Substrate−Membrane Interactions: Mechanisms for Imposing Patterns on a Fluid Bilayer Membrane

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A variety of new techniques are emerging that require the use of a patterned substrate to impose micropartitions on a supported fluid bilayer membrane. The barrier-forming characteristics of aluminum oxide, indium−tin oxide (ITO), chrome, and gold patterns on silica substrates have been examined. All four materials form effective barriers to lateral diffusion within the supported membrane; however, two distinctly different mechanisms were observed. Aluminum oxide inhibits vesicle fusion, thus restricting membrane formation to the exposed silica surface. In contrast, vesicles will fuse with ITO, chrome, and, to some extent, gold; however, the resulting membrane is effectively immobile over the time scale of several hours. These materials partition the supported membrane by selectively immobilizing membrane that adsorbs to their surface. The two mechanisms of membrane partitioning described here provide additional flexibility in the design and application of micropatterned membranes.

Introduction

The interaction between a solid substrate and a thin fluid film can be used to modify the fluid film. For example, micron-scale modification of surface wettability has been used to create arrays of tiny beads of water.[1] Recently we have used patterned substrates to impose micropartitions or corrals on fluid lipid bilayer membranes.[2] The bilayer membrane consists of two opposed leaflets of phospholipid molecules and is the basic structure of all membranes in living cells. With an appropriate solid surface as a support, a thin (∼10 Å) layer of water is trapped between the membrane and the substrate (see schematic diagram in Figure 1).[3−5] The membrane flows over the surface conformally, tightly trapped by a balance among electrostatic, hydration, steric, and long-range van der Waals forces.

Supported membranes are self-assembling, two-dimensional fluid systems. They can be conveniently formed by spontaneous fusion of unilamellar phospholipid vesicles with an appropriate substrate such as silica. Supported bilayers were originally developed for cell recognition studies, where they have proven highly useful. It was shown that a supported membrane doped with the appropriate proteins can effectively replace the antigen-presenting cell when interacting with a helper T-cell.[6,7] As evidenced by this remarkable ability, supported membranes preserve many of the physical and biological properties of free membranes. One of the most notable physical features is long-range lateral fluidity: both leaflets of a supported bilayer freely diffuse over the entire surface of a homogeneous substrate.

Lateral fluidity of supported membranes is an important characteristic that distinguishes them from other surfaces.

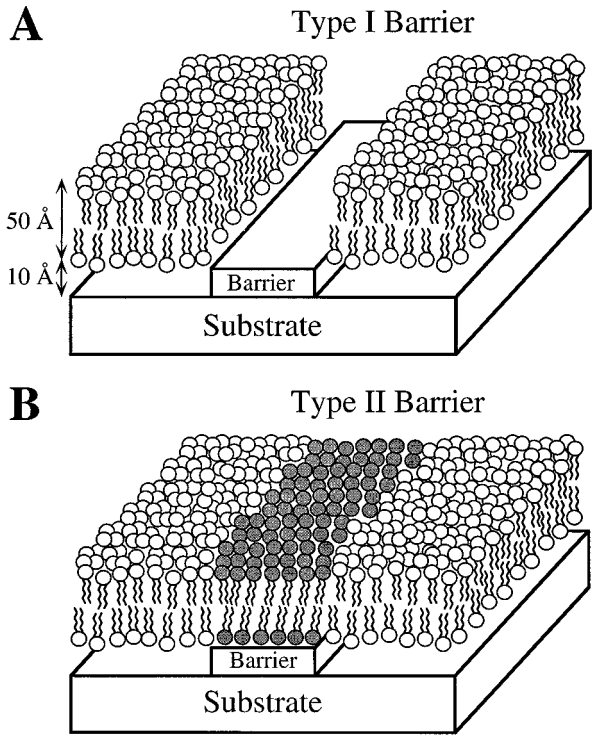

Figure 1. Schematic diagram of supported bilayer membranes partitioned by the two mechanisms described in the text. (A) Depiction of the type I mechanism whereby vesicle fusion is prevented in the barrier region, leaving this area exposed. The structure of the edge of the membrane at the barrier is not drawn, and the barrier is not drawn to scale. It is expected that the two leaflets bend around and meet at the membrane edge, thus avoiding exposure of the hydrophobic tail groups to the aqueous phase. Under the conditions described, barriers made of aluminum oxide function by this mechanism. (B) Illustration of a type II barrier in which lateral diffusion is blocked by selective immobilization of the lipid over the barrier material. Immobilized lipids are drawn in gray. The precise structure of lipids in this immobilized region is not known and is drawn this way only for convenience. Under the conditions described, barriers made of chrome, ITO, and gold function by this mechanism.

Fluidity is essential for membrane function in a cell. On a solid support, however, it presents a challenge to

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controlled experiments, since membrane components are continually mixing. Micropatterned substrates can be used to impose barriers to the diffusive mixing and flow of molecules in a supported membrane. This technique offers control over long-range diffusion while preserving the membrane's distinctive properties in well-defined corrals.[2] In this paper the mechanisms by which different barrier-forming materials impose structure on the supported membrane are examined. Comparison is made based on the macroscopic functionality of the barrier. In particular, we make the distinction between materials and conditions which prevent vesicle deposition and those to which vesicles will adsorb, but the resulting lipid film lacks long-range lateral mobility.

Materials and Methods

Fused silica wafers were used as the substrate for supported bilayer formation. Standard positive photoresist was spun onto wafers to a thickness of 1 μm. The wafers were exposed to ultraviolet light through a photolithographic mask and developed. The patterned photoresist was then used as a template for lift-off of electron-beam-evaporated thin films of chrome, gold, aluminum oxide, or ITO. A typical cleaning procedure involved boiling in a 1:4 dilution of ICN 7X (Costa Mesa, CA) for 20 min and rinsing extensively with deionized water followed by a 3-min etch in argon plasma. Other methods such as soaking in pirhana solution (3:1 $H_2SO_4-H_2O_2$) or baking at 400 °C were used as well when compatible with the materials.

Planar supported bilayers were formed by spontaneous fusion of small unilamellar vesicles (SUVs) with the micropatterned substrates. The SUVs were made roughly according to the Barenholz procedure.[8] A lipid solution in chloroform was evaporated onto the walls of a round-bottom flask, which was then evacuated overnight. Lipids were resuspended in distilled water by vortexing moderately for several minutes. The lipid concentration at this point was around 6 mg/mL. The lipid dispersion was then probe-sonicated to clarity on ice. The SUVs were separated from other lipid structures by ultracentrifugation for 3 h at 192000*g*. The supernatant contained the SUVs with yields of 50−75%. SUVs were stored at 4 °C and could typically be used for a few weeks to several months. Prior to use, the SUV suspensions were exchanged into the desired buffered salt solution, typically 5 mM Tris, pH 8.0, 50 mM NaCl. The SUVs were composed of L-α-phosphatidylcholine from egg (egg-PC) (Avanti Polar Lipids, Alabaster, AL) with 1% by mole of the fluorescent probe *N*-(Texas Red sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (Texas Red DHPE) (Molecular Probes, Eugene, OR).

The bilayer was allowed to self-assemble by placing the substrate over an 80-μL drop of vesicle suspension in a Petri dish for several minutes. The dish was then carefully filled with distilled water, and the excess vesicles were rinsed away by shaking gently. The patterned supported membrane was next assembled into a sandwich with a cover slip, taking care not to expose the membrane-coated surface to air. For electrophoresis experiments, this cover slip sandwich was mounted in a membrane electrophoresis cell[9] and field strengths of 10−20 V/cm were applied. Membranes were observed in a temperature-controlled room (21 °C) with an epifluorescence microscope (Zeiss, Oberkochen, Germany) using 10× and 40× objectives. Images were monitored with a low-light-level video camera (Cohu, San Diego, CA) and recorded with an S-VHS VCR (JVC, Elmwood Park, NJ). The camera's γ factor ($I_{out} = I^{\gamma}$) was set at $\gamma = 1$, providing linear imaging of the fluorescence intensity. Color photographs were taken on a Nikon (Tokyo, Japan) Labophot epifluorescence microscope fitted with a Nikon F3 camera.

Results and Discussion

Four barrier-forming materials (aluminum oxide, ITO, chrome, and gold) were examined under the experimental conditions described above. It was found that the mechanisms by which these materials partition the membrane fall into two basic classes. Aluminum oxide forms a barrier by preventing vesicle fusion and membrane spreading, thus leaving a gap in the membrane (Figure 1A). This directed fusion mechanism will be referred to as type I. In contrast, under these conditions vesicles will fuse with ITO, chrome, and, to some extent, gold. However, the resulting membrane lacks long-range lateral mobility over the time scale of several hours. Hence, these materials also produce barriers to lateral diffusion in the supported membrane (Figure 1B), but by a mechanism of selective immobilization (type II).

These two mechanisms of substrate-imposed partitioning of supported membranes have distinctly different properties. The type I mechanism genuinely patterns the membrane, allowing vesicle fusion only in selective regions. This mechanism is necessary to deliver membranes and membrane-associated proteins to a surface in patterns with specified areas and shapes. Additionally, all sections of the membrane on a type I patterned surface will have the same diffusion characteristics, limited only by the connectivity of the various regions. The type II mechanism modulates the long-range diffusion within an otherwise continuous membrane. The primary distinction between a type I and a type II barrier as described here is the absence or presence of lipid material in the barrier region. In some cases vesicles may coat the barrier region by adsorption without completely fusing to form a fully connected membrane. We will include this case in our definition of a type II mechanism, since it produces the same macroscopic effect: Control over diffusive mixing and flow of membrane components is achieved without leaving exposed portions of the substrate. This could be particularly important for applications that involve interactions between living cells and patterned membranes. Both mechanisms could be combined on a single substrate to produce patterned membrane surfaces with the desired combination of properties.

Figure 2A illustrates sections of a supported membrane partitioned by patterns of aluminum oxide on a fused silica substrate. The membrane appears bright due to incorporation of 1% by mole of the fluorescent probe lipid Texas Red DHPE. The bilayer, which has formed on the exposed silica surface, is fully fluid, as can be seen by the electric-field-induced reorganization shown in Figure 2B. The Texas Red probe lipid carries a net negative charge and is driven toward the anode by the field.[10] These concentration profiles are completely reversible and relax back to uniformity when the field is turned off. The effectiveness of the aluminum oxide barrier is confirmed by its ability to contain the probe lipid within the corralled region. If any breaks in the barrier are present, the probe lipid can readily be seen streaming through them. The dark appearance of the barriers indicates that aluminum oxide inhibits vesicle fusion, thus restricting membrane formation to the exposed silica surfaces. This is an example of the type I mechanism.

Lipid vesicles were found to adsorb to the highly hydrophilic chrome surface. Despite its tendency to adsorb membrane, chrome forms effective barriers to lateral diffusion within the bilayer. This is demonstrated in Figure 3 by its ability to trap photobleached regions of membrane. The membrane in each 20 μm by 20 μm corral is fully fluid while there is no intermixing between different corrals. Figure 3A depicts the array before photobleaching. Figure 3B shows the patterned mem-

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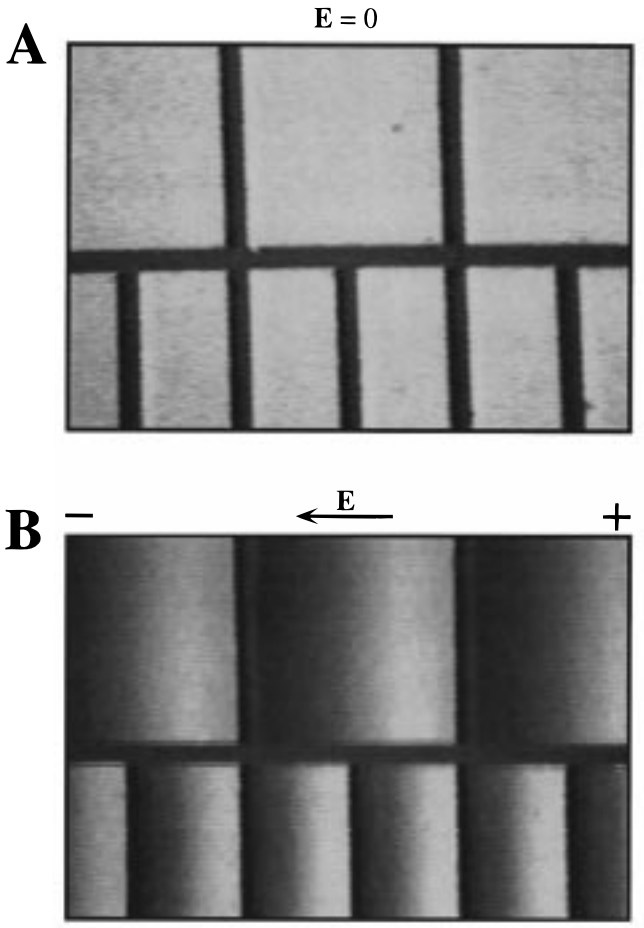

Figure 2. (A) Corrals of fluid bilayer partitioned by aluminum oxide barriers viewed by epifluorescence microscopy of 1 mol % Texas Red DHPE doped into egg PC. The supported bilayer appears as continuous fluorescence (bright), while the barriers appear dark due to a lack of adsorbed membrane in these regions. The lower corrals are 100 μm across. (B) Similar set of corrals with steady-state electric-field-induced concentration gradients of the negatively charged Texas Red probe lipid.[10] The field-induced redistribution of the probe demonstrates long-range fluidity within the corrals as well as the ability of the aluminum oxide barriers to confine membrane-associated molecules. These gradients were achieved at a field strength of 15 V/cm.

brane after photobleaching with a circular spot roughly 60 μm (about three corral widths) in diameter. Different fluorescence intensities result from the fractional area of each particular corral that was exposed to the photobleaching beam. The four central corrals were entirely contained within the beam and received the largest dose whereas the peripheral corrals were only partially exposed, thus receiving smaller doses. The composition within each individual corral becomes homogeneous in a matter of seconds due to rapid diffusive mixing. The chrome barriers prevent mixing between separate corrals. There was no visible leakage across the 2-μm-wide chrome barriers over the course of 24 h. This indicates that any lipid adsorbed to the chrome surface lacks long-range lateral mobility. These images are views of the membrane from the back, through the silica substrate. The opaque chrome thus blocks fluorescence from the membrane adsorbed to it, facilitating observation of the fluid membrane on the silica surface.

Similar grids of membrane corrals separated by chrome barriers are illustrated in Figure 4, viewed from the front. Bright fluorescence can clearly be seen coming from adsorbed lipid on the chrome regions. The fact that the

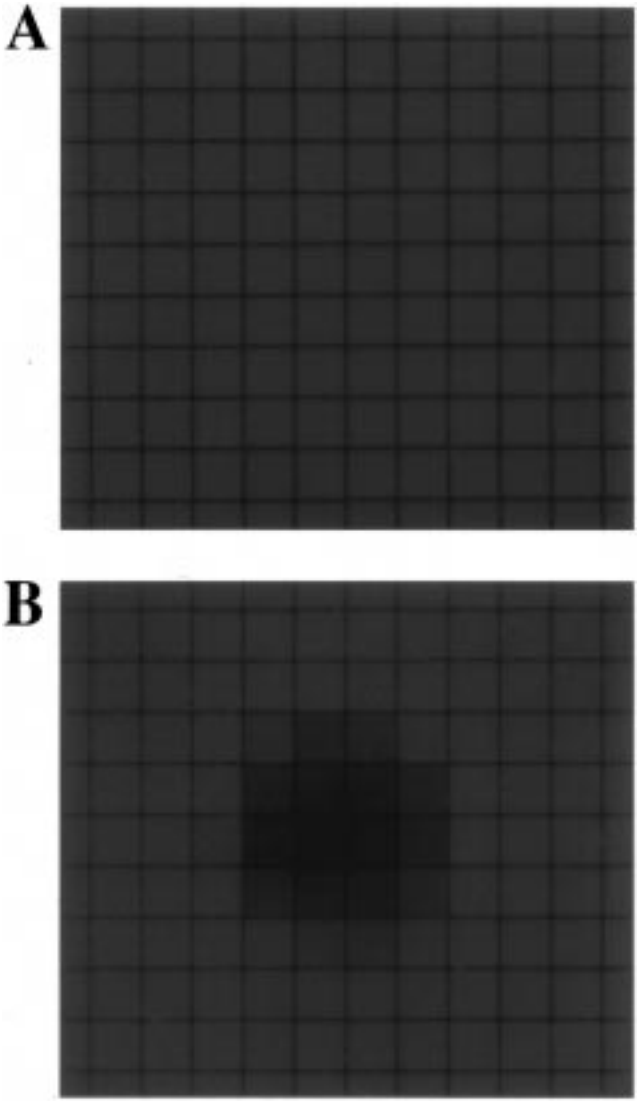

Figure 3. Epifluorescence photographs of an array of 20 μm by 20 μm corrals of fluid bilayer membrane partitioned by a microfabricated grid of chrome lines. The images were taken through the back of the silica substrate; hence, the opaque chrome barriers appear dark. (A) Photograph of the partitioned membrane before photobleaching. (B) Photograph taken after partial photobleaching with a circular spot of light approximately 60 μm in diameter (about three corral widths) from the microscope illumination roughly in the center of the grid. The photobleached dye spreads to fill each corral with a level of darkness related to the fraction of the corral area exposed during the photobleaching.

membrane appears brighter on the chrome than on the silica is due, at least in part, to the fluorescence reflected from the chrome surface. The chrome-supported membrane appears to contain a significant number of adsorbed vesicles in the vicinity of the chrome, which may also contribute to its bright appearance. Control experiments without membranes confirm that the chrome itself makes no contribution to the fluorescence observed at this wavelength (620 nm). It can be seen from Figure 4B that photobleached lipid on the chrome barriers remains fixed in position, indicating a lack of long-range diffusion. We conclude that patterns of chrome on a silica substrate form barriers to lateral diffusion within the membrane by selectively immobilizing lipids that adsorb to the chrome surface.

ITO is another interesting material which can be used to partition a membrane by the type II mechanism. The

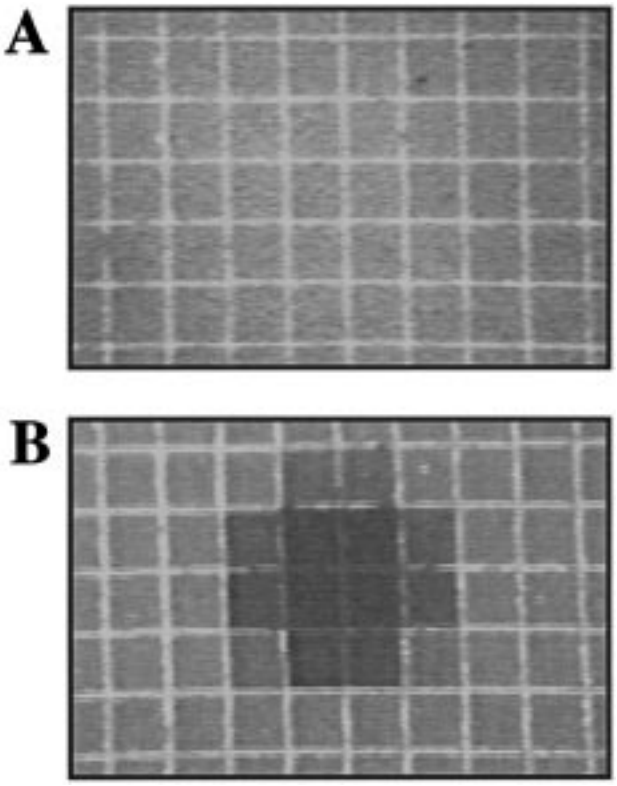

Figure 4. (A) Image of 20 μm by 20 μm membrane corrals partitioned with chrome barriers taken from the side where the membrane is bound to illustrate the presence of membrane material on the chrome surface. The bright fluorescence along the chrome is due, at least in part, to fluorescence reflected from the chrome surface and may also contain contributions from vesicles that tend to adsorb tightly in this region. (B) Image of a similar partitioned membrane after photobleaching a circular region as in Figure 3B. Fluorescent lipid on the barrier region as well as within the corral has been photobleached. Lack of fluorescence recovery on the connected barrier region confirms the lack of long-range fluidity on the barrier.

surface of this transparent semiconductor, like chrome, is very hydrophilic and adsorbs lipid vesicles. The resulting membranes appear uniform and continuous but lack long-range lateral diffusion under our conditions.[11] We have previously demonstrated that the amount of lipid adsorbed to an ITO surface by the vesicle fusion method is consistent with formation of a single bilayer.[12] Under the conditions used here, patterns of ITO form type II barriers with the added benefit of transparency, simplifying visualization of the membrane, and the possibility to exploit the electrical conductivity of ITO.

Not all barrier-forming materials function by a distinctively type I or type II mechanism. The behavior of gold is much more sensitive to the cleaning procedure than those of the other materials mentioned above. Although patterns of gold form highly effective barriers to lateral diffusion, the mechanism by which they function seems to vary between type I and type II. In earlier work, photoresist was also used to pattern supported membranes;[2] however, the specific mechanism by which this polymeric resin partitions the membrane is not well understood. Merely presenting a hydrophobic surface is known to be insufficient to impose a barrier to membrane diffusion. For example, patterns of octadecyltrichlorosilane (OTS) monolayers created by microcontact printing have been observed to allow lipid mixing between separated bilayer corrals.[13] This presumably results from vesicle fusion to the hydrophobic OTS surface, creating a supported monolayer which connects the bilayer regions. In general, the ability to interrupt the long-range fluidity of a supported membrane is not a specialized property; many materials are expected to function as barriers to lateral diffusion. The more specialized situation occurs when this partitioning is achieved through a well-defined mechanism.

Conclusions

A variety of new techniques are emerging that require the use of a patterned substrate to impose micropartitions on a fluid bilayer membrane. The barrier-forming characteristics of aluminum oxide, ITO, chrome, and gold have been examined and shown to fall into two basic classes. Aluminum oxide exhibits a distinctively type I mechanism whereby it inhibits vesicle fusion, thus restricting membrane formation to the exposed silica surface. The type II mechanism of barrier formation can be found in the behavior of chrome and ITO patterned membranes. Although vesicles fuse to these materials, the resultant supported membrane lacks long-range fluidity under these conditions. Barriers to lateral diffusion are formed by the selective immobilization of lipids on the chrome or ITO surfaces. Whereas gold is an effective barrier-forming material, the mechanism involved can range from type I to type II depending on the cleaning procedure and storage of the substrate. The characteristics of these materials and the different mechanisms by which they impose patterns on a supported bilayer provide added flexibility in the design and application of micropatterned membranes.

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