Writing and Erasing Barriers to Lateral Mobility into Fluid Phospholipid Bilayers

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We describe a method for making and erasing barriers to the lateral diffusion of membrane components in fluid lipid bilayers supported on glass substrates. When a bilayer is mechanically partitioned by scratching the membrane-coated surface at basic pH, barriers to lateral diffusion are formed which prevent mixing between the regions separated by the scratches. Upon lowering the pH, the bilayer is observed to spread over the scratch boundary, allowing diffusive mixing between the previously separated regions. This is exploited in combination with electrophoresis within the membrane to separate fluorescently labeled charged lipid probes, partition them with a scratch, and allow remixing to occur when the scratch is healed. This method for membrane manipulation can be used to transform a homogeneous membrane into an array of corrals with different compositions while preserving the ability to allow subsequent remixing. This approach should be useful for examining the kinetics of reactions and the assembly of fluid membrane-associated components in a native setting, and for investigating the dynamics of two-dimensional fluids.

Introduction

Planar supported lipid bilayers have been shown to possess many properties that are similar to those of natural cell membranes.[1] In particular, bilayers assembled on glass supports are cushioned by a thin (10−20 Å) layer of water,[2−7] so that both leaflets retain the fluidity that is an essential feature of biological membranes. Because this fluidity leads to mixing of membrane-associated components by lateral diffusion, the patterning of supported membranes presents an intrinsic difficulty. We have developed methods for confining and organizing *fluid* membrane components in defined regions either by scratching the bilayer-coated surface[8] or by photolithographic patterning of barriers on the solid support in combination with electrophoresis of charged components in the plane of the bilayer.[9]

In the case of patterned barriers on surfaces, two primary mechanisms have been found to partition the bilayer, depending on the precise chemical composition of the barrier.[10] The first mechanism exploits the patterning of a material that prevents membrane deposition onto a membrane-compatible substrate, for example, alumina patterned on silica. In the second mechanism, materials that lead to the immobilization of phospholipid membranes are patterned onto membrane-compatible substrates, for example, chrome on silica. In the second case the entire substrate is coated with lipid material; however, membrane material over the chrome portion lacks long-range fluidity while the rest of the bilayer behaves like a fully connected two-dimensional fluid.

The mechanism responsible for partitioning membranes by scratching the surface is somewhat more complicated and is described in detail elsewhere.[11] Briefly, barriers can be erected *after* the formation of the planar membrane, in contrast to the case for lithographically patterned substrates where preexisting patterns induce barriers by directing the assembly of the membrane. Scratching the membrane-coated surface produces barriers to lateral diffusion and membrane spreading by a combination of topographical and tribological interactions. Scratching the surface not only removes lipid bilayer material but also permanently scores the underlying substrate. Variations in surface topography require the membrane to bend in order to remain in contact with the support. As the surface curvature increases, it becomes less energetically favorable for the bilayer to spread laterally. Tribological effects due to the alignment of water at the substrate/water interface also prevent membrane spreading. The alignment of near-surface water molecules arises from the large electric field near the oxide/water interface. On a silica surface, the field is predominantly caused by surface charges from titratable hydroxyl groups that terminate oxide surfaces. Such highly ordered water molecules are a poor lubricant and prevent the membrane from spreading. However, they can be regulated by varying the bulk pH of the buffer, allowing the bilayer spreading process to be turned on and off.

In this paper the scratch method of creating boundaries in a supported membrane is combined with electrophoresis to generate gradients of charged membrane components. Once charge gradients have been formed, additional partitions are scored into the supported lipid bilayer, allowing high concentrations of charged constituents to be permanently corralled. At this point, the supported membrane consists of a mosaic of fluid corralled regions with substantially different compositions. The mosaic pattern is stable in the absence of an electrophoretic field since the scratch boundaries prevent mixing between separate corrals. The corrals can then be opened simply by lowering the pH of the bulk solution, allowing remixing to occur. This combination of scratching and membrane electrophoresis provides a system of membrane manipu-

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lation that is exceedingly simple and convenient. Furthermore, it is possible to produce membrane compositions of lipids and proteins by this method that are not stable in vesicular form. These techniques may be of general use for the study of mixing and assembly of membrane-associated components.

Experimental Section

Experiments were performed with egg phosphatidylcholine (egg PC, Avanti Polar Lipids, Alabaster, AL) containing the appropriate concentrations of *N*-(Texas Red sulfonyl)-1,2-dihexadeconoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red DHPE) and 4-(4-(didecylamino)styryl)-*N*-methylpyridinium iodide (D291) (Molecular Probes, Eugene, OR) as charged fluorescent probe. Supported bilayers were formed using the vesicle fusion method on clean borosilicate cover slips (VWR Scientific, Media, PA).[1]

Cover slips were prepared by cleaning in 1:3 solutions of 7X detergent (ICN, Costa Mesa, CA) in deionized water, rinsing in Millipore water, and subsequently baking at 425 °C for 4 h. Lipid samples were prepared by mixing egg PC with dye-labeled lipids in chloroform, drying under a nitrogen stream, and desiccating under house vacuum for 1−2 h. The lipids were resuspended in 50 mM sodium phosphate buffer at pH 8.0 and small unilamellar vesicles (SUVs) were formed by probe sonication for several minutes and centrifugation to remove heavier lipid structures. To form the supported bilayer, a cover slip was brought into contact with a 60 μL drop of SUV solution on the bottom of a crystallization dish. The dish was then filled with buffer solution and the cover slip was briefly shaken to remove excess lipids.

Electrophoresis was performed on supported bilayer samples using the methods of Groves and Boxer.[8] The apparatus consisted of a Teflon holder with two aqueous wells containing platinum wire electrodes. After a bilayer-coated cover slip was prepared, a second cover slip was placed over it to form a sandwich with a thin water layer (~50 μm). The cover slip sandwich was then placed in contact with the two aqueous wells. Fields of up to 100 V/cm could be applied parallel to the supported bilayer using a conventional power supply. All electrophoresis experiments were performed in distilled water; typical currents were always less than 2 μA, which produces a negligible amount of resistive heating.

A Nikon E800 fluorescence microscope equipped with a Photometrics Sensys CCD camera was used to image the bilayers. Image Pro Plus data acquisition software from Media Cybernetics was used to obtain images and take line profiles across images. Further processing, including false color imaging, was done with Photoshop 4.0 from Adobe. Fluorescent images taken through the Texas Red filter set were assigned the color red, while images taken with the D291 filter set were assigned the color green.

Results

(i) Writing and Erasing Patterns in Lipid Bilayers. Figure 1a shows the epifluorescence false color image of a planar supported egg PC bilayer formed with 1 mol % of the fluorescently labeled lipid Texas Red DHPE in a 50 mM, pH 8.0 sodium phosphate buffer. The membrane appears highly uniform, and fluorescence recovery after photobleaching (FRAP) experiments revealed that the bilayer was fluid with no detectable immobile fraction (i.e. less than 1% immobile constituents).

The bulk solution above the immersed bilayer was exchanged for a 50 mM, pH 10.3 sodium phosphate buffer. The cover slip was then scratched with a tweezers in the vertical and horizontal directions to create a checkerboard pattern of boxes a few hundred square microns in size separated by sharp barriers, one of which is shown in Figure 1b. The boundaries remained stable in basic buffer (above pH 8) or ultrapure Millipore water for several weeks, and the phospholipids inside each individual surface patch remain fluid.

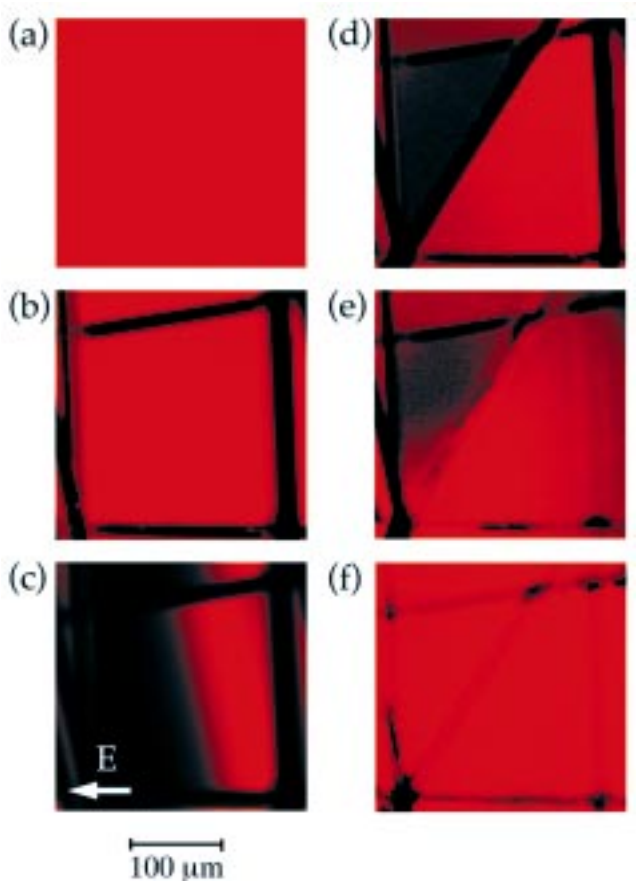

Figure 1. Writing and erasing on a membrane surface (see schematic diagram in Figure 2). (a) An egg PC bilayer was formed on borosilicate with 1% Texas Red DHPE (red color observed by epifluorescence microscopy). (b) The bilayer was immersed in 50 mM, pH 10.3 sodium phosphate buffer and mechanically scratched with a pair of #5 tweezers to form boxes, one of which is shown. (c) The bulk solution was replaced with deionized water and an electric field of 50 V/cm was then applied parallel to the membrane for 60 min; (d) the field was turned off, and a diagonal scratch was made across the box so that the Texas Red molecules were corralled off into the lower right-hand side of the box when the system had relaxed. (e) The system was then immersed in acidic buffer (pH 4.5, 50 mM), and the membrane began to creep over the bare portion of the surface, healing the scratch mark. (f) After 10 h the scratch mark was completely healed and the Texas Red probe molecules were again evenly spread out.

The buffer solution above the scratched membrane was then replaced with distilled water in preparation for placement in the electrophoresis cell. Upon applying an electric field of 50 V/cm parallel to the membrane surface, the negatively charged Texas Red DHPE lipids began moving in the opposite direction of the field and formed a region of high concentration on the right-hand side of the boxes, as shown in Figure 1c. Previous work by Groves and Boxer has shown that such gradients are completely reversible and the field can be switched back and forth numerous times.[8] The field was applied for 60 min, and the current remained steady at 0.4 μA over the entire course of the experiment. If the field were turned off, this gradient would relax back to uniformity (Figure 1b) over a period of about 20 min. Instead, immediately after the field was turned off the support was scratched again to create a diagonal barrier in the middle of the box. The probe concentration then relaxed back to equilibrium in about 20 min. A line profile across the image reveals that the Texas Red DHPE molecules are concentrated by about a factor of 2 in the lower right-hand region while they are

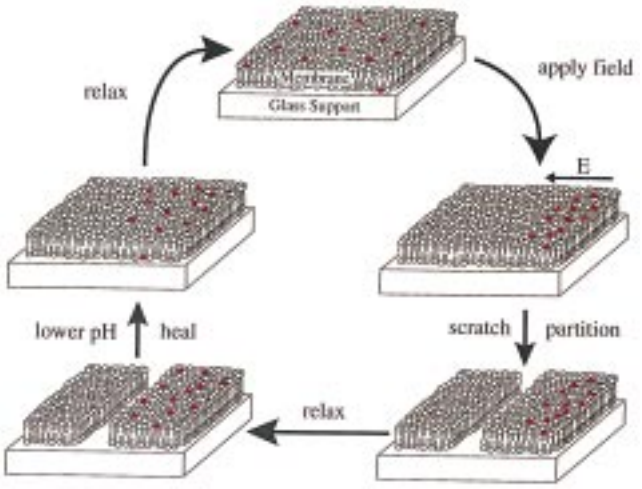

Figure 2. Schematic representation of the bilayer partitioning, electrophoresis and healing processes shown in Figure 1.

depleted by an order of magnitude on the upper left (Figure 1d). Corralled domains made in this manner were stable in basic solution over a period of many weeks.

At this point the bulk solution above the membrane was acidified by immersion of the system into a 50 mM, pH 4.5 buffer. When this was done, the lipid materials began to creep over the scratch boundaries and fuse back together (Figure 1e). The bilayer healed one section at a time, rather than at an equal rate for all scratch boundaries; however, once a scratch boundary began to reseal, the concentration gradient of Texas Red fluorophores in two adjoining membrane patches came to equilibrium within a matter of minutes. This process continued in an apparently random fashion as membrane patches healed together over the entire silica surface. After 10 h the vast majority of scratch boundaries had been resealed (Figure 1f). The essential features of patterning, electrophoresis, probe confinement, and remixing are depicted schematically in Figure 2.

After the entire healing process was completed, a few open patches and faint dark traces could still be seen where the original scratch marks had been made (Figure 1f). These darker portions persist over a period of several days without further change and are probably an indication that the bilayer does not completely fill in the deformed areas of the surface. Nevertheless, it should be emphasized that the regions that have been scratched do at least in part heal and that the initially separate bilayer patches are fully reconnected. FRAP[12] experiments indicate that the darker regions are fully fluid and pose no barrier to long-range lateral lipid diffusion in the membrane.

(ii) Separation by Charge. Following the methods described above, an egg PC bilayer membrane containing 1% Texas Red DHPE and 2% D-291, a positively charged membrane probe, was formed on a float glass cover slip and barriers were created by scratching. The membrane was imaged with two different filter sets. Exciting the Texas Red probes near 560 nm yielded fluorescence in the red peaked near 630 nm, whereas excitation of the D291 probes near 455 nm yielded fluorescence in the green peaked near 530 nm. These separate images were then superimposed to form the two-color image shown in Figure 3a. The yellow appearance of the combined image is a result of mixing red and green fluorescence colors in Photoshop. Figure 3b shows the two-color fluorescence

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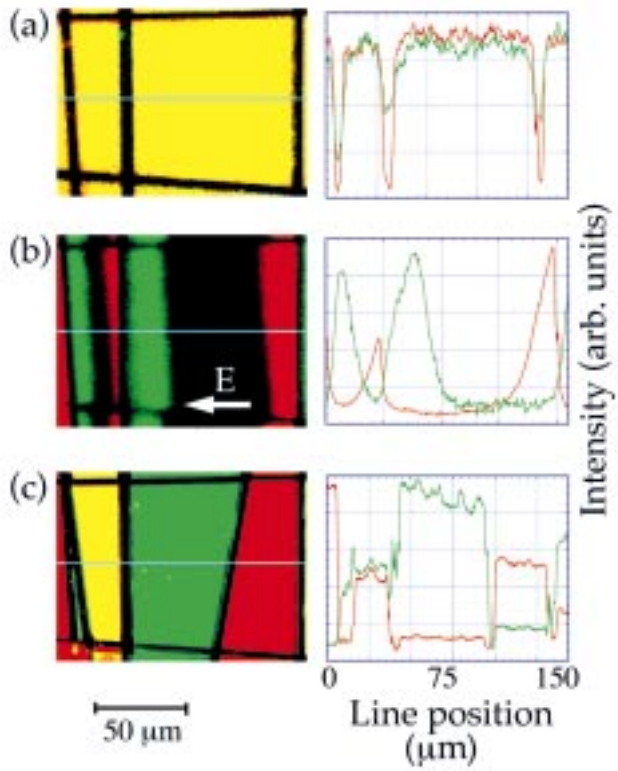

Figure 3. Separating oppositely charged probes: (a) two-color fluorescence image of a supported egg PC bilayer containing both 1% Texas Red DHPE and 2% D-291 which has been patterned into boxes by scratch marks; (b) the same systems after applying an electric field of 50 V/cm for 60 min which separates the oppositely charged probes. (c) The field was turned off and a second scratch was made in each box to corral the negatively charged Texas Red probes (red area on the right) and the positively charged D291 probes (green area on the left). The smaller box on the left side is partitioned much further into the green region; consequently, upon remixing the right-hand section of this box contains both red and green probes and appears yellow.

image after electrophoresis. The positively charged D291 probes moved to the left, while negatively charged Texas Red probes moved to the right in both boxes shown. The field was then turned off and a vertical scratch mark was made though each box, roughly in the center of the larger box on the right and close to the left barrier in the smaller box on the left. Once the probe concentrations had relaxed back to equilibrium, it is evident that the newly erected barrier in the larger box prevents remixing of the Texas Red and D291 lipids. The line profiles revealed that a high level of separation has been achieved. The situation in the smaller box on the left is also interesting, as the newly introduced scratch only partitions off a small region containing the positively charged green D291 probe in the lower left corner of this box. After the system has relaxed, this is nearly completely green, whereas the much larger region to the right still contains a mixture of the two dyes and appears yellow.

The system presented in Figure 3c is quite remarkable because it contains patches of predominantly different composition. For example, the surface charge on adjacent patches containing positively and negatively charged components should be quite different, although the surface potential is still dominated by the charge concentration of the underlying silica support. Simple Guoy−Chapman calculations[13] reveal that adjoining fluid membrane patches may differ by more than 10 mV in potential just

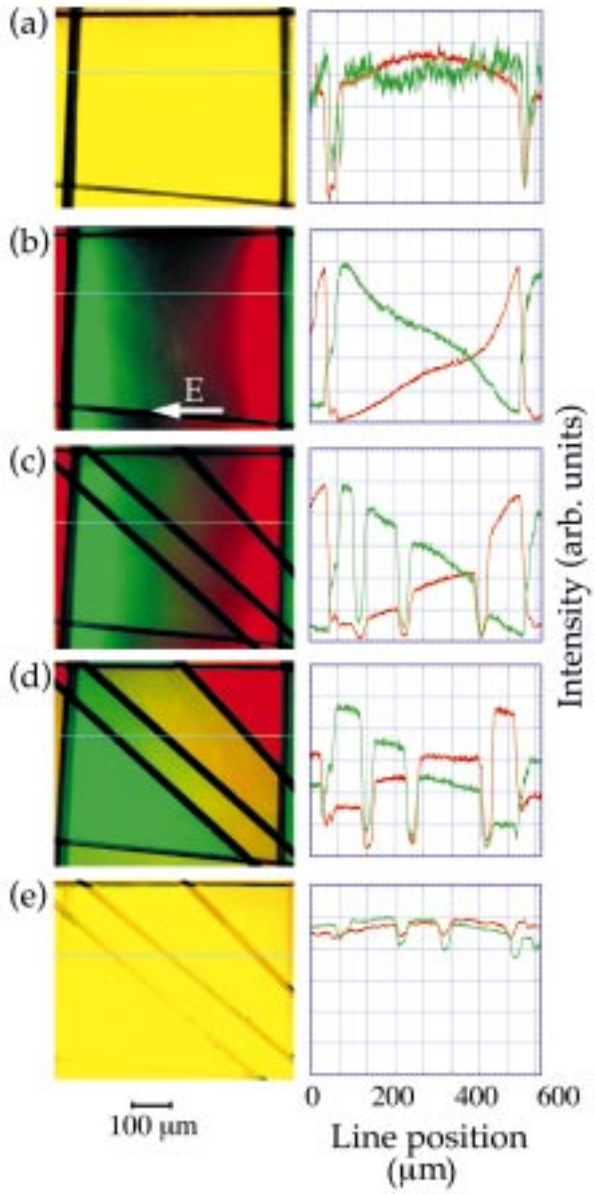

Figure 4. Making composition gradients of oppositely charged probes: (a) two-color fluorescence image of a supported egg PC bilayer containing both 1% Texas Red DHPE and 2% D-291 which has been patterned into boxes by scratch marks (one box is shown). (b) After applying an electric field the Texas Red probes move right while the green D291 probes move to the left. (c) The field is turned off, and a second set of three scratches is made at about 45° with respect to the first. This divides the original box into four pieces. (d) After 1 h the probe concentrations have relaxed to equilibrium in their respective boxes. The Texas Red DHPE concentration is highest in the upper right-hand corner and lowest in the lower left-hand corner. The concentration gradient for D291 is reversed from that for Texas Red. (e) The system is then immersed in acidic buffer (pH 4.5, 50 mM), and partitions heal, leading to remixing of the components.

above the membrane. The system remains intact for weeks with no detectable degradation in either membrane fluidity or barrier width.

Additional experiments performed with other lipids and lipid-linked proteins (NBD-PE, GPI-linked MHC,[14] biotinylated lipid head group linked to fluorescently labeled streptavidin) demonstrated that this technique is widely applicable. Membrane-associated proteins in these systems could be corralled on the basis of either electrostatic or hydrodynamic forces. In all cases membrane and membrane-bound components could be concentrated from less than a factor of 2 to well over an order of magnitude depending upon the positioning of the first and second sets of scratches.

(iii) Gradients of Charged Probes. Starting again with both D291 and Texas red probes incorporated into a supported egg PC bilayer, boxes were formed by scratching and an electric field was applied to separate the probes (Figure 4a and b). This time three scratches were made through a single box at approximately 45° with respect to the original etch marks (Figure 4c) and the system was then allowed to relax back to equilibrium (Figure 4d). This allowed varying probe concentrations to be corralled into elongated patches. A line profile through the image demonstrates that the Texas Red concentration is about four times higher in the lower right-hand triangle than it is in the upper left-hand triangle. The inverse probe concentration profile now exists for the D291 labeled lipids. Acidifying the buffer solution above the membrane then leads to remixing the probes (Figure 4e).

Discussion

We have demonstrated a simple method for separating mobile membrane constituents in phospholipid bilayers into isolated corrals. Barriers to lateral mobility can be written and also erased. In principle, this technique should be applicable to a wide range of mobile bilayer components either possessing a charge or having a significant hydrodynamic drag force. Potential applications for this technique range from purifying and concentrating membrane-associated proteins to the separation of large and/or charged membrane components. In particular, the methods presented in this paper will allow low concentrations of membrane-tethered proteins to be corralled for easy visualization. Adjoining membrane patches presenting varying concentrations of peptides or proteins can be prepared for assaying the effect of concentration upon fusion of cells or proteins from the bulk solution. The reversible scratch barriers can be combined with fixed barriers prepared by lithographic patterning so that selected separations and mixing can be carried out. Finally, it is often difficult to form vesicles or fuse vesicles to oxide supports when the concentration of charged components is high. The methods presented here readily allow high-concentration domains to be formed by electrophoresis and corralled into regions that are stable for weeks.

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