Tailoring interleaflet lipid transfer with a DNA-based synthetic enzyme

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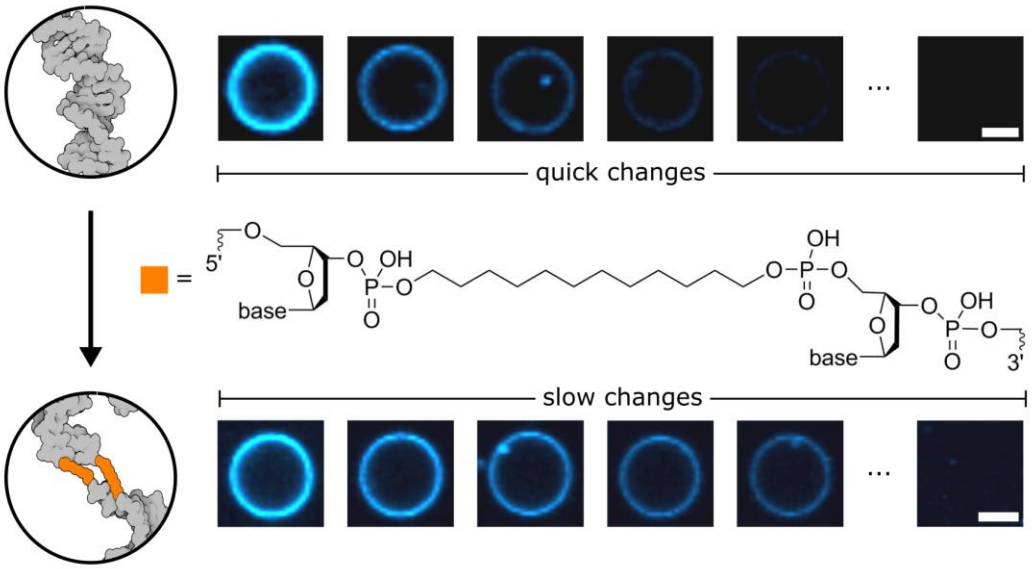

Lipid membranes, enveloping all living systems, are of crucial importance, and control over their structure and composition is a highly desirable functionality of artificial structures. However, the rational design of protein-inspired systems is still challenging. Here, we have developed a highly functional nucleic acid construct that self-assembles and inserts into membranes, enabling lipid transfer between inner and outer leaflets. By designing the structure to account for interactions between the DNA, its hydrophobic modifications and the lipids, we successfully exerted control over the rate of interleaflet lipid transfer induced by our DNA-based enzyme. Furthermore, we can regulate the level of lipid transfer by altering the concentration of divalent ions, similar to stimuli-responsive lipid-flipping proteins.

Keywords: DNA nanotechnology, lipid membranes, synthetic ion channel, dodecane, lipid flipping, molecular dynamics

Phospholipid membrane envelopes every cell, and is an important signaling platform for all organisms. Embedded within it, transmembrane proteins are molecular machines responsible for membrane's properties[1]. Their relatively complex structure allows for a high level of control over bilayer shape[2] and lipid composition[3]. Such degree of control makes protein-inspired transmembrane structures a desirable tool, and the development of these a vibrant field of study[4–13].

One of the materials currently explored as a prospective protein-mimicking building block is DNA, due to the ease with which it may be modified, designed and assembled using complementary base pairing. Starting with a synthetic DNA-origami membrane channel[14], a whole variety of structures was developed[15-21], all designed to insert into the membrane *via* hydrophobic anchors; the nucleic acid scaffold is rarely modified[22]. Due to the DNA's charge, the hydrophilic headgroups, rather than the hydrophobic tails of the lipids, face towards the membrane-spanning DNA. This results in the formation of a toroidal pore, shaped by the merged bilayer leaflets. Such arrangement of lipids has many implications, namely the ion flow on the DNA-lipid interface[21] or lipid transfer between bilayer leaflets[23]. Here, we report dependency of both these effects on the hydrophilicity of the DNA-based constructs' membrane-spanning domain, and present an artificial, protein-inspired structure exhibiting control over the rate and the level of interleaflet lipid transfer.

Knowing that leaflet merging results from DNA-lipid interactions, we hypothesized that the shape of the pore formed by transmembrane nanostructures depends on the hydrophilicity of the membrane-spanning domain. Indeed, the effect has been attributed to the mechanisms of naturally occurring lipid-flipping proteins[24]. To confirm this idea, we have designed a membrane-inserting DNA duplex with four nucleotides in the central part of one of its strands replaced by a 12-carbon chain (dodecane). The membrane-spanning part of the structure is therefore half-hydrophilic and half-hydrophobic. An illustration of this construct embedded in a bilayer is shown in Fig. 1a. The schematic was created based on the results of all-atom molecular dynamics (MD) simulations, shown in Fig. 1b. Upon 1 μs of simulation, lipids were found with their headgroups predominantly pointing towards the unmodified strand, while no lipids were present within the pore on the side of the hydrophobic domain.

Realizing that the toroidality of the pore can be disrupted by modifying the membrane-spanning part, we investigated three versions of the aforementioned DNA duplex: featuring none (0D), one (1D) or two (2D) dodecane spacers placed in the central site of the structure. All tested duplexes were additionally modified with two cholesterol moieties (2C) (Fig. 1c, Supplementary Table 1), which facilitate their attachment and insertion into the bilayer (Supplementary Fig. 1). In the absence of cholesterol modifications, no attachment caused by the presence of the C12 chain (0C 2D) was observed (Supplementary Fig. 2). The three structures were physically folded using a commercially available C12-modified strands. The structures were characterized and tested experimentally *via* ionic current recordings, while an additional fluorescent tag allowed optical measurements described further in this work. Additionally, all-atom MD simulations enabled thorough understanding of the details of the systems.

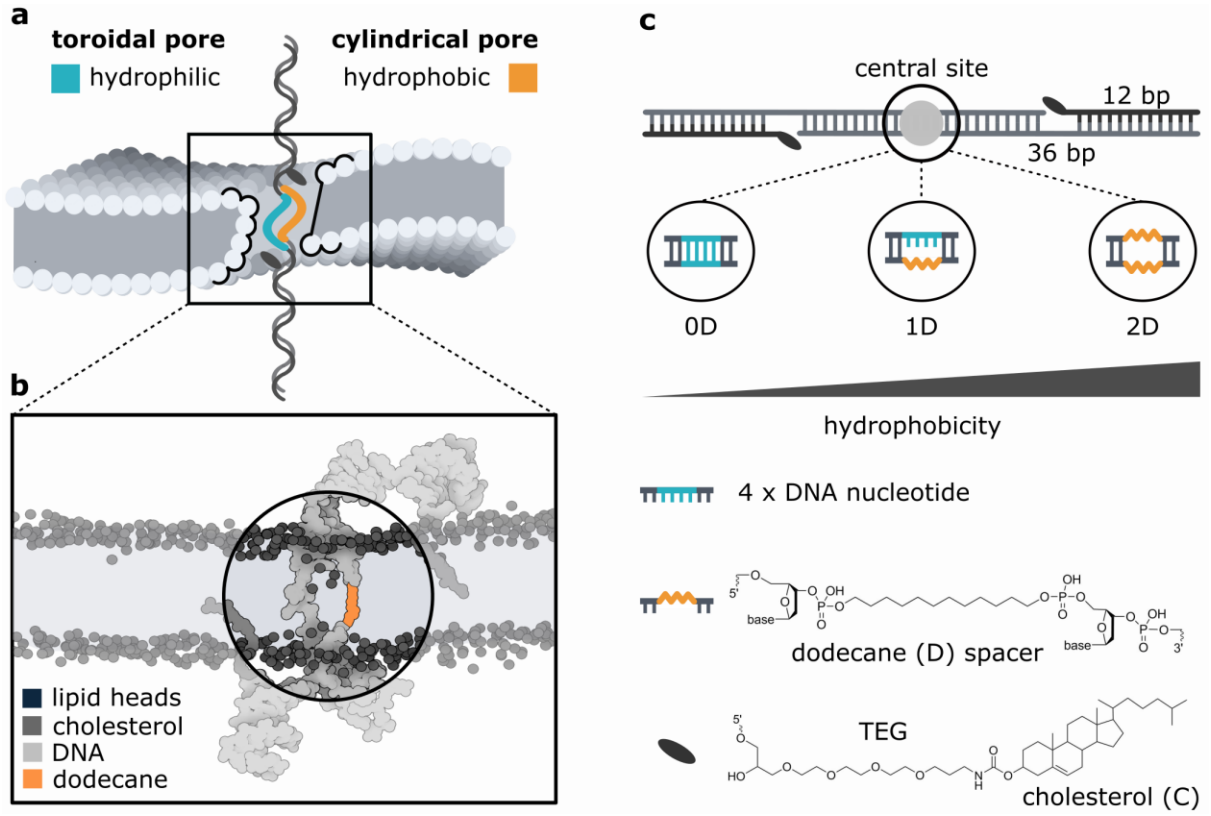

Figure 1. Schematic representation of the designed DNA nanostructure. (a) The hydrophilicity of the membrane-spanning domain determines the structure of the DNA-lipid interface. (b) Snapshot from an all-atom MD simulation of the 1D construct. No lipid headgroups are present in the proximity of the dodecane spacer. (c) Schematic representation of the double-stranded DNA construct, highlighting its membrane-anchoring (cholesterol) and internal (dodecane) modifications. Three different designs varying in hydrophobicity were used: with either none (0D), one (1D) or two (2D) dodecane spacers placed in the structure's central site.

The design of the duplexes, especially the long separation of the hydrophobic anchors (24 bp ≈ 8 nm), causes transient insertions of the structure, which has been reported earlier for a similar construct[21]. The mode of insertion can be studied by recording the ionic current across a lipid membrane in the presence of structures. Figure 2a shows three representative current traces, one for each of the studied designs. The results indicate that: (I) the structures do not form a stable pore, but induce current bursts while spanning the bilayer, and (II) with an increasing number of internal dodecane modifications the total amount of ions transported across the membrane is reduced. The experimental results suggest quantitative differences in the size of water channels formed during the insertion, controlled by construct's hydrophobicity as expected (see also Supplementary Fig. 3).

To further investigate the interactions between the inserted structures and surrounding lipids, we performed all-atom MD simulations. An initial examination of a 2D construct in the absence of a lipid membrane showed that, when in an aqueous solution, the dodecane modifications adapt a contracted conformation, rather than appearing fully stretched (Supplementary Fig. 4). However, after being placed in a membrane, the C12 chains extend to span through the hydrophobic core, while DNA moves out from the hydrophobic region.

Therefore, even though a toroidal pore was formed around each construct, the induced water channels differed noticeably between each system (Fig. 2b); the number of water molecules in the pore decreased with the increasing hydrophobicity of the central site, agreeing with the observed differences between experimentally obtained conductance traces. After 1 μs of simulations, dodecane was fully stretched, which caused closure of the pore and subsequent cessation of water and lipid transfer (Supplementary Fig. 5). The presence of a stretched dodecane spacer affected the molecular arrangements of the created pore, resulting in a smaller number of permeated water molecules (Fig. 2c), as well as fewer lipids transferred between merged leaflets (Supplementary Fig. 6).

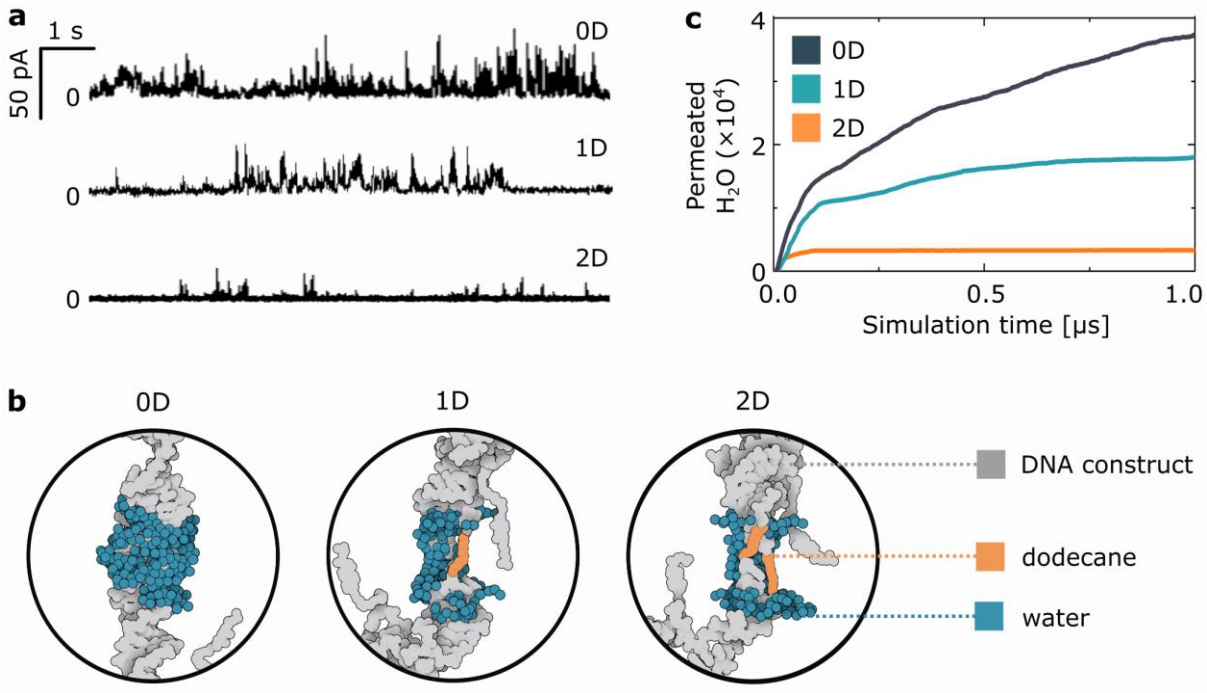

Figure 2. Experiments and simulations reveal the DNA-induced transient water channel in a lipid bilayer. (a) Representative current trace for each of the three designs. (b) Snapshots highlighting the number of water molecules in the channel after 0.8 μs of MD simulations of DNA constructs in a lipid bilayer. Lipids and ions are not shown for clarity. (c) Results of all-atom MD simulations, showing the number of water molecules permeated through the membrane as a function of simulation time.

To further observe the disruption of the toroidal pore, and its effects on the rate of lipid transfer, we experimentally examined the movement of lipids between the inner and outer leaflet of the bilayer. This movement is intrinsically rare due to a high energy barrier[25]. However, when the toroidal pore is formed upon DNA duplex insertion, the lipids can move unhindered between both - now merged - leaflets[23]. The change in the bilayer's composition is studied *via* an optical assay based on a redox reaction. The system under study consisted of giant unilamellar vesicles (GUVs) made of 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) lipids containing a 0.5 wt% addition of NBD-labelled PC lipids. Upon addition of a reducing agent, the fluorescent NBD molecule turns into its non-fluorescent derivative[25].

When no toroidal pore is formed, in the presence of the membrane-impermeable reductant, the fluorescence intensity of the vesicle decreases to around 50 %, since only the outer leaflet labels are bleached. Once the toroidal pore is formed, NBD-lipids from the inner leaflet diffuse to the outside of the vesicle, where they too are reduced. Consequently, a complete loss of the fluorescence signal is observed (Fig. 3a). In these studies, dithionite ($[S_2O_4]^{2-}$) was chosen as the reductant, due to its negative charge ensuring membrane impermeability[23,26]. The charge also prevents its translocation through the DNA-induced pore, as barely any transport of negative ions has been observed during MD simulations (Supplementary Fig. 7). The fluorescence stability of occasionally appearing internal vesicles is an additional confirmation of dithionite membrane impermeability in the presence of the inserting DNA (Supplementary Fig. 8). Furthermore, in a previous study by our group, dithionite leakage through pores induced by much larger DNA construct was shown to be negligible at the time scale of the experiment[23].We studied the effect of the designed duplexes on the composition of a lipid bilayer by incubating vesicles with each of the structures, and recording their fluorescence upon the addition of dithionite. After the experiment, two populations of vesicles can be distinguished, with optical signals decreased to either 50 % or 0 % (Supplementary Fig. 9). The first group was assigned to the vesicles with no toroidal pore, indicating the absence of DNA insertions. These results can be compared with control experiments performed for non-inserting 0C and 1C structures (Supplementary Fig.10). The population of vesicles that exhibited complete loss of fluorescence represents the membranes with merged leaflets – and therefore with a DNA duplex spanning through the bilayer.

The fluorescence decay traces, averaged for the vesicles affected by the DNA insertion, are shown in Fig. 3a. The observed rates of bleaching (0.25 ± 0.01, 0.21 ± 0.01 and 0.09 ± 0.01 min^{-1} for 0D, 1D and 2D structures, respectively) correlate with the hydrophobicity of the central site (Supplementary Table 2). We hypothesize that the presence of the C12 spacer impedes the formation of a pathway for the lipids' interleaflet movement - its effect being especially striking when comparing the vesicles incubated with 0D and 2D constructs, as presented in Fig. 3b. Even the structures with an entirely hydrophobic central site (2D) cause lipid transfer across the membrane, which suggests the transient merging of the leaflets during the insertion, agreeing with the ionic current measurements (Fig. 2a).

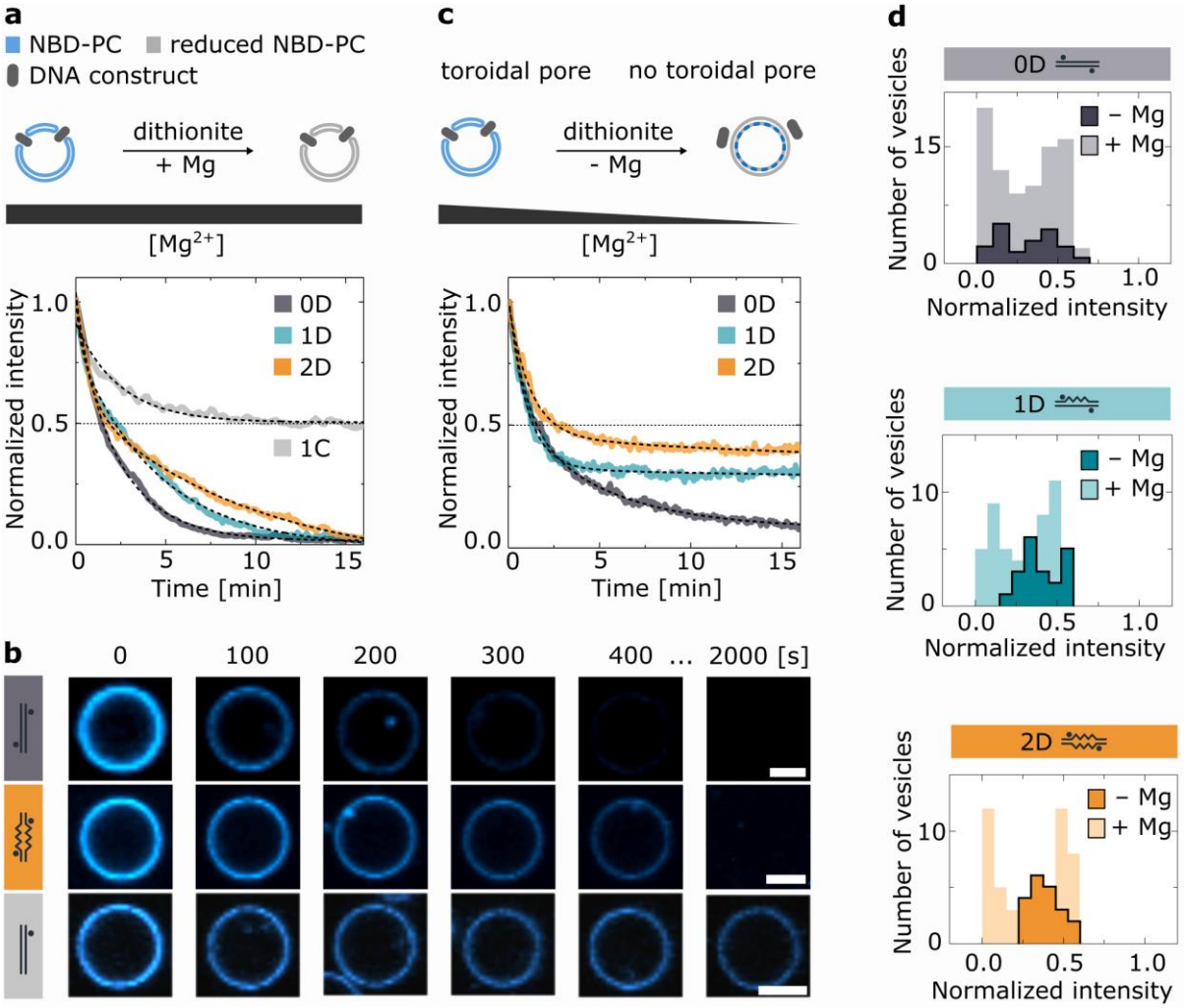

Figure 3. Controlling the rate and the level of lipid flipping through DNA nanostructure's architecture and the concentration of divalent cations. (a) Experimental results from the schematically illustrated bleaching assay, with magnesium concentration constant (4 mM, +Mg) throughout the experiment. The plot shows fluorescence intensity time traces collected for the three constructs and the non-inserting (1C) control upon dithionite addition at t = 0. Each plot is an average of at least three traces indicative of the leaflet merging (Supplementary Fig. 11). The black dashed lines represent biexponential fit (Supplementary Table 2). (b) Representative confocal microscopy image sequences at +Mg conditions, showing the difference in fluorescence decay rates of 0D and 2D structures, alongside the non-inserting 1C structure. The scale bars indicate 5 µm. (c) Experimental results analogous to (a), with magnesium concentration decreasing by 1.5 mM (-Mg) throughout the experiment. Each plot is an average of at least three traces, indicated by the respective peaks in the histograms of the final intensities presented in (d) (Supplementary Fig. 12). (d) Histograms of the final intensity values collected from three experiments for each DNA construct, for +Mg (N_{0D}=82, N_{1D}=49, N_{2D}=50) and -Mg (N_{0D}=24, N_{1D}=N_{2D}=20) conditions (Supplementary Fig. 9).

The experiments involving DNA constructs are most commonly performed in the presence of magnesium ions to ensure the stability of DNA-based nanostructures[27,28]. The stability of internally modified constructs shows higher sensitivity to changes in magnesium concentration, which we confirmed by spectroscopic measurements (Supplementary Figs. 13-14, Supplementary Table 3) and polyacrylamide gel electrophoresis (Supplementary Fig. 15, Supplementary Table 4). We concluded that the presence of Mg^{2+} may provide an additional handle to control DNA-induced lipid movements. Further experiments were conducted, as presented in Fig. 3c, where the magnesium concentration was rapidly decreased upon the addition of dithionite. Therefore, the observed effects resulted from a combination of: (I) reducing agent bleaching the fluorophores, (II) DNA-induced pores influencing the accessibility of fluorophores to the bleaching factor, and (III) changes in ionic concentration affecting the stability of the DNA structure.

We compared the final intensities of the traced vesicles from the experiments previously described - with constant magnesium concentration (+ Mg) - with the ones that were exposed to the decreasing magnesium concentration (- Mg) (Fig. 3d). When there are no changes in divalent cation concentration, all three systems exhibit similar distributions of final fluorescence intensities. 50 %, 46.9 % and 40 % of vesicles studied in the presence of 0D, 1D and 2D structures respectively had their final fluorescence intensity indicating the presence of lipid flipping (> 70 % bleaching, see Supplementary Fig. 9 for details). However, when the concentration of Mg^{2+} is reduced, both 1D and 2D constructs exhibit only partial bleaching of inner-leaflet lipids, suggesting that the DNA-induced lipid transfer was stopped during the experiment. We attribute this to the decreased stability of these structures in the reduced Mg^{2+} concentration (Supplementary Figs. 13-15) and subsequent disruption of lipid flipping occurring during the assay. As a result, only a fraction of lipids changes their interleaflet position - reminiscent of the effects of natural scramblases, which exhibit control over the amount of transferred lipids[29]. The level on which the lipid movement is terminated depends on the number of dodecane modifications, with 0D showing the lowest level of residual fluorescence. The change in cation concentration by 1.5 mM resulted in bleaching of around 92%, 70% and 61% of lipids, caused by the 0D, 1D and 2D structures respectively (Supplementary Fig. 12, Supplementary Table 2). Our measurements indicate that we can not only control the rate of lipid flip-flop movements using hydrophobic modifications, but also use an external stimulus to vary the level of interleaflet transfer.

We report synthetic DNA-based nanostructures that insert into membranes and exhibit a control over their interaction with surrounding lipids, mimicking natural membrane-spanning molecules. By introducing a protein-inspired hydrophobic domain, we changed the rate of lipid flipping induced by the DNA. Additionally, we demonstrated a fundamental connection between the design of the DNA structure and its ability to flip lipids in response to external stimuli like divalent salts. The removal of Mg^{2+} allowed for stopping lipid flipping which until now was the ability of only specialized transmembrane proteins. The results of our experiments and simulations emphasize the importance of contextual design when creating new synthetic constructs. We show that the architecture of the membrane-targeting structure should not be treated in the isolation, but rather in the context of its interactions with the surrounding environment - and in a broader sense, its prospective applications[30] – which is essential for the creation of a new generation of complex protein-mimicking molecular machineries.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
DNA sequences, experimental details, supporting figures and tables can be found in the supporting information.

Competing interests

The authors declare no competing financial interests.

Acknowledgments

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