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DNA Nanostructures as Smart Drug-Delivery Vehicles and Molecular Devices

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DNA molecules can be assembled into custom pre-designed shapes via hybridization of sequence-complementary domains. The folded structures have high spatial addressability and a tremendous potential to serve as platforms and active components in a plethora of bionanotechnological applications. DNA is a truly programmable material and thus its nanoscale engineering opens up numerous attractive possibilities to develop novel methods for therapeutics. The tailored molecular devices could be used in targeting cells and triggering the cellular actions in the biological environment. In this review, we focus on the DNA-based assemblies – primarily DNA origami nanostructures – that could perform complex tasks in cells and serve as smart drug-delivery vehicles for *e.g.* cancer therapy, prodrug medication and enzyme replacement therapy.

Emerging DNA nanotechnology

The field of structural DNA nanotechnology started around 30 years ago when Ned Seeman performed pioneering research with DNA junctions and lattices [1]. To date, a cornucopia of different DNA nanostructures and shapes based on Watson-Crick basepairing [2] have been designed and demonstrated, and the whole research area has enjoyed a rapid progress especially during the past decade [3]. The key player in the fast development of DNA nanotechnology has been the invention of DNA origami in 2006 [4]. The DNA origami method is based on folding a long single-stranded "DNA scaffold strand" into a customized shape with a set of short synthetic staple strands. The robustness of the technique has made it widely accessible. Since then, the method has been generalized to 3D-fabrication [5-10], and it enables shapes with custom curvatures, twists and bends [11,12]. Very recently, techniques for modular scaffold-free fabrication [13,14], 3D meshing and wireframe-based approaches [15-17], as well as shape-complementarity-based construction [18] of DNA objects have been introduced. In addition, powerful computational tools for designing and analyzing DNA nanostructures have been developed [19-21], which appreciably help researchers to create their own DNA nanoarchitectures for any conceivable application. Table 1 lists and

describes the novel design strategies that can be used for fabricating diverse DNA origami nanostructures and other complex DNA-based shapes for various nanotechnological purposes.

The platonic DNA nanostructures and DNA origamis are by all means remarkably impressive examples of precise engineering and constructing at the nanoscale. However, the attractiveness of the origami method lies in the fact that one can add any desired functionality to the tailored DNA shapes by assembling other biomolecules and molecular components to them with nanometer precision. Importantly, DNA is inherently biocompatible, and its stability can be further tuned by the optional functionalization [20,22-24]. Aforementioned superior features can be eventually exploited in designing artificial DNA-based biomachinery, such as nucleic acid devices [25] and protein-DNA hybrid structures [26] for nanomedicine and biosensing. In this focused review, we discuss the recent progress of the nanomedical applications of self-assembled DNA systems; especially the drug delivery vehicles and nanomachines based on the DNA origami technique.

Towards DNA-based drug delivery vehicles and advanced therapeutics

The tremendous self-assembly properties of DNA can be exploited in creating various programmable shapes and larger assemblies for cellular delivery for e.g. cancer and enzyme replacement therapy. The very first DNA origami nano-objects proposed to work as molecular containers for drug delivery applications were single-layer origamis, which were further assembled into 3D shapes - a tetrahedron [5] or hollow cubes [6,7] (Fig. 1A). Since then, cellular uptake for various DNA structures (based on different design strategies) such as nanotubes [27,28], cages [29] and cubes [30] has been demonstrated both *in vitro* and *in vivo.*

The successful delivery of DNA-based structures into cells opens new avenues for tackling diverse medical tasks. Jiang *et al.* [31] and Zhang *et al.* [32] managed to deliver doxorubicin – an anticancer drug and a DNA intercalator – into cells *in vivo* using rod-like DNA origamis or DNA origami triangles as carriers (Figure 1B). Zhao *et al.* [33] demonstrated a similar system, except that they used a twisted 3D rod as a vehicle, which enabled tunable release of the doxorubicin molecules (Figure 1B).

Besides delivering drug molecules, small DNA-based assemblies can be used in functional *in vivo* imaging [34] and inhibiting protein expression by degrading mRNA *in vitro* [35]. The structures can also be utilized for transfecting small interfering RNA molecules (siRNA) for silencing genes in a controllable way. Kocabey *et al.* [36] tried to silence a target gene by equipping a small PEGylated folate -modified DNA nanotube (Figure 1C) with siRNA molecules, but did not succeed with that particular setup. However, Lee *et al.* [37] showed that folate -or peptide –modified tetrahedral DNA nanoparticles loaded with siRNAs (Figure 1C) can silence the target genes in tumors. Essentially, they noticed that the delivery of siRNAs strongly depends on the density and the spatial orientation of the cancer-targeting ligands of the nanoparticle. Similarly, it has been observed that the membrane receptor-mediated signaling in cancer cells can be regulated by adjusting the spatial orientation of the membrane-binding ligands with DNA origami “nanocalipers” [38]. These observations elegantly show the potential of DNA-based delivery systems, since the vehicle can be easily programmed and functionalized in a user-defined way.

Recently, a significant effort has been put in studying the DNA-based nanostructures as potential programmable immunostimulants. Schüller et al. [39] decorated a tubular DNA origami with tens of cytosine-phosphate-guanine (CpG) sequences (Figure 1D), and subsequently monitored the induced immune responses in spleen cells. They observed that the origami covered with CpG sequences ended up in the endosomes and induced a higher immunostimulation than the same amount of CpG sequences delivered into cells using a standard transfection reagent (Lipofectamine) system. In addition, origami-CpG-complexes were noncytotoxic, opposite to the Lipofectamine-based delivery. Later on, Sellner *et al*. [28] performed analogous and successful experiments with CpG-coated origamis *in vivo*. In addition, Li *et al.* [40] and Mohri *et al.* [41,42] have reported similar results when using RAW264.7 cells and tetrahedral DNA cages (Li *et al.*), polypod-like structured DNA (Figure 1D, Mohri *et al.*) or DNA dendrimers (Mohri *et al.*) as carriers for immunostimulatory CpG motifs.

These aforementioned results show that DNA-based designs have tremendous potential in fully tunable and triggered cell-targeting tasks and they could open up entirely new opportunities e.g. in cancer therapy. One of the most striking examples of highly sophisticated delivery systems is a logic-gated DNA origami nanorobot (Figure 1E) by Douglas *et al*. [43]. The nanorobot can sense the target cell surface proteins, which trigger conformational change of the robot. Thus, based on the regulation of the pre-programmed aptamer-based logic-gates, the robot can selectively transport the molecular payload to the cells. In the proof-of-principle experiments, it was shown that the nanorobots equipped with specific antibodies were able to manipulate target cell signaling (in several different cell types) when released.

Despite all the recently reported achievements in the field, an efficient transfection method for the DNA-based assemblies is still urgently needed. Due to the polar nature of DNA origami structures, it has been noticed that they are poorly transfected [44], and they often need peptide-, cationic polymer- or lipid-modifications for successful transportation into cells. However, there exist several ways to improve the delivery efficiency. Recently, Brglez *et al.* [45] showed that the specific DNA intercalators can modify the surface properties of DNA origami objects, and subsequently improve the transfection rates.

Other alternative approaches are based on either directly taking advantage of the virus particles or mimicking virus performance [46]. Mikkilä *et al.* [47] combined virus capsid proteins (CPs) of cowpea chlorotic mottle virus (CCMV) with rectangular DNA origami sheets, resulting in CP-origami complexes with different morphologies (Figure 1F). The highly flexible DNA rectangle could adopt either a rolled-up or a completely encapsulated form depending on the amount of CPs mixed with DNA origamis. The transfection rates of these CP-origami complexes to the human cell line HEK293 were significantly higher compared to bare origamis (bare origamis showed negiligible rates even when combined with the common Lipofectamine-system). Importantly, there was no detectable toxicity present in the experiments. In addition, the cellular transfection rates continuously improved with the increasing amount of added CPs. This might be attributed to the common assumption that the rigid and compact DNA structures are transfected more efficiently than the highly flexible ones. Equally inspired by the viruses, Perrault and Shih [48] ingeniously encapsulated spherical DNA origami designs in PEGylated lipid membranes (Figure 1G). The coating provided a proper protection for DNA origami nanostructures against nuclease digestion,

and in the *in vivo* experiments, immune activation of the objects was significantly decreased, whereas pharmacokinetic bioavailability of the particles improved remarkably.

As a final note to this section, there exist yet more compelling DNA-assembly –based techniques for therapeutic delivery purposes. Most of them rely on aptamer-based assemblies, such as those demonstrated by Wu *et al.* [49] (Figure 1H) and Charoenphol and Bermudez [50]. As a conceivable alternative to utilizing discrete DNA nano-objects in molecular transporting, the drugs can be also released from structurally stable and large self-assembled thin DNA films as Cho *et al.* [51] have proposed (Figure 1H).

Smart DNA nanodevices and molecular platforms

Besides serving as nanocarriers for drug molecules, DNA-based structures can act as tiny molecular devices in living systems. DNA nanodevices could be used in assembling pivotal molecules, such as proteins and nucleic acids in cells and in performing complex pre-programmed tasks with them. These molecular-scale platforms could help us to construct entire "nanofactories" with fully tailored assembly lines [52]. Fascinating examples of nanoscale engineering include the possibility to organize intracellular reactions using RNA-scaffolds [53] and activate enzyme cascade reactions with DNA-based nanoassemblies [54].

DNA origami – with its high spatial addressability – provides an impeccable platform for designing customized biomachinery and controlling reactions at single-molecule level. Voigt *et al.* [55] demonstrated this by controlling and imaging the successive chemical reactions on the rectangular DNA origami platform (Figure 2A). In addition, DNA-based nanodevices can be utilized in controlling and actuating enzymatic reactions in sophisticated manner [56]. Liu *et al.* [57] built a tweezer-like DNA device and placed an enzyme and its cofactor to the tips of the arms (Figure 2B). They were able to control the enzymatic activity by opening and closing the tweezers using additional DNA oligonucleotides as fuel. Fu *et al.* [58] fabricated multienzyme cascades on DNA templates, where substrate channeling was realized using a DNA strand as a flexible swinging arm between the immobilized enzymes. Linko *et al.* [59] showed how modular DNA origami building blocks could be assembled together to form a tubular enzyme cascade nanoreactor (Figure 2C) for detecting glucose. The device could find use as a biosensor or a delivery vehicle for enzymatic payloads e.g. in enzyme replacement therapy.

A slightly different but equally attractive perspective is to take advantage of DNA origami objects for studying the properties of motor protein ensembles. The work by Derr *et al.* [60] demonstrated how a programmable 3D DNA origami template with specific binding sites was used as a cargo mimic for the proteins (Figure 2D). By selecting the amount and types of motor proteins attached to the cargo mimic, they managed to arrange a molecular tug-of-war for studying the collective motility of the motor protein ensembles *in vitro*.

One of the implementations that highlights the impact of the DNA origami technique is the work by Langecker *et al.* [61], which demonstrates the possibility to build artificial ion channels in lipid membranes using DNA origami devices (Figure 2E). The DNA origami can be anchored to vesicles by cholesterol linkers, which enable the needle-like device to punch a hole in the membrane and form a channel for molecular transportation. In addition, Burns *et al.* [62] showed that similar DNA

structures equipped with hydrophobic ethyl phosphorothioate belts (Figure 2F) can be inserted into cell membranes. Interestingly, these artificial nanopores can cause the death of the cells after insertion, indicating that this kind of structures could be used in controlling the viability of cancer cells.

For therapeutic uses of the nanomachines, the work by Amir *et al.* [63] promisingly extends the biocomputer-inspired methods previously presented by Douglas *et al.* [43]. Amir *et al.* showed an elaborate example of creating controllable devices by building dynamic DNA origami nanobots capable of interacting with each other in living animals. The nanobot-interactions generate logical outputs, which are subsequently used to tweak the release of the molecular payloads in cells (Figure 2G). These achievements in creating computerized living systems make precisely targeted drug delivery and prodrug medication accessible, and eventually they open up completely new opportunities for health applications.

Concluding remarks and future perspectives

In this review we have briefly discussed the recent and the most potent approaches of using DNA- and DNA origami-based nanostructures in pharmaceutical applications. In structural DNA nanotechnology, the transition from platonic structures to nano-objects that can perform predefined tasks and be used in sophisticated implementations has been extremely fast. However, there are still plenty of challenges as well as unexplored opportunities (see the "Outstanding Questions" Box). The advantages in using DNA-based nanostructures in therapeutics over the other accessible nanosized systems are not only limited to their intrinsic biocompatibility and biodegradability. The most important aspect is the modularity; the size of an object and the positions of modifications (ligands and other molecules) can be precisely controlled at nanometer scale and moreover, the shape and the flexibility of the object can be fine-tuned. These superior and adjustable properties facilitate straightforward characterization of the DNA nanostructures (labeling/bioimaging) and entirely engineered targeting and releasing features for delivery purposes. Therefore, we believe that the proposed and imminent DNA-nanoassemblies will have a huge impact on advanced health sciences and clinical chemistry.

Undoubtedly, despite all the advantages and expectations, there are some obstacles that need to be tackled. One of them is to improve the pharmacokinetic bioavailability of the DNA-based structures *in vivo*. DNA origami structures can survive in cellular milieu [22] and against nucleases [20], but the circulation times of the objects could be increased by adjusting their modular properties (see above) or by creating novel protecting strategies such as protein- [47] or lipid membrane coatings [48].

Another challenge in creating DNA-based delivery vehicles and nanomachines is arguably the relatively expensive synthesis of the starting materials. However, since the entire research field is constantly growing and developing, it is likely that scaling up of the production and simultaneous knockdown of the price will take place rapidly [3,64]. Very recent examples [65-68] promisingly show that production quantities of pure DNA origami objects are currently about to upgrade from micro-/milligram scale to gram scale. Today, 1 gram of scaffolded megadalton-sized DNA origamis costs approximately 100,000 $. However, if the trailblazing printing techniques for sequence synthesizing and efficient enzymatic amplification and purification methods will be further developed, in the near future 1 gram of pure DNA origami objects could be purchased for

as low as ~1,000 $ [64]. On the other hand, the expected reduction of the price of synthetic DNA would mean that larger and therefore even more complex DNA structures could become affordable. These attainable structures could find use in biomimetic systems; artificial enzyme-like objects and innovative sensing devices could be created. By combining a new level of structural complexity with rapidly evolving biocomputing techniques one could possibly develop artificial immune systems; fully programmable nanorobots capable of detecting various agents *in vivo* and subsequently reacting / protecting against diseases.

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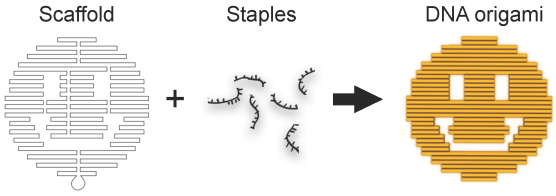
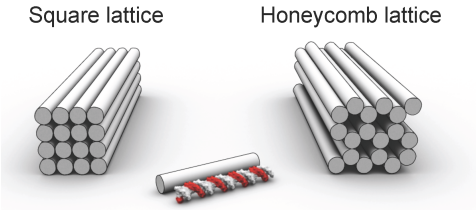
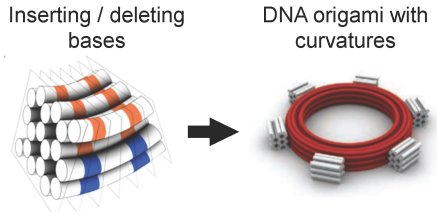
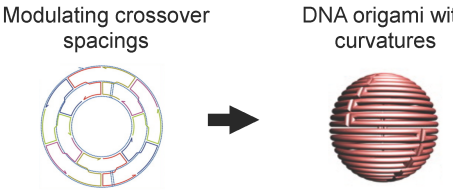
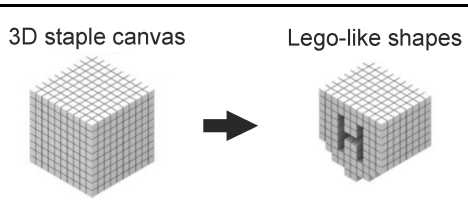
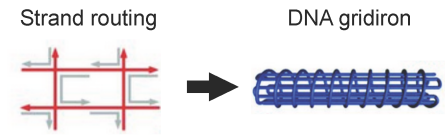
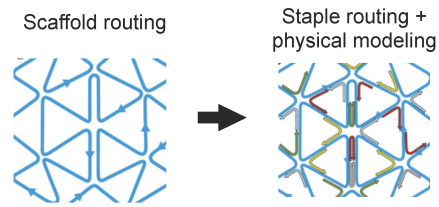
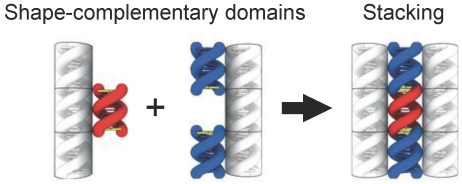
Table 1. Design strategies for creating diverse DNA nanostructures

	**2D DNA origami** [4]: ~7000 nucleotides long single-stranded DNA "scaffold" is folded into a desired shape with the short synthetic ssDNA strands called "staples" in a thermal annealing process. Yellow cylinders represent dsDNA domains formed *via* Watson-Crick basepairing of the DNA strands.
	**3D DNA origami** [8,20]: The method described above can be extended to 3D fabrication. Multilayered DNA origamis are commonly designed either in a square [9] or a honeycomb lattice [8] using caDNAno software [18] (hexagonal and hybrid lattices also possible [10]). Gray cylinders represent dsDNA domains as depicted in the foreground.

	**DNA origami with bends and twists** [11]: By inserting or deleting bases in the design (modulating the distances between crossovers), it is possible to induce twists and bends to the structures. Yellow segments have less, and blue ones more than 10.5 basepairs (bp) per turn (natural B-form of DNA).
	**DNA origami with curvatures** [12]: Similarly to the above, by adjusting the crossover spacings between the adjacent helices (distance between the crossovers increases from inner to outer circles), one can create DNA origamis with different curvatures.
	**Scaffold-free DNA shapes** [13,14]: Short ssDNA strands, a.k.a. "DNA bricks" form a cubic-like molecular canvas. By selecting subsets of the strands, one can create hundreds of different shapes. Each cube (voxel) represents an 8 bp interaction between the neighboring strands.
	**DNA gridiron** [15]: Complex DNA structures can be created *via* 3D meshing by applying it to nanoscale engineering. Four-arm junctions are used as vertices, and the scaffold strand can be directed through vertices in multiple directions.
	**DNA rendering of polyhedral meshes** [17]: A scaffold is routed using an algorithm that takes into account the rules and constrains of a 3D polyhedral meshing. Staple strand routing is added and, before the staple sequences are computed, the physical model is generated to permit strain in the structure to be relaxed.
	**Shape-complementarity-based construction** [18]: DNA origamis can be programmed into larger assemblies by taking advantage of non-basepairing interactions. A red dsDNA protrusion fits to the designed recession shown in blue. The domains are "glued" together *via* short-ranged nucleobase stacking bonds.

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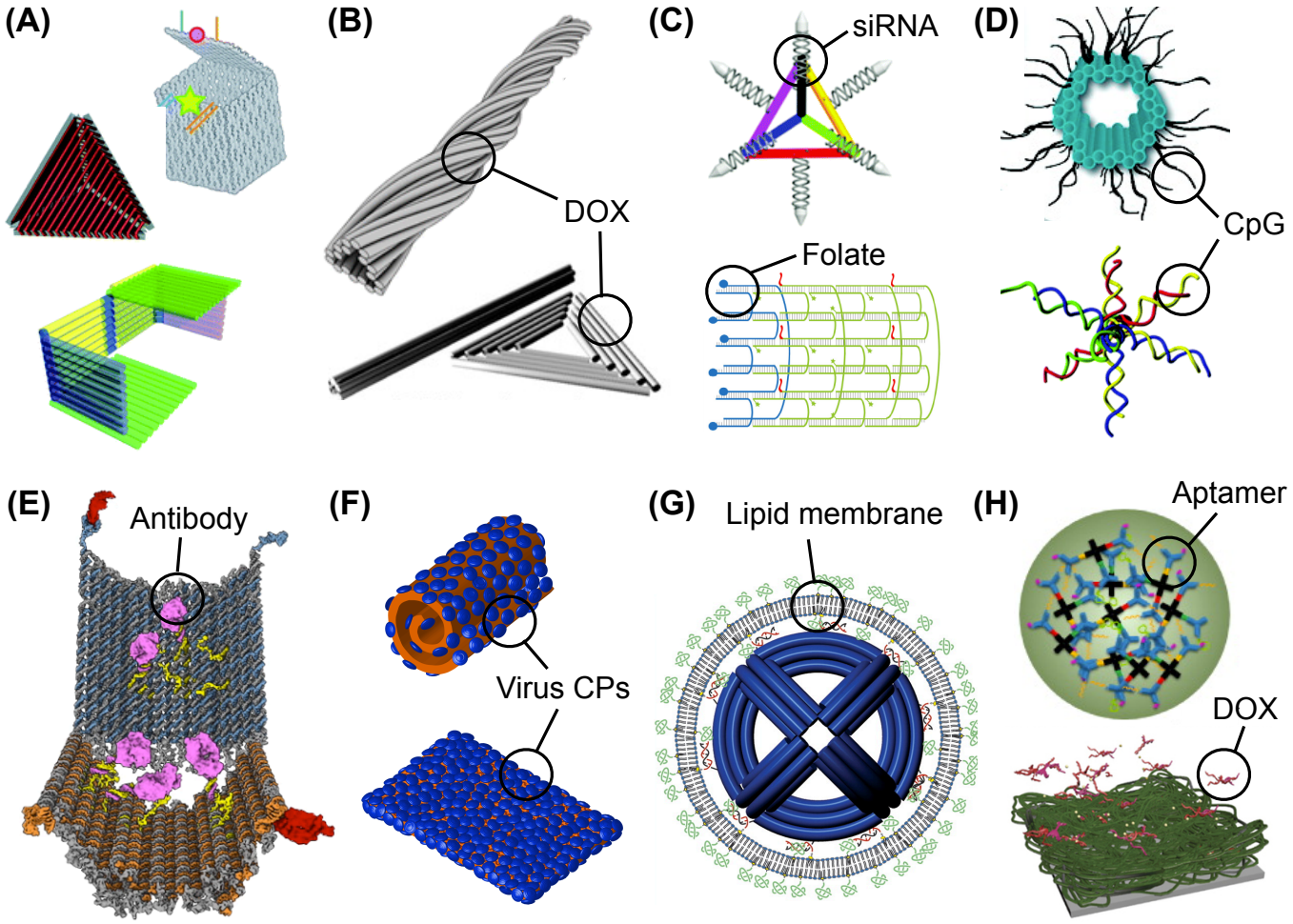

Figure 1: Potential DNA-based drug delivery vehicles and devices for triggering cell signaling. (A) DNA origami molecular containers: a box with a switchable lid [6], a tetrahedron [5] and a two-state box [7]. **(B)** DNA origami nanostructures for delivering doxorubicin (DOX) into cancer cells: an under-twisted rod-like shape [33], a straight rod [31] and a triangle [31,32]. **(C)** DNA structures for small interfering RNA (siRNA) delivery: a cage with cell-targeting ligands (folate or peptide) at the end of the siRNA motifs [37], and a PEGylated folate-modified nanotube [36]. **(D)** DNA structures for CpG-triggered immunostimulation: a DNA origami tube [39] and a polypod-like structure [41]. **(E)** A smart logic-gated DNA origami nanorobot for targeting cells and subsequently displaying the molecular payload [43]. **(F)** Rectangular DNA origamis coated with virus capsid proteins (CPs) for efficient cellular delivery: the complexes can adopt different conformations depending on the added capsid protein concentration [47]. **(G)** A virus-inspired membrane-encapsulated spherical DNA origami vehicle for decreasing immune activation and enhancing pharmacokinetic bioavailability [48]. **(H)** DNA-based assemblies for cancer therapy: an aptamer-based DNA nanosystem for targeted delivery [49] and a thin DNA-structured nanofilm for controlled release of doxorubicin in a serum environment [51]. *A tetrahedron was adapted with permission from Ref. [5]; Copyright (2009) American Chemical Society. A box with a lid was adapted with permission from Ref. [6]; Copyright (2009) Nature Publishing Group. A two-state box was adapted with permission from Ref. [7]; Copyright (2009) Royal Society of Chemistry. A straight rod and a triangle were adapted with permission from Ref. [31]; Copyright (2012) American Chemical Society. An under-twisted rod was adapted with permission from Ref. [33]; Copyright (2012) American Chemical Society. A folate-modified nanotube was adapted with permission from Ref. [36]. siRNA cage was adapted with permission from Ref. [37]; Copyright (2012) Nature Publishing Group. A CpG-DNA origami tube was adapted with permission from Ref. [39]; Copyright (2011) American Chemical Society. A polypod was adapted with permission from Ref. [41]; Copyright (2012) American Chemical Society. A nanorobot was adapted with permission from Ref. [43]; Copyright (2012) The American Association*

for the Advancement of Science. Rectangular DNA origamis coated with virus proteins were adapted with permission from Ref. [47]; Copyright (2014) American Chemical Society. A membrane-encapsulated origami was adapted with permission from Ref. [48]; Copyright (2014) American Chemical Society. An aptamer-based DNA nanosystem was adapted with permission from Ref. [49]; Copyright (2013) American Chemical Society. A DNA nanofilm was adapted with permission from Ref. [51].

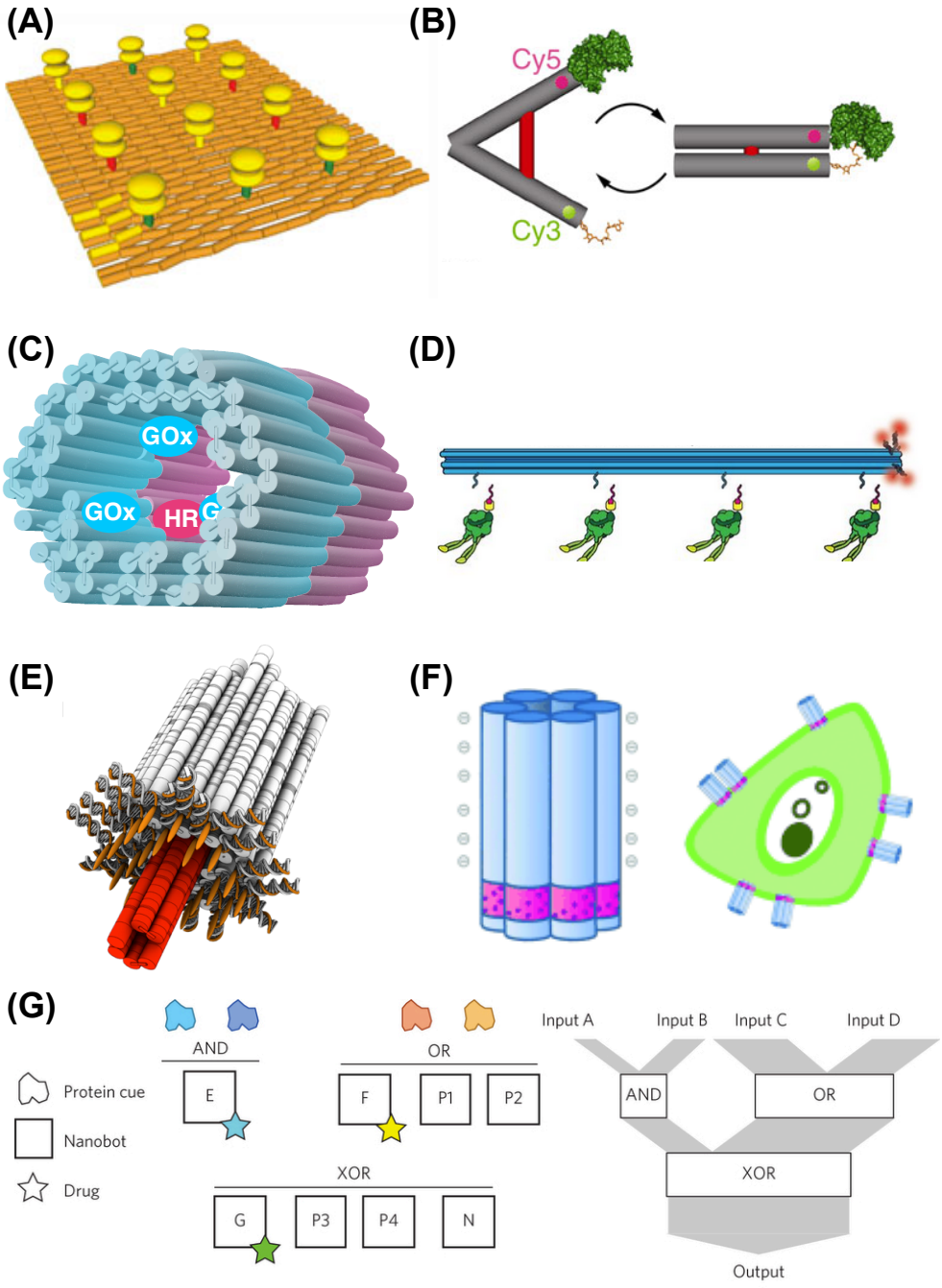

Figure 2: Smart DNA nanodevices and molecular scale platforms. (A) A DNA origami platform for controlling single-molecule chemical reactions [55]. **(B)** A switchable tweezer-like enzymatic nanoreactor [57]. **(C)** A tubular DNA origami-based enzyme cascade nanoreactor for biosensing [59]. **(D)** A cargo mimic for dynein motor proteins [60]. **(E)** A DNA origami that can form an ion channel in a lipid membrane [61]. **(F)** A DNA nanopore with a hydrophobic belt [62]. The structures can be inserted into cell membranes, and the pores can kill cancer cells. **(G)** DNA origami nanorobots can interact with each other in living animals and emulate various logic gates for medical tasks such as a tailored release of molecular payloads [63]. *A DNA origami platform was adapted with permission from Ref. [55]; Copyright*

(2010) Nature Publishing Group. DNA-tweezers were adapted with permission from Ref. [57]; Copyright (2013) Nature Publishing Group. A DNA origami nanoreactor was adapted from Ref. [59] with permission from Royal Society of Chemistry. A DNA origami as a cargo mimic was adapted with permission from Ref. [60]; Copyright (2012) The American Association for the Advancement of Science. A DNA origami – based ion channel was adapted with permission from Ref. [61]; Copyright (2012) The American Association for the Advancement of Science. DNA nanopores with hydrophobic belts were adapted with permission from Ref. [62]; Copyright (2014) John Wiley and Sons. Logic gate operations for DNA origami nanorobots were adapted with permission from Ref. [63]; Copyright (2014) Nature Publishing Group.

"Trends" Box:

* Rapid evolving of structural DNA nanotechnology: from platonic DNA structures to functional DNA devices
* Tailored DNA-based assemblies can be used as advanced drug delivery vehicles in various therapeutic applications
* DNA nanodevices can target cells and trigger single-molecule level reactions in biological environment
* Functional DNA nanostructures can serve as versatile molecular machines and preprogrammed templates in cells

"Outstanding Questions" Box:

* How to improve the delivery of DNA-based structures and control their stability *in vivo*? For some applications, protection mechanisms are urgently needed since the structures are often sensitive to cellular milieu and depletion of salt ions. However, some studies show that DNA structures are relatively stable in cell lysates and against nucleases. Protection and customized properties of DNA structures (size, shape, rigidity, ligand modifications, control over the surface charge, etc.) are key elements in improving pharmacokinetic bioavailability.

* How to scale-up the fabrication of DNA origami nanostructures (to gram/kilogram scale) and how to knock down the relatively high cost of synthetic DNA strands? At present, 1 gram of DNA origami objects (origami size at megadalton regime) costs ~100,000 $. However, production costs of synthetic DNA strands are constantly decreasing. The price per synthesized base on small scales can be expected to come down up to 100-fold if advanced sequence-printing techniques as well as efficient amplification and purification methods become widely accessible.

* How to create even more sophisticated DNA-based biodevices, circuits and systems? If the price of synthesis can be significantly reduced, larger and more complex DNA structures can be created. This could open up new avenues in developing user-defined biomimetic systems, e.g. synthetic enzymes and complex organelles, hybrid DNA-protein nanopores, actuators, smart sensing devices, fully programmed drug-delivery systems and artificial immune systems based on DNA nanorobots.