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Patterning Barriers to Lateral Diffusion in Supported Lipid Bilayer Membranes by Blotting and Stamping

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Two new methods are introduced for patterning fluid lipid bilayer membranes on solid supports. These methods are based on the observation that supported membranes undergo self-limiting lateral expansion when bilayer material is removed from the surface or when it is deposited in a pattern on a surface. Spatially selective (patterned) removal of bilayer material can be achieved by using a poly(dimethylsiloxane) (PDMS) stamp. Following slight lateral expansion into the bilayer-free region created by this blotting process, stable barriers to lateral diffusion are formed. Inspection of the barrier regions indicates that nearly all of the bilayer material is removed, implying that it has been transferred to the stamp. As a consequence, it also proves possible to transfer the lifted material from the stamp onto a fresh surface. The transferred material retains the original pattern from the stamp and is also laterally mobile, and the mobility is confined to the printed region. Alternatively, bilayers assembled on a PDMS stamp can be printed onto fresh surfaces. Together these methods constitute a simple and powerful approach for preparing patterned fluid bilayers in nearly any geometry.

Introduction

The ability to both functionalize and pattern surfaces is of widespread interest. Lipid bilayers on solid supports are especially challenging because they are two-dimensional fluids. When bilayers are assembled on glass supports, they are cushioned by a thin (10−20 Å) layer of water[1−3] so that both leaflets retain the fluidity that is an essential feature of biological membranes. The result is that the components are continually mixing and are free to diffuse across the entire surface. Such supported membranes retain many of the physical properties of natural cell membranes, and they can interact with living cells if the necessary components are present.[4,5] Thus, the functionalization and patterning of fluid supported lipid bilayers have applications in the fabrication of biosensors,[6] to achieve separations,[7] for fundamental studies of membrane biophysics,[8,9] and to construct an interface between hard surfaces and living cells.[4,5]

Our lab has previously developed two methods to partition or corral regions of supported bilayers, allowing diffusive mixing within the corrals but preventing mixing between corralled regions.[10−14] The supported membrane can be partitioned by scratching the surface of a previously assembled continuous bilayer.[10,13,14] Scratches function as barriers to lateral diffusion by a combination of topographical and tribological interactions.[14] The second method involves patterning the properties of the solid support using photolithography or electron beam lithography before the membrane is assembled.[11,12] Depending on the chemical composition of the patterned material, lipids either do not assemble on the patterned regions or they assemble but are immobile.[12] In either case, the effect of patterning is to confine diffusive mixing to the corralled regions. Scratching is a simple method to apply, but it is not well controlled or entirely understood. Surface patterning requires the application of a second material to the surface, and the interaction of the lipids with the materials used is poorly understood. In both cases, the glass support must be chemically and/or physically altered.

In the following we demonstrate that it is not necessary to alter the glass support to partition lipid bilayers, rather we can exploit the properties of the membrane itself to create barriers, in essence, from nothing. Lipids assemble into bilayers as a result of a balance of hydrophobic interactions, interfacial surface tension, and repulsive intermolecular interactions; the interaction between planar bilayers and oxide supports involves a balance of van der Waals, electrostatic, hydration, and steric forces. As a consequence of the subtle balance among these forces, supported lipid bilayers can expand only over a limited range on the surface.[14,15] In particular, Cremer and Boxer[14] have shown that supported lipid bilayers made from egg phosphatidylcholine and formed by vesicle fusion on glass supports will expand to approximately 106% of their original area after removal of bilayer material from part of the glass support by exposure to air. The 106% expansion proceeds by fingering at the edge and was found to be independent of the initial amount of material.[16] Expansion does depend on pH: at high pH expansion is arrested, while at low pH it is initially quite rapid but then slows down over the course of a few minutes. The pH effect is

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(16) We note that the 106% expansion was seen for egg phosphatidylcholine bilayers. It is likely that the exact amount of expansion varies with different lipid compositions.

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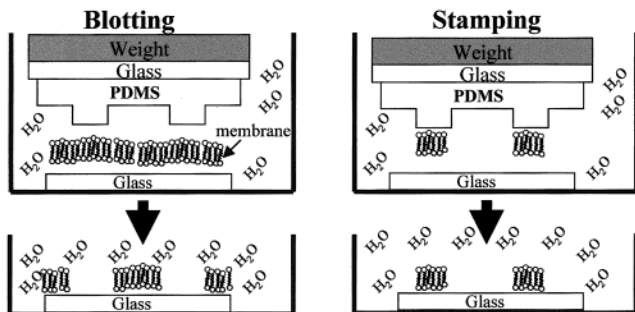

Figure 1. Schematic illustration of the two methods used to create patterned bilayer and bilayer-free regions on the surface. The left side illustrates the blotting method while the right side illustrates the stamping method. The supported membrane is shown schematically in cross section; no attempt is made to describe the edge of the bilayer adjacent to the barrier region as little is known at molecular dimensions. The bilayer is separated from the glass support by a 10−20 Å layer of water.

likely related to the protonation state of the glass surface and the resulting water structure at the interface. Expansion is ultimately self-limiting because the lipids lose favorable interactions with each other.

In this Letter we exploit this self-limiting lateral expansion by gently removing bilayer material using a patterned polymer stamp. Once material is removed, the remaining supported membrane expands laterally, but the expansion halts leaving an essentially free-standing but bounded, stable, and fluid region of bilayer material. Remarkably, it also proves possible to transfer the material that is removed onto a fresh surface, thereby stamping fluid bilayers in any desired pattern.

Experimental Section

Vesicles were prepared from egg phosphatidylcholine (egg PC) from Avanti Polar Lipids with 1 mol % *N*-(Texas Red sulfonyl)-1,2-dihexandeconoyl-*sn*-glycero-3-phosphoethanolamine (Texas Red DHPE) from Molecular Probes. The preparation of supported lipid bilayer from vesicles has been outlined in detail elsewhere.[10,14] A Nikon E800 fluorescence microscope equipped with a Photometrics Sensys CCD camera was used to image the bilayers. Electrophoresis within the membrane was performed using methods described previously[10] and with Millipore water so that currents never exceeded 2 μA producing a negligible amount of resistive heating.

Poly(dimethylsiloxane) (PDMS) stamps were formed by curing Sylgard 184 (Dow Corning) on silicon masters with patterned photoresist. The masters were created using Shipley 3612 positive photoresist, 1 μm thick, on silicon wafers which were vapor primed with hexamethyldisilazane. The patterning was achieved using standard photolithographic techniques.

Results and Discussion

The underlying principle of our partitioning scheme is that supported membranes undergo self-limiting lateral expansion when bilayer material is removed from the surface. Thus, we need a method for selectively removing lipids from the surface or alternatively for selectively placing them on the glass support, creating bilayer regions and bilayer-free regions in a controlled manner. The two methods we chose to investigate in this Letter are illustrated schematically in Figure 1. They were inspired in part by previous nanofabrication work using printing[17−19] and embossing.[20] In both cases a PDMS stamp is attached to a weight (<50 g) and brought into contact with a surface under water. The weight was necessary to ensure contact with the glass support in the water environment; without the weight the PDMS stamp would float away. Because PDMS is hydrophobic, air bubbles would often form on the PDMS surface under water, and it was necessary to shake them off before bringing the PDMS into contact with the surface. In the first method, called blotting, a lipid bilayer is formed on a surface by the vesicle fusion method, and a patterned PDMS stamp is brought into contact with it. After the PDMS stamp is removed, an imprint of the pattern on the stamp is left behind. In the second method, called stamping, a PDMS stamp which has a lipid bilayer on its surface, either by blotting or by self-assembly directly on the stamp surface, is brought into contact with a glass slide. The lipid bilayer is then transferred from the raised pattern on the PDMS to the glass slide.

Blotting. For all experiments shown in this Letter, the pH was such that spreading is expected to occur.[14] As a result it is necessary to chose the amount of material removed vs amount of material left such that after expansion a bilayer-free region remains. In all examples shown in this Letter the pattern on the PDMS is a square grid 1 μm high; the width of the grid lines is 15 μm and the lines are 215 μm apart. Using this stamp to blot a lipid bilayer should yield grid lines that are about 9 μm wide and 221 μm apart after the remaining bilayer has expanded 106%.[21]

Figure 2a shows an epifluorescence image of a supported lipid bilayer after it was blotted for 10 min in Millipore water. The image was taken approximately 30 min after blotting, at which point the bilayer has essentially finished expanding into the region where the membrane was removed. A dark grid pattern is clearly visible in the image where the lipids have been removed, and the pattern was shown to be stable under water for at least 1 week. The expansion of the bilayer is responsible for the ragged edges of the pattern, as the expansion front of a bilayer proceeds in fingerlike projections on glass surfaces.[14,15,22] The fluorescence intensity in the grid (dark) areas is very close to background levels, indicating that lipid material has been nearly completely removed from the grid area, that is, *both* leaflets of the bilayer appear to be removed. The grid lines are approximately 19 μm wide rather than the expected 9 μm. However, as one of the properties of PDMS is that it is deformable, it would not be surprising if the area of the PDMS in contact with the bilayer during blotting was larger than expected.

To determine if this pattern functions as a barrier to lateral diffusion, we applied an electric field parallel to the plane of the bilayer.[10,23] Electrophoresis causes the negatively charged Texas Red-labeled lipids to be drawn toward the positive electrode, forming a gradient at steady state as they build up against the boundary of a patterned

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(22) At the edges of the pattern the top and bottom leaflets must connect in some fashion to keep the hydrophobic interior away from water. The organization of the bilayer at the edges is not known; however, it is reasonable to assume that an unfavorable line energy results. This line energy should cause an isolated square section of bilayer to reorganize into a circular shape. We have observed that square grid patterns created by blotting and printed squares retain their shape for extended periods of time, suggesting that such large-scale reorganization on the surface is prevented, possibly by pinning defects on the surface.
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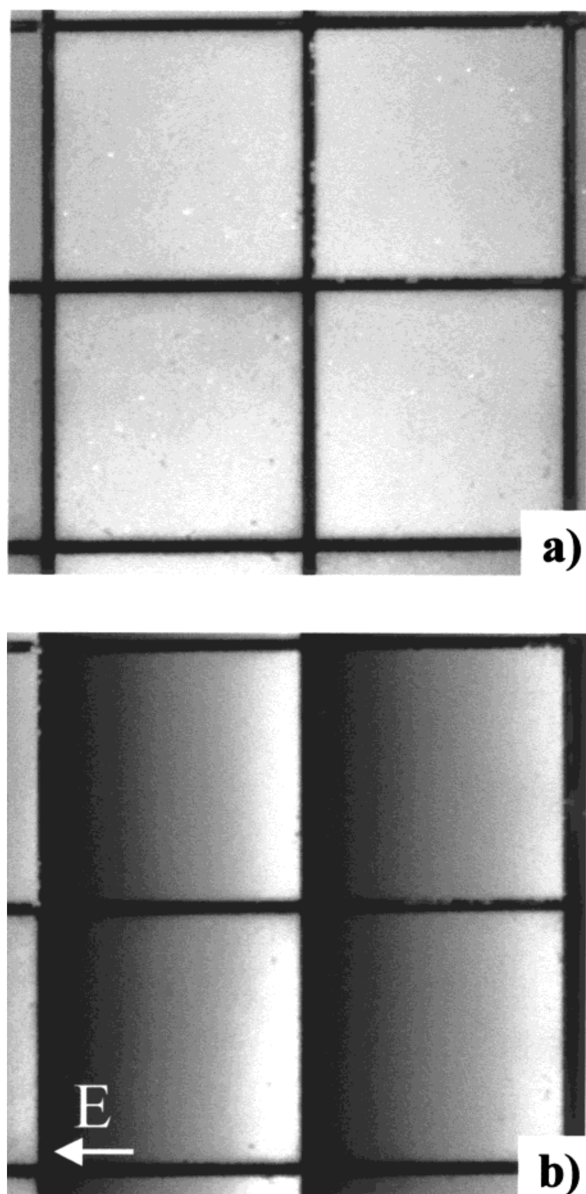

Figure 2. (a) Epifluorescence image of a supported lipid bilayer after blotting for 10 min in Millipore water with a PDMS stamp containing a grid pattern and waiting for 30 min for self-limiting lateral expansion to occur into the regions where lipid was removed. The bright regions are fluorescence from Texas Red-labeled lipids; the dark grid pattern is where the bilayer has been removed. This pattern is indefinitely stable if the sample is kept under water. (b) Epifluorescence image taken after an electric field (11 V/cm and 1 μA) was applied parallel to the bilayer plane for 65 min creating a steady-state gradient in the concentration of the negatively charged Texas Red-labeled lipid component. If the field was turned off, the gradient relaxed back to uniformity, and if the field direction was reversed, the gradient formed against the left boundaries in each corralled region. The dimensions of both images are 480 μm × 470 μm.

region. Figure 2b shows the same area as Figure 2a after an electric field of 11 V/cm and 1 μA was applied for 65 min. A concentration gradient of bright fluorescence is seen on the right side of each partitioned region demonstrating that the lipids are mobile within the square corrals but are confined. Upon removal of the field, the lipids relax back to uniformity; the electric field direction can be reversed and the gradient forms along the opposite side. Thus, it is seen that the bilayer-free regions created by removing lipids with the PDMS stamp serve as stable barriers to lateral diffusion. Fluorescence recovery after photobleaching (FRAP) experiments were also performed (data not shown), and these demonstrated recovery within the patterned regions but not across boundaries.

An interesting situation arises at the outside borders of the blotted region. A stamp half the size of the bilayer-covered region was used, so that only half the bilayer area was patterned. Because the patterned areas at the borders were adjacent to a larger reservoir of material then those in the interior, as the bilayer expands the gaps closest to the edges should be and are erased.[24] This result means that all that is necessary to create barriers to diffusion is to remove enough material so that after the subsequent expansion the bilayer regions are left unconnected. Additionally, it is possible to erase all of the barriers to diffusion by incubating with vesicles, i.e., adding material back in. After incubation the lipids are once again free to diffuse across the entire surface.

Stamping. We have explored two different ways to "ink" the PDMS stamp. In the first method a supported lipid bilayer is blotted as described above; that is, a supported bilayer acts as an ink pad, and the material is transferred by stamping onto a fresh surface for several minutes. In the second method, vesicles are placed in contact with the PDMS leading to assembly of PDMS-supported membranes. This requires at least 1 h for hydrophobic PDMS and 1−5 min for hydrophilic (oxygen plasma treated) PDMS. Following assembly, the excess vesicles are shaken off, and the self-assembled material is stamped onto a fresh slide under water. We have performed preliminary FRAP and quantitative fluorimetry studies that suggest that either inking procedure leads to fluid bilayer assembly on the PDMS surface, that is, the lipids are not soaked up into the PDMS; these results will be discussed in a subsequent paper.

Figure 3 shows epifluorescence images of the material that is stamped using the first inking procedure and a grid pattern that is identical to that shown in Figure 2. The bright pattern of fluorescence corresponds to the region that was removed in Figure 2a, giving further evidence that the blotting method actually removes bilayer material. To test whether the transferred lipids are themselves assembled into a fluid bilayer, a region of the fluorescence just below the center (Figure 3a) was photobleached. Figure 3b is the same region 46 min later, and it is evident that much of the fluorescence in the bleached region has recovered. Close inspection indicates that recovery is not complete; we suspect that the removal and deposition processes as currently practiced leave some regions that are not fully covered, though sufficiently connected to permit long-range diffusion. The fluorescence level of the transferred lipids is approximately 60% that of the fluorescence level of the surface from which the lipids were removed; of the 40% lost approximately 10% was lost in the removal and 30% was lost in the stamping. Given that the lipids adsorb to the PDMS, it is interesting that they will transfer back to the glass surface. That the transfer is incomplete suggests that there is a competition for the lipids between the glass surface and the PDMS. We have done little to optimize this system, and variations in the methods, the stamp material, or the stamp topography may prove fruitful. What is clear is that the most complete transfer of lipids from PDMS to glass occurs when an oxygen plasma treated PDMS stamp that has been inked by vesicle fusion is used.

(24) The number of barriers erased is a determined by a balance between the fractional expansion and the increased reservoir size that occurs every time a barrier is erased. This expansion does not occur indefinately. After less than a hour it terminates, resulting in the erasure of several barriers and leaving a much larger number intact.

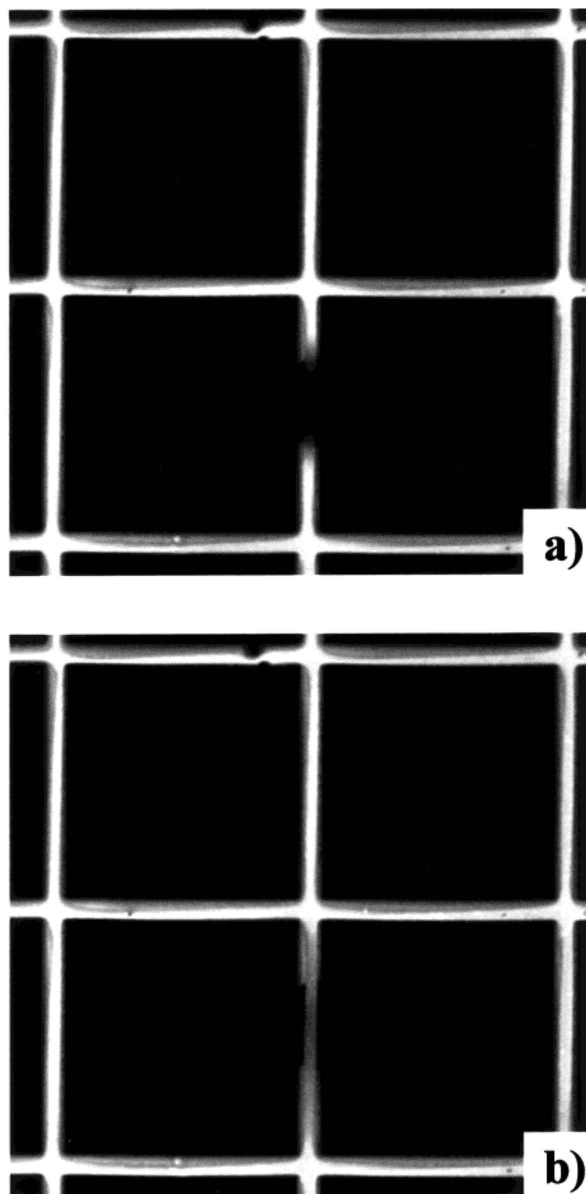

Figure 3. (a) Epifluorescence image of a grid pattern formed by stamping. A sample like that in Figure 2a was blotted with a PDMS stamp, and the material that lifted off the surface onto the stamp was then transferred onto a fresh glass surface. The bright lines correspond to a high level of fluorescence that was transferred from the bilayer that was used to ink the stamp. A region of the vertical grid line just below the center of the figure was photobleached, and the image shown was obtained immediately after photobleaching. (b) Epifluorescence image taken 46 min after photobleaching showing partial recovery of the bleach as a result of lateral diffusion within the printed line. The dimensions of both images are 480 μm $\times$ 470 μm.

We note in passing that there is an inversion of the membrane leaflet that is in contact with the receiving surface in these two inking methods. In the first method, the side of the supported membrane originally in contact with the glass remains in contact after transfer and printing. In the second method, where the assembly is initially on the PDMS surface, the transfer should place the side that is in contact with the bulk solution when on PDMS in closest contact with the receiving glass surface. This may prove useful for inverting the orientation of self-assembled systems.

Patterns formed by stamping could also be erased by incubating with vesicles as was the case for patterns formed by blotting. The addition of more material to a surface that is clean except in the patterned region is another demonstration that the patterns are maintained by the self-limiting lateral expansion of supported lipid bilayers.

Conclusions

We have demonstrated a new method for partitioning supported lipid bilayers that exploits self-limiting lateral expansion into regions from which bilayer is selectively removed. The barriers so created are intrinsic to the physical properties of the supported membrane itself, not to any alteration of the physical or chemical properties of the surface, in contrast to earlier approaches. For example, it is then possible to fill in the gaps between adjacent bilayer regions with other materials such as proteins.[25] It is interesting that PDMS appears to pick up both layers of the supported membrane; presumably a single monolayer exposed to water would be unstable. Remarkably, it appears that this fragile structure can be transferred intact to another surface. The details of this process need to be investigated further. This methodology is a simple extension of PDMS stamping methods that have been applied in other areas. Therefore it should be possible to use the bilayer blotting and stamping methodology in combination with some of those methods or with secondary photolithography to further control the lateral composition of fluid bilayer membranes on solid supports. Finally, by stamping bilayers of defined areas into regions that are partitioned with permanent barriers[11] and then filling in the remaining unstamped area with vesicles followed by lateral mixing within the regions defined by the permanent barrier, it is possible to create membrane arrays of variable composition.[26]

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