served as an internal control. The relative ratio of the expression level of the firefly luciferase to the Renilla luciferase was used to evaluate the targeted gene silencing effect in MCF-7 cells upon transfection. The results revealed that the tetrahedron harboring four luciferase siRNAs with a concentration achieved <90% gene silencing which is similar to the positive control of luciferase siRNA only at 4 x 10^{-8} M concentration. In contrast, the tetrahedron only and scrambled control did not show any noticeable gene silencing effects (Figure S6). These results demonstrated that luciferase siRNA still retained their gene silencing ability after incorporation into the RNA tetrahedrons, suggesting that RNA tetrahedrons could serve as an efficient vehicle for intracellular siRNA delivery.

For effective cancer therapy, it is critical to guide therapeutics to specific cancer cells. The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTK) is highly prevalent in both primary tumors and metastatic breast cancer cells, making them an ideal candidate for targeted therapies. RNA aptamers have been developed to bind to EGFR receptors with high selectivity and sensitivity. Here we incorporated EGFR targeting RNA aptamers into RNA tetrahedrons (Figure S7, Supporting Information) and evaluated their cellular binding by confocal microscopy (Figure S7). Alexa47 (red) labeled RNA strand was incorporated into tetrahedrons for fluorescence imaging. Tetrahedrons without EGFR aptamer were used as negative control. Confocal imaging showed that tetrahedron nanoparticles with EGFR aptamers strongly bound to EGFR(+) MDA-MB-231 cells (Figure S7a), as revealed by the co-localization of the Alexa47-labeled RNA (red) and cellular actin (green) (Figure S7a). In contrast, negative control “naked” tetrahedrons without the EGFR aptamer showed negligible cellular binding. The results suggest that the EGFR aptamer could facilitate the binding of RNA tetrahedrons to EGFR-expressing cancer cells.

For studying the in vivo cancer-targeting properties of the RNA tetrahedrons, we generated orthotopic breast cancer mouse models by injecting MDA-MB-231 cells directly into the mammary fat pad of athymic nude mice to generate xenografts. Nuclease-resistant 2'-F RNA tetrahedrons harboring EGFR aptamers and Alexa47 were systemically injected into mice and...
Figure 5. In vitro and in vivo evaluation of RNA tetrahedrons harboring siRNA and cancer-targeting aptamers. a) Confocal images showing RNA tetrahedron (with and without EGFR aptamers) binding to MDA-MB-231 cells. b) Luciferase siRNA silencing effects assayed by dual luciferase assay. The error bars indicate mean ± S.D. c) Biodistribution assay in orthotopic MDA-MB-231 tumor-bearing mice after systemic tail vein injection of RNA tetrahedrons harboring EGFR aptamers.

Their biodistribution was monitored by whole-body imaging. The mice were sacrificed 8 h post injection and their organs were collected for ex vivo imaging (Figure 5c). RNA tetrahedrons were not detected in any other organs except the breast tumor, indicating that the nanoparticles were cleared from normal organs quickly and did not accumulate in the liver, lung, spleen, or kidney after systemic injection. In another confirmatory animal trial by using an EGFR-expressing KB cells xenograft mouse model, similar cancer-targeting ability of the RNA tetrahedrons was also observed (Figure S8, Supporting Information). The selective cancer-targeting ability of the RNA tetrahedrons would make this delivery system an attractive candidate for future targeted cancer imaging studies and/or cancer therapy.

In conclusion, here we report the design and self-assembly of multifunctional 3D RNA tetrahedrons based on the ultrastable pRNA-3WJ motif. The constructed RNA tetrahedrons have defined 3D structures as revealed by both AFM and single-particle cryo-EM. The size of the RNA tetrahedrons can be easily tuned by changing the number of RNA base pairs per edge. Melting experiments revealed its high thermodynamic stability. Aptamers, ribozyme, and siRNA were successfully incorporated into the RNA tetrahedron with their correct folding and optimal functionality. Cellular binding and biodistribution study showed that the RNA tetrahedron functionalized with EGFR-aptamer targeted orthotopic breast tumors without detectable accumulation in other healthy organs. These results suggest that 3D RNA tetrahedron nanoparticles have the potential to escort imaging modules and therapeutics for in vivo cancer diagnosis and therapy.

Supporting Information
Supporting information is available from the Wiley Online Library or from the author.

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Enhancing immunomodulation on innate immunity by shape transition among RNA triangle, square and pentagon nanovehicles

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ABSTRACT

Modulation of immune response is important in cancer immunotherapy, vaccine adjuvant development and inflammatory or immune disease therapy. Here we report the development of new immunomodulators via control of shape transition among RNA triangle, square and pentagon. Changing one RNA strand in polygons automatically induced the stretching of the interior angle from 60° to 90° or 108°, resulting in self-assembly of elegant RNA triangles, squares and pentagons. When immunological adjuvants were incorporated, their immunomodulation effect for cytokine TNF-α and IL-6 induction was greatly enhanced in vitro and in animals up to 100-fold, while RNA polygon controls induced unnoticeable effect. The RNA nanoparticles were delivered to macrophages specifically. The degree of immunostimulation greatly depended on the size, shape and number of the payload per nanoparticles. Stronger immune response was observed when the number of adjuvants per polygon was increased, demonstrating the advantage of shape transition from triangle to pentagon.

INTRODUCTION

One area of biomimetic nanotechnology involves the construction of nano-scale, supramolecular architectures utilizing modular units of functional nucleic acids. The aim is to design nanostructures that undergo self-assembly in a controllable fashion. Ribonucleic acid (RNA) was discovered as an attractive material to build nanoparticles via nanotechnology (1), offering a variety of structural modules and motifs that can be manipulated into 1D, 2D and 3D architectures (for review see (2)). In the past decade, a variety of geometric RNA nanoparticles and nano-scaffolds have been obtained via the approaches of hand-in-hand (1,3–5), foot-to-foot (6–9), branch extension (10–14), loop–receptor contact (15–17), ‘sticky’ or ‘dangling’ ends (6,18,19) and synthetic RNA–protein complex interactions (20). These motifs are available in databases and can be used to build artificial nanostructures by manipulating their interchangeable units (21). Recently, RNA rolling cycle transcription has been utilized to generate RNA sponges (22,23). In RNA tectonics approach, structural motifs like double helices, loops and junctions can be isolated from large and complex RNA molecules appearing in structural databases and used to build artificial nanostructures by manipulating their interchangeable units (24,25). As such, previously reported designs of RNA nanoparticles, e.g. tecto-square (26), square-shaped nano-scaffolds (27,28), RNA nanoring (1,5,7,9) or pRNA dimmers, tetramers and hexamers (1,7,9,29,30), as well as RNA nano-cubes (19), RNA polyhedron (14), RNA bundles (6,31) and filaments (15,16) utilize fundamental principles of RNA structure and folding (32–36). Overall stability of conventional constructs though, mainly relies on the stability of canonical and non-canonical base pair (bp) forming by loop–loop, receptor–loop, or ‘sticky-ends’ with a number of pairing nucleotides usually not exceeding six. A new approach is needed to increase overall stability of RNA nanoparticles, one that uses naturally-selected stable RNA building blocks for structure building, and the example is the 3WJ motif from pRNA of bacteriophage phi29 DNA packaging motor. In addition to discovering that the pRNA-3WJ shows exceptional stability under physiological conditions and in the presence of strong
denaturing agent (10,11), recent studies also suggest that the thermodynamic stability of the 3WJ is entropy driven (37).

In this report, we introduce a conceptual approach to the rational design of stable RNA architectures by stretching the 60° AO3 angle (L) (Figure 1) of the thermodynamically stable pRNA 3WJ motif. We demonstrate that it can be stretched to wide conformations resulting in different 3D polygons: triangle (AOB = 60°) (18), square (AOB = 90°) and pentagon (AOB = 108°). Intermolecular interactions such as kissing loops, receptor loop, or ‘sticky-ends’ were avoided by introducing linkages through base pairing between corners of the polygons using RNA double helices. Therefore, this system is advantageous with an increased thermo-stability in the overall construct.

We further demonstrate that the RNA polygons have the potential to serve as a new generation of delivery systems for immunomodulators. Synthetic unmethylated cytosine-phosphate-guanine oligodeoxynucleotides (CpG ODNs) are immunostimulatory DNA molecules that mimic the immunostimulatory activity of bacterial DNA (38,39). CpG DNA motifs strongly activate the mammalian innate immune system by interacting with various immune cells via endosomal Toll-like receptor 9 (TLR9) (40,41). Upon the stimulation by CpG DNA, immune cells could secret a variety of proinflammatory and antiviral cytokines including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-12 and interferon (IFN), which leads to potent immune response. The therapeutic potential of CpG DNA has also been extensively explored in both basic research and human clinical trials, including the development of new vaccine adjuvants, anticancer agents, immunoprotective agents and anti-allergic agents (42–45). Recently, several groups have reported the utilization of DNA nanostructures such as DNA tetrahedrons (46), DNA polypropid-like structures (47), DNA origami structures (48), Y-shaped DNA (49) and DNA dendrimers (50) to deliver immunostimulatory CpG DNA. In this work, to the best of our knowledge, we report the first use of RNA nanostructures to deliver CpG DNA in vitro and in vivo. The immunostimulatory efficacy of RNA polygons was evaluated by measuring the release of cytokines. We found that the induction of cytokines is highly dependent on the number of CpG per polygon. With increasing number of CpG per polygon, stronger immune response was observed, demonstrating the advantage of the transition from a triangle to a pentagon that can carry five CpGs.

**MATERIALS AND METHODS**

**RNA nanoparticles design, synthesis and self-assembly**

The 3WJ crystal structure of the pRNA molecule (PDB ID: 4KZ2) was primarily used for designing polygon models using Swiss PDB viewer, as previously described (18). RNA strands for corresponding triangle, square and pentagon, were synthesized by in vitro T7 transcription using polymerase chain reaction (PCR) generated DNA templates. RNAs were purified by denaturing polyacrylamide gel electrophoresis (PAGE) and were either Cy5 whole body RNA labeled (Mirus Bio LLC) or 5'-end [γ-32P] ATP (PerkinElmer) labeled, as previously described (8).

RNA polygons were assembled in one pot by mixing equimolar concentrations (1 μM) of four RNA strands for the triangle, five RNA strands for the square and six RNA strands for the pentagon in 1× TMS buffer (50 mM TRIS pH 8.0, 100 mM NaCl and 10 mM MgCl2). Samples were annealed for 1 h at a thermocycler with controlled, slow cooling (1°C/min) from 80 to 4°C. All RNA polygons harboring CpG ODNs were assembled from their corresponding 2′-F-U/C modified strands in one pot procedure.

**Native PAGE, temperature gradient gel electrophoresis (TGGE) and boiling resistance assays**

RNA assemblies were evaluated on 7% (29:1) native polyacrylamide gels in the presence of 0.5 × TMS buffer. Gels were run at constant 90 V, 44°C. Gels were imaged with Typhoon FLA 7000 (GE Healthcare) to visualize RNA strands. TGGE analysis was performed on 7% native PAGE in a buffer containing 50 mM TRIS pH 8.0, 100 mM NaCl and 0.2 mM MgCl2, as previously described (14,26,28). A gradient temperature of 30–70°C was applied perpendicular to electrical current and the experiment was run for 1 h at 20 W. A total RNA concentration of 100 nM was used in TGGE analysis. Apparent Ts values corresponded to the temperature at which half of the polygons fractions were dissociated and apparent Kd values for multiple RNA strands were calculated, as described previously (7).

Boiling resistance assay was performed in 10 μl containing 1 μM preassembled polygons in TMS buffer or in the presence of 8 M urea. Samples were incubated at 100°C for several minutes, then snap cooled on dry ice to prevent refolding following evaluation on 7% native PAGE at 4°C. Individual experiments were repeated several times to reduce error.

**Quantification analysis**

Quantification analysis was performed using ImageJ (52). Equal-sized boxes were drawn around the lanes corresponding to the triangle, square, or pentagon complexes and corresponding quantified values for each type of polygon were divided by the sum of the values presented in the corresponding lane.

**Cell cultures**

Mouse macrophage-like RAW 264.7 cells were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 mg/ml streptomycin at 37°C in humidified air containing 5% CO2. Cells were then seeded on 24-well plates or 96-well
plates at a density of $5 \times 10^5$ cells/ml and cultured overnight before use.

**Cytokine secretion from RAW264.7 Cells**

RAW 264.7 cells were plated into 24-well plates with the density of $2.5 \times 10^5$ per well and cultured overnight. Then, RNA nanoparticles harboring different numbers of CpG ODNs were diluted in Opti-MEM medium (Life Technologies Corporation, Carlsbad, CA, USA) and added to the cells. The cells were continually cultured for 8 h at 37°C in humidified air containing 5% CO$_2$, and the cell culture supernatant were collected and stored at $-80^\circ$C until use. The concentration of TNF-α and IL-6 in the supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using Mouse ELISA MAXTM Deluxe sets (BioLegend, Inc., San Diego, CA), following protocols provided by the manufacturer.

**Cytokine secretion from mice**

Male CD-1 mice (4–5 weeks old) were purchased from Charles River Laboratories. All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Kentucky and were performed in accordance with guidelines issued by the National Institutes of Health for the care of laboratory animals. For *in vitro* immunostimulation, RNA triangular nanoparticles harboring CpG ODN, RNA triangular nanoparticles, or CpG ODN were dissolved in phosphate buffered saline (PBS) and administrated to the mice via tail vein injection at 2 mg/kg (CpG ODN per body weight). The same volume of PBS was injected into a mouse as a control. Blood samples were collected 3 h post-injection by cardiac puncture. Serum was prepared by centrifugation at 12,000 g for 10 min. Serum TNF-α and IL-6 levels were determined by enzyme-linked immunosorbent assay (ELISA) using Mouse ELISA MAXTM Deluxe sets (BioLegend, Inc., San Diego, CA, USA), following protocols provided by the manufacturer.

**Confocal microscopy imaging**

RAW 264.7 cells were seeded on glass coverslips in 24-well plates and cultured at 37°C in humidified air containing 5% CO$_2$ overnight. The culture medium was removed and the cells were washed with Opti-MEM medium twice to remove dead cells. RNA nanoparticles harboring Cy3-labeled CpG DNA or Cy5-labeled CpG DNA only were diluted in Opti-MEM medium and added to the cells. After 4 h incubation at 37°C in humidified air containing 5% CO$_2$, the cells were washed twice with PBS and fixed with 4% formaldehyde. ProLong® Gold Antifade Reagent with DAPI (Life Technologies Corporation, Carlsbad, CA) was used to stain the cell nucleus and mount the samples. Alexa Fluor® 488 phallodin (Life Technologies Corporation, Carlsbad, CA, USA) was used to stain actin. The images were obtained on a Olympus FV1000 confocal microscope (Olympus Corporation, Tokyo, Japan).

![Figure 2. Design of Polygons and assembly properties.](image)

**RESULTS**

**RNA polygons: triangle, square and pentagon fabrication and self-assembly**

The structural features of the recently discovered ultrastable pRNA 3WJ module from the bacteriophage Phi29 DNA packaging motor were utilized for *in silico* design of the RNA triangle, square and pentagon 2D polygons. During the computer modeling we used the particular angle of the 3WJ formed by H1 and H2 as an inner angle of the polygons as we hypothesized that the angle could be stretched to a more open conformation. Throughout this report the intra-helical angle between H1 and H2 is denoted as $\angle$AOB, as shown in figure 1. Each RNA model contained a pRNA 3WJ motif at each vertex, and the inner angles correspond to $\angle$AOB. The resulting 3D models exhibited flat conformations, as expected from the plane geometry of the 3WJ motif (S1) (Figure 2A).

Each polygon was composed of a different number of RNA strands classified as short strands (external) and long strands (internal) (Figure 2B). By increasing the number of external strands and the propagation of the central or internal strand, the tension on the inter-helical $\angle$AOB increased to 60°, 90° and 108°, allowing for 2D formation of corresponding triangle, square and pentagon shapes. The measured widths, from one corner to another, were 10.2 nm, while the heights differed as follows: triangle = 9.1 nm, square = 10.2 nm and pentagon = 12.7 nm. Following
the transcription of individual RNA strands, self-assembly properties of the triangle, square and pentagon designs were evaluated on 7% native polyacrylamide gel electrophoresis (PAGE) (Figure 2C). All polygon formations were obtained by one-step self-assembly (7,10,11,19). Each RNA component of corresponding nanoparticles were whole chain labeled with Cy5 to evaluate participation of all RNA strands in their corresponding assemblies. Yield of correctly assembled polyplexes was estimated to be >90% based on native PAGE gel evaluations. Equilibrium constants of dissociation were obtained from apparent \( K_d \) gels, and \( K_d \) values were determined to be 18.8, 20.3 and 22.5 nM for triangles, squares and pentagons, respectively (Supplementary Figures S1 and S2).

These results demonstrate that each RNA nanoparticle assembles into the desired nanostructure and indicated by a stretching of the 60° \( \angle \text{AOB} \) to wider conformations. The assembly of RNA strands into specific-shaped nanoparticles based on the 60° \( \angle \text{AOB} \) of the prRNA 3WJ motif was controlled by modulating the number of short external strands and the length of the long internal strand.

**Structural characterization of polygons by atomic force microscopy (AFM) and dynamic light scattering (DLS)**

To further evaluate the size and shape of the resulting RNA assemblies, structural characterization of each polygon was conducted by AFM. AFM images of the prRNA 3WJ based polyplexes revealed that the shapes of resulting polygons were similar to their predicted, theoretical 3D models (Figure 3A). The estimated average dimensions were found to be 13 ± 1.1, 14 ± 1.8 and 17 ± 1.6 nm for triangles, squares and pentagons, respectively. These values do not reflect the true sizes of the RNA polygons due to the AFM tip convolution, but rather demonstrate that the average size of the nanoparticles increases from triangle to pentagon. In addition, the central cavity of each RNA shape is visible, and the size of the cavity gradually increases with the number of polygon vertices. The measured heights for all nanoparticles was found to be ~2 nm, in agreement with previously reported heights of nucleic acid double helices (26,53).

Quantification analysis was performed to compare apparent yields between polygons observed on AFM mica surface. Equal concentrations (1 nM) of the polyplexes were deposited on a mica surface and correctly folded polyplexes were manually counted in a 0.5 µm² area, resulting in 48 triangular particles, 33 square particles and 17 pentagon nanoparticles (Supplementary Figure S3). The estimated number of triangular nanoparticles adsorbed on the mica was ~1.5 times more than that of the square and three times more than that of the pentagon. However, native PAGE revealed that the yields among the three polygons were almost equal. The difference in adsorption amounts between polyplexes on the mica surface was presumably due to variation in their sizes and 3D conformations, resulting in different dynamic and physical properties.

DLS was performed to determine the apparent hydrodynamic diameters for each of the polyplexes. The diameters were found to be 8.5, 11.1 and 13.5 nm for triangles, squares and pentagons, respectively (Figure 3B). The decrease in number of 3WJ cores corresponds with the larger observed diameter. The measured diameters agreed with their corresponding 3D models. However, there was a discrepancy between polyplex sizes determined by AFM and DLS. This could be attributed to the fundamentally different techniques, as DLS determines the average size distribution profile of nanoparticles in solution assuming that the polyplexes have globular shapes [refer to manual at http://www.malvern.com], while AFM imaging can produce images larger than the real diameter due to tip size used (34) and the resolution of imaging equipment. Nevertheless, the two techniques demonstrated that the relative size of the nanoparticles increased from triangle to square to pentagon.

Accordingly, native PAGE, AFM and DLS showed the formation of compact molecular 2D assemblies of triangle, square and pentagon based on the prRNA 3WJ \( \angle \text{AOB} \). Consequently, the naturally preserved 60° \( \angle \text{AOB} \) could be stretched to reach the formation of square and pentagon. The stretching, or tension, that the angle underwent could have had a significant impact on the overall stability of the nanoparticles. Therefore, it was of great interest to evaluate and compare the polyplex's stabilities.

**Stability comparison between triangle, square and pentagon**

The stabilities of polyplexes were studied using a perpendicular TGGE (Biometra GmbH). This convenient technique has garnered widespread use for measuring melting temperatures of RNA nanoparticles with multiple strands (5,7,14,26,28,55). The preassembled polyplexes were subjected to 7% native TGGE with a gradient temperature of 30–70°C perpendicular to electrical current. The following
apparent $T_M$ values were obtained for the polygons at 100 nM total concentration ($C_t$) in presence of 0.2 mM MgCl$_2$; triangle $T_M = 56.5 ^\circ C$, square $T_M = 53 ^\circ C$ and pentagon $T_M = 50 ^\circ C$ (Figure 4A). The triangular nanoscaffolds were more stable than squares and pentagons, although the number of RNA bp was much higher in the pentagon construct, as compared to the square and triangle. Usually, the stability of nucleic acids with the same base-pair content is directly dependent on metal ion and total nucleic acid concentrations. Since these two criteria were the same, it was assumed that the higher the number of bp in a given RNA structure the higher the stability. Therefore, the most stable shape produced should be the pentagon. However, based on TGGE data the opposite was found. This was likely due to the extension caused by the stretching of the native RNA 3WJ 60$^\circ$ $\triangle$AOB. The triangular construct angle was preserved ($60^\circ$), the square and pentagon angles were stretched to the wider conformations of $90^\circ$ and $108^\circ$, respectively. Previously it has been shown that any nucleotide mutations or deletions within the native core structure of the pRNA 3WJ motif would also result in the loss of its thermodynamic stability (10). Interestingly, the measured triangle and square $T_M$ values differed by $+3^\circ$C, as did the square and pentagon. Boiling resistance assay in the presence and absence of 8 M urea further confirmed that the triangle was the most stable nanoparticle (Figure 4B). The quantification of nanoparticle bands after heating to 100$^\circ$C resulted in 75 $\pm$ 4% recovery of the triangular assembly, suggesting a $T_M > 100^\circ$C. By definition, $T_M$ is the measured temperature when half the RNA concentration has melted, i.e. 50% recovery. The percentage of recovery for square was 28 $\pm$ 2% and for pentagon was 16 $\pm$ 5%, much lower than the value estimated for triangle recovery. The experiment with the presence of 8 M urea in boiling solution showed that the overall trend of stability remained the same, but the percentage of recovery was 55 $\pm$ 4% for triangle, 8 $\pm$ 3% for square and no pentagon fraction was detected. Overall, the nanoparticle with fewer 3WJ motifs (triangle) resulted in a higher thermostability and resistance in chemical degradation and the change in stability was in large part due to the stretching of the $\triangle$AOB.

The modulation effect of triangle, square and pentagon harboring immunological adjuvant

CpG oligonucleotide is an immunological adjuvant popularly used as vaccine adjuvant or immunotherapy reagent for disease control and treatment (42). To evaluate whether RNA polygons can enhance the immunomodulation effect, CpG oligonucleotide was incorporated with each RNA polygon using one-pot self-assembly (Supplementary Figures S4-S6). The toxicity assay for the resulting complexes revealed no toxicity; moreover the RNA polygon-CpG complexes induced cell proliferation during the incubation period, as compared to the cell only control (Supplementary Figure S7).

The extracellular immunostimulatory efficacy of RNA polygons was evaluated by measuring the release of cytokines TNF-α and IL-6 after addition to mouse macrophage-like RAW 264.7 cells (Figure 5A and B), as previously described (56,57). The triangular RNA nanoparticle coupled with only one CpG exhibited the highest level of cytokine induction for both TNF-α and IL-6 compared to square and pentagonal RNA nanoparticles. Increasing the number of CpG per nanoparticle yielded the opposite effect, as pentagonal RNA nanocarriers showed the highest level of the induction of both TNF-α and IL-6 presumably due to the increased local CpG concentration. The results suggest that the cytokine release by CpG coupled to RNA polygons with different shapes remarkably increases the immunostimulatory activity compared to CpG alone (Figure 5). RNA particles with the size of about 10 nm, such as the triangle, induced the greatest amount of TNF-α and IL-6. In addition, the induction of cytokines was highly dependent on the number of CpG per polygon. With increasing number of CpG per polygon, a stronger immune response is observed (Figure 5C), demonstrating an advantage of transiting from triangle to pentagon that can carry five CpG oligonucleotides.

Enhancement of modulation effect in animal by immunological adjuvant incorporated into RNA triangle

To examine whether RNA nanoparticles retain their immunostimulatory activity in vivo, nanoparticles were administered to CD-1 mice by injection into the tail vein at 2 mg/kg (CpG oligonucleotide per body weight), following level determination of cytokine TNF-α and IL-6 levels after 3 h post-administration in collected blood serum. Figure 5D shows that free triangle nanoparticles and free CpGs did
not induce any cytokine production, whereas the complex triangle-CpG resulted in elevated levels of both cytokines. The difference in immunostimulatory activity of triangle-CpG was estimated to be 10-fold compared to free CpG in vitro. These data are in agreement with the in vitro stimulation of murine RAW 264.7 cells.

**Comparison of cellular uptake by different Cpg-RNA polygons**

Previously, it has been demonstrated that the CpG oligonucleotide can be readily recognized by TLR9 on the endosomal membrane of macrophages, resulting in cellular uptake of the CpG adjuvants (58, 59). To investigate whether there is a difference between the efficiency of RNA polygons binding to the cells, we quantified the cellular uptake of polygons-CpG using flow cytometry assay (9, 10). Figure 6A demonstrates the binding of different RNA polygon-CpG to the RAW264.7 cells in a dose-dependent manner. There was an increase in binding efficiency from triangle to pentagon with more CpGs (Supplementary Figure S8). Notably, all RNA polygons-CpG complexes remain intact after 16 h incubation in 10% fetal bovine serum (FBS) indicating robustness of the assembled complex in extracellular environment (Supplementary Figure S9). Overall, RNA polygon-CpG complexes exhibit significantly more binding efficiency to cells compared to CpG oligonucleotides alone.

This observation was further confirmed by confocal microscopy images (Figure 6B) revealing that RNA nanoparticle harboring CpGs are localized in the cytoplasm exclusively and that there are much higher amounts of triangular nanoparticles inside the cell compared to free CpG, suggesting that RNA nanoparticles could efficiently enhance the cellular uptake of the CpG adjuvants.

Collectively, both flow cytometry and confocal imaging demonstrated that all RNA nanoparticles with different shape and harboring CpGs have much stronger binding and cellular entry to the macrophage-like RAW 264.7 cells compared to free CpGs. In addition, cellular uptake was highly dependent on the number of CpG per polygon. With the increasing number of CpG per polygon, more efficient cell binding and entry was observed (Figure 5C and Supplementary Figure S8), demonstrating the advantage of the transition from triangle to pentagon nanoparticles that can carry five CpG.

**DISCUSSION**

Artificial construction of RNA nanoparticles requires knowledge of the structural properties of RNA motifs, such
as interhelical or intrahelical distances, X–Y and X–Y–Z angles, number and orientations of RNA branches in multiway junctions, canonical and non-canonical interactions, binding sites for proteins, metal ions and small molecules (2,10,16,25,51,60). Progression in RNA structural biology allowed for the analysis of RNA 3D motifs from existing RNA structures at atomic resolution and various databases, including www.pdb.com, RNA multi-way junction (61) and Find RNA 3D motif (62). This study, based on the previously reported versatile pRNA 3WJ 3D motifs (PDB accession ID: 4KZ2) (10,51), shows that the 3WJ structure can be folded into desired conformations based on the dynamics of the 60° /AOB, demonstrating the ability to tune the physical and structural properties of RNA polygons for a variety of technological, biological and medicinal needs. By using the 3WJ, a strategy has been reported to tune the size of a square by altering the length of each side of the polygon (63). This approach, based on the propagation of the central RNA strand used to direct the folding of corresponding short or external strands into planar triangle, square and pentagon conformations, resulted in the 60°, 90° and 108° bending of interhelical /AOB of the pRNA 3WJ. This is especially important for medical applications where one needs to construct different RNA nano-scaffolds based on a non-toxic and thermodynamically stable building block. The following advantages result from this technique: (i) the number and combination of therapeutic molecules can be tuned to an RNA-based nano-carrier; (ii) the nano-scaffold has a controllable size and shape and (iii) variable thermodynamic and RNase resistance properties can be applied depending on the application of the nano-scaffold.
In addition to the discovery of the unique approach for the rational design of stable RNA nanoparticles, it has been demonstrated that each RNA polygon has the potential to serve as multivalent nanocarriers for vaccine adjuvants, particularly of CpG oligodeoxynucleotides. The designed RNAs self-assemble into distinct, non-toxic homogeneous nanoparticles with high chemical, thermal and intracellular stability. We found that the size and shape of the RNA nanostructures are important factors for the induction of immunostimulatory processes in vitro and in animal models and that there is a correlation between the cytokine induction and the local CpG concentration effect. The highest level of secretion of pro-inflammatory cytokines TNF-α, IL-6 was obtained with the smallest nanoparticle (triangle ~ 9 nm size) harboring one CpG compared to square (~11 nm) and pentagon (~13 nm). However, upon increasing the numbers of CpG per RNA nanoparticle the cytokine induction was affected more by pentagon as the number or local concentration of CpG is highest in the pentagon. This study illustrates the importance of the size and shape of RNA nanoparticles for improvement of activity of CpG based vaccines adjuvants targeting infectious diseases and cancer cells, as well as for increasing immune responses by the innate and adaptive immune systems. The RNA nanoparticles harboring CpGs are safe, effective, versatile and easy to manufacture, offering new solutions to address the unmet needs in current vaccines and adjuvants design and development. Recent findings on the thermodynamically unstable (10,11) and heat-resistant (18) RNA nanoparticles have expanded the potential for application of pRNA 3WJ derived nanoparticles in the fields of biomedical, nanotechnology, or polymer industries.

REFERENCES

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References


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Cui, D., et al., Regression of gastric cancer by systemic injection of RNA nanoparticles carrying both ligand and siRNA. Scientific Reports, 2015. 5.


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