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Review

Nucleic acid-based nanoengineering: novel structures for biomedical applications

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Nanoengineering exploits the interactions of materials at the nanometre scale to create functional nanostructures. It relies on the precise organization of nanomaterials to achieve unique functionality. There are no interactions more elegant than those governing nucleic acids via Watson–Crick base-pairing rules. The infinite combinations of DNA/RNA base pairs and their remarkable molecular recognition capability can give rise to interesting nanostructures that are only limited by our imagination. Over the past years, creative assembly of nucleic acids has fashioned a plethora of two-dimensional and three-dimensional nanostructures with precisely controlled size, shape and spatial functionalization. These nanostructures have been precisely patterned with molecules, proteins and gold nanoparticles for the observation of chemical reactions at the single molecule level, activation of enzymatic cascade and novel modality of photonic detection, respectively. Recently, they have also been engineered to encapsulate and release bioactive agents in a stimulus-responsive manner for therapeutic applications. The future of nucleic acid-based nanoengineering is bright and exciting. In this review, we will discuss the strategies to control the assembly of nucleic acids and highlight the recent efforts to build functional nucleic acid nanodevices for nanomedicine.

Keywords: DNA; origami; self-assembly; nanomedicine; templated display; drug delivery

1. INTRODUCTION

As a vital part of modern nanotechnology, nanoengineering aims at manipulating and developing components, devices and systems at the nanoscale. It has the potential to miniaturize existing products, build new materials with novel properties, and more importantly, help us gain deeper insights into nature and life. Over the past decades, the concepts and tools of nanoengineering have been widely applied to life sciences: micro- and nano-fluidic devices are used to control the cellular microenvironment [1–4]; both top-down and bottom-up strategies are used to create specific surface patterns to modulate cell behaviour [5–10]; and multi-functional nanoparticles (NPs) are applied to improve drug and gene therapies [11–17]. To build these enabling materials or devices, polymers, lipids, ceramics and metals have been commonly used. In this review, we will instead focus on the use of nucleic acids as building materials for nanoengineering. We will discuss the self-assembly of nucleic acid-based nano-architectures and how their properties can be tailored for novel analytical and biomedical applications.

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One contribution of 9 to a Theme Issue 'Nanoengineering life: from cell to tissue'.

2. PLAYING LEGO: NUCLEIC ACID-BASED SELF-ASSEMBLY

Nucleic acids (DNA and RNA) are fundamental 'molecules of life' as they play essential roles in gene heredity, regulation and expression. Beyond their biological functions, they also possess properties that make them versatile materials for nanoengineering. For example, the DNA double helix is inherently a nanoscale object. It has a diameter of approximately 2 nm, a helical repeat of 3.4 nm (10.5 base pairs) and a persistent length of approximately 50 nm [18,19]. While being more chemically labile, RNA molecules have structural components that are very similar to DNA. In addition, most RNAs are single-stranded molecules with complex nanoscale motifs that mediate stereochemically precise and intra- and inter-molecular tertiary interactions [20,21]. More importantly, both of these molecules have remarkable sequence recognition capabilities, and can be conveniently synthesized with a nearly infinite number of sequences. All these unique properties

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have made DNA and RNA intriguing building blocks for nanoengineering.

2.1. Nanoengineering DNA: nature's building blocks go exotic

Although DNA seems to be the ideal nanomaterial that chemists dream about, the practice of building complex DNA nanostructures is far from easy. As linear DNA lacks the complexity and flexibility needed for nanoconstruction, Seeman and co-workers first proposed to combine branched DNA motifs with sticky ends to create two-dimensional arrays. After examining natural DNA primitives with branched junctions, they designed and constructed small building blocks called tiles, which bear three [22], four [23] or more double-helical arms [24] radiating from one single focal point. Once equipped with matching sticky ends, multiple tiles can associate with each other to form larger complexes. However, because of the inherent flexibility of the junction regions, it is extremely difficult to assemble large arrays from these tiles. To solve this problem, Seeman and co-workers designed more rigid building blocks using paired four-arm junctions, also known as crossovers [25,26]. By facilitating strand exchange between neighbouring double helical domains, these crossovers can hold the structures tightly in one focal plane. The building blocks, according to the number, orientation and layout of their crossover pairs, are further classified as DX [25,27], TX [28] and PX tiles [29] (figure 1*a*,*b*). More complex tile designs have been presented more recently, such as the double-double crossover tiles [44] and the 3-, 4-, 6-, 8- and 12-helix DNA tile complexes with non-coplanar DNA helices joined together by multiple crossovers [45–48]. These stable DNA building blocks, and the ability to combine them arbitrarily with sticky-end cohesions, gave rise to a wide variety of structures, such as periodic two-dimensional lattices [27,28], DNA nanoribbons and nanotubes [31,49], etc. (figure 1*a*,*b*). Factors governing the circumferences and lengths of the assembled tubes were further studied [30,31,45], and various other strategies to creating DNA nanotubes were reported [50–52].

Another versatile DNA tile, the 4 × 4 cross-tile, was developed in 2003 [34] (figure 1*c*). With four four-arm junctions that are cross-aligned, the tile can self-assemble into either nanotubes or two-dimensional lattices called nanogrids. A further developed cross-tile design with two tile types was used to synthesize larger two-dimensional lattices and fixed width nanotrack structures (figure 1*c*) [33]. These principles were later extended by introducing symmetry into the tile design, thus demonstrating an increase in the long-range order of the fully assembled lattices [53,54]. Sequence symmetry was also implemented in the design of three-point-star and six-point-star motifs, which resulted in stable hexagonal [37] and multi-layer nanoarrays [55] (figure 1*d*). Finite-size addressable arrays, either with or without sequence symmetry, were also reported, such as the molecular pegboard [35], the 4 × 4 arrays [32] and the 5 × 5 arrays [36] (figure 1*c*). For the preparation of these arrays, multiple unique tile sets and multi-step annealing are required. Therefore, it becomes unrealistic to build addressable arrays with much larger sizes. To solve this problem, Rothemund *et al.* [56] proposed an alternative strategy, the algorithmic assembly strategy. With the smart use of local, tile-to-tile interactions, this method only needs a limited number of tile sets to generate sophisticated patterns, such as the Sierpinski triangles and lattices with binary counting patterns [56,57]. More recently, Hamada & Murata [39] described the construction of the 'T-shaped' junction with two interconnecting duplexes. This tile does not have any designed crossover points, but it works surprisingly well in the formation of complex nanostructures, such as orthogonal coordinated ladders, lattices and polar coordinated wheels (figure 1*e*).

Although tile-based DNA engineering can produce large supramolecular structures, the design process is tedious, the assembly requires strictly balanced stoichiometry, and the achievable structures are often limited by the length of the DNA that can be chemically synthesized. To address these issues, a new scaffold-assisted self-assembly approach was invented. This technique, also known as nucleated assembly, uses single-stranded DNA (ssDNA) as a molecular scaffold and employs multiple small 'staple' strands to fold the scaffold into an addressable shape. An early example was described in 2003 for computational purposes and was prototyped as two-dimensional barcode lattices [58]. In 2004, Shih *et al.* [59] adopted a virus-derived 1.7 kb ssDNA fragment as the scaffold and created three-dimensional tetrahedra with only five short DNA strands as 'staples'. A general and more powerful approach was presented by Rothemund in 2006 [40]. This strategy, known as DNA origami, uses about 200 DNA staple strands to knit together selected parts of a 7.3 kb single-stranded M13 viral genome, thus creating non-periodic two-dimensional architectures, such as rectangles, squares, snowflakes and smiley faces (figure 1*f*). Similar approaches were taken to create other two-dimensional DNA origami structures, such as the map of China [60], dolphins with flexible tails [61] and origami tape with defined wells [62,63].

There is no doubt that the origami strategy can significantly increase the complexity and sophistication of DNA nanoengineering. However, because of the length limit of the scaffold strand, most of the origami structures fall below 100 nm in size, which is much smaller than those structures achievable by tile-based assembly. In the past 5 years, many efforts have been made to scale up the sizes of DNA origami structures. For example, Zhao *et al.* [41] used rectangular-shaped DNA tiles instead of ssDNA as staples to create two-dimensional origami structures that were four times the size of the original origami (figure 1*g*). Multiple origami monomers can also be linked together through blunt-end or sticky-end cohesions. By introducing shape and sequence complementarity as well as employing the π-stacking interactions between the side edges of DNA origami, the Sugiyama group demonstrated the assembly of multiple rectangular origami structures into 'DNA jigsaw pieces' (figure 1*g*) [42,43]. Using a similar strategy, Li *et al.* [64] achieved the assembly of zigzag DNA origami into nanoarrays and tubular structures. More recently, Endo *et al.* [65] used a four-way connector to help

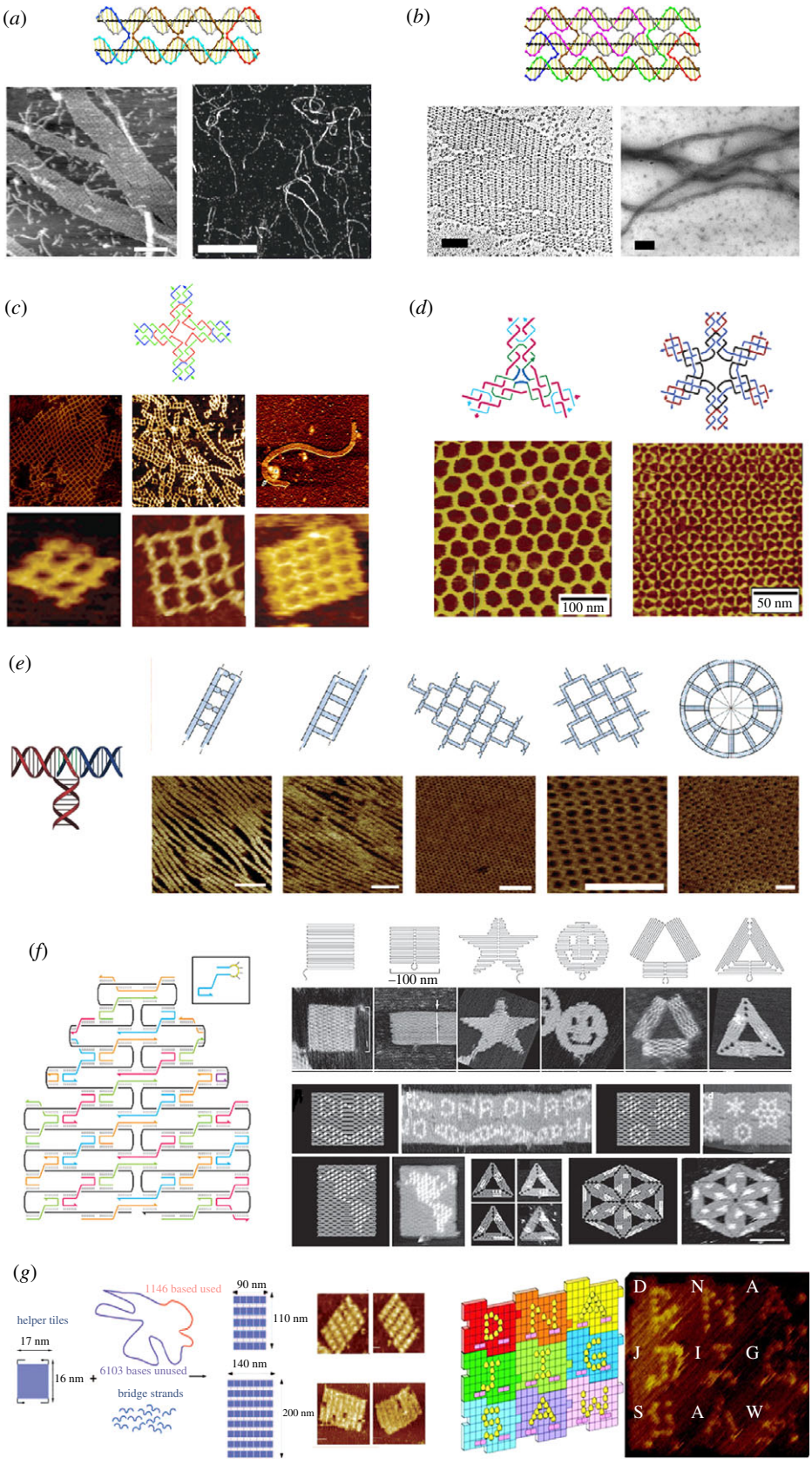

Figure 1. Two-dimensional DNA nanoarchitectures. (*a*) DNA double-crossover tile and their self-assembled two-dimensional arrays and tubes visualized by atomic force microscopy [27,30]. (*b*) DNA triple-crossover tile and their self-assembled two-dimensional arrays and tubes visualized by transmission electron microscopy [28,31]. (*c*) 4 × 4 cross-tile and their nanoassemblies. Top: from left to right, nanogrid (500 × 500 nm), nanotrack (500 × 500 nm) and nanoribbon (1 × 1 μm) [32–34]; bottom: from left to right, finite-sized 3 × 3 [35], 4 × 4 [32] and 5 × 5 arrays [36]. (*d*) Three-point-star, six-point-star tiles and their respective nanoassemblies [37,38]. (*e*) T-shaped junction and their nanostructures self-assembled on mica surface [39]. (*f*) DNA origami. A long scaffold strand is folded into the desired structure via the help of 'staple' strands [40]. This approach is used to approximate different two-dimensional architectures (top) or to draw specific two-dimensional shapes via stem–loop protrusion (bottom). (*g*) Origami with extended dimensions. Left: origami using folded tiles as staples [41]; right: origami jigsaw pieces [42,43].

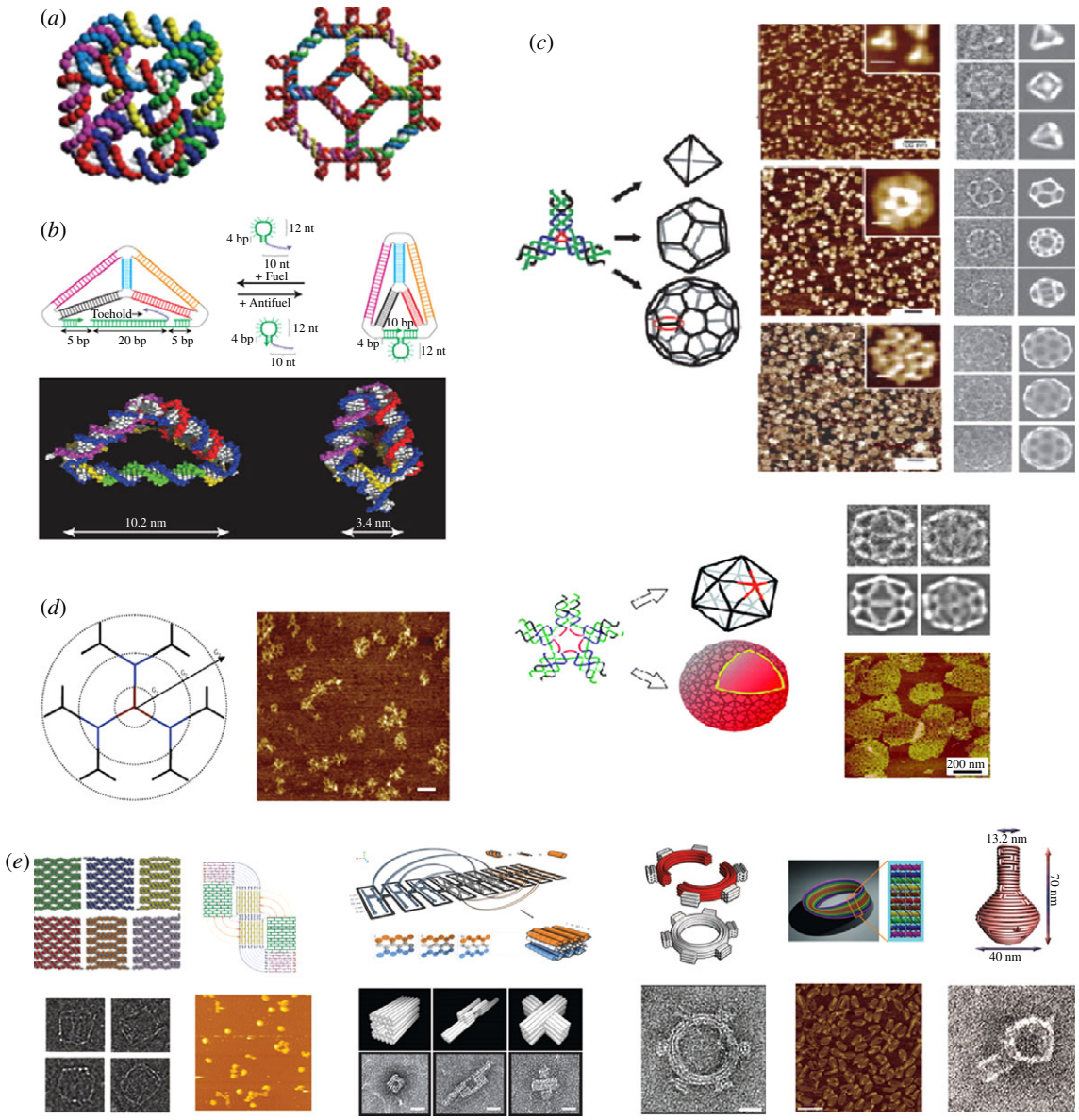

Figure 2. Three-dimensional DNA nanoarchitectures. (*a*) DNA molecules with the connectivity of a cube [69] and truncated octahedron [70]. (*b*) DNA tetrahedron that can be expanded or contracted via the addition or removal of a DNA strand [71]. (*c*) DNA tetrahedra, dodecahedra, buckyballs and icosahedron assembled from three-point-star and five-point-star motifs [73,74]. (*d*) Dendrimer-like DNA assembled from Y-shaped DNA [75]. (*e*) Libraries of three-dimensional origami structures. From left to right: origami box with two different designs [76,77], honeycomb lattices and their assemblies [78], nanogears [79], Möbius strips [80] and origami nanoflasks [81].

facilitate the assembly of rectangular origami monomers into a cross and a hollow square. Such strategies of combining origami structures with hierarchical assembly can potentially build very large structures, but the assembly efficiency needs to be further improved. Alternatively, surface-mediated self-assembly [39,66] and a lithographically patterned template [67] can also be considered to facilitate the assembly of larger structures.

Another unique architectural strategy, the 'weave tile' strategy, was recently proposed by the LaBean group [68]. Unlike the previous tile-based structures and origami structures that rely on crossovers to maintain their correct structural conformation, the weave tile does not employ any Holliday junction-like crossovers and therefore has a lower complexity and a higher flexibility. By using only two oligonucleotides to form the woven pattern that resembles the origami routing, the group has successfully assembled two-dimensional DNA patches that are several hundred nanometres in size.

The molecular-level control demonstrated by these two-dimensional nanostructures represents a major step towards engineering DNA-based controllable systems. However, many applications in nanomedicine and nanorobotics require additional capabilities such as controlled three-dimensional assembly and movement. To meet this challenge, scientists have attempted the engineering of three-dimensional nucleic acid objects. Some of the earlier examples include the DNA cube [69] and truncated octahedron [70] (figure 2*a*) designed by the Seeman group, the rigid and chiral DNA octahedron described by Shih *et al.* [59], and the tetrahedron described by Goodman *et al.* [71,82] (figure 2*b*). In 2008, the Mao Group [73] used symmetric three-point tiles for the assembly of a number of DNA polyhedral structures, such as tetrahedra,

dodecahedra and buckyballs (figure 2*c*). Later, a five-point-star motif was used to assemble DNA icosahedral structures [72,74]. In 2009, nearly 30 years after the original idea of using DNA to construct three-dimensional crystals was proposed, Seeman and co-workers [83] successfully demonstrated the growth of tensegrity triangles into crystals of several hundred micrometres in size. This type of designer DNA crystal can be further used to host proteins and other biomolecules to facilitate the study of their structures and host–guest interactions. Instead of using the tile-based assembly strategy, Oliveira *et al.* showed that stable and covalently closed three-dimensional DNA octahedra can be assembled from eight or 12 ssDNA [84]. More recently, Yan and co-workers [85] generated a tetrahedral structure from only one DNA strand that is 286 nucleotides long. With a simplified assembly process and less dependence on stoichiometry, this assembly strategy resulted in a product yield that is more than 90 per cent.

Another interesting type of three-dimensional network of DNA is the dendrimer-like DNA (DL-DNA) formed from controlled assembly of Y-shaped structural motif (Y-DNA) (figure 2*d*). As suggested by Li *et al.* [75], DL-DNA is stable, monodispersed and robust in nature. The same group also designed and synthesized DNA hydrogels using X-shaped and Y-shaped DNA motifs and cross-linked plasmids [86,87]. These gels not only showed the ability to produce proteins in a cell-free environment, but also demonstrated higher protein-production efficiency.

The origami assembly strategy has also been exploited for the engineering of complex three-dimensional nanostructures. Andersen *et al.* [76] recently constructed a DNA box with a well-defined shape and internal cavities. This is achieved by assembling six DNA origami faces and then connecting them via staple strands at the edges (figure 2*e*). Kuzuya & Komiyama [77] (figure 2*e*) presented an alternative strategy to assemble a DNA nanobox. In their approach, the box is generated in two steps, with the second step closing two sets of three walls using a total of nine face-sharing staple strands. Using a similar strategy, Sugiyama and co-workers [88] have folded multi-arm DNA structures into novel hollow prisms. Yan and co-workers [89] recently presented quite a different approach to assemble three-dimensional structures with tetrahedral geometry. Instead of using modular domains formed from the scaffold for the assembly, they made the scaffold to pass one helix at a time through each face, so that only two hairpin turns were present in the entire structure. In contrast to the previously described three-dimensional nanostructures, all these origami-based nanocages have solid faces made of single-layer origami.

Three-dimensional objects can also be achieved by piling up multi-layer origami structures. Shih and co-workers [78,90] developed a general approach to roll DNA origami into a range of discrete three-dimensional structures. This method involves folding the scaffold strand with staple strands to create a honeycomb lattice (figure 2*e*), in which parallel DNA duplexes are connected by Holliday junctions at specific locations to ensure the relative position of one helix with respect to the next. Three-dimensional objects with different morphologies were rationally designed using this method, such as monoliths, square nuts, railed bridges, slotted crosses and stacked crosses. Yan and co-workers [91] recently described a more compact design of three-dimensional origami. With layers of helices packed on a square lattice, this type of DNA origami has a higher material density than the honeycomb lattices and can be prepared through a one-step annealing process.

Besides origami with rigid edges, twisted and curved three-dimensional origami structures can also be made. For example, Shih and co-workers [79] demonstrated the assembly of twisted DNA bundles, curved shapes with well-defined bend angles, and more complicated curved structures such as nanogears (figure 2*e*) and spherical wireframes. Yan and co-workers [80] recently assembled an origami Möbius strip, a topological ribbon-like structure that has only one side. They also showed that this strip can be reconfigured through strand displacement to create topological objects such as supercoiled ring and catenane structures. In a more recent study, they further demonstrated the level of fine control that can be achieved over origami surface curvatures. A variety of complex curved origami was reported, such as DNA origami spheres, ellipsoids and nanoflasks (figure 2*e*) [81].

2.2. Nanoengineering RNA: inspired by nature

Despite a chemical structure that is similar to DNA, RNA often adopts two- and three-dimensional conformations that are far more complex than its DNA counterpart. Analysis of the rapidly growing structural and sequence databases for RNA has revealed novel RNA structural motifs and RNA–RNA interactions, such as loop-receptor-interacting motifs, RNA loop–loop interactions, three-way junctions and K-turn motifs [92–96]. These discoveries have inspired scientists to pursue RNA-based nanoengineering.

Unlike DNA nanoengineering, which relies heavily on artificial building blocks and sticky-end cohesions, RNA-based nanoengineering focuses more on using the tertiary interactions between structural motifs. In the past decade or so, various modular RNA motifs have been designed, such as RNA dimers [97,98], H-shaped [99] and square-shaped RNA units [99]. By exploiting the specific, non-Watson–Crick interactions between these motifs, or 'tecto-RNAs', a variety of nanostructures have been built, such as RNA particles [99–101], filaments [102–104] and many programmable planar nanostructures [105,106].

Among all these examples, the Jaeger group's square-shaped tectoRNA might be the best-known design [105]. In 2004, Jaeger and co-workers [106] designed a tectoRNA with two interacting hairpin loops joined by a small structural motif of 11 nucleotides. This motif, known as the right angle (RA) motif, specifies a 90° angle between the adjacent helices, and therefore is used to guide the assembly of the tectoRNAs into tetramers (tecto-squares) via kissing loop interactions. With specially designed tail connectors, multiple tecto-squares can self-assemble into complex multi-square lattices that resemble jigsaw puzzles (figure 3*a*). In 2009, they further showed that the RA motifs can be replaced with three-way junction motifs, which yielded even more complex nanostructures such as the triads and other non-uniform square antiprisms (figure 3*c*).

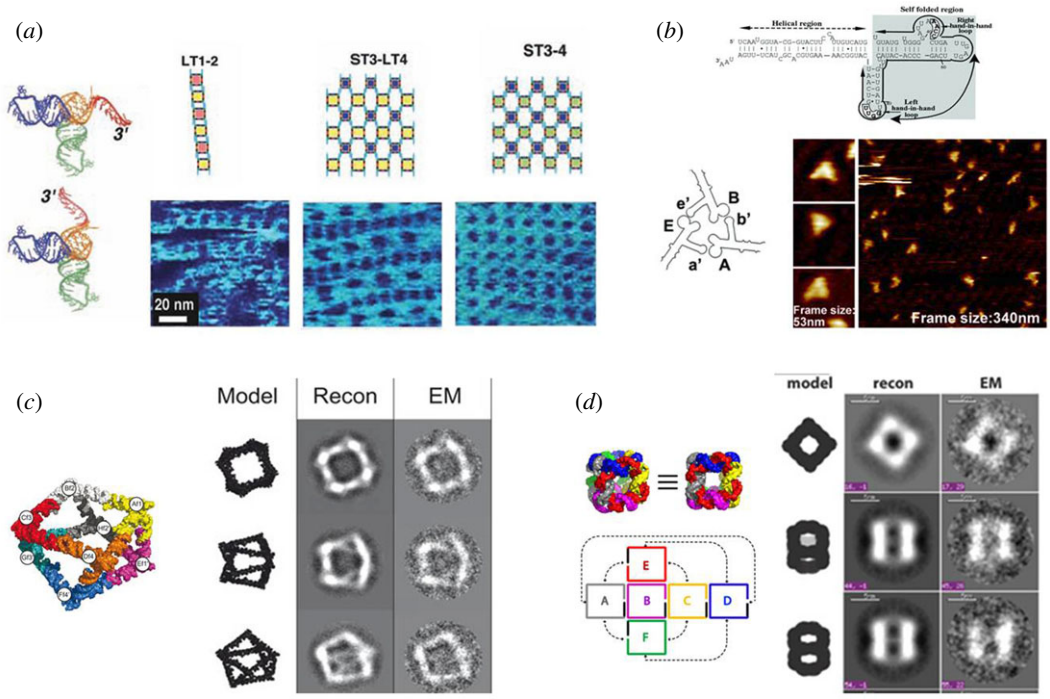

Figure 3. RNA-based nanoarchitectures. (*a*) RNA tectosquares and selected two-dimensional assemblies [105]. (*b*) phi29 pRNA and selected trimeric assemblies [107]. (*c*) t-RNA antiprism nanoparticles [245]. (*d*) RNA nanocube assembled by sticky-end cohesion [108].

Another RNA structural motif that is being widely studied is the DNA-packaging motor *pRNA* of bacterial virus *phi29*. Guo and co-workers [107,109] demonstrated that by making complementary mutations in four nucleotides of pRNA's left and right loops, they were able to engineer dimers and trimers very efficiently (figure 3*b*). Further modifications, such as extensions, deletions and circular permutations of this domain might give rise to defined self-assembled nanostructures of various sizes and shapes, such as hexamers, rods, triangles and even three-dimensional arrays. In addition, such arrays are stable and resistant under a wide range of pH, temperatures and salt concentrations [104].

Although not as commonly used as tertiary interactions, sticky-end cohesion is also explored for the engineering of complex RNA nanostructures. For example, Jaeger and co-workers [108] have created RNA nanocubes that are assembled by sticky-end hybridization. As illustrated in figure 3*d*, these cubic structures have an edge length of around 13 nm. In addition, they can be created in a one-pot procedure at 37°C, which offers an exciting opportunity of generating defined RNA nano-object at ambient temperature and even *in vivo*. Cayrol *et al.* [110] also reported that DsrA, an 87-nt non-coding RNA of *Escherichia coli*, can self-assemble into a hierarchy of nanostructures through antisense interactions of three contiguous self-complementary regions. This is the first time that natural RNAs have been shown to form extended nanostructures.

2.3. *Engineering hybrid nanostructures: two is better than one*

For all the previously mentioned examples, DNA or RNA molecules are the sole material used in the engineering process, which typically results in dense, rigid assemblies. An alternative approach to build more versatile nucleic acid nanostructures involves the combined use of multiple materials, such as DNA, RNA, protein and synthetic molecules.

Ohno *et al.* [111] showed that RNA and the ribosomal protein L7Ae can form a nanostructure shaped like an equilateral triangle (figure 4*a*). The construction of this complex relies on the proteins binding to the kink-turn (K-turn) motifs in the RNA, which allows the RNA to bend by approximately 60° at three positions to form a triangle. Mao and co-workers [113] recently constructed three different RNA–DNA hybrid branched nanomotifs, which readily self-assembled into one-dimensional nanofibres, extended two-dimensional arrays and discrete three-dimensional objects (figure 4*c*). Mayer *et al.* [115] described the efficient synthesis of double-stranded DNA (dsDNA) minicircles containing bespoke ssDNA gap regions, which allowed the integration of RNA aptamer motifs and polypeptide struts.

Another strategy of building hybrid nanostructures involves the use of synthetic molecules. For example, natural DNA bases can be replaced with supramolecular building blocks to bring additional interactions therefore achieving more structural varieties: Chaput & Switzer [116] showed that DNA-bearing non-standard nucleobase iso-guanines can form a pentameric assembly in the presence of caesium ions; Wang *et al.* [117] demonstrated that foldable polymers with alternating ssDNA and planar conjugated organic perylene tetracarboxylic diimide units can form nanostructures through π–π stacking; and Goritz & Kramer [118] showed that a bis(terpyridine)-modified ssDNA can form a stable cycle by Fe^{2+}-assisted ring closure.

Synthetic molecules can also be used as junctions to covalently link DNA structures. In the pioneering work

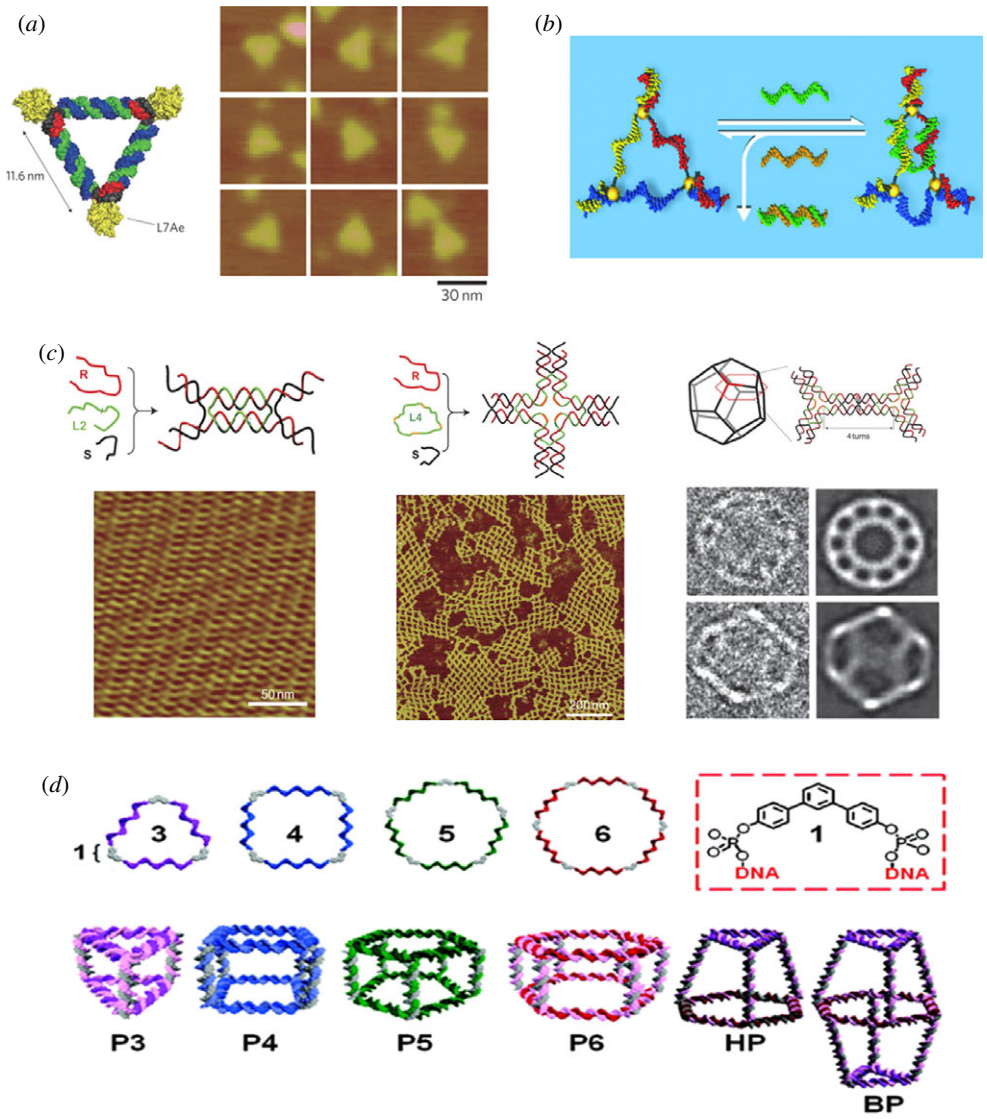

Figure 4. Hybrid nanostructures employing DNA and RNA molecules. (*a*) Triangular RNA–protein assemblies [111]. (*b*) Triangular metal–DNA assemblies [112]. (*c*) DNA–RNA hybrid structural motif and their assembly into one-, two- and three-dimensional nano-objects [113]. (*d*) Three-dimensional triangular prism, cube, pentameric and hexameric prisms, heteroprism and biprism generated from ssDNA triangles, squares, pentagons and hexagons with organic vertices [114].

by Shi & Bergstrom [119], the assembly of DNA rings connected by arylethynylaryl moieties was reported. The Shchepinov [120] and von Kiedrowski [121] groups further studied branched DNA structures with organic corner units and showed that they can self-assemble into well-defined nanostructures. More recently, Tanaka *et al.* [122] showed that azobenzene molecules can be inserted into the sticky ends of three-point-star motifs to facilitate their assembly into nanocapsules with increased stability. Metal complexes can also be used as vertices to allow for more flexible geometries. The self-assembly of ssDNAs modified with metal–organic modules into discrete DNA triangles and hexagons were reported by the Han group [123] and the Sleiman group [112,124] (figure 4*b*), respectively. Using a related approach, Sleiman and co-workers [114,125] further demonstrated the assembly of metal-linked branched DNA motifs into various three-dimensional nanocages (figure 4*d*).

By combining the precise programmability of DNA/RNA molecules and the diverse geometry and functionality of protein and other supramolecule entities, these hybrid nanostructures will provide a robust platform for the engineering of complex multi-functional nanodevices.

2.4. *DNA nanomachine: a dynamic dance partner*

Apart from building well-defined static nanostructures, DNA/RNA molecules can also be used to engineer dynamic nanomachines. Driven by environmental stimuli such as changes in ionic strength, pH or catalysing molecules, these nanomachines can undergo structural transitions, exert pulling or stretching forces and perform unidirectional or rotational motions.

The earliest DNA nanomachine can be dated back to 1999, when Seeman and co-workers [126] constructed a nanodevice with two DX molecules connected by a double-helical linker. This linker contains the base-paired sequence $d(CG)_{10}$, a 'proto-Z' sequence that could be converted to left-handed Z-DNA at high ionic concentration. When the B-Z transition occurs, a rotary motion is created, which in turn changes the relative position of

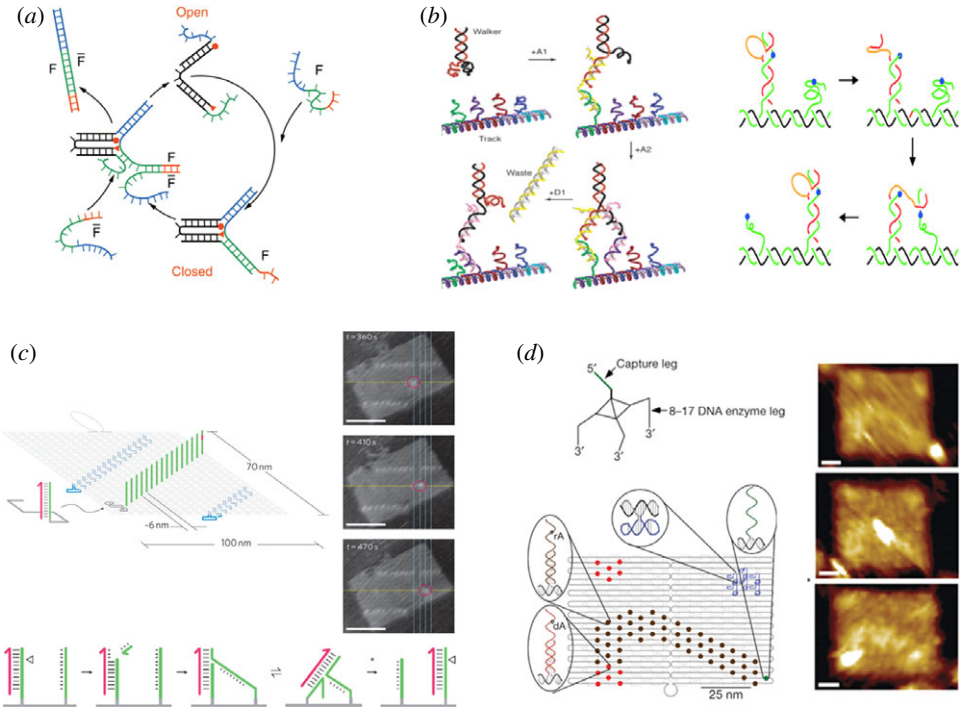

Figure 5. DNA nanomachines. (*a*) DNA molecular tweezer [127]. (*b*) DNA nanomachines that execute stepwise movements along linear tracks. Left: DNA walker driven by sequential addition of two control strands, one that lifts the back foot from the track and one that binds it to a new anchorage ahead of the stationary foot [128]. Right: DNA walker with autonomous movements driven by enzymatic hydrolysis [129]. (*c*) Design of a DNA motor and AFM observation of discrete steps of its movements along a track on an origami scaffold [130]. (*d*) Design of a DNA molecular spider and AFM observation of its movement along predesigned tracks with turns [131].

the two DX molecules. The following year, Yurke *et al.* [127] developed DNA tweezers, the first DNA nanomachine driven by hybridization force. As shown in figure 5*a*, the tweezers are pushed to the closed state by a fuel strand (F) which is complementary to the toehold overhangs of strands B and C. The addition of the removal strand, strand F', which is fully complementary to strand F, leads to the reopening of the device. An optimized device was further developed by Seelig *et al.* [132], where they introduced a DNA catalyst strand and a fuel strand with a kinetically trapped metastable configuration. With improved understanding of the metastable states of DNA structures, scientists were able to build more complex systems with higher rates of catalysis [133,134]. Apart from these examples, there are numerous other DNA nanodevices that have relied primarily on hybridization-induced actuation, such as the single-stranded nanomotor relying on the formation of a G-rich quadruplex [135], and molecular gears with a pair of DNA nanocircles that were 6.5 nm in diameter [136]. However, nanomachines that rely on hybridization-induced mechanical motions are inherently limited by the rate of the hybridization reaction, and the performance decreases with the build-up of the DNA waste after a number of cycles. To address this issue, Bishop & Klavins [137] developed a motor based on a DNAzyme, which enabled them to selectively digest DNA/RNA hybrid waste products.

Meanwhile, DNA devices have also been built exhibiting adaptive responses to a variety of other stimuli such as changes in pH [138–141], DNA hydrolysis [142–144], DNA polymerization [145], light [146,147] and even electric field [148].

Another focus in the field of DNA nanomechanical devices is the development of DNA motors that can move along a defined path. In 2004, the Pierce group reported a bidirectional DNA walker with two distinct feet and a series of four single-stranded anchorages (figure 5*b*). Each step is driven by sequential addition of two control strands, one that lifts the back foot from the track and one that binds it to a new anchorage ahead of the stationary foot [128]. In 2005, Mao and co-workers [129] developed a DNA walker that autonomously and unidirectionally moves along an engineered DNA track (figure 5*b*). The walker mechanism combines thermodynamically driven DNA hybridization interactions with an integrated DNAzyme that has the ability to specifically cleave a di-ribonucleotide built into the stations of the track. Yin *et al.* [134] applied modular DNA stem-loops and strand displacement into their design and reported the controlled assembly of X-, Y- and dendrimer-shaped DNA as well as the stochastic movement of a bipedal DNA walker along a DNA track. More recently, He & Liu [149] demonstrated that DNA walkers with amine groups at their ends can perform a series of acylation reactions while moving along their designated track. This is the first time that advanced chemical reactions have been incorporated into the walker system, which could lead to the development of more complex chemical assembly lines at the nanoscale.

The notion of combining walker device and defined tracks are further extended using two-dimensional origami as the supports. By using a nicking enzyme, Turberfield and co-workers [130] demonstrated the

autonomous movement of a DNA motor along a 100 nm-long DNA track prescribed by a rectangle origami. They also used real-time atomic force microscopy (AFM) for the direct observation of the 16 individual steps of motor movements (figure 5*c*). Lund *et al.* [131] recently reported an autonomous system where the movement of a DNA walker known as a molecular spider is controlled by one anchor strand and three leg strands made with DNAzymes. As shown in figure 5*d*, the surface traversed by the spider is a sheet of DNA origami, designed to contain cleavable DNA strands whose base sequences are complementary to those of the spider's legs. Gu *et al.* [150] recently reported a walker system with additional arms to carry and release NP cargoes while walking. This is the first time that multiple, rather than individual devices, have been used to perform coordinated operations, which constitute a crucial advance in the development of DNA-based nanomachines.

Although the functions of these nanomachines are still far away from the possibilities imagined in science fictions, the creativity in the development of autonomous nucleic acid systems is inspiring nonetheless.

3. LAYERING IT UP: NUCLEIC ACID NANOSTRUCTURES AS SCAFFOLDS FOR DIRECTED SELF-ASSEMBLY

One of the key challenges in nanoengineering is to precisely control and manipulate matter at the nanoscale. The aforementioned self-assembled nucleic acid nanostructures represent a promising avenue to meet this challenge. Their unique sequence and spatial addressability allow precise positioning of proteins, NPs and other nanomaterials with sub-10 nm features, which far surpasses the resolution of current top-down methodologies. This ability to create arrays of nanoentities with nanometre scale spacing has important implications in nanomedicine, especially in biosensing and detection.

3.1. Nucleic acid-directed organization of protein and small molecules: more than just pretty decorations

Because of the delicate yet diverse architectures of protein molecules, it is difficult to devise one generally applicable method for their selective coupling onto DNA scaffold. Instead, a variety of different strategies have been attempted in the past, such as reversible antibody–antigen interactions [151,152], aptamer binding [153,154], nucleic acid hybridization of DNA-tagged proteins [155,156] and predominantly biotin–streptavidin interactions [34,35,58,62,157].

The biotin–streptavidin pair was first employed as a molecular connector for the fabrication of both nano- and micro-structured protein arrays, as reviewed by Niemeyer [158]. The tetravalent nature of streptavidin was further exploited in the construction of complex DNA–protein networks and nanocircles with conformational switching mediated by DNA supercoiling [159,160]. This approach was later applied to self-assembled DNA nanostructures, where selected DNA strands are chemically modified with biotin and used as docking sites for the organization of streptavidin molecules. A variety of two-dimensional streptavidin arrays with programmable inter-protein spacing were obtained this way, with TX arrays [157], DNA nanotracks [33], DNA nanogrids [33,58] (figure 6*a*) and two-dimensional origami structures [62,63] as scaffolds. Finite-sized arrays with individual addressability have also been used to achieve precise placement of streptavidin molecules, as reported independently by the Yan group [35] and the LaBean and Dwyer groups [32] (figure 6*a*). The multivalent nature of the streptavidin molecules also provides the opportunity for further binding of other biotinylated entities such as biotinylated nanotubes [163].

A second strategy for DNA-directed protein assembly involves special DNA sequences or motifs. Malo *et al.* [164] developed a RuvA array, where RuvA, a Holliday junction binding protein, not only binds to the four-arm junctions of the two-dimensional assemblies but also stabilizes the conformation of the structure. DNA or RNA aptamers, with their intrinsic binding capacities towards selected targets, are also widely used for templated protein display. For example, aptamer-tagged TX tile arrays and nanogrids were used to direct the assembly of thrombin molecules [165,166]. Chhabra *et al.* [153] further demonstrated that spatially addressable multi-protein nanoarrays can be constructed by incorporating different aptamer sequences into both DX tile arrays and DNA rectangle origami (figure 6*b*). The same group also designed a multi-helix tile array with two different aptamers that bind to opposite domains of the thrombin molecule [154]. The spatial separation of the aptamers is further tuned to determine the ideal inter-ligand distance for efficient binding of the target protein. Weizmann *et al.* [167] reported the simultaneous assembly of thrombin, fluorophores and NPs onto the rings of ladder-shaped polycatenane chains, which also involved the use of thrombin-binding aptamers. Li *et al.* [168] further extended this approach and demonstrated aptamer-mediated display of single-chain antibodies on a variety of DNA templates. Because a virtually unlimited number of aptamer–antibody pairs can be identified through *in vitro* selection, the technology is highly modular, and can be extended to build hetero-bifunctional single-chain antibody dimers as universal adaptors for nanoengineering.

The nucleic acid hybridization reaction is also widely used for the creation of DNA-templated protein nanoarrays. For example, Williams *et al.* [152] demonstrated that tailor-made peptide and protein nanoarrays can be directed by hybridization between DNA capture probes on DX lattices and DNA–peptide conjugates bearing complementary sequences (figure 6*c*). Recently, Stephanopoulos *et al.* [169] demonstrated the assembly of bacteriophage MS2 capsids onto two different types of DNA origami by modifying the exterior of the capsid with 20-nt poly-T sequences and the origami with complementary probes.

Apart from the three general strategies mentioned above, there are various other templating techniques available. For example, He *et al.* [151] reported the assembly of anti-fluorescein antibodies into periodic tetragonal two-dimensional arrays along fluorescein-modified DNA

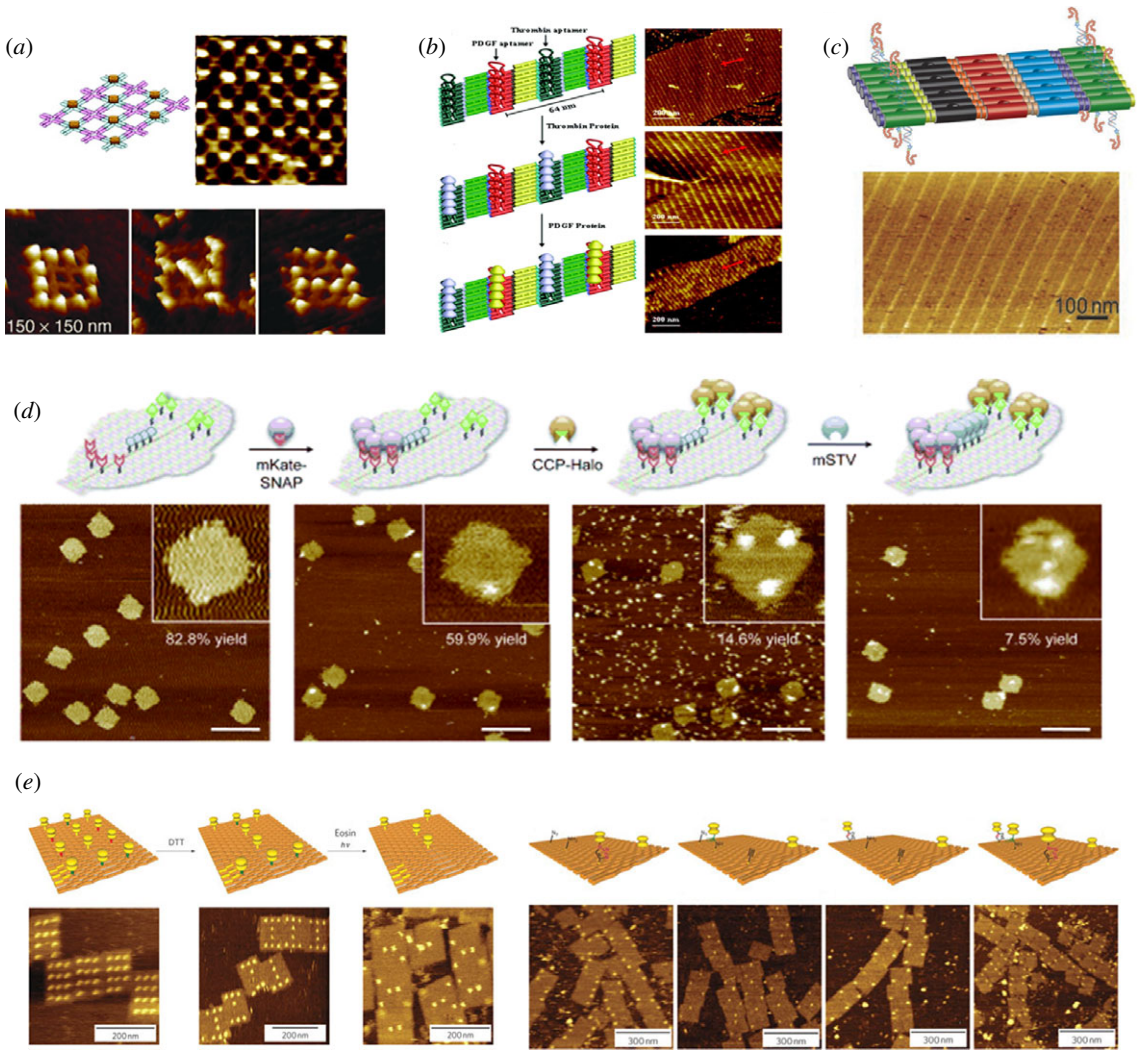

Figure 6. DNA nanostructure-directed self-assembly of protein molecules. (*a*) Programmable assembly of streptavidin arrays on biotinylated nanogrids and addressable 4 × 4 cross-tile lattices [32–34]. (*b*) Periodic two-dimensional arrays of thrombin and platelet-derived growth factor directed by aptamer tags on DX nanolattices [153]. (*c*) Peptide nanoarray directed by hybridization to DX lattices [152]. (*d*) Sequential binding of mKate-Snap, CCP-Halo and streptavidin molecules on DNA origami bearing benzylguanine, chlorohexane and biotin groups. The binding of these proteins leads to the decoration of the origami with features resembling a mouth, eyes and a nose, respectively [161]. (*e*) Single-molecule detection on DNA origami scaffolds. The disappearance (left) and attachment (right) of streptavidin molecules caused by the cleavage and coupling reactions are detected via AFM imaging [162].

lattices. The Dervan group [170] demonstrated the use of polyamide–biotin conjugates to recruit streptavidin onto the minor grooves of DX nanoarrays. Shih and co-workers [171] reported the design and construction of a detergent-resistant liquid crystal of 0.8 μm long DNA-nanotubes that can be used to induce weak alignment of membrane proteins via residual dipolar coupling effects. Norton and co-workers [172] reported the fixation of a His-tagged protein on a DNA origami structure containing nitrilotriacetic acid (NTA) bearing staple strands. More recently, Niemeyer and co-workers [161] demonstrated that benzylguanine and chlorohexane groups can be chemically incorporated into DNA origami structures for the site-specific coupling of fusion proteins containing the self-labelling protein tags hAGT and HaloTag (figure 6*d*).

The ability to assemble multiple molecules with well-controlled spacing makes DNA nanostructures an ideal platform for studying protein–protein or protein–ligand interactions. A pilot step was taken by Niemeyer and co-workers [173], where they assembled two enzymes—NAD(P)H : FMN oxidoreductase and luciferase—using ssDNA as a guide. They found that the overall catalytic activity of the two enzymes is significantly higher when assembled in close proximity on a DNA template when compared with a random distribution on a surface. The work is a proof-of-concept demonstration that an artificial multi-enzyme complex can work on a DNA scaffold. In a later experiment, Willner and co-workers [156] attached glucose oxidase and horseradish peroxidase to self-assembled ladder-like DNA scaffolds and studied the cascade reactions mediated by these two enzymes. They found that the well-ordered organization of the biomolecules leads to the activation of enzyme cascades that normally do not proceed in a random, non-organized system. Furthermore, the bi-enzyme complexes assembled on the 'two-ring' scaffolds show higher activity than those assembled on the 'four-ring' scaffolds, owing to the closer proximity of the two enzyme components.

DNA origami scaffold has also been used to study enzyme kinetics, as reported by Endo *et al.* [174].

Besides enzymatic reactions, chemical reactions with single molecules can also be performed and monitored on DNA nanostructure scaffold. Voigt *et al.* [162] recently reported the use of a rectangular origami structure as an addressable support to visualize chemical coupling and cleavage reactions. In their design, biotin molecules are linked to the surface of the origami via different types of linkers, and the formation or cleavage of the chemical bonds in the linkers result in either attachment or removal of the streptavidin molecules from the origami surfaces, which can be easily visualized by AFM (figure 6*e*). This direct monitoring of chemical reactions at the single-molecule level is a stunning demonstration of the versatility of DNA origami platforms.

3.2. Nucleic acid-directed organization of inorganic materials: precision rules

Apart from being used to organize biomacromolecules, nucleic acids and their self-assembled nanostructures can also dictate the assembly of inorganic nanomaterials.

Traditionally, non-specific interactions such as electrostatic interactions, groove binding and intercalation have been used for DNA-templated display of metal and semiconductor ions. Silver [175], gold [176], palladium [177], platinum [178], and semiconductor [179] NPs and nanowires have been templated on DNA using this approach. With advances in structural DNA nanotechnology and the highly addressable two- and three-dimensional structures, it becomes more and more feasible to employ specific molecular recognition to achieve site-specific organization of inorganic materials.

The earliest examples of DNA-directed assembly of NPs can be traced back to 1996. In the first paper, Mirkin and co-workers described the assembly of networked particle arrays via the use of two types of gold NPs (AuNPs) bearing multiple oligonucleotide modifiers complementary to each other [180]. The second report, from Alivisatos' group, outlined a method to align monovalent NP–DNA conjugates on DNA templates for the creation of small, periodic NP assemblies [181]. Both of these examples harnessed the base complementarity of the DNA linker molecules. Similar strategies have been applied to achieve DNA-directed formation of two- and three-dimensional NP assemblies. For example, Le *et al.* [182] patterned AuNPs on DX tile arrays by hybridizing oligonucleotide (ODN)-functionalized NPs with pre-assembled DX tiles that carry complementary overhangs. Yan and co-workers [183] later demonstrated AuNP nanoarrays templated along cross-tile-based two-dimensional lattices by a similar hybridization strategy, or by allowing the NP-conjugated DNA to directly participate in the formation of the lattices (figure 7*a*) [184]. Seeman and co-workers [186,187] further extended this approach and achieved precise arrangement of two differently sized AuNPs on a triangular-shaped DNA scaffold (figure 7*b*).

Another commonly used strategy for nanomaterial organization involves covalent attachment of functional groups to selected DNA strands and the subsequent binding of their ligands. For example, by using biotinylated DNA TX tile arrays as templates, LaBean and co-workers [157] demonstrated linear display of streptavidin-functionalized AuNPs. More recently, the specific sulphur–gold interaction was exploited to mediate three-dimensional NP assembly. Alivisatos and co-workers [188] constructed a DNA pyramidal nanocage with four hexanethiol linker-modified vertices, which are used to direct the assembly of AuNPs with four different sizes (figure 7*c*). By placing three thiol groups and one biotin group at the vertices, Howorka and co-workers [193] further made DNA tetrahedron that has affinity for both gold substrates and streptavidin molecules in the surrounding media. By manipulating the DX array system and the attachment sites of AuNPs with different sizes, Yan and co-workers. created DNA nanotubes with different AuNP-loading patterns: single-, double- and nested-spirals [189] (figure 7*d*). In this case, the NPs are not just passively displayed along the scaffold; instead, they are found to influence the conformations of the DNA nanotubes through size-dependent steric repulsion effects. Apart from biotin and sulphur groups, DNA nanostructures can also be conjugated with gold-binding peptides (figure 7*a*) [185,194] or amines [124] to achieve site-specific binding of AuNPs.

Besides tile-based DNA nanoarrays, DNA origami structures have also been used for NP organization. For example, by using AuNPs monofunctionalized with lipoic acid-modified DNA oligos, Yan and co-workers [190] achieved a discrete number of AuNPs displayed on rectangle DNA origami (figure 7*e*). Ding *et al.* [191] used a triangular DNA origami to organize three different types of AuNPs (5, 10 and 15 nm in diameter) in a linear fashion, with less than 10 nm spacing between neighbouring particles (figure 7*f*). Later, similar triangular origami scaffold was used by Pal *et al.* [192] to achieve site-specific assembly of silver nanoparticles (AgNP) and dimeric AgNP–AuNP nanoarchitectures (figure 7*f*).

RNA nanoscaffold has also been explored for the arrangement of AuNPs. As Jaeger and co-workers [102] reported, they synthesized ladder-like nanocrown structures from tectoRNAs. With their negatively charged, central openings, these structures were used to bind cationic NPs based on electrostatic, size, and shape recognition.

Nucleic acid-programmed assembly of materials has not been limited to metal NPs. DNA or self-assembled DNA nanoscaffolds have been successfully used for the arrangement of nanorods [195], mesoscale particles [196,197], dendrimers [198], semiconductor ions [199] and carbon nanotubes [163,200]. This ability to efficiently and precisely programme the assembly of other materials represents an ideal platform for future applications in nanoelectronics.

4. PUTTING NUCLEIC ACIDS TO WORK: ENGINEERING NUCLEIC ACIDS FOR THERAPEUTIC AND DIAGNOSTIC APPLICATIONS

4.1. DNA nanostructure in biosensing and detection: a good multi-tasker

The specific molecular recognition capability of DNA not only makes it an ideal structural material but also renders it suitable for biosensing applications. In fact, many

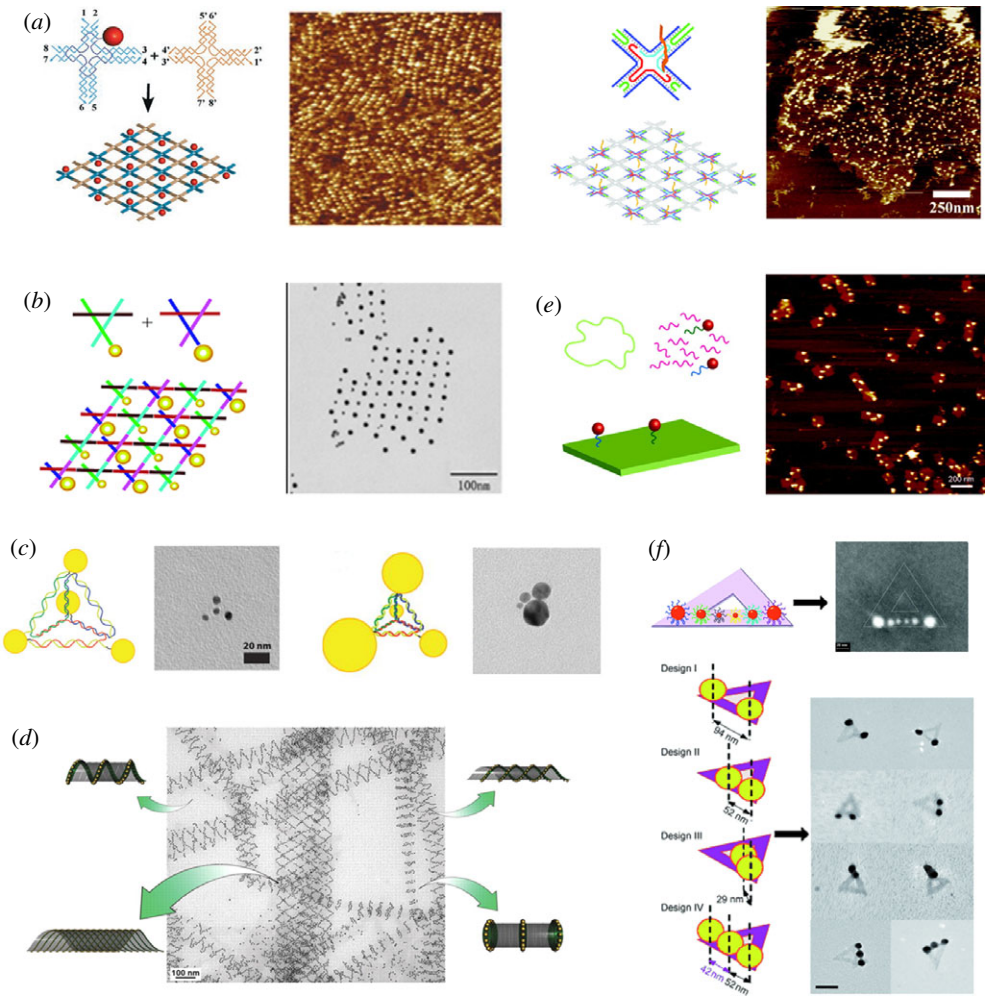

Figure 7. DNA nanostructures as scaffolds for templating inorganic metallic nanoparticles. (*a*) Gold nanoparticle arrays templated by nanogrids. Left: gold nanoparticles are conjugated with one 109-nt ssDNA that participates in the formation of the lattices [184]. Right: DNA nanolattices are modified with gold-binding peptides [185]. (*b*) Formation of alternating 5 nm and 10 nm gold nanoparticle arrays on triangular-shaped nanoarray [186,187]. (*c*) DNA pyramids with four gold nanoparticles at the ends and chiral pyramids with the assembly of gold nanoparticles of different sizes [188]. (*d*) Three-dimensional assembly of gold nanoparticles using various configurations of tubular DNA templates [189]. (*e*) Assembly of gold nanoparticles on rectangular origami [190]. (*f*) Triangular DNA origami-directed assembly of gold nanoparticles (top) [191] and silver nanoparticles (bottom) [192].

detection systems have been built taking advantage of the hybridization power of linear DNA, such as molecular beacons [201,202], and colorimetric detection systems based on AuNP–DNA conjugates [180,203] (figure 8*a*,*b*). Compared with ssDNA or dsDNA used in these systems, complex DNA–RNA nanostructures offer many advantages in biosensing, especially with regards to multiplex detection. For example, as discussed in the previous section, a rich set of molecular probes (DNA, RNA, proteins and inorganic materials) can be precisely engineered into DNA–RNA nano-architectures using modular approaches, and the distance between different ligands can be further finely tuned to fit individual needs. In addition, these nanostructure-based systems can be fabricated in a highly efficient way, making parallel multiplex detection possible.

Taking advantage of these unique properties, Luo and co-workers [204] developed a DNA 'nanobarcode system' for multiplex detection. In this system, Y-shaped DNA self-assembles into DL-DNA. The terminal ends of each 'DL-DNA' are modified with detection probes and different combinations of fluorophores. By altering the number of fluorophores that each DL-DNA carries, a unique colour code can be generated. This unique nanobarcode system is coupled with a microbead-based detection system to achieve simultaneous detection of multiple DNA oligos of pathogenic origins (figure 8*c*). Later, the Luo group developed a target-driven polymerization strategy based on Y- and X-shaped DNA [87,205]. Similar to the nanobarcode system, subunits of the monomers are labelled with different moieties, such as quantum dots, photocrosslinkable groups and single-stranded oligonucleotide probes that are complementary to the target DNA. The monomers will only dimerize in the presence of the target DNA. Upon exposure to ultraviolet light, signals from their photoreactive groups can be read and used as an indicator of the target concentration. As target-driven polymerization happens very fast, this detection method enables rapid signal amplification with high specificity and sensitivity.

Armitage and co-workers [207] also employed Y-shaped DNA in designing their fluorescent nanotags. Instead of conjugating fluorophores to the terminals of DNA helices, they used special dye molecules that can be intercalated into the DNA base pairs. The resulting

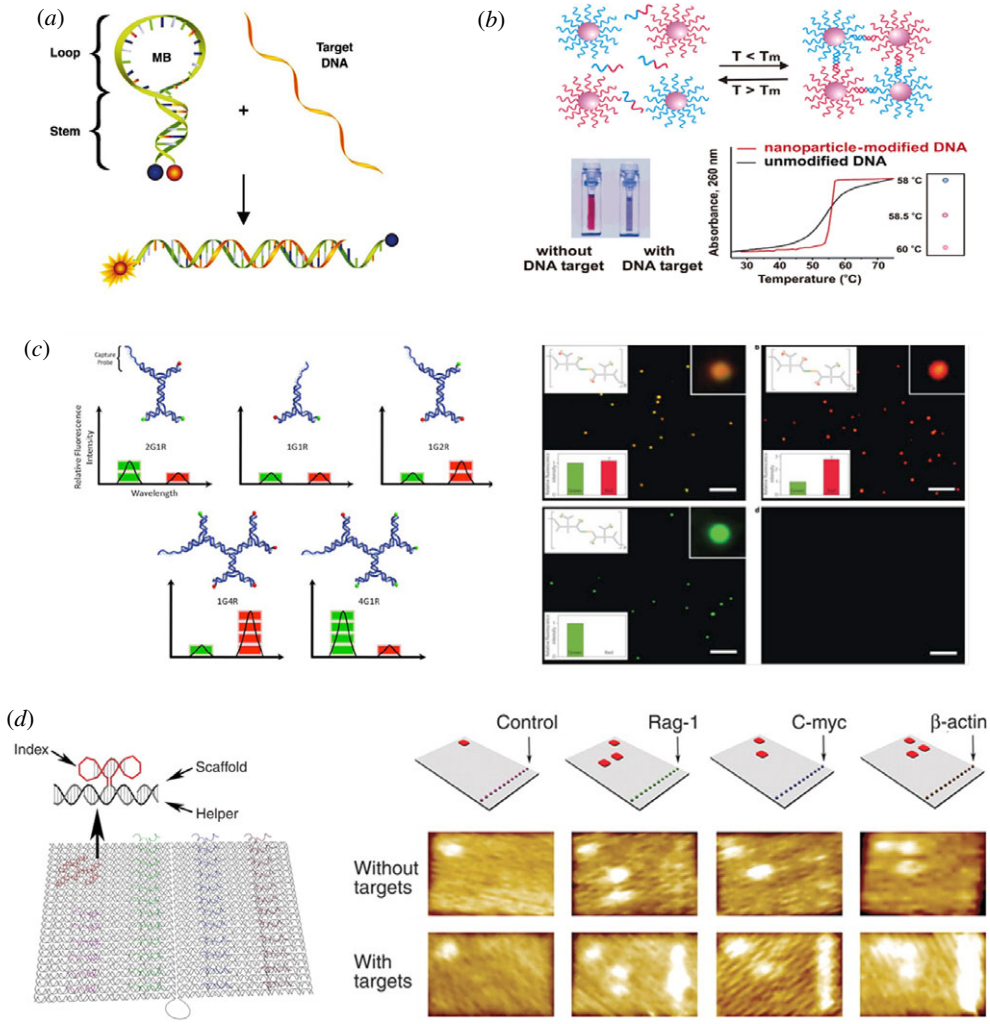

Figure 8. Nucleic acid-based nanostructures with biosensing capabilities. (*a*) Molecular beacon [201]. (*b*) dsDNA-AuNP conjugates for colorimetric detection of target DNA–RNA sequences. In the presence of complementary target DNA, ODN-functionalized gold nanoparticles aggregate, resulting in the change of solution colour from red to blue [180,203]. The aggregation process can be monitored using UV-vis spectroscopy or simply by spotting the solution on a silica support. (*c*) DNA-based fluorescence nanobarcode (left) [204] and target-driven polymerization of ABC monomers for the detection of pathogen DNA [205]. (*d*) DNA origami-based nucleic acid probe for label-free detection of RNA [206].

self-assembled multi-chromophore arrays exhibit high extinction coefficients and efficient energy transfer behaviour. They further applied the dye molecules to a three-dimensional DNA tetrahedron and created a novel nanotag with improved photostability and enhanced resistance to nuclease digestion [208].

Yan and co-workers [165] reported a different barcoding nanoarray, where two-dimensional nanogrid is used as a platform and multiple aptamers are attached as the signalling units. In their design, the aptamer is integrated into the lattices as protruding stem-loops. One of its nucleotide is replaced with a fluorescent analogue, which displays increased fluorescence upon target-binding. The same two-dimensional grid is also used in the construction of a multiplexed sensing array, where its tiles are labelled with either one of the two encoding dyes (red and green) or the detection probes carrying a blue dye [209]. A target-binding event displaces the dye-labelled probes (blue) causing a colour change of the grid from the blue-masked colour to a mixture of the two remaining encoding colours. By using this system, they were able to detect severe acute respiratory syndrome virus, human immunodeficiency virus, and protein targets such as thrombin and adenosine triphosphate simultaneously. More recently, the same group used DNA origami to construct a multiplex RNA detection system, where selected positions of the origami are extended by single-stranded overhangs [206]. As shown in figure 8*d*, upon hybridization with the target RNAs, DNA/RNA duplexes form, and the increase in stiffness and height caused by the hybridization event can be readily imaged by AFM. Targets such as Rag 1, C-myc and β-actin were successfully detected using this system. More recently, Seeman and co-workers [210] developed an elegant strategy to use DNA origami as a molecular chip to detect single nucleotide polymorphism, which further demonstrates the potential of DNA nanostructures for biological sensing applications.

4.2. DNA and RNA nanocontainers: cargoes anybody

Besides using two-dimensional nanostructures to organize nanomaterials and build multiplex sensing devices, many groups have started to explore the possibility of employing

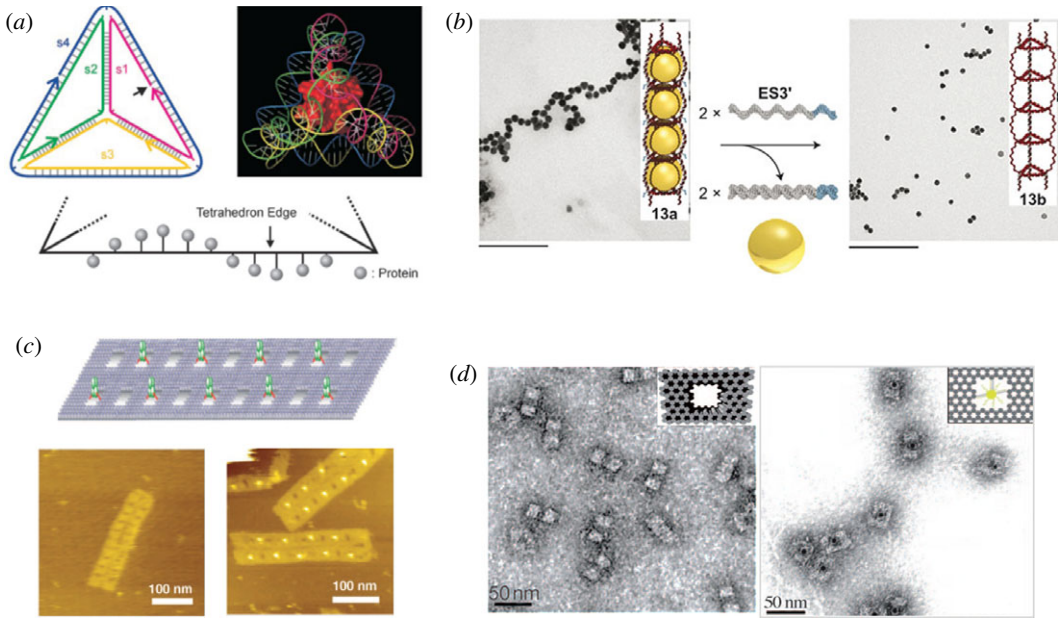

Figure 9. Nucleic acid-based nanostructures for cargo encapsulation. (*a*) DNA tetrahedron with its protein cargo, cytochrome c. The amine attachment site of the protein on the edge determines the position of the protein relative to the cage [211]. (*b*) DNA nanotubes, with alternating large and small capsules along the tube length, exhibit size-selective encapsulation of AuNPs into the large capsules. The cargo can be spontaneously released with externally added control strands [212]. (*c*) Punched DNA origami for the encapsulation of streptavidin molecule. The formation of the periodical two-dimensional streptavidin array is dictated by both biotin–streptavidin interaction and size selection [62]. (*d*) DNA origami cages encapsulating AuNPs [213].

complex three-dimensional nanocontainers as delivery vehicles. For example, Turberfield and co-workers [211] demonstrated the encapsulation of cytochrome c within a DNA tetrahedral cage (figure 9*a*), where the protein molecule is conjugated to the 5′-end of one of the component DNA strands via a surface amine. By altering the sequences of the DNA strand, they were able to control the position of the protein relative to the cage, which made it possible to choose whether the protein was held on the inside or on the outside of the tetrahedron. Recently, they further demonstrated the operation of a reconfigurable DNA tetrahedron, whose shapes change precisely and reversibly in response to specific molecular signals [214]. This reconfigurability holds great promise for controlled cargo encapsulation and release.

Recently, Sleiman and co-workers [212] constructed a DNA nanotube with alternating large (14 nm) and small (7 nm) capsules (figure 9*b*). With this design, they were able to achieve size-selective encapsulation of AuNPs. In addition, the tubes can be opened by externally supplied DNA strands, which causes a fast and complete release of the NP cargoes.

In other experiments, the strategies of chemical conjugation and size-selection were combined to achieve better capture of the cargo molecules. For example, Kuzuya *et al.* [62] constructed a punched DNA origami tape with periodic nanometre-scale wells (6.8 × 12 × 2.0 nm) (figure 9*c*). Two edges of the wells are modified with biotin for the binding of streptavidin molecules. Because of the size constraint imposed by the well, only one streptavidin molecule is allowed per well, thus forming linear arrays with regular spacing. By using similar structural scaffold and the well-known thiol–gold interactions, they later demonstrated selective encapsulation [215] and on-demand release [63] of AuNPs. More recently, Yan and co-workers [213] demonstrated the ability of a DNA origami nanocage to encapsulate AuNPs with various sizes. In their experimental setting, AuNPs modified with ssDNAs and an origami nanocage bearing complementary strands on its inner wall are used (figure 9*d*). By taking advantage of the spatial addressability of the origami capsule, they were able to attach AuNPs to both the inside and the outside of the cages.

Meanwhile, RNA nanoassemblies have also been explored as potential drug carriers. For example, Guo and co-workers [216] have used pRNAs to build functionalized dimeric and trimeric NPs for the delivery of siRNAs to cancer cells via CD4 targeting and for targeted delivery of ribozymes against the hepatitis B virus [217]. Grabow *et al.* [218] reported the assembly of polygonal RNA nanorings with increased serum stability. They also demonstrated the incorporation of multiple siRNAs into the complex and their recognition by the recombinant Dicer *in vitro*.

These initial studies are very promising as they reflect the ability of self-assembled nanostructures to load and release cargoes as well as respond to external stimuli. However, researchers still need to learn how to bring these constructs back to biology to solve real problems such as drug delivery and tissue engineering.

5. PUTTING DRUGS IN A DNA NANOBOX: HOW FAR ARE WE FROM REAL BIOMEDICAL APPLICATIONS?

Despite all the excitement about engineering nucleic acids for *in vivo* biosensing and drug delivery, significant challenges remain before their full potential can be realized.

First of all, to successfully detect its target molecules or deliver its therapeutic cargoes, a nucleic acid-based nanocarrier needs to reach its site of action in a biologically active form. Substantial studies have shown that for the delivery of plasmid DNA, antisense oligos and siRNAs, there are at least four different systemic barriers: degradation by plasma nucleases, opsonization by charged serum components, uptake by the reticuloendothelial system and distribution to non-targeted tissues [219–222]. Considering the chemical similarities between DNA–RNA molecules and their nanoassemblies, these barriers will probably put similar constraints on the successful delivery of DNA–RNA-based nanostructures. On the other hand, these nanostructures often have size, shape and folding pattern that are completely different from their natural DNA–RNA counterparts, and thus may be handled differently by the body. Therefore, before any claim can be made regarding their suitability for *in vivo* biomedical applications, a systematic study of the nanostructures' serum half-life, biodistribution profile and their interactions with blood components is needed. Meanwhile, general strategies to increase the stability of DNA–RNA, such as the incorporation of unnatural nucleic acids, should also be considered. In fact, previous studies have shown that locked nucleic acids [223], peptide nucleic acid [224], glycol nucleic acid [225] and L-DNA (the mirror image of natural D-DNA) [226] can be used to generate highly stable nanostructures. In addition, covalent linkages of DNA–RNA to other molecules, such as cholesterol and polyethylene glycol (PEG), can also be considered to increase the serum stability of DNA–RNA nanostructures [227–230].

Another potential problem with the *in vivo* usage of nucleic acid nanostructures is their poor permeability. Because of the existence of multiple phosphoric acid groups, self-assembled DNA–RNA nanostructures are highly negatively charged. As a result, the electrical repulsion forces between the nucleic acids and the negatively charged cell membrane hinder their interactions. Therefore, a proper delivery scheme is probably needed for more efficient cellular targeting and entry. Recently, Koyfman *et al.* [231] demonstrated that DNA hexagonal nanoarrays coupled with streptavidin can be successfully directed to biotinylated cell membranes. Although this strategy is simple and effective, it requires the use of an additional linker pair (biotin–streptavidin) and the modification of cell membranes. A more straightforward method will be the direct conjugation of cell-specific targeting modules, such as cell-penetrating peptides [232,233], DNA–RNA aptamers [234–237] and folate [238,239], to the nanostructures. Considering that this type of modification has been widely used in the delivery of siRNAs and other small molecule therapeutics, it should be readily adapted to the self-assembled systems. For example, Mao and co-workers [240] recently reported that folate can be conjugated to the surface of DNA nanotubes to facilitate efficient transport of Cy3 molecules into cancer cells.

Apart from cellular uptake, the intracellular stability of nucleic acid-based nanostructures will also need to be tested. Although DNA nanostructures are generally viewed as stable, they can disassemble under many conditions with the breakage of individual strands. The intracellular environment, with its reducing nature and abundance in DNases and RNases, definitely poses a threat to the overall stability of the self-assembled nanostructures. Interestingly, Mei *et al.* [241] recently reported that folded DNA origami structures confer increased stability in cell lysates when compared with its ssDNA and dsDNA counterparts. The finding that the DNA origami and its nanoprobes are stable in cell lysates for at least 12 h is encouraging. To fully elucidate the mechanisms of this increased stability and further validate the potential of DNA–RNA nanostructures for *in vivo* usage, a real-time intracellular trafficking study and a cytotoxicity study will be needed.

Last but not least, the immunostimulatory effects of self-assembled nanoarchitectures need to be thoroughly studied. CpG (cytosine-phosphate-guanine) DNA has a long history of being used as an immunostimulatory compound which exhibits its biological activity through interaction with Toll-like receptor 9 [242]. For most of the self-assembled nucleic acid nanostructures, synthetic DNA and RNA molecules are being used, many of which bear CpG islands, which might render the structures highly immunostimulatory. In addition, several groups recently showed that Y-shaped DNA induced greater amounts of proinflammatory cytokines from RAW264.7 cells irregardless of their CpG contents [243,244]. Therefore, the size and geometry of self-assembled DNA–RNA structures might also play an important role in immunostimulation, which also requires further study. Nonetheless, this unusual immunopotency suggests us that DNA–RNA nanostructures can also be studied as potential immunoadjuvants. As these structures can be easily modified using cell-targeting ligands, and they have shown increased serum stability, they can be engineered to target specific tissues such as lymph nodes to achieve greater immunostimulatory effects. Alternatively, a DNA–RNA nanostructure-based carrier device can be designed for the on-demand release of CpG than free oligonucleotides.

6. CONCLUSIONS

In summary, although its full potential still remains to be explored, nucleic acid nanoengineering is nothing short of revolutionary. In the past three decades, we have stripped the DNA–RNA molecules from their preconceived biological roles and created astonishing one-, two- and three-dimensional nanostructures. These nanostructures can be further used to pattern biomolecules, NPs, nanotubes, transition metals and numerous other functional materials. The implications of being able to precisely manipulate the position and orientation of materials at the atomic level are enormous. It could potentially lead to a plethora of novel materials and products that can impact nanoelectronics, aerospace and nanomedicine. Along this avenue, delicate multi-component systems employing nucleic acid nanostructures have already been built which have intriguing applications ranging from biosensing to detection and to drug delivery. Integration of DNA–RNA aptamers and nanomechanical devices into these systems may further achieve targeted and dynamic

functionalities in response to biologically relevant signals. With collective efforts from scientists across disciplines, the field of nucleic acid nanoengineering is sure to flourish, leading to a new era in biomedical nanotechnology.

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