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Rationally designed DNA-based nanocarriers

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ABSTRACT

Nanomaterials employed for enhanced drug delivery and therapeutic effects have been extensively investigated in the past decade. The outcome of current anticancer treatments based on conventional nanoparticles is suboptimal, due to the lack of biocompatibility, the deficient tumor targeting, the limited drug accumulation in the diseased region, *etc.* Alternatively, DNA-based nanocarriers have emerged as a novel and versatile platform to integrate the advantages of nanotechnologies and biological sciences, which shows great promise in addressing the key issues for biomedical studies. Rather than a genetic information carrier, DNA molecules can work as building blocks to fabricate programmable and bio-functional nanostructures based on Watson Crick base-pairing rules. The DNA-based materials have demonstrated unique properties, such as uniform sizes and shapes, pre-designable and programmable nanostructures, site-specific surface functionality and excellent biocompatibility. These intrigue features allow DNA nanostructures to carry functional moieties to realize precise tumor recognition, customized therapeutic functions and stimuli-responsive drug release, making them highly attractive in many aspects of cancer treatment. In this review, we focus on the recent progress in DNA-based self-assembled materials for the biomedical applications, such as molecular imaging, drug delivery for *in vitro* or *in vivo* cancer treatments. We introduce the general strategies and essential requirements for fabricating DNA-based nanocarriers. We summarize the advances of DNA-based nanocarriers according to their functionalities and structural properties for cancer diagnosis and therapy. Finally, we discuss the challenges and future perspectives regarding the detailed *in vivo* parameters of DNA materials and the design of intelligent DNA nanomedicine for individualized cancer therapy.

Contents

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1. Introduction

Cancer diagnosis and therapy remain to be the major challenges for modern medicine [1,2]. Numerous anticancer drugs and imaging probes have been extensively investigated over the past few decades. However, targeted delivery of the functional agents to tumors still needs to be improved [1,2]. The majority of these functional agents, such as small molecular chemotherapeutic drugs and imaging probes, usually displayed insufficient therapeutic effects due to limited delivery to cancerous cells induced by the complicated tumor microenvironment (TME) [3–5]. Compared to the vessels in normal tissues, tumor vessels are more permeable to large molecules and nanoparticles, which can be retained in the tumor region due to limited clearance [3–5]. Regarding these unique properties of TME, the construction of nanoplatform for cancer diagnostic sensors and anti-tumor therapeutics has been considered as an attractive way to improve cancer treatment [1,2]. In recent years, nanotechnology for pharmaceutics has been extensively developed and is now being applied to enhance drug delivery and to reduce the toxicity. Drug carriers based on nanoparticles (10–1000 nm) are emerging as a class of cancer diagnostic and therapeutic tools, which have demonstrated increased antitumor efficiency with decreased side effects. The advanced delivery in the diseased region by nanoscale carrier systems can be attributed to size- and shape-dependent cellular uptake, and the enhanced permeability and retention (EPR) effects in tumors [3–5].

The novel drug design for cancer treatments can be facilitated by a further understanding of the interaction between nanomaterials and the TME. To effectively deliver the molecular payloads to cancer cells both *in vitro* and *in vivo*, several properties of nanocarriers are desired:

(1) The nanocarriers can be synthesized or assembled to possess uniform sizes and shapes for enhanced systemic delivery due to EPR effects in tumor regions. (2) The nanocarriers are able to remain stable in the physiological environments, e.g., serum, and protect the payloads from the unexpected degradation, allowing for drug delivery *in vivo*. (3) The nanocarriers can load (*via* non-covalent attachment, covalent coupling or encapsulation) the desired cargo molecules or co-deliver two or more functional components for synergetic treatment. (4) The nanocarriers can be targeting delivered to tumor region and be internalized by diseased cells. (5) The nanocarriers can release the drug in response to the internal or external triggers. (6) The nanocarriers are synthesized or assembled by biocompatible materials which can be safely degraded and cleared after drug delivery and release.

Great efforts have been devoted to develop new materials and fabricate functional vectors for improving drug delivery and reducing toxicity. The conventional nanoparticle-based carriers for cancer treatment are mainly fabricated from lipids, polymers, or inorganic materials, and have been explored for encapsulating various imaging and therapeutic agents, such as organic dyes, quantum dots, upconversion nanomaterials, molecular cytotoxic drugs, therapeutic peptides, and nucleic acids [1–3,6]. Despite considerable laboratory and preclinical efforts, the nanoscale carrier systems for cancer treatments are still suffering from low efficacy and biocompatibility, deficient tumor targeting and accumulation, as well as limited cellular uptake and drug release *in vivo* [7–9]. In contrast to conventional materials, novel DNA-based nanosystems hold promise for versatile applications including drug delivery, due to their intrigue properties, such as uniform sizes and shapes, surface addressability, multi-functionality and intrinsic biocompatibility [10,11].

The development of a DNA-based drug delivery system has demonstrated the potential to improve cancer therapy. Compared to other classic nanosystems, DNA-based nanomaterials have several attractive properties that will be a promising candidate for drug delivery [10–16] (Fig. 1).

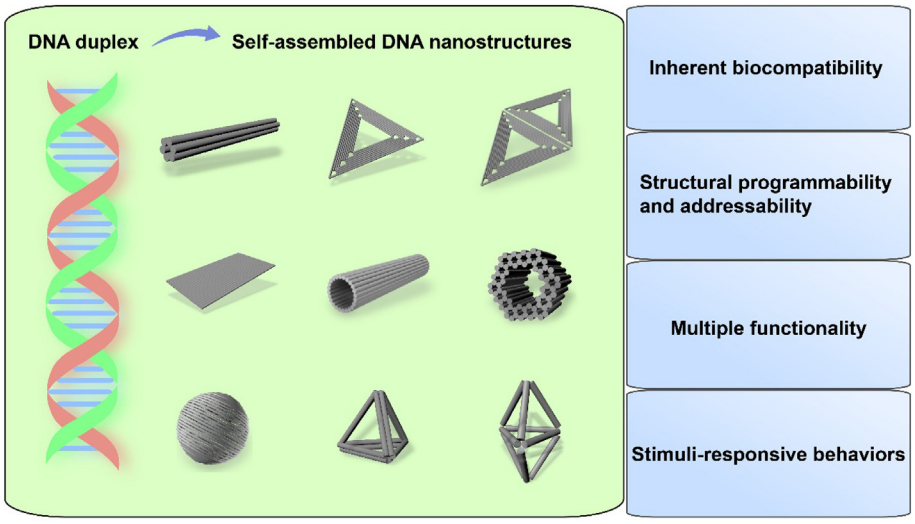


Fig. 1. The superior properties of DNA nanostructures for biomedical applications. As a promising candidate for nanocarrier, self-assembled DNA architecture holds distinct advantages, e.g., inherent biocompatibility, precisely designed nanoscale shapes and sizes, tailored functionality, and responsive reconfiguration. With these outstanding features, DNA nanostructures provide an unparalleled platform for optimized cargo delivery functioned as cancer diagnosis and therapy.

Through programmable DNA hybridization, the DNA nanostructures can be easily fabricated into pre-designed sizes and shapes with high yield [10–16]. Unlike ssDNA and dsDNA, self-assembled DNA nanostructures with certain geometries are able to enter tumor regions and can surpass cell membranes, which can realize complicated functions as DNA nanocarriers. The hydrodynamic sizes of different DNA nanostructures are typically in the range around 10–200 nm, which allows them to take advantage of EPR effect of the tumor region. In addition, DNA nanostructures possess suitable sizes and hydrophilic surfaces, which may partially reduce the opsonization reactions and subsequent clearance by macrophages during their circulation [17]. Compared with single-stranded or double-stranded DNA (ssDNA or dsDNA) ravels, self-assembled DNA nanostructures show enhanced stability against degradation in physiological environments. For further protection of nanostructures *in vivo*, surface modification with hydrophilic poly (ethylene glycol) (PEG) chains can be provided and gives DNA nanostructures stealth-shell characteristics, resulting in their prolonged circulation time and reduced immunogenicity [18–20].

Besides the structural programmability, DNA nanostructure can be conveniently decorated with multiple functional moieties [10,11,13–16]. These functional groups (including targeting aptamers, anticancer drug molecules, gene sequences, protein payloads, imaging contrast probes, *etc.*) exhibit high loading capacity and precise location on DNA nanostructure, which make it an even more attractive material of creating nanovehicles for drug loading and targeting delivery.

Furthermore, DNA nanostructure can be programmed to be responsive to external stimuli [15,21,22], such as near-IR light, acidic pH in tumor region or lysosome, malignant cells receptors, tumor vessel markers, intracellular glutathione, *etc.* Thus, DNA nanostructure can respond to the diseased environment, triggering the mechanical change of its structure and activating drug release on-demand.

DNA nanostructure will eventually be degraded and cleared after cargo transport [23–26]. As a natural material existing in all living creatures, DNA molecules show inherent biocompatibility, which provides an unparalleled platform to fabricate highly ordered and well-controlled system for optimized drug delivery. Several reported DNA nanostructures have been proved to be safe and immunologically inert in cells and animal levels [25–30], demonstrating DNA nanostructures represent a promising nanovehicle for precise drug delivery in cancer therapy. These key properties of DNA-based nanomaterial together provide huge opportunities for efficiently and safely delivering therapeutic agents to the tumor.

Herein, the brief history of DNA-based nanostructures and recent advances of self-assembly approaches in this field are discussed. We highlight the requirements and current stage of DNA-based nanocarrier as an engineerable novel drug delivery system (DDS) for cancer diagnosis and therapy. The recent advances and strategies of DNA-based DDS for biological applications are described. After incorporating specific functional moieties on addressable surfaces and cavities, DNA nanocarriers have been facilitating to improve stability in circulation, deliver cargoes to target diseased cells or region, responsively release or expose the loaded drug, even work as robotic systems. Finally, we outline the challenges and future perspectives regarding the key information of *in vivo* parameters of DNA nanocarriers, their potential immune responses and the advanced design of intelligent DNA nanomedicine.

2. Structural design of DNA assembly and requirements for biomedical applications

In the nanomedical field, the ability to generate a variety of complex nanostructures as molecular carriers and other therapeutic agents by efficient self-assembly reactions is an advantageous feature for emerging technologies [1,2]. Today, one of the most promising methods is to achieve the assembly of nanostructures with well-controlled geometries relying on DNA self-assembly [13], including tile-based procedures, rolling circle application (RCA)-derived approaches and DNA origami techniques. (Fig. 2A–C). The specific Watson-Crick base pairing between multiple DNA strands allows the design and fabrication of 2D and 3D structures, as well as the arrangement of components and functions at the nanoscale with high programmability.

Many biological applications require large amounts of DNA nanostructures with high purity and stability for desired functions both *in vitro* and *in vivo* (Fig. 2D). After decades of research, DNA nanotechnology now enables the mass production of a variety of complex nanostructures with a diverse range of sizes and geometries. Considerable DNA nanocarriers with different structural complexities and tailored biomedical functionalities have been designed and constructed. Recently, purification and stabilization of functional DNA carriers have been developed for a variety of biomedical applications. Several examples for large-scale synthesis, high-yield purification, and stabilization of functional DNA architectures will be introduced.

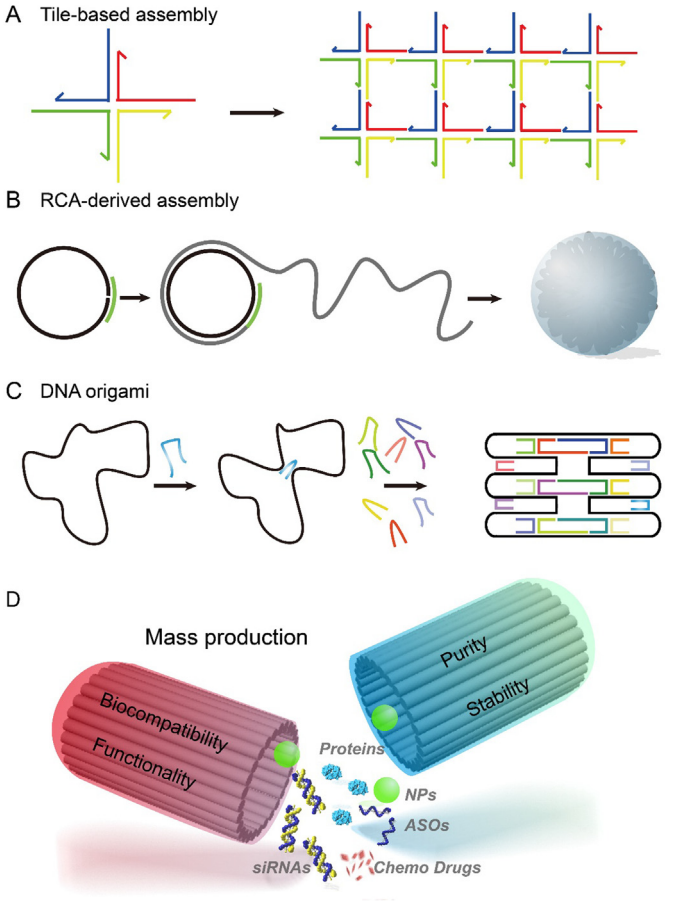


Fig. 2. Structural design of DNA assembly and advantages for biomedical applications. (A) In tile-based assembly, four ssDNAs form holiday junctions that can be combined into a network. (B) In RCA-derived DNA assembly, the linear DNA template is first ligated to form a circular template for RCA. Then RCA process generates a large amount of elongated DNA, forming mono-dispersed, densely packed DNA nanostructures. (C) In DNA origami, a long scaffold ssDNA strand (typically M13 genome DNA) is folded into arbitrary shapes by hundreds of short staple strands. (D) The advantages of DNA-based nanocarriers for biomedical applications. *In vitro* and *in vivo* evaluations require large amounts of DNA nanocarriers with high purity and stability to execute desired functions in a controlled fashion. DNA nanostructures are biocompatible in cellular and animal levels. DNA nanotechnology enables the mass production of a variety of complex nanostructures. Various functional moieties can be organized to DNA nanostructures for the particular biomedical functionalities. A series of purification and stabilization approaches for functional DNA carriers can be realized.

2.1. Self-assembly of DNA-based nanostructures

DNA serves as a genetic information carrier and plays a central role in expressing and regulating the biological functions of most living organisms. In the early 1980s, Seeman group proposed that DNA molecules can be used for fabricating programmable and multifunctional nanostructures [31,32]. By the aid of the self-recognition properties, DNA architecture has become a new player in material science [10–16]. In structural DNA nanotechnology, the strict base pairing principles allow DNA molecules to serve as building blocks for self-assembly into nanostructures with various sizes and geometries [12,13]. Through the rational design of appropriate sequences, DNA strands acting as both stick and glue spontaneously assemble into almost any desired 2D or 3D shape (Fig. 3), which has attracted tremendous interests in recent years.

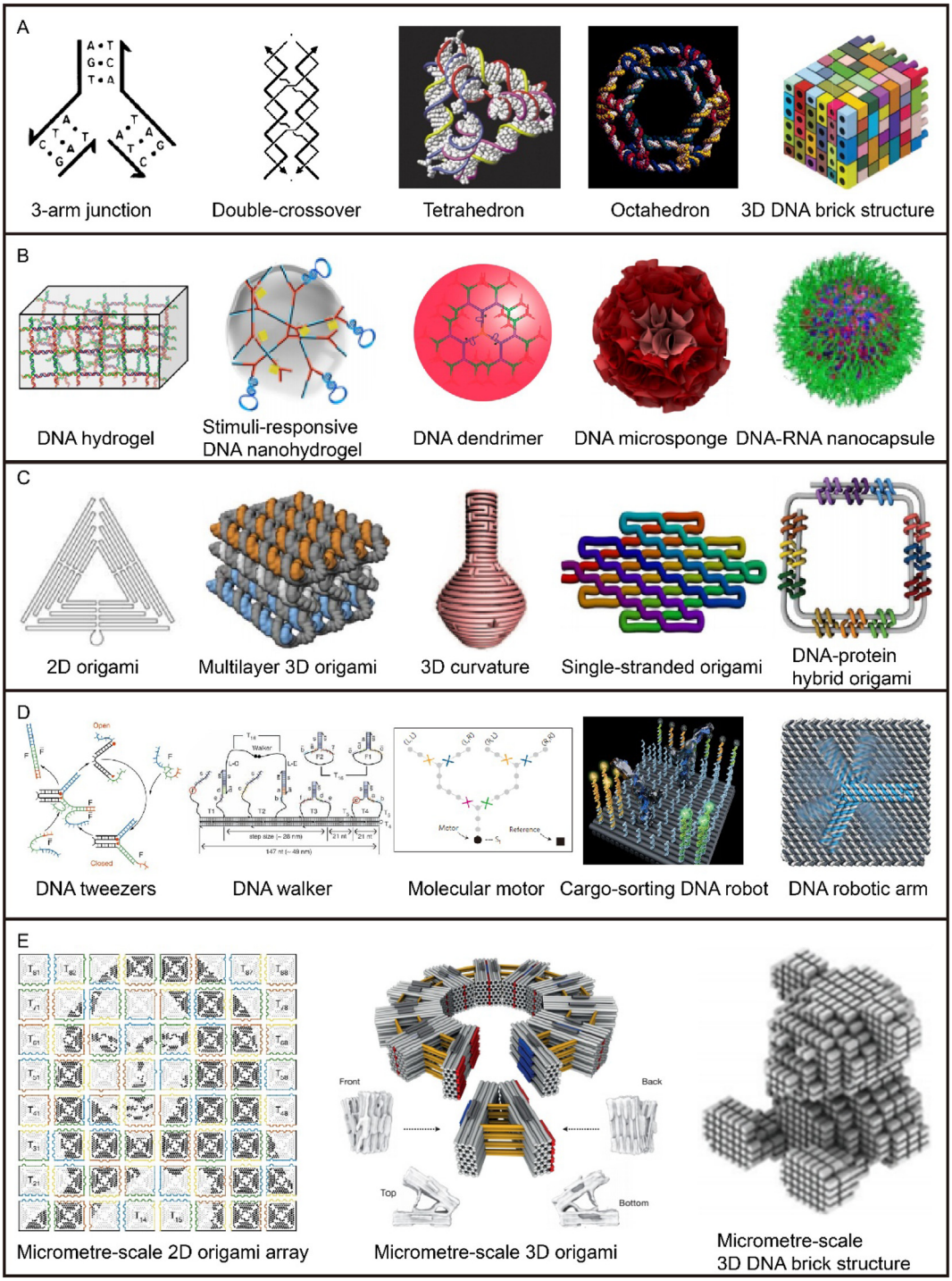


Fig. 3. Examples of self-assembled DNA nanostructures. (A) DNA tile-based self-assembly: 3-arm junction [31], Double-crossover [34]. Tetrahedron [38]. Octahedron [39]. 3D DNA brick structures [78]. (B) Hydrogels, dendrimers and RCA based DNA nanostructures: DNA hydrogels [53]. Stimuli-responsive DNA nanohydrogel [54]. DNA dendrimer [50]. DNA microsponge [57]. DNA-RNA nanocapsule [61]. (C) DNA origami-based nanostructures: 2D origami [62]. Multilayer 3D origami [65]. 3D curvature [63]. Single-stranded origami [76]. DNA-protein hybrid origami [75]. (D) Dynamic DNA nanoarchitectures: DNA tweezers [40]. DNA walker [42]. Molecular motor [43]. Cargo-sorting DNA robot [71]. DNA robotic arm [72]. (E) Micrometer-scale DNA objects: Micrometer-scale 2D origami array [73]. Micrometer-scale 3D origami [74]. Micrometer-scale 3D DNA brick structure [79]. The panels are reprinted with permission from the publisher.

2.1.1. Junctions, crossovers, and wire-framed DNA nanostructures

Different DNA motifs, such as individual DNA junction structures (Y-shapes and X-shapes junctions) [31–33], crossover structures (double-crossover (DX) [34], triple-crossover (TX) [35,36]), and other topological building blocks [37] were created by hybridization of multiple DNA oligonucleotides (ODNs) containing carefully designed sequences (Fig. 3A). Based on that progress, multi-crossover structures were designed and fabricated [34,36], which contain crossover sites in double-helical domains that can form rigid 2D wire-framed nanostructures. A variety of 3D DNA nanostructures were also successfully constructed in high yield, including various DNA polyhedral wireframe cages (cube [37], tetrahedron [38], octahedron [39]) (Fig. 3A). Dynamic nanostructures with multi-stranded junctions and multi-crossovers have been self-assembled into artificial, machine-like DNA devices [22,40–42]. DNA nanodevices, such as DNA tweezers [40], DNA walkers [42], molecular motors [43], *etc.* were constructed and operated by hybridization topology and showed controlled mechanical movement in molecular-scale.

2.1.2. Lattices, hydrogels, and dendrimers

Using sticky-end cohesion to assemble multi-arm junction as building blocks (DNA tiles), higher-order 2D periodic superstructures or lattices [44–48] can be prepared. The tile-based 3D self-assemblies include dendrimer-like DNA nanostructures [49–51], hydrogels [52–54], DNA crystals [55], *etc.* Usually, these DNA assemblies with high yields and uniform sizes can be reliably fabricated by enzyme-free methods with exact stoichiometry and purity control of involved ODNs and tiles (Fig. 3B).

2.1.3. DNA nanostructures based on rolling circle application

As an isothermal enzymatic strategy, rolling circle amplification (RCA) is usually used to form a long single-stranded DNA or RNA depending on a circular DNA template, a short primer and special DNA or RNA polymerases [56]. Recently, RCA has been employed to generate complex DNA nanostructures such as DNA microsponges [57], nanoflowers [58,59], nanoclews [60], nanocapsules [61], *etc.* (Fig. 3B). These self-assembled RCA-based nanostructures displayed exceptional stability under denaturing and physiological environments [58,59]. Moreover, fluorescent tags and other functional elements can be incorporated into RCA assemblies during the isothermal enzymatic process or by hybridization. These features make RCA-driven fabrication a highly desirable and scalable strategy for assembling DNA nanostructures for biomedical applications.

2.1.4. DNA origami-based nanostructures

One of the milestones in DNA nanotechnology is the invention of DNA origami. The DNA origami technique was established by Rothemund in 2006 [62]. In this technique, the desired origami shape is made by a long "scaffold" DNA (usually viral genome ssDNA) to fold into an arbitrary architecture, and hundreds of "staple" strands to hold the scaffold in place. The staple strands are rationally designed to be complementary to the particular regions of the long DNA scaffold, guiding the self-assembly process. Besides serving as the constructing elements of DNA origami, the staple strands also act as the addressable units for further functionalization. Up to now, a wide variety of predesigned DNA origami nanostructures has been constructed [63–68], characterized with controlled geometries, precise addressability, ease of modification, and intrinsic biocompatibility (Fig. 3C). All of these features make DNA origami as a versatile and promising candidate for loading a wide range of diagnostic and therapeutic elements.

Through rational structural design, dynamic DNA origami nanorobots have been achieved [30,69–72] (Fig. 3D), which promise cargo-sorting functions and controlled mechanical reconfiguration for payloads release. Recently, Qian group and Dietz group reported constructing 2D [73] or 3D micrometer-scaled DNA structures [74], by which the large DNA origami arrays (about half a micrometer across) or objects (up to 450 nm in diameter) can be produced (Fig. 3E). Alternatively, Dietz group provided a molecular origami method using double-stranded DNA scaffolds and special protein staples to create hybrid nanostructures [75]. Yan group described a framework to design and synthesize single-stranded DNA and RNA that can self-fold into diverse, stable, user-defined structures [76]. The single-stranded origami was self-assembled from a single-stranded scaffold, which can be replicated in living cells and allow for low-cost production at large scales with high purities.

2.1.5. Single-stranded tiles (SST)-based DNA nanostructures

Another important design strategy in DNA nanotechnology is single-stranded tile (SST) assembly [77]. As a molecular Lego, each SST (DNA "brick") comprises a single-stranded DNA with four short binding domains only fitting with specific and predefined partners, enabling individual SST to assemble through the formation of DNA duplexes at the tile/tile interface. Collections of SSTs have been reported to form 2D sheets [77] or 3D blocks [78] (Fig. 3A) with different patterns and shapes, which can be created by selectively including or omitting specific SSTs. Yin group recently reported a new one-pot approach that employed the self-assembly of DNA bricks for the construction of 3D DNA nanostructures of great size (at the micrometer scale) and complexity [79] (Fig. 3E).

Above all, the development of structural DNA nanotechnology is stimulating us to manufacture DNA-based drug carriers with desired size, shape and molecular precision for further functionalization.

2.2. Requirements of DNA-based nanomaterials for biomedical applications

A broad range of DNA nanostructures with diverse sizes, architectures and surface properties have been designed [13]. Due to ease and reliability of programming their shapes, site-specific functionalization and responsive behaviors, DNA nanostructures have evoked great interest as potential therapeutics and diagnostics in the biomedical area [10,11,14–16]. In order to bring self-assembly DNA nanostructures to the practical application, great efforts are necessary to overcome existing limitations in large-scale production, functionalization, purification and stabilization. With the rapid development in recent years, several approaches have been established for preparation of larger amounts, pure and stable DNA nanostructures for biomedical applications (Fig. 4).

2.2.1. Scale-up productivity of DNA nanostructures

Many biological applications require milligram-scale and even gram-scale amounts of DNA nanostructures so that more practical experiments rather than solely proof-of-concept investigations could be performed. For origami self-assembly, the availability of the raw materials (DNA scaffold and staple strands) limits the amount of DNA origami objects that can be produced.

Shih group provided a method for nanomole-scale production of DNA scaffold strands (M13 bacteriophage-derived genomic DNA) by bacteriophage proliferation and ssDNA isolation [80,81]. To achieve a more scalable and efficient ssDNA production, Weuster-Botz group developed an advanced approach for bacteriophage proliferation [82]. They kept high-cell-density fermentation of bacteriophage-infected *E. coli* in a stirred-tank bioreactor under controlled pH, dissolved oxygen and substrate supply conditions. Their approach yield gram-scale amounts of purified ssDNA (Fig. 4A). In addition, a stretagy introduced by Hogberg group on the enzymatic production of monoclonal, single-stranded DNA oligonucleotides can be utilized for the efficient production of staple strands [83]. Their "monoclonal stoichiometric" (MOSIC) method was scalable and provided large batches of very pure, monoclonal, single-stranded DNA ODNs in precisely controlled stoichiometric ratios. Dietz group recently provided a biotechnological method for mass production of single-stranded DNA scaffolds and staples [84]. They successfully demonstrated that the single-stranded DNA raw

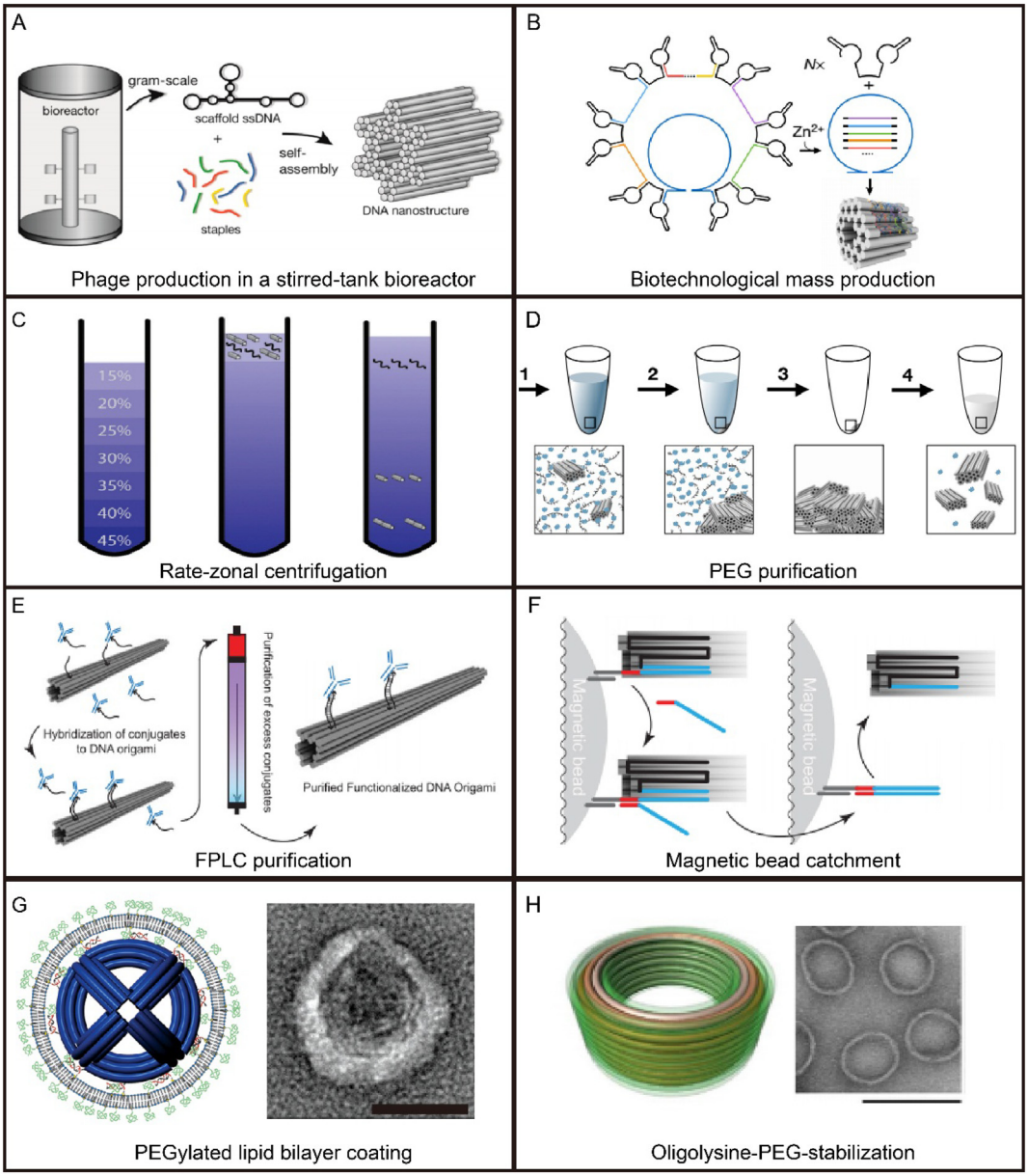


Fig. 4. Several examples of mass production, purification, and stabilization of DNA nanostructures. (A-B) Methods of scale-up production of DNA nanostructures: (A) Phage production in a stirred-tank bioreactor [82]. (B) Biotechnological mass production of DNA origami [84]. (C-F) Purification strategies: (C) Rate-zonal centrifugation [87]. (D) PEG-induced precipitation [88]. (E) FPLC separation [89]. (F) Magnetic bead catchment [89]. (G-H) Approaches for improvement of the stability: (G) PEGylated lipid bilayer encapsulation [18]. (H) Oligolysine-PEG decoration [19]. The panels are reprinted with permission from the publisher.

materials for origami assembly were produced using bacteriophages, which generated precursor DNA that contained target strands (hundreds of staple-strand and scaffold sequences, or SST strands) interleaved with self-excising sites. After cleavage by a "DNAzyme", the target sequences were formed and self-assembled into pre-designed DNA nanostructures (Fig. 4B).

Besides of self-production and purification of raw DNA strands, Hogberg group presented a method for origami directly assembled from intact phages [85]. The phage particles were mixed with the pre-designed staple strands and denaturants. After the phages were denatured, the released scaffold DNA and staple strands were assembled into the DNA origami structure *via* thermal annealing in a one-pot reaction. Genetically encoding DNA nanostructures provide another route for their production as well as applications in living cells. Voigt group generated a genetic encoding DNA nanostructure, which can form structures in living bacteria or can be purified for *in vitro* assembly [86].

Using these production methods, mass production of DNA nanostructures can be achieved in a scalable and cost-efficient manner, enabling large-scale biological applications such as molecular imaging and drug delivery.

2.2.2. Multi-functionality of DNA nanostructures

A collection of convenient approaches have been established to construct DNA-based nanomaterials that exhibit various geometric, topological complexity and specific biological functions [10–16]. Various moieties could be attached to DNA nanoarchitectures to realize the particular biomedical functionalities, such as precise tumor recognition, efficient drug loading, and controlled drug release. The functional cargo molecules, such as chemotherapeutic drugs or imaging agents, can be directly loaded *via* intercalation or covalent linkage. Hybridization between the DNA strands attached to functional cargoes and capture strands on DNA nanostructure templates offers a vital approach for site-specific loading of a range of functional cargo molecules (such as

aptamers, small interference RNA, microRNA, antisense oligonucleotides, CpG sequences, and DNA-modified fluorescent dyes, peptides, proteins, nanoparticles, *etc.*). The capture strands can be extended site-selectively on the DNA nanostructures, and hybridize with the complementary DNA strands modified molecular or nanoscale cargoes. The hybridization of captures and cargoes enables the geometrically-controlled organization of the cargoes of interest on the nanoscale level, producing the complex with desired functionalities. Aptamer sequences-targeted binding and biotin-streptavidin interaction provide alternative ways for specific cargo molecules loading by site-selectively recognition and anchoring. There are also several cargoes that can be encapsulated by DNA nanostructures for facilitating their delivery.

2.2.3. High yield purifying steps of DNA nanostructures

As mentioned above, various approaches open up the possibility to arrange diverse functional elements with nanometer precision in DNA nanostructures for biological applications. Before *in vitro* and *in vivo* application, the biomedical DNA nanostructures require the purification step and the excessive functional material could be removed from the desired DNA nanostructure. Great efforts have been devoted to generating reliable and high-yield approaches for purification of functionalized DNA nanostructures.

Previously reported methods for purifying DNA nanostructures usually rely on agarose-gel electrophoresis (AGE) for separation. Such laborious and low-yield AGE procedure would suffer the next step application. Another commonly used purification method is ultrafiltration by spin columns, the membrane pores of which might be potentially blocked by functionalized proteins on the nanostructures and reduce the efficiency. Thus several new methods have been developed to purify functionalized DNA nanostructures.

Shih group presented a readily scalable purification approach utilizing rate-zonal centrifugation [87], which provided comparable separation resolution as agarose gel electrophoresis. Utilizing a linear glycerol solution (15–45%, *v*/v) as a density gradient media, the desired DNA structures were easily recovered through the fraction with consistently high yield (40–80%) after high-speed centrifugation (Fig. 4C). For purification and concentration of the target nanostructures, Dietz group described a method based on poly (ethylene glycol) (PEG)-induced precipitation [88]. They demonstrated that PEG method was applicable to a wide variety of DNA architectures with excellent recovery yields (>90%), providing efficient separation from non-integrated DNA strands (Fig. 4D). Hogberg group developed two another purification strategies which were applied in the modification of DNA origami with either small molecules, antibodies, or larger proteins [89]. One purification method was based on magnetic bead catchment and strand-displacement (Fig. 4E). The other approach was applied by fast protein liquid chromatography (FPLC) system using a size-exclusion chromatographic column to elute the target nanostructures, which is commonly used to purify plasmid DNA or proteins (Fig. 4F). Compared to the previously reported methods (include gel extraction, size exclusion columns, glycerol gradient ultracentrifugation, and PEG precipitation), the magnetic-bead capture was a potential universal purification method for DNA origami with a comparable recovery yield with other methods. FPLC approach was a powerful and automated strategy for scale-up production of functionalized DNA nanostructures. Fan group introduced HPLC strategy to purify multi-armed DNA tetrahedron and successfully utilized the purified structures for *in vivo* imaging [26].

2.2.4. Improvement of the stability of DNA nanostructures

After self-assembly, functionalization, purification, and concentration, DNA nanostructure has become a prime candidate that promises utility in various areas of nanomedicine. However, the possible instability of DNA nanostructures in biological environments may hamper their *in vivo* applications. Several natural or artificial nanoparticles have been utilized as polymer shells to maintain structural integrity or protect the inner cargoes. A series of studies based on encapsulation of DNA nanostructures have been reported.

Kostiainen group demonstrated an approach for the coating of DNA origami nanostructures with purified cowpea chlorotic mottle virus capsid proteins, which can bind and self-assemble on the origami surface through electrostatic interactions and further pack the origami nanostructures inside the viral capsid [90]. The report indicated that the protein protection process enhanced cellular attachment and intracellular delivery efficacy (13-fold) compared to bare origami nanostructures. They recently provided another coating method of origami with precisely defined one-to-one protein-dendron conjugates for enhancing stability against endonucleases. As a result, the transfection into the human embryonic kidney (HEK293) cells by the protein-dendron coated origami was enhanced [91].

Shih group provided a virus-inspired enveloping approach of DNA origami octahedron with PEGylated lipid bilayer [18]. In their work, the DNA wire-framed nanostructures were wrapped tightly by mixing surfactant-lipid micelles in high yield and showed improved resistance to nuclease digestion. Compared to non-enveloped structures, polymer shell-protected DNA nanostructures displayed significantly prolonged half-life *in vivo* after tail injection. Recently, Shih group introduced another structure-independent protecting strategy of DNA nanostructures by coating oligolysine-PEG copolymer [19]. They demonstrated that the encapsulated DNA origami enabled potent protection against digestion by serum nucleases (1000-fold) and can survive when uptaken into endosomal compartments in cells. In a mouse model, oligolysine-PEG-stabilized DNA nanostructures exhibited a modest increase in pharmacokinetic bioavailability. Schmidt group provided a similar coating approach for DNA origami using a cationic PEG-polylysine block copolymer through electrostatic interaction [20]. They demonstrated that the protection strategy of DNA nanostructures did not hamper their functionalization with DNA-modified gold nanoparticles or streptavidin-modified quantum dots.

Sleiman group recently developed a DNA nanostructure for high-affinity binding to human serum albumin (HSA) [92]. HSA is the most abundant protein in the human blood and has already been successfully applied for the delivery of small-molecule drugs and nanoparticles [93–95]. In their report, DNA strands or nanocages were conjugated to dendritic alkyl chains, creating amphiphiles as binding sites to HSA. They also confirmed that the affinity of the DNA cage to HSA can be modulated by altering the number and orientation of the amphiphilic ligand on the DNA structures in a site-dependent manner. In contrast to unconjugated single-stranded DNA (several minutes), the HSA-bound DNA cage showed prolonged serum half-life (22 h).

3. DNA-based nanocarriers for cancer diagnosis and therapy

New approaches for cancer diagnosis and therapy are being designed to specifically deliver drugs or functional cargoes to the tumor at higher concentrations with minimal damage to normal tissues. Over the past decades, a collection of convenient methods have been established to construct DNA-based nanomaterials with particular biological functions. After modifications with various functional components, DNA nanoarchitectures can be customized with specific tumor recognition, efficient and multiple payloads delivery, triggered activation and controlled drug release. The current stage of DNA-based nanocarrier as the engineerable novel drug delivery system both *in vitro* (Figs. 5–7) and *in vivo* (Fig. 8) will be summarized.

3.1. Targeting moieties for facilitated uptake in vitro and in vivo

To deliver the loaded cargoes to the diseased site while reducing the distribution to normal tissue or cells, tumor-targeting drug delivery system shows great promise in addressing the associated problems and providing superior therapeutic benefits. The targeting molecules, namely ligands with the purpose of tumor recognition and binding,

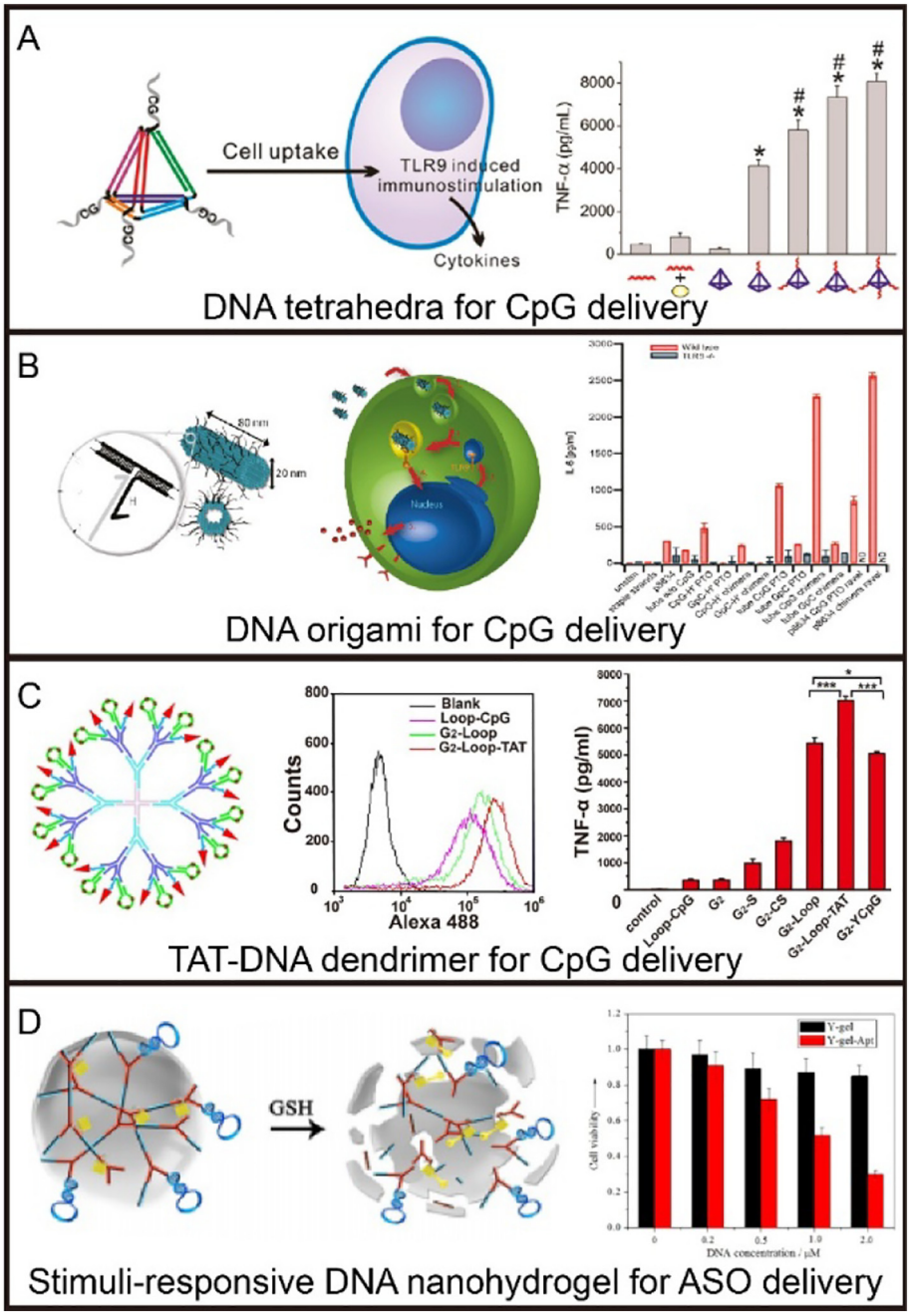


Fig. 5. DNA nanostructures as vehicles for intracellular delivery of functional nucleic acids and cancer therapy. (A) DNA tetrahedra for CpG delivery to mouse macrophage cell line Raw 264.7 [104]. (B) Cellular delivery and immuno-stimulation by CpG-coated DNA origami after incubation with isolated splenic macrophages [105]. (C) TAT-dendrimer for efficient CpG delivery to Raw 264.7 cells [99]. (D) Aptamer-modified and stimuli-responsive DNA nanohydrogel for therapeutic ASO (ISIS5132) delivery to lung adenocarcinoma epithelial A549 cells [54]. The panels are reprinted with permission from the publisher.

including nucleic acid aptamers, peptides, antibodies and small molecules, could modify DNA nanostructures *via* covalent or non-covalent linkage. For DNA or RNA aptamers, both the nanocarrier and the targeting elements are essentially the same kind of biomolecules, so the loading processes are usually reliable and convenient. Through base-pair hybridization or just simply integrating targeting sequences with the structural sequences, aptamers can be anchored to the DNA nanoarchitectures. For peptides and antibodies, DNA strands can be modified to their surface through several approaches, such as amine coupling reaction, click chemistry, biotin-streptavidin affinity, aptamer-protein recognition, *etc.* Then DNA nanocarriers can load those DNA-functionalized peptides and antibodies by hybridization between the DNA strands attached to targeting moieties and the captures on the carriers. Additionally, small molecular targeting ligands can be loaded to DNA nanostructures by covalent cross-linkage with structural DNA strands.

Aptamers, which are usually single-stranded DNA or RNA molecules, generate from an *in vitro* process known as SELEX (systematic evolution of ligands by exponential enrichment) [96]. By folding into specific nanostructures, aptamers are able to recognize multiple biomarkers in complicated biological environments, which are attractive candidates for nanomedicine. Tan group reported a series of self-assembly DNA nanostructures that incorporated aptamers for targeting delivery. Through rolling circle replication by using two DNA strands (one template and one primer), DNA nanoflowers (NFs) were generated. The long linear template ssDNA were designed to encode complementary

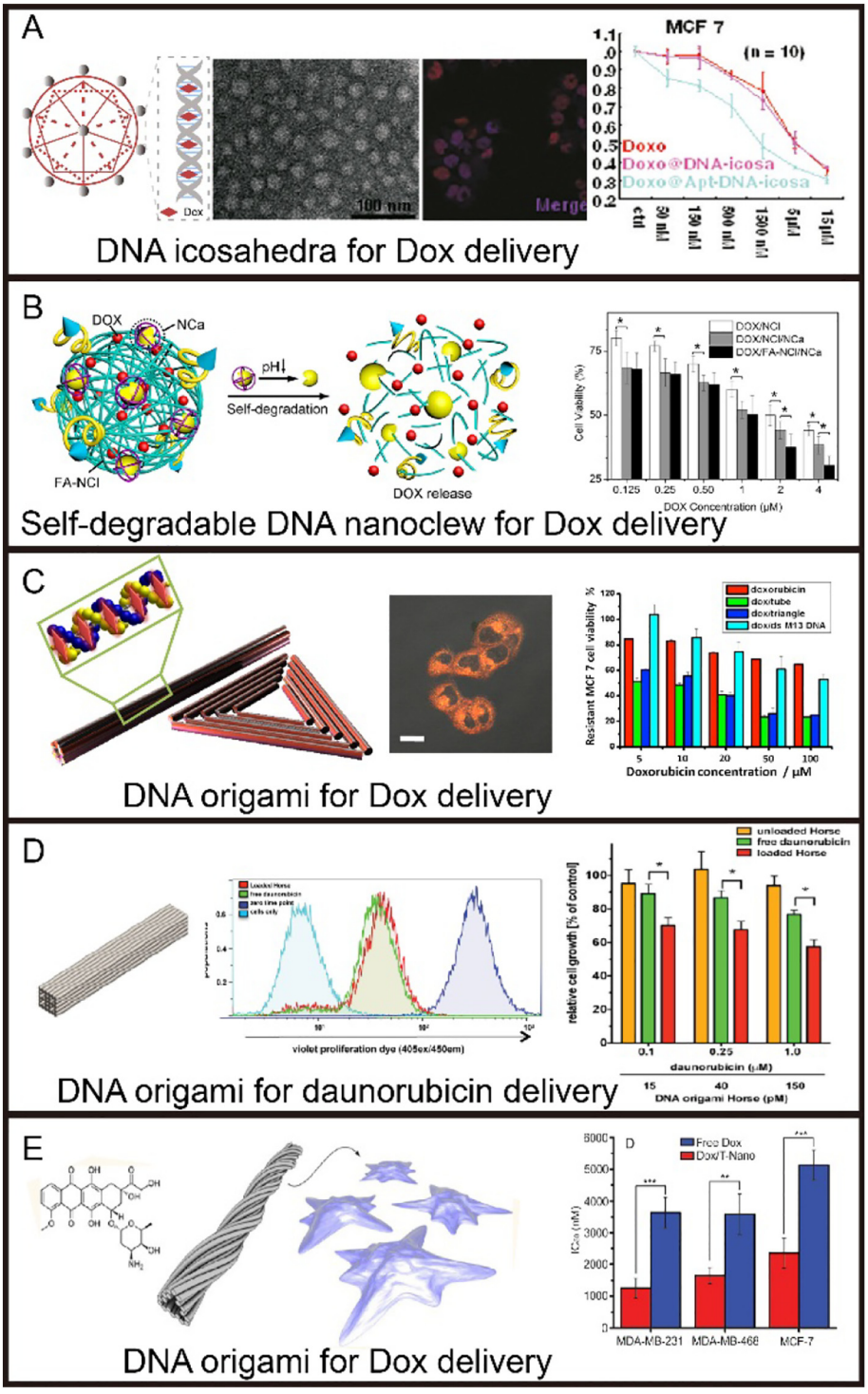
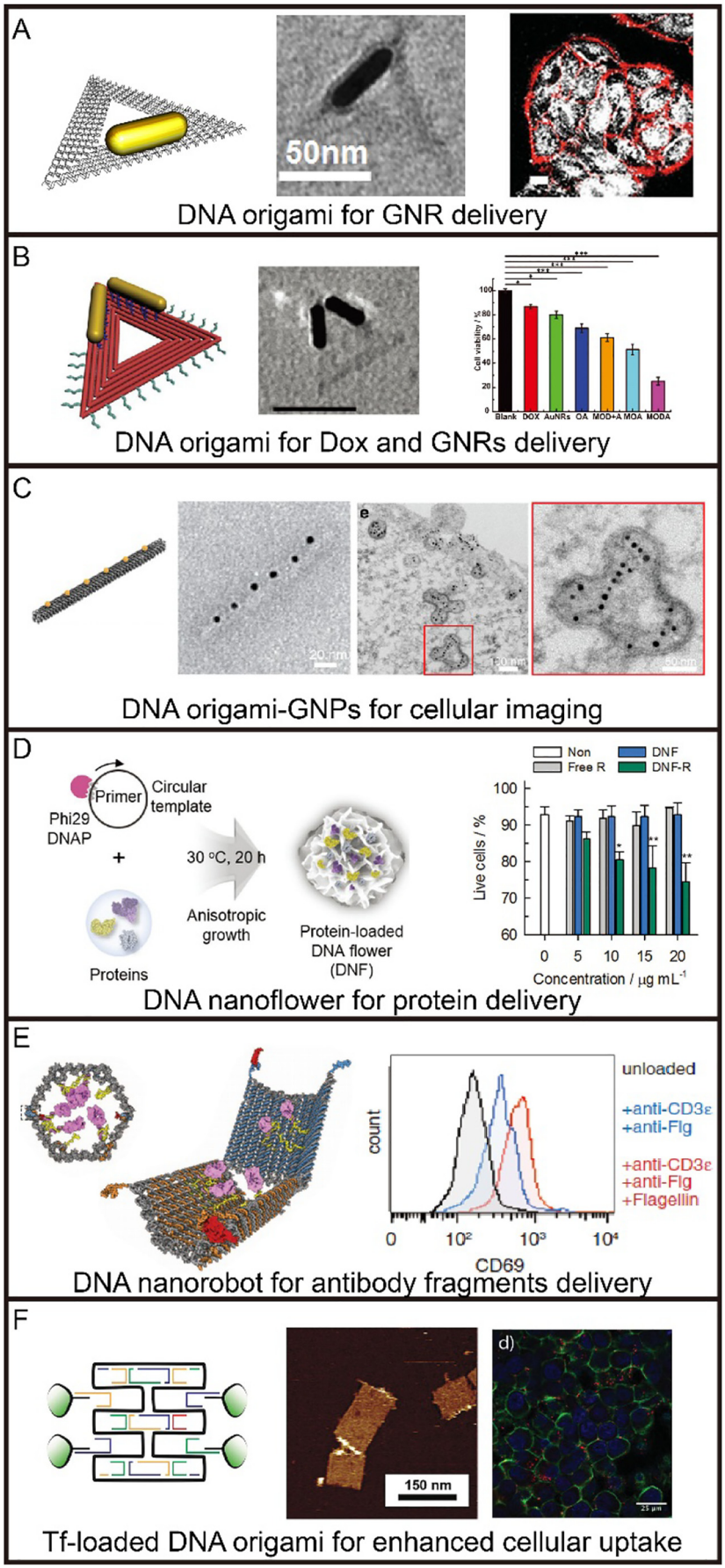


Fig. 6. DNA nanostructures as delivery tools of chemotherapeutic drugs for cancer therapy *in vitro*. (A) MUC-1 aptamer-DNA icosahedra for efficient doxorubicin delivery to human breast cancer cell line MCF-7 [114]. (B) Folic acid attached self-degradable DNA nanoclew for doxorubicin delivery and controlled release in MCF 7 cells overexpressing folate receptors [60]. (C) DNA origami as doxorubicin vehicle for prominent cytotoxicity in drug-resistant MCF 7 cells [115]. (D) Daunorubicin-loaded DNA origami rods for enhanced anti-tumor efficacy in drug-resistant leukemia cells [116]. The panels are reprinted with permission from the publisher. (E) DNA origami as doxorubicin delivery carrier for cancer therapy with tunable release properties [117].

sequences of the aptamers (sgc8 and MUC1). Since DNA NF assembly *via* liquid crystallization of the resulting long building blocks, all aptamer sequences can be used for specifically recognizing tumor cells to facilitate targeted cellular imaging and traceable drug delivery [59]. In their report on self-assembly of DNA nanohydrogel, aptamer S6 was constructed as a building block, which targeted A549 cancer cells (human lung adenocarcinoma epithelial cell line) and provided the specific tumor cell uptake for the antisense sequences loaded DNA carriers [54]. Recently, they reported the design of circular bivalent DNA aptamers with dumbbell structures that enabled *in vivo* stability and recognition [97]. The two sgc-8 aptamers (one aptamer with 5′-phosphate group and the other with 3'-OH group) were designed with additional complementary sequences and could hybridize to form a bivalent aptamer with two nicks. By sealing the two nicks with T4 DNA ligase,


A
50nm
DNA origami for GNR delivery
B
Cell viability / %
Blank DOX AuNRs OA MOD+A MOA MODA
DNA origami for Dox and GNRs delivery
C
DNA origami-GNPs for cellular imaging
D
Primer
Circular template
Phi29 DNAP
+
30 °C, 20 h
Anisotropic growth
Proteins
Protein-loaded DNA flower (DNF)
Non
Free R
DNF
DNF-R
Live cells / %
Concentration / μg mL-1
DNA nanoflower for protein delivery
E
unloaded
+anti-CD3ε
+anti-Flg
+anti-CD3ε
+anti-Flg
+Flagellin
count
CD69
DNA nanorobot for antibody fragments delivery
F
150 nm
d)
Tf-loaded DNA origami for enhanced cellular uptake

circular bivalent DNA aptamers were constructed. The cyclization process of circular bivalent DNA aptamers ensured the conformational integrity of the nanostructure and enhanced their thermal stability and nuclease resistance. Compared with the regular aptamer sequences, the circular aptamers showed improved physical stability in biological media, which led to their stronger binding affinity, recognition, accumulation, and retention in tumors of xenograft nude mice.

Peptides, short amino acid chains (approximately 50 amino acids or less in length) are emerging as a tool in the development of drug delivery systems. Peptide-modified drug delivery systems provide new opportunities to cross endothelial and epithelial barriers and enter the cytoplasm of target cells. By incorporating with DNA functionalized peptides, DNA nanostructures are able to display unique functionalities. Fan group reported a method to modulate the cellular fate of the DNA nanostructures by the conjugation of the functional peptide [98]. The tetrahedral DNA nanostructures (TDNs) were attached to nuclear localization signals (NLSs) peptides through click chemistry, which efficiently coupled azide-modified ssDNAs to NLS peptides containing a propargylglycine. After peptide modification, the NLS-TDNs were observed within the nucleus in Hela cells, whereas TDNs without NLSs were mostly restricted to the lysosomes. Ding group has utilized cell penetrating peptides (CPPs) to facilitate the cellular delivery of the dendrimer-like DNA nanostructures [99]. TAT peptide derived from trans-activator of transcription, a protein encoded by human immunodeficiency virus type 1 (HIV-1). As one of the outstanding CPPs, TAT peptide molecules were attached to DNA dendrimers, which substantially improved the cell membrane permeability of the nanocarriers and accumulation to endosomes. Recently, Fan group designed a tetrahedral DNA nanocage functionalized with angiopep-2(ANG) for brain tumor targeting and imaging [100]. ANG (a 19-mer peptide derived from human Kunitz domain of aprotinin) modified single-stranded DNA (ANG-ssDNA) were synthesized by click action kit. The complementary single-stranded arms that extended from the DNA tetrahedron were hybridized with ANG-ssDNA, forming ANG-functionalized TDNs (ANG-TDNs). Exhibiting high binding efficiency with low-density lipoprotein receptor-related protein-1 (LRP-1), ANG-TDNs showed potent cellular uptake in brain capillary endothelial cells (bEnd.3) and human glioblastoma Uppsala 87 Malignant Glioma cells (U87MG). Mediated by ANG peptides, ANG-TDNs can pass through the blood-brain barrier (BBB) *in vitro* and *in vivo*. The *in vivo* imaging demonstrated that ANG-TDNs can be used as tumor targeting probes in U87MG xenograft nude mice.

As another ligand for targeting cancer cells in drug delivery nanocarriers, transferrin (Tf) is a key player in the metabolism of iron transportation and delivery to cells *via* receptor-mediated endocytosis. It has successfully been used to transport DNA nanoarchitectures into cells. Kjems group introduced a rectangular DNA origami nanostructures functionalized with transferrin-oligodeoxynucleotide conjugates (Tf-ODN) [101]. Tf-ODN was produced by DNA-templated protein conjugation approach, which kept biological functions of the conjugated Tf molecules. After purification, Tf-ODNs were incubated with origami structures at room temperatures overnight for efficient protein incorporation. The evaluation of Tf-loaded DNA rectangle *in vitro* demonstrated that Tf enhanced the internalization of DNA origami structures into KB carcinoma cell line (highly expressed Tf receptor) in a ligand dose-dependent manner. Krishnan group showed another DNA-based nanomachine functionalized with Tf, which were molecularly programmed to enter cells *via* the transferrin endocytic/recycling pathway [102]. The ligands modified DNA machines were able to map pH changes within well-defined subcellular environments along pathway inside the same cell, which may have implications for sensing and therapies in a diverse range of contexts.

Folate receptors (FRs) are highly expressed on the surfaces of various cancer cells such as ovarian, breast, colon, renal and malignant nasopharyngeal carcinomas *etc.*, and are emerged as new targets for specific recognition and localization of nanoscale drug delivery systems. Folic acid (FA) is a small molecule, which can be directly conjugated to DNA molecules and used as a specific ligand. Mao group designed tubular DNA nanostructures that were functionalized with folic acid molecules to target receptors at the surface of cancer cells, resulting in efficient cell uptake [103]. For DNA nanostructures based on RCA, Gu group introduced a multifunctional cocoon-like DNA nanocomposite (nanoclew, NCl) [60]. To achieve tumor-targeting delivery of the chemotherapeutic drugs, folic acid was conjugated to an NCl complementary DNA oligomer followed by hybridization to the DNA nanocomposite. Then the FA-modified DNA nanoclew entered the human breast cancer (MCF-7) cells overexpressing FR through the endocytosis pathway extremely fast even within 10–30 min. Fan and co-workers incorporated folic acid onto their multi-armed tetrahedral DNA nanostructures (TDNs) to facilitate the tumor targeting and *in vivo* imaging [26].

3.2. Versatile payloads for powerful and selective therapeutic strategies

Rapidly advancing synthesis and modification technologies enable novel biological applications for DNA-based nanomaterials. The highly addressable properties of DNA nanostructures lead to precise control over the valence and the position of cargo molecules. Usually, functional nucleic acids, chemotherapeutic drugs, proteins, peptides, and nanoparticles can serve as functional payloads for DNA nanocarriers.

3.2.1. Functional nucleic acids

The concept underlying functionalization for DNA nanostructures is relatively straightforward: nucleic acids with unique biomedical function are able to be integrated with the DNA nanovehicles by hybridization. As molecular cargoes, several types of functional nucleic acids, such as CpG sequences, antisense sequences, siRNA, miRNA, *etc.*, have been engineered to the DNA nanoarchitectures (Fig. 5).

3.2.1.1. CpG ODNs for immunostimulation. Unmethylated cytosine-phosphate-guanine (CpG) ODNs are a type of therapeutic nucleic acids with strong immunostimulatory activities. Specifically recognized by endosomal toll-like receptor 9 (TLR 9), CpG sequences can induce an immune response, which has been actively explored in both basic research and clinical trials as a type of potent vaccine adjuvant in immunotherapy for cancer [29]. However, naked and linear-structured CpG ODNs show the poor efficiency of cell internalization and are prone to be degraded by nucleases before implementing their immune function. A series of DNA nanocarriers have been designed for efficient CpG ONDs delivery and tailored immunotherapy both *in vitro* and *in vivo* [15,61,99,104–108].

Fan group described an approach to develop CpG-containing DNA tetrahedron (Fig. 5A). Four DNA strands (containing core sequences for tetrahedral nanostructure assembly and CpG sequence) were assembled for CpG-bearing DNA nanostructure (~10 nm) with a simple annealing procedure [104]. Tetrahedron with a different valence number of CpG sequences was constructed by a similar process. Compared to free CpG sequences, the tetrahedron loading CpG ODNs showed

Fig. 7. The utility of DNA nanostructures in cellular experiments for nanoparticles or proteins delivery and cancer therapy. (A) DNA origami as a carrier of gold nanorod for intracellular photothermal therapy [125]. (B) DNA origami-gold nanorod-doxorubicin complex inducing a remarkable reversal of phenotype resistance [127]. (C) Gold nanoparticles-tagged DNA origami rod for the study of the cellular uptake and intracellular trafficking [128]. (D) Multiple protein-encapsulated DNA nanoflowers for enhanced intracellular protein delivery [132]. The panels are reprinted with permission from the publisher. (E) DNA nanorobot for targeted delivery of molecular payloads [133]. (F) Transferrin-loaded DNA origami for enhanced intracellular delivery into KB cells [101].

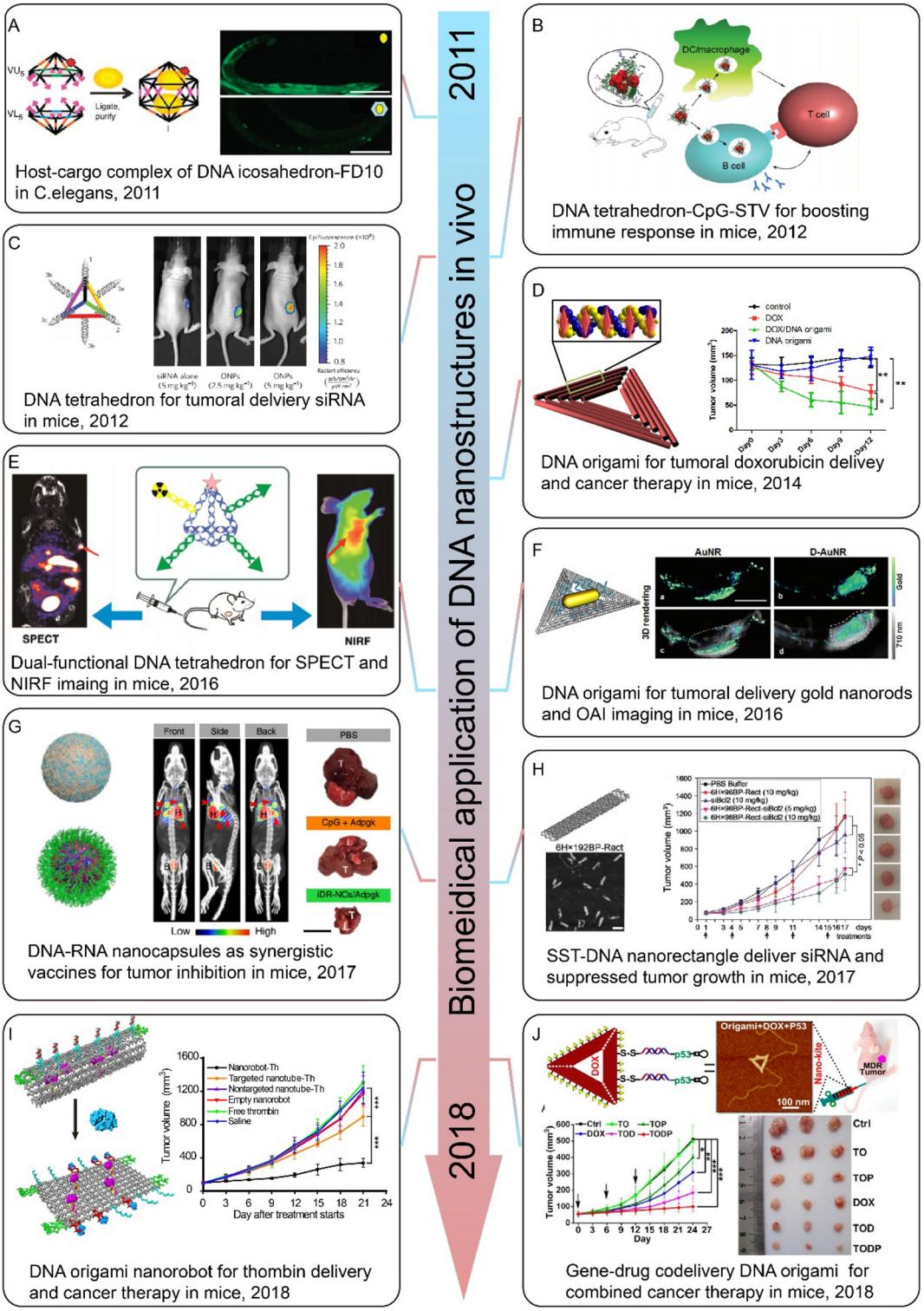


Fig. 8. Several examples of DNA nanostructures for *in vivo* cargo delivery. (A) DNA icosahedron-based host–cargo complex for functional *in vivo* imaging [123]. (B) Antigen and adjuvant conjugated DNA tetrahedron as a synthetic vaccine complex for immuno-stimulation *in vivo* [106]. (C) Multi-functional DNA tetrahedron for targeted *in vivo* siRNA delivery [25]. (D) DNA origami as an *in vivo* doxorubicin delivery vehicle for cancer therapy [27]. (E) Multiple-armed DNA tetrahedron for tumor-targeting, dual-modality *in vivo* imaging [26]. (F) DNA origami for tumoral delivery of gold nanorod and enhanced *in vivo* optoacoustic imaging [126]. (G) DNA-RNA nanocapsules loaded with tumor neoantigens as synergistic vaccines for cancer immunotherapy [61]. (H) DNA nanostructures derived from SST method as carriers for systemic delivery of Bcl 2-targeting siRNA and gene therapy [113]. (I) DNA origami nanorobot as an intelligent carrier of thrombin for on-target tumor infarction for cancer therapy [30]. (J) DNA origami nanokite as a co-delivery carrier of therapeutic p53 gene and doxorubicin for cancer therapy [144]. The panels are reprinted with permission from the publisher.

improved stability, and can be efficiently uptaken by macrophage-like RAW264.7 cells without transfection agents. After TLR 9 recognition, CpG-bearing tetrahedron triggered high-level production of pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-12, demonstrating the potent immunostimulatory effect of the nanostructures.

Liedl group provided another DNA carrier for CpG ODNs loading and immune stimulation [105] (Fig. 5B). They introduced a 30-helix tubular

DNA origami to serve as an efficient vehicle for CpG motif delivery in mammalian cells and immune stimulation. Each origami tube carried 62 CpG ODNs at the pre-designed binding sites by hybridization of the extended capture strands with the complementary anchor sequences of the CpG payloads. The DNA constructs displayed the increased loading efficacy of the molecular payloads and the enhanced cellular internalization after incubation with splenic macrophages. More efficient cellular uptake of CpG-containing DNA origami structures over free CpG ODNs might be due to the compactness and size of folded origami structures. The CpG-DNA constructs were located in the endosome of immune cells and the elevated production of cytokines was observed, indicating that the origami nanostructures decorating with CpG motifs greatly enhanced the immunostimulatory activities.

Ding group developed a self-assembled DNA dendrimer aiming at enhanced macrophage internalization and immune stimulatory effects [99] (Fig. 5C). By decorating TAT peptide and loop structured-CpG onto the surface of DNA dendrimer, a dual-functional nanocarrier was constructed. After modification with TAT peptides, DNA dendrimers loaded CpG ODNs exhibited increased intracellular accumulation. In contrast to DNA nanocarriers loaded CpG with linear-structure, DNA dendrimers decorated with CpG-containing hairpin-loops triggered stronger immune response characterized by pro-inflammatory cytokines production, which demonstrated that TAT-DNA dendrimers could serve as efficient vehicles for delivery of immunostimulatory CpG hairpin-loops motifs.

Besides those *in vitro* investigations of CpG loading DNA nanostructures, Rehberg group investigated the use of DNA-based nanotubes as carrier systems for CpG delivery and their effect on immune cells *in vivo* and in real time [109]. The 8-helix DNA nanotubes were designed by the single-stranded tiles (SST) methods, and 24 tiles in every tube were extended by CpG motifs. Similar to other DNA nanocarriers above, the CpG DNA tubes showed enhanced internalization and TNF-α response in RAW 264.7 macrophages. Next, the CpG-tubes were microinjected into skeletal muscle of anesthetized mice. Investigated by *in vivo* microscopy, CpG-DNA tube was observed to be internalized by tissue-resident macrophages and localized in their endosomes. In contrast to naked DNA nanotubes or unloaded CpG ODNs, the microinjection of CpG-decorated DNA nanotubes induced a significant recruitment of leukocytes into the muscle tissue as well as activation of the NF-κB pathway in surrounding cells, suggesting that DNA nanotubes were promising delivery vehicles to target tissue macrophages.

3.2.1.2. ASO and RNAi for gene therapy. Antisense oligonucleotides (ASOs), an important type of functional nucleic acid for regulating the expression, are synthetic DNA/RNA-like ODNs. Typically comprised of 16–21 nucleotides, ASOs bind to mRNA through sequence-specific Watson-Crick base pair interactions and exert their therapeutic functions by disrupting target gene expression [110].

Sleiman group generated DNA cages for ASO delivery and gene knockdown. They constructed 3D DNA nanocages with binding sites that were able to brace 1, 2, 4, and 6 ASO strands [111]. Phosphorothioated firefly luciferase ASO (PS-TOP4005) was chosen in their model system. Programmable assembly of these ASO-DNA cages was achieved and resulted in highly mono-disperse structures with controllable particle size and ASO location and density. In mammalian cells, significant and robust gene knockdown was triggered by these ASO-DNA cages. Because of increased stability of bound antisense units, ASO-DNA cages maintained gene knockdown levels more effectively than single and double-stranded controls. For another example of efficient delivery ASO and gene therapy, Tan group introduced size-controllable and stimuli-responsive DNA nanohydrogels as targeted delivery vectors [54] (Fig. 5D). Nanohydrogels were fabricated by three kinds of Y-shaped DNA building blocks. By incorporating targeting aptamers S6, disulfide linkages, and therapeutic ASO (ISIS5132) into different building blocks, the assembled nanohydrogels can be used for targeted and stimuli-responsive gene therapy. After incubation with S6 targeted human lung adenocarcinoma epithelial A549 cells, DNA nanohydrogels resulted in specific tumor cell internalization, significant inhibition of cell proliferation and migration. With properties of targeted cellular delivery, enhanced nuclease resistance and superior biocompatibility, DNA nanohydrogels can serve as efficient and safe nanocarriers for gene therapy.

RNA interference (RNAi) is a powerful and selective gene therapeutic strategy that induces target gene silencing. As it can be designed to regulate the expression of genes involved in all stages of tumor development (initiation, growth, and metastasis), the potential of RNAi has attracted tremendous interests in cancer therapy. Small interference RNAs (siRNA) are synthetic mediators of RNAi. They usually are dsRNA molecules (21 to 23 base pairs in length) and are designed to silence the expression of genes. By targeting disease-causing mRNA, siRNA can lead to their removal through degradation pathways. As a promising tool for cancer therapy, siRNA has been limited in clinical application for the lack of robust *in vivo* delivery systems.

Anderson group developed multifunctional DNA tetrahedron (~10 nm) for decorating therapeutic siRNA and targeted *in vivo* delivery [25]. Through programmable self-assembly of short DNA fragments for therapeutic siRNA, the siRNA-tetrahedral nanoparticles were prepared. The spatial orientation and density of cancer-targeting ligands (such as peptides and FA molecules) on the tetrahedron surface can be controlled by the overhang design of the DNA strands. With well-defined particle sizes and precisely controlled target ligands, the DNA tetrahedral nanoparticles can deliver siRNAs into tumor cells and down-regulate the target genes. In the KB tumor xenograft mouse model, FA-DNA tetrahedral nanoparticles showed longer blood circulation time ($t_{1/2} \approx 24.2$ min) than the unloaded siRNA ($t_{1/2} \approx 6$ min) and enhanced tumor accumulation. After systemic injection of a tetrahedron with FA-conjugated anti-luciferase siRNA into KB xenograft tumors, robust gene silencing was observed, without any detectable immune response.

Sleiman group designed another DNA cages for encapsulation of siRNA and conditional release the cargo molecules upon recognition of ODN triggers [112]. They constructed a DNA nanoswitch for siRNA encapsulation and selectively release. A DNA scaffold was designed to incorporate siRNA and formed a closed prism, with two gates recognizing a chosen trigger (mRNA or miRNA). When recognized by the marker strand, the two gating strands unwind by strand displacement, releasing an encapsulated siRNA. The siRNA encapsulated DNA cages showed a high production yield (~100%), which can sustain in biological conditions and remain responsive at the molecular level in a complex cellular environment (in fixed cells). This design of DNA nanoswitch for siRNA encapsulation and controlled release can be engineered to respond to genetic markers present in the disease cells or can readily be made to act as a dual therapeutic (ASO or miRNAs work as gating strands).

Ke and Shin used SST-DNA nanostructures (20–100 nm) to deliver siRNAs *in vivo* and demonstrated the therapeutic efficacy [113]. Rectangular or tubular DNA nanostructures of varied dimensions were assembled by the SST method. ssDNA captures that extended from the surface of DNA nanostructures were used for anchoring siRNA that targeted anti-apoptotic protein Bcl2 (siBcl2) through hybridization between handles and overhangs protruding from siBcl2. Both *in vitro* and *in vivo* experiments confirmed that the siRNA decorating DNA nanostructures can target and inhibit tumor cell proliferation with no toxicity, and the suppressed tumor growth in a xenograft model was specifically correlated with Bcl2 down-regulation.

3.2.2. Chemotherapeutic drugs and other small molecules

3.2.2.1. Doxorubicin and daunorubicin. As small molecular chemotherapeutic agents, the anthracyclines (doxorubicin and daunorubicin, *etc.*) are used to kill tumor cells by intercalating with DNA base pairs and inhibiting macromolecular biosynthesis. Utilizing the intercalating properties of drugs, several notable drug-loaded DNA nanoarchitectures

have been constructed, and the *in vitro* and *in vivo* therapeutic effects have been reported (Fig. 6).

Huang group introduced a DNA icosahedra nanostructure for doxorubicin loading and cancer therapy [114] (Fig. 6A). They created sticky-ended five or six-point-star motifs for construction of MUC-1 aptamer-DNA icosahedra through a sticky-end association between the tiles. Doxorubicin was then incorporated into DNA icosahedra where dsDNA served as the docking site for drug molecules. After incubated with MUC1-positive human breast cancer cell line (MCF-7; MUC 1^{+}), drug-loaded MUC-1 aptamer-DNA icosahedra showed improved cellular internalization and prominent cytotoxicity compared with bare drug or untargeted DNA icosahedra loaded ones. As mentioned above, Gu group introduced a multifunctional cocoon-like DNA nanoclew (NCl) for doxorubicin delivery and controlled drug release [60]. They utilized RCA method for nanoclew assembly, which integrated with multiple GC-pair sequences into the NCl for enhanced doxorubicin intercalation (Fig. 6B).

Ding group utilized triangular and tubular DNA origami nanostructures as carriers for doxorubicin delivery [115] (Fig. 6C). After drug molecules non-covalently attachment to DNA origami nanostructures through intercalation, doxorubicin-loaded DNA origami architectures were prepared and administrated to drug-sensitive or resistant human breast cancer cell line (regular MCF-7 or drug-resistant MCF-7). As "trojan horses", DNA origami nanocarriers enhanced drug accumulation in the diseased cells, and exhibited prominent cytotoxicity not only to regular MCF-7 but more importantly to doxorubicin-resistant cancer cells, inducing a remarkable reversal of phenotype resistance. In similar fashion, daunorubicin can be loaded into DNA nanostructures for cancer therapy. Castro group reported DNA nanostructures that were utilized as nanocarriers for circumvention daunorubicin drug resistance in a leukemia cell line model [116] (Fig. 6D). They fabricated rod-like DNA origami nanoarchitectures for drug incorporation. As another DNA "trojan horse", daunorubicin-loaded DNA nanostructures enhanced drug efficacy in leukemia cells displaying multi-drug resistance (MDR). The drug-loaded DNA nanostructures circumvent MDR1-mediated drug resistance in a resistant model of leukemia at clinically relevant drug concentrations, providing a rationale for exploring DNA origami as a drug delivery system in leukemia and other hematologic malignancies. Hogberg group introduced another doxorubicin-loaded DNA origami delivery system for cancer therapy [117] (Fig. 6E). They designed different DNA nanostructures with varying degrees of global twist for tuning the drug encapsulation efficiency and the release rate. Compared to free drug, doxorubicin-loaded DNA origami tubes showed enhanced cytotoxicity and lowered the intracellular elimination rate in breast cancer cell lines (MDA-MB-231, MDA-MB-468, and MCF-7). By tuning the origami nanostructure design, the drug loading efficacy and release rate can be controlled. The DNA carriers with tailored drug release kinetics were achieved.

Based on those *in vitro* reports, Ding group utilized different DNA origami nanostructures and investigated the shape-dependent tumor accumulation *in vivo* [27]. After intravenous injection, DNA origami displayed enhanced tumor passive targeting and long-lasting properties in tumor region. The triangular DNA origami exhibited optimal tumor passive targeting accumulation. Drug-loaded DNA origami (DOX/origami) was administered to nude mice bearing a human MDA-MB-231 orthotopic breast tumor and showed more specific anti-tumor efficacy without any observable systemic side effects *in vivo* compared to free drugs. The results suggest that DNA origami had immense potential as an efficient, biocompatible drug delivery vehicle for the treatment of cancer.

For enhancing the stability of DNA carriers, Lee and Ahn group reported mirror DNA tetrahedron nanostructures for tumor-specific delivery of doxorubicin [118]. L-DNA, a mirror form of natural D-DNA, was utilized for self-assembling DNA tetrahedron. The L-DNA tetrahedron showed identical intercalating properties of doxorubicin molecules D-DNA nanostructures, while possessing improved serum stability. This optimal stability of the L-DNA tetrahedrons provided the prolonged circulation time and better biodistribution. The L-DNA nanostructures delivered doxorubicin selectively to tumors with enhanced cellular and tissue penetration, offering greater anticancer effects as compared to conventional PEGylated liposomes. Next, Ko group studied *in vitro* and *in vivo* behavior of DNA tetrahedron as drug delivery carriers [119]. They conducted a pharmacokinetic study to examine the potential of L-DNA tetrahedron as a nanocarrier for *in vivo* tumor-targeted delivery of a low dose of doxorubicin (DOX). Compared with free drugs, doxorubicin-loaded L-DNA tetrahedron showed increased circulating half-life and decreased clearance, owing to their small sizes and prominent stability.

3.2.2.2. Radioactive isotope. Radioactive isotope technetium-99 m can be covalently attached to single-stranded DNA, next assembled to DNA nanostructures and exerted its function. Fan group developed multiple-armed DNA tetrahedral nanostructures (TDNs) as *in vivo* contrast agents to facilitate dual-modality imaging by near-infrared (NIR) fluorescence and single-photon emission computed tomography (SPECT) [26]. NIR dye Dylight-755 was conjugated to one of the core strands and self-assembled to TDN structures. The multiple-arms of TDNs were then utilized for decorating of FA (the targeting ligand)-labeled ssDNA and ^{99m}Tc (radioactive isotope)-labeled ssDNA. The dual-labeled molecular imaging tetrahedral probes exhibited the capability of targeted imaging in cancer cells and noninvasive tumor-targeting imaging in xenograft mice using both NIR and SPECT modalities. Using NIR fluorescence imaging, the pharmacokinetics of TDNs in mice was evaluated, which exhibited a distinctly different pattern of *in vivo* biodistribution and longer circulation time compared with those of dsDNA. Wang group reported a similar strategy for DNA bipyramid nanostructures (DBNs) anchoring with ^{99m}Tc as *in vivo* imaging probes [120]. DNA bipyramids with an overhang were assembled and then radiolabeled by ^{99m}Tc tagged single-stranded DNA. ^{99m}Tc attached DNA bipyramids as imaging probes were administrated in normal KM mice and the biodistribution and SPECT/CT imaging were performed. The *in vivo* imaging showed that ^{99m}Tc tagged DNA bipyramids mainly concentrated in the intestine, liver, and kidneys.

3.2.2.3. Other small molecules. Previously, Ding group developed a label-free imaging probe by utilizing DNA origami nanotubes anchored with carbozole-based biscyanine molecules. With "turn-on" fluorescence after biscyanine molecules absorbing to the DNA nanostructures due to restrict intra-molecular rotational motions (RIR emission characteristics), the DNA origami-RIR probe was successfully applied to intracellular imaging [24]. Liang and colleagues utilized triangular DNA origami nanostructures as carriers to load a similar carbazole derivative, 3, 6-bis[2-(1-methylpyridinium) ethynyl]-9-pentylcarbazole diiodide (BMEPC). The origami-BMEPC nanocomplex exhibited enhanced internalization and played the roles of both imaging and photosensitizing agents inside MCF-7 cells [121].

Similar to their CpG-DNA tubes assembled by SST method, Rehberg group recently developed dexamethasone-conjugated DNA nanotubes functioned in a target-specific and stimuli-responsive manner [122]. They utilized glucocorticoids (GCs) dexamethasone molecules as an anti-inflammatory agent, which reacted with amine-modified ssDNA (whose sequence was complementary with pH-sensitive i-motif) to form Dex-ODN conjugates. DNA nanotubes were constructed from 15 single-stranded DNA tiles, extending with a pH-responsive i-motif for dexamethasone-ssDNA assembly and the intracellular release of the anti-inflammatory drug dexamethasone. It is demonstrated that the dex-nanotubes were internalized by MH-S macrophages *in vitro* and by tissue-resident macrophages in the mouse cremaster muscle *in vivo* and localized in their endosomes. Microinjection of dex-nanotubes into muscle tissue of anesthetized mice triggered in a marked reduction of ischemia-reperfusion-elicited leukocyte transmigration and diminished vascular expression of the endothelial adhesion molecules. The

results indicated that DNA nanotubes could serve as an efficient and safe vehicle for *in vivo* delivery of the anti-inflammatory agent.

3.2.3. Nanoparticles and proteins

DNA nanostructures have been utilized as containers for large particles assembly. A variety of nanoparticles (quantum dots, gold nanoparticles, *etc.*), proteins (model antigens, immunoglobins, enzymes, *etc.*) and peptides have been used as functional payloads. Several nanoparticle or protein incorporated DNA materials and their biological applications will be summarized (Fig. 7).

3.2.3.1. Nanoparticles. Krishnan group introduced encapsulation of molecular cargo within DNA polyhedron structures for *in vivo* imaging in *Caenorhabditis elegans* (*C. elegans*), which is the first report on *in vivo* experiment by DNA nanostructures [123]. DNA icosahedral carriers were designed to host fluorescein isothiocyanate (FITC)-dextran (FD) as a cargo, uptaken by cell specifically, quantitatively and spatially mapping pH changes associated with endosomal maturation within the coelomocytes of *C. elegans*. The similar DNA icosahedron loading quantum dot and functionalized with endocytic ligands were designed and applied for monitoring compartmental dynamics and endocytic pathways, representing a new class of molecular probes for quantitative functional imaging [124].

Ding group provided a self-assembled DNA origami-gold nanorod (GNR) complex to work as a dual-functional nanotheranostics, by decorating gold nanorods onto the surface of DNA origami [125,126]. Capture DNA strands were extended from one arm of the triangular origami template serving as binding units to precisely organize one gold nanorod modified by complementary DNA strands of the captures (Fig. 7A). Compared with bare particles, DNA origami loading gold nanorod complex showed enhanced cell internalization, which may attribute to the size and shape of assembled triangular and tubular origami template. The structured DNA origami-gold nanorod complex was then utilized to perform photothermal therapy *in vitro* and *in vivo*. The complex worked as a dual-functional nanotheranostics and offered two-photon imaging and photothermal ablation in one DNA nanoplatform, suggesting a promising candidate for cancer diagnosis and therapy [125]. As optoacoustic signal contrast agent and photothermal generator, they next utilized those DNA origami-gold nanorod complex as multifunctional nanoplatforms to perform the *in vivo* cancer diagnosis and therapy simultaneously [126]. As an efficient probe of optoacoustic imaging (OAI), improved imaging quality and decreased dose of DNA origami-gold nanorod complex were achieved when the complex was administrated in the tumor-bearing mice. Simultaneously, DNA origami-gold nanorod complex responded to NIR irradiation for the photothermal therapy and effectively inhibited tumor regrowth, prolonging the survival of diseased mice. Ding group recently reported multiple functional DNA origami platforms integrating doxorubicin, gold nanorods and MUC-1 aptamer for circumvention of drug resistance [127] (Fig. 7B). The multi-functional DNA nanostructures showed enhanced delivery of drugs and nanoparticles, as well as down-regulated expression of P-glycoprotein, a multidrug resistance pump on the cell surface of multidrug-resistant MCF-7 cells upon near-infrared (NIR) laser irradiation. Enabling the synergistically chemotherapeutic and photothermal effects, the multi-functional DNA nanoplatform displayed improved tumor cell inhibition and circumvention of drug resistance.

Another example of nanoparticle decoration is used for visualization of the cellular uptake and trafficking of DNA origami. Barcoded gold nanoparticles onto the DNA origami nanostructures, Ke group provided a general methodology to track origami *in vitro* and to assess the cellular uptake efficiency of origami nanostructures influenced by sizes, shapes, and cell lines [128]. The gold NPs attachment enabled high-resolution cellular imaging at a single particle level by transmission electron microscopy (TEM) (Fig. 7C). Utilizing this methodology, distinct stages of internalization of origami tubes were directly investigated in cancer cells.

Chen group introduced ruthenium polypyridyl complexes (RuPOP) loaded tetrahedral DNA cages as unique nanoplatforms for cancer therapy [129]. The RuPOP was entrapped by DNA molecules and the conjugation of biotin was involved in the DNA cage, forming the cancer-targeted, biocompatible DNA nanosystem of metal complexes. Compared to free metal complex, the RuPOP-DNA cages showed enhanced specific cellular uptake, drug retention and cytotoxicity against HepG2 cells. The DNA nanosystem transported metal complex to cell nucleus after internalization, where the DNA cages were cleavage in response to DNases, leading to triggered drug release and induction of ROS-mediated apoptosis. In the tumor-bearing nude mice, the RuPOP-DNA cages exhibited tumor delivery of metal complex, and potent *in vivo* inhibition of tumor growth without observable toxicities.

3.2.3.2. Proteins and peptides. The organizational properties of DNA nanostructures have been used as synthetic hosts for proteins or peptides encapsulation. Yan and Chang group provided an approach for DNA tetrahedron as platforms to assemble model antigens (streptavidin molecules, STVs) and adjuvants (CpG ODNs) simultaneously [130]. Compared with a mixture of antigen and CpG molecules, the co-assembled STV-CpG-DNA tetrahedron (antigen-adjuvant complexes) triggered strong, specific and long-lasting immune responses *in vivo*, without eliciting an undesirable response against the DNA scaffold itself. Chen group recently reported RCA-driven DNA-RNA nanocapsules that incorporating CpG, *Stat3* short hairpin RNA adjuvants and tumor-specific peptide as nanovaccines for synergistic cancer immunotherapy [61]. RCA process produced tandem CpG and shRNA, which were then self-assembled into DNA-RNA nanocapsules. After shrunk by PPT-g-PEG, DNA-RNA nanoflower was further loaded with neoantigen *via* hydrophobic interactions. As the synergistic nanovaccines, DNA-RNA nanocapsules were delivery into antigen presenting cells (APCs) in draining lymph nodes (LNs), elicited potent and durable neoantigen-specific T cell responses and inhibited tumor progression.

Similar to Chen's DNA-RNA nanocapsules, Gu group generated self-assembled DNA nanoclews based on RCA for Cas9 protein and single guide RNA (sgRNA) co-delivery [131]. The DNA nanoclew structure was synthesized by RCA and then loaded with the Cas9/sgRNA complex through Watson-Crick base pairing. After coated with PEI for stabilization and enhanced endosome escape, the Cas9/sgRNA co-loaded DNA nanoclews were delivered efficiently to the nuclei of human cells for genome editing. While maintaining cell viability, the Cas9/sgRNA co-loaded DNA nanoclews enabled targeted gene disruption *in vitro* and *in vivo* (intratumoral injection), providing a potent and versatile strategy for delivering proteins. Further, they used another RCA nanoclews for delivery CpG and anti-PD1 antibody (aPD1) for cancer immunotherapy [108]. Through RCA reaction specifically based on a template encoded with the CpG sequence, the DNA nanoclew was assembled by a long-chain single-stranded DNA repeatedly containing interval CpG sequences and cutting sites of restriction enzyme *Hha*I. HhaI molecules were caged into triglycerol monostearate (TGMS, can be cleavage by esterases and matrix metalloproteinases (MMPs)), formed nanoparticles (TGMS HhaI NPs) and attached to CpG-containing nanoclews. The aPD1 could be loaded into TGMS-CpG-containing nanoclews by ultrasonication. The whole complex can be triggered by the inflammatory condition occurring in the wound site of the tumor resection incision, where TGMS can be enzymatically cleaved and release the HhaI. This can further sequentially digest DNA nanoclews to CpG ODNs and release aPD1. For remaining or metastasis tumors after the resection of primary tumors, the antitumor efficacy was substantially improved by the local injection of these multifunctional DNA nanoclew. This microenvironment-responsive controlled release of CpG and aPD1 was more effective than free CpG nucleotides and aPD1, while preventing the potential risk of toxic peak dosage of aPD1 in the body. Stevens

group also reported multiple protein-encapsulated DNA nanoflowers (derived by RCA) and their application in intracellular protein delivery [132]. In their report, cytotoxic protein RNaseA was used as model molecules to be delivered to the cells without a loss in its biological function and structural integrity, resulting in highly increased cell death in contrast to the free protein (Fig. 7D).

DNA origami nanostructures have been utilized as containers for protein encapsulation. Douglas group described a DNA origami barrel, which can incorporate proteins or nanoparticles to the DNA nanostructure and delivery the payload to specific cells [133] (Fig. 7E). As mentioned above, Kjems group introduced a strategy for construction of DNA origami rectangles modified by transferrin molecules, which functioned as tumor-targeting ligands [101] (Fig. 7F). Through the rational design, several origami nanostructures have been self-assembled for organizing multiple protein cargoes and exerting functions by induced conformational changes [134–136]. Andersen group introduced a DNA origami device that functioned as a nanoscale vault with controllable "lid" for containing an enzyme molecule [137]. A single enzyme was loaded into the cavity at the cargo-anchoring site (CAS) of the compact 3D DNA nanovault. After cargo loading, the openly stated DNA vault was then closed by adding a sequence-specific locking component. When a sequence-specific opening key was present in solution, the structures of the DNA vault was reconfigured, followed by exposure of the encapsulated enzyme. With the unique properties of reversible opening/closing, cargo loading and wall porosity, the DNA vault showed the control over the enzymatic reaction catalyzed by an encapsulated protease. The responsive system represented a general concept to control enzyme-substrate interactions by mediating conformational changes in a rationally designed DNA nanodevice.

3.2.4. Intelligent DNA nanodevices for stimuli-triggered structural reconfiguration and controlled drug release

Natural macromolecular systems with stimuli-responsive properties have evolved to play various essential roles in biological systems. Nature needs to spatiotemporally manage the conformational changes of self-assembled biomolecules, which are operated in dynamic conditions and responded to subtle biological cues to realize their functions. Inspired by natural macromolecular systems, the bottom-up design, construction, and operation of artificial devices and machines on the molecular scale are popular topics in nanoscience and technology [138]. As excellent building blocks for the design and construction of mechanical molecular devices, DNA molecules have been demonstrated to sense, actuate and exert critical functions. Several dynamic DNA nanostructures and artificial DNA molecular system have achieved fully controllable and complex movement of the molecular machine [69–72]. Besides those developments, DNA-based nanodevices and robots as imaging probes and cargo delivery vehicles have been constructed and applied *in vitro* and *in vivo*.

Krishnan group provided a series of molecular devices for quantitative functional imaging and cargoes delivery based on nucleic acids [139]. They utilized several synthetic DNA strands for self-assembly and engineered artificial functions into the DNA nanostructures. For functional imaging, they previously constructed DNA devices incorporating cytosine-rich oligonucleotides and named them as "I-switch", which can be triggered by protons and functions as a pH sensor based on fluorescence resonance energy transfer (FRET) inside living cells [140]. Next, they molecularly programmed the two DNA devices with different endocytic pathway markers, furin for retrograde endocytic pathway and transferrin for endocytic/recycling pathway [102]. Their work demonstrated that both nanomachines could successfully localize into the organelles for which they were designed, mapping pH changes within well-defined subcellular environments along both pathways inside the same cell. Differing from the pH-dependent approaches, they also used three strands (containing RNA aptamer and peptide nucleic acid modules) to construct DNA nanodevice and precisely measure chloride in organelles of living cells in a pH-independent manner. The DNA devices for chloride, (called "clensor"), were composed by sensing module (PNA sequence conjugated with chloride-sensitive fluorescent dye), normalizing module (DNA sequence bearing Alexa 647 fluorophore) and targeting module (DNA sequence expending with transferrin RNA aptamer). The nanodevice can precisely localize within organelles along the endolysosomal pathway and measure the activity and location of subcellular chloride channels and transporters in living cells. For normalizing and sensing moieties incorporated in one DNA device, the clensor was successfully used in quantitating the resting chloride concentration in the lumen of acidic organelles in *Drosophila melanogaster*.

Besides those imaging nanodevices, they introduced encapsulation of molecular cargoes within DNA icosahedral carriers for *in vivo* imaging (fluorescein isothiocyanate (FITC)-dextran (FD) [123] or quantum dot [124], Fig. 8), which were mentioned above. They next triggered the release of small molecules into their DNA nanocapsules for *in vivo* imaging probes or cellular signaling tools. Stimuli-responsive polymers (SRPs) bearing the small-molecule payload were synthesized and loaded in icosahedral-DNA nanocapsules [141]. When receiving a light stimulus, chemically modified dextrans entrapped in DNA nanocapsules released fluorophores which diffused out of the nanocapsule, or released a non-fluorescent protecting group leaving behind a fluorescent cargo within the nanocapsule. As imaging probes, DNA icosahedron showed cytosolic delivery of small molecules with the spatial resolution of single endosomes within a single cell *in vivo* (in *C. elegans*). These DNA nanocapsules reported on the extent of small molecules release after photo-activation, and highlighted the location where uncaging of the molecules occurred. These photo-responsive DNA nanocapsules were applied to release dehydroepiandrosterone (DHEA, a neurosteroid that promotes neurogenesis and neuron survival) and determined the timescale of neuronal activation, which can potentially generalize diverse stimulatory nanodevices to a variety of cells and pathways.

Those *in vitro* and *in vivo* reports about DNA nanodevices are all small assemblies (< 20 nm) fabricated by several DNA strands. Based on DNA origami nanostructures, Douglas group described a DNA nanorobotics system, which can transport molecular payloads to specific cells, sense cell surface inputs, trigger activation and reconfigure its structure for payload delivery [133]. They introduced a hexagonal DNA origami barrel (~40 nm), which can be noncovalently fastened in the front by staple strands modified with DNA aptamer-based "locks". Inspired by aptamer beacons and structure-switching aptamers, these DNA "locks" were designed to open in response to binding antigen "keys". Molecular payloads such as DNA modified-gold nanoparticles or antibody Fab' fragments can be loaded inside the nanorobots through hybridization with DNA linker strands inside the barrel structures. When confronted the human leukocytes with antigens expressed on their surfaces, the DNA nanorobots can be unlocked. The inside antibodies were then allowed to bind to cell-surface receptors and inhibit the growth of the target cells or induce cell signaling. Moreover, the two aptamer locks could also be programmed to recognize two different inputs (thus equivalent to a logical AND gate), and both keys were needed to activate the DNA robot. The DNA robots strategy could inspire new designs with different selectivities and biologically active payloads. Bachelet group studied the barrel-shaped DNA nanorobots and expended the bio-computing application in living cockroaches (*Blaberus discoidalis*) [142]. They utilized the aptamer-conjugated nanorobot systems to create architectures that emulated various logic gates (AND, OR, XOR, NAND, NOT, CNOT and a half adder). The dynamic DNA nanorobots showed that Boolean computations can be performed in living insects when suitable "keys", platelet-derived growth factor and vascular endothelial growth factor were triggered. In a recent study of this system, Bachelet group showed that DNA nanorobots inside cockroaches can be temporally controlled by human thoughts [143]. EEG patterns associated with cognitive states were recorded to control the state of an electromagnetic field [143]. The field controlled and induced local heating effects of DNA nanorobots by adding metal

nanoparticles to the robotic locks. Remotely triggered by human brain activities, DNA origami robots can be activated and subsequent exposure of a bioactive payload.

DNA-based nanodevices and robotics have been utilized as imaging probes and cargo delivery vehicles in cultured cells, multicellular organisms, and insects. However, both *C. elegans* and cockroaches showed differences from vertebrates, such as different circulatory blood system and body temperature, which could not provide direct information and prediction when DNA robotic system is utilized in mammals. To better mimic the environment of the human body, Zhao group recently used tumor-bearing mice as model animals, described a self-assembly DNA nanorobot and intravenously injected it into diseased mice to investigate its biological behavior and therapeutic efficacy [30]. The tubular DNA nanorobots functionalized with tumor endothelium-specific DNA aptamers on its external surface, and the blood coagulation protease thrombin within its inner cavity initiated tumor vessel occlusion and induced tumor necrosis when they are targeting transported to tumor vessels highly expressed of nucleolin, a tumor vascular endothelial marker protein. The nucleolin-targeted DNA aptamers (AS1411) were utilized as both targeting and triggering molecules for the mechanical opening of the DNA nanorobot, revealing thrombin to activate coagulation at the tumor site. The nanorobot-induced thrombosis of tumor-associated vessels resulted in tumor necrosis and inhibition of tumor growth for tumor-bearing mouse models of breast cancer, ovarian cancer, melanoma and lung cancer. With potent therapeutic effects of on-site tumor blood vessel infarction, the nanorobot has been proved safely and immunologically inert in normal mice and Bama miniature pigs. Recently, Ding group introduced a universal strategy to construct DNA origami-based co-delivery nanodevice [144]. These kite-like DNA nanostructures were rationally designed to incorporate p53 genes and doxorubicin molecules for cancer therapy. Combining tumor targeting delivery and controlled release of the therapeutic cargoes (genes and drug molecules), DNA nanokites exhibited effective inhibition of tumor growth *in vitro* and *in vivo* without apparent systemic toxicity. All these results showed that DNA nanorobots represent a promising strategy for precise drug delivery in cancer therapy.

4. Discussion and perspectives

Over the past decade, DNA nanocarriers have emerged as a novel and versatile platform to integrate the advantages of nanotechnologies and biological sciences. In this review, we summarized recent advances in self-assembly of DNA materials in nanomedicines. There has been a burst of interests in this rapidly progressing field with the development of biological materials for cancer treatment. DNA-based nanostructures have unique properties, given their uniform sizes and shapes, pre-designable and programmable nanostructures, site-specific surface functionality and excellent biocompatibility. These features allow DNA nanostructures to load therapeutic components such as small molecular drugs, DNA, RNA, proteins, and imaging contrast agents with high efficiency. Their intrigue features enable them to have precise tumor recognition, customized functions and optimal stability, making them highly attractive in many aspects of cancer treatment. Stimuli-triggered DNA nanodevices enable the presentation of active therapeutics at defined regions, which allows drug molecules to exert their functions at the desired sites. The “magic bullets” engineered by DNA self-assembly for intelligent drug delivery and controlled release could be expected.

However, the *in vivo* usage of DNA-based nanomaterials for cancer therapy is still in infancy. More studies about DNA nanoagents are needed to obtain key information on their potential clinical applications. The detailed *in vivo* parameters of DNA nanocarriers must be investigated and elucidated, including the circulating half-life, their pharmacokinetics, the size and shape-dependent properties for passive tumor targeting, the uptake and intracellular fate and the clearance mechanism. Naked DNA nanostructures possess negative charge densities. The relative high charges may affect their behaviors of systematic circulation, cellular trafficking and clearance. By functionalizing structures with positively charged molecules, PEGylated or other polymer shells coated DNA nanostructures have shown to improve stability and systematic circulation [18–20]. The polymer coating strategy of DNA nanostructures is compatible with further functionalization by ssDNA stabilized-gold NPs or streptavidin-modified quantum dots. The thickness of polymeric shells on DNA nanostructures can be controlled by monitoring the encapsulation conditions and processes. Choosing the suitable molecular weight of the polymer for shielding layers and the length of oligonucleotide linker on DNA nanostructures would ensure the well-defined shape and the control over ligand positioning.

The immune response of DNA nanostructures *in vivo* is yet another concern. Currently, most biological studies showed the immunogenic inert property of DNA nanostructures, which indicate their safety for *in vivo* application. Although DNA is a natural biopolymer, several conditions, such as CpG sequences or nucleotide modifications, may elicit immune responses. The desired functions can be achieved for self-assembled DNA architectures that either activate or down-regulate the immune response by controlling their sequences and structures. For instance, the immunogenicity of a DNA nanodevice could be tuned by varying the contents of immunostimulatory moieties (CpG motifs, dsRNA sequences, model antigens, *etc.*) in the nanostructure. Its immunostimulatory response can either be enhanced to produce potent immune adjuvants or decreased to fabricate inert carriers. Custom-designed immune responses of smart DNA architectures can be expected.

With the increasing knowledge on the design of DNA structures, the behaviors of DNA materials *in vivo* and the molecular interactions with cancer, it is highly possible that DNA-based nanocarriers could be customized to provide safe and efficient individualized applications for cancer treatment in the future. This will require the production of intelligent DNA nanostructures that can be assembled with multiple cargoes to facilitate drug combination or cocktail therapy. Furthermore, more specific recognition of the single diseased cells could be achieved by profiling the multiple membrane receptors, thus enabling more accurate disease diagnosis and therapy. The programmability and spatial addressability make DNA nanostructure to be one of the best candidates to control the structure-function relationship. The targeting ligands can be organized at pre-designed sites on the DNA platform. The number and distances of the targeting elements are able to be precisely controlled. In addition, logic gated dynamic DNA nanodevice with multiple targeting ligands could be designed to autonomously and precisely target cancer cells, which is able to advance the cell discrimination properties, especially in the aspect of reducing off-target toxicities of the drug-loaded DNA delivery system [133,145]. In addition, the tumor microenvironment heterogeneity should be considered for the intelligent nanocarrier design, because targeting both the tumor stroma and tumor cells may help increase the efficacy of anti-cancer drugs [146,147]. The DNA nanostructure-based delivery system can also be designed for cascaded drug delivery, spatiotemporally controlled release, and stimuli-responsive degradation based on their triggered structural reconfiguration. Up to date, the DNA-based nanocarriers have not been explored clinically. They are currently in rapid development and show great potential in designing new treatment strategies in the future. DNA-based nanocarriers would offer a flexible and powerful platform for both mechanism studies and possible clinical applications.

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