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DNA-Based Patterning of Tethered Membrane Patches

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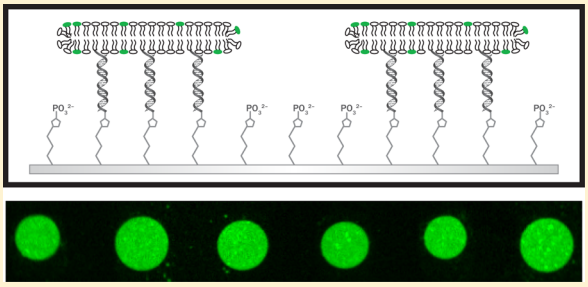
Supporting Information

ABSTRACT: Solid-supported lipid bilayers are useful model systems for mimicking cellular membranes; however, the interaction of the bilayer with the surface can disrupt the function of integral membrane proteins and impede topological transformations such as membrane fusion. As a result, a variety of tethered or cushioned lipid bilayer architectures have been described, which retain the proximity to the surface, enabling surface-sensitive techniques, but physically distance the bilayer from the surface. We have recently developed a method for spatially separating a lipid bilayer from a solid support using DNA lipids. In this system, a DNA strand is covalently attached to a glass slide or SiO_2 wafer, and giant unilamellar vesicles (GUVs) displaying the complement rupture to form a planar lipid bilayer tethered above the surface. However, the location of the patch is random, determined by where the DNA-GUV initially binds to its complement. To allow greater versatility and control, we sought a way to pattern tethered membrane patches. We present a method for creating spatially distinct tethered membrane patches on a glass slide using microarray printing. Surface-reactive DNA sequences are spotted onto the slide, incubated to covalently link the DNA to the surface, and DNA-GUVs patches are formed selectively on the printed DNA. By interfacing the bilayers with microfluidic flow cells, materials can be added on top of or fused into the membrane to change the composition of the bilayers. With further development, this approach would enable rapid screening of different patches in protein binding assays and would enable interfacing patches with electrical detectors.



INTRODUCTION

Supported lipid bilayers (SLBs) are model systems that mimic several properties of cell membranes, in particular the lateral mobility of the lipids.[1] These synthetic systems are stable and allow control over the membrane composition, and the location, size, and interactions of the bilayer can be controlled using a variety of membrane patterning techniques.[2−16] Additionally, the planar geometry is well suited for a wide range of surface-sensitive analytical techniques.

Despite these advantages, SLBs also have strong interactions with the substrate and therefore are often incompatible with integral membrane proteins which may stick to the substrate.[2,17−21] As a result, many variations on tethered or cushioned lipid bilayers have been developed to distance lipid bilayers from solid surfaces.[13,22−33] We have recently introduced a method for spatially separating a lipid bilayer from a solid support using DNA−lipid conjugates.[34] DNA−lipid molecules have been used in a variety of self-assembled membrane architectures, exploiting the well-defined specificity and length of DNA.[35−40] In our system, the essential features of which are illustrated in Figure 1, a DNA strand is covalently attached to a silane-modified glass slide or SiO_2 wafer. The complementary DNA strand conjugated to a lipid moiety[41] is inserted into giant unilamellar vesicles (GUVs), and the GUVs displaying DNA are allowed to interact with the surface. At a sufficiently high DNA density on the surface, the DNA hybridization induces flattening and rupture of the GUV to form a lipid bilayer patch whose dimensions correspond approximately to the surface area of the GUV. This DNA-tethered bilayer system separates the planar lipid bilayer patch from the surface by a distance precisely controlled by the length of the DNA sequence (e.g., 24-mer = 8 nm, 48-mer = 16 nm). We anticipate that this height and the average spacing between the DNA tethers of >11 nm are sufficient to accommodate membrane proteins that protrude from the bilayer surface. Since methods to incorporate functional proteins into GUVs are being developed,[42−47] this system has the potential to produce integral membrane protein arrays. Additionally, imaging the bilayer is simply due to the planarity of the tethered patch, the membranes are stable, and a wide range of compositions can be used.

While this system creates patches that can be unique from each other, the location of the patch is random, determined by where the GUV displaying DNA initially binds with its complement on the surface. We sought, therefore, a method for patterning tethered membrane patches. Control over the placement of the tethered membrane patches would enable the rapid screening of interactions with membranes in a format that is compatible with membrane proteins. Furthermore, the control over the placement of the tethered lipid bilayer potentially allows interfacing them with electronic and/or optical elements patterned on the surface.

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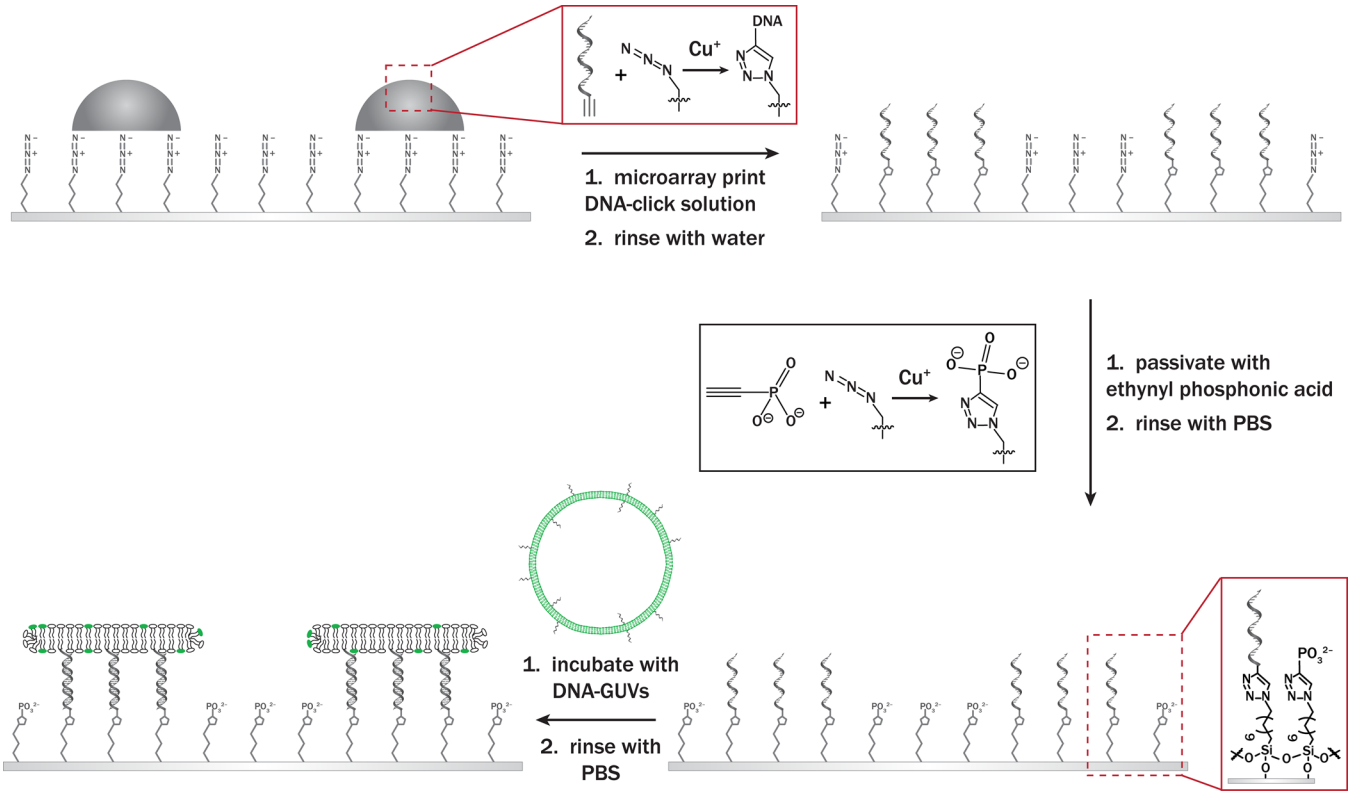

Figure 1. General scheme for assembling patterned tethered membrane patches using microarray printing (not to scale). A solution containing a DNA–alkyne and Cu^+ ions formed *in situ* is deposited over a glass slide displaying azide groups on the surface. DNA strands covalently attach to the surface where the click cocktail is deposited, and any remaining azide groups are passivated with ethynylphosphonic acid. GUVs containing a DNA–lipid complementary to the strand attached on the surface are incubated with the slide, and tethered patches form selectively over the DNA pattern.

In this paper, we present a method for creating spatially distinct tethered membrane patches above a glass slide. Surface-reactive DNA sequences are spotted onto the slide using a microarray printer, incubated to covalently link the DNA to the surface, and stable DNA–GUV patches are formed selectively on the printed DNA. Moreover, variations across the array can be created by integrating the membrane patch arrays with a microarray flow cell. Using laminar flow, molecules can be selectively tethered above membrane patches, and the composition within the patch can be altered using DNA-mediated vesicle fusion. These complex self-assembled systems require systematic optimization to achieve high quality arrays, described in detail in the following.

MATERIALS AND METHODS

Patterning Covalently Linked DNA Spots. A 24-mer DNA sequence with a 5′ terminal hexynyl moiety (alkyne-DNA1; sequence information provided in the Supporting Information) was covalently attached to an azide-terminated glass coverslip using a copper-mediated cycloaddition reaction. Briefly, 2.8 mM sodium ascorbate, 560 μM 1,1′,1″-tris(1*H*-1,2,3-triazol-4-yl-1-acetic acid ethyl ester) trimethylamine ligand (TTMA, kindly provided by Professor Christopher Chidsey, Stanford University) complexed to 280 μM $CuSO_4$, 80–170 μM alkyne-DNA1, and 1 mM β-octylglucoside in 5% glycerol–95% water were combined. The click mixture was spotted onto the azide-terminated slide using a GeneMachines OmniGrid Accent microarrayer with SMP3 Telecam stealth pins (Arrayit) at 50% relative humidity (RH). The reaction was allowed to proceed at 50% RH for 3–5 h after spotting and was terminated by rinsing with copious amounts of water. The remaining azide groups on the slide were reacted with 1.6–3.2 mM ethynylphosphonic acid in 1 mM sodium ascorbate, 100 μM TTMA, 50 μM $CuSO_4$ in water for 1.5–2 h at room temperature and rinsed with water and phosphate buffered saline (PBS; 10 mM sodium phosphate pH 7.4, 240 mM NaCl). This passivation with ethynylphosphonic acid greatly reduces the non-specific adsorption of GUVs and has been found to be superior to passivation with PEG for this application.

Formation of Tethered Membrane Patches. GUVs displaying DNA–lipid conjugates complementary to the strands on the surface were formed using gentle hydration.[48] To form tethered patches, these GUVs were incubated with the surface at 6–20 μM in PBS for 1–2 h at RT and rinsed gently with PBS. The patches were further incubated for 5–15 min at RT and rinsed thoroughly with PBS to remove loosely associated GUVs.

Varying Patch Composition. To create adjacent patches of different composition, a Y-shaped flow cell made from PDMS elastomer was sealed using vacuum grease to a slide with tethered lipid patches already assembled. To introduce variation into the pre-existing patches, a DNA lipid molecule (5′lipid–DNA2; sequence provided in Supporting Information) was flowed across half of the channel at a rate of 0.9–2 mL/h for 30 m to insert the DNA–lipid into the membranes, held apart by laminar PBS flow in the other inlet. Any free DNA lipids were rinsed away with PBS in both inlets at a rate of 2–4 mL/h for 30 min. To probe the difference in sequence created by flow, 2:1:1 DOPC:DOPE:cholesterol vesicles containing Texas Red–DHPE and DNA–lipids complementary to the inserted DNA (5′lipid–DNA2′ or 3′lipid–DNA2′) were incubated with the entire sample for 10–20 min, rinsed with PBS, and imaged. Vesicles containing DNA lipids coupled at the 5′ end tethered the SUVs on top of the membrane patches, while SUVs with DNA lipids coupled at the 3′ end primarily underwent DNA-mediated vesicle fusion to the patches.

Further details for all the methods are available in the Supporting Information.

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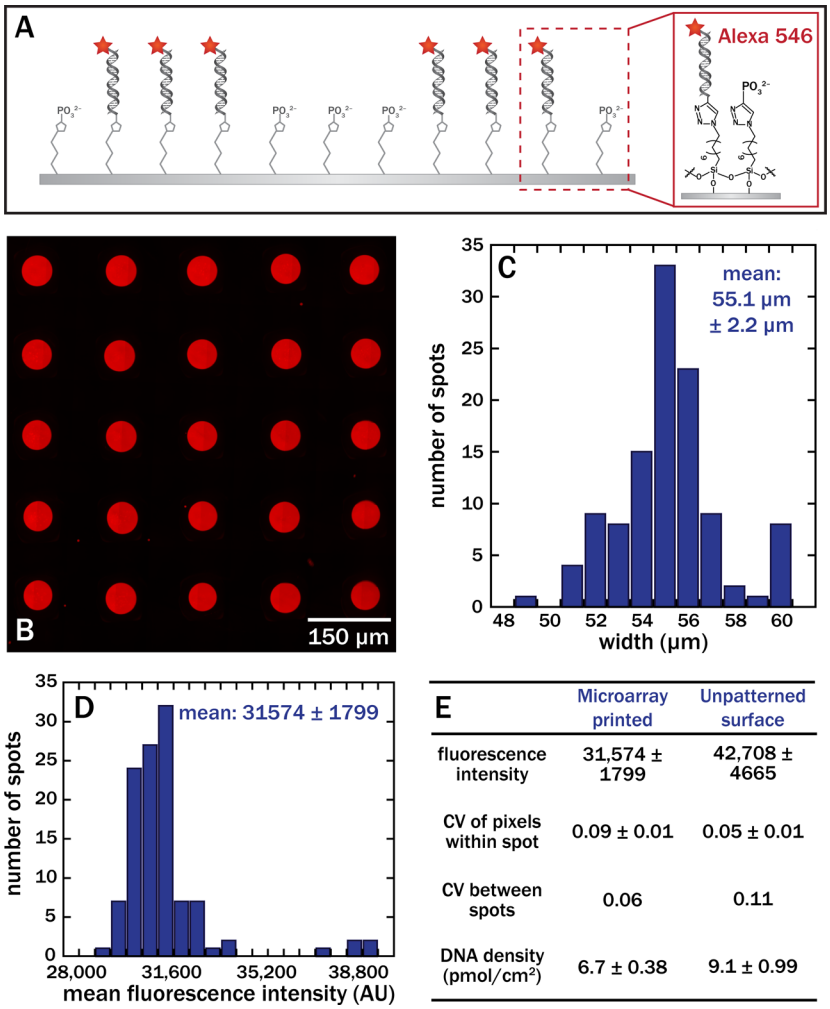

E	Microarray printed	Unpatterned surface
fluorescence intensity	31,574 ± 1799	42,708 ± 4665
CV of pixels within spot	0.09 ± 0.01	0.05 ± 0.01
CV between spots	0.06	0.11
DNA density (pmol/cm^2)	6.7 ± 0.38	9.1 ± 0.99

Figure 2. Covalent patterning of DNA using microarray printing. (A) To probe the patterned DNA density, Alexa 546-labeled DNA was hybridized to its complement covalently attached to the surface. (B) Fluorescence micrograph of Alexa 546-labeled DNA complementary to the DNA reacted on the surface. After optimization of the DNA-coupling reaction, spots are highly uniform and consistent. (C) Distribution of the width of the patterned spots. (D) Mean fluorescence intensity of the antisense strand over each printed spot. (E) Statistics on microarray-printed DNA spots and unpatterned DNA surfaces. CV is the coefficient of variation (standard deviation/average). Standard deviation is reported over 113 spots for the microarray-printed spots, and over 120 48.8 μm × 48.8 μm areas for the macroscale surfaces.

RESULTS AND DISCUSSION

DNA Patterning Using a Copper-Mediated Click Reaction. To form stable, patterned bilayer patches, we sought a method which could covalently attach DNA on a surface in patterns tens of micrometers in size. This size range enables easy visualization by fluorescence microscopy and is on the same order of magnitude as the diameter of GUVs. With the DNA at a sufficiently high density, tethered bilayers can then be formed when GUVs displaying the complementary DNA sequence are incubated with the surface.[34] The location of the membrane patches is therefore dictated by the underlying DNA patterning, and the membrane patches are separated from the surface at a distance controlled by the length of the DNA sequence. Our goals differ from traditional DNA arrays as the DNA anchors are being used to assemble a soft material at a precise distance from the surface in a defined area, while minimizing nonspecific binding of lipids elsewhere on the surface.

While other methods of DNA patterning have been used successfully, such as photomasked DNA synthesis[49] and dip pen nanolithography,[50] we chose contact microarray printing for its simplicity and its feature size (tens to hundreds of micrometers). To covalently attach the DNA to the substrate, we selected a copper-mediated cycloaddition reaction of a DNA alkyne with an azide-modified glass coverslip. This reaction is moderately fast (reaction saturating within an hour) and, more importantly, can be combined with a passivation step. The successful formation of stable DNA-tethered membrane patches requires efficient passivation of unreacted sites on the glass slide with ethynylphosphonic acid, which caps the hydrophobic azide groups with a small negatively charged group.[34] This passivation resists nonspecific adsorption of GUVs displaying DNA without blocking DNA hybridization[51] and in our experience has been more successful than PEG-based passivation for this application. To achieve patterning, therefore, droplets of the DNA−click cocktail were spotted on the surface using a microarray printer, allowed to react, and any remaining azide groups on the surface were capped with a click reaction containing ethynylphosphonic acid (Figure 1). At sufficiently high DNA density on the surface, GUVs containing the complementary DNA sequence will hybridize to the surface, flatten, and form tethered membrane patches.

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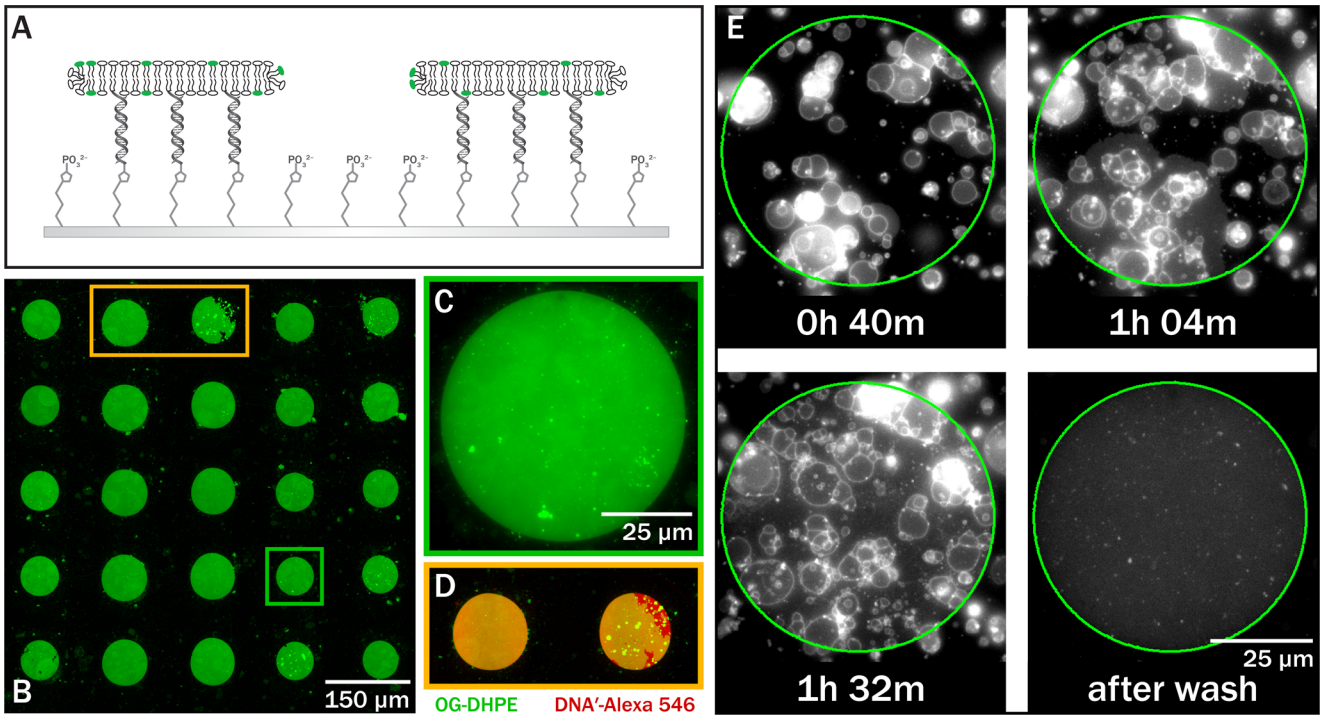

Figure 3. Fusion of GUVs to microarray-printed DNA spots and tethered patch formation. (A) Membrane patches are formed on the patterned DNA areas. (B) GUVs displaying a DNA sequence complementary to the one printed on the surface tether to the surface selectively over the patterned areas and rupture to form membrane patches. (C) Zoom-in of the membrane patch in the bottom right corner of (B), outlined in green. (D) Overlay of two membrane patches with their underlying DNA pattern, outlined in yellow in (B). Patches form selectively over the regions patterned with DNA. The DNA pattern was visualized first by binding an Alexa 546 conjugate to the DNA complement of the printed DNA (shown in red), and the membrane was visualized with OG-DHPE (green). (E) Images of the patch formation process. GUVs bind to the DNA pattern (outlined in green), flatten, rupture, and merge to form a tethered lipid bilayer. The contrast in the final image, after washing, is increased to account for photobleaching during the washing process. Time-lapse videos of the process are provided in the Supporting Information.

After optimizing the click reaction for microarray deposition (see Supporting Information), arrays of uniform DNA spots were formed over mm^2 areas (Figures 2B,D), as measured by the fluorescence of the Alexa 546-labeled antisense strands. This fluorescently labeled DNA conjugate to the printed sequence was incubated with the surface to probe the surface DNA density and location, and unbound antisense strands were rinsed away. While this metric does not report on the actual DNA density, as high DNA densities are known to sterically and electrostatically prevent the complement from binding,[52−55] it does indicate the number of available DNA tethers which could bind to GUVs displaying DNA.

On average, the spots were 55 μm in diameter, circular, and similar in size (Figure 2C). The size of the spots can be adjusted by using different diameter printing pins or by changing the surface tension of the spotted droplet using surfactants. The DNA spots were all similar in average fluorescence intensity between and within the spots (Figures 2D,E), indicating that each spot contains a similar and uniform DNA density.

The fluorescence intensity can be converted to the DNA density by dividing the average intensity over the spot by the intensity of a single Alexa 546 photobleaching step (Figure S3). As this measure assumes that the photophysical properties of the fluorophores, which may experience different environments in the two measurements, are comparable, it is not intended as an absolute measure of the DNA density on the surface; however, it does provide an estimate for the DNA density on the surface. In previous work, the DNA density required to support stable DNA-tethered patches was estimated to be greater than 1 pmol/cm^2.[34] For the patterned DNA areas in this work, the average density of each spot was measured to be 6.7 pmol/cm^2, comparable to the density and evenness on unpatterned surfaces which are known to spontaneously form tethered bilayer patches (Figure 2E).

GUV Fusion To Form Tethered Lipid Patches. When GUVs displaying the complementary DNA are incubated with the DNA-patterned surfaces, they spontaneously form arrays of tethered bilayer patches over mm^2 areas (Figure 3B and Figure S4). At high magnification (100×), these bilayers appear as a uniform plane of fluorescence with no visible defects (Figure 3C), consistent with a tethered bilayer patch. Each patch displays similar average fluorescence intensities (Figure S5) which are consistent with the layer being a single lipid bilayer. As with tethered bilayers formed on unpatterned surfaces,[34] dye-labeled lipids in the patterned tethered patches are mobile when photobleached (Figure S6).

These continuous tethered patches primarily form and are only stable on the regions patterned with DNA (Figures 3D,E, Figure S7, and Supporting Information movies). As shown in the supporting movies, as GUVs flatten to form tethered bilayers, they follow the edge of the DNA pattern (Figure S7A), and if the GUVs initially land near but not within the DNA pattern, the GUVs are drawn into the patterned region as the GUV flattens (Figure S7B). The overlap between the DNA pattern and the tethered bilayer can also be visualized directly by incubating the surfaces with an Alexa 546-labeled DNA complement (Figure 3D). The majority of the DNA was then

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dehybridized by rinsing the samples in pure water, and patches were formed on these surfaces by incubating with GUVs containing OG-DHPE and 5′lipid−DNA1′. The OG-DHPE signal colocalizes with the Alexa 546 intensity, indicating that the patches form selectively over the patterned DNA pads. Outside the patterned region, little lipid absorption is observed; most GUVs that settle outside of the patterned region remain as intact GUVs which can be rinsed away with buffer (see Supporting Information).

Optimization of Assembly. Successful assembly of arrays of stable tethered patches is dependent on three primary factors: the density of the DNA on the surface, efficient coverage of the patterned area with GUVs, and the effective passivation of unreacted groups with ethynylphosphonic acid. A detailed discussion of these factors and the optimization of tethered bilayer assembly are provided in the Supporting Information.

Introducing Variation in the Lipid Patches. For applications of arrays of tethered membrane patches to make multiplexed measurements, controllable variation of patch composition would be useful. Ideally, variation could be introduced by patterning two or more different DNA sequences on the surface and incubating the sample with two sets of GUVs, each displaying one of the DNA−lipid complements. However, patches formed by sequentially incubating two sets of GUVs with two different DNA patterns leads to mixed patches, with ∼10% of the patches containing a small portion of the lipids from the noncomplementary GUVs (<10% of the noncomplementary lipids; Figure S9). While the interaction between the DNA strands hybridized on the surface and the DNA−lipids in the GUVs is specific, GUVs settle over the entire surface during the patch formation process. As a result, they will occasionally leave behind small membrane bits during washing, presumably from interactions of the GUVs with defects in the passivation layer. When GUVs containing complementary DNA−lipids are added to these samples, the residual noncomplementary lipid debris incorporates into the GUVs patches that form. It remains possible that with more complete surface functionalization that the cross-reactivity could be minimized, or in situations where a small population of mixed patches is tolerated, this strategy could be used to introduce variation into the patches.

Since the patches are formed from a variable number and size of GUVs, stochastic variation in the patch composition can be introduced by mixing together two populations of GUVs that both contain DNA−lipids complementary to those on the surface. Each patch contains a different percentage of the two (or more) GUV types (Figure S10); however, the ratio of each individual patch cannot be controlled *a priori* and can only be assayed by a secondary marker, such as ratio of two fluorescent lipid dyes in the two GUV populations.

To introduce controlled variation into the patch arrays, therefore, the tethered patch arrays were interfaced with a PDMS flow channel containing two inlets (Figure 4A). Liquid flowing through the flow cell was held apart by laminar flow; as such, bilayers on the right side of the channel were only exposed to the solution delivered by the right inlet. When this solution is comprised of DNA−lipids in solution, the lipid tails of these molecules will spontaneously insert into the pre-existing lipid bilayers. The selective DNA−lipid insertion to a portion of the array can be probed by hybridizing the DNA complement, and this complement can be coupled to something else to deliver cargo, such as SUVs. If the DNA is

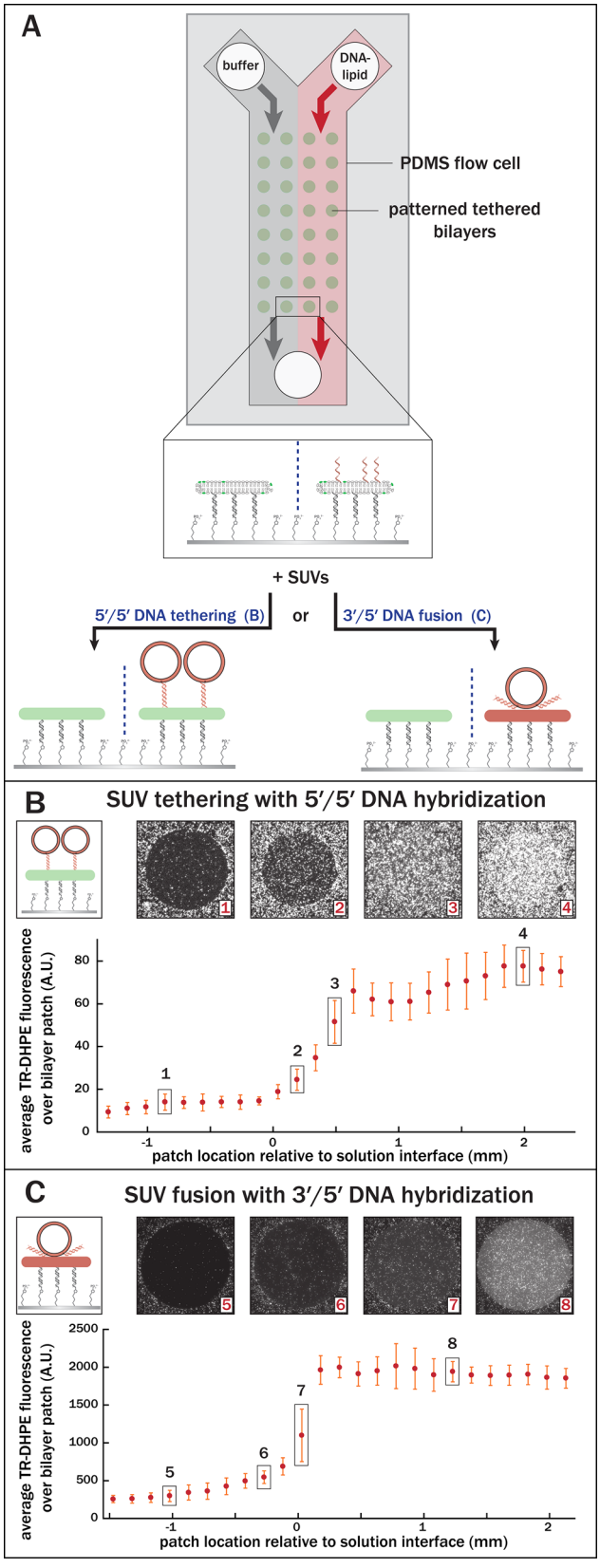

Figure 4. Insertion of DNA−lipid molecules from solution into preformed tethered patch arrays to introduce variation between the patches. (A) Cartoon schematic of the process. Arrays of tethered bilayers are first formed and then interfaced with a PDMS flow cell. The flow cell delivers DNA−lipids selectively to the right side of the array by laminar flow, where they spontaneously insert into the membrane patches. Excess DNA lipid molecules are rinsed away. The

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Figure 4. continued

inserted DNA−lipids can then be used to either tether SUVs containing the complementary strand (left) or to fuse other materials into the existing bilayers using DNA-mediated fusion (right). (B) Tethering of SUVs to DNA strands inserted into membrane patch arrays. On the left side of the interface between the buffer and DNA−lipid flow, where the bilayers were exposed to buffer followed by SUVs, few vesicles stick to the bilayer surface, though many nonspecifically stick on the surface outside the tethered bilayer. The tethered bilayer which lacks the complementary DNA acts as a better passivating agent than ethynylphosphonic acid. On the right side of the interface, which was exposed to a DNA−lipid solution and then to complementary SUVs, many SUVs bind to the tethered membrane patches, shown by an increase in fluorescence over the bilayer relative to the bilayers only exposed to buffer. Fluorescence microscopy images of the boxed data points are shown above the graph. Error bars represent the standard deviation in the average TR-DHPE intensity across 17−37 patches located at a similar position in the gradient. (C) Fusion of SUVs to DNA strands inserted into membrane patch arrays with the complementary DNA sequence in the zippering orientation. Similar to (B), DNA−lipids are inserted into the right portion of the patch array, and all the sample is exposed to SUVs containing TR-DHPE. When the orientation of the DNA−lipid in the SUVs is changed from 5′ to 3′, the vesicles undergo DNA-mediated vesicle fusion and merge the SUV membrane with the existing tethered patch membrane which transfers the dye into the patch.[41,56−58] Fluorescence microscopy images of the boxed data points are shown above, and the error bars represent the standard deviation across 24−40 patches.

coupled to the lipid moiety at its 5′ end in the SUV, the molecule will simply tether the SUV above the bilayer; however, if the lipid is attached at the 3′ end of the DNA, the SUVs can undergo DNA-mediated membrane fusion to merge the contents of the SUV with the tethered patch.[41,56−58]

When SUVs containing complementary DNA−lipids coupled to the 5′ end were added to the entire flow chamber after the insertion of DNA−lipids, SUVs selectively tether over the region that has been exposed to the DNA−lipid solution (Figure 4B). Some SUVs nonspecifically stuck to both the tethered bilayer and the ethynylphosphonic acid-passivated surface. This nonspecific adsorption is more significant on the unpatterned surface: the tethered lipid bilayer acts as a better passivating agent than ethynylphosphonic acid. As a result, few SUVs tether on the portion of the membrane array not exposed to DNA−lipids, shown by the low average TR-DHPE fluorescence over these bilayers. In the region exposed to the DNA−lipids, the average fluorescence increases substantially. On both sides of the interface between the lipid−DNA and buffer solutions, the fluorescence intensity is constant; at the interface, an intermediate fluorescence is observed over a distance of ∼500 μm. The transitional region and the noise in the measurement are due to two factors: diffusion between the two solutions which allows some mixing to occur, and slight misregistration of the PDMS flow cell with the patterned regions. If there is a slight angle between the edge of the channel and the patterned bilayers, adjacent bilayer patches will be at slightly different positions with respect to the solution interface. In these experiments, the degree of tilt was estimated to be ∼60 μm from the left edge of the pattern to the right edge.

At the edge of the transitional region, the average fluorescence intensity over the bilayer is approximately equal to the amount of nonspecific adsorption to the unpatterned surface (see bilayer 3 in the inset, Figure 4B). Consequently, the edge of the bilayer becomes difficult to differentiate from the background. Over the plateau region where the DNA−lipid insertion saturates, the concentration of tethered SUVs is significantly higher than the background, and the boundary of the lipid bilayer can be clearly observed over the background (bilayer 4, Figure 4B). Taken together, the tethering of the SUVs, indicative of DNA−lipid incorporation, is selective for the regions of the array exposed to the DNA−lipid solution and can selectively introduce variations into portions of membrane arrays.

Instead of tethering SUVs, DNA−lipids can also be used to fuse SUVs to patches when the lipid is anchored to the DNA at the 3′ end in the SUV. After 5′lipid−DNA2 is flowed over half of the membrane array, SUVs containing TR-DHPE and 3′lipid−DNA2′ is incubated over the entire array (Figure 4C). Upon DNA hybridization, vesicles that dock to the membrane can undergo one of three fates: they can remain docked with their membrane adjacent to the tethered bilayer; they can hemifuse, merging their outer leaflets with the underlying bilayer; or they can fully fuse, combining both their inner and outer leaflets with the tethered bilayer and releasing their contents into the solution below the patch.[41,56−58] Punctate fluorescence is therefore indicative of either docked or hemifused vesicles; diffuse fluorescence within the bilayer indicates hemi- or full membrane fusion and lipid mixing. Similarly to when 5′/5′ SUV docking is used to probe the selective insertion of DNA−lipids, an increase in fluorescence over the background only occurs in the portion of the array exposed to the DNA−lipid solution. Over regions exposed only to buffer, a small number of nonspecifically adsorbed vesicles are observed on the bilayer, and little to no nonspecific membrane fusion is observed (bilayer 5). Over the region exposed to the DNA−lipid solution, membrane fusion is observed, indicated by an increase in the uniform fluorescence over the patch (bilayers 7 and 8). The number of docked vesicles also increases markedly, since the probability of docking scales with the density of the complementary DNA−lipids in the bilayer. In sum, the selective insertion of DNA−lipids into an area of the membrane array can be used to incorporate lipids into a subset of the membrane patches. Interfacing a flow cell with an existing lipid patch array, therefore, provides a mechanism for introducing controlled variation into arrays of tethered membrane patches. While this study creates an array of two different lipid compositions, this concept can be extended to transfer membrane proteins from SUVs to patches where, for example, the variation across the array of bilayer patches could be in the number density or identity of the protein.

■ CONCLUSIONS

Large arrays of reproducible DNA spots can be patterned on glass slides using microarray printing of a copper-mediated click reaction mixture. Under the optimized reaction conditions, the DNA density within these patterns is dense and uniform. Arrays of tethered membrane patches can be readily assembled by incubating the surfaces with GUVs that display DNA complementary to the sequence on the surface. These tethered patches separate the bilayer from the surface by the length of the DNA sequence, and stable patches only form over the DNA patterns. Currently the surface passivation is insufficient to completely prevent GUVs from leaving small amounts of contamination on the surface. However, it is possible to differentially modulate the composition of the patches in a

controlled manner using microfluidic flow devices. The selective addition of DNA–lipids to a portion of the array can be used to bind materials above a membrane or to introduce new materials into the lipid bilayer via DNA-mediated vesicle fusion. Furthermore, though technical challenges remain, the tethered platform should enable the incorporation of functional integral membrane proteins and the controlled integration of these patches into more complex devices. Current work is focusing on integrating these proteins into tethered patches to enable their rapid screening and characterization and interfacing the patches with flow cells containing more than two inlets.

■ ASSOCIATED CONTENT

Supporting Information

Additional materials and methods, supplemental characterization of DNA patterning and tethered bilayer formation, and videos of the tethered patch formation process. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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