Patterning and Composition Arrays of Supported Lipid Bilayers by Microcontact Printing

Jennifer S. Hovis and Steven G. Boxer*

Department of Chemistry, Stanford University, Stanford, California 94305-5080

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Fluid-supported lipid bilayers self-assemble on glass and SiO_2 surfaces. We have found that it is also possible to assemble fluid bilayers on plasma-oxidized polydimethyl siloxane (PDMS) surfaces. Furthermore, it is possible to transfer or print the supported bilayer from raised PDMS surfaces, such as are typically used for microcontact printing, to fresh glass surfaces creating a supported bilayer membrane replica of the patterned PDMS surface on glass. These patterned islands of bilayer are fully fluid and indefinitely stable under water. The pattern is erased upon addition of more vesicles leaving a continuous bilayer surface. By printing membrane islands of various sizes onto a glass surface that is prepatterned with a material that forms permanent barriers to lateral diffusion and then backfilling the open region with vesicles, it is possible to create arbitrary concentration or composition arrays of membrane-associated components. These arrays may be useful for studies of membrane biophysics, for high throughput screening of compounds that target membrane components, and for probing and possibly controlling living cell−synthetic membrane interactions.

Introduction

Lipid bilayers comprise the basic structural motif of cell membranes. Supported lipid bilayers are formed by the self-assembly of lipids into two opposing leaflets on hydrophilic surfaces, such as glass. Biophysical studies of membranes have used supported lipid bilayers extensively as they provide a convenient 2-D platform.[1−4] A thin (10−20 Å) layer of water cushions bilayers on glass. This water layer allows the lipids to retain their lateral fluidity, an essential feature of biological membranes.[5−7] One consequence of this fluidity is that the components are continually mixing and are able to diffuse over long length scales. Thus, there is great interest in methods for partitioning membrane components, that is, to create containers for this 2-D fluid, and for controlling the composition within each container.[8−12]

When bilayer material is removed from the solid support, the remaining bilayer expands to ∼106% of its original size (depending on the precise composition and conditions) and then arrests.[13] We termed this phenomenon self-limiting lateral expansion.[8] The spreading arrests because of at least two factors: there is an energetic cost associated with bilayer spreading over the glass surface,[14] and the lipids lose favorable interactions with each other as they spread apart. In a previous paper,[8] we reasoned that if it were possible to selectively remove or place lipids on a surface with dimensions such that the features would not connect after expansion, then it would be possible to create arbitrary patterns of lipid bilayers without modifying the underlying surface. To do this, we brought a patterned polydimethyl siloxane (PDMS) stamp into contact with a supported lipid bilayer for a short time (<10 min). After removal of the PDMS stamp from the surface, approximately 90% of the lipids were removed from the areas in contact with the stamp. The resulting surface had large patches of lipid bilayer separated from one another by only the open regions on the glass support. We then showed that if the stamp which was used to blot away the bilayer was brought into contact with a clean glass slide, bilayers could be printed onto the slide, again leaving patterned islands of lipid bilayer separated by only the uncovered areas on the glass support.

The printing process is a variation on microcontact printing, a highly versatile technique that has been employed to pattern a wide variety of molecules, from alkane thiols (simple organic molecules) to proteins (complex biomolecules).[9,15−18] That lipid bilayers could be patterned with this method was a surprising finding because the lipid bilayer is a rather fragile structure. In the course of this work, we also found that bilayer material preassembled directly onto oxidized PDMS surfaces could be transferred to glass and that this method, illustrated in Figure 1, is a superior way to "ink" the PDMS stamp. In the following, we have investigated the PDMS/supported membrane system in much greater detail and worked out conditions for efficient and reliable transfer of membrane patches to glass. Additionally, by combining

* To whom correspondence should be addressed. E-mail: sboxer@stanford.edu.

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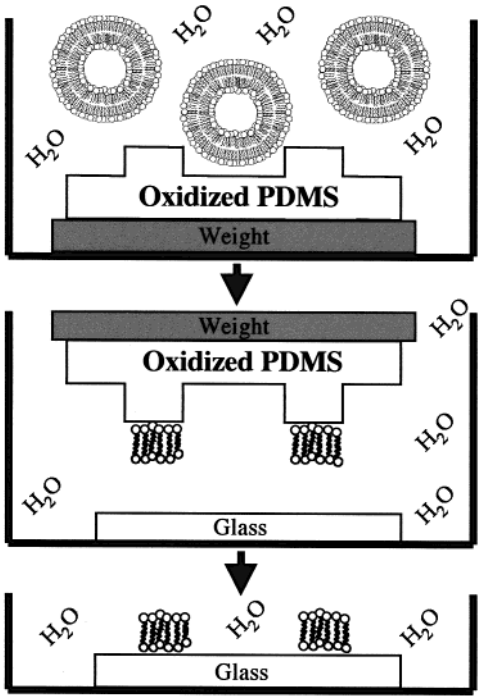

Figure 1. Schematic illustration of the printing process. Vesicles are brought into contact with an oxidized PDMS stamp and fuse to form supported bilayers. The pattern is then transferred to a glass surface where the shape is retained. Typically, the raised PDMS features are on the order of 1 μm and the bilayer is on the order of 5 nm, so the drawing is not to scale. Likewise, the supported lipid bilayer is shown schematically in cross section and shown only on the raised portion of the stamp (presumably the bilayer coats the entire surface of the PDMS stamp); no attempt is made to describe the edge of the bilayer.

this printing methodology with fixed barriers on the glass surface,[11,19] we have developed a general method for creating arrays of patterned membranes where the composition of each corralled region can be varied.

Experimental Section

Small unilamellar vesicles were prepared from egg phosphatidylcholine (eggPC, Avanti Polar Lipids) with 1 mol % Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE, Molecular Probes), or 3 mol % Marina Blue 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Marina Blue DHPE, Molecular Probes). The appropriate amounts of each kind of lipid were mixed together in chloroform, dried under N_2, and put under vacuum for at least 40 min. The lipids were then reconstituted with Millipore (18 MΩ) water and passed 19 times through an Avanti extruder containing a membrane with 50 nm pores. The extruded vesicles were stored at 4 °C and used within 3 days.

A Nikon E800 fluorescence microscope equipped with a Photometrics Sensys CCD camera was used to image the bilayers. Texas Red fluorophores were imaged using a Texas Red filter set (Chroma Technology Corp.), and the Marina Blue fluorophores were imaged using a Cascade Blue filter set (Chroma Technology Corp.). The Cascade Blue filter set matches the spectrum of Marina Blue reasonably well. Electrophoresis within the membrane was performed, using methods laid out previously,[10] in Millipore water with currents of $<2\,\mu$A which produce a negligible amount of resistive heating. Diffusion coefficients of the fluorescent probes were determined by Fourier analysis of the time evolution of a fluorescence profile using a custom fitting program.

PDMS stamps were formed by curing Sylgard 182 (Dow Corning) on silicon masters with patterned photoresist at 70 °C for 80 min. The masters were created using Shipley 3612 negative photoresist, 1 μm or 1.65 μm thick, on silicon wafers, which were vapor primed with hexamethyldisilazane. The patterning was achieved using standard photolithographic techniques. After developing, the wafers were vapor primed again with hexamethyldisilazane to assist in the subsequent removal of the PDMS. Flat PDMS stamps were formed by curing on silicon wafers that were vapor primed with hexamethyldisilazane. Surface oxidation of PDMS was carried out using a plasma cleaner (Harrick Scientific) under high power for 15−60 s while a small amount of air was leaked into the chamber. Glass slides were prepared by washing in ICNx7 detergent (ICN, Costa Mesa, CA) followed by exhaustive rinsing in distilled water and then baking in a kiln at 400 °C for at least 4 h.

Microcontact printing of the lipid bilayers was carried out as follows. Within 30 min of oxidation, the PDMS was brought into contact with a solution of lipid vesicles (either in water or mixed with 5 mM Tris, 50 mM NaCl, at pH 8 buffer) for 1 min, and then the excess vesicles were washed away with large amounts of water. Keeping the PDMS in water at all times, the inked PDMS was then brought into contact, using a light weight (5.2 g), with a glass surface for 10−15 s in a solution of 5 mM Tris, 50 mM NaCl, at pH 8.

Permanent grid patterns, which serve as barriers for membrane partitioning, were produced on glass by microcontact printing bovine serum albumin (BSA) as described in detail elsewhere.[9] In brief, a 20 μL solution of 80 μg/mL of Alexa488 labeled BSA (Molecular Probes) in a 17 mM, pH 8 phosphate buffer was placed on an oxidized PDMS stamp and allowed to sit for 10 min; the excess was shaken off, and then the stamp was dried under N_2. The stamp was brought into contact with a glass slide using a light weight (14 g) for 30 s. The slide was rinsed vigorously in deionized water to remove the excess BSA and dried under N_2.

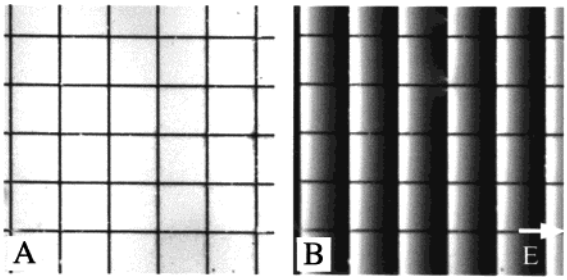

Figure 2. (A) Epifluorescence image of a patterned supported lipid bilayer that was printed onto a glass surface (eggPC with 1 mol % Texas Red DPHE). The bright regions are fluorescence from the Texas Red labeled lipids, and the dark grid pattern is the bare glass surface. (B) Epifluorescence image taken after an electric field (13.3 V/cm, $<2\,\mu$A) was applied parallel to the bilayer plane for 18 min creating a steady-state gradient of the negatively charged Texas Red labeled lipids and demonstrating long-range mobility. The dimension of both images is 560 μm $\times$ 560 μm.

Results and Discussion

Printing Bilayers from Oxidized PDMS. Figure 2a shows an epifluorescence image of a printed array of lipid bilayer squares (eggPC with 1 mol % Texas Red DHPE whose fluorescence is visualized), each 100 μm $\times$ 100 μm and separated by 10 μm gaps. In the gaps between the square patches, the fluorescence intensity drops to background levels indicating that nothing is present there except the glass support and water. Figure 2b shows the effect of a lateral electric field on the organization of the negatively charged Texas Red labeled lipids (the image returns to that of panel A when the field is turned off and the lipids diffuse back to uniformity). The bright Texas Red fluorescence moves toward the positive electrode and builds up at the edge of each individual square but does not go beyond. This is an important observation for three reasons. First, it shows that the lipids are connected over

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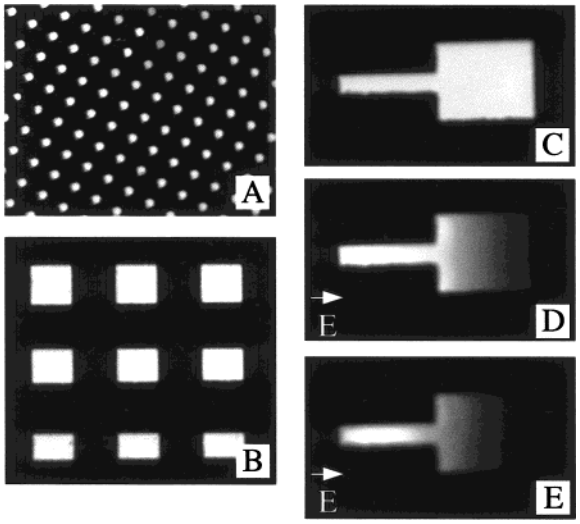

Figure 3. Epifluorescence images of a variety of patterns of lipid bilayers printed onto glass surfaces (eggPC with 1 mol % Texas Red DPHE). The bright regions are fluorescence from the Texas Red labeled lipids, and the dark regions are the bare glass surface. (A) Array of 10 μm dots. Image size 900 μm × 720 μm. (B) Array of rectangles, which increase in size proceeding lower right to upper left. Image size is 290 μm × 290 μm. (C) More complex structure, 200 μm long, 20 μm wide on the left half and 80 μm wide on the right half. (D and E) Structure in C after an electric field (22.2 V/cm, <2 μA) was applied parallel to the bilayer plane for 24 and 48 min, respectively, creating a steady-state gradient of the negatively charged Texas Red labeled lipids. Image size 280 μm × 170 μm.

a long length scale, ruling out the presence of clumps of lipids or vesicles, implying that either a monolayer or bilayer formed. Second, a comparison (using epifluorescence) of the amount of fluorescence in the printed areas to the amount of fluorescence from a bilayer made by vesicle fusion shows that the printed lipids are approximately 85% as intense, ruling out monolayer formation. Third, it shows that barriers to diffusion and electrophoresis can be created in lipid bilayers without modifying the underlying surface chemically and/or physically, a direct consequence of self-limiting lateral expansion. The diffusion coefficient of the labeled lipids is 1.21 $\mu m^2/s$, which is typical for bilayers on glass.[9,10] Despite the lower density, the properties of the printed lipids are essentially the same as those of lipids self-assembled directly by vesicle fusion on glass, and we can conclude that lipid bilayers are printed on the surface with a high level of homogeneity over large areas. Close inspection of the edges of the squares in the images reveals slightly ragged edges where the bilayer has expanded by fingering.[13,20] When vesicles are added to the patterned surface, the gaps erase and a continuous bilayer is formed across the entire glass surface.

Figure 3 illustrates several examples of the generality and versatility of this printing technique: arrays of dots in panel A, variable-sized rectangles in panel B, and a shovel-shaped structure in panel C (all eggPC with 1 mol % Texas Red DPHE whose bright fluorescence is visualized).[20] Panels D and E show the same structure as panel C during the application of an electric field for 24 and 48 min, respectively. Long-range motion of the Texas Red labeled lipids can clearly be seen (the image returns to that of panel C when the field is turned off and the lipids diffuse back to uniformity).

(20) The organization of the bilayer at the edges is not known. Presumably, the top and the bottom leaflets connect in some manner to keep the hydrophobic interior away from water. The result is an unfavorable line energy that should cause an isolated square such as the ones in Figure 2 to become circular. However, we have not observed any reorganization for any of the shapes printed or blotted, indicating that large-scale reorganization is prevented, probably by entanglement in pinning defects on the surface.

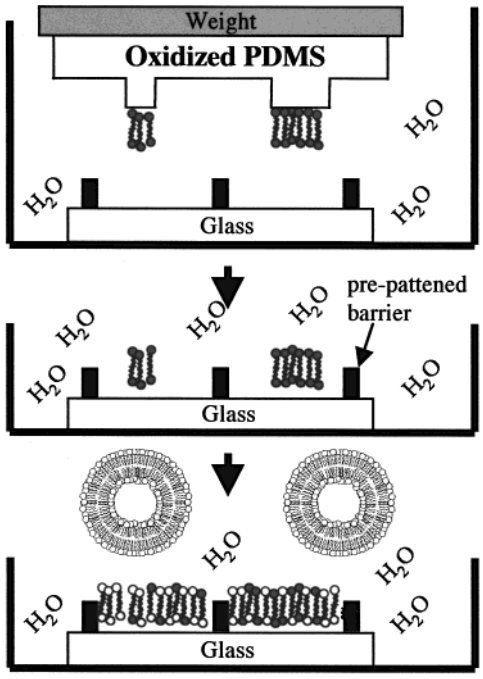

Figure 4. Schematic illustration showing the creation of concentration and composition arrays of lipids. Different sized areas of lipid-bilayer-coated PDMS are brought into contact with a prepatterned surface (the dark vertical bars are fixed barriers; these can be made from a variety of materials). The printed substrate is incubated with vesicles containing lipids of a different composition. The vesicles form a bilayer on the empty areas of the substrate and connect with the printed bilayer, and the two components (dark and light) mix together. This can be used to create concentration arrays if either the printed or vesicle bilayer contains the component that is being diluted or binary composition/concentration arrays (as shown in Figure 5) where different components are present in the printed part versus the added vesicles.

Composition Arrays on Prepatterned Surfaces. In addition to partitioning lipid bilayers, we would like to be able to change the composition of the bilayer in each partitioned region. For example, this would make it possible to address questions regarding the effect of the lipid and/or membrane-associated protein composition in a parallel format. First steps toward this include secondary photolithography[19] and patterning by flow.[21] Figure 4 shows schematically a general method for making arbitrary membrane composition arrays based on the tools described above and the original concept of introducing fixed barriers on the surface.[11,19] As illustrated in Figure 3B, it is straightforward to print membrane patches with variable areas. Such an array can be printed onto a surface that is prepatterned with a material that leads to membrane corralling. Then, as illustrated in Figure 4, the remaining open areas within each prepatterned region can be backfilled with vesicles. The material(s) of interest can be in the originally printed patches, in the vesicles added to backfill the open regions, or both. Because the vesicles fill in the open areas, fuse with the existing bilayer patches and then mix within, but not between, the prepatterned corrals, concentration variations or mixture compositions can be prepared by varying the area of the

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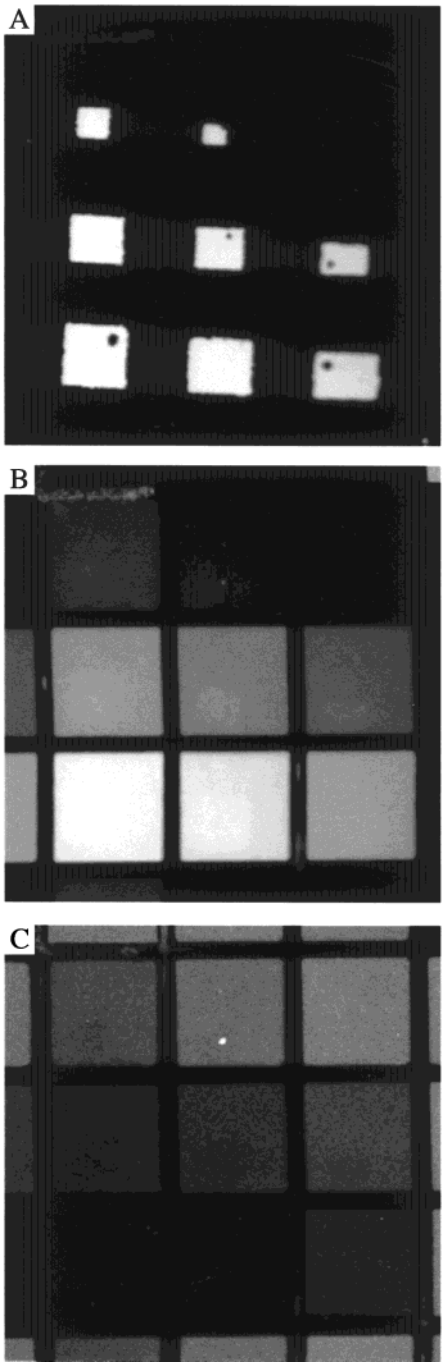

Figure 5. (A) Epifluorescence image of a supported lipid bilayer that was printed onto a glass surface patterned with grid lines of BSA (not seen). The printed lipids were eggPC with 1% Texas Red DHPE. (B and C) Epifluorescence images of the same place on the surface after it was incubated with vesicles containing eggPC with 3 mol % Marina Blue DPHE; image B was taken using the Texas Red filter set, and image C was taken with the Cascade Blue filter set (which matches well with the Marina Blue spectrum). The dark grid lines in the image are the patterned BSA. All three images are 330 μm × 330 μm.

originally printed patch and/or the dimensions of the fixed barriers.

Figure 5 shows a realization of this idea. Figure 5a shows eight printed rectangles of lipid bilayer (eggPC with 1 mol % Texas Red DHPE), which vary in size from 48 μm × 48 μm to 24 μm × 12 μm. The lipids were printed onto a glass surface prepatterned with a square grid pattern of BSA (not seen; the grid lines are 10 μm wide and are separated by 100 μm; see Experimental Section for details of the BSA printing). We know from prior work that lipid bilayers do not form on top of BSA nor can they cross over it making it an effective barrier to diffusion (numerous other materials can be used as fixed barriers; BSA was chosen because it is convenient).[9] The surface was then incubated, for approximately 2 min with vesicles of eggPC with 3 mol % Marina Blue DPHE in a 5 mM Tris, 50 mM NaCl, pH 8 buffer. The images in Figure 5b,c were taken of the same area as in Figure 5a after incubation of the surface with vesicles. The Texas Red filter set was used for Figure 5b, and the Cascade Blue filter set was used for Figure 5c. One can see that composition arrays of both the Texas Red labeled lipids and the Marina Blue labeled lipids have been created and that, as expected, they are the inverse of each other.

Table 1. Result of Fluorimetry Experiments on Lipids Extracted from Either Glass or Oxidized PDMS Surfaces after Vesicle Fusion[a]

surface	glass	oxidized PDMS
sample 1	$3.35 \pm 0.04 \times 10^4$	$3.14 \pm 0.04 \times 10^4$
sample 2	$3.26 \pm 0.04 \times 10^4$	$3.23 \pm 0.04 \times 10^4$
sample 3	$3.45 \pm 0.04 \times 10^4$	
weighted average	$3.35 \pm 0.04 \times 10^4$	$3.18 \pm 0.04 \times 10^4$

[a] The Texas Red dye was excited at 585 nm, and collection occurred at 605 nm once a second for 60 s.

Self-Assembly of Bilayers on Oxidized PDMS. One question raised by these results is, what is the nature of the material that self-assembles on oxidized PDMS? There have been several studies of supported membranes on polymer supports,[22,23] though most do not examine lateral mobility. To characterize the product of vesicle fusion on oxidized PDMS, we determined both the number of lipids on the oxidized PDMS surface (by fluorescence) and the mobility of the lipids (by fluorescence recovery after photobleaching, FRAP, experiments), both in direct comparison with identical samples on glass. The samples were made by allowing vesicles of eggPC with 1 mol % Texas Red DPHE in a 5 mM Tris, 50 mM NaCl, pH 8 buffer to come into contact with either a glass or an oxidized PDMS (cut to be the same size as the glass) surface for 1 min. The excess vesicles were rinsed away, and each sample was quickly checked with the microscope to ensure that uniform fusion occurred.

To determine the number of lipids on each surface, we extracted them using a detergent solution of 10 mM Tris, 100 mM NaCl, 0.1% LDAO at pH 8. Each sample of detergent solution plus lipids was placed in a fluorimeter, and the intensity of the Texas Red fluorescence, excited at 585 nm, was measured at 605 nm every second for 60 s; results are shown in Table 1. The fluorescence from the oxidized PDMS sample is slightly lower; however, because of experimental constraints, the edges of the PDMS samples were exposed to air, thus decreasing slightly the available surface area. We can conclude that after vesicle fusion the oxidized PDMS surfaces have the same number of lipids per unit area on them as the glass surfaces. As it is established that bilayers form on glass by vesicle fusion,[24] this result, then, indicates that bilayers form on oxidized PDMS. To confirm, we performed FRAP experiments; FRAP is a standard method to determine whether the lipids exhibit long-range lateral mobility, indicative of either monolayer or bilayer formation. We performed FRAP experiments on bilayers assembled on oxidized PDMS, and the bleached spots were found to fully recover

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Table 2. Measured Diffusion Coefficients ($\mu m^2/s$) for Lipids on Three Different Glass Surfaces and Three Different Oxidized PDMS Surfaces

surface	glass	oxidized PDMS
sample 1	1.70 ± 0.01	2.25 ± 0.02
sample 2	1.37 ± 0.01	2.21 ± 0.02
sample 3	2.13 ± 0.01	2.28 ± 0.02

and showed no significant immobile fraction. The diffusion coefficients for experiments on three different glass surfaces and three different oxidized PDMS surfaces are shown in Table 2. For consistency, the same batch of vesicles was used for all six experiments and the experiments were all conducted within 8 h. The diffusion coefficients are very similar. The coefficients on oxidized PDMS are a little higher and more consistent from one experiment to another. The increased consistency is probably due to the fact that the PDMS is molded against a silicon wafer which results in a smoother surface than that of glass. Taken together, the FRAP and fluorimetry results show that a mobile bilayer forms on oxidized PDMS surfaces.[25]

The oxidation of the PDMS surface is a temporary effect and has been described qualitatively in the literature.[26] With time, either the modified groups of the polymer turn inward exposing the hydrophobic groups or bulk transfer through the polymer occurs and the hydrophilic strands at the surface are replaced by hydrophobic stands. We have not investigated this phenomenon in detail but note two observations that may be of interest. First, even after short plasma etches (<30 s) the PDMS becomes hydrophilic enough for bilayer formation up to at least 1 h afterward. Second, if the PDMS is kept under water, bilayers are stable on the surface for hours to days, depending on the initial oxidation time. Slowly though, small patches of bilayer will peel away from the surface, an effect that is not seen on glass.

Practical Considerations. That efficient transfer of lipids from the oxidized PDMS stamp to the glass surface occurs at all is surprising because the bilayers seem to be stable, at least over the short term, on oxidized PDMS. Although the precise mechanism is not understood, we close with some observations that may relate to the mechanism and will hopefully be of use to others interested in using this method. In most applications of microcontact printing, there is a large excess of the material being stamped; in our case, there is no excess, and an entire fragile structure is being transferred. Thus, the efficiency of transfer is very critical. It is always possible to transfer some lipids, but transferring a uniform bilayer is nontrivial. After much iteration, we have been able to optimize the procedure so that the transfer occurs evenly and at better than 80%. Here are some of our observations. First, the fresher the 50 nm extruded vesicles, the better, with 3 days being approximately the upper limit. Second, cleaned and baked glass substrates should also not be more than 3 days old. Third, printing works better in a buffer solution of 10 mM Tris, 100 mM NaCl, pH 8 than in Millipore (18 MΩ) water. Fourth, the pattern makes a difference: large features separated by small spaces typically transfer better than small features separated by large spaces. Additionally, we found that placing the pattern in a checkerboard arrangement was more effective than a continuous arrangement. Fifth, we found that switching from Sylgard 184 to 182 helped. Finally, if good transfer has not occurred within 15 s, it will not occur.

All of these points except the last are fairly easy to understand. The first three factors typically lead to the best results for self-assembly by vesicle fusion on glass as well and thus are probably intrinsic to the bilayer formation process. That different patterns transfer more effectively than others is due to the fact that the pressure exerted over each pattern needs to be uniform; this can be more or less easy to achieve depending on the pattern, as has been described for other microcontact printing strategies.[27] The weight used to do the printing should be quite light but not too light (5.2 g was used for the work shown in this paper); this is more of an issue for small features separated by large areas than for the inverse. Sylgard 182 is better than 184 because 184 will continue to harden at room temperature whereas 182 will harden only above 65 °C. This indicates that the softness of the PDMS is important.

The only puzzling observation is the last one, that after approximately 15 s, increasing the printing time does not improve the amount of transfer; in fact, in our hands it appears to make matters worse. Our initial hypothesis was that the printing occurs because of a delicate balance of changing forces. The PDMS is slowly becoming hydrophobic; thus, the glass surface becomes a slightly more attractive place to be. The transfer happens very quickly, and if the stamp is left in contact with the surface for much longer than 15 s, the slightly more hydrophobic stamp will blot the lipids. So although the natural inclination is to leave the stamp down longer if complete transfer has not occurred, it in fact only makes matters worse. To look into this hypothesis further, we explored whether it is possible to print a lipid membrane from a patterned oxidized PDMS to a flat oxidized PDMS surface. In fact, it is possible, and the transfer efficiency is similar to that of printing onto glass. This indicates that the PDMS becoming hydrophobic is not a major contributor to the transfer. The driving force for this transfer, then, remains unknown. It appears to be a collective process; once part of the membrane transfers, it transfers entirely when the surfaces are separated.

In our prior work on membrane blotting, we showed that after removal of the lipid bilayer the remaining bilayer would expand over the open region on the glass surface and then arrest.[8] We termed this phenomenon self-limiting lateral expansion and for eggPC bilayers observed that the amount of expansion was roughly 106% under the conditions described here. When bilayers are printed, we would expect that they too would expand over the empty glass surface. We do observe expansion of the printed bilayers, but the amount of spreading is less than 106%, and it varies quite a bit from experiment to experiment. This is an indication that the amount of transfer is not 100% and that it is quite variable. As it is difficult to make quantitative measurements with epifluorescence microscopy, we can only roughly estimate the efficiency of transfer. We do this by comparing the amount of fluorescence in the printed regions to the amount of fluorescence in the same region from a bilayer on glass made by vesicle fusion (using the same vesicle preparation in each experiment). For the experiments shown in this paper, we estimate that the amount of transfer varied from 80 to 95%. This is probably an underestimation; in

(25) We note that we have also looked at vesicle fusion on unoxidized PDMS. From fluorimetry and FRAP measurements, there are approximately 40% the number of lipids on these surfaces as on glass and there is long-range diffusion, with diffusion coefficients slightly slower than those on glass. These results indicate that it is possible to form a mobile monolayer on hydrophobic PDMS.

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the comparison, we do not take into account the fact that the printed bilayer has spread and is therefore less dense than one formed by vesicle fusion. Examination of the PDMS stamps after printing shows low levels of fluorescence, indicating that a small percentage of the lipids are left behind. The amount of transfer could in principle be greater than 100% because the edges of the pattern are also coated with lipids and those lipids could spread from the sides onto the glass surface. Although in theory this is true, the printing time is not sufficient for any significant spreading to occur.

Conclusions

We have shown that it is possible to form supported lipid bilayers on oxidized PDMS by vesicle fusion and that the lipids are mobile. It is also possible using oxidized patterned PDMS stamps which have been inked by vesicle fusion to transfer lipid bilayers onto glass surfaces in arbitrary patterns and to retain these patterns on the accepting surface because of the self-limiting lateral expansion of bilayers. This ability to pattern arbitrary shapes allows a general method for creating composition arrays of membrane components by printing variable-sized membrane patches of one composition onto a prepatterned surface and backfilling the open regions with vesicles of a different composition. Following lateral mixing within but not between corralled regions, mixtures of the two membrane compositions result with the relative concentrations depending on the areas printed and the size of the corrals. The transfer efficiency of printing ranges from 80 to 95% and depends on a number of factors. Although some elements have been characterized, further systematic development should lead to a generally robust method for preparing membrane arrays with compositions that are useful for biophysical and biological studies.

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