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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 unstability. RNA can serve as a unique polymeric material to build varieties of nanostructures including nanoparticles, polygons, 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 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 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 nucleobase, and a phosphate group. The nucleotides are covalently linked together through $3' \rightarrow 5'$ 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 macro-molecule 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 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 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) thermostabilty. 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_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 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 (G^0) required for double helix formation, or conversely, double helix unwinding ($G^0 = -G^0$helix formation $= G^0$helix unwinding). Remarkably, the RNA double helix is more thermodynamically stable than the DNA double helix considering G^0 for RNA double helix formation is, on the average, -3.6 to -8.5kJ/mol per base pair stacked and G^0 for DNA double helix formation is -1.4kJ/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^{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 ribonucleases (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, 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$_2$) 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 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, oxidoreduc-tases 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(lactic-*co*-glycolic 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 biodegrad-ability [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 (45–50nm) 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 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 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 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 IIa-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. 1I). 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-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 [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, pen-tamer, 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 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 [85,114,115] (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 tectoR-NAs 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 molecule folding. A palindrome sequence reads the same whether from $5′ \rightarrow 3′$ direction on one strand or from $5′ \rightarrow 3′$ 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 palindrome sequences can also be utilized to bridge RNAnanostructures, 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 RNA nanoprisms 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 metalions [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 retain 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 knock-down 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, Khisamutdinov 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° angle) and pentagonal shape (108° angle) 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 (K_D) for triangle, square and pentagon are 18.8 nM, 20.3 nM and 22.5 nM, and the 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 [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 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 [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 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 [114,115,144–146], and the spilt 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 spherulitic 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 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 spherulitic 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. 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 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 boranophosphate, phosphoramidate 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 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 [163]. The chemically modified pRNA-based nanoparticles have shown promising applications in cancer therapy [36,66,164–167]. Recent study also showed that 2′-F modified pRNA nanoparticles are resistant to I-125 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 into 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 (ASO) 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]. (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 [139]. 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 [155,176,177]. (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 [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 phi29 motor pRNA was achieved *via* experience and brainstorming without computer algorithm 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 [182–184]. 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 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 [92,186]. 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/h, 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 palindrome sequences (nucleotide sequences that read the same $5' \to 3'$ on one strand and $3' \to 5'$ 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 confirmed with both polyacrylamide 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 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 [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 siRNA targeting surviving [108]. 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 [111]. 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 [188]. 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) [189]. 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 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 8M 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 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. 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 siRNA 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 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 functioned 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 siRNA 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 Bcl2Like12 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 (1000copie/μm^2) 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 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 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) [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 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 [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 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 [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 ($t_{1/2}$) of the MG aptamer containing pRNA-3WJ inside living cells to be ~4.3h.

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 (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. Then, each hammerhead ribozymes cleaved off their 3′ fragment according to addtion of individual analytes. They also showed the quantitave and qualitive measurement of cAMP in culture media from *E. coli* strand.

RNA can also serve as the detection analytes to detect viable *E. coli* as an indicator organism in drinking water, which was proposed by Baeumner 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. Gothelf group developed a RNA-based electrochemical biosensor for theophylline in serum with a ferrocence (Fc) redox probe [225,233]. In this study, the thiol group-modified and amino group-modified RNA aptamer was 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-caroxylic acid NHS reaction. Usually, the RNA aptamer stays on the open conformation in absence of theophylline. However, when the theophylline added to RNA apatmer-modified electrode, the aptamer folds into the conformationally restricted hairpin strucutre. 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 [232].

In an alternative approach, Hammond group used a RNA-based fluorescent biosensors 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 DFHBI fluorescent molecule for cell imaging. This system provided selective and sensitive detection for each cyclic dinucleotide. Thus, there are many examples demostrating 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 DNA [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. 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 [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, 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, 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 genes 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 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 [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, 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 [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 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 [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(O-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 pi–pi 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 demostrated 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 sp^2-bonded carbon atoms. It can be used as a fundamental building block for constructing other graphite-based structures such as fullerenes 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 anticancer 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 nanoparticle has been successfully demonstrated by a series of studies for nanosized 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 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 [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 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

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Sijin Guo, B.S., received his B.S. in chemistry from Wuhan University, China in 2014. He is currently a Ph.D. candidate in Prof. Peixuan Guo's lab at University of Kentucky. His research interests include the study of function, mechanism and application of bacteriophage phi29 DNA-packaging nanomotor as well as applications of RNA nanoparticles derived from phi29 packaging RNA (pRNA) in therapeutics delivery, targeting and disease treatment (RNA nanotechnology).

Dr. Jing Xu, Ph.D., is an associate professor at the National Center for Nanoscience and Technology, Beijing, China. She received her B.S. in Chemistry in 2004 and M.S. in Polymer Science in 2007 from Nankai University, Tianjin, China. In 2012, she received her Ph.D. in Chemistry from the University of North Carolina at Chapel Hill with Professor Joseph M. DeSimone. Her work focuses on development of drug delivery systems for treatment of cancers and prevention of infectious diseases.

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Dr. Farzin Haque, Ph.D., is a Research Assistant Professor in the University of Kentucky College of Pharmacy, Department of Pharmaceutical Sciences. He received his B.A. degree in Biochemistry and Mathematics (2004) from Lawrence University and a Ph.D. degree in Chemistry (2008) from Purdue University. He held a postdoctoral appointment (2009–2011) at the University of Cincinnati, with Professor Peixuan Guo. Dr. Haque's scholarly interest broadly focuses on Nanoscience and Nanotechnology in Biology and Medicine. These include, nanopore-based technology for single molecule detection and sensing of chemicals and biopolymers; and RNA Nanotechnology - construction of RNA nanoparticles for therapeutic and diagnostic applications.

Dr. Xing-Jie Liang, Ph.D., got his Ph.D. at National Key Laboratory of Biomacromolecules, Institute of Biophysics at CAS. He finished his postdoc at Center for Cancer Research, NCI, NIH, and worked as a Research Fellow at Surgical Neurology Branch, NINDS. He worked on Molecular imaging at School of Medicine, Howard University before he became deputy director of CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology of China. He is a founder member of International Society of Nanomedicine, member of American Association for Cancer Research, and member of American Society of Cell Biology. He is current Associate Editors of 'Biomaterials' and 'Biophysics Report'; Advisory editorial board member of 'ACS Nano'; Editorial member of 'Advances in Nano Research', 'Current Nanoscience', 'Biomaterials Research', Theranostics' and guest editor of 'Biotechnology Advances'. Developing drug delivery strategies for prevention/treatment of AIDS and cancers are current program ongoing in Dr. Liang's lab based on understanding of basic physiochemical and biological processes of nanomedicine.

Dr. Peixuan Guo, Ph.D., is William Farish Endowed Chair in Nanobiotechnology, director of University of Kentucky Nanobiotechnology Center and director of NIH/NCI Cancer Nanotechnology Platform Partnership Program: "RNA Nanotechnology for Cancer Therapy". He obtained his Ph.D from University of Minnesota, and postdoctoral training at NIH, joined Purdue University in 1990, was tenured in 1993, became a full Professor in 1997, and was honored as a Purdue Faculty Scholar in 1998. He constructed phi29 DNA-packaging motor, discovered phi29 motor pRNA, pioneered RNA nanotechnology, incorporated phi29 motor channel into lipid membranes for single-molecule sensing with potential for high-throughput dsDNA sequencing. He is a member of two prominent national nanotech initiatives sponsored by NIH, NSF, NIST, and National Council of Nanotechnology, director of one NIH Nanomedicine Development Center from 2006–2011. His work was featured hundreds of times over radio, TV such as ABC, NBC, newsletters NIH, NSF, MSNBC, NCI, and ScienceNow. He was a member of NIH/NCI intramural site-visit Review Panel at 2010 and 2014, and a member of the Examination and Review Panel (Oversea Expert) of the Chinese Academy of Sciences since 2014.
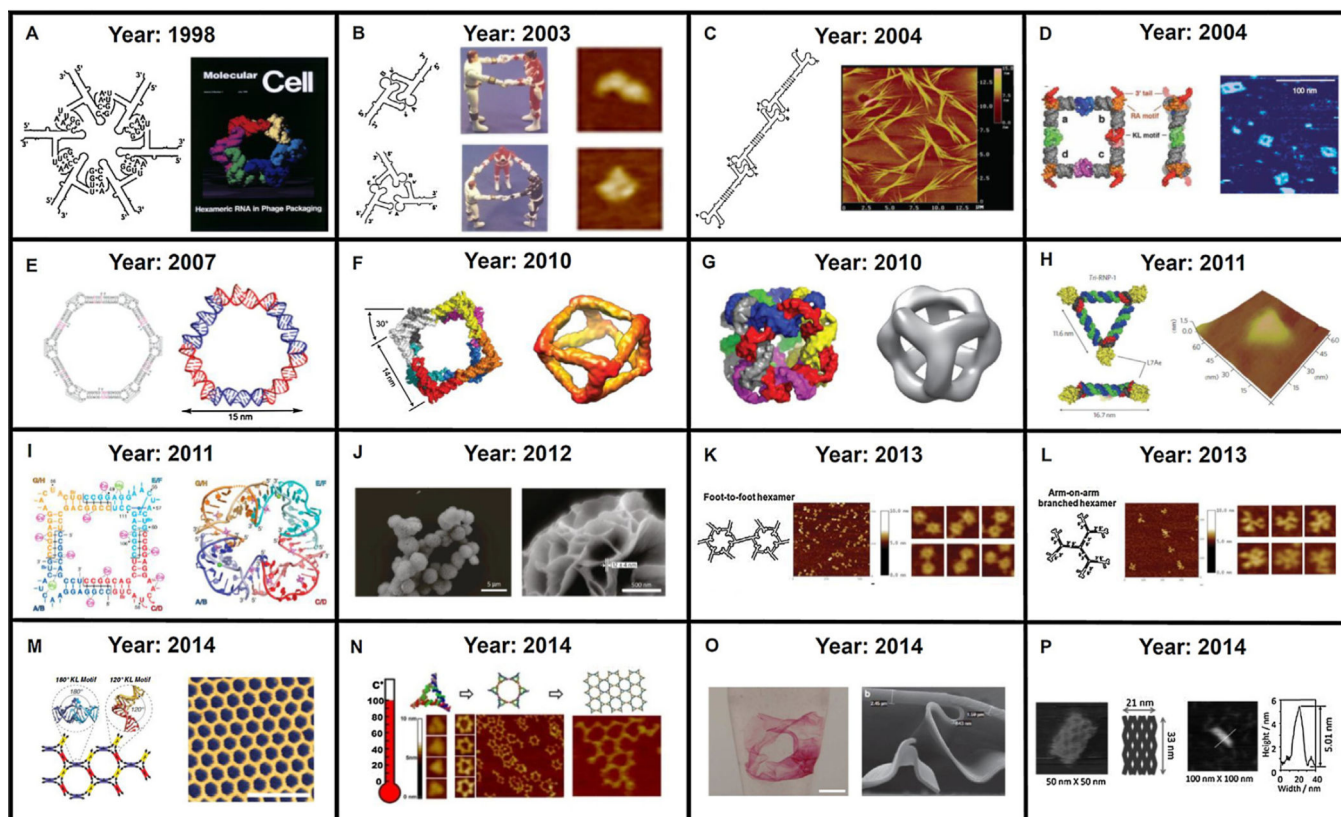
Figure 1.
Design and construction of RNA nanostructures. (A) Phi 29 DNA packaging motor pRNA hexamer [110]. Reprinted with permission from Ref. [110]. Copyright 1998 Elsevier. (B) pRNA dimer and trimer [106]. Adapted with permission from Ref. [106]. Copyright 2003 American Scientific Publishers. (C) pRNA arrays [112]. Reprinted with permission from Ref. [112]. Copyright 2004 American Chemical Society. (D) RNA tecto squares [116]. Reprinted with permission from Ref. [104]. Copyright 2004 The American Association for the Advancement of Science. (E) The RNA nanoring [114]. Reprinted with permission from Ref. [114]. Copyright 2007 American Chemical Society. (F) The tRNA-based polyhedron [118]. Reprinted with permission from Ref. [118]. Copyright 2010 Nature Publishing Group. (G) Cubic RNA-based scaffolds designed *in silico* [144]. Reprinted with permission from Ref. [144]. Copyright 2010 Nature Publishing Group. (H) Triangular RNA—protein complex [127]. Reprinted with permission from Ref. [127]. Copyright 2011 Nature Publishing Group. (I) The self-assembling RNA square [93]. Reprinted with permission from Ref. [93]. Copyright 2011 The National Academy of Sciences of the United States of America. (J) RNA microsponges for siRNA delivery [147]. Reprinted with permission from Ref. [147]. Copyright 2012 Nature Publishing Group. (K) pRNA nanoparticles fabricated via hand-in-hand and foot-to-foot tool kits [113]. Reprinted with permission from Ref. [113]. Copyright 2013 RNA Society. (L) pRNA nanoparticles fabricated via branch extension tool kit [113]. Reprinted with permission from Ref. [113]. Copyright 2013 RNA Society. (M) Single stranded RNA origami [136]. Reprinted with permission from Ref. [136]. Copyright 2014 The American Association for the Advancement of Science. (N)

Boiling-resistant RNA triangles and the array based on RNA triangles [36]. Reprinted with permission from Ref. [36]. Copyright 2014 American Chemical Society. (O) The self-assembled RNA membrane [152]. Reprinted with permission from Ref. [152]. Copyright 2014 Nature Publishing Group. (P) RNA origami tile and tube [139]. Reprinted with permission from Ref. [139]. Copyright 2014 John Wiley and Sons.
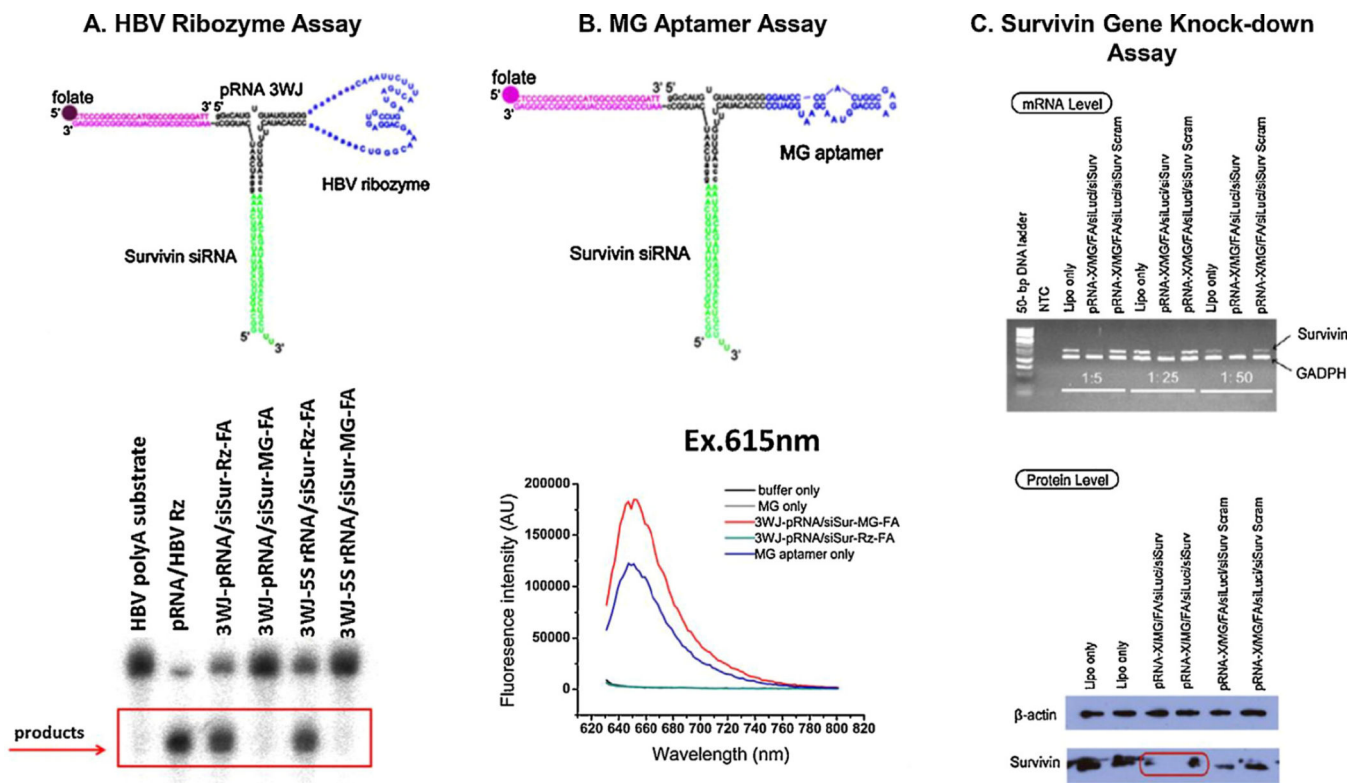
Figure 2.
Construction and functional assays of thermodynamically stable pRNA-(three-way junction) 3WJ or pRNA-X nanoparticles. (A and B) HBV ribozyme assay and MG aptamer assay of pRNA-3WJ nanoparticles [123]. Adapted with permission from Ref. [123]. Copyright 2011 Nature Publishing Group. (C) Survivin gene knock-down assay of pRNA-X nanoparticles [73]. Reprinted with permission from Ref. [73]. Copyright 2012 Elsevier.
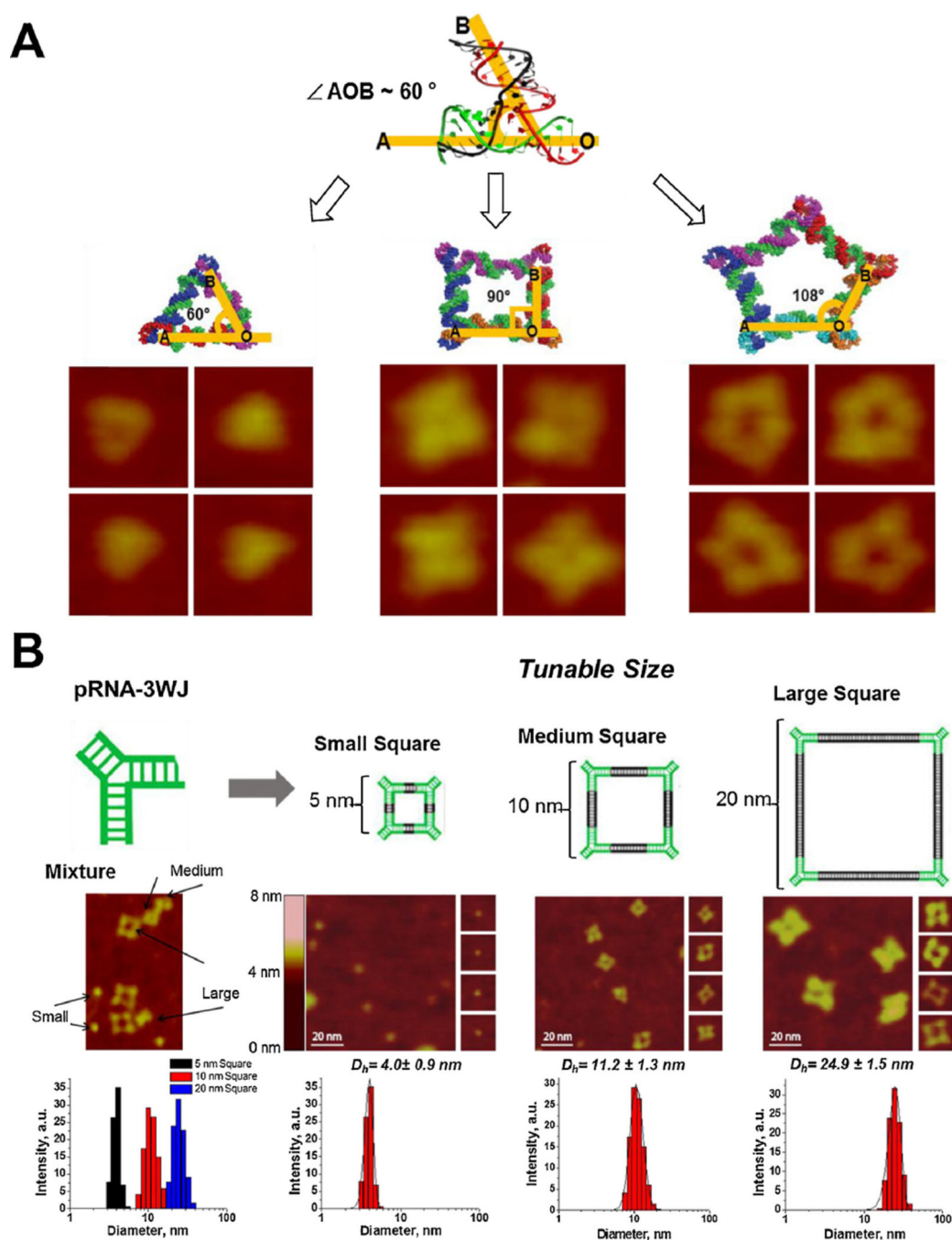
Figure 3.
Construction of RNA polygons based on pRNA-3WJ. (A) Construction of RNA triangle, square and pentagon by tuning the interior pRNA-3WJ angle [92]. Reprinted with permission from Ref. [92]. Copyright 2014 Oxford University Press. (B) Construction of RNA squares with tunable sizes [125]. Reprinted with permission from Ref. [125]. Copyright 2014 American Chemical Society.
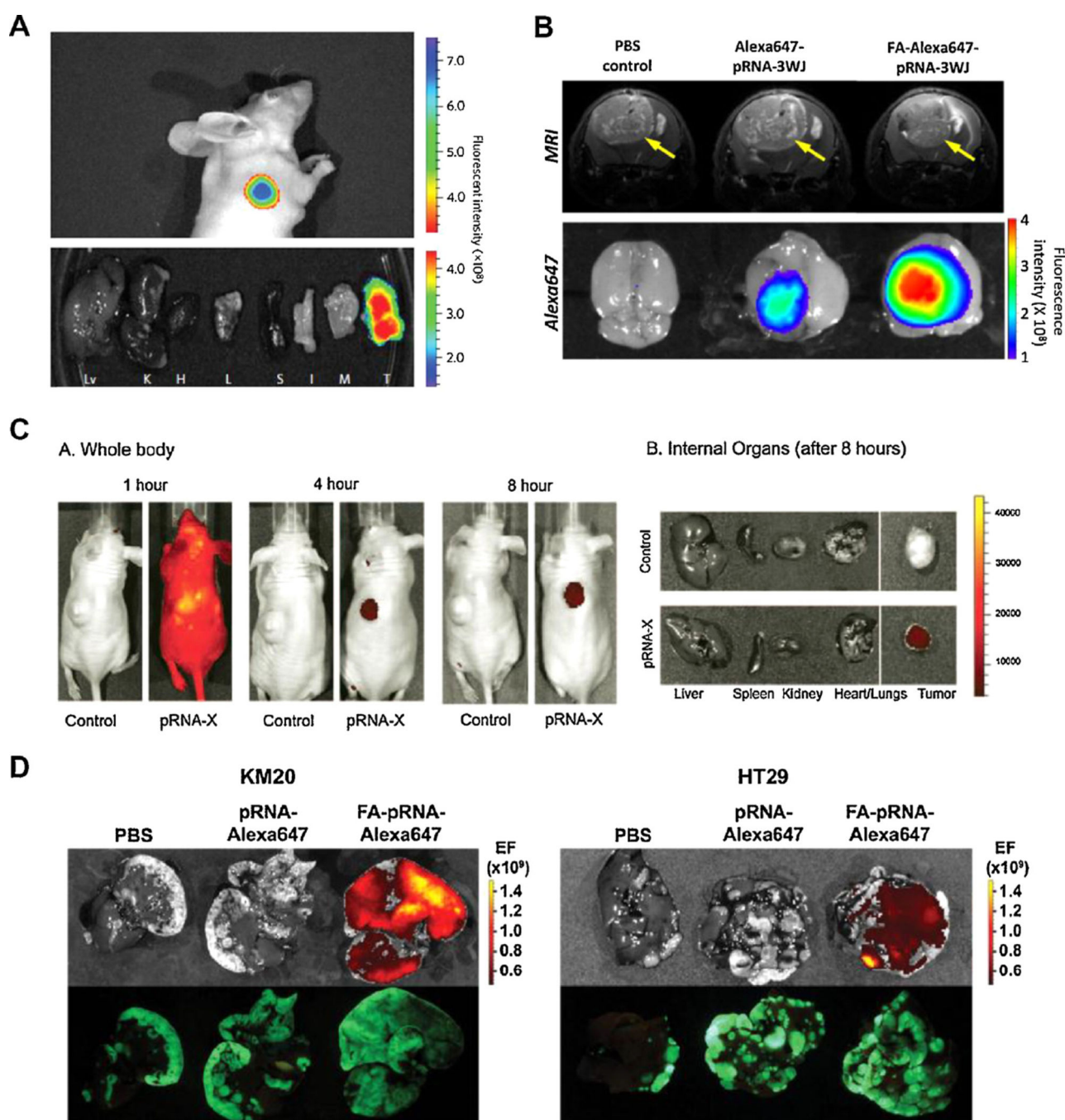
Figure 4.
pRNA nanoparticles for cancer targeting. (A) pRNA-3WJ nanoparticles target folate-receptor positive tumor xenografts [123]. Reprinted with permission from Ref. [123]. Copyright 2011 Nature Publishing Group. (B) pRNA-3WJ nanoparticles target glioma [164]. Reprinted with permission from Ref. [164]. 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) pRNA nanoparticles targeting

colorectal cancer metastases [165]. Reprinted with permission from Ref. [165]. Copyright 2015 American Chemical Society.
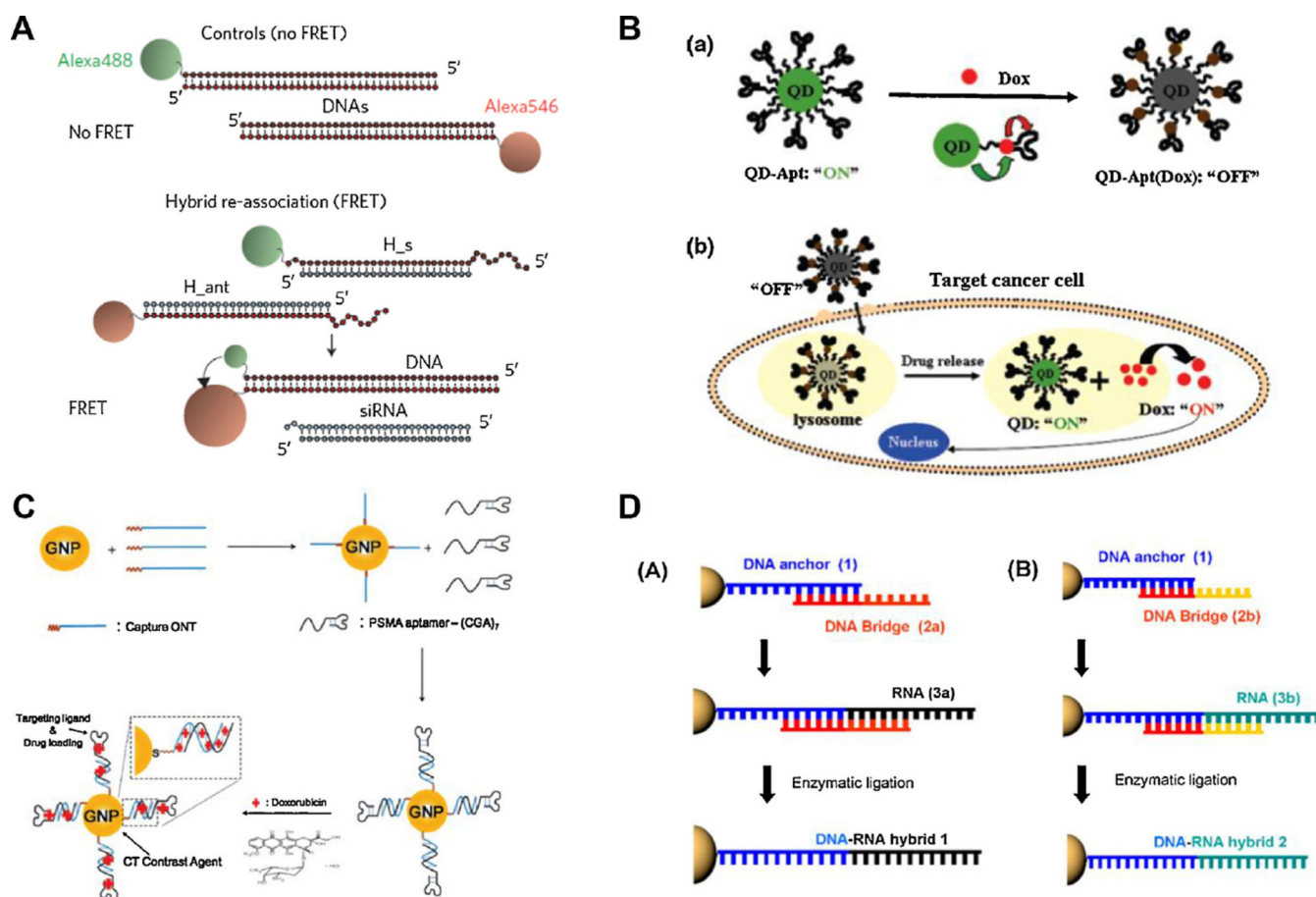
Figure 5.
Functional RNA nanostructures for therapeutics delivery and medical detection. (A) Triggered siRNA delivery based on RNA/DNA hybrid nanostructures [140]. Reprinted with permission from Ref. [140]. Copyright 2013 Nature Publishing Group. (B) QD-aptamer-doxorubicin conjugate nanoparticles as a cancer-targeted imaging, sensing and treatment platform [210]. Reprinted with permission from Ref. [210]. Copyright 2007 American Chemical Society. (C) PSMA aptamer-conjugated gold nanoparticles for targeted molecular CT imaging and therapy of prostate cancer [213]. Reprinted with permission from Ref. [213]. Copyright 2010 American Chemical Society. (D) Spherical nucleic acids scaffold for loading RNA therapeutics [191]. Reprinted with permission from Ref. [191]. Copyright 2014 American Chemical Society.
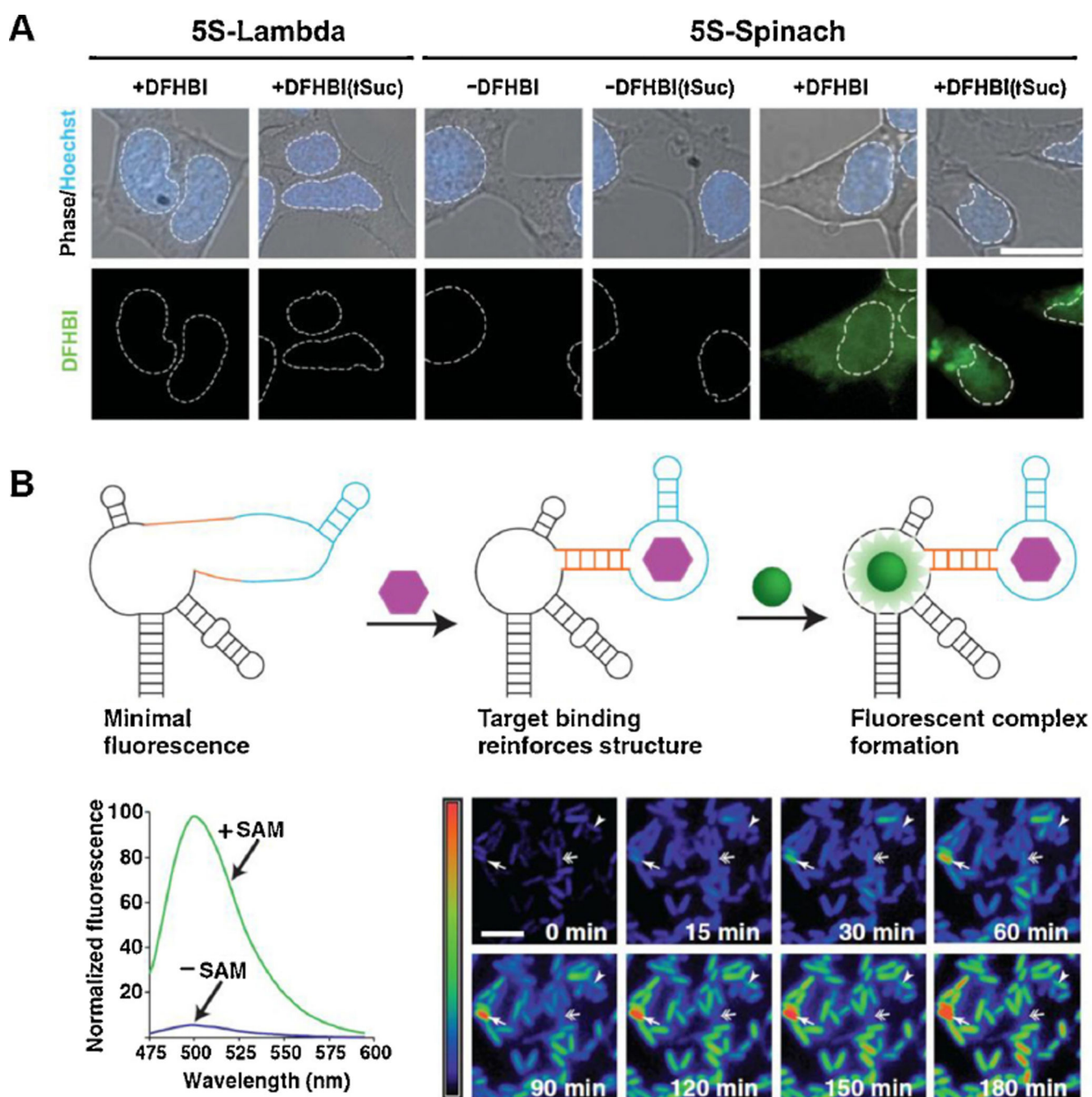
Figure 6.
Spinach RNA aptamer for intracellular imaging and sensing. (A) Live-cell imaging of Spinach-tagged 5S RNA[214]. Reprinted with permission from Ref. [214]. Copyright 2011 The American Association for the Advancement of Science. (B) Imaging cellular metabolites in *E. coli* with sensor RNA[215]. 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.
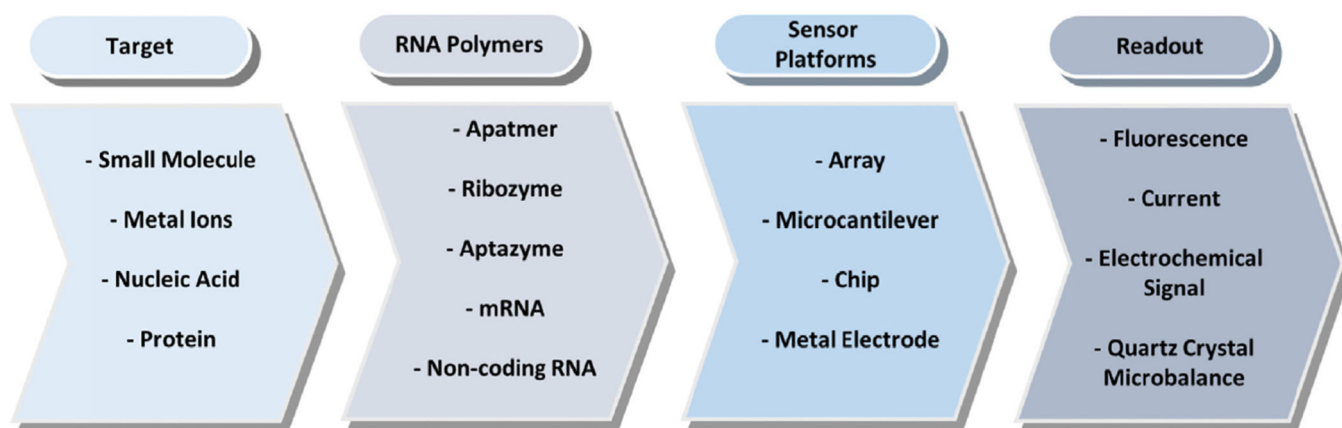
Figure 7.
The basic constitution of RNA-based biosensor.

Advantage of RNA-based Biocomputation

- Multi-functionality.
- Modularity by using aptamer, ribozyme, siRNA, miRNA, riboswitch, and other long or short non-coding RNAs.
- Well-defined structure, size, and stoichiometry.
- Controllable and predictable self-assembly and hybridization.
- Capacity of operating inside cells or tissue.

Figure 8.
Advantages of RNA-based biocomputation

Table 1

Summary of chemical modifications of RNA.

Chemical modifications	Advantages	Disadvantages	References
Phosphate backbone			
• Phosphorothioate (PS) • Boranophosphate (BO) • Phosphonoacetate (PACE) • Phosphoramidate • Methylphosphonate	• Improve nuclease resistance • Combine with other modifications to dramatically improve RNA property	• Destabilize siRNA duplexes (e.g. decreases T_m by 0.5 °C per PS linkage) • Extensive modification causes cytotoxic effect	[157–162]
2′-OH group			
• Small 2′-substituents (e.g. 2′-O-methyl (2′-OMe), 2′-fluoro (2′-F), 2′-aminoethyl, 2′-deoxy-2′-fluoroarabinonu-cleic acid (2′-F-ANA)) • Bulky 2′-modifications (e.g. 2′-O-MOE, 2′-O-allyl)	• 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 thermo-stability and duplex asymmetry, and also give higher binding affinity	• 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	[36,66,161–169, 173]
Locked nucleic acid (LNA) Unlock nucleic acid (UNA)	• 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	• 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	[170–175]
Ribose moiety			
• Altritol nucleic acid (ANA) • Hexitol nucleic acid (HNA) • 2′-deoxy-2′-fluoroarabino-nucleic acid (2′-F-ANA) • Cyclohexenyl nucleic acid	• Enhance thermostability (e.g. 2′-F-ANA increases T_m of RNA duplex by 0.5–0.8°C per modification) • Improve nuclease resistance	• Modification at seed region would slightly reduce siRNA potency	[178]
Ribonucleotide base			
(5-bromo-, 5-iodo-, 2-thio-, 4-thio-uracil, dihydro-, pseudo, 5-biotinylated, 5-aminoallyl-uracil, diamino-purine)	• Stabilize base-pairing and to enhance binding specificity • Particularly, 2-thio- and pseudo-uracil reduce cellular immune response	• Some base modifications (e.g. 5-bromo- and 5-iodo-uracil) will affect siRNA potency	[139,177]

Table 2

Classification of RNA polymer-based bioelectronics.

RNA-based bioelectronics		References
Biocomputation devices	Biologic gate	[238,243]
	Biomemory	[238,243,266]
	Bioinformation processor	[242,243,245,247,248,250]
Biosensors	Biomedical Sensor	[215,217,221,222,225–227]
	Environmental sensor	[228,229]