Stability of DNA-Tethered Lipid Membranes with Mobile Tethers

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Supporting Information

ABSTRACT: We recently introduced two approaches for tethering planar lipid bilayers as membrane patches to either a supported lipid bilayer or DNA-functionalized surface using DNA hybridization (Chung, M.; Lowe, R. D.; Chan, Y. H. M.; Ganesan, P. V.; Boxer, S. G. J. Struct. Biol. 2009, 168, 190–9). When mobile DNA tethers are used, the tethered bilayer patches become unstable, while they are stable if the tethers are fixed on the surface. Because the mobile tethers between a patch and a supported lipid bilayer offer a particularly interesting architecture for studying the dynamics of membrane–membrane interactions, we have investigated the sources of instability, focusing on membrane composition. The most stable patches were made with a mixture of saturated lipids and cholesterol, suggesting an important role for membrane stiffness. Other factors such as the effect of tether length, lateral mobility, and patch membrane edge were also investigated. On the basis of these results, a model for the mechanism of patch destruction is developed.

INTRODUCTION

We recently introduced two approaches and architectures for tethering planar lipid bilayers to either a fluid supported lipid bilayer (SLB) or DNA-functionalized surface using DNA hybridization as illustrated schematically in Figure 1. In both cases, giant unilamellar vesicles (GUVs) displaying the antisense DNA based on a DNA–lipid conjugate are first tethered either to a supported bilayer displaying mobile DNA tethers or to a fixed and sparse layer of DNA bound to the substrate. These tethered GUVs flatten and spread as more DNA hybrids are formed. If the DNA density on the surface is low (0.01–0.1 mol %, equivalent to about 0.26–2.6 nmol/m² surface density), then the GUV remains tethered indefinitely in either design, and this represents a useful tool for probing interactions with GUVs that will be described elsewhere. If, on the other hand, the DNA density is higher, the tethered GUV is observed to rupture and patches of tethered membrane whose area roughly corresponds to the surface area of the original GUV are formed as illustrated in Figure 1 (see Supporting Information Figures S1 and S2 for further illustration of this process). Unfortunately, such patches tethered to an SLB (mobile tethers) composed of simple phospholipids such as egg phosphatidylcholine (EggPC) were observed to be dynamically unstable, spontaneously breaking apart and disappearing, while patches formed using fixed DNA on the surface and flattened GUVs tethered to an SLB (with a low density of DNA) remain stable irrespective of their composition.

Because the tethered bilayer patches formed by mobile DNA-tethers offer a unique opportunity to model membrane—membrane junctions with ligand–ligand interactions, for example, segregated DNA hybrid patterns or topological domains when two different lengths of DNA tethers are used to tether the two membranes, and because even more complex architectures built upon the patches could be interesting, we have systematically investigated the sources of instability. In the following, we describe approaches to make stable DNA-tethered lipid bilayer patches with mobile DNA-tethers, focusing in particular on the effects of lipid composition on stability, and some aspects of the mechanism by which tethered patches are lost.

MATERIALS AND METHODS

SLB and DNA-Immobilized Surface Preparation. In a typical experiment, SLBs are formed by vesicle spreading. The lipid vesicles used to form the SLB were made by extrusion with a 100 nm polycarbonate membrane (Avanti) using 98 mol % of egg phosphatidylcholine (EggPC, Avanti Polar Lipids) and 2 mol % of 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoazadiazol-4-y1)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC, Invitrogen). A 0.1–0.5 mol % portion of DNA-lipid conjugates, (C₄₆)₂-polyA (typically a 24mer; see the Supporting Information for structure and sequence details), were added to the vesicle suspension, so the SLBs display lipid-bilayer-anchored DNAs for tethering their counterpart. As previously described in detail, the DNA-lipid conjugates were synthesized by adding lipid-phosphoramidite to a DNA synthesizer so that the last nucleobase is coupled (Supporting Information Figure S3). After the lipid-oligonucleotide conjugate was cleaved from resin and deprotected, conjugates were purified by HPLC and analyzed by MALDI. This system has been...
A, the tethering DNA is covalently attached to the substrate and so the DNA tethers are not mobile (the lateral density can be controlled; the remainder of the surface is passivated with phosphate groups). In part B, the tethering DNA is displayed on the surface of a supported lipid bilayer so the DNA tethers are laterally mobile. This design brings two fluid bilayers into close proximity defined by the length of the DNA tether (∼8 nm for a 24mer hybrid; ∼16 nm for a 48mer hybrid). While tethered lipid bilayer patches formed using immobilized tethers are stable irrespective of their composition, those formed from lipids such as EggPC using mobile tethers are not. See Supporting Information Figures S1 and S2 for the mechanism of GUV tethering and patch formation.

Figure 1. Schematic diagram of DNA-tethered lipid bilayer patches formed by rupture of giant unilamellar vesicles on two different substrates. In part A, the tethering DNA is covalently attached to the substrate and so the DNA tethers are not mobile (the lateral density can be controlled; the remainder of the surface is passivated with phosphate groups). In part B, the tethering DNA is displayed on the surface of a supported lipid bilayer so the DNA tethers are laterally mobile. This design brings two fluid bilayers into close proximity defined by the length of the DNA tether (∼8 nm for a 24mer hybrid; ∼16 nm for a 48mer hybrid). While tethered lipid bilayer patches formed using immobilized tethers are stable irrespective of their composition, those formed from lipids such as EggPC using mobile tethers are not. See Supporting Information Figures S1 and S2 for the mechanism of GUV tethering and patch formation.

Figure 2. Schematic diagram of the architecture used to create a second tethered lipid membrane patch by GUV rupture on top of a first bilayer patch which is tethered using fixed DNA on the surface and whose composition can be varied at will (c.f. Figure 1A). The GUVs used to form the first story tethered patch display both the antisense sequence to bind the GUV and patch to the fixed DNA on the surface (black DNA) and a second DNA sequence, orthogonal to the first, which is used to tether the second story GUV and patch (red DNA). In the example described in the text, the lower tethered bilayer is a 60:40 mixture of DPPC and cholesterol, while the upper bilayer is EggPC. This strategy is used to test the dependence of the second story patch stability on DNA hybrid diffusion, but also is the basis for much more complex designs.

studied extensively: the lipid-anchored DNAs are mobile on the SLB surface, and small vesicles tethered using the antisense DNA are observed to be mobile parallel to the plane of the SLB. In the case of immobilized DNA (Figure 1A), DNA is covalently attached to the glass surface by click chemistry and available for tethering, as previously described. Briefly, following the vapor-deposition of azidosilane monolayers on a glass coverslip (VWR), the azide-modified glass surfaces were treated by 5 alkynyl modified oligonucleotide (alkynyl-terminated DNA), either a 24 or 48mer. The remaining unreacted azides were passivated by ethynyl phosphonic acid which effectively repels vesicles and GUVs.

**GUV Preparation and Tethered Membrane Patch Formation.** GUVs were grown in a 250 mM sucrose solution by using the electroformation technique. Lipid mixtures composed of varying fractions of cholesterol and saturated phospholipids—including 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipentadecanoyl-sn-glycero-3-phosphocholine (D15PC), and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)—were used with 1 mol % Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (TR-DHPE, Molecular Probes, Eugene, OR) for visualization. The maximum cholesterol concentration used in this work is 50 mol %, which is below the maximum solubility in a DPPC bilayer. For the mixtures of unsaturated phospholipids and cholesterol, 1-palmitoyl-2-octenoyl-sn-glycero-3-phosphocholine (POPC) or EggPC was used. In the case of GUVs containing single chain lysolipids, a small amount (~0.2 mol %) of 1-lauroyl-2-hydroxy-sn-glycero-3-phosphocholine (Avanti Polar Lipids), which is sufficient to populate the small edge region of the patches (see below), was added to the EggPC. Electrosionelling was performed at 60 °C, above the chain melting temperature. A 1 mol % portion of DNA-lipid conjugate was added to the lipid mixture before electroformation for GUV tethering and patch formation as previously described. As a result, the DNA is displayed on both the inner and outer surface of the GUV (and the top and bottom of the resulting patch). For tracking the location of the DNA tethers during patch disintegration, Cy5 was added on the 3' end of the DNA, with the 5' end bound to the lipid. For this measurement, the GUVs did not contain additional dye-labeled lipids.

Patches are formed by rupture of the GUVs on either the DNA immobilized surfaces or DNA presenting SLBs (see Supporting Information Figures S1 and S2). When GUVs make contact with a DNA displaying surface, the bound GUVs flatten, then rupture as DNA hybridization progresses, and this process has been visualized by confocal and epifluorescence microscopy [1, Supporting Information Figure S2]. The lipid membrane of the ruptured GUV then rapidly spreads to form a planar tethered lipid bilayer patch. Although not the subject of this paper, GUVs and patches stably tethered to fixed DNA on the surface (Figure 1A) are an ideal platform for studying many membrane-dependent processes, complex lipid phases can be formed, and the tether length can be varied at will to control the distance between the lower leaflet and the solid support, which is ideal for the incorporation of integral membrane proteins.

Another interesting hybrid architecture in which a "second story" bilayer is tethered to a patch that is stably tethered to fixed DNA on the surface is illustrated in Figure 2. The first patches are made by rupturing GUVs containing two orthogonal DNA sequences—one for tethering the first story to the DNA immobilized on the surface, and the second for binding the second story, through mobile tethers, to the first (see the Supporting Information for sequences). After the first story patches are formed and excess GUVs removed, the second set of GUVs displaying the antisense sequence of DNA is added and allowed to land on the first story patches. While this could be done more precisely by micropipet manipulation, to date these second story patches are largely formed by chance and the yield is not very high. Note that as with the mobile tether design (Figure 1B), the two membranes are formed by independent processes, so their compositions can be different and they can be independently labeled with dye-labeled lipids.

**Classifying Tethered Patch Stability.** As described above, the EggPC bilayer patches tethered with mobile tethers (Figure 1B) disintegrate over time in a stochastic fashion. While it is possible to capture interesting behavior prior to their destruction, the transient nature makes this an unreliable system. A precise definition of stable and unstable bilayer patches is somewhat arbitrary, so the approach we have taken is best
illustrated by documenting the actual process of disintegration as shown in Figure 3. A patch formed by GUV rupture and visualized by epifluorescence microscopy. A tethered lipid bilayer patch composed of EggPC lipids and containing a small amount of Texas Red labeled lipid is formed by GUV rupture and spreading on a mobile supported bilayer as illustrated in Figure 1B. The time of formation is defined as \( t = 0 \) (see Supporting Information Figure S2). The initial area is outlined, and it is observed that the patch shrinks from this boundary over time. The bright spots outside of the patch are bound midsize GUVs.

In order to describe and classify this diverse behavior, we have developed the following criterion: if most of the patches maintain their original form for 30–60 min or longer, it is possible to do further analysis, and if more than 80% of the initial area remains after 30 min, this will be denoted a “stable patch”. To compare patch stability for different lipid compositions and architectures, tethered bilayer patches were prepared by GUV rupture as described above, and the number of stable patches and unstable patches was counted. After the GUVs rupture and form patches, an initial image of each patch was made, followed by a further measure 30 min later to determine the percent of remaining area. For each sample, we explored a 1.2 mm\(^2\) area and examined 30–50 patches; data from 5 samples were averaged to obtain meaningful statistics. It is simple to classify limiting cases, e.g. when most of the patch remains intact, they are classified as stable; likewise, when more than 30% of initial area is lost, these are classified as unstable. However, for patches with 70–90% remaining area, we had to measure the area left behind carefully by counting pixels so that the area of irregularly shaped patches could be quantified.

**Diffusion Coefficient of Lipids by FRAP.** As previously described,\(^1\) diffusion coefficient measurements of lipids in tethered bilayer patches by fluorescence recovery after photobleaching (FRAP) is challenging. For valid FRAP analysis, only a small fraction of the total area should be bleached in order to assume an infinite reservoir of fluorescent lipids. This means that the radius of the circular bleached spot should be smaller than 3 μm for the 20–40 μm radius of tethered bilayer patches. Moreover, the small bleached spot recovers in a few seconds because the lipids in these patches are highly mobile, so the recovery profile must be recorded immediately. The automated FRAP module of the Leica TCS SP2 AOBS laser scanning confocal microscope meets these requirements. The acquired images are processed to yield an integrated intensity recovery curve, and the diffusion coefficient and mobile fraction data are obtained from the fit of the solution of Fick’s law.

**RESULTS AND DISCUSSION**

**Dependence of Patch Stability on Composition.** The tethered lipid bilayer patches and underlying supporting lipid bilayer in our initial work were both made of EggPC, a widely used natural lipid. Because the majority of the EggPC is composed of unsaturated phospholipids, the EggPC lipid bilayers have a low chain melting temperature and are relatively loosely packed. Therefore, we expected that the destruction of patches might be prevented if we made the patches stiffer by using saturated lipids and cholesterol, so the stability of tethered patches composed of varying fractions of DPPC and cholesterol (always tethered to an EggPC SLB) was tested.\(^13\) The phase behavior of lipid bilayers composed of DPPC/cholesterol binary mixtures has been extensively characterized. While different investigators differ somewhat on the exact phase boundary, temperature, and composition, the low-cholesterol DPPC bilayer is in the gel phase until about 25 mol % cholesterol, and those with more than 30 mol % cholesterol are in the liquid-ordered (\(L_0\)) phase at room temperature.\(^14,15\) When tethered patches were formed with more than 70% DPPC, the lipids in these patches lost lateral mobility, as expected for the gel phase, and the patches retained their original shape over time. These are not interesting for probing membrane–membrane dynamics; however, the result is consistent with the suggestion that stiff membrane patches are likely to be more stable. Patches with 60% DPPC and 40% cholesterol were stable—90% of these patches retain more than 80% of their initial area after 30 min (as defined in Materials and Methods), and the lipids in the patches were fluid, characteristic of the \(L_0\) phase. With less than 50% DPPC, the patches are unstable. Therefore, the dense and stiff DPPC bilayers indeed prevent the patches from disintegrating, and those with more cholesterol tend to be more stable and fluid.

To test the individual effects of DPPC and cholesterol, we measured the stability of EggPC/DPPC and EggPC/cholesterol mixtures. It has been reported that there is about a 4-fold increase in the bending modulus from 0.27 × 10\(^{-19}\) J for pure EggPC to 1.81 × 10\(^{-19}\) J in EggPC bilayers upon inclusion of 15–50% cholesterol.\(^16\) If this were the primary factor determining stability, the stiffer EggPC/cholesterol bilayers might be expected to have increased stability, though they are still less stiff than saturated lipid–cholesterol mixtures, e.g. a bending modulus of 4.2 × 10\(^{-19}\) J has been reported for a 7:3 DMPC/cholesterol mixture.\(^17\) Similarly, we would expect that the EggPC/DPPC bilayers should be stiffer than pure EggPC bilayers because the saturated DPPC will make the bilayer structure denser, though we are not aware that mechanical properties have been reported for this lipid mixture. As seen in Table 1, even when EggPC is mixed with more than 50% cholesterol, the patches were not observed to be stable. We also tested the stability of patches composed of 1:1 and 3:1 EggPC/DPPC lipid mixtures. The majority of patches with 3:1 EggPC/DPPC are unstable (25% stability), while a 1:1 mixture of EggPC/DPPC shows higher stability (75%), but at the expense of lateral mobility of the lipids in these patches. This result suggests that we cannot make stable and fluid patch bilayers by adding DPPC or cholesterol independently to EggPC bilayers. On the basis of these observations, DPPC appears to be important for stability but has to be combined with cholesterol to maintain fluidity. This is illustrated
Table 1. Stability of Patches Made from a Variety of Lipid Compositions Using Mobile Tethers

<table>
<thead>
<tr>
<th>lipid compositions of tethered patches</th>
<th>stability [%]</th>
<th>diffusion coefficient [μm²/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>EggPC only</td>
<td>5</td>
<td>5.6 ± 1.0</td>
</tr>
<tr>
<td>EggPC/DPPC 3:1</td>
<td>25</td>
<td>4.8 ± 1.2</td>
</tr>
<tr>
<td>EggPC/DPPC 1:1</td>
<td>75</td>
<td>gel phase</td>
</tr>
<tr>
<td>EggPC/Chol 6:4</td>
<td>15</td>
<td>2.9 ± 0.8</td>
</tr>
<tr>
<td>DPPC/Chol 7:3</td>
<td>95</td>
<td>gel phase</td>
</tr>
<tr>
<td>DPPC/Chol 6:4</td>
<td>90</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>DPPC/Chol 5:5</td>
<td>70</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>D15PC/Chol 8:2</td>
<td>95</td>
<td>gel phase</td>
</tr>
<tr>
<td>D15PC/Chol 7:3</td>
<td>85</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>D15PC/Chol 6:4</td>
<td>30</td>
<td>3.6 ± 0.8</td>
</tr>
<tr>
<td>DPPC/Chol 9:1</td>
<td>65</td>
<td>gel phase</td>
</tr>
<tr>
<td>DMC/Chol 8:2</td>
<td>25</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>DMC/Chol 7:3</td>
<td>20</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>EggPC only with 48mer</td>
<td>10</td>
<td>N/A</td>
</tr>
<tr>
<td>EggPC patch on DPPC/Chol patch</td>
<td>70</td>
<td>N/A</td>
</tr>
<tr>
<td>EggPC patch on EggPC/Chol patch</td>
<td>20</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Data of second-story patches. Others are formed on EggPC supported bilayers.

in Figure 4 in a typical side-by-side comparison of a disintegrating patch formed with EggPC and a stable patch formed with DPPC/cholesterol.

To test further for the effect of saturated lipids and cholesterol mixtures, D15PC and DMC were also investigated (see Table 1). We observed that a 7:3 D15PC/cholesterol mixture showed a comparable stability as a 6:4 DPPC/cholesterol mixture, while patches containing less than 70% D15PC were unstable, and those with more than 70% were gel phase. Patches made from DMC/cholesterol mixtures were unstable up to 80% DMC and were in the gel phase when more DMC was added. Interestingly, no DMC/cholesterol mixture was as stable as the 6:4 DPPC/cholesterol mixture. This may be attributed to the higher stiffness of DPPC/cholesterol bilayers than DPPC/cholesterol bilayers. The reported area compressibility of DPPC/cholesterol mixtures is in the range 640–2000 dyn/cm from molecular dynamics simulations and 1281 dyn/cm for a 6:4 DPPC/cholesterol mixture from micropipet aspiration. The area compressibility for 7:3 DMPC/cholesterol mixtures was reported as 600 dyn/cm. Because the relation between area compressibility and bending modulus and the membrane hydrophobic thickness is well-established, the stiffness of these bilayers are compared by compressibility due to limited bending modulus data. Though an exact correlation between patch stability and the stiffness of bilayers is not possible due to insufficient independent data on mechanical properties, the overall trend supports the suggestion that stiff bilayers made of saturated lipids increases the stability of the tethered patches.

Dependence of Patch Stability on Tether Mobility. Not surprisingly, there is also a correlation between patches whose lipids have lower diffusion coefficients and stability (Table 1). The mobility of the hybridized DNA tethers, which are anchored both to the supported and tethered lipid bilayers, will decrease when at least one anchored lipid bilayer has a low diffusion coefficient. Since patches with immobile tethers are stable irrespective of composition, we might expect that more slowly moving DNA hybrid tethers would not tend to dissipate and disintegrate the patches. This can be investigated by taking advantage of the independent assembly of the supported bilayer and the tethered GUV. Specifically, we would predict that an EggPC bilayer patch tethered to a DPPC/cholesterol supported bilayer should be as stable as a DPPC/cholesterol patch on an EggPC supported bilayer. Unfortunately, it proved difficult to make uniform 6:4 DPPC/cholesterol supported bilayers. Instead, we take advantage of the more complex architecture shown in Figure 2 where a wide range of compositions can be used on the “first story”, including 6:4 DPPC/cholesterol tethered patches. Once the 6:4 DPPC/cholesterol tethered patch was assembled, a “second-story” EggPC bilayer patch was added using an orthogonal DNA tethering sequence, as illustrated schematically in Figure 2. In this configuration, 70% of the second-story EggPC bilayer patches were stable, but not as stable as “one-story” 6:4 DPPC/cholesterol bilayer patches tested above with mobile tethers (Figure 1B) despite the same linker mobility. This implies that both the mobility of the DNA hybrid tethers and also the stiffness of the bilayer patches contribute to stability. As a comparison, the stability of second-story tethered EggPC patches on an EggPC first story bilayer tethered with immobile tethers was observed to be as unstable as tethered EggPC patches on EggPC SLBs. Thus, the different quality of the lower bilayer, for example, due to possible defects of SLBs or the hard solid support, has no effect on the stability of tethered patches.

Tether Length, Concentration, and Salt Dependence. In order to test whether the length of the tether matters, we compared the stability of tethered patches with same mole percent of 24mer (~8 nm) and 48mer (~16 nm) DNA tethers. As shown in Table 1, there is no significant difference in the stability of tethered bilayer patches with 24 or 48mer hybrids. This suggests that the interaction between the two lipid membranes is not a major factor for patch stability.

Repulsion between highly negatively charged DNA tethers which are confined inside a small patch might enhance the dissipation of DNA tethers; however, if this is the case, the
patches should be more stable with a smaller mole percent of DNA, but the stability was not affected. Moreover, lowering the salt concentration did not change the patch stability, unless the salt concentration reached the level that cannot maintain DNA hybridization. Because the average distance between DNA tethers is on average about 8 nm for 1 mol % DNA, the charge interaction should be well screened by the salt.

**Mechanism of Patch Disintegration.** While useful compositions have emerged from these empirical observations, it would be desirable to at least speculate on the possible mechanism of instability. The observations reported above and in the earlier work\(^1\) provide some hints about the sources of tethered membrane patch instability. Tethered, flattened GUVs were stable (Supporting Information Figure S2),\(^21\) even if composed of unsaturated lipids, but as soon as they rupture and form planar bilayer patches, they start to disintegrate. The obvious difference between tethered GUVs and bilayer patches is that the patches have edges. The edge of a planar lipid bilayer might be expected to be unstable because the hydrophobic interior of the bilayer is more exposed to the aqueous solvent.\(^22\) As with patterned SLBs,\(^23\text{--}25\) the precise nature of the edge is not entirely clear, though it is likely that some sort of micellar structure stabilizes the edges.

To test this hypothesis, we added a small amount (\(\sim0.2\) mol %) of a single chain lysolipid, which is known to favor a micellar structure\(^26,27\) and could make the edge more stable; however, this had no effect on the patch stability. This may imply that the patches with edge are unstable, not because of the instability of the bilayer edge, but because it allows bilayer fragments dissipated.

The patch edge is obviously important for another reason, as nearly all loss is observed at the edge (Figure 3). Tethered GUVs form a continuous closed system without an exposed edge, while the patches have open edges from which pieces of lipid membranes can be fragmented and dissipated. The mobile DNA hybrid tethers crowded in patches are expected to be evenly distributed, and this may be why the mobility is related with the patch stability. Using dye-labeled DNA, we can track where the DNA hybrid linkers from the patch are\(^28\) during the patch destruction process, and this is shown in Figure 5. Initially, the DNA appears homogeneous over the patch, but as it dissipates, a halo of fluorescence appears around the patch and this diffuses away over time. A similar halo is also observed with dye-labeled lipid during patch destruction, though it is not visible in Figure 3 due to its low brightness relative to the clear outline of the patch. As reported earlier,\(^1\) if the underlying supported bilayer is patterned and a patch forms within the patterned region, the disintegrating membrane in the patch is confined to the patterned region. It was not clear from the earlier work whether the dye-labeled lipid from the patch ended up in the underlying SLB or remained in tethered fragments. It unlikely that the SLB, which is initially formed in the presence of excess vesicles, has enough room to absorb a significant amount of lipid molecules derived by destroyed tethered bilayers. Though the lipids could detach into the bulk solution by blebbing, no such changes in structure perpendicular to the tethered bilayer from the edge or the middle of the patch have been observed. The tracking of the DNA tethers suggests the lipids remain, but as tethered fragments.

Putting these elements together, we can speculate further on what distinguishes different compositions. The motion of DNA hybrid tethers away from the patch is entropically favored over those whose motion is arrested in a patch (illustration in Figure 5); however, fragmentation of the tethered lipid membrane is disfavored because small lipid membrane fragments create edges that must be accommodated.\(^29\) Lipid membranes composed of saturated lipids have reduced mobility and a more densely packed structure, while unsaturated lipids form more relaxed structure with higher mobility. One expects, therefore, that the penalty for breaking apart a patch made from saturated lipids would be greater. While this is far from a quantitative or predictable result, this is a complex assembly, and its dynamics are, not surprisingly, complex.

**ASSOCIATED CONTENT**

Supporting Information. Schematics and microscopy pictures describing tethered patch formation by GUV rupture along with sequence information. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES


(3) Alternatively, the DNA-lipid conjugate can be added to the preformed SLB; the properties and stability of the resulting tethered patches is independent of the method used.


(10) The DNA-lipid conjugate can be added exclusively to the outer leaflet of the GUV in which case it will be initially on the lower side of the tethered membrane patch given the formation mechanism; however, we have observed that DNA is eventually found on both surfaces because vesicles displaying the antisense DNA can be tethered to the patch irrespective of how the DNA-lipid conjugate was initially incorporated into the GUV. This suggests that DNA-lipid conjugates can move around the edges of the patch as we expect flip-flop through the bilayer with a DNA headgroup would never occur.


(12) If the GUV that forms the second story lands on a patch formed from a GUV that was smaller, it typically does not rupture. Though a continuous tethered bilayer would not have this limitation and would be useful for many other applications, we have never succeeded in making a completely continuous tethered bilayer by fusion among patches. We believe the reason for this is the inevitable small vesicles (∼5 nm) and debris associated with GUV formation that bind the area outside of patches and inhibit successive patch formation. If highly purified populations of GUVs displaying DNA could be made, perhaps this could be achieved.

(13) Tethered GUVs composed of DPPC/Chol are relatively stiff and hard to rupture spontaneously. We usually wait until they are flattened and induce rupture by mechanical or osmotic stress.


(21) Some flattened GUVs do not undergo spontaneous rupture and remain intact for hours even though the DNA density is sufficiently high that most rupture.


(28) Cy5-labeled DNA-lipid conjugates were only included in GUVs, while the complementary strand DNAs on the supported bilayer are not dye-labeled, so that the Cy5 signal originates only from the GUV. Because GUVs have a higher mole percent of DNA, a fraction of them might remain unhybridized and indistinguishable from those that are hybridized. However, since the DNA-lipids on the supported bilayer diffuse rapidly, what matters is the total pool of DNA, not the mole percent or the number density, as diffusion brings fresh DNA from outside the patch into the patch region. This was simply proved by monitoring patch formation on a supported bilayer containing dye-labeled DNA, which causes the labeled DNAs that were originally evenly distributed across the supported bilayer surface, to accumulate under the patches by hybridization with excess antisense DNA-lipid on the patch. This gathering effect is a simple consequence of the diffusive nature of the DNA-lipid conjugates in this dynamic system. This intrinsic effect has led to misleading conclusions about the number density dependence of membrane processes, e.g. in the membrane fusion literature.

(29) If the bilayer patch edge disintegrates into small vesicles, tethered bilayer fragments and small vesicles can, in principle, be distinguished by fluorescence microscopy if the fragments are large enough. We have not observed vesicles large enough to be clearly distinguished as such as the patches disintegrate. This may be because the number of DNA hybrid tethers are approximately 1 out of every 100 lipid molecules, and from what we know in earlier studies of small (50–200 nm) tethered vesicles, only a single DNA-lipid hybrid tethers the vesicle to a supported bilayer. Furthermore, if small vesicles were formed, they would appear as bright spots (below the diffraction limit), but what we observe is that the edges of the patches shrink leaving more diffuse fluorescence which diffuses away—this is more consistent with pieces breaking off, rather than tethered vesicles.
