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Self-Assembled Nucleic Acid Nanostructures for Biomedical Applications

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Abstract: Structural DNA nanotechnology has been developed into a powerful method for creating self-assembled nanomaterials. Their compatibility with biosystems, nanoscale addressability, and programmable dynamic features make them appealing candidates for biomedical research. This review paper focuses on DNA self-assembly strategies and designer nanostructures with custom functions for biomedical applications. Specifically, we review the development of DNA self-assembly methods, from simple DNA motifs consisting of a few DNA strands to complex DNA architectures assembled by DNA origami. Three advantages are discussed using structural DNA nanotechnology for biomedical applications: (1) precise spatial control, (2) molding and guiding other biomolecules, and (3) using reconfigurable DNA nanodevices to overcome biomedical challenges. Finally, we discuss the challenges and opportunities of employing DNA nanotechnology for biomedical applications, emphasizing diverse assembly strategies to create a custom DNA nanostructure with desired functions.

Keywords: Nucleic acid nanotechnology, self-assembly, DNA Origami, reconfiguration, nanomaterials, biomedical application

1. INTRODUCTION

Ever since the rise of nanotechnology, scientists have been seeking materials that can be manipulated with nanoscale precision. Sources of inspiration for such purposes can be drawn from the abilities of cells to produce biomacromolecules with well-defined functions. For example, synthesizing and assembling biocompounds in an ordered manner is the basis for smooth-running complex bio-machinery and efficient regulation over cellular interactions. During these processes, the self-assembly of biomolecules happens continuously and plays an essential role. The major driven forces are the weak non-covalent bonds during self-assembly, including hydrogen bonds, electrostatic forces, hydrophobic interactions, etc. Examples of self-assembly process employed by nature include the hybridization of DNA strands to a helical DNA duplexes, the folding of peptide chains to a functional protein, and lipid membrane fusion between plasma membrane systems and etc.

Among examples above, the excellent candidates for building programmable assemblies and achieving molecular manipulation in nanoscale are oligonucleotides. Unlike their natural roles, DNA and RNA are used as programmable building blocks in nucleic acid nanotechnology, relying on the robust Watson−Crick base pairing rules (adenine (A) pairs thymine (T) (uracil, U in RNA) and cytosine (C) pairs guanine (G) base). The physicochemical properties of nucleic acid chains are also well studied. For instance, a double-stranded B-form DNA is a right-handed duplex with ~10.5 base pairs (~3.4 nm) per turn and a width of ~2 nm. These properties provide a solid foundation for constructing structures of almost arbitrary shapes at nanoscale resolutions. In the past four decades, structural DNA nanotechnology has created an ever-increasing number of nanoassemblies, including motifs, arrays, crystals, discrete 3D shapes, scaffolded DNA origami, supramolecular 3D shapes, and dynamic nanomachines and devices. Many of these nanostructures found their applications in nanorobots [1], material engineering [2, 3], biomedical research [4-6], and other applications [7, 8].

Compared with other nanomaterials, DNA nanotechnology has unique advantages that make it an outstanding candidate to solve biological and medical problems. First, DNA can be programmed to diverse shapes and sizes with nanometer precision, and thus offers a great pegboard for organizing vast types of materials with exact positions and orientations. This spatial addressability feature has been well demonstrated in novel design methods such as DNA origami, where hundreds of sequence-unique staple strands can be unambiguously identified and modified with different moieties on origami surfaces, including signal molecules, dyes, enzymes, and antibodies. With number-defined and address-defined functional species on DNA nanostructures, attractive applications can be achieved such as disease sensing and mechanisms study of biomolecular

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interactions. Second, DNA structures are ideal nano-scaffolds to guide the growth and modulate the fabrication of other materials. Benefiting from the programmability and biocompatibility of DNA, biomolecules, such as liposomes and proteins, can be synthesized and manipulated using DNA nano-templates, thus allowing studying of their synthesis processes, understanding their functional mechanisms, and regulating their activities for biomedical purposes. Third, the dynamic features of DNA nanostructures have enabled applications in 'smart' nanomachines and nanorobots such as switchable sensors, protein activity regulators, and targeted drug delivery vehicles for therapeutics. By incorporating Toehold-mediated strand displacement reactions, logic gate cascades, and aptamers, programmable switching or reconfiguration can be achieved and benefit the development of medical studies. In this review, we will discuss the development of design strategies and biomedical applications of DNA nanotechnology with an emphasis on integrating the three unique properties of DNA nanostructure to solve biomedical problems.

2. Fundamentals of Structural DNA Nanotechnology

2.1 DNA Tile Assembly

The field of structural DNA nanotechnology originated from the idea, proposed by Nadrian Seeman, of building 3D lattices with immobile Holliday junctions with sticky-ends [9]. Inspired by the flexible Holliday junction found in a biological recombination process, Seeman (1993) synthesized five types of DNA double-crossover motifs that consisted of two double-helical DNA strands connected by double-crossovers [10]. Depending on the directions of the two continuous helical strands flanking the ends of multi-arm junctions, the double-crossover (DX) motifs were classified into two categories, antiparallel motifs, referred to as DAX motifs, and parallel motifs known as DPX motifs nowadays. The sequences of the DNA double-crossover motifs were optimized to form immobile DNA four-arm (branched) junctions, which led to more rigid motifs than the Holliday junction motifs. Since then the rigid and robust DNA DX motifs have been used as fundamental building blocks for constructing DNA nanostructures.

In 1999, Winfree and coworkers reported the first periodic 2D DNA arrays using different sets of DX motifs as building blocks [11]. In 2003, Yan and coworkers managed to connect the central strands of four-arm DNA branched junctions to one strand inserted poly-T single-stranded loops to prevent base-stacking interactions between adjoining four-arm junctions, and thus successfully constructed a 4 by 4 tile with four branched arms [12]. Mao et al. extended the idea of multi-arm junctions and assembled three-dimensional supramolecular DNA polyhedral nanostructures [13]. In their design, three-point star DNA tiles connected by sticky ends served as universal units. By varying the length of T loops between multi-arm junctions and the concentration of the three-point star tiles, DNA tetrahedra, dodecahedra and buckyballs were formed based on four, twenty, and sixty tiles, respectively. The symmetrical assembly of the three-point star DNA motif simply needs three types of DNA strands accounting for the robustness and advantage of this design strategy. Multi-arm tile systems were then proved to be a useful tool for more complicated 2D assemblies. For instance, Zhang et al. reported the Archimedean tessellation in periodic polygonal patterns assembled by three-arm and four-arm DNA junctions, suggesting the possibility of establishing quasi-crystals or more complex tiling by DNA building blocks[14]. The 12-fold 2D quasicrystalline patterns were successfully formed by mixing 5-arm and 6-arm junction motifs with the help of a structural strut between adjacent arms[15]. Besides multi-arm tiles, 2D and 3D designer DNA architectures have been created by using single-stranded tile (SST) method, in which more than 10,000 unique short single strands self-assemble to custom 3D structures, suggested by Ke et al [16, 17].

Overall, tile assembly methods rely on exploiting robust DNA tiles as units and establishing matching rules encoded in sticky-end hybridization between tiles. Such assembly methods have provided a simple means to fabricate 2D and 3D DNA nanostructures to serve as templates for conjugation or deposition of other molecules (e.g., metal nanoparticles [18], proteins [19] and aptamers [20]).

2.2 DNA Origami

2.2.1 Single-layer DNA Origami

Besides the traditional tile assembly method, another approach to build DNA nanostructures was reported by Shih in 2004 [21]. In this new approach, a long strand was introduced into the structural design. A DNA octahedron was constructed by hybridizing a long single-stranded DNA (~1.7 kilobases) with 5 short DNA oligomers serving as body supports. Paranemic hybridization was employed to complete the polyhedral shapes. In 2006, Paul Rothemund presented the method of scaffolded DNA origami technology, in which a ~7.3 kilobase DNA strand from the M13 phage genome was employed as a scaffold with hundreds of short DNA oligomers as staples to build a variety of DNA nanostructures with different shapes and sizes [22] (Figure 1a). In this work, the target shapes were transformed into a cylinder view, then into duplex DNA patterns with periodic crossovers. Staple strands were generally 20 to 60 nucleotides in length between crossover sites and traveled through multiple scaffold DNA cylinders. The scaffold strand was converted into antiparallel helices during the annealing process and formed designed shapes.

The key to the success of scaffolded DNA origami nanotechnology is the employment of a long circular scaffold strand with short staples. The reduction of entropy in the assembly process enables the high quality and quantity production of the designed DNA structure without the requirements for precise stoichiometry control and tedious sequence design. Another factor leading to the popularity of this method is the availability of materials and software. With the commercialized solid-phase synthesis of staple DNA and the polymerase replication of genome sequences for scaffolds, materials for DNA origami synthesis become easily obtainable. The development of design programs (e.g., caDNAno) have facilitated the design process in creating DNA nanostructures without the need for high expertise in DNA nanotechnology and enabled the wide adoption of this user-friendly technique by researchers with various backgrounds. With all these benefits, DNA origami nanotechnology stimulates the field of biomedical science. It provides potential materials made up of DNA origami

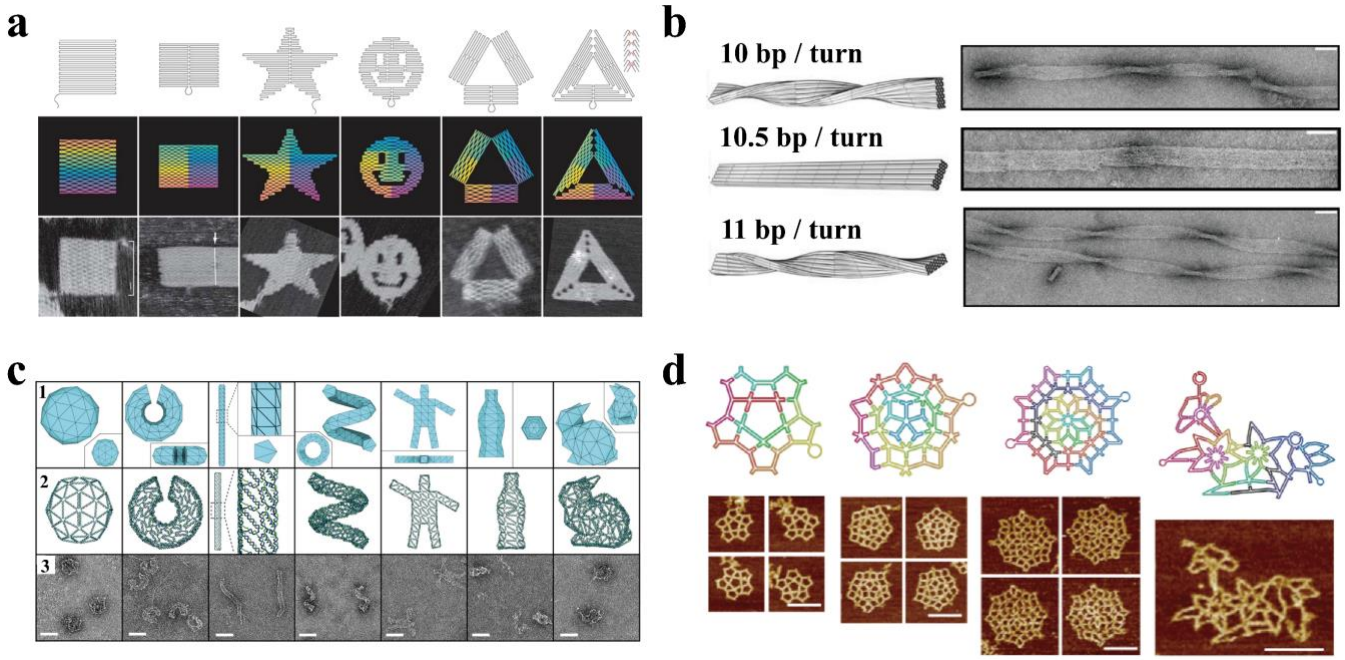

Figure 1. Single layer, multilayer and wireframe DNA Origami. **(a)** Shapes and patterns assembled by Scaffold DNA Origami. Reproduced with permission from [22]. Copyright 2006 Springer Nature. **(b)** Twists and curvature on a 10-by-6-helix DNA origami bundle achieved by deleting or inserting bases in a DNA turn. Scale bar: 20 nm. Reproduced with permission from [29]. Copyright 2009 AAAS. **(c)** Multiple 3D wireframe DNA origami mesh objects assembled by routing one DNA duplex in most edges. Scale bar: 50 nm. Reproduced with permission from [30]. Copyright 2015 Springer Nature. **(d)** Wireframe DNA origami employing multi-arm junctions as vertices and two DNA duplexes as edges for the construction of complex wireframe shapes. Scale bar: 100 nm. Reproduced with permission from [31]. Copyright 2015 Springer Nature.

structures with customizable shapes and precise spatial control features that are crucial for both making biosensors and drug carriers and for performing biointeraction studies.

2.2.2 Multi-helix Bundle DNA Origami

Although diverse geometric DNA nanostructures have been achieved by Rothemund's scheme [23-25], the construction of 3D objects with structural complicity and enhanced mechanical properties remains challenging. To make a compact 3D DNA structure, the scaffold routing and staple crossing should be rationalized with an advanced tool. CaDNAno software has become widely used in DNA nanotechnology to produce scaffold routing with appropriate staple crossover sites [26]. In the honeycomb lattice of the caDNAno program, crossovers are allowed to be built every 7 bps, resulting in a 240^o angle (B-form DNA duplex has 21 bps in 2 turns) between two neighboring helices connected by crossovers. The scaffold helices are then rolled up by staples in a planer arrangement to a compact honeycomb shape with multilayers. Douglas and coworkers first reported 3D DNA origami structures as well as the design program caDNAno [27]. Six 3D structures were achieved, and an icosahedron with six-helix DNA bundle edges was built through sticky-end connections of three DNA origami isomers. In addition, Ke et al. reported a square-lattice layout caDNAno software version by allowing crossovers to be built every 8 bps (-90^o rotation to the adjacent helix) [28]. The complexity of DNA origami multihelix bundles was advanced by programming curvatures and enabling twist of the DNA bundles by Dietz et al [29] (Figure 1b). Curvatures of DNA origami bundles were tuned by systematically inserted or deleted nucleotides between crossovers in each helix. Both DNA structures with bending angles ranging from 30^o to 180^o and intricate nonlinear shapes were folded following this design principle.

2.2.3 Wireframe DNA Origami

A DNA wireframe shape can be built with DNA motifs representing edges and vertexes. Unlike DNA origami based on parallel alignments or packed arrangement of DNA helices, robust DNA motifs such as DNA multi-arm junctions and DX motifs, are employed as buiding blocks for the construction of a wireframe shape. For example, various 2D and 3D wireframe structures were illustrated by Han et al. with a gridiron pattern by four-arm junctions [32]. Moreover, Benson and coworkers reported a method that rendered the most of DNA polyhedral mesh edges in DNA duplexes [30] (Figure 1c). By adopting an A-trail routing algorithm based on a graph theory, they provided a highly automated paradigm for transforming the target structure into digital DNA meshes. In the same year, the strategy of using double-crossover tiles as edges was reported to construct wireframe DNA origami objects with reconfigurable ability and relatively high rigidity [31] (Figure 1d). In this work, the edges of DNA wireframe shapes consisted of two DNA duplexes, which were combined with antiparallel crossovers, and contained a scaffold strand travelling through all edges inside the shape. Each vertex was designed with controllable angles by adjusting the length of T_n loop and the unpaired scaffold within the vertex junctions. This strategy allowed the successful construction of highly complex DNA wireframe origami such as a bird-flower painting, curvilinear arrays, and a 3D snub cube, demonstrating the versatility of this strategy. However, the optimization of both sequence and routing pathways for wireframe shapes was time-consuming, thus limiting the use of wireframe shapes in many applications. To facilitate a

wider adoption of this design technique, automated design algorithms were reported, including DAEDALUS [33] and PERDIX [34], assisting the automation of scaffold routing in a top-down manner. By inputting the target wireframe shapes, design programs render the closed surface representation of the wireframe shapes and provide the sequence information needed for the shapes. Compared with the close-packed DNA nanostructures that require high cation concentrations to fold, the wireframe nanostructures generally exhibit higher stability in physiological solutions such as phosphate buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM).

2.2.4 Single-stranded Origami

Besides the scaffolded DNA origami systems, the unimolecular self-assembly—the way most biomacromolecules fold into higher orders in nature—has been achieved and optimized to create new shapes of DNA and RNA origami nanostructures. By applying the design rule of DNA (B-form) DX motifs to create RNA (A-form) motifs, Cody et al. reported an approach to building single-stranded RNA architectures [35] (Figure 2a). Compared to DNA, RNA has many unique structural motifs (kissing loops, junctions, RNA aptamers, etc.) and can be co-transcriptionally assembled in cells. A variety of single-stranded RNA antiparallel DX motifs were achieved via both annealing and co-transcriptional folding. These RNA DX tiles were intramolecularly connected by RNA kissing loops, the tertiary structures of RNA found in HIV genome and cells [36], to accomplish the single-stranded routing. Additionally, the kissing loops also replaced the role of sticky ends to form connections between the single-stranded RNA DX tiles. RNA arrays were successfully assembled, and three assembly protocols including mica anneal, co-transcription, and transcription on mica were demonstrated using this assembly method. In addition, another single-stranded assembly approach was reported for creating large DNA and RNA origami structures with thousands of nucleotides [37] (Figure 2b). Instead of antiparallel crossovers used in DNA origami systems, parallel crossovers were adopted in a single-stranded assembly system to fold into unknotted nanostructures with DNA up to ~10000 nt and RNA up to ~6000 nt. The nucleic acid strands for the unknotted nanostructures were produced by both in vitro transcription with enzymes and replication in living cells. One year later, the same authors demonstrated a knotted strategy to construct single-stranded DNA and RNA nanostructures [38]. The routing pathway for their nanostructures was elaborately designed and optimized to overcome topological difficulties within the knotted structure. By adopting this knotted strategy, they successfully constructed both knotted nucleic acid nanostructures with up to 57 crossings and multiple 3D knotted wireframe objects.

2.2.5 Hierarchical Assembly of DNA Origami

The design space and potential applications of DNA origami nanotechnology are largely determined by the size of the assembled structure. Although there have been many intricate 2D and 3D objects produced by DNA origami nanotechnology, the sizes of the objects are generally limited by the length of scaffolds, usually 7249-nt M13mp18, a single-stranded, circular DNA vector from a bacteriophage. Both artificially synthesizing a long scaffold by bioengineering methods [39, 40] and performing the hierarchical assembly of DNA origami tiles in a bottom-up approach play key roles in scaling up the size of DNA nanostructures. By connecting DNA origami tiles using sticky end hybridization connection or base stacking interactions, a variety of DNA origami structures were created with their sizes scaled up. In 2010, Seeman's group reported DNA arrays generated by a double-layer DNA origami tile with sticky ends [41]. This tile had two orthogonal domains with parallelly aligned DNA helices and underwent self-assembly into two-dimensional DNA arrays with edges in dimensions of 2-3 μm. This robust tile with DNA helices arranged side by side was preferred for their base stacking and inspired later scaling up design strategies based on base stacking. The hierarchical assembly of 3D DNA objects was also practicable with DNA sticky end hybridization. Ke et al. designed the DNA multihelix bundle tripods and assembled DNA polyhedral nanostructures with molecular weight up to 60 megadaltons [42]. To support such large objects, sixteen-helix bundles with high stiffness were employed as building blocks, and a strut consisting of DNA double helices was adopted to maintain the angles between each arm. Multiple sticky ends were hybridized at the ends of each arm, ensuring the monomer connection.

Similar to the sticky-end connections for DNA origami monomers, the blunt-end base-stacking interactions were also adopted to assist in the hierarchical assembly process. In the original paper on DNA origami [22], most of the synthesized origami shapes suffered from uncontrolled base-stacking interactions until poly-T tails were added at both ends of DNA helices. DNA helices with blunt ends provided sufficient energy for origami stacking in a parallel alignment as a single layer origami or parallelly packed in a multilayer origami. This unfavored base-stacking interaction was later found to be useful for programming origami-origami interactions. For example, by programming the edges into binary sequences of both blunt ends and scaffold loops, Woo and Rothemund were able to achieve the sequence recognition assembly of DNA origami rectangles with different patterns [43]. Inspired by this and the orthogonal DNA origami tiles, Tikhomirov et al. developed a square DNA origami tile with four symmetric triangles zipped by bridge staples and performed a random tiling array. Random loops, mazes, and trees were programmed on the surface of the DNA origami array in a micrometer size [44]. This concept was further extended to the arbitrary pattern assembly of DNA origami arrays. Tikhomirove et al. designed groups of tile building blocks with specific blunt stacking end patterns to control the stacking order between these tiles [45, 46] (Figure 2c). The authors constructed a DNA origami array with 8x8 unique tiles and up to 8704 pixels enabling the rendering of drawings such as the Mona Lisa. The employment of an artificial arm robot for strand mixing largely improved the assembly efficiency. In addition, the base stacking assembly of multilayer DNA origami was achieved by Gerling et al [47]. The authors also built a controllable 3D DNA dynamic device via shape-complementary base-stacking interactions inside of a multilayered DNA origami. Following this, Wagenbauer and coworkers demonstrated the hierarchical assembly of giga-dalton sized DNA structures by shape complementary base stacking [48] (Figure 2d). This method exploited the modularity of building blocks during assembly and enabled the self-assembly of structures approaching viral size.

3 Using DNA Nanostructure to Address Biomedical Challenges

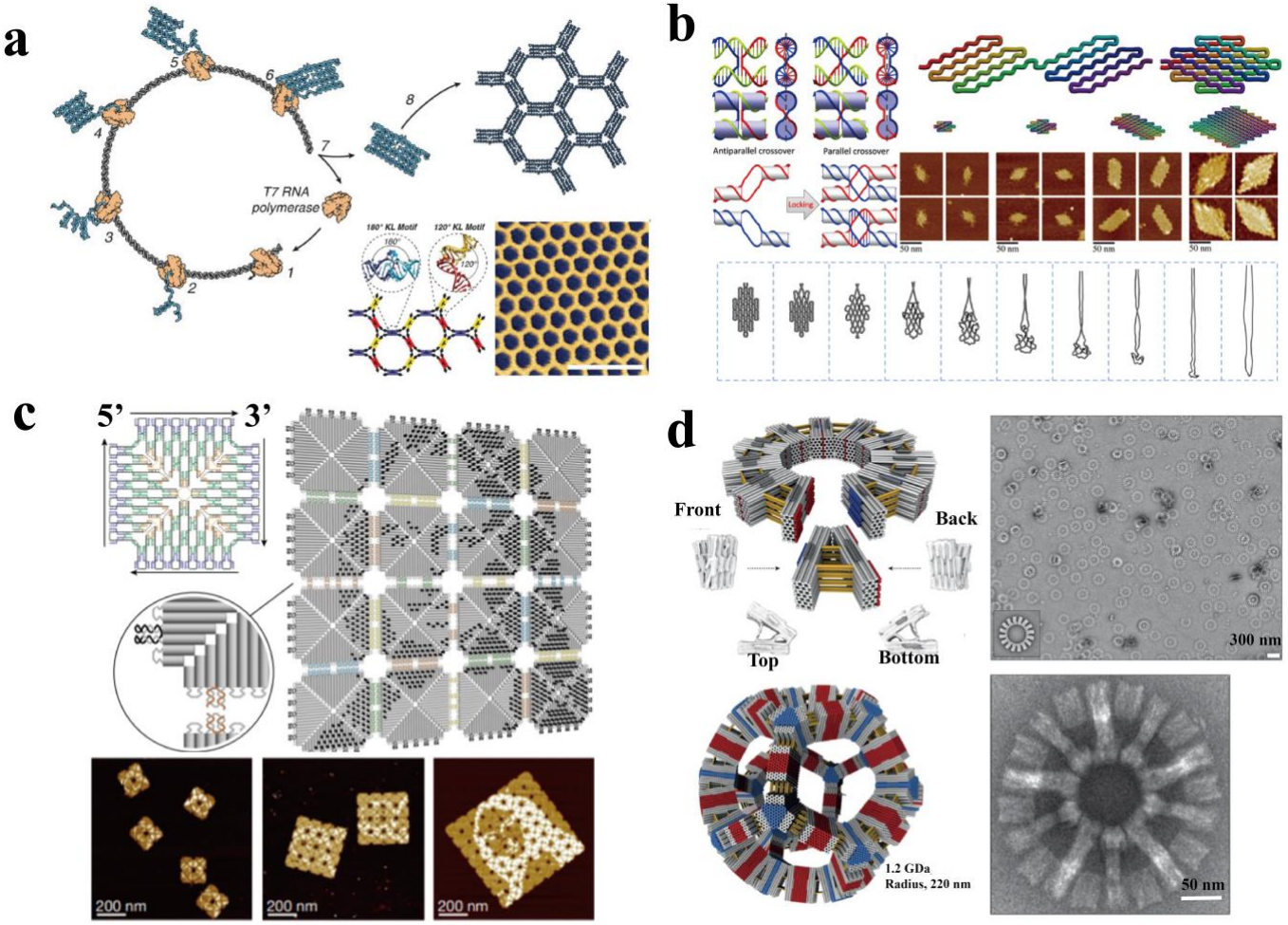

Figure 2. Single-strand Origami and hierarchical assembly of DNA Origami. **(a)** Co-transcriptional folding of single-stranded RNA Origami and the growth of single-stranded RNA origami array. 120° and 180° kissing loops were adopted in both single-stranded RNA origami routing and connecting RNA origami tiles on arrays. Reproduced with permission from [35]. Scale bar: 100 nm. Copyright 2014 AAAS. **(b)** Single strand DNA and RNA Origami based on parallel crossover. In this case, the knotting number of single-strand DNA/RNA routing is zero, accounting for the robust assembly of DNA nanostructures. Reproduced with permission from [37]. Copyright 2017 AAAS. **(c)** Mona Lisa assembled from 8-by-8 identical square DNA origami tiles. In the routing diagram, the square DNA origami tile has blunt ends aligned to four directions (on the edges) and possesses an addressable recognition pattern on each tile, thus enabling hierarchical origami tile assembly for arbitrary patterns. Reproduced with permission from [45]. Copyright 2017 Springer Nature. **(d)** Building giga-Dalton self-limiting DNA nanoarchitectures with shape-complementary DNA origami monomers assembled via blunt end base stacking. Reproduced with permission from [48]. Copyright 2017 Springer Nature.

DNA has been proved to be a powerful engineering material with reconfiguration ability and spatial addressability after rapid development. Those incredible features have made it an excellent candidate as a versatile nanoplatform to meet the urgent needs of biomedical research. In this section, we will discuss the diverse applications of structural DNA nanotechnology in biomedical fields, focusing on structural design methodologies and related contributions to respected applications.

3.1 Controlling Spatial Parameters in Biomedical Study Using Nucleic Acid Assemblies

3.1.1 Using DNA Tiles and DNA Origami to Control Spatial Parameters

An essential factor for biological and chemical studies is the spatial distribution of molecules. For example, the numbers, distance, and orientation between substrate and catalyst significantly influence the kinetics or products of a reaction. The same idea can be applied to a living system as well. Multiple receptors on the cell surface function together to recognize external substances and determine subsequent cellular behaviors. These receptors are subtle, and any minor change in distance, orientation, or number may result in the weakening of interactions or even leading to the activation of cell apoptosis. Therefore, the spatial factors should be considered and systemically studied to answer biomedical questions. With the rapid growth of structural DNA nanotechnology, manipulating molecules or proteins with nanoscale resolution becomes attainable. DNA structures with custom shapes serve as structural scaffolds with multiple precisely designed binding sites. Biomolecules can be integrated with DNA structures at these sites by conjugating a short DNA strand that is non-covalently hybridized with the main structure, or by forming covalent bonds between functional groups at terminals [49]. Given the predictable structure of DNA duplexes based on Watson-Crick base pairing rules, molecules can be anchored in the same orientation every 10.45 nm (length of a full turn) in a DNA

duplex. While in a parallelly arranged DNA multihelix bundle, the distance between two units anchored at the same end of neighboring helices can be controlled as ~2 nm. In 2008, Yan et al. reported an approach to study the multi-valency binding of thrombin (a coagulation protein involved in blood clotting) by varying the distance between aptamers on multi-helix DNA tile arrays [50] (Figure 3a). An aptamer is a short single-stranded DNA or RNA oligomer selected by systematic evolution of ligands by exponential enrichment (SELEX) with binding affinity specific to target molecules [51, 52]. DNA nanostructures can be conveniently modified with an aptamer because the aptamer sequences can be introduced to DNA architecture by encoding the sequence in its building strands such as stable strands. In Yan's work, two aptamers for two different epitopes of thrombin were displayed on a five-helix bundle with the separated distance of 2, 3.5, 5.3, and 6.9 nm. The optimal distance for multi-aptamer and thrombin binding was validated to be 5.3 nm in this arrangement, where shorter and longer distances reduced the binding affinity. This is the first example of using DNA nanostructures to demonstrate the impact of aptamer spatial distribution on aptamer binding affinity. However, for monovalent aptamers selected by the traditional SELEX method, the binding affinity is typically modest with K_d values in the μM range. In another work, the same research group developed a novel strategy to generate bivalent aptamers in a structure-assisted SELEX process and discovered a bivalent aptamer pair with femtomolar K_d values and potent anticoagulant activity using a two-helix DNA tile [53] (Figure 3b). The DNA tile had a pair of loop regions at the end of each helix for aptamer screening and defined the distance and orientation of the bivalent aptamer. The selected bivalent aptamer pair, ThAD, bound to a different site of the substrate and suppressed the coagulation cascade for about 1 hr in human plasma with a concentration of 5 nM against 5 nM thrombin. Apart from aptamers, glucose oxidase (GOx)/horseradish peroxidase (HRP) enzyme cascade [54], myosin movements on actin filaments [55], and ephrin ligand-receptor functions [56] were also investigated and reported by different research teams using specifically designed DNA structures.

The spatial addressability of structural DNA nanotechnology can be applied to studies not only on individual proteins or ligands in test tubes but also on cancer cells and infectious pathogen surfaces. The surface of cells and viruses presents a great number and range of character antigens with certain distribution patterns. The binding efficiency of ligands towards these antigens largely depends on matching levels to the patterns. Multivalent arrangement of ligands mimicking the global distribution of receptors increases the recognition efficacy. Wang and coworkers suggested a DNA architecture with multivalent recognition mimicking the dengue (DENV) virus surface protein patterns [57] (Figure 3c). According to the reported dengue envelop protein domain III (ED3) cluster distribution on viral surface, the authors designed a star-shaped DNA template displaying ten aptamers for multivalent pattern matching recognition and five fluorescent molecular beacon motifs for signal outputting. It is worth mentioning that the DNA device was flexible enough to perform dynamic reconfiguration upon aptamer-protein binding, triggering the fluorescent beacon. Multiple designs of DNA architecture, including bivalent, linear, and hexagon shapes, were compared and ended up with a lower targeting ability in the lack of proper antigen pattern matching. The authors then described the viral inhibition by confocal microscopy and antiviral plaque-forming half-maximum effective concentration (EC_{50}) assays. This method was summarized as a customized molecular platform with several design principles for different types of ligands. Among the protein patterns in plasma membranes, the death-inducing receptors were widely studied as well. Several works suggested identical DNA structures decorated with the death receptor ligand Fasl [58] and necrosis-related ligand mimicking peptides TRAIL [59]. Both observed a high apoptosis rate by mimicking the hexagonal geometries of receptors within 10 nm spacing.

The hieratical assembly of DNA origami assists the process of trapping some dangerous pathogens including viruses. Based on the previous breakthrough of megadalton DNA assemblies [47, 48], Dietz group proposed the DNA icosahedral shell system for antivirus purposes recently [60] (Figure 3d). The overall architectures were constructed with base stacking interactions between modular units, pseudosymmetric triangular DNA origami structures. These stacking units were divided and assigned to individual shape-complementary patterns to assemble various shell objects with user-defined geometries and apertures. After the assembly steps were optimized and characterized, 90 antibodies against hepatitis B virus (HBV) core particles were conjugated inside a half-shell DNA nanostructure, and virus trapping was observed by both preassembly method and postassembly from the virus surface method. The cytotoxicity of half-shell structures was quantified by cell viability following the incubation of HEK293T cells (human embryonic kidney cell line) with half-shell mixtures. No effect was found across any of the concentrations used in this study. The authors then illustrated the virus neutralization effect of the DNA half icosahedron using green fluorescent protein-encoded adeno-associated virus serotype 2(AAV2) virions. A step of UV point linking and poly(ethylene glycol) (PEG)–oligolysine/oligolysine modification to enhance the stability in physiological solutions was carried out. The cell infection rate of DNA shell neutralizing group, quantified from GFP fluorescent signals, was significantly lower than that of the virus only and free antibody group, indicating the potent antivirus activity of this DNA assembly.

3.1.2 Manipulating Spatial Parameters Using RNA Assemblies In addition to DNA, RNA nanostructures have also been proved to be useful tools for biomolecular interaction studies. Unlike DNA, a stable genetic information carrier, a bunch of natural existing functional RNA motifs and dynamic RNA structures (e.g., kissing loops) vastly enrich the diversity and functionality of structural RNA technology [61]. RNA aptamers are short RNA oligomers that can fold into certain tertiary structures to recognize target molecules. Many aptamers were selected to have specific functions to their targets such as stimulation and inhibition [52, 62]. LaBean and coworkers described a functional RNA origami structure bearing multiple thrombin-binding RNA aptamers and possessing high anticoagulant activity with high stability for a long period of time [63] (Figure 3e). A family of two-helix RNA origami with two RNA aptamers appended at different distances and orientations were established. The stability of the RNA anticoagulant structure was improved by incorporation with 2'-fluoro-modified C- and U-nucleotides on RNA scaffolds. The anticoagulant activity was sevenfold

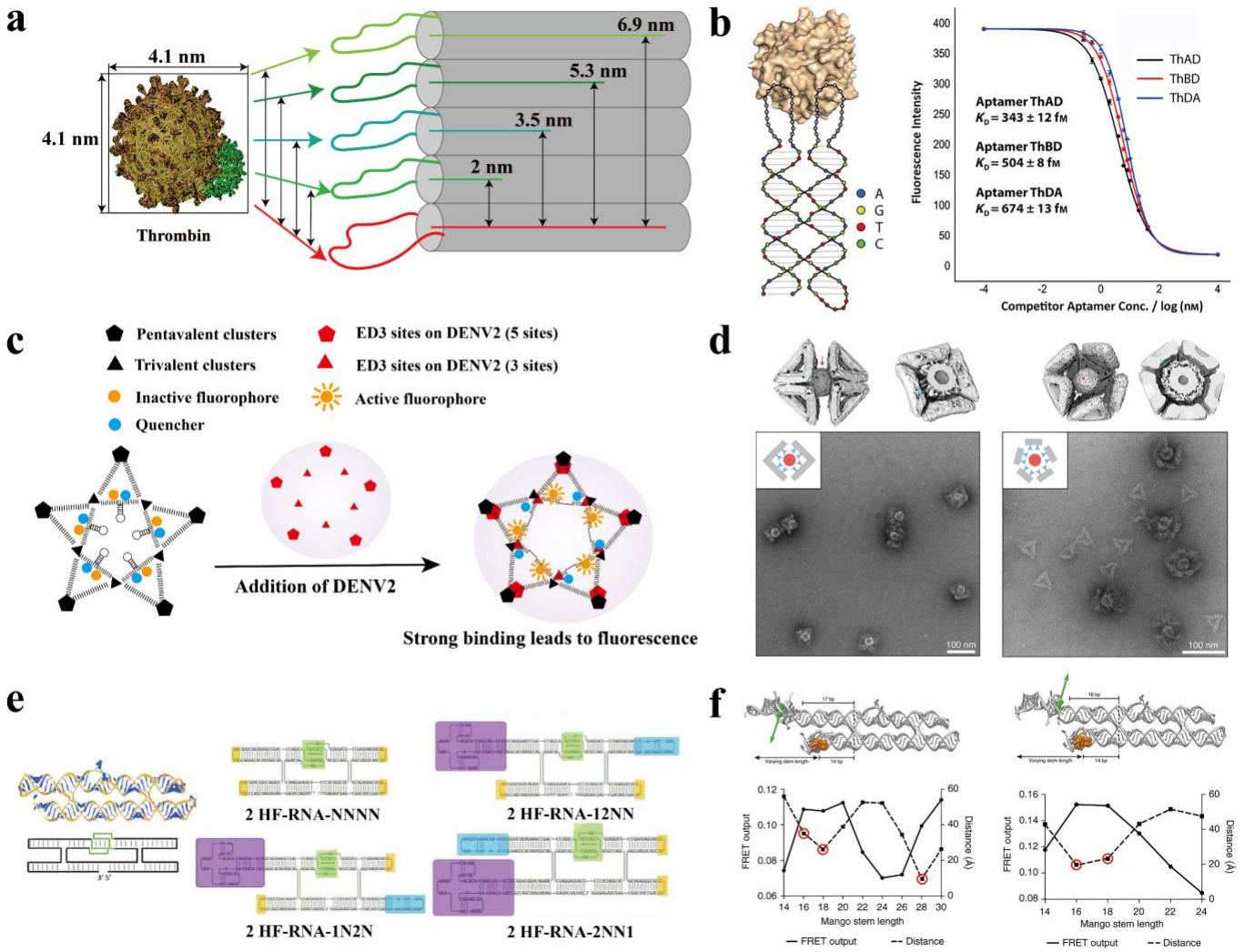

Figure 3. Controlling spatial parameters in biomedical studies. **(a)** Varying the distance between two thrombin DNA aptamers on multihelix DNA tiles to study the multi-valency binding between thrombin and aptamer. Reproduced with permission from [50]. **(b)** Bivalent aptamer SELEX process for compatible and high anticoagulant activity thrombin aptamer pairs screening using two-helix DNA tile. Reproduced with permission from [53]. Copyright 2019 Wiley-VCH Verlag GmbH & Co. KGaA. **(c)** Controlling aptamer patterns on DNA templates to match the dengue virus surface antigen distribution for virus targeting and antiviral study. Reproduced with permission from [57]. **(d)** Trapping and neutralizing the virus with antibody incorporated DNA icosahedral nanoshells assembled by base stacking of modular pseudosymmetric triangular DNA origami units. Reproduced with permission from [60]. Copyright 2021 Springer Nature. **(e)** A genetically encoded functional bivalent anticoagulant composed of thrombin RNA aptamers and single-strand RNA origami with 2'-fluoro-modified C- and U-nucleotides. Reproduced with permission from [63]. Copyright 2019 Wiley-VCH Verlag GmbH & Co. KGaA. **(f)** Varying the distance between two florescent RNA aptamers to control the Förster resonance energy transfer (FRET) effect of the genetically encodable FRET nanodevice. Reproduced with permission from [66]. Copyright 2018 Springer Nature.

greater than the free aptamer and could be maintained at least 90 days with storage in buffer at 4 ºC. The ligand-protein domain can be replaced by other interactions of interest, enabling the study of their spatial-dependent features. Especially, the conformation-sensitive Förster resonance energy transfer (FRET) effect [64] has been investigated based on the precise distance control of RNA/DNA origami [65]. Mette et al. developed an RNA origami FRET system using a fluorescent RNA aptamer, a structured RNA molecule binding a fluorophore and switching on the fluorescence [66] (Figure 3f). The aptamer Spinach and Mango were appended on the same side of a two-helix RNA origami where the distance was determined by the nucleotide numbers of the stem structures from the crossover. The dipole moment orientation of the fluorophore bound to Spinach could be reversed by extending the stem from 16 bp to 17 bp. The conformational changes when tuning the length of the Mango stem were then transferred to FRET signals. An RNA invader strand causing a branched kissing loop and a SAM riboswitch responding to S-adenosylmethionine (SAM) were also demonstrated by this RNA origami FRET device. The RNA device was then genetically encoded and co-transcriptionally folded in the cells, indicating a promising real-time ratiometric sensor in vivo.

3.2 Molding and Guiding Biomolecules with DNA Scaffolds

Molding the shape and size of synthesized materials in a controllable manner is challenging yet appealing because of

its diverse applications. As a programmable tool, nucleic acid nanotechnology assists the synthesis and processing of a wide range of non-DNA materials from inorganic materials, organic molecules, to proteins and lipids. Liedl and colleagues used a set of DNA structures to study the gold cluster metallization on DNA origami [67]. In 2014, Yin and Seidel developed a DNA origami barrel as a mold to host gold nanoparticle seeds in its cavity for gold nanomaterial casting [68, 69]. For the molding of organic materials, Gothelf and coworkers synthesized DNA conjugated polymers following the designed route on DNA origami [70]. A comprehensive discussion of this topic can be found in a recent review paper reported by Weil, Schubert and coworkers [71].

3.2.1 Biomembrane Formation and Regulation by DNA Nanostructure

The top priority of most living systems is the ability to program the structure and conformation of essential biomacromolecules such as lipids and proteins. The correct function of these macromolecules determines the operation of fundamental cellular activities such as cell respiration and proliferation. With structural DNA nanotechnology, researchers can program and guide the assembly of these essential fundamental biomacromolecules, creating exciting opportunities in biomedical studies. Liposomes are excellent models for studying the dynamic plasma membrane as well as promising platforms for medicine delivery. However, the homogeneity of liposomes produced by the conventional extrusion method is typically poor, limiting its quality and efficiency as a delivery platform. By developing a DNA nanoring template, Yang and coworkers suggested an approach to produce liposomes in precise nanometer size and investigated the assembly mechanisms of DNA templated liposomes [72] (Figure 4a). With a DNA origami ring template, the liposome was nucleated and confined to the DNA template, generating homogeneous products inside the template. Four distinct sized liposomes were produced with nanoscale precision as well as high monodispersity. The liposome assembly process was further investigated by varying the number and angle of lipid seeds and monitoring the product during dialysis. The fluid plasma membrane performs crucial cell functions such as exocytosis and endocytosis. However, the manipulation of such dynamic features is hard to achieve. Inspired by the DNA nanoring template, researchers in Lin's group described a reconfigurable modular DNA cage for modeling the assembly and arrangement of liposomes both statically and dynamically [73] (Figure 4b). The authors designed a DNA nanocage made from two rings with overhangs for lipid anchoring and four DNA four-helix bundle pillars for height control. The nanocages could further be stacked for higher-order assembly by blunt end base stacking, and the length of each pillar was controllable by adding or deleting base pairs. This nanocage served as a modular unit for fabricating discrete, fused, curved, and even helical liposomes. By employing toehold mediated SDR, the authors managed to reshape the DNA cages, thereby fusing or bending the liposomes assembled in these units. The presented method of producing curved lipid bilayer and programmed membrane fusion was predictable and controllable, offering the opportunity for the systematic study of dynamic lipid bilayers.

Zhao et al. adopted the same approach [74] to assemble the membrane proteins and study the early viral entry into DNA templated liposomes (Figure 4c). Lipid bilayers were synthesized with DNA nanocorralled templates. Two membrane protein clusters, human Voltage-Dependent Anion Channel 1 (hVDAC-1) and the Rhodobacter sphaeroides photosynthetic reaction center protein (RC), were incorporated into the lipid bilayers during synthesis. The authors observed that poliovirus tethered to the model membrane and created pores in the membrane facilitated by the decoration of CD155, a poliovirus receptor inducing infection. Besides the synthesis and molding of liposomes, researchers also used DNA origami as scaffolds to sculpt and regulate the lipid membrane. Franquelim et al. designed a set of DNA origami with distinct curvatures (form linear to half circle) and validated their performance in binding and manipulating the transformation of lipid membrane [75] (Figure 4d). The origami structures mimicked the function and assembly of BAR protein, a 'banana-like' shape protein family aiding scaffolding and tubulation of the membrane. Outwards tubular deformation of the membrane, similar to the reports about several F-BAR proteins, was observed on the liposome with quarter curvature DNA origami. Three requirements for membrane deformation in terms of scaffold elements were summarized: affinity to membranes, curvature, and certain surface density.

3.2.2 Proteins Regulation and Assembly Guided by DNA Origami

Apart from liposomes, the assembly of proteins on DNA nanostructures has also been widely studied. To organize the assembly of tubular amyloid fibrils, a type of protein aggregate related to Alzheimer's, Udomprasert et al. designed a DNA origami tube grafted with amyloid peptide appendant inside to nucleate the amyloid fibrils into tubular shapes [76]. The studies of nuclear pore protein properties aided by DNA nanoring were published by two cooperation teams—the Lusk and Lin team and the Dekker and Dietz team—at almost the same time in 2018 [77, 78] (Figure 4e). Both probed the functions of FG-nups domains—disordered repeat protein domains controlling the permeability of the central transport channels in the cell nuclei—by housing them at a specific position on DNA origami nanoring with tunable protein density and topology. The transient occlusion of FG-nups proteins was observed and was found to be dependent on the chemical composition and their geometry on the nuclear pores (DNA-ring mimics). Tobacco mosaic virus (TMV) coating proteins assembled on DNA origami were another example of organizing protein assembly by DNA nanostructure. Zhou et al. described that the assembly of tobacco mosaic virus (TMV) coat proteins could be controlled on DNA origami [79]. Rather than anchoring the protein directly on DNA origami, a TMV genome-mimicking RNA, which could translate to TMV capsid protein around the strand, was hybridized to DNA staples at the chosen regions. Anisotropic DNA-protein hybrids could be generated with this method, indicating the capability of DNA structure as molding scaffolds. Moreover, Zhou and coworkers reported an approach to dynamically program the TMV capsid protein assembly route following the preassigned routing pathway recently [80] (Figure 4f). The translation process of genome mimicking RNA was hindered by DNA hybridization along the path of DNA origami and could be activated by removing the DNA strands by toehold

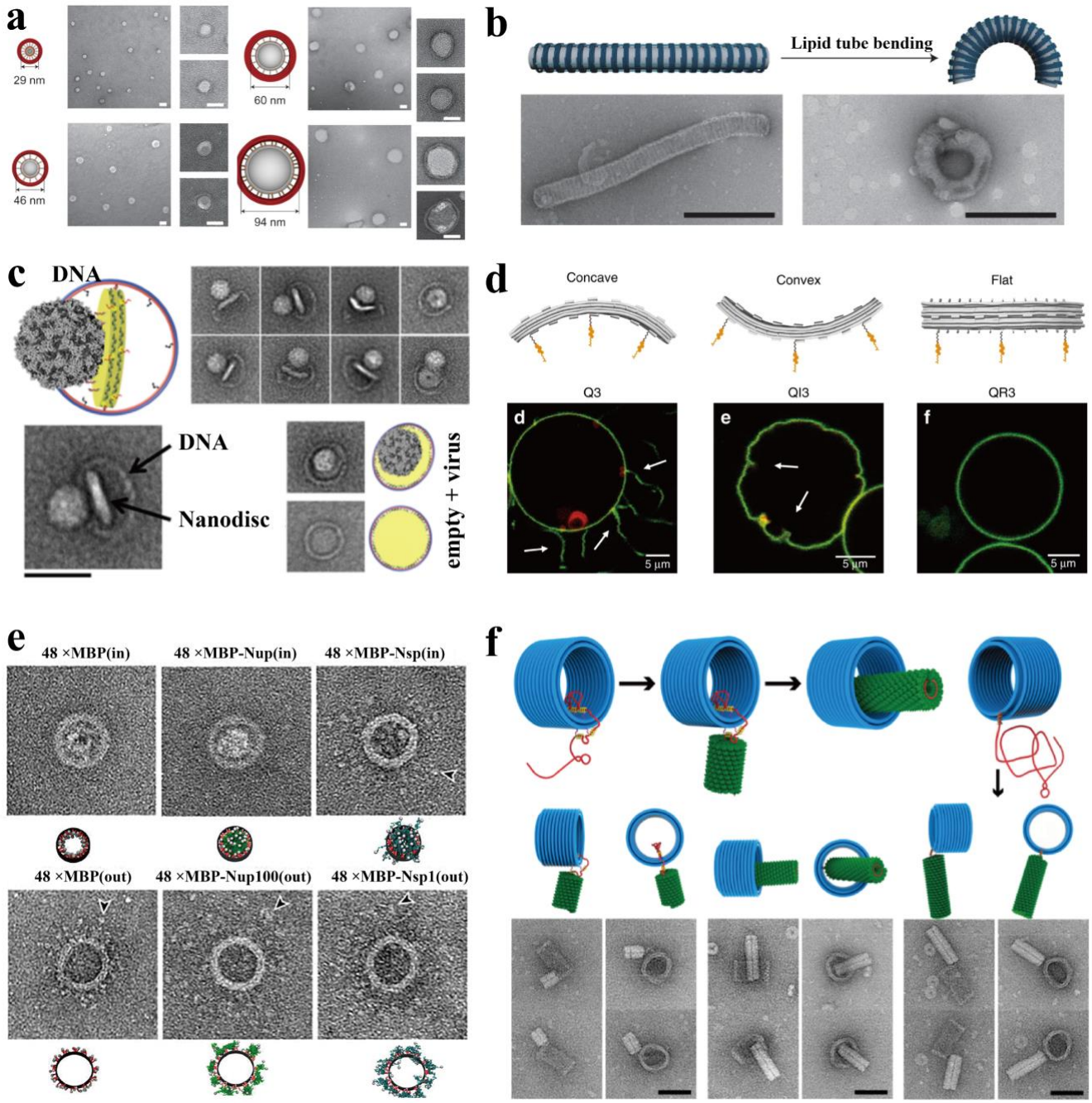

Figure 4. Molding and programming biomolecules with DNA scaffolds. **(a)** Templating liposomes with precise size control using DNA origami nanorings. Reproduced with permission from [72]. Copyright 2016 Springer Nature. **(b)** Programming liposomes with reconfigurable modular DNA nanocages. The reconfiguration of DNA cages is driven by toehold mediated SDR. Discrete, fused, curved and helical liposomes are fabricated. Scale bar: 50 nm. Reproduced with permission from [73]. Copyright 2006 Springer Nature. **(c)** Molding lipid bilayers by DNA nanodiscs for membrane protein clustering and viral entry study. Scale bar: 50 nm. Reproduced with permission from [74]. Copyright 2018 American Chemical Society. **(d)** Sculpturing membrane with curved DNA origami scaffolds. Tubular deformation of the membrane was observed upon incubating with quarter-curved DNA origami. Reproduced with permission from [75]. Copyright 2018 Springer Nature. **(e)** Controlling the geometry of nuclear pore proteins by nuclear pore-mimicking DNA nanoring confinement to study the permeability of the nuclear pore proteins. Scale bar: 50 nm. Reproduced with permission from [77]. Copyright 2018 American Chemical Society. **(f)** Programing the dynamics of virus capsid assembly on DNA origami by unfastening and allowing the transcription of RNA origin of assembly sequence (OAS) hybridized to DNA origami. Scale bar: 50 nm. Reproduced with permission from [80]. Copyright 2020 American Chemical Society.

mediated SDR. The authors also designed a DNA barrel to demonstrate that the translation and extension of TMV proteins on DNA barrels could be preprogrammed and well-controlled.

3.3 Using Reconfigurable DNA Nanodevice to Overcome Biomedical Challenges

The ability of a nanodevice to change its configuration in a controllable fashion allows researchers to advance medical applications such as multivalent molecular recognition,

conformational-based sensing, and conditional drug release [81-83]. Thanks to the base pairing rules, the configuration of DNA is easy to be predicted and programmed. After understanding DNA chemical and physical characters, multiple approaches to program dynamics on DNA have beenproposed, including toehold mediated strand displacement (SDR) [84], toehold exchange [85], enzyme mediated transformation [86], aptamer tertiary structure transformation, and etc. Among them, the most widely used method is SDR. In an SDR, a template strand is partially hybridized with a short strand, leaving a piece of unpair region at one end, also known as a toehold. An invader strand then attacks the partially hybridized template DNA from the toehold and replaces the weak complementary strand by strand migration in a short period of time. The outcome of this process is the formation of a longer and more stable template DNA duplex and a short DNA oligomer output.

3.3.1 Studying Biomolecules with Switchable DNA Devices

Prior to DNA origami technique, small dynamic nanostructures have been demonstrated such as DNA walkers and molecular tweezers [87, 88]. Peng and coworkers suggested a programable DNA assembly pathway and verified them with a bipedal walker [89]. An earlier example was the DNA mechanical device developed by Yan et al. based on PX and JX2 tile transformation achieved by SDR [90]. Recently, Ke et al. reported reconfigurable DNA 'domino' nanoarrays powered by DNA input and used the array as an information relay to transfer input signals [91]. To The scaffold DNA origami expands DNA assembly methodology and enables scientists to program nanoarchitectures with dramatically increased complexity and functionality [1, 92].

Encouraged by classic molecular DNA tweezers, Komiyama research group reported the first switchable DNA origami device for single-molecule detection [93]. The authors designed tweezer-like 'single-molecule beacon' using DNA origami and turned the interaction processed with various target molecules to shape transitions. This versatile platform provides an easy solution to the sensing of various targets ranging from ions to proteins. In the meantime, dynamic DNA origami multihelix bundles were used to amplify signals as biosensing devices. Using a switchable DNA origami device decorated with gold nanorods, Liu and coworkers were able to create dynamic three-dimensional plasmonic metamolecules [94]. The gold nanorods were positioned on the cross-like DNA origami bundles, and the conformational change was transduced to plasmonic circular dichroism (CD) changes. Liedl and colleagues applied a similar device to virus RNA detection [81] (Figure 5a). The CD signal shifted because of the replacement of the blocking strand by hepatitis C virus RNA through SDR and the subsequent hybridization between the DNA overhangs on neighboring arms. The detection concentration of the RNA was below 100 pM. Further improvement of serum stability can be achieved by silica and block copolymer protection.

Besides switchable devices, the mechanically dynamic DNA nanostructures were also intensively studied. Marras et al. reported the programmable motion of several 2D and 3D DNA origami mechanisms by applying macroscopic machine design principles to DNA nanostructures. This review paper [95] by Castro team who systematically studied mechanical DNA nanodevices covers the development of DNA origami mechanisms and is recommended. Ke and coworkers applied mechanically dynamic DNA nanostructures for biomedical purposes [96] (Figure 5b). They built a rhombus-shaped DNA nanoactuator with allosteric regulation of fluorescent behavior of split enhanced green fluorescent protein (eGFP). Ten-helix bundles were used to build inner hinged edges with improved mechanical stiffness, while the struts connected the left two edges with unpaired scaffold region. The shape of the actuator changed once the strut was hybridized with complementary strands of different lengths. This trend could be propagated to the non-strut half due to the rigidity of the edges and the flexibility of the joints. Therefore, the fluorescence resulted from split eGFP pendants on the unstrut arms was tuned to several states by controlling the hybridization of the strut on the other half. By adding responsive domains at the joints, the nanoactuator was also able to sense various targets ranging from ions to miRNA.

The dynamic nature of DNA hybridization could be used to alter the accessibility of DNA nanocontainers. The first example of dynamic DNA origami objects was the 3D DNA box with a controllable lid suggested by Anderson et al. [25] (Figure 5c). The origami box was made from six inner hinged rectangle DNA faces whose neighbor edges were bridged by staples. The lid face of the box was able to open in the presence of a 'key' strand by SDR. Encapsulating cargos in such DNA boxes was subsequently achieved by the Andersen team as well. They proposed a DNA nanovault loading enzyme in its cavity with a reversible opening and closing feature [97] (Figure 5d). With the layered arrangement, a compact design capable of caging molecules out of external access was possible. The DNA nanovault could fold in the middle and had a cavity there for deposit as well. Opening key and closing key could reset the hybridization of multiple DNA 'locks' by SDR, controlling the interactions between deposited enzymes and the exterior substrate. The DNA wall porosity was characterized, and the enzyme reactions were regulated by reconfiguration.

3.3.2 Programable DNA Devices for Cancer Diagnosis and Treatment

DNA aptamer provides an alternative way to realize conformation dynamics and introduce new functions to DNA devices. Aptamer sequence in one staple hybridizes with a complementary overhang in another staple by design, creating strains to maintain the structure in a close configuration. Once the target units are presented, the aptamers interacting with the targets will fold to a tertiary structure, releasing the overall strain to turn the device to an open configuration. A prominent prototype of DNA nanorobot capable of reconfiguration for payload delivery was described by Douglas et al. in 2012 [82] (Figure 5e and 5f). The nanorobot consisted of a barrel DNA origami hinged by a scaffold in the rear and fastened by aptamer locks in the front. The logic gates comprised of various aptamer patterns enabled the recognition of different leukemia cancer cell lines and regulated the delivery of fluorescent labeling molecules or antibody cargos. High precision cell discrimination was achieved, and antibody-induced apoptosis/immune cell activation was observed.

Encouraged by the seminal example of DNA nanorobot for intelligent cell recognition and payload delivery in vitro, multiple research teams devoted dedicated use of

reconfigurable DNA nanodevices for cancer treatment. A collaboration work by Li et al. reported a DNA nanorobot targeting tumor endothelial cells with thrombin payloads. The DNA nanotube was locked by aptamers against nucleolin, a specifically expressed marker on tumor vessel [83]. After transporting to tumor cells, aptamer-protein binding unrolled the DNA nanotube and exposed interior thrombin proteins. The toxicity of DNA nanorobots was demonstrated in cells, mice and Bama miniature pigs. The Murine endothelial bEnd3 cells were cultured with different concentrations of DNA nanorobots andno significant cytotoxicity was observed. The nanorobot was also proved to be immunologically inert because the cytokine levels (IL-6, IP-10, TNF-α and IFN-α) of mice were barely changed after nanorobot injections.

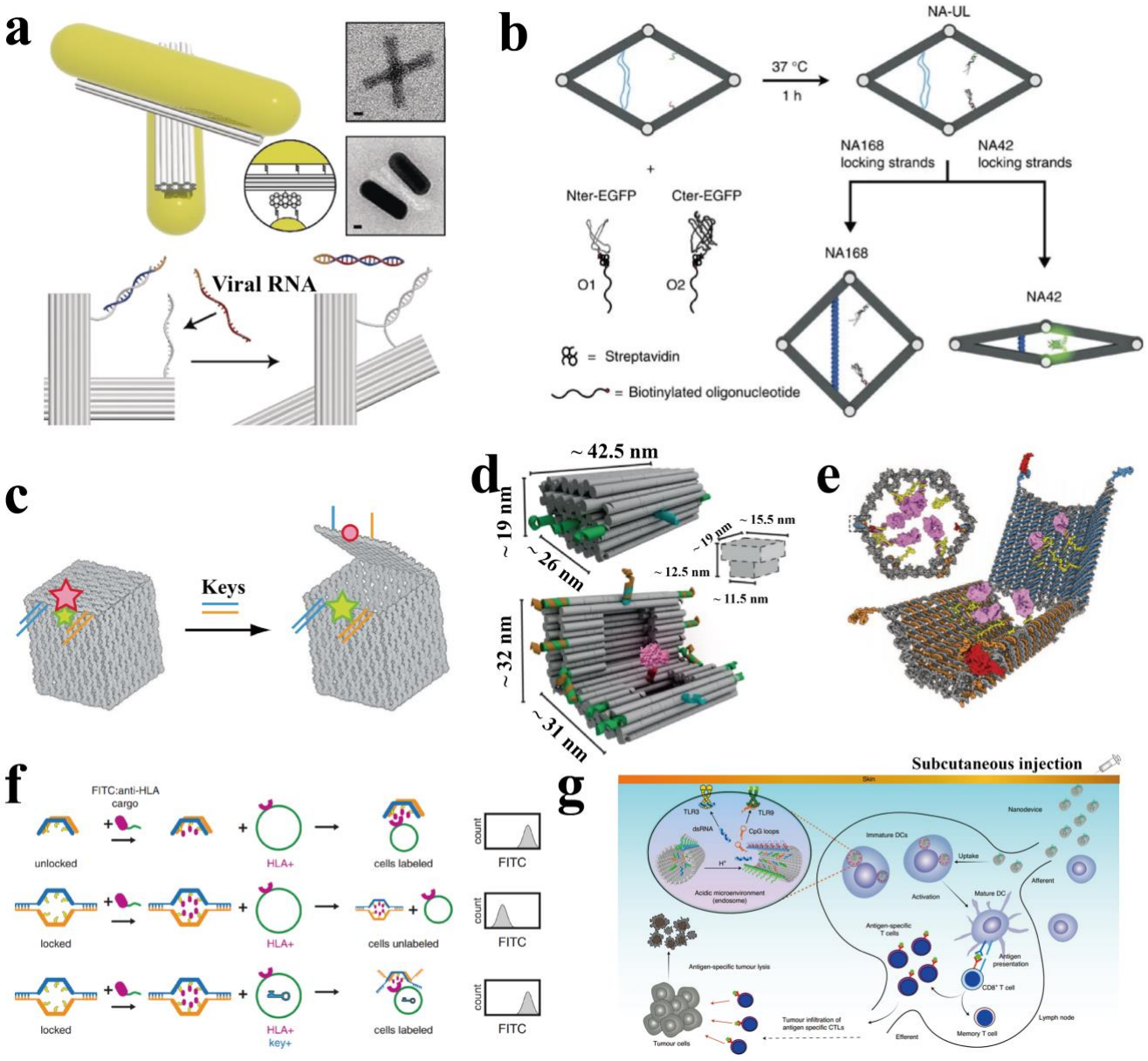

Figure 5. Switchable and reconfigurable DNA devices for biomedical purposes. **(a)** Sensing picomolar virus RNA using a switchable plasmonic DNA device. The SDR triggered by virus RNA results in chirality changes and transduces to circular dichroism (CD) shifting. Scale bar: 50 nm. Reproduced with permission from [81]. Copyright 2018 Wiley-VCH Verlag GmbH & Co. KGaA. **(b)** Mechanically dynamic DNA nanoactuator regulating the distance of split enhanced green fluorescent protein eGFP and probing biomolecules. Reproduced with permission from [96]. Copyright 2016 Springer Nature. **(c)** Switching between open and close states of an inner-hinged DNA origami box with key strands. Reproduced with permission from [25]. Copyright 2009 Springer Nature. **(d)** Controlling enzyme–substrate reaction by switching between open and close state of a DNA origami multihelix bundle nanovault. Reproduced with permission from [97]. Copyright 2017 Springer Nature. **(e and f)** The DNA nanorobot with aptamer 'locks' for conditional delivery of payloads (e) and operation schemes of the recognition patterns on the DNA nanorobot with fluorescent labels/non-label on target cells/control cells (f). Reproduced with permission from [82]. Copyright 2012 AAAS. **(g)** Activating immune cells against cancer by a pH-responsive DNA nanovaccine loaded with antigens and adjuvant. Reproduced with permission from [98]. Copyright 2021 Springer Nature.

Besides, this treatment method showed no effect on blood coagulation parameters (including platelet activity, plasma thrombin and fibrin concentrations, and circulating platelet numbers) and didn't induce thrombosis in major organs after intravenous injections to normal Bama miniature pigs with equivalent dose to mice.. Recently, a similar structure was employed as a vaccine for cancer immunotherapy [98] (Figure 5g). Instead of using aptamers, a pH-induced conformational changing sequence SWITCH was adopted to lock the DNA device and shielded the interior antigens and adjuvants. After the nanorobot was internalized into antigen-presenting cells through the endocytic pathway, the acid endosome environment would unlock the SWITCH and expose the cytosine-phosphate-guanine dsRNA, thereby activating toll-like receptor 9 and 3 pathway. The activated immune system was potent to bring about tumor regression and provided long-term immune memory against tumor reoccurrence in vivo.

CONCLUSIONS

Researchers in the field of DNA nanotechnology have proven that DNA, beyond its hereditary role, is a material to create almost arbitrary shaped and sized nanostructured with ever-increasing complexity. The unique features of DNA nanostructures, including geometric diversity, precise spatial addressability, and programmable reconfiguration, have enabled their broad applications in many research disciplines such as material engineering, chemistry, physics, and biology. In particular, DNA nanotechnology becomes a powerful tool to tackle the remaining tasks in biomedical science, such as implementing nanoscale control in biology studies, scaffolding and engineering biomaterials, targeting drug delivery, and diagnosing diseases at cellular levels.

A long-standing goal of DNA nanotechnology is to create functional nanodevices that rival natural machinery. Such structures can be programmed with a wide variety of biological functions such as manipulating biomolecules at the atomic level, synthesizing and regulating essential biomolecules in living cells, recognizing external substances, activating defenses against pathogens, etc. Although the ability of DNA nanotechnology to build nanodevices with complicated configurations and intricate functions has been developed rapidly, there are still several challenges of using DNA nanotechnology to solve biomedical problems. For example, to advance DNA nanodevices with biomedical functions, efforts are needed in improving the complexity and performance of functional domains that are assigned to a DNA nanodevice. Robust chemical or biological interactions are also required to transfer substrates or information efficiently between multiple domains or different nanodevices.

Overcoming such challenges relies on the development of DNA nanoscience as well as many other research efforts. Due to the interdisciplinary nature of this topic, the real-world applications of nucleic acid nanotechnology depend on continuous endeavor and collaborations between researchers in nanoscience, cell biology, computer science, etc. Nucleic acid nanotechnology will continuously contribute to biomolecular science and biomedical studies.

CONSENT FOR PUBLICATION

All authors gave consent to the publication.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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