Stiffness and Membrane Anchor Density Modulate DNA-Nanospring-Induced Vesicle Tubulation

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Supporting Information

ABSTRACT: DNA nanotechnology provides an avenue for the construction of rationally designed artificial assemblages with well-defined and tunable architectures. Shaped to mimic natural membrane-deforming proteins and equipped with membrane anchoring molecules, curved DNA nanostructures can reproduce subcellular membrane remodeling events such as vesicle tubulation in vitro. To systematically analyze how structural stiffness and membrane affinity of DNA nanostructures affect the membrane remodeling outcome, here we build DNA-origami curls with varying thickness and amphipathic peptide density, and have them polymerize into nanosprings on the surface of liposomes. We find that modestly reducing rigidity and maximizing the number of membrane anchors not only promote membrane binding and remodeling but also lead to the formation of lipid tubules with better defined diameters, highlighting the ability of programmable DNA-based constructs to controllably deform the membrane.

KEYWORDS: DNA nanotechnology, DNA origami, membrane deformation, self-assembly, vesicles

INTRODUCTION

Intracellular space is full of intricate and dynamic membrane architectures that segregate biochemical reactions and facilitate cellular metabolism.1,2 Tubular shapes are found as common intermediates during membrane remodeling and are often maintained by well-organized, however transient, protein complexes.3−9 Creating biomimetic constructs that recapitulate membrane dynamics, such as tubulation in a cell-free environment, is a powerful approach to the mechanistic understanding of protein-mediated membrane remodeling. Further, controllable membrane manipulating techniques may enable the development of drug delivery and cell reprogramming tools. Structural DNA nanotechnology, which takes advantage of the defined DNA structures and base-pairing rules to build nanoscale objects with customizable geometry and chemically addressable surfaces,10−15 presents an opportunity to dynamically shape membranes.16−22 For example, a number of studies showed that DNA nanostructures modified with membrane anchors (e.g., cholesterol, amphipathic peptides) were able to drive lipid tubule formation on artificial membranes, including supported lipid bilayers,20 as well as large and giant unilamellar vesicles (LUVs and GUVs).21−23 Besides proving the concept, this body of work also provides general guidelines for the design (e.g., curvature and membrane anchor placement) and deployment (e.g., osmolality, membrane surface coverage, etc.) of the membrane-sculpting DNA structures. However, the curved DNA structures used in previous studies to imitate membrane-deforming proteins (e.g., BAR and ESCRT) featured high structural rigidity, yet the resulting lipid tubules were somewhat polydispersed in diameter (i.e., curvature).22,23 This prompted us to study the correlation between the stiffness of DNA structures and their vesicle-tubulation products.

Mechanical properties of multihelix bundle DNA nanostructures greatly depend on the shape and area of their cross-sections.24,25 Increasing the number of helices in a DNA-origami rod can lead to a quadratic increment in its persistence length.26,27 We recently built membrane-anchor decorated DNA-origami 24 helix bundle (24HB) curls that form spring-like filaments (DNA nanosprings) on vesicles in response to the addition of linker strands, generating nanospring-scaffolded membrane tubules.28 Using similar design principles, here we constructed thinner 6HB and 12HB DNA curls that can polymerize into left-handed nanosprings with helical pitches (53 and 54 nm) and diameters (26 and 22 nm) nearly identical to those of 24HB nanosprings (Figure 1a and Figures S1 and S2). Additionally, we outfitted the interior of each monomeric DNA curl (approximately three-quarters of a turn, 100 nm contour length) with a maximum of 24 (24HB) or 16 (12HB) single-stranded DNA “handles” for the attachment of amphipathic peptides (adapted from the N-terminus α-helix motif of snf7) as membrane anchors (Figure 1b).23,28 We
expect the three types of nanosprings to have distinct stiffness and tunable membrane anchor density, thus allowing for a systematic investigation of the DNA structures’ membrane binding and tubulation behaviors.

**RESULTS AND DISCUSSION**

We first examined the assembly and labeling of the DNA nanosprings in aqueous solutions. Negative-stain transmission electron microscopy (TEM) showed that the purified, well-folded DNA curl monomers exhibited contour lengths (24HB: 101 ± 4 nm, 12HB: 81 ± 5 nm, and 6HB: 81 ± 5 nm), thicknesses (24HB: 13 ± 1 nm, 12HB: 8 ± 1 nm, and 6HB: 7 ± 1 nm), and linker-strand-triggered polymerization capability in accordance with the design (Table S1 and Figure S3). All three types of nanosprings showed comparable helical pitches (24HB, 80 ± 9 nm; 12HB, 70 ± 5 nm; and 6HB, 75 ± 5 nm) that are larger than the designed values, likely due to the global left-handed twisting not accounted for by our bending model and the DNA structures splaying on TEM grids. The inner diameters of 24HB, 12HB, and 6HB springs measured 26 ± 5 nm, 16 ± 6 nm, and 8 ± 2 nm, respectively, suggesting DNA filaments with smaller cross sections were more prone to structural distortion (Table S2 and Figure S3). The increased flexibility associated with thinner DNA filaments is qualitatively corroborated by the measured persistence lengths of the nanosprings (Table S3). We note that the DNA springs’ stiffness can also be reduced by self-assembly errors that occur during the folding and polymerization of the DNA curls. Hybridizing Cy5-modified DNA-peptide conjugates to the inner handles of DNA curls yielded mostly monomeric DNA structures with Cy5 signal proportional to the number of handles (0, 7, 16, or 24) per monomer, as confirmed by non-denaturing agarose gel electrophoresis (Figure 1c and Figure S4). Next, we tested the binding affinity of the 24HB, 12HB, and 6HB DNA curls (4 nM) with 0, 7, or 16 peptides toward rhodamine-labeled LUVs (39.7 μM 1,2-dioleoyl-sn-glycero-3-phosphocholine or DOPC, 0.3 μM 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) or Rhod-PE), extruded through a polycarbonate membrane with a pore size of 200 nm (mean vesicle diameter: ∼134 nm, determined by TEM). This 10 000:1 lipid-to-curl ratio translates to ∼15 curls per average-sized LUV (consisting of ∼150 000 lipid) in a hypothetical scenario where all DNA curls were associated with membrane. Using our previously established protocol, the DNA curls and vesicles were incubated for 1 h at 4 °C in 10 mM MgCl₂ and 400 mM KCl buffer solution to allow DNA structures to bind to membrane without changes in osmolarity (Figure 2a). Immediately following, mixtures underwent an isopycnic centrifugation to isolate the LUV-bound DNA structures from the unbound ones. Fluorescence scans revealed that stronger lipid-DNA colocalization is generally associated with increasing number of peptide labels and decreasing DNA-curl thickness (Figure 2b, with the complete data set and quantification shown in Figure S5). This can be explained by the better accessibility of the peptides on the thinner DNA curls, consistent with the notion that membrane anchors can be sterically blocked by bulky DNA-origami structures. Additionally, the flexibility to “breath” may augment the readiness of the less stiff curls to
interact their amphipathic interiors with membranes of different curvatures and tensions. A negative-stain TEM study on unpurified mixtures of DNA curls and LUVs corroborated the centrifugation results. Here, thinner DNA curls labeled with more peptides left noticeably fewer unbound monomers on the TEM grids; in all TEM specimens there were no apparent reduction in the concentration of LUVs to suggest DNA-curl induced aggregation. Interestingly, only 12HB and 6HB curls carrying 16 peptides per monomer led to tubule formation after 1 h of incubations in the absence of linker strands (Figure S6). This positive correlation between membrane affinity and linker-independent vesicle tubulation is consistent with previous findings that high membrane coverage by curved DNA structures is a prerequisite for vesicle tubulation.22,23

After the initial binding tests at 4 °C for 1 h, we held the mixture of vesicles and DNA curls at 30 °C for another 16 h in the presence or absence of linker strands (Figure 2a) and analyzed the resulting lipid tubules using negative-stain TEM. In general, without linker strands, tubulation efficiency of DNA curls positively correlated with their membrane binding affinity (Figure 2c and Figures S5 and S7–S9). Namely, all three types of curls labeled with 16 peptides generated appreciable amounts of tubules, with more flexible structural designs resulting in higher tubulation efficiency (24HB, 2.7%; 12HB, 6.5%; 6HB, 8.5%; quantified as number of tubules/number of vesicles), whereas their 7-peptide-labeled counterparts generated little to no tubules (24HB, 0.3%; 12HB, 1.2%; 6HB, 0.6%). When the DNA coatings were discernible on the electron micrographs, we found that these lipid tubules were densely covered by irregular patterns of DNA curls. In contrast, having linker strands during the extended incubation period induced the formation of nanosprings and improved tubulation efficiency in all cases (Figure 2c, d). For example, the 16-peptide-labeled 24HB, 12HB, and 6HB nanosprings led to tubulation efficiency of ~7.1, ~11.1, and ~9.0%, respectively. However, polymerizing handle-less DNA curls with free peptides did not generate tubulation (Figure S10).

Surprisingly, polymerized 12HB curls produced more tubules than the 6HB curls, despite the latter being stronger LUV binders in the monomeric form. We initially speculated that this is because the linker strands promote tubulation partially by recruiting additional DNA-curl monomers to the membrane; thus, tubulation by stiffer springs benefits from a larger supply of free monomers left over in solution after the initial binding. This also seemed to explain the larger boost in tubulation efficiency when stiffer DNA curls were polymerized on membrane—24HB, 12HB, and 6HB curls equipped with 16 peptides produced 160, 71, and 5.8% more tubules with linker strands added, respectively. However, this hypothesis does not explain the tubulation efficiencies of 7-peptide-labeled structures, where the thinner, more lipophilic curls saw a larger tubulation boost upon polymerization (Figure S11). Therefore, it seemed that all curls must reach certain surface coverage for membrane remodeling to happen efficiently22,23 and once this requirement is met, stiffer structures may have advantages in tubulation. For example, with 7 or 16 peptides per monomer, tubulation by the 24HB curl was inferior to that by the 12HB and 6HB curls because of its poor membrane affinity. However, modifying each monomer with 24 peptides brought 24HB curl’s membrane coverage to a level comparable to the 7-peptide-labeled 12HB and 6HB curls; under this condition, the 24HB structure produced 4–8 fold more tubes than the 12HB and 6HB curls (Figure S5). Nevertheless, with the same peptide-labeling density, DNA-to-lipid ratio, and polymerizing condition, we found the 12HB curls to be the best-performing membrane-deforming structure. Increasing Mg2+ concentration from 10 to 15 mM further enhanced the 12HB curls’ tubulation efficiency from 11.1 to 15.6% (16 peptide per monomer) and from 4.7 to 10.8% (7 peptide per monomer) as well as producing longer tubules with average lengths beyond 500 nm (Figures S12–S15 and Table S4), likely due to more efficient linker-induced nanospring formation under higher divalent cation concentration.

Although the TEM studies do not permit direct visualization of tubulation kinetics, examining the morphologies of lipid
tubules can give clues as to their formation mechanism. For example, although polymerizing 24HB curls on LUVs yielded tubules with considerable width heterogeneity, the tubules with a dense DNA coat generally had widths that conform to the inner diameter of the nanosprings, suggesting that the nanosprings control the tubule width only when sufficient surface density is achieved. Therefore, we expected the 12HB and 6HB nanosprings, because of their thinner filaments and greater compressibility (i.e., smaller spring constant), to cover the membrane surfaces with high density and result in better controlled lipid-tubule diameters. Our analyses on lipid-tubule widths and apparent DNA-nanospring pitches (peak-to-peak distance of the DNA coat) showed that this was indeed the case (Figure 3). Labeled with 16 peptides per monomer, 12HB and 6HB nanosprings produced tubules with diameters of 28.2 ± 4.4 and 27.1 ± 3.6 nm, respectively, which are notably more homogeneous than those produced by the 24HB nanosprings (23.7 ± 7.2 nm) in the same conditions (Figure 3b). The 12HB nanosprings on lipid tubules also showed much smaller and monodispersed apparent pitches (19.2 ± 2.5 nm) relative to 24HB nanosprings (34.9 ± 17.7 nm), consistent with our expectation (Figure 3c). These comparisons held true when peptide labels were reduced to seven per monomer, albeit with overall reduced tube abundance and homogeneity (Figure S16 and Table S4). Enhancing the membrane affinity of 24HB curls by labeling each monomer with 24 peptides and doubling the DNA-to-lipid ratio produced more tubes with widths approximating the inner diameter of the nanosprings. However, the overall tube-width homogeneity was still less than those produced by the 12HB and 6HB springs (Figure S16). High-magnification TEM images revealed that the majority of lipid tubules formed by the 12HB nanosprings were coated by intertwined triplexes of modestly compressed nanosprings, each with a mean helical pitch ≈ 47 nm that translates to an apparent pitch of ~16 nm. This means such tubules were nearly completely covered by DNA, considering the ~11 nm designed thickness of the 12HB filaments. By contrast, 24HB nanosprings compressed to a comparable level (mean helical pitch ≈ 42 nm) formed primarily duplexes of ~21 nm apparent pitches (Figure 4); in fact, most 24HB springs remained uncompressed, leading to a larger percentage of uncovered membrane surfaces (Figure S17). In other words, comparing to the thicker, stiffer 24HB nanosprings, the thinner, more flexible 12HB nanosprings enforced stricter confinement of tubule width by forming a denser DNA-spring coating on lipid tubules as a result of higher membrane affinity, and greater susceptibility to compression and multiplex formation. This also embedded nearly 50% more peptides into the same length of tube (Figure 4), which introduced greater leaflet asymmetry to promote tubulation (Figure S18). Although most of the 6HB filaments did not generate enough contrast under TEM to be measured on membrane, we can extrapolate and expect a dense DNA coating on the lipid tubules, explaining the best uniformity of tube diameter produced by the three types of nanosprings.

Figure 3. Impact of DNA-nanospring thickness on lipid-tubule morphologies. (a) TEM images of lipid tubules covered by 24, 12, and 6 helix-bundle (HB) nanosprings. Scale bars = 100 nm. (b) Average tubule widths as a result of polymerizing 24, 12, and 6HB DNA curls (16 amphipathic peptides per monomer) mixed with LUVs. Means are weighted for the tubule lengths. Error bars show standard deviations. (c) Scatter plots showing the widths of lipid tubules versus the apparent pitches of the tubule-associating 24 and 12HB nanosprings (defined as the length of nanospring-coated tubule divided by the cumulative helical turns of DNA filaments).

Figure 4. Cartoon models and the corresponding TEM images contrasting the compressed 24 and 12 helix-bundle (HB) DNA nanosprings (gray, blue, and red) intertwining around lipid tubules (tan). The measured apparent and actual pitches of nanosprings are labeled at the bottom and top of each model, respectively. The “see-through” box reveals the different surface densities of peptides (purple dots) introduced by the 24 and 12HB nanosprings. Scale bar = 100 nm.
CONCLUSIONS
Nature has evolved membrane-deforming proteins that are not only efficient and dynamic, but also under layers of regulation.\textsuperscript{6,7,34,35} When engineering artificial systems to mimic such elegance of nature, it is therefore important to understand the design parameters that modulate the membrane remodeling in vitro. By varying the curvature of a stiff (20HB) DNA structure labeled with cholesterol, Franquelim et al. found that a modest bend led to the most effective GUV tubulation.\textsuperscript{22} Here we showed that the filament thickness of DNA nanosprings profoundly impacted the frequency of LUV tubulation events and the morphology of the lipid tubules. Clearly, further investigation is needed to fully elucidate the DNA-nanospring-induced membrane deformation mechanism (e.g., by controlling membrane curvature and tension, by monitoring the deformation kinetics). With this in mind, the main contribution of this work is establishing stiffness as a tunable parameter that affects vesicle tubulation outcome and providing a practical guide for designing and choosing from an assortment of “strong” and “weak” DNA nanosprings for membrane deformation. Our findings implied that the abilities to (i) promiscuously bind membranes with various curvatures and (ii) readily propagate into a dense coat of nanosprings on membrane surfaces are key for curved DNA structures to efficiently draw structurally defined lipid tubules from vesicles. This insight will inform the future implementation of more advanced membrane deforming machineries that could, for example, carry specific ligands to target curved biological membranes and constrict membrane tubules to enable scission.

EXPERIMENTAL SECTION

Materials. DNA oligonucleotides were purchased from Integrated DNA Technologies (see Supporting Information for sequences). DNA-peptide conjugates were purchased from Bio-Synthesis. Lipids and liposome extrusion filters were purchased from Avanti Polar Lipids. TEM grids and uranyl formate were purchased from Electron Microscopy Sciences. JM109 \textit{E. coli} cells and VCSM13 helper phage were purchased from Agilent Technologies. Ultracentrifuge tubes were purchased from Beckman Coulter. Iodixanol was purchased from Stemcell Technologies. Other chemicals and Amicon filters were purchased from Millipore Sigma.

DNA-Nanospring Design and Preparation. Three-dimensional models of DNA-origami curls were initially built in Autodesk Maya, which guided the DNA-origami design by caDNAno (cadnano.org).\textsuperscript{36} The models and design diagrams are available in Figure S1 and S2. M13-derived scaffold DNA strands (8064 and 7308-nt long) were produced using \textit{E. coli} and bacteriophages as previously described.\textsuperscript{37} The pBlueScript-derived scaffold (3024-nt long) was prepared with JM109 cells and VCSM13 using a single-stranded phagemid rescue protocol recommended by the manufacturer. The DNA curls were folded from a mixture of scaffold strand (40 nM) and staple strands (240 nM each) in a TE buffer (pH 8) containing 10 (for 6HB) or 15 mM (for 12 and 24HB) MgCl\textsubscript{2}, using a 36-h thermal annealing (80–24 °C) program. Correctly folded DNA curls were purified and concentrated by rate-zonal centrifugation and Amicon filtration.\textsuperscript{38} To functionize the DNA structures, purified curls displaying handles were then incubated with peptide or Cy5-modified antihandles at antihandle-to-handle ratio of 3:1. The excessive antihandles were removed by polyethylene-glycol (PEG) fractionation.\textsuperscript{39} To form nanosprings, we added linker strands to the peptide/Cy5-labeled DNA curls in 20-fold molar excess; the mixture was incubated at 30 °C for 16 h.

Gel Electrophoresis. Equal molar amount of DNA curl monomers (with or without peptides and Cy5 labels) were loaded into separate lanes of a 1.8% agarose gel casted with 0.5 × TBE buffer containing 10 mM MgCl\textsubscript{2}. The gel was run in the same buffer solution at 5 V/cm for 3 h before being imaged on a Typhoon FLA 9500 scanner using laser and filter settings for the Cy5-fluorescence channel. It was then stained by ethidium bromide (EtBr), destained, and imaged again using settings for the Cy5 and EtBr channels.

DNA-Curl-Mediated Vesicle Tubulation. LUVs (99.2% DOPC, 0.8% Rhod-PE, 15 mM total lipid) were prepared via lipid-film rehydration followed by extrusion. Unless noted otherwise, these vesicles (final lipid concentration = 40 μM) were mixed with peptide-labeled, PEG-fractionated DNA curls at 10 000:1 lipid-to-curl ratio in a 25 mM HEPES buffer (pH 7.5) containing 400 mM KCl and 10 mM MgCl\textsubscript{2}, incubated for 1 h at 4 °C (to allow binding), and brought to 30 °C for an 16-h incubation in the presence (20-fold excess, to trigger polymerization on membrane) or absence (for the study of linker-independent tubulation) of linker strands.

Isopycnic Centrifugation. One hundred microliters of preincubated LUVs and DNA curls (prepared as described above) was diluted to a final volume of 300 μL, which consisted of 30% iodixanol, 25 mM HEPES (pH 7.5), 10 mM MgCl\textsubscript{2}, and 400 mM KCl. This mixture was transferred to a 0.8 mL ultracentrifuge tube, on top of which six layers (60 μL each) of solutions with decreasing iodixanol concentrations (26–6%, with 4% decrement per layer) were added. The content of this tube was spun at 48 000 rpm at 4 °C in a swing bucket rotor (SW 55 Ti) for 5 h, fractionated from top to bottom into 13 wells of a 96-well plate (~52 μL per fraction), and imaged on a Typhoon FLA 9500 scanner in Cy5 and Cy3 channels.

Transmission Electron Microscopy. A drop (~5 μL) of sample was deposited on a glow-discharged carbon-coated copper grid and allowed to adsorb for 1–2 min before blotted away. Negative-stain was achieved by adding 2% uranyl formate solution to the grid and incubating for 1–3 min. After blotting away the stain, the grid was air-dried and imaged using a JEOL JEM-1400 plus microscope operated at 80 kV. Images were acquired by a 4k × 3k CCD camera (Advanced Microscopy Technologies) and analyzed by ImageJ (National Institutes of Health) for tube abundance and dimensions, and EasyWorm\textsuperscript{40} for persistence lengths.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.9b05401.

- Designs of DNA-origami curls, DNA sequences, materials and methods, and additional data and models (PDF)

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Notes
The authors declare no competing financial interest.

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