Nano Today 26 (2019) 98–107

Contents lists available at ScienceDirect

Nano Today

journal homepage: www.elsevier.com/locate/nanotoday

Review

DNA nanostructures *in vitro*, *in vivo* and on membranes

Wooli Bae [1], Samet Kocabey [2], Tim Liedl *

Ludwig-Maximilians-Univeristy, Geschwister-Scholl-Platz 1, 80339, Munich, Germany

ARTICLE INFO

Article history:
Received 17 December 2018
Received in revised form 1 February 2019
Accepted 18 March 2019
Available online 25 April 2019

Keywords:
DNA nanostructures
DNA origami
DNA structure stability
Lipid membrane
Supported lipid bilayer
Cell membrane
DNA nanopores

ABSTRACT

Recent developments in DNA nanotechnology brought rich structural and functional diversity. However, for DNA nanostructures to perform in a biologically relevant context, obstacles such as nuclease activity and low divalent ion concentrations have to be addressed. For drug delivery or gene therapy applications, ultimately the lipid membrane barriers must be targeted and overcome. In this article, we highlight efforts and achievements in enhancing the stability of DNA nanostructures including chemical modifications, covalent crosslinking and coating with protective layers composed of polymers or lipids. We then review interactions between DNA nanostructures and lipid membranes, which are often mediated by ligands for membrane receptors or hydrophobic domains incorporated into the structure. Finally, we present applications of DNA nanostructures on and in lipid membranes, including higher order assembly, controlling membrane curvature, targeting and arranging membrane proteins in living cells and DNA-based synthetic lipid channels.

Contents

Introduction

The development of nanomachines that autonomously act inside the human body to diagnose and cure diseases is a long-standing dream in natural sciences. Consequently, countless efforts of researchers from a variety of disciplines aim towards fulfilling these goals with one approach being based on the molecular recognition properties of complementary DNA strands. DNA, the molecule encoding genetic information, was identified as a building material for self-assembling nanostructures with Ned Seeman's proposal to design two- and three-dimensional networks from DNA junctions [1]. Today, DNA is one of the most popular building materials for self-assembly as it is commercially available, biocompatible, programmable in sequence and has intrinsic nanoscale features. With recent developments of the field, it is now possible to design and analyze DNA nanostructures with the help of a variety of software packages [2] and assemble almost any desired shape and functionality.

For biological or future medical applications, DNA nanostructures and DNA devices must perform in demanding conditions. The

* Corresponding author.
E-mail address: tim.liedl@physik.lmu.de (T. Liedl).
[1] Current address: Department of Bioengineering, Imperial College London, SW7 2AZ, London, United Kingdom.
[2] Current address: Department of Medicine, University of Fribourg, Ch. du Musée 18, 1700 Fribourg, Switzerland.

https://doi.org/10.1016/j.nantod.2019.03.001
1748-0132/

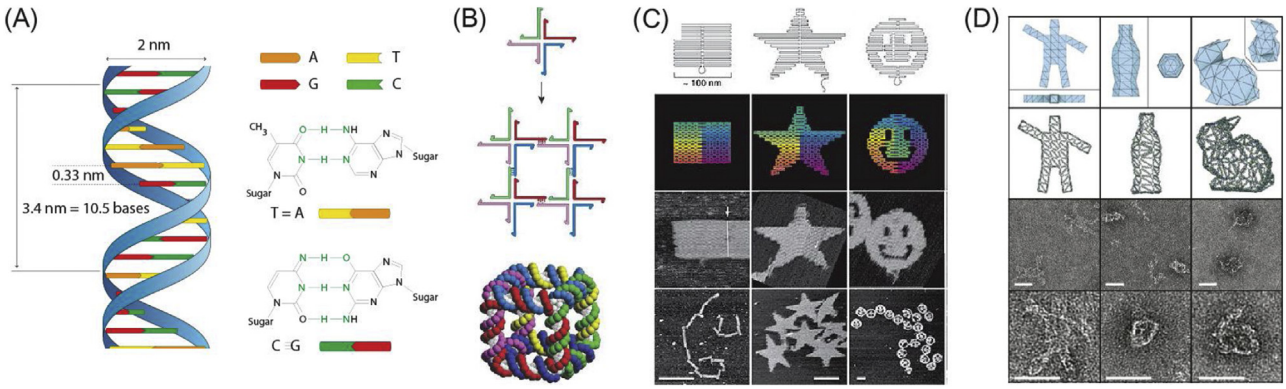

Fig. 1. DNA nanostructures. (A) Structure and base pairing of B-form double-stranded DNA. (B) Ned Seeman's initial approach to build lattices from Holliday junctions (top) and his DNA cube (bottom). Adapted with permission [18]. Copyright 2003, Springer Nature. (C) Scaffold path (upper two rows) and AFM images (bottom rows) of DNA origami sheets, stars and smilies. Adapted with permission [19]. Copyright 2006, Springer Nature. (D) 3D meshes rendered in DNA of a human figurine, a bottle and the Stanford bunny. Adapted with permission [20]. Copyright 2015, Springer Nature.

structures are usually assembled in specific folding buffers with high concentrations of Mg^{2+} ions ($\geq$ 10 mM) but are required to maintain structural and functional integrity inside biological fluids or tissues, where typically less than 2 mM of divalent ions are present [3]. Only if enduring this challenge, a DNA structure can interact with a targeted cell type where the structure encounters the next hurdle: the cellular membrane, which separates the cell from the outer environment and also serves as the interface and selective gate for the cells. Thus, targeting DNA nanostructures to cellular membranes and membrane-associated proteins provides a possible point of control of cellular viability and function.

The trafficking of nutrients, drugs, and neurotransmitters into living cells across the cellular membrane is mediated by an elaborated system of membrane-associated proteins, such as SNARE [4,5] or Clathrin [6]. These helper-proteins facilitate the engulfment of membrane patches to form intracellular vesicles or enable the fusion of vesicles with membranes. Membrane-associated proteins further act as reporter molecules to guide the transport of incorporated content within the endosome. There also exist a wide variety of membrane-spanning receptor proteins that can trigger intracellular pathways that lead to cAMP signaling, immunostimulatory responses, or even apoptosis. Examples are the family of G protein-coupled receptors [7], the multi-protein complexes forming fas-receptor-mediated Death Inducing Signaling Complexes (DISC) [8], the tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptors (TRAIL-R) [9] and the family of toll-like receptors that recognize structurally conserved molecular motifs from pathogenic organisms [10].

DNA nanostructures can be designed in such a way that they specifically bind to and interact with lipid bilayers. One of the opportunities offered by DNA nanotechnology is to study the intricate mechanisms that allow membrane-associated proteins to alter the physical behavior of cellular membranes. The possibility to artificially mimic biological carrier systems or cascade-triggering events will on the one hand improve the understanding of molecular trafficking and will on the other hand be of great interest for the establishment of concerted drug delivery systems. Once these cellular mechanisms become engineerable, it will be possible to create new pathways to direct nucleic acids to the genetic machinery of the interior of the cell and to overcome current barriers to pharmaceutical substances [11,12].

The nanoscale dimensions and designable functions of DNA structures render them also suitable for studying lipid domains in biological membranes which otherwise are difficult to access due to their small size and transient nature [13].

In this article, we will first introduce DNA nanostructures with complex geometries and then discuss approaches to increase their stability in biologically relevant environments. After touching on the composition and properties of biological lipid membranes we will review recent findings obtained from DNA nanostructures on and in lipid membranes. We refer the reader to other review articles on DNA structures interacting with living cells as molecular drug delivery vehicles or stimulants of immune responses [14–17].

DNA nanostructures

DNA is a biopolymer encoding genetic information for most living organisms. It consists of four different bases; Adenine (A), Thymine (T), Guanine (G) and Cytosine (C) with A-T and C-G forming selective Watson-Crick base pairing through hydrogen bonding and planar stacking of neighboring base pairs (Fig.1A) [21]. With this highly sequence-specific binding, two DNA strands with complementary sequences hybridize in an antiparallel fashion to form the well-known right-handed double helix, which is the prevailing form of DNA in nature. The famous X-ray scattering experiment by Franklin and Wilkins and its interpretation by Watson and Crick further revealed that the distance between base pairs in the DNA double helix is 0.34 nm with DNA making one full helical turn every 10.5 base pairs [21,22]. Single strands branching from one helix and entering another one can connect DNA double helixes in many possible ways. A specific branching motif, which naturally occurs during homologous recombination or repair of double strand breaks is the Holliday junction (Fig. 1B) [23]. Ned Seeman suggested using this and other DNA multi-arm junctions assembled from short DNA oligonucleotides to construct 2D and 3D DNA lattices (Fig. 1B) [1,18,24]. In such DNA crystal designs, Holliday junctions are connected in specific geometries by short single DNA overhangs with specific sequences, so-called "sticky ends". The first 3D DNA nanostructure that caught extensive attention was a cube assembled from six oligonucleotides in 1991 [25]. Since then, the field has evolved rapidly to the current state, where numerous software packages [2,26–32] allow the easy design of structures of any shape and with dimensions ranging from a few nanometers up ~1 μm [25,33–37]. A particularly robust method for building structures with complex geometry is scaffolded DNA assembly, where a long single-stranded DNA, usually derived from bacteriophages, is folded into a desired shape by hundreds of short, synthetic oligonucleotides (Fig. 1C, D) [19,20,38–41]. With the so-called single-stranded tile or DNA bricks methods, structures of rivaling complexity can be also assembled from synthetic oligonucleotides only [32,42].

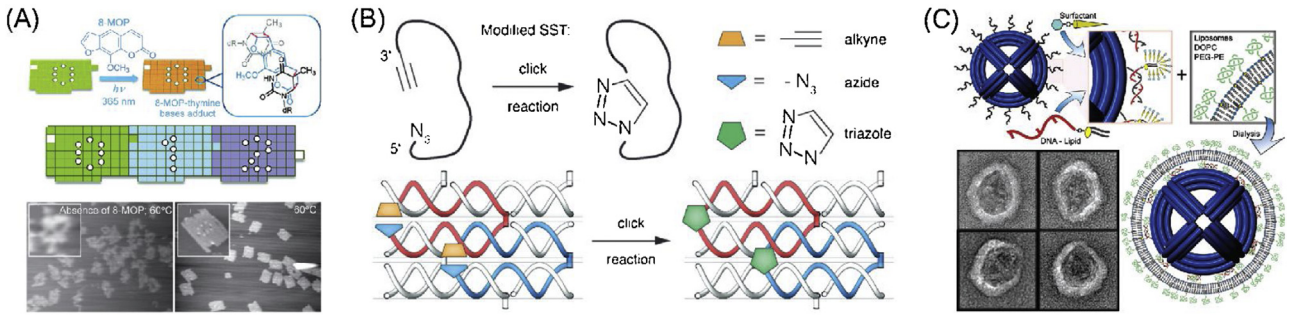

Fig. 2. Approaches to overcome stability issues. (A) Adducts formed by photo-cross-linking 8-MOP (light blue) and thymine bases (gray and black) stabilize DNA origami sheets at elevated temperatures. Adapted with permission [72]. Copyright 2011, American Chemical Society. (B) DNA oligonucleotides interlocked by covalent click reaction form catenanes stabilizing single-stranded tile assemblies. Adapted with permission [73]. Copyright 2015, WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (C) DNA octahedrons with PEGylated lipid bilayer surviving against nuclease attacks and in-vivo environment. Adapted with permission [74]. Copyright 2014, American Chemical Society.

Stability of DNA nanostructures

Due to their nanoscale dimensions, addressable surfaces and inherent biocompatibility, DNA-based devices hold great potential for biological applications including therapeutic cargo delivery and specific activation of cellular receptors [43,44]. For the DNA structures to perform their intended functions in biologically relevant environments, they should maintain structural integrity *in vitro* and *in vivo* during their journey to the cellular membranes and often also beyond this point. Two major factors that potentially inhibit this goal in biological fluids and tissues are the low concentrations of free divalent ions (often below 2 mM) [3] and the presence of (poly)nucleotide-degrading enzymes such as endo- and exonucleases (Fig. 2A). During the thermal annealing of the DNA structures, the folding solution is usually supplemented with a high concentration of Mg^{2+} (> 10 mM) [45]. This helps to screen electrostatic repulsion between tightly packed adjacent DNA double helices, whose phosphate backbones are negatively charged. Consequently, small angle X-ray scattering experiments, for example, revealed disassembly of the 24-helix nanorods in TE buffer below 2 mM Mg^{2+} [46]. If the salt-containing folding buffer is, however, replaced with Tris buffer (no EDTA) or even pure water, the structural integrity of DNA nanostructures can be maintained for several weeks [47,48]. This at first sight counter-intuitive behavior can be explained by considering two effects: i) Mg^{2+} (or other ions) are strongly associated with the folded DNA structure and continue to stabilize the structure after buffer exchange; ii) commonly used buffer constituents such as EDTA or Na_2HPO_4 compete for these ions and thus destabilize the structures. Several studies reported the fate of DNA-based nanostructures in cell culture media or in *in vivo* experiments where free divalent ions are scarce [49–52]. Perrault and colleagues, for example, tested the structural integrity of various DNA origami structures including a DNA octahedron, a 6-helix bundle (6HBs) and the 24-helix nanorod in unmodified Roswell Park Memorial Institute (RPMI) tissue culture medium that contains 0.4 mM Mg^{2+} [53]. The structures showed different levels of stability scaling with their packing density. Strikingly, only the least dense 6HB remained intact after 24 h. Supplementing the medium with 6 mM Mg^{2+} preserved the structural integrity of all structures while maintaining cellular viability. In one of our studies, we have observed that 6-helix bundle tubes made of 42-base single-stranded DNA tiles are stable in as low as 2 mM Mg^{2+} concentration in PBS buffer and also in Dulbeccoś Modified Eagle Medium (DMEM) for 8 h [54]. However, when these 6HB tubes were modified with single-stranded DNA overhangs for siRNA hybridization, the structures required more than 4 mM of Mg^{2+} for structural integrity and also disassembled in DMEM medium after 8 h. Using 84 nt-long single-stranded tiles as building blocks recovered the structure's stability even without any Mg^{2+} supplemented to the buffers. Benson et al. showed that their DNA polyhedral meshes, which consist of loosely packed single helices, stay intact in biologically relevant buffers such as PBS or DMEM at low salt concentrations and elevated temperatures [20]. DNA nanostructures also showed enhanced stability against chaotropic agents like urea and guanidinium chloride compared to double-stranded DNA [55,56]. In short, the structural integrity of DNA nanostructures is closely related to the temperature, the ion concentrations present in the surrounding media, the length of the DNA building blocks and the packing density of DNA helices with tightly packed DNA structures requiring higher Mg^{2+} concentrations and less packed ones surviving at physiological concentrations of divalent ions and at 37 °C.

The stability of DNA nanostructures against catalytic enzymes can be tested by mixing DNA structures with isolated nucleases, cell lysate or blood serum [45,52,57–71]. Mei et al. imaged several DNA origami structures by TEM and atomic force microscopy (AFM) after exposing them to cellular lysate [61]. The structures were intact after 12 h of incubation at room temperature. Single or double-stranded DNA, in contrast, degraded within 1 h of incubation. Another study by Castro et al. demonstrated the stability of DNA origami bundles against several nucleases including DNAse I, T7 endonuclease I, T7 exonuclease and MseI restriction enzyme [45]. The authors of this study found that one unit of DNAse I degrades 2 ng of DNA origami structures in 60 min at 37 °C, while the complete degradation of 65 ng of plasmid DNA takes only 5 min. Other enzymes did not inflict any structural damage to the origami structures. Unlike plasmid DNA, DNA nanostructures have densely packed DNA double helices that inhibit access of the nucleases. Bermudez et al. showed that DNA tetrahedrons are stable against the restriction enzyme DdeI, when the recognition site is close to the vertexes. The decay time of the structures was increased by a factor of 50 compared to simple double-stranded DNA when incubated in 10% FBS. If the recognition sequence was located in the middle of an edge the degradation progresses rapidly [59]. Overall, there is compelling evidence that DNA nanostructures show enhanced resistance against nucleases by rendering the binding or recognition site for the nucleases inaccessible.

The stability of DNA nanostructures can be enhanced by covalently linking DNA strands using chemical groups or by encapsulating the entire structures with a lipid bilayer [72–93]. Rajendran et al. enhanced the thermal stability of DNA origami tiles up to 60 °C by photo-cross-linking thymine bases and 8-MOP (8-Methoxypsoralen) (Fig. 2A) [72]. Photo-cross-linking staple strands by forming thymine dimers further enhanced the stability of 3D DNA origami objects to survive at 90 °C and pure water [93]. A widely used method to achieve covalent bonds is the (copper(I)-catalyzed) alkyne-azide cycloaddition, usually referred as a "click reaction". Copper-free click reactions on 3′-alkyne and 5′-azide-modified oligonucleotides lead to the formation of branched

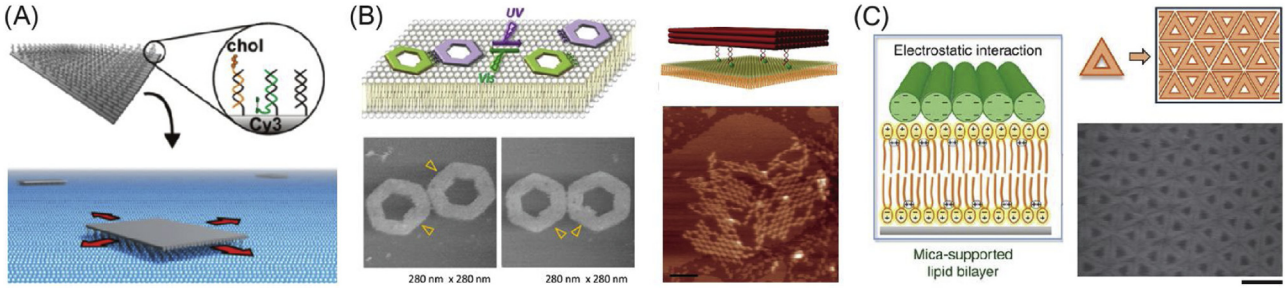

Fig. 3. DNA origami nanostructures on supported lipid bilayers. (A) DNA origami nanostructures can associate to lipid membranes *via* cholesterol and undergo 2D diffusion. Adapted with permission [115]. Copyright 2014, American Chemical Society. (B) Reversible binding and unbinding between origami structures on lipid bilayers: Under UV irradiation, hybridization of azobenzene-modified oligonucleotides is inhibited by the azobenzene trans-state. Illuminating with visible light induces the transition to cis-form azobenzene, which restores hybridization of the modified strands. Adapted with permission [116]. Copyright 2014, American Chemical Society. (C) Cholesterol anchors allow lattice assembly of DNA origami nanostructures on membranes. Adapted with permission [121]. Copyright 2015, American Chemical Society. (D) Also, electrostatic interaction between DNA backbone and lipid head groups (polar or charged) mediates binding of DNA nanostructures onto lipid membranes. Adapted with permission [129]. Copyright 2015, Springer Nature.

hexagons resistant to thermal and formamide-induced degradation [76]. Cassinelli et al. showed that single-stranded tile structures can be exploited as templates for the formation of up to 24 interlocked rings (catenanes) (Fig. 2B) [73]. Strikingly, 6-helix tubes consisting of 24 catenanes maintain their integrity at extremely unfavorable conditions such as temperatures of 95 °C, lack of any ions in the buffer as well as the presence of exonuclease I. Another strategy to increase the stability of DNA structures is the formation of disulfide bonds between adjacent DNA strands. The Gothelf group showed that also these DNA objects are stable under heating conditions (65 °C) and in the presence of denaturing agents [81]. The chemical reaction used here can be reversed in the reducing cytoplasm of cells, which could be exploited for cellular cargo delivery by DNA nanostructures. Perrault and colleagues observed that DNA origami structures were digested within 24 h when the cell medium was supplemented with more than 5% FBS [53]. The authors showed that a viable way to prevent the nuclease activity during *in vitro* experiments is to heat-inactivate the nucleases at 75 °C and to add actin proteins to inhibit the nuclease activity while not affecting cell growth and viability. In an alternative approach to protect DNA structures from nuclease activity, Perrault and Shih encapsulated DNA octahedrons with PEGylated lipid bilayers by using lipid-modified oligonucleotides on the outer part of the origami structures as a nucleation site for membrane formation (Fig. 2C) [74,88]. They showed that the lipid envelope protected the structures efficiently against nuclease attacks. Specifically, 85% of the structures remained intact after incubation for 24 h with 20 units of DNAse I. Encapsulated DNA octahedrons showed 17 times greater half-life in comparison to DNA oligonucleotides and bare octahedrons. After injection into mice the lipid-modified structures were distributed over the whole body whereas the DNA origami octahedrons lacking the envelope accumulated in the bladder.

All in all, the good news is that DNA nanostructures of complex shapes can be modified such that they withstand conditions of low divalent ions, presence of nucleases and elevated temperatures. Nevertheless, further cost-efficient strategies to increase the stability of DNA nanostructures in cell media or blood serum are desirable to open the door for employing these structures as potent carrier systems with designable functionality in biological studies.

Biological membranes

On its way to the interior of a cell, the cellular membrane is the next barrier a DNA nanostructure encounters after surviving in physiological conditions. Such lipid bilayers consist of amphiphilic phospholipids, glycolipids, and sterols and together with a host of embedded proteins they compartmentalize the cell and its cellular organelles, providing additionally communicating interfaces and functional platforms. Membranes enable the cell to maintain different pH values in different compartments and support neurotransmitter and ion gradients, which are essential for many core functions including photosynthesis [94], ATP synthesis [95] and synaptic transmission [96]. In aqueous environments, lipid molecules form bilayers with their hydrophilic heads facing outside and hydrophobic tails facing inside away from the water molecules. This non-covalent hydrophobic interaction is weak and allows lipid molecules to undergo 2D diffusion with diffusion coefficients of several $\mu m^2/s$, strongly dependent on the local environment [97–99]. Owing to this fluid mobility, proteins embedded into these membranes can fulfill their various biological tasks ranging from cellular recognition [100], membrane fusion [96] to ion-selective passages [101]. Membrane proteins represent 30% of all transcribed reading frames and 50% of the total mass of eukaryotic membranes [102]. The classical model of a biological membrane is that of a 2D mosaic with freely diffusing functional membrane proteins floating on and in the lipid bilayer [103]. However, the discovery of detergent-resistant lipid domains gave rise to the concept of lipid rafts, functionally active membrane regions highly enriched by membrane proteins, sphingolipids and other functional lipid molecules [104–106]. Nowadays, the lipid raft concept has evolved to a picture of smaller and transient domains with diameters of ~20–200 nm [13,107,108]. With their rich physical and biological behavior, lipid membranes offer a unique fluidic platform for studies with self-assembled DNA structures that can act as artificial floating rafts or penetrating pores. Using amphiphilic moieties as anchor elements, DNA nanostructures are envisioned to mimic the function of membrane proteins and lipid nano-domains with all their structural diversities.

Interaction of DNA nanostructures with lipid membranes

There are several ways to facilitate interactions of DNA nanostructures with lipid membranes. One approach relies on introducing hydrophobic groups on the surfaces of the DNA nanostructures. During synthesis, DNA strands can easily be modified with cholesterol-TEG (triethylene glycol) on their 3′ ends to facilitate its spontaneous insertion into the lipid membrane [109–113]. This functionalization is commercially available and hence it is the most commonly used anchor group thus far. This coupling strategy was applied in a variety of studies and it could be shown that DNA nanostructures on membranes undergo 2D diffusion (Fig. 3A) [114–121]. For example, DNA nanorods with eight cholesterol anchors showed a diffusional speed of 1.4 μm^2/sec on DOPC (1,2-dioleoyl-sn-glycero- 3-phosphocholine) membranes [114].

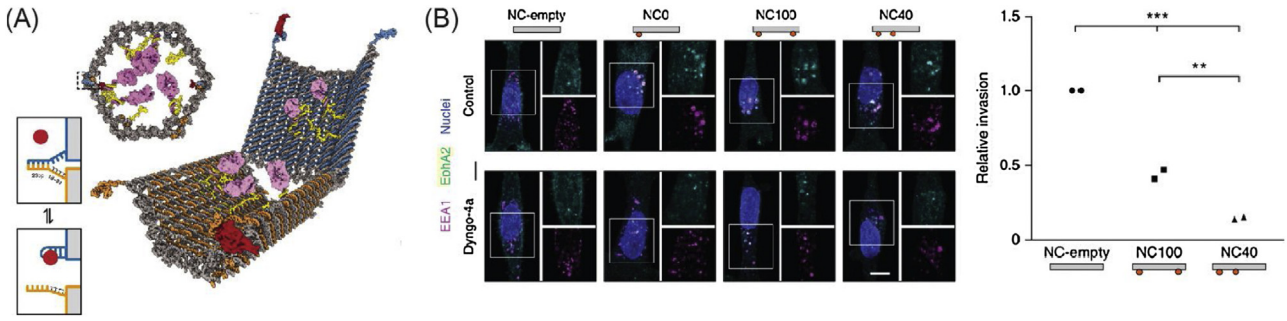

Fig. 4. DNA nanostructures interacting with membrane-associated proteins. (A) The molecular cargo inside a DNA nanostructure becomes accessible when an aptamer lock recognizes a specific cell type *via* its membrane-bound receptors. Adapted with permission [135]. Copyright 2012, American Association for the Advancement of Science. (B) Controlling the distribution of ligands and associated membrane receptors with DNA nanocalipers modulates EphA2 mediated responses. Adapted with permission [136]. Copyright 2014, Springer Nature.

Cholesterol-mediated hydrophobic interactions can be reversed by adding detergent molecules. For example, two-layered DNA sheets initially hiding their cholesterol modifications in their interior can be opened by detergent molecules or directly by lipid bilayers [122]. In addition to cholesterol, other hydrophobic molecules such as porphyrin ethyl phosphorothioate [123], polypropyleneoxide [122,124] and α-tocopherol [125] were successfully used to introduce DNA nanostructures into lipid membranes. Also, electrostatic interactions can mediate the binding between DNA nanostructures and lipid membranes. For example, cationic lipid molecules like DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane), which both carry a positive charge on their head group, have a high affinity to DNA whose phosphate backbone is negatively charged [126]. Interestingly, in the presence of divalent ions like Mg^{2+} and Ca^{2+}, DNA also binds to zwitterionic lipid headgroups with zero net charge [127,128]. This ion-mediated interaction can be switched reversibly by changing the ion concentration of the buffer [114].

DNA structures on supported lipid bilayers

Placing an object of interest onto or into a lipid bilayer offers two inherent advantages: i) the object is confined to diffusion in two dimension, which, compared to diffusion in 3D, strongly increases the likelihood of encounters between the objects; ii) the object can be designed to associate with the membrane in a specific orientation again leading to better chances of geometrically defined interactions. As a consequence, lipid membranes provide a unique platform for higher order assembly of DNA nanostructures. Suzuki et al. first used hexagonal DNA origami sheets with azobenzene-modified oligonucleotides protruding from the sides, which allowed for reversible multimerization of DNA origami structures on supported lipid bilayers (SLBs). By using alternating illumination with UV and visible light, the azobenzene molecules were switched between their cis- and trans-form, which in turn enabled or disabled hybridization of the azobenzene strands (Fig. 3B) [116]. In the same year, Johnson Buck et al. presented a study that investigated the 2D diffusion of "DNA origami barges" and their assembly into linear polymers on lipid bilayers [115]. Our group then demonstrated 1D and 2D assembly of rectangular DNA origami blocks on SLBs and on small unilamellar vesicles using connector DNA staples, leading to visible deformations of the vesicles (Fig. 3C) [121]. In the same study we also assembled a Y-shaped DNA origami structure into homotrimeric triskelions and further observed weakly ordered arrays of hexagons and pentagons, which resembled the surface arrangements of clathrin-coated pits. Suzuki et al. performed sequential self-assembly on SLBs by first assembling a DNA origami lattice followed by the recruiting of a second type of origami tile into the existing lattice [130]. Using high speed atomic force spectroscopy, the same group also observed dynamic processes like fusion of lattice patches and defect filling in real-time (Fig. 3D) [129]. Employing blunt end interactions, Sleiman et al. achieved impressive long-range ordering of small DNA tiles [131].

Next to mere assembly on lipid bilayers, DNA nanostructures have also demonstrated their potential to investigate membrane-associated proteins. As expected, DNA origami assembly on lipid bilayer depends on the phase of the lipid membrane [132]. As a side note, Douglas, Chou and Shih exploited the small interactions between membrane proteins and DNA nanostructures for NMR structure analysis by weakly aligning the proteins of interest in a liquid crystal formed from six-helix bundles [133]. Xu et al. used DNA origami rings with defined protein anchor sites on SLBs to study the number of SNARE proteins required for membrane fusion. Here, the authors took advantage of the stoichiometry control that DNA origami offers and found that single SNAREs can already trigger the fusion process [134].

Cell (membrane) interactions

DNA nanostructures modified with functional moieties can target and cluster membrane proteins on living cell thus influencing cellular receptor signaling [136] and stem cell differentiation [137,138]. Another excellent example of DNA nanostructures interacting with membrane-associated proteins is the DNA origami nanorobot presented by Douglas et al. (Fig. 4A) [135]. Their barrel-like nanorobots were loaded with fluorescently labeled antibody fragments against human leukocyte antigen (HLA-A/B/C) and aptamer-encoded gates controlled the opening of the barrel. Consequently, the structure only opened and presented its antibodies to HLA-A/B/C antigens when specific cell surface markers were expressed on the cells and recognized by the aptamers. With different aptamer combinations, six different cell lines that express different antigen combinations could be targeted. In a study by Shaw et al. 18-helix DNA origami bundles, termed nanocalipers, were used to investigate how the spatial organization of ephrin-A5 ligands modulate cellular binding and subsequent signaling with EphA2 receptors (Fig. 4B) [136]. Structures with two ephrin ligands placed with 40 nm distance were more effective in receptor activation and inhibiting the invasive properties of breast cancer cells compared to structures offering the two ligands at 100 nm distance. This strikingly demonstrates the importance of spatial organization of membrane proteins to trigger cellular pathways. Angelin et al. combined top-down micropatterning of solid substrates with self-assembled epithermal growth factor (EGF) – DNA nanostructures to analyze the activation of EGF receptors in MCF7 cells [139]. Cell membranes have also been coated with DNA nanostructures offering, for example, specific DNA sequences for programmed cell-cell adhesion [140]. With the goal to control the oligomeriza-

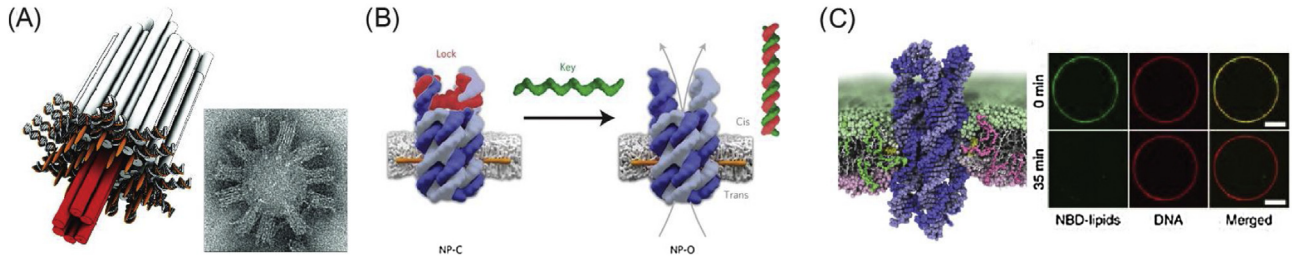

Fig. 5. DNA-based synthetic lipid channels (A) DNA nanostructures penetrating the bilayer of a lipid vesicle. Adapted with permission [143]. Copyright 2012, American Association for the Advancement of Science. (B) DNA channels controlled by the binding of key and lock sequences. Adapted with permission [144]. Copyright 2016, Springer Nature. (C) 4-hellix bundle DNA channels ("flipases") that flip lipid molecules across lipid bilayers. Adapted with permission [145]. Copyright 2018, Springer Nature.

tion states of membrane protein complexes, a DNA scaffold was used to assemble different numbers of alpha-hemolysin monomers into protein nanopores with varying sizes and conductances [141]. Also Kurokawa et al. used DNA origami templates to arrange Kir3 channel subunits and observed significantly intensified electrophysiological signals in HEK293 T cells [142].

DNA nanopores

DNA nanopores are among the most interesting DNA nanostructures and usually consist of one or several membrane-associating regions and a hollow channel that can span the lipid bilayer. Owing to their programmable surfaces and selective gating functions, such structures will potentially find application in nanopore-based DNA sequencing or as a new class of antibiotic or anticancer agents that perforate cellular membranes [146–150]. The first DNA nanopores were developed to fit into or onto silicon-nitride nanopores [151,152] and later also into nanocapillaries [153,154]. To achieve penetration of lipid bilayers, Langecker et al. designed a DNA origami structure with a hydrophobic region consisting of 26 cholesterol moieties that anchors the structure onto the membrane and forces the channel region through the bilayer (Fig. 5A) [143]. The conductance of this membrane-penetrating DNA nanopore was around 1 nS, orders of magnitude higher compared to that of protein ion channels [155]. Simmel and Dietz et al. also demonstrated DNA nanopores with a diameter of ~4 nm, which was used to translocate and simultaneously probe the passing DNA strands [150]. Generally, there is a large energy barrier for the hydrophilic part of a nanostructure to go through the entire hydrophobic core of the lipid membrane. This energy barrier can be lowered by adding detergents to destabilize the membrane [144] or by applying a voltage [143,156] to add favorable force. To simplify the assembly processes, the groups of Howorka and Keyser developed DNA tile-based nanopores capable of ion transportation across lipid membrane [123,144–148,156–161]. First, Burns et al. demonstrated the membrane-insertion of a 6-helix bundle only 15 nm in length and with a 2.2 nm thick hydrophobic ring in the center, matching the thickness of the bilayer. By employing their ethylphosphorothioate modified nanopores to living cells, Burns et al. claim the insertion of DNA nanopores into cellular membranes and triggering the death of cancer cells. The authors speculate, that the effect could result from the general damage to the membrane or from an alteration of the ion influx/exflux [123,144]. Tethering cancer-specific markers such as aptamers or antibodies could further increase the potential of these nanopores. Göpfrich et al. developed a 4-helix nanopore with a length of 11 nm and an inner diameter of 0.8 nm, which is similar to those of biological ion channels [158]. Ion flow through such DNA channels can be controlled by blocking the pore region with the incorporation of a system of key and lock sequences (Fig. 5B) [144]. Next to transmitting ions, such types of DNA nanopores can also accelerate flipping of lipid molecules across the bilayer (Fig. 5C) [145]. Interestingly, the state of incorporation as well as the orientation of small DNA nanopores can be distinguished by analyzing the activity of nucleases, which can only digest freely accessible parts of the pores while parts embedded in the membrane or inside a vesicle are spared [161]. The Keyser group very recently also accomplished the insertion of a funnel-shaped DNA origami porin with an inner diameter larger than 6 nm into a membrane and recorded conductances of tens of nanosiemens, which is ten-fold higher than that of previously reported man-made channels [146]. This work also features matching molecular dynamics simulations by the group of Aleksei Aksimentiev, who successfully modeled currents through a variety of DNA nanopores in previous work [148,159,162].

DNA nanostructures controlling membrane shape

In living cells, proteins like clathrin and BAR domains precisely control the shape and topology of lipid membranes through intricate membrane – protein and protein – protein interactions [166]. Inspired by these and other natural examples, also DNA nanostructures have been designed to manipulate the shape of lipid membranes [160,163–165,167–175]. Already simple hybridization of two types of complementary single-stranded DNA immobilized on two batches of vesicles can induce the fusion of pairs of these vesicles [167–171]. Czogalla et al. showed that 2D assembly of DNA origami structures triggered the deformation of GUV lipid membranes (Fig. 6A) [172]. This effect has also been observed on small unilamellar vesicles (SUVs) by Kocabey et al [121]. and on polymer-supported membranes by Birkholz et al. (Fig. 6B) [160]. Generally, membrane deformation occurs easily in the presence of any membrane-interacting objects, so careful data analysis needs to be performed. One fascinating illustration of the assembly power of DNA nanostructure is their use as a platform for the *de novo* genesis of membrane structures: Yang et al. used ring-shaped DNA nanostructure with different diameters as scaffold to generate vesicles with precisely defined diameters (Fig. 6C) [164]. In a similar approach, the groups of Hao Yan and Dongsheng Liu constructed shape and size-controlled cuboid and dumbbell-shaped heterovesicles with amphiphiles and DNA origami frames [173]. Inspired by the mechanism of dynamins and endosomal sorting complexes required for transport (ESCRT) machineries, Grome et al. designed DNA origami curls that induce the formation of membrane tubules [175]. In comparison, standard *in vitro* vesicle forming methods like sonication, electrophoresis and detergent depletion result in particles that are note shape-controlled and exhibit far less homogeneity. Notably, extrusion can lead to the formation of tolerably uniform SUVs, however, only within the range between 30 nm and 100 nm of diameter [176]. In a unique application by Lin's group, lipid membranes coupled to DNA nanostructures were dynamically reshaped by reconfiguring the geometry of the shape-defining DNA nanostructure (Fig. 6D) [165].

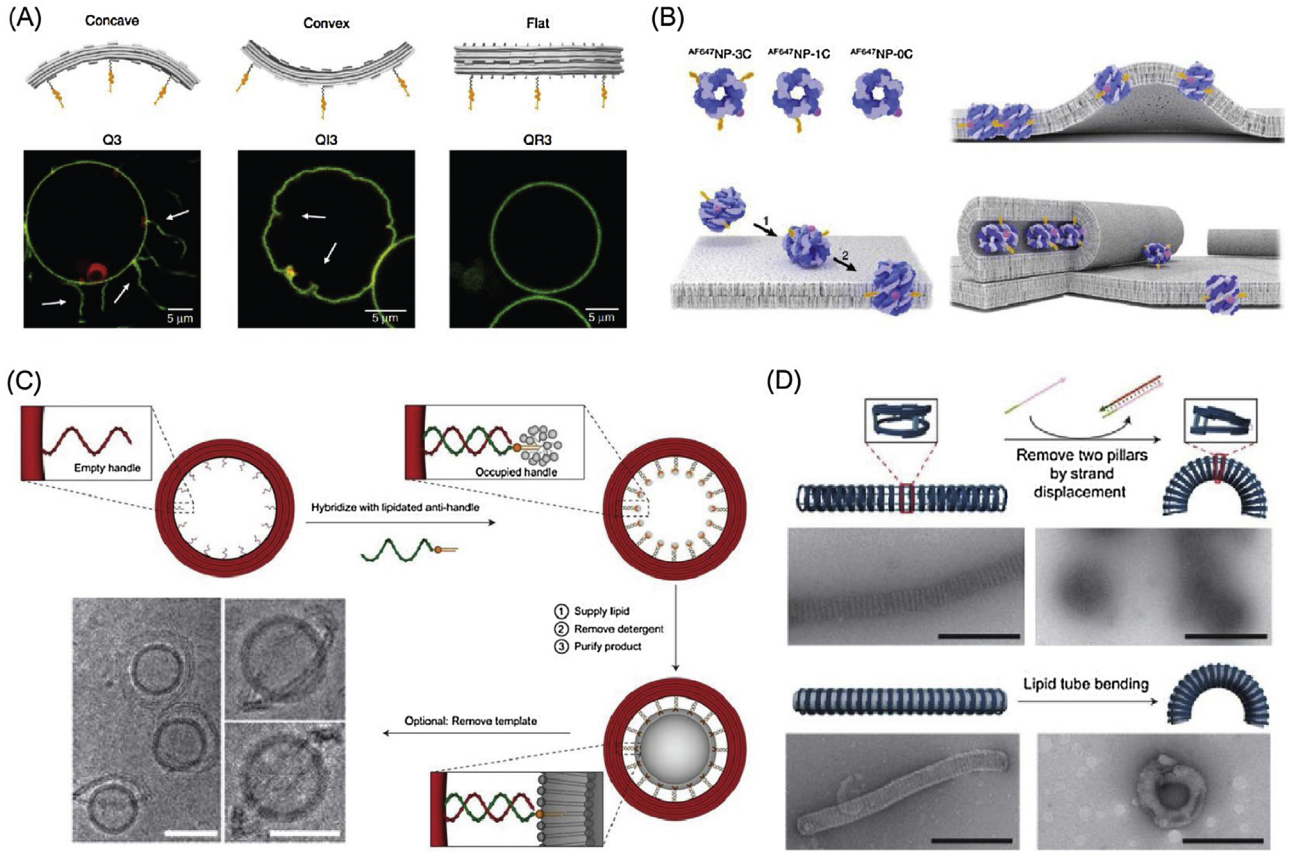

Fig. 6. DNA nanostructures controlling membrane shape. (A) DNA nanostructures on giant unilamellar vesicles (GUVs) deform their shapes. Adapted with permission [163]. Copyright 2018, Springer Nature. (B) Models of DNA nanopores that puncture and remodel lipid membranes. Adapted with permission [160]. Copyright 2018, Springer Nature. (C) DNA nanostructures carrying multiple hydrophobic anchors act as scaffolds for the formation of size-controlled vesicles. Adapted with permission [164]. Copyright 2016, Springer Nature. (D) Liposomes are assembled, arranged and remodeled by reconfigurable DNA nanocages. Adapted with permission [165]. Copyright 2017, Springer Nature.

Conclusion and perspective

We here have outlined how recent advances in designing and folding DNA nanostructure into increasingly complex and larger 3D structures can help to provide scaffolds for nanodevices that act autonomously in cellular environments. Computer-aided design tools that semi-automatically generate DNA sequences for user-defined structures [2,20,42] greatly lowered the barrier to enter the field of DNA Nanotechnology. Moreover, the price for synthesizing oligonucleotides has decreased steadily and recent biotechnological progress has led to enzymatic and large-scale production of oligonucleotides in bacteria [177,178]. In parallel, more and more (bio)chemical modifications and moieties are being developed for DNA chemistry enabling researchers to easily plug and assemble the desired functionalities. It is therefore already possible to combine several modifications into a single nanostructure that targets specific cell types, delivers a cargo or performs a programmed function *in vivo*. However, the yields of complex and multi-component modifications are sometimes low. Consequently, cost-efficient multifunctional modifications and increased conjugation yields are required to make such complex nanomachines accessible to clinical trials. Additionally, questions on how to efficiently deliver intact DNA structures or merely their contents into the cytoplasm or the cell nucleus persist. It also remains a challenge to overcome efficiently and selectively the membrane barriers, which demands further research elucidating the intricate interactions of nanoscale objects with natural membranes or model bilayer systems. A biological membrane is not a trivial target to explore and address because of its small thickness, its heterogeneity in molecular components and its inherent dynamic nature. Encouragingly, with their dynamically controllable geometry and their ability to carry functional groups in defined arrays, DNA nanostructures provide a platform to exploit and control the shape and function of biological membranes. For example, by using reconfigurable DNA nanostructures that first bind and then actively arrange membrane-bound receptor or other molecules, it could be possible to control membrane-associated cargos dynamically. Another prospect is the potential to adhere to and shape or penetrate the membranes of living cells with the goal to mimic endocytotic processes or viral cell entry. With such approaches it could also become feasible to induce membrane curvature-dependent cell responses. DNA-based nanopores could be further developed into antibiotic agents specifically punctuating microbial membranes, which are often highly resistant to mechanical or chemical stress. Synthetic DNA nanopores that connect two different cells could facilitate selective cell-cell communication. These are just a few entry points to the many directions of research that have opened up through the rapid and striking developments brought forward through DNA nanostructures interacting with lipid membranes.

Acknowledgement

W.B., S.K., and T.L. acknowledge support from the European Commission through the ERC Grant Agreement No. 336440, ORCA and the DFG through the SFB1032, Project A06.

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Dr. Wooli Bae is a research associate at Bioengineering department of Imperial College London. He received his B.S. and Ph.D in physics under supervision of Tae-Young Yoon from KAIST, South Korea in 2007 and 2014, respectively. His Ph.D research focused on single molecule studies of SNARE proteins and DNA origami. Between 2014 and 2017, he was a postdoc fellow working with Tim Liedl in the physics department of Ludwig-Maximilians-University (LMU) Munich, Germany, where he studied DNA origami structures interacting with lipid membranes and proteins. His current research interest is developing a ribozyme module for RNA nanotechnology.

Dr. Samet Kocabey, Ph.D. received his B.Sc degree in molecular biology and M.Sc degree in materials science from Bilkent University, Turkey in 2010 and 2012, respectively. Then, he joined the group of Prof. Tim Liedl and received his Ph.D degree in physics from LMU Munich, Germany in 2016. His research interests are focused on DNA self-assembly and cellular applications of DNA-based nanomaterials, with an emphasis on targeted delivery and immune therapeutics. He is currently pursuing his postdoctoral research at the Department of Medicine, University of Fribourg, Switzerland.

Dr. Tim Liedl is professor for experimental physics at LMU since 2009. He received his diploma in physics in 2004 in the group of Wolfgang J. Parak at LMU where he worked on the development of hydrophilic coatings for fluorescent semiconductor nanoparticles. In 2007 he obtained his Ph.D. in the group of Friedrich C. Simmel studying DNA-based nanodevices and switches, which are driven by chemical oscillations. From 2007 to 2009 he visited William M. Shih's laboratory at Dana-Farber Cancer Institute / Harvard Medical School where he used the DNA-origami method to construct self-assembling two- and three-dimensional structures. The research of Tim currently focuses on the application of DNA-based nanostructures in biology and for self-assembled plasmonic materials.