Patterning Hybrid Surfaces of Proteins and Supported Lipid Bilayers

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Two methods for patterning surfaces with supported lipid bilayers and immobilized protein are described. First, proteins are used to fabricate corrals for supported lipid bilayers. Poly(dimethylsiloxane) stamps are used to deposit arbitrarily shaped patterns of thin layers of immobilized protein onto glass surfaces. This is followed by vesicle fusion into the regions that are not coated with proteins. Second, supported bilayer membranes are blotted to remove patterned regions of the membrane,[1] and the blotted regions are filled in or caulked with protein from solution. In both cases, the lipid bilayer regions exhibit lateral fluidity, but each region is confined or corralled by the protein. These two methods can be combined and used iteratively to create arrays with increasing lateral complexity in both the fixed protein and mobile-supported membrane regions for biophysical studies or cell-based assays.

Introduction

Cell membranes are composed of complex and dynamic patterns of lipids and proteins. Supported lipid bilayers are useful models for these biological membranes.[2] Bilayers self-assemble onto surfaces such as silica, trapping a thin water layer about 10−20 Å between the solid support and the bilayer.[3−5] As a consequence, the lipids in both leaflets are free to diffuse laterally, preserving an important dynamical property of biological membranes.

The fluidity of the membrane leads to continuous mixing of membrane constituents. During the past several years, we have introduced a series of methods for partitioning supported membranes into corralled regions each of which retains the fluid properties, but where lateral diffusion is confined to regions on the surface defined by the pattern. Methods for achieving this include scratching the surface,[6] lithographic patterning of a variety of hard materials such as plastic, alumina, titania, and a number of metals,[7] and blotting away or printing bilayer patterns with poly(dimethylsiloxane) (PDMS) stamps,[1] a variation on microcontact printing.[8] It occurred to us that it might be possible to combine protein and membrane patterning so that surfaces with both physical and chemical lateral complexity might be formed. Proteins can be deposited directly on glass surfaces or through the use of linkers and retain some degree of their native function.[9] Such surfaces have been used to study protein assemblies such as biological motors by force and single molecule spectroscopic methods[10] and to study cellular processes and properties.[11,12]

In this paper, we demonstrate two methods for patterning mixed lipid bilayer and protein surfaces. In the first method, barriers of protein are patterned onto glass by microcontact printing,[13−15] followed by vesicle fusion on unmodified regions of the surface to form lipid bilayer membranes that are confined laterally by the printed protein. The second method involves microblotting away patterned regions of a continuous supported lipid bilayer,[1] taking advantage of self-limited lateral expansion,[16] which prevents the lipids from spreading into the blotted regions. These blotted areas can then be filled in when proteins are added to solution as proteins often bind nonspecifically to exposed glass surfaces. These two complementary methods can be used to create hybrid bilayer−protein surfaces of high complexity for interactions with biological molecules or cells. They can also be combined with other tools that have been developed for manipulating the composition of the supported membrane, such as patterning with barriers made from hard materials, membrane stamping and blotting, secondary photolithography,[17] and electrophoresis.[18]

Experimental Methods

Small unilamellar vesicles (SUVs) comprised of egg phosphatidylcholine (egg PC) and typically 2 mol % NBD headgroup-labeled phosphatidylethanolamine (NBD-PE) (Avanti Polar Lipids, Alabaster, AL) were prepared by extrusion (LiposoFast, Avestin) through 50 or 100 nm filters. Formation of supported lipid bilayers on glass by vesicle fusion and subsequent application of lateral electric fields have been described previously.[18,19] Bovine serum albumin (BSA, Boehringer Ingelheim GmbH, Germany) was labeled with Texas Red X-SE (TR-X, Molecular Probes, Eugene, OR), following the protocol provided by the manufacturer,

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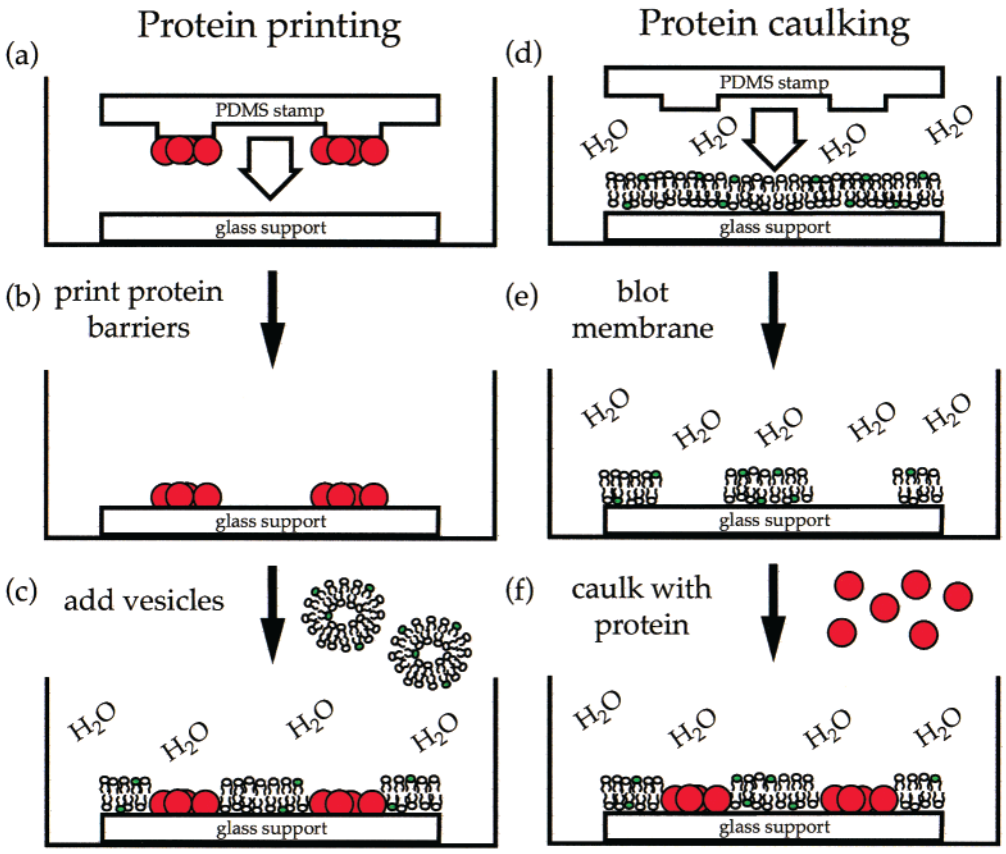

Figure 1. Schematic of two methods for creating patterned hybrid protein-supported membrane surfaces: left column, protein patterning by microcontact printing followed by vesicle fusion; right column, supported membrane blotting followed by caulking with protein. In printing, a PDMS stamp inked with protein (shown schematically as filled red circles to represent the Texas Red labeled BSA used in this paper) is contacted with a glass surface (a). The stamp is removed (b) and vesicles are added to solution leading to self-assembly of planar lipid bilayers on the exposed regions on the glass. Excess vesicles are rinsed away, to achieve the final structure (c). In caulking, a surface with a continuous lipid bilayer is patterned by blotting using a PDMS stamp (d) to remove lipids selectively.[1] The stamp is removed leaving a patterned surface with exposed glass regions that are incubated with a protein that nonspecifically binds to the newly exposed regions of the surface (e). Excess protein is then rinsed away (f) to realize the final structure. The actual height of the protein deposited by both methods is approximately a monolayer, so the diagram is approximately to scale in the vertical direction (see text). The shape of the edge of the bilayer at the boundary with the barrier is shown schematically as it is not known.

with an efficiency of ~1.2 TR-X molecules per BSA. The labeled protein (TR-BSA) was purified by ultrafiltration (Microcon-30 unit, Millipore) and diluted to 250 μg/mL in 10 mM phosphate buffer, pH 7.4. Silicon wafers patterned with photoresist were made by standard photolithographic techniques. Poly(dimethylsiloxane) stamps were formed by curing Sylgard 184 (Dow Corning) for 1 h at 70 °C.[20]

A Nikon E800 epifluorescence microscope and a Photometrics Sensys CCD camera were used to image the samples. Images were taken under two filter sets (one for TR-BSA protein and one for NBD-PE lipid) and combined following contrast adjustment and false color assignment using Adobe Photoshop. Diffusion constants of the NBD-PE probe were determined by Fourier analysis of the time evolution of a fluorescence profile after electrophoresis[21] with a custom fitting program.

Results

Microcontact-Printed Protein Barriers. This method for patterning and corralling supported membranes by patterned proteins is shown schematically in the left column of Figure 1. The surface of the PDMS stamp is oxidized for approximately 30 s in a plasma cleaner (Harrick Scientific, Ossining, NY) and immediately incubated with a solution containing 250 μg/mL TR-BSA for 10 min. The stamp was then dried under a nitrogen stream, removing excess protein solution from the stamp. The stamp was then placed in contact with a clean silica surface under a ~20 g weight for 5 min. The protein-patterned glass slide was then incubated with lipid SUVs to form supported membranes on the exposed regions, and finally the surface was rinsed extensively in water to remove excess vesicles and weakly bound protein.

This technique results in a patterned surface of supported membrane and protein regions. A few examples of surfaces patterned with printed TR-BSA and lipid membranes are shown in Figure 2. Epifluorescence images have been false-colored such that the TR-BSA is red, while the 2% NBD-PE doped egg PC lipid membranes are green. In Figure 2A, 20 μm square regions are separated by 15 μm wide grids of patterned protein; narrower protein features are shown in Figure 2B where 10 μm boxes are separated by grids of patterned protein 5 μm in width. Figure 2C shows an example of a larger area patterned with 40 μm squares bordered by 10 μm grids of protein.

The patterned protein acts as a barrier to lateral diffusion of the corralled lipid bilayer membranes. This is demonstrated by the confinement of a photobleached pattern covering several corrals as shown in panels C and D of Figure 2. An octagonal pattern was bleached for 30

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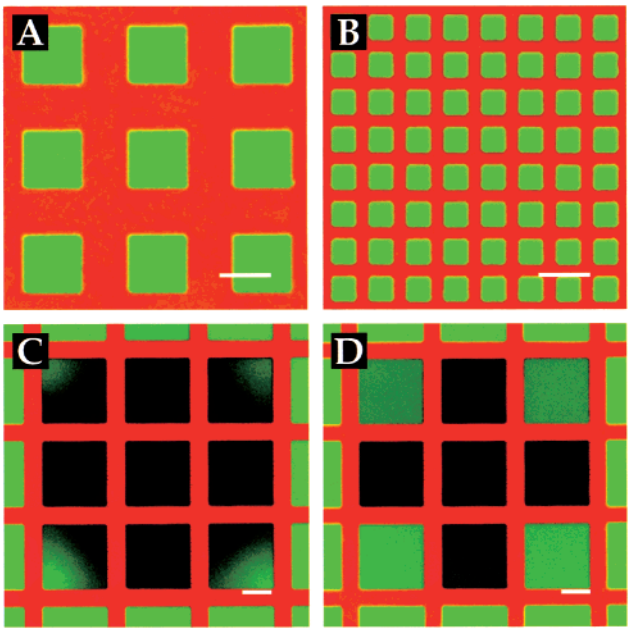

Figure 2. Epifluorescence images of supported membranes patterned by printing proteins. In each panel the protein is TR-BSA and is colored red, while the supported membrane regions are 2% NBD-PE doped egg PC and are colored green. The scale bars are each 20 μm across. (A) 20 μm bilayer regions separated by 15 μm protein; (B) 10 μm bilayer regions separated by 5 μm protein grids; (C) and (D) 40 μm bilayer regions separated by 10 μm protein grids. In panel C an octagonal spot was photobleached for 30 s centered on the middle corral. After irradiation has ceased, the lipids mix freely within each corral, creating corrals of uniform but intermediate fluorescence intensity, as seen in (D) after 360 s of recovery.

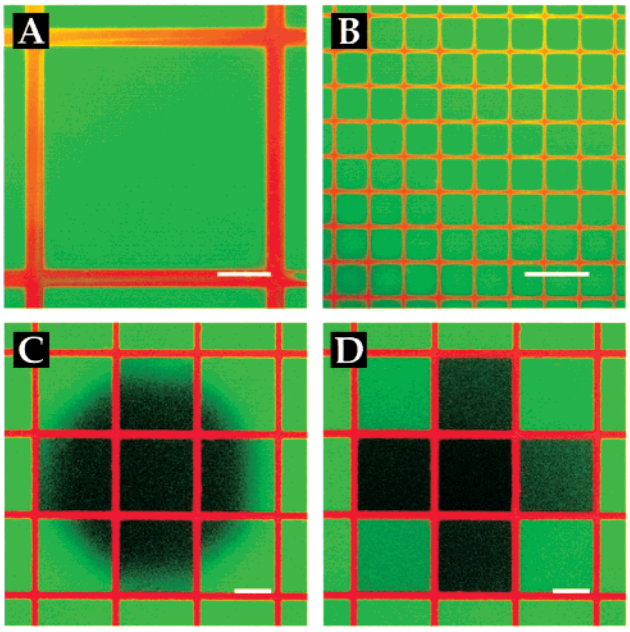

Figure 3. Epifluorescence images of supported membranes patterned by blotting, then caulking protein. The materials and color coding are given in Figure 2; the scale bars are each 50 μm across. (A) 200 μm bilayer regions separated by 16 μm protein grids; (B) 25 μm bilayer regions separated by 2 μm protein grids; (C) 100 μm bilayer regions separated by 8 μm protein grids. In panel C an octagonal spot was photobleached for 30 s centered on the middle corral. After irradiation has ceased, the lipids mix freely within each corral, creating corrals of uniform but intermediate fluorescence intensity, as seen in (D) after 240 s of recovery.

s onto the nine-box region shown in Figure 2C, completely bleaching the fluorescently labeled lipids in the middle box, but only partially bleaching the fluorescence in the outer boxes. After irradiation is ceased, the lipids undergo lateral diffusion and mixing within each confined region. After about 6 min, the differential effect on the composition of each box is apparent in Figure 2D. Each box has recovered uniformity in fluorescence due to mixing within each confined region, but there is no mixing between corralled regions. Thus, the membrane patches are both laterally mobile and confined by the printed protein barriers.

The negatively charged NBD-PE can be subjected to lateral electrophoresis leading to a concentration gradient in the confined region.[18] The approach to steady state by lipids in these regions has been analyzed quantitatively to obtain the diffusion constant $D = 1.2 \pm 0.6$ μm^2/s for the lipids. The membranes in these regions also exhibit a small amount of fluorescence viewed through the Texas Red filter set, and we suspect that there are small amounts of TR-BSA which have adhered to defects in these regions[22] (see caulking results below). However, the patterning of membranes with printed protein does not affect the mobility of the lipids, as membranes which are not patterned with proteins exhibited $D = 1.1 \pm 0.4$ μm^2/s. These diffusion constants are consistent with previously reported values for supported lipid membranes.[23]

Caulking with Proteins. The second method used to confine regions of supported bilayers with proteins is illustrated schematically on the right side of Figure 1. We recently demonstrated that it is possible to blot away a bilayer from a preassembled supported membrane on glass using a patterned PDMS stamp.[1] Following limited lateral expansion into the blotted region, stable barriers are formed leaving open regions on the glass surface. Since many proteins bind to glass, we reasoned that it should be possible to fill in or caulk the blotted regions between the supported membranes where the glass is exposed simply by adding protein in solution as illustrated. Supported lipid bilayers consisting of egg PC containing 2% NBD-PE were formed by vesicle fusion. The membrane was subsequently blotted with a patterned PDMS stamp for 60 s and incubated within a few minutes with a solution containing 100 or 250 μg/mL TR-BSA for 10 min. The surface was then rinsed and shaken vigorously to remove excess protein. The sample was kept under water (or buffer) for the entire procedure.

Figure 3 shows a few examples of lipid bilayers that were patterned via caulking. Patterns of various sizes can be created, as shown in Figure 3a, where 200 μm boxes of membrane are separated by 16 μm grids of caulked protein, and in Figure 3b where 25 μm boxes are separated by 2 μm grids. The incubation with protein adds a small but significant amount of fluorescence not just to the exposed grid areas but also to the corrals with the supported membrane. This suggests that there may be a number of defect sites in the bilayer to which the protein is adhering. Caulking may thus be a method for passivating the glass surface to subsequent nonspecific binding interactions by first covering these defects with protein. The incubation with protein does not significantly alter the diffusion of membrane components. This is shown in Figure 3C, where an octagonal spot is photobleached in a region of 100 μm boxes of supported membrane with 8 μm grids of protein. The same region is shown after 240 s in Figure 3D, where diffusive mixing has homogenized

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the fluorescence in each corral; differential effects of photobleaching are retained in proportion to the area of each box that was bleached. Diffusion constant measurements taken on corralled membranes after the caulking process yielded diffusion constants which were similar to those for stamped or unmodified bilayers, with $D = 1.1 \pm 0.2\ \mu m^2/s$. The similarity of the diffusion constants for all three types of samples indicates that the membranes are unaffected by either the stamping or caulking process.

Discussion

The use of proteins as barriers has several advantages over the previous methods of scratching and lithographically patterned barriers. Scratches introduce a high degree of surface roughness and alter the surface chemistry.[16] Lithographically fabricated barriers are typically on the order of 100 nm in height, and such hard barriers require complex surface manipulations for fabrication. Because proteins are soft materials, they can be patterned under conditions that are compatible with many biological systems and it is relatively easy to pattern as desired. Unlike some fabricated barriers, proteins are electrically nonconducting and so are well-suited for electrophoresis. We have examined the topography of proteins stamped on the surface by atomic force microscopy in air. Features are observed in the protein-patterned regions of 2−3 nm in height that are consistent with a monolayer of protein. Thus, the protein barriers are of roughly the same dimensions as the lipid membrane, and the schematic diagram in Figure 1 is approximately to scale in the verticle direction. Finally, proteins can be used for patterning membranes on expensive substrates such as lithium niobate, which has a high refractive index and can be used to assemble supported membranes for evanescent wave experiments.[24]

The use of PDMS stamps to remove lipids from the bilayer leads to barriers to lateral diffusion due to the self-limiting lateral expansion of the membrane. Thus, for relatively large feature sizes and small regions of intervening supported membranes, the proteins subsequently deposited in the blotted regions surfaces do not add barrier functionality but are useful if protein function can be exploited (see below). In cases where the feature sizes are small or the intervening lipid reservoirs are large, blotting alone is insufficient because the exposed regions will heal over upon expansion on a time scale of minutes.[16] For example, this is the case for the geometry illustrated in Figure 3B: without caulking, the 2 μm blotted lines heal over to form a homogeneous bilayer (data not shown). The relatively slow time scale of the healing process compared to protein adsorption allows caulking to increase the effective resolution limits of patterning by blotting. As with stamping, the caulked protein may introduce useful functionality as well.

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Printing and caulking proteins with the use of PDMS stamps are quite versatile. The flexibility of the process allows for a wide range of feature sizes. For example, we have printed proteins with large distances between membrane patches. This can be useful for experiments where membrane corrals are probed separately, and interaction between each corral can be minimized. We have also patterned thin features if it is desirable to have membrane regions in close proximity or thin lipid regions between protein areas. With optimized stamping techniques, patterned features can be scaled down to lengths of about one micrometer or smaller with the use of electron-beam lithography in the fabrication of the stamp patterns. Protein printing and caulking can be combined to pattern surfaces of increasing complexity. After protein printing, the supported membrane can be thought of as a substrate for subsequent blotting and caulking steps. For example, a PDMS stamp with a different pattern can then blot away specific lipid regions, exposing fresh silica surface to binding of a different protein or incubation with more vesicles to deliver different membrane-associated components. Repeated cycles of protein printing and blotting require registration of the PDMS stamps and the surfaces with the use of an aligner.

Obviously, proteins with interesting biological functions can be printed or caulked or the proteins used to pattern the surface can be further functionalized. There are many examples of proteins that retain some degree of functionality after adsorption onto silica surfaces. For example, we have shown that fibronectin can be used both to pattern and corral fluid bilayer membranes and to provide patterned sites for the adhesion of endothelial cells.[22] Iterations of blotting and stamping to control the composition of both the membrane and immobilized protein regions on the surface can then be used to assemble surfaces where cell-supported membrane interactions can be examined quantitatively and with control. Molecules other than proteins can, of course, also be printed or caulked in following blotting, so these methods should be generally applicable to many different membrane-patterning strategies.

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