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RNA nanotechnology for computer design and *in vivo* computation

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Molecular-scale computing has been explored since 1989 owing to the foreseeable limitation of Moore's law for silicon-based computation devices. With the potential of massive parallelism, low energy consumption and capability of working *in vivo*, molecular-scale computing promises a new computational paradigm. Inspired by the concepts from the electronic computer, DNA computing has realized basic Boolean functions and has progressed into multi-layered circuits. Recently, RNA nanotechnology has emerged as an alternative approach. Owing to the newly discovered thermodynamic stability of a special RNA motif (Shu *et al.* 2011 *Nat. Nanotechnol.* **6**, 658–667 (doi:10.1038/nnano.2011.105)), RNA nanoparticles are emerging as another promising medium for nanodevice and nanomedicine as well as molecular-scale computing. Like DNA, RNA sequences can be designed to form desired secondary structures in a straightforward manner, but RNA is structurally more versatile and more thermodynamically stable owing to its non-canonical base-pairing, tertiary interactions and base-stacking property. A 90-nucleotide RNA can exhibit 4^{90} nanostructures, and its loops and tertiary architecture can serve as a mounting dovetail that eliminates the need for external linking dowels. Its enzymatic and fluorogenic activity creates diversity in

computational design. Varieties of small RNA can work cooperatively, synergistically or antagonistically to carry out computational logic circuits. The riboswitch and enzymatic ribozyme activities and its special *in vivo* attributes offer a great potential for *in vivo* computation. Unique features in transcription, termination, self-assembly, self-processing and acid resistance enable *in vivo* production of RNA nanoparticles that harbour various regulators for intracellular manipulation. With all these advantages, RNA computation is promising, but it is still in its infancy. Many challenges still exist. Collaborations between RNA nanotechnologists and computer scientists are necessary to advance this nascent technology.

1. The end of Moore's law for the electronic computer

Silicon-based computer and embedded systems are becoming ubiquitous in our daily lives [1]. We need processors to implement computation, communication and control to meet the demands of new applications and paradigms. Computer chip manufacturers frantically compete to make future microprocessors that are increasingly difficult to continue scaling with Moore's law, the number of electronic devices on the microprocessor every 18 months, doubling the demand to break the quickest record. Silicon microprocessor speed and miniaturization will eventually reach their own limits. Sooner or later, this chip competition is bound to reach stalemate. Scientists and engineers are wondering whether Moore's law can be continued for the next 10 years, and whether the capacity of transistors in the unit area of a chip can be doubled every 2 years (or every 18 months). There are three reasons for this critical challenge: (i) the accuracy of computing will be affected if we make a transistor at the atomic level, because two wires in the circuit will be too close and they will affect each other; (ii) the heat generated in such a small area with too many concentrated transistors will greatly affect the functions of the transistors; (iii) the energy consumption to cool the circuit board would be too high a burden.

By 2011, US data centres were predicted to consume 100 billion kWh at a cost of $7.4 billion per year [2]. Unfortunately, much of this energy is wasted by systems that are idle when current servers still draw about 60% of peak power. In typical data centres, average utilization is only 20–30%. Chip temperature impacts circuit reliability, energy consumption and system cost. Research has shown that every 10–15°C increase in operating temperature reduces the lifetime of the chip by half. With increasing temperatures, the leakage current of a chip increases exponentially. In addition, the cooling cost increases significantly, which amounts to a considerable portion of the total cost of the whole computer system. The cross-sectional power density increases linearly with the number of stacked silicon layers, causing a serious thermal problem. Chip manufacturers need a new kind of material in order to produce faster computing microprocessors.

Living entities have the most complicated computational systems in the world. The calculation capacity of cells, living organisms and the human brain is incomparable with machines used in the outside world. The supercomputer of biological systems, including the human body, relies on the interaction of DNA, RNA and proteins. Currently, the computational mechanism of the human brain has not been completely elucidated, but it is believed that biomimetic approaches following the realism of RNA, DNA and protein would be a new horizon that would solve electronic computing problems.

2. Molecular-scale computing

Molecular computation uses bottom-up approaches to create biological and chemical computers at the nanoscale [3–7]. Comparatively, electronic-integrated circuit systems comprise logic gates that perform Boolean logic by receiving true (1, high voltage) and false (0, low voltage) values resulting in a Boolean output [8]. Although the computational DNA logic gate first appeared in 1989 [9,10], the actual beginning of molecular-scale computing was when Adleman's group, in

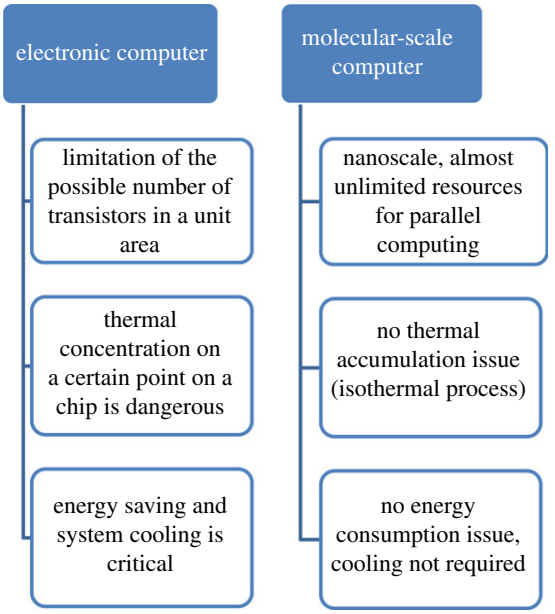

Figure 1. Comparison of the electronic computer and a molecular-scale computer.

1994, used a DNA-based design to solve a seven-city Hamiltonian path problem [11]. This began the new era of DNA computing.

The basic mechanism of the DNA-based computer is a single-strand of DNA that undergoes a certain reaction upon the arrival of an input signal. This process is controllable, and has led to DNA strand displacement circuits [12–16]. The advantage of using DNA is that DNA is stable, and DNA hybridization reactions can be used to represent computational steps, with dynamic DNA nanostructures representing computational states. Figure 1 shows a detailed comparison between an electronic computer and a molecular-scale computer. The molecular-scale computer has many promising features that have the potential to overcome the restraints of Moore's law for electronic computers. Based on concepts borrowed from electronics, such as Boolean values, signal processing, signal amplification and feedback, multi-layered biocircuits have been created since the middle of the 1990s.

Boolean frameworks have been used in the measurement of biological interactions, and the classification of genes has been represented under diseased conditions [17,18]. Currently, most Boolean logic gates operate by fluorescence detection, a method that is complex and physically inconvenient [14,19–23]. Alternatively, a homogeneous colorimetric detection method has been employed that uses high extinction coefficients and the distance-dependent optical properties of gold nanoparticles to reduce the complexity and cost of the procedure and increase the ease of operation [24,25]. As exemplified in figure 2, metal-ion-mediated DNA logic gates (using AND, NAND and NOR) have been fashioned based on electrochemical outputs that use the unique features of Ag^+ ions that interact with the cytosine–cytosine mismatch, and of Hg^{2+} ions that interact with the thymine–thymine mismatch, in DNA duplexes [26]. The AND logic system was based on the proximity-dependent surface hybridization between thiolated T-/C-rich DNA on a gold electrode surface and T-/C-rich DNA labelled with ferrocenecarboxylic acid (Fc), in which the Hg^{2+} and Ag^+ ions are used as inputs and the current of the Fc as output. Subsequently, an NAND logic gate was constructed based on the strand dissociation as well as the conformational switch of T-/C-rich DNA triggered by Ag^+ and Hg^{2+} ions. In addition, it was found that the $C-Ag^+-C$ and $T-Hg^{2+}-T$ base pairs can trigger the structural conversion of multiple nucleic acid helices from triplexes to duplexes, which motivated the fabrication of another NOR logic gate.

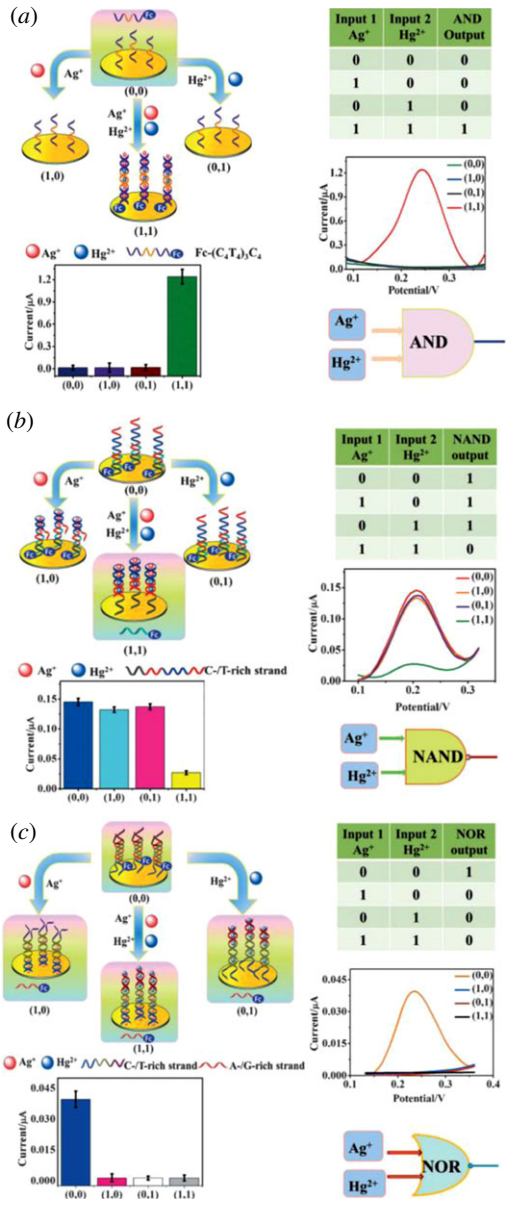

Figure 2. Logic-gate systems with Ag^{+} and Hg^{2+} ions as inputs and electrochemical signals as output detected by differential pulse voltammetry (DPV). (*a*) Schematic of the two-input logic gate and equivalent electronic circuit for the AND logic operations. (*b*) A schematic presentation of a 'NAND' gate. (*c*) Schematic of a 'NOR' gate. (Adapted from [26]. Copyright © 2013 with permission from Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.)

DNA electrochemical logic gates that can be made with minimal reagents, fewer working steps and a simpler optical set-up are optimal. Compared with silicon-based elements used in electrical computation, nucleic acids are structurally simple, have straightforward sequence-specific hybridization between complementary strands, and can capture certain target molecules (e.g. metal ions, small molecules and proteins in a highly specific manner [27,28]; as such, in order to compute DNA, DNA logic gates must be created [29]). Zhang and co-workers [30] have designed a system of colorimetric logic gates (OR, AND and INHIBIT) using Pb^{2+} and Mg^{2+} ions as the DNAzyme cofactors to activate respective scission DNAzymes, and Zhang *et al*. have

constructed a complete set of two-input logic gates by designing a series of circular substrates (OR, AND, INHIBIT, XOR, NOR, NAND and XNOR), where ion-dependent DNAzymes were the functional components and the respective cofactor ions were used as the inputs [31].

Another step forward was the introduction of toehold-mediated DNA strand displacement. A toehold is a single-stranded domain that leads to enzyme-free DNA machinery that is automated by hybridization [6,32]. The most recent results are from the Caltech laboratory [33]. Qian and Winfree [33] used a DNA hybridization reaction to represent the logic operations AND and OR. The digital values were controlled through threshold gates. The signal recognition is amplified through fluorophores and quenchers. The designed seesaw circuits with three layers of gates demonstrated the possible progression of DNA computation to more layers of gates.

However, there are many limitations of DNA-based computation. The most obvious is that the execution time of a logic gate such as AND or OR is too long to tolerate. It usually takes 1–2 h to finish one AND or OR operation through biochemical reactions. Three-layer seesaw circuits require more than 6–10 h to reach a stable state and to achieve the final value represented. The second challenge of DNA-based computation is the limiting proteins required in the DNA reactions, such as DNA-binding and DNA-cutting proteins. Nevertheless, scientists and engineers have started to use molecular-scale computing devices to build new-style computers, such as quantum computing and neurocomputing. But, DNA-based computation is still a promising approach.

3. Historical evolution of RNA nanotechnology

Adleman [11] proposed and introduced the idea of using DNA to solve complex mathematical problems. DNA coding is very similar to how permanent personal genetic information is stored in an electronic computer's hard drive. DNA Logic is a team from Rochester, NY, whose DNA-based logic gate achieved the first step in the creation of computing systems that have the same structure as a conventional computer. They used an electronic signal instead of a DNA genetic code to carry out a calculation. They used the genetic materials as input and a combination of two fragments as an output. Two DNA inputs were linked and crossed by a chemical to form an end-to-end concatemer structure to serve as an 'AND GATE'. They reported that these logic gates could be combined with DNA microchips to execute a new approach for DNA computation.

The main driving force of RNA-based computing lies in the concept of RNA nanotechnology. To uncover the role of RNA nanotechnology in network computing devices, it is important to briefly provide terminology and a history of the RNA nanotechnology concept. RNA nanotechnology is a relatively recent field of science that uses bottom-up or top-down approaches to build artificial RNA architectures that are nanometres in scale. It involves the characterization of the physical, chemical, biological and pharmaceutical properties of artificial RNA scaffolds or nanoparticles that can be used in nanobiotechnology, synthetic biology, nanomedicine and computing devices. RNA nanotechnology is a unique field that is distinct from the classical studies on RNA structure. The concept began in the late 1990s with the pioneering work led by Dr Peixuan Guo and his laboratory. In 1998, they demonstrated the construction of RNA nanoparticles using the re-engineered natural packaging RNA (pRNA) derived from a bacteriophage ϕ29 DNA packaging motor to self-assemble by hand-in-hand interaction into multimeric RNA nanoparticles (figure 3*a*). This finding was published in Zhang *et al*. [39], and was featured in Hendrix [40]. In 2004, the same group reported the systematic formation of pRNA nanoparticles using technologies of palindrome sequence-mediated self-annealing [37] (figure 3*b*). In the following years, they successively showed that pRNA molecules could be conjugated with various therapeutic functionalities, including aptamers, siRNA, ribozymes and microRNA, and serve as polyvalent vehicles to deliver these molecules [35,38,41–50] (figure 3*c*). These findings have paved the way for RNA nanotechnology to develop into a novel area of therapeutics for the treatment of various diseases such as cancer, viral infections and genetic diseases. This is pioneer work to prove the concept of RNA nanotechnology and intracellular computation and raises the possibility of intracellular computation.

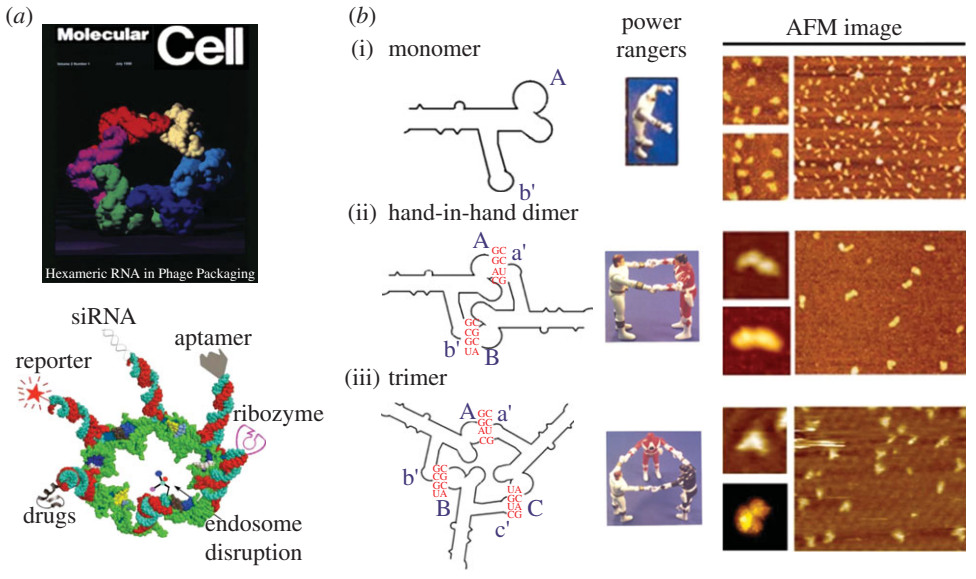

Figure 3. Historical outline of RNA nanotechnology. (*a*) Re-engineered pRNAs form hexamers, one of the first pieces of evidence for the application of the RNA nanotechnology concept [34,35]. (Upper panel, adapted with permission from F. Major and from © Elsevier 1998; lower panel, adapted from [35] with permission from © Mary Ann Liebert, Inc. 2005.) (*b*) Re-engineered pRNA (i) monomer can assemble to (ii) dimers and (iii) trimers [36–38]. AFM, atomic force microscopy. (Adapted from [38] with permission from © Elsevier 2010.)

The development of multi-valent pRNA nanoparticles in the Guo laboratory is just one facet of the rapidly emerging field of RNA nanotechnology and therapeutics. Elucidation of the structure and folding mechanism of RNA motifs and junctions has laid a foundation for the further development of RNA nanotechnology. In its early stage, significant contributions to the fundamental studies of RNA structural motifs were made by Eric Westhof, Neocles Leontis, Luc Jaeger, David Lilley and their laboratories [51–65]. Advances in RNA three-dimensional computation from traditional intra-molecular interaction to inter-molecular interaction were promoted by Bruce Sharpiro and co-workers (figure 3*d*) and have brought new features into the RNA nanotechnology field [59,66–69].

Currently, RNA nanotechnology is becoming a popular and rapidly developing branch of science, as evidenced by the burst of publications on RNA nanostructures in the past 5 years, indicating a strong interest in RNA nanotechnologies in diverse fields such as chemistry, biophysics, biochemistry, structural biology, microbiology, cancer biology, pharmacy, cell biology and nanomedicine. New perspectives on the application of the RNA nanotechnology concept are slowly developing into a more sophisticated era of RNA computing.

4. Significance and uniqueness of RNA nanotechnology

RNA biopolymers are very important to RNA nanotechnology, beginning at the atomic level of these intriguing molecules. Composed of four different nucleotides—A, C, G and U—RNA is capable of Watson–Crick base pairing (as DNA does) as well as a variety of non-canonical base pairing [55,58,70–72]. This property mainly promotes RNA folding into either rigid or flexible structures containing tertiary interactions that are distinct from those of double-stranded DNA (figure 4) [54,55,73–76]. RNA tertiary interactions mediate bulges, internal hairpin loops, multi-way junctions and inter- and intra-RNA–RNA components. A 90-nucleotide RNA can display up to 4^{90} different sequences with a very large number alternative secondary and tertiary structures. Such a huge pool of rich structural conformations could ease the search for

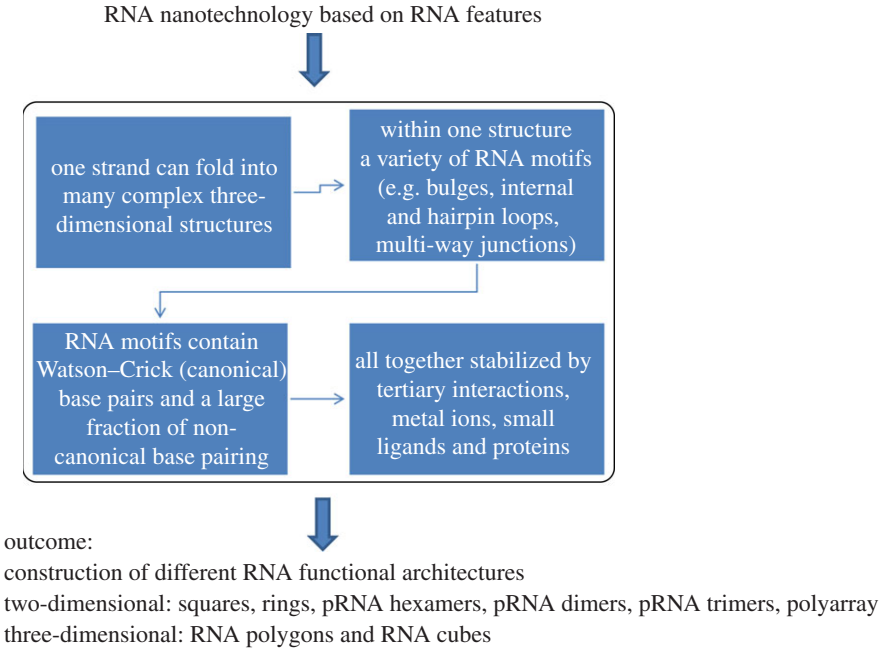

Figure 4. Significance and uniqueness of RNA nanotechnology.

viable partners in particle assembly, substrate binding, architecture building and manufacture engineering.

In addition, the versatility and low-energy folding of RNA deliver a significant advantage over DNA, such as high thermodynamic stability [77–79] and various *in vivo* attributes [34,39,45,80–84]. At the same time, RNA can be designed and manipulated with a level of simplicity characteristic of DNA. But it displays a structural flexibility and functional diversity similar to that of proteins, including enzymatic activities. Although RNA nanotechnology can be regarded as a subdivision of nucleic acid nanotechnology, the uniqueness of RNA properties when compared with DNA will advance the emerging field of RNA nanotechnology. The discovery of diverse RNA functions in ribozyme, riboswitch, aptamer, siRNA and miRNA and the methods to produce fluorogenic RNA in the cell [85,86] suggest the immense potential of intracellular computation.

5. Fabrication of RNA nanoparticles with potential as computer parts

Artificial construction and assembly of RNA molecules into more complex and functional systems requires the use of programmable, addressable and predictable building blocks. The thermodynamic stability, specificity, affinity, flexibility and folding rules of RNA structural motifs need to be known, so that possible advantages can be found or difficulties overcome. RNA folding into various three-dimensional structures dictating its function is the result of complex hierarchical self-organization of modular elements. Self-assembly of RNA building blocks in a predefined manner to form larger two- and three-dimensional structures is a prominent bottom-up approach and represents an important means by which biological techniques and biomacromolecules can be successfully integrated into nanotechnology [35,37,43]. Figure 5 provides the main concepts of RNA nanoparticle fabrication based on the following methods [54,55,73–76,91].

First is the biomimetic method, which mimics RNA constructs from naturally occurring atomic resolution X-ray or NMR structures. The structures of RNA motifs and the mechanisms of RNA folding and sequence interactions have been investigated for many years. A rich resource of

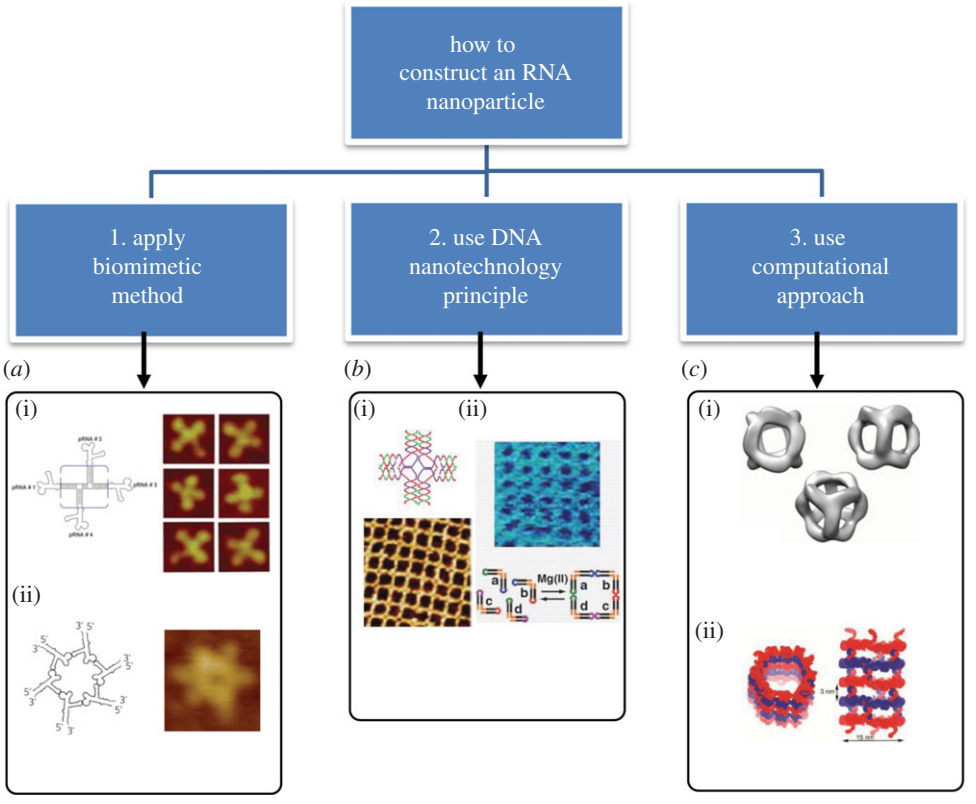

Figure 5. Fabrication of RNA nanoparticles. (*a*) (i) pRNA four-way junction constructed by mimicking the observed natural pRNA 3WJ core motif [42]. (Adapted from [42], © 2012 with permission from Elsevier.) (ii) pRNA hexamer was built via loop–loop interaction by naturally occurring pRNA hexamers [34,87]. (Left panel, adapted from [34]. © 1998 with permission from Elsevier. Right panel, adapted from [88]. © 2013 with permission from Elsevier.) (*b*) Illustration of the similarity between DNA and RNA structural arrays: (i) AFM image of cross-shaped tiles possessing a four-arm formation through the sticky end [89,90]. (Adapted from [89]. © 2008 with permission from the American Association for the Advancement of Science.) (ii) An example of RNA square-based structural arrays obtained from six sticky nucleotides [62]. (Adapted from [62]. © 2008 with permission from the American Association for the Advancement of Science.) (*c*) Examples of RNA nanostructures designed with the help of computer programs. (i) Computer-aided design of an RNA nanocube [59]. (Adapted from [59]. © 2010 with permission from Nature Publishing Group.) (ii) An example of an RNA nanotube [66]. (Figure courtesy of Dr Bruce A. Shapiro.)

well-developed databases can be used to extract known RNA structural units for construction of novel RNA nanoparticles with the desired properties [82,92,93]. One such example is based on the structural features of the pRNA of the bacteriophage ϕ29 DNA packaging motor, which uses a hexameric RNA ring to gear the machine [94–96]. The basic methodology to produce different RNA nanoparticles based on the pRNA sequence is illustrated in figure 6 (Shu Yi nature protocols). Thus, Guo's group has extensively re-engineered pRNA to form dimers, trimers, tetramers, hexamers (with proteins) and arrays via hand-in-hand or foot-to-foot interactions between two interlocking loops of pRNA, as summarized in figure 7 [36,37]. In addition, the dimer and trimer nanoparticles have been used successfully as polyvalent vehicles to deliver a variety of therapeutic molecules as well as for constructing RNA arrays [37].

Another example that uses the structures of known RNA structural elements is called 'RNA architectonics' [62]. The strategy is based on the rational design of artificial three-dimensional RNA constructs guided by specific loop–loop interactions. This can be decomposed and reassembled to create new RNA nanoscopic architectures by inverse folding.

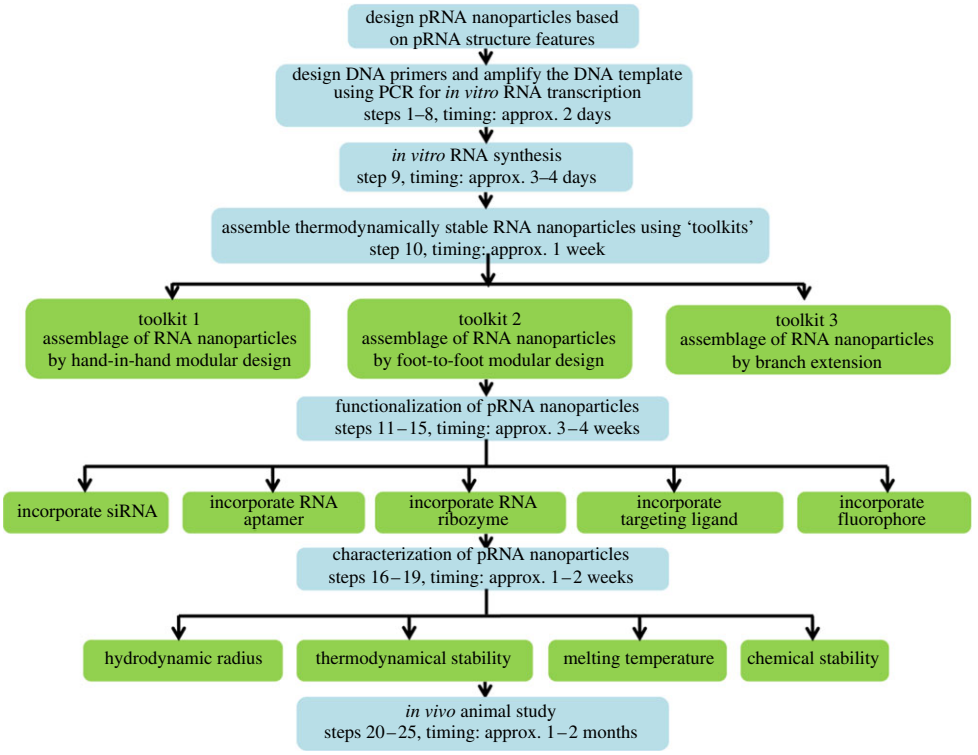

Figure 6. Workflow diagram of functional RNA construction based on the pRNA molecule. PCR, polymerase chain reaction.

Application of a three-way junction (3WJ) [41] and a four-way junction (4WJ) [42] that are selected from known RNA structures is another exciting example of a biomimetic approach to create functional nanoparticles [60,67]. There are several examples in the literature: RNA structural motif (from rRNA) to guide the tetramer assembly of L-shaped tectoRNAs; 3WJ motif (from 23S rRNA) to construct a T-shaped arrangement of three helices; and tRNA motifs consisting of 4- and 5-WJ to fold L-shaped tertiary structures [60,82]. However, crystallography and NMR are not the only sources of RNA functional elements—many of them have been identified by *in vitro* selection, for instance aptamers [97,98].

The second method is to inherit the principles of DNA nanotechnology. Because DNA and RNA share some common structural and chemical features, DNA methods can provide viable models for RNA nanotechnology development. Direct DNA self-assembly is predictable, and complex nanostructures can be created with precise addressability. This is demonstrated by assembling DNA into a variety of elegant shapes with precise control over their geometries, periodicities and topologies (figure 5). Branched DNA tiles, tensegrity triangles (rigid structures in periodic array form) [99], algorithmic self-assembled Sierpinski triangles (aperiodic arrays of fractal patterns) [100], nanotubes, helix bundles [101], polycatenated DNA ladders [102] and three-dimensional cubes, polyhedrons, prisms and buckyballs are some of the examples.

Rothemund's [103] DNA origami is an exciting demonstration of the addressable and programmable property of DNA. Behind the principle of DNA origami lies a long single-stranded viral DNA which is used as a scaffold for binding shorter strands to generate well-defined two- and three-dimensional configurations. This strategy was applied to build three-dimensional boxes that can be locked and unlocked [104], nanoarrays for label-free detection of substrates [105], multi-layered three-dimensional DNA nanostructures and for structure elucidation of organized proteins [106]. Rationally designed supramolecular DNA assemblies can be conjugated with organic and inorganic molecules, such as conjugation of porphyrins

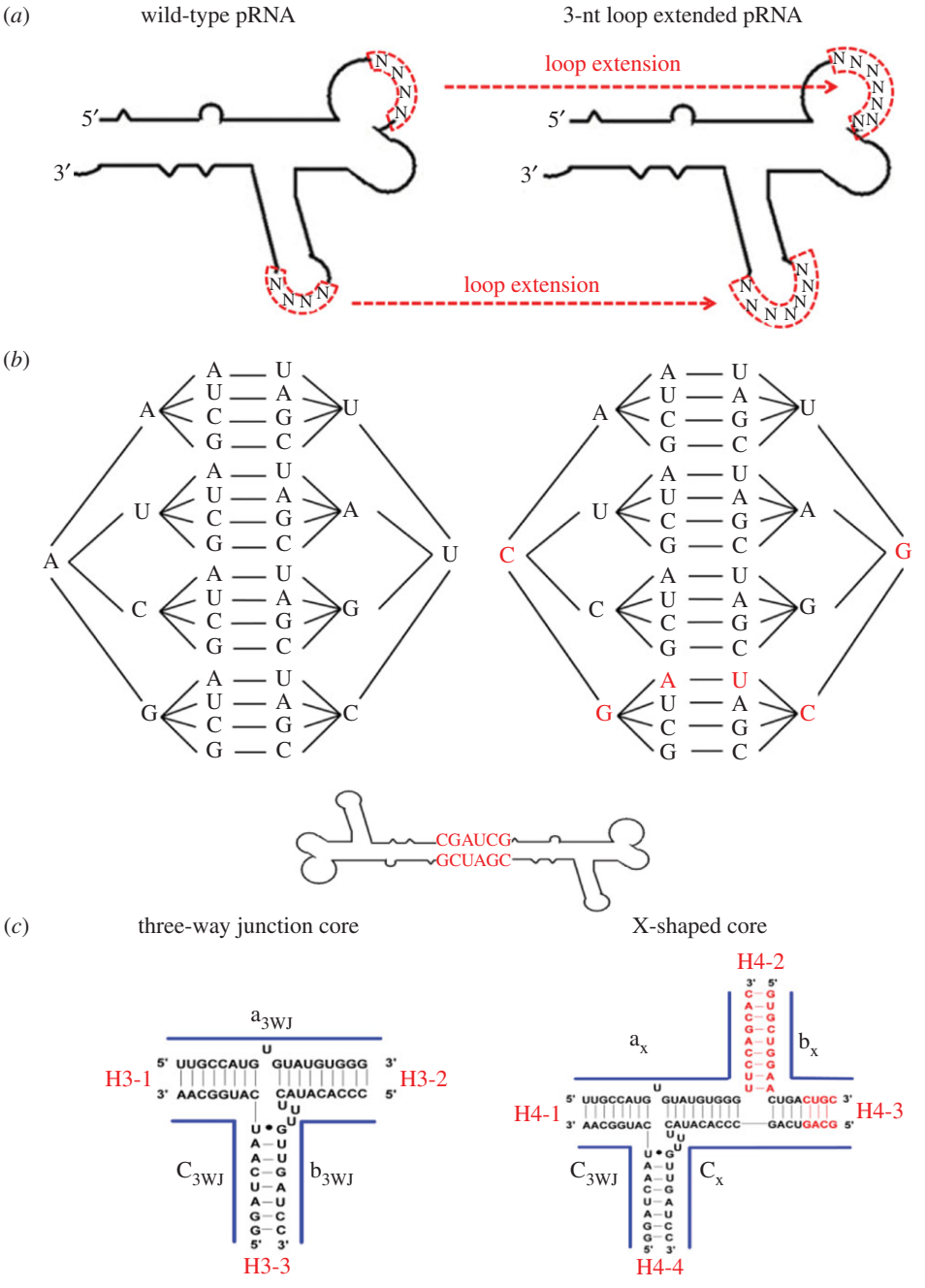

Figure 7. Illustration of toolkits to obtain RNA nanoparticles. (*a*) pRNA extended interlocking loop–loop interaction 'hand-in-hand', toolkit no. 1; (*b*) pRNA interaction via a palindrome sequence introduced at the 3′-end 'foot-to-foot', toolkit no. 2; and (*c*) 3WJ motif of pRNA 3WJ used to construct branched RNA nanoparticles, toolkit no. 3 [87]. (Adapted from [87]. © 2013 with permission from Cold Spring Harbor Laboratory Press.)

on parallel DNA helix bundles [107], nanomagnets [108] and elegant nanomachines [109,110]. Recently, DNA fold-and-cut methodology was used to build a reconfigurable topological surface with only one side and only one boundary called a Möbius strip [111].

The above-mentioned DNA nanotechnology principles have been successfully applied to RNA nanotechnology (figure 5). The formation of jigsaw puzzles and bundles was demonstrated

in both RNA and DNA [37,61,62,112–114]. To introduce branching into structures, multi-helix junctions are used as monomers in DNA constructs [115,116], enabling similar novel and diverse RNA-based architectures to be built [55,60].

The final method is to use computational tools to design RNA nanoparticles. As mentioned above, RNA molecules display diverse structures mediated by both canonical and non-canonical base pairing. In contrast to traditional methods in which raw materials are harvested from three-dimensional databases rather than designed for a given application, the next generation of building blocks can be designed *a priori* for programmed assembly and synthesis. RNA secondary structures are stabilized largely by nearest-neighbour interactions that can be measured in model systems [77,78].

Application of this method, however, requires a large data bank of parameters for the nearest-neighbour interactions. Fortunately, advances in RNA synthesis make it possible to study molecules with many different structural motifs. The use of computational methodologies can significantly reduce the time and expense required to build RNA-based functional nanoparticles to address experimental needs. However, prediction of RNA structure or folding for particle assembly is a great challenge, owing to the unusual folding properties involving non-canonical interactions. Single-base modifications can result in folding alterations and loss of function. Currently, using Zuker's RNA secondary structure dynamic programming algorithm, typically only 70% of the two-dimensional folding prediction is accurate [117,118]. Obviously, predicting the RNA three-dimensional structures is even more difficult.

Although there are novel strategies that predict the self-assembly of nucleotide sequences into three-dimensional RNA nanoparticles, presently, new and efficient computational approaches are required. Generally, there are two steps in building RNA nanoparticles: (i) the computational approach (e.g. using *Kinefold*) [119], using the spontaneous self-folding property of RNA into defined structures via base–base interactions based on their characteristic ΔG, and (ii) spontaneous assembly of the resulting RNA building blocks into larger assemblies based on the predicted architecture. This creates an effective computational pipeline for generating molecular models of RNA nanostructures. The RNA junction database [82], NanoTiler [67], RNA2D3D algorithms [120], RNA dynamics [121,122] and FR3D [123] are used to build RNA nanoparticles that incorporate individual RNA motifs to defined user specifications [66] and have been shown to self-assemble *in vitro* (e.g. nanocubes; figure 4 and [59,68]).

6. Application of RNA nanotechnology for computer design

Thus far, we have shown the capabilities of RNA nanotechnology to provide one of the few ways to form designed, complex structures with precise control over nanoscale properties. The field is beginning to see application in the design of logic gates as building blocks for computer construction. Here, we briefly emphasize some important aspects of why it is critical to apply RNA nanotechnology into 'RNA computers'.

One of the main advantages of RNA is that it can carry catalytic (e.g. ribozymes [124]) and gene regulation functions (e.g. riboswitches [124]) within the cell as well as performing detection, signalling and sensing functions (e.g. aptamers). Thus, the RNA molecule has advantages over the DNA molecule not only in versatility of different structures but also in functionality (figure 8*a–d*). Unlike traditional electronic computers, which use electric current as inputs and outputs, these RNAs use the concentrations of specific chemical species as signals. These RNAs can replace DNA to implement logic functions and to build up the multiple layer Boolean networks with AND/OR/NOT logic gates [91,127,128].

The design and synthesis of basic functional circuits are the fundamental tasks for developing a fully functional computing device. Before it is possible to engineer higher order genetic networks that can perform complex functions, basic functions should first be realized. RNA nanotechnology-based devices can perform cellular information-processing operations from standard components. These devices can exhibit logic operations (AND, NOR, NAND or OR gates) and signal filtering. RNA-based devices process and transmit molecular inputs to

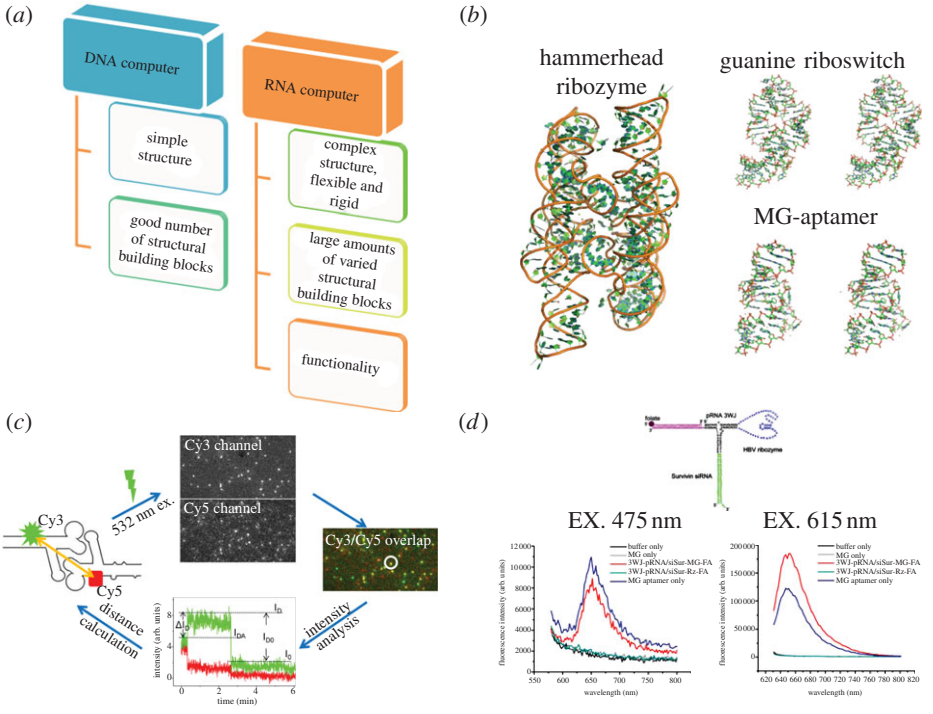

Figure 8. Application RNA nanotechnology for computer design. (*a*) Comparison between DNA and RNA computers. (*b*) Atomic resolution structures of some functional RNAs (shown are the hammerhead ribozyme PDB ID: 1GID, guanine riboswitch PDB ID: 3GER and malachite green (MG) aptamer PDB ID: 1F1 T). (*c*) Example of the application of RNA nanoparticles to calculate a distance [125]. (Adapted from [125]. © 2010 with permission from the American Chemical Society.) (*d*) Application of MG aptamer-functionalized 3WJ pRNA nanoparticles *in vitro*, showing the different emissions based on different excitation wavelengths [126]. (Adapted from [126]. © 2011 with permission from Nature Publishing Group.)

targeted protein outputs, linking computation to gene expression and thus the potential to control cellular function. Several information-processing systems have been developed based on RNA computing. For example, protein-based systems can perform logic operations to convert molecular inputs to regulated transcriptional events. In addition, a framework for the construction of single input–single output RNA devices based on the assembly of three functional components—a sensor component which is made of an RNA aptamer; an actuator component, made of a hammerhead ribozyme; and a transmitter component, made of a sequence that couples the sensor and actuator components [129]—was proposed.

RNA nanostructures will represent different inputs. The output, such as the activation of a pathway, is based on logic functions of input RNA concentrations. The beauty of the RNA approach is that there are many more variations in RNA than in DNA, as emphasized in figures 9– 11. As an example, using only one specific type of RNA structure as an input, we can construct a tremendous variety of different structures and functionalities through different methods, e.g. hand-in-hand (figure 9), foot-to-foot (figure 10) and junction core extension toolkits (figure 11). RNA logic gates can lead to a computer capacity that is comparable to the world's currently most powerful supercomputer. The future RNA computer will be compact; for example, a 1 cm^3 space can hold 10 trillion RNA molecules. With this small amount of RNA, a computer would be able to hold 10 terabytes of data, and perform 10 trillion calculations at a time. By adding more RNA, more calculations could be performed. The RNA computer will be fast. In contrast to conventional computers, which operate linearly, RNA computers can perform parallel calculations. Such parallel computation allows RNA to solve in 1 h complex mathematical problems that would require hundreds of years for conventional computers to compete the task.

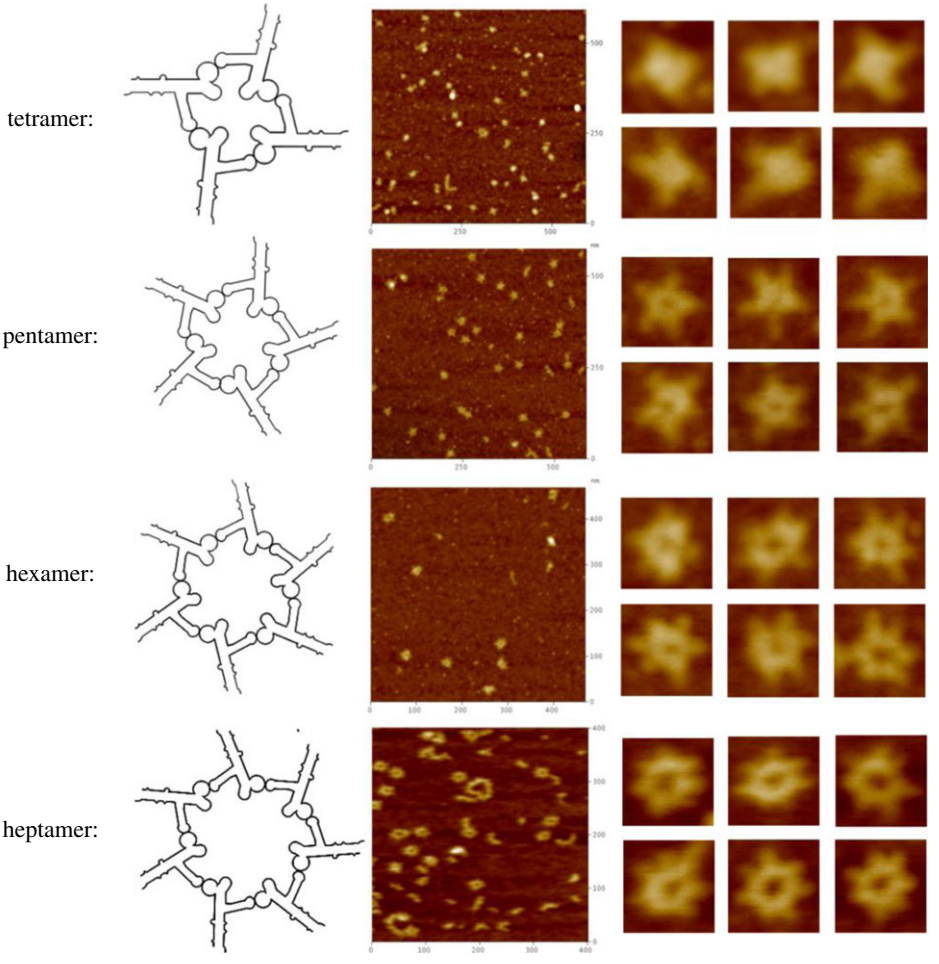

Figure 9. AFM images of pRNA nanoparticles obtained by 'hand-in-hand' interaction following toolkit no. 1 [87]. (Adapted from [87]. © 2013 with permission from Cold Spring Harbor Laboratory Press.)

In addition, there are numerous small RNA regulators available [130,131]. By the *trans*- and *cis*-actions, we can use varieties of small RNA regulators to build *in vivo* products and functional pathways and control them with induction or repression. Three types of working methods are generally available: cooperative, synergistic and antagonistic, to produce computational logic circuits as conjunctive or disjunctive normal forms, or other kinds of logic operation [91,127,128].

7. RNA *in vivo* computation

Recent advances in RNA-based therapeutics have broadened the scope of therapeutic targets for a variety of human diseases ranging from genetic disorders to HIV infection and extending to various cancers. The concept of nanotechnology application to living systems has already been proven when molecular computers were found to be able to operate in, and directly communicate with, a biological environment [6,14].

In vitro demonstration of this computing device has been shown before [4] when DNA was the software encoded with input and output and DNA-manipulating enzymes were the hardware.

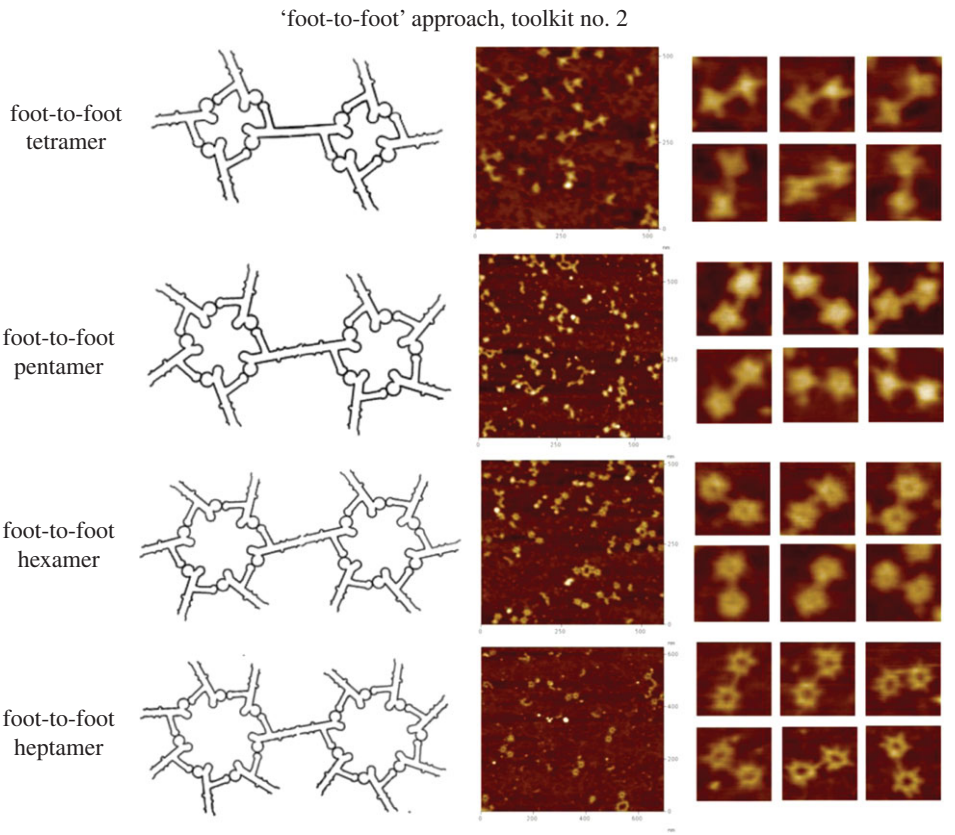

Figure 10. AFM images of pRNA nanoparticles obtained by 'foot-to-foot' interaction following toolkit no. 2 [87]. (Adapted from [87]. © 2013 with permission from Cold Spring Harbor Laboratory Press.)

Simple tasks could be performed; for example, determining whether a list of zeroes and ones had an even number of ones. Later, it was programmed to sense specific biomolecular concentrations (messenger RNA (mRNA)); perform a simple computation; and release a biomolecule (DNA) in response [19]. From this, abnormal concentrations of mRNA could be sensed, cancer diagnosed and an agent released to treat it *in vitro*. Seelig *et al*. [14] inserted Boolean logic gates over microRNA (miRNA) by DNA strand displacement and created multi-layered circuits based on electronic concepts such as signal restoration, amplification and feedback.

Rinaudo *et al*. [132] was able to program a biomolecular computing device to work inside a living cell. They inserted a plasmid with specifically encoded DNA and used an external program with the ability to control the computation inside the host cell. The mRNA was used to encode a fluorescent protein, and the target sequences for small interfering RNA (siRNA). They found that siRNA was able to control the level of fluorescence exhibited by the cell because of mRNA degradation that allowed siRNA the control to do so.

Win & Smolke [129] developed *in vivo* programming with computation independent of the cell machinery, yet that can respond to both endogenous and exogenous molecular signals. In their work, they used a combination of ribozymes and RNA aptamers [133,134]. The whole idea was simply to use the cleaving ability of ribozyme, which was regulated by binding aptamer RNA to a specific molecule, so that aptamer binding allowed cleavage or blocked cleavage by the ribozyme. They demonstrated Boolean logic operations using the concentrations of two proteins as input and the expression of green fluorescent protein (GFP) as output, implemented by the ribozyme–aptamer molecules using yeast. Their modular and easy to program system consisted

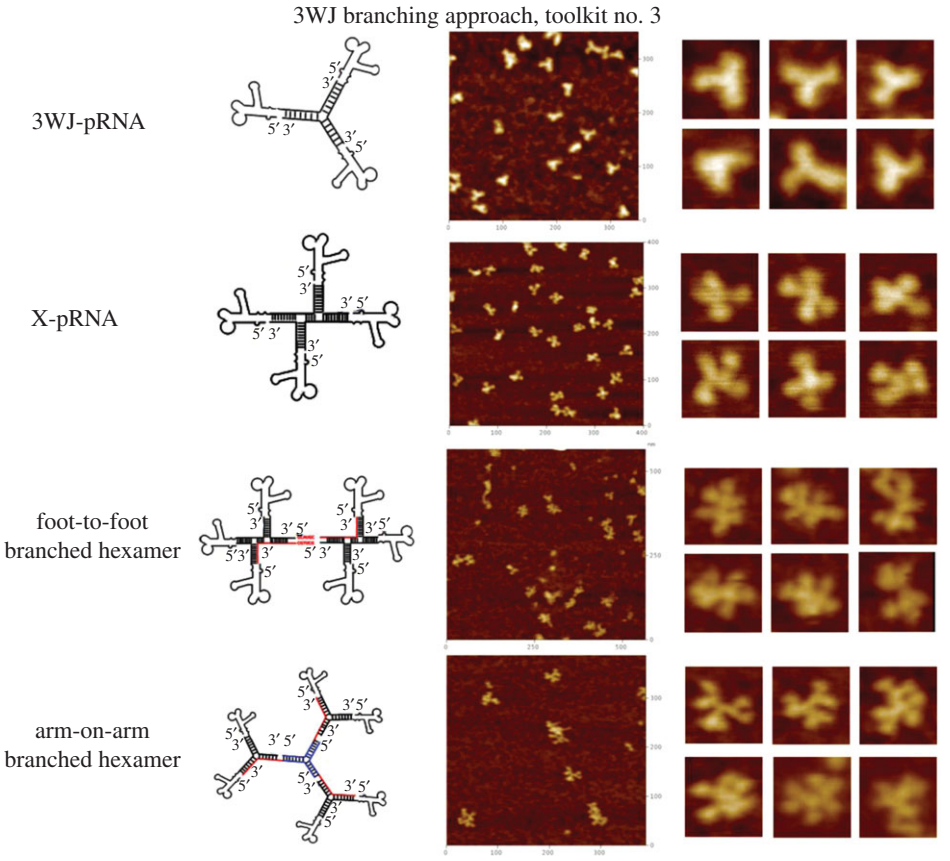

Figure 11. AFM images of pRNA nanoparticles obtained by 'foot-to-foot' interaction following toolkit no. 3 [87]. (Adapted from [87]. © 2013 with permission from Cold Spring Harbor Laboratory Press.)

of mRNA that encoded GFP with a modified control region (3′ untranslated region); one or more ribozyme–aptamer molecules were embedded in the region.

Recently, Auslander *et al*. [135] used the advances in RNA synthetic biology and designed standardized control devices using trigger-controlled transcription factors and RNA-binding proteins. These combinatorial circuits can be integrated as a two-molecule input and can perform digital computations with NOT, AND, NAND and N-IMPLY expression logic in single mammalian cells. Importantly, they showed that individual mammalian cells capable of executing basic molecular arithmetic functions isolated or coordinated to metabolic activities in a predictable, precise and robust manner. As the outcome, this information may provide new treatment strategies and bioelectronic interfaces in future gene-based and cell-based therapies.

Lou *et al*. [136] developed a memory module which is a toggle switch with two mutually repressed repressors, CI and CI434 genes from the lambda and 434 phages, respectively. The memory module is expected to function as follows: when CI is present, it activates transcription and represses the transcription of CI434, thus establishing a stable high CI/low CI434 state, which is defined as the 'ON' state of the memory module. In this case, red fluorescent protein is expressed. Alternatively, when CI434 is present, it can repress the transcription of CI, thus maintaining the transcription of itself and establishing a stable low CI/high CI434 state, which is defined as the 'OFF' state. Another approach used a scalable transcriptional/post-transcriptional synthetic regulatory circuit composed of HeLa cancer cell 'classifier' that senses expression levels of a customizable set of endogenous microRNAs and triggers a cellular response only if the

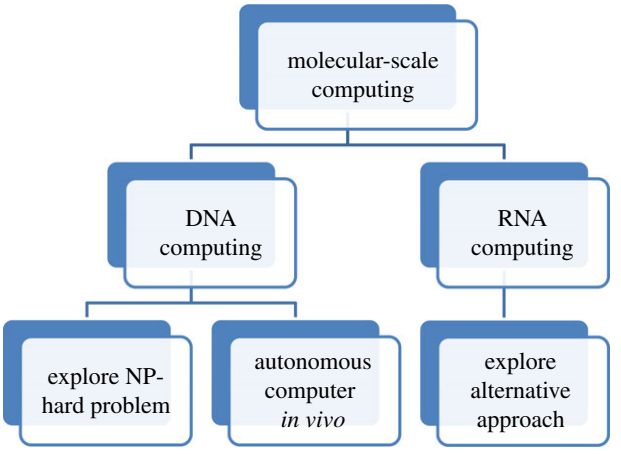

Figure 12. The development path of molecular-scale computing.

expression levels match a predetermined profile of interest. This cancer cell classifier selectively identifies HeLa cells and triggers apoptosis without affecting non-HeLa cell types [137].

Based on these developments, we believe that RNA computing will provide a way forward for future biocomputing. Artificial RNA computers operating inside living cells would enable us to control cellular physiology. Our knowledge of RNA-based cell regulations is the most promising tool to construct *in vivo* computers. Nature has already given us all the information required to translate these RNA functions into digital molecular networks that embody standardized forms of logic functions.

8. Challenges for future RNA-based computation

RNA-based computation has the advantage of more variations and flexibility than DNA-based computation. But, the biochemistry reaction time is still a critical challenge. Inspired by the results and progress in DNA-based computing, RNA-based computing is promising. Xie *et al*. [138] reported that the observed rate constants of the siRNA output production are one to two orders of magnitude lower than those measured with model DNA strand-exchange substrates. But they have not built a scalable RNA computer and determined whether the RNA computer is slower or faster than the DNA computer at system level.

The interdisciplinary approach combining computer engineering and biochemistry is critical to the success of RNA-based computation. There are two complementary motivations for constructing molecular computing [138]. One is to solve the famous 'NP-hard' computational problem [11,139,140], the other is to build autonomous molecular computers that could potentially operate *in vivo* [6]. Figure 12 shows the development path of molecular-scale computing. We can borrow many concepts from electronic computer science and engineering to learn how to build a complicated molecular-based computer. Although by designing the logic circuit of AND/OR/NOT gates in a cell an 'RNA computer' can theoretically be designed and implemented in bacterial, yeast and mammalian systems [127], the way to harness the computer in a cell to our benefit is still a very long way off. The biggest challenge is that protein engineering is still in its infancy [141] and we are still very far away from building a molecular-based computer comparable to current electronic computers.

One of the biggest challenges associated with RNA-based intracellular computation is its instability. Despite the fact that RNA therapeutics such as ribozymes, aptamers and siRNAs have been shown to demonstrate exceptional versatility inside the body, delivery vehicles are still required for therapeutic moieties to efficiently transport them to the targeted cells. Because natural RNA is prone to RNase degradation this has hindered its application as a delivery vehicle.

However, some progress has already been reported. This includes artificial modification of the RNA bases, phosphate backbones and the C-2′ functional group [68,142]. The introduction of peptide nucleic acids, locked nucleic acids (LNAs) and their respective derivatives polycarbamate nucleic acids [143] or LNA with a bridge at different positions (2′–4′, 1′–3′) also significantly improves the chemical and enzymatic stability of RNA structures [144]. However, it is believed that the most prominent is the replacement of the 2′-hydroxyl group with fluorine as this has a minimal detrimental effect on RNA folding and functions.

In addition to instability issues, the challenges of *in vivo* computation using RNA [127,145] include scaling the logic operations with a large number of inputs, extending input signal types and non-specific actions resulting in targeting unexpected or undesired pathways resulting in toxicity effects. The results of modifications related to RNA folding and *in vivo* toxicity of the nucleotide derivatives remain to be explored. Owing to the metabolism and biocompatibility issues, the most stable RNA might not necessarily be the most desirable; retention of particles within an appropriate time period is more attractive.

Another challenge associated with RNA 'computers' is the yield and cost of RNA production. As has been reported before [91], commercial RNA chemical synthesis can only offer 40 (conservative) to 80 (with low yield) nucleotides. Acetalester 2′-OH protecting groups, such as pivaloyloxymethyl, have been reported to enhance chemical synthesis of RNA. RNase ligase II has been shown to be a good alternative to the traditional T4 DNA ligase to generate longer RNA by ligation of two shorter synthetic RNA fragments [146]. Heterogeneity of the 3′-end of RNA products obtained during *in vitro* transcription is another issue [147]; this can be addressed by extending the transcribed sequence beyond the intended end and then cleaving the RNA at the desired site using ribozymes, DNAzymes or RNase H [146–148]. Large-scale RNA complexes produced in bacteria escorted by a tRNA vector have also been reported [149,150]. Based on the rapid reduction of cost over the history of DNA synthesis, it is expected that the cost of RNA synthesis will gradually decrease with the development of industrial-scale RNA production technologies.

In conclusion, natural or synthetic RNA molecules can fold into predefined structures that can spontaneously assemble into nanoparticles with multiple functionalities. The field of RNA nanotechnology is emerging but will play more and more important roles in medicine, biotechnology, synthetic biology and nanotechnology.

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