Two-Component Membrane Lithography via Lipid Backfilling

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Herein, we describe the development of a novel lithographic technique to pattern artificial lipid bilayer microdomains by exploiting the limited mobility of gel-phase phospholipids in two-component mixtures. Reconstituted supported phospholipid bilayers are formed by the self-assembly of lipids into two opposing leaflets on a hydrophilic surface. They are useful model systems for studying the physics and chemistry of biological membranes because of similarities with natural analogues, as well as accessibility to fluorescence microscopy and atomic force microscopy.[1] Spatially addressable supported lipid bilayers hold great promise for the development of integrated biological solid-state devices, such as rapid screening assays for proteins or other biomolecules that associate with membranes, enabling new high-throughput studies for many important cellular functions.[2,3] They are also highly ordered structures that effectively resist nonspecific binding of many molecules.[4]

Patterning of supported lipid bilayer microarrays has been demonstrated with various lateral diffusion barriers, such as metals or metal oxides,[5] proteins,[6,7] photoresist,[8] mechanical scratches,[7] and voids following either resist lift-off[9–11] or photo-oxidative degradation of lipids.[12] Patterning has also been demonstrated by direct deposition or removal of lipid material with polymeric stamps.[6,7] Recently, metastable membrane microdomains with lipid mixtures that resemble the composition of putative natural lipid rafts[13] were patterned photolithographically into supported bilayers.[14]

Our approach is to use kinetically "trapped" separation of components in a binary mixture to form stable lipid patterns. Previous investigators have shown that microdomains can spontaneously form in supported bilayer membranes, which contain two or more lipid components.[15–19] Lipid phases segregate due to different head groups, chain lengths, and/or varying degrees of saturation of their hydrocarbon chains.

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), which has a longer chain length and a higher transition temperature ($T_m \approx 41\,^\circ C$), was used to form ordered gel-like regions, while 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC) ($T_m \approx -1\,^\circ C$) was used to form liquid crystalline regions. Differential scanning calorimetry of multi-bilayers,[20] as well as confocal microscopy and fluorescence correlation spectroscopy of giant unilamellar vesicles,[21] have shown that DLPC and DPPC lipids have limited miscibility and can mix over time to form a two-phase region of coexisting ordered (rich in DPPC) and fluid (rich in DLPC) phases at equilibrium. However, the lateral mobility of lipid molecules in the ordered phase is 250 times below that of the fluid phase.[21] Lipid mixing in patterned areas in this case will be an activated process with a large energy barrier, which suggests that, at room temperature, segregated regions of pure DLPC and pure DPPC will remain kinetically stable.

Poly(dimethylsiloxane) (PDMS) stamps were used to create both positive and negative pattern transfers of bilayer microdomains, as depicted in Figure 1. Fluid microdomains could be patterned into a solid gel-like matrix (Figure 1 A). Alternatively, gel-like lipids could be patterned in a sea of fluid lipids (Figure 1 B).

For the first scenario shown in Figure 1 A, a PDMS stamp with square features in bas relief was held in contact with a clean planar glass coverslip in aqueous solution. This served as a mold around which the gel-phase DPPC bilayer was cast, using the vesicle fusion method.[22] A solution containing small unilamellar vesicles (SUVs) of DPPC at 45 °C, which is above the transition temperature for this lipid, formed a fluid bilayer around the PDMS posts on the coverslip. Upon cooling to room temperature, the DPPC bilayer solidified into a gel. After rinsing away any residual debris and removal of the PDMS, a solution containing DLPC vesicles was introduced, which resulted in the formation of fluid bilayers in the areas previously protected by the PDMS posts.

Generally, the fluid-to-gel phase transition in supported phospholipid bilayers results in the formation of submicron

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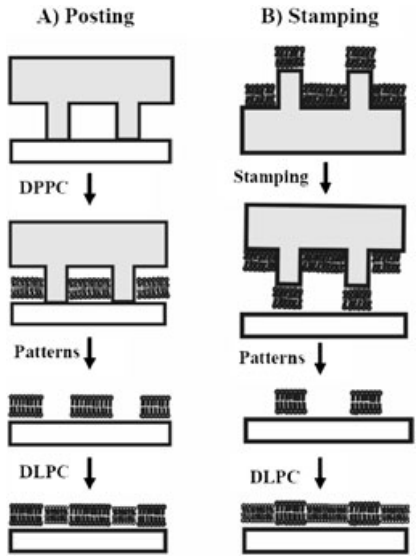

Figure 1. *Schematic diagrams of lipid patterning by PDMS posting (A) and stamping (B).*

scale cracks and other foamlike defects in the gel phase, due to lateral contraction caused by the ordering of the hydrocarbon tails.[23,24] These defects are typically too small to be observed by optical microscopy but can be easily seen with AFM. The cracks may then be unintentionally filled by fluid-phase DLPC lipids or other molecules, compromising our ability to pattern pure gel-like phases that can resist nonspecific binding of molecules. However, in our case, the DPPC bilayer was allowed to cool while in the presence of excess DPPC SUVs in solution. AFM images of the resulting gel-like bilayer, taken at room temperature, showed that all the defects had been filled in by aggregates of unfused vesicles of DPPC (see Supporting Information), consistent with the results reported by Kumar and Hoh.[25] We will show below that the DPPC lipid aggregates effectively passivated the defects in the gel.

For the pattern in Figure 1B, the stamping method described by Boxer's group[6,7] was adopted to generate gel-phase patterns surrounded by liquid crystalline phase barriers. A solution of DPPC vesicles at 45 °C ruptured and formed a fluid bilayer on the PDMS surface. After cooling below the phase-transition temperature, the bilayer was transferred onto a clean coverslip by stamping, followed by the introduction of DLPC vesicles to backfill the unpatterned areas.

Figure 2 shows combined epifluorescence images of supported membranes consisting of either fluid-phase DLPC bilayer areas patterned in a gel-phase DPPC bilayer background (Figure 2A), or gel-phase DPPC patterned areas surrounded by fluid-phase DLPC bilayer lipids (Figure 2B). In both panels, the DLPC phase was doped with 2 mol% of the fluorescently labeled lipid NBD-DOPE [1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2–1,3-benzoxadiazol-4-yl)] and the DPPC phase was doped with 1 mol% TR-DHPE [Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine]. Fluorescence from the two phases was collected through red and green filter sets and combined into false color images using Adobe Photoshop. At room temperature, there was no mixing of lipid molecules between the two phases. Heating the patterned membranes formed by either method to 45 °C for 30 minutes resulted in the disappearance of the patterns due to lipid mixing, as shown in Figure 2C. For both methods (Figures 2A and 2B), the patterns were stable under aqueous solution for several days.

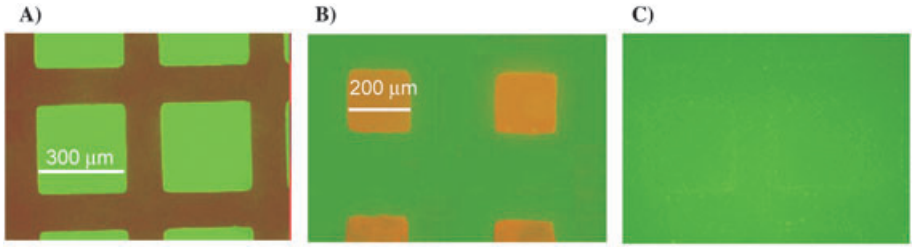

Figure 2. *Combined epifluorescence images of patterned bilayers corresponding to the processes shown in Figure 1. A) Fluid-phase DLPC bilayer patterned in gel-phase DPPC bilayer. The size of each fluid square was 300 μm × 300 μm. B) Gel-phase DPPC patterned in fluid-phase DLPC bilayer. The size of each gel square was 200 μm × 200 μm. C) The epifluorescence image of (A) after 30 minutes at 45 °C. The same behavior was seen for (B).*

Electrophoresis was performed by applying an electric field across sandwiched coverslips to test the lateral mobility of the segregated lipids in the two phases.[26] Pure water (> 18 MΩ) was used for these measurements. Figure 3A shows fluorescence images from corrals of 2 mol% NBD-DOPE-labeled DLPC lipids, surrounded by gel-phase DPPC. After an electric field of 25 V cm^{-1} was applied parallel to the interface, the negatively charged NBD-DOPE lipids migrated toward the positive elec-

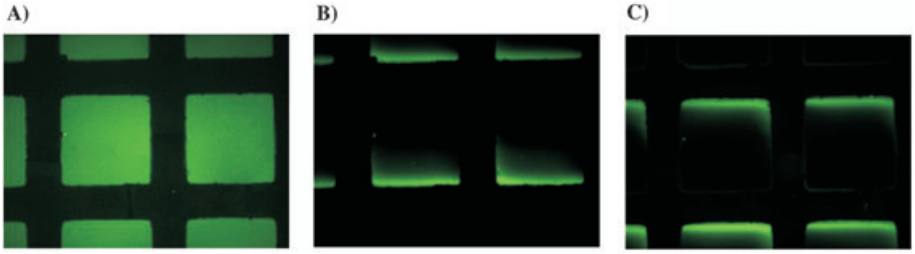

Figure 3. *A) Epifluorescence image of patterned corrals before applying electric field. Fluorescence was from a 2% NBD-DOPE label incorporated in the DLPC phase. B) Same region after applying an electric field for 8 minutes (25 V cm^{-1}). C) After reversing the direction of the field for additional 10 minutes.*

trode, resulting in the fluorescent intensity gradient seen in Figure 3B. Switching the electrode polarity resulted in reversal of the gradient, shown in Figure 3C. In both cases, the DPPC matrix blocked the migration of the fluorescent lipids. When the electric field was removed, the fluorescent probes returned to their original uniform density within 50 minutes. Close inspection of the images revealed that a small amount of NBD-DOPE remained localized at the interfaces between the two phases, presumably due to electric-field-induced penetration of the charged probe molecules into the DPPC barrier. This indicates that, although separable domains with different physicochemical properties could be spatially defined, the bilayer itself remained a single cohesive entity, and was contiguous at the interfaces between the two phases. A separate control experiment (not shown) revealed that, at room temperature, the electric field did not result in significant movement of 2 mol% NBD-DOPE lipids that had been previously homogeneously mixed with DPPC above 45 °C.

To show that patterned membrane arrays were functional for molecular recognition-mediated protein immobilization with minimal nonspecific binding, fluid phases consisting of 5 mol% Cap-Biotin-PE [1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(Cap-Biotinyl) (Sodium salt)], 2 mol% NBD-DOPE, and 93 mol% DLPC were patterned in a pure DPPC-gel background. Figure 4A is an image captured through a green filter set from the NBD-DOPE label in the DLPC fluid bilayer phase. The membrane was then incubated for 30 minutes in a solution of 0.05 mg mL^{-1} Cy3-labeled streptavidin, followed by rinsing of unbound protein in phosphate buffered saline (PBS). The image through the red filter set due to fluorescence from the Cy3 probe, Figure 4B, exactly matches the image from the green channel in Figure 4A. Figure 4C is a fluorescence intensity profile of the line in Figure 4B indicating that the streptavidin probe bound exclusively to the biotin-labeled lipids in the patterned areas. The lack of nonspecific binding of streptavidin to the DPPC gel phase is explained by the presence of lipid aggregates consisting of unfused DPPC vesicles that passivate the defects in the gel, which is consistent with the results from AFM described earlier.

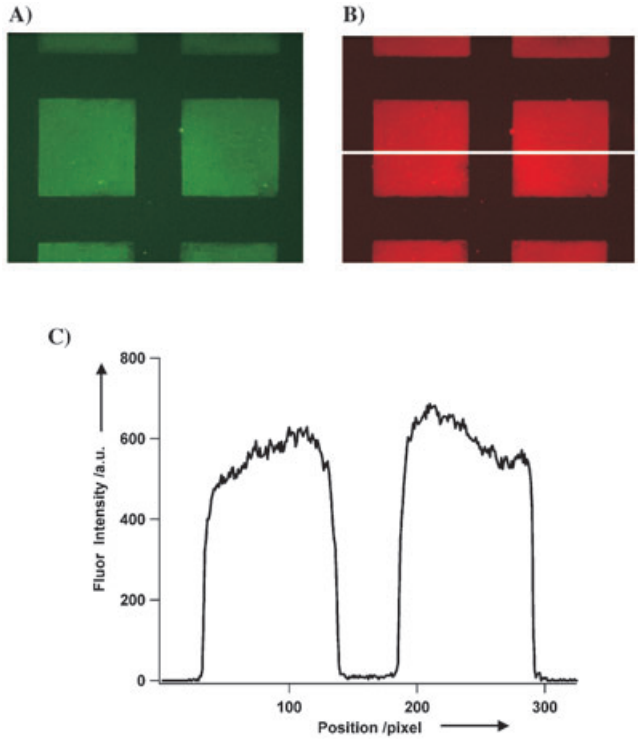

Figure 4. *A) Epifluorescence image of 2 mol% NBD-DOPE, 5 mol% Cap Biotin-PE, and 93 mol% DLPC bilayer surrounded by 100 mol% DPPC bilayer. B) After incubation of the patterned membrane with 0.05 mg mL^{-1} Cy3-labeled streptavidin. C) Fluorescence intensity profile of the line drawn across (B).*

Because the array sites and the diffusion barriers that define them form a single, continuous lipid bilayer supported on a 1–2 nm cushion of water on the substrate,[1] we believe our methodology may be ideally suited for patterning lipid domains at much smaller and physiologically more relevant length scales by mechanical nanografting or by nanoshaving selected bilayer regions with an AFM tip, without damaging the underlying surface,[23,27,28] in conjunction with backfilling of cleared areas with SUVs containing different lipids. Clean removal of DPPC-bilayer material at room temperature by an AFM tip has been demonstrated,[23] without the need for cumbersome electron beam lithography steps (see Supporting Information). The result will be a means to spatially manipulate the composition of lipid domains in bilayer membranes at the nanoscale.

Experimental Section

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2–1,3-benzoxadiazol-4-yl) (NBD-DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(Cap-Biotinyl) (Sodium salt) (Cap-Biotin-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TR-DHPE) was obtained from Molecular Probes (Eugene, OR). Cy3-conjugated Streptavidin was purchased from Zymed Laboratories (South San Francisco, CA).

Small unilamellar vesicles (SUVs) were prepared by the extrusion method.[29] The appropriate amounts of each kind of lipid were mixed and dissolved into chloroform solution. Solvent was evaporated under a gentle steam of nitrogen and desiccated under vacuum overnight. The dried lipid mixture was hydrated in PBS buffer (pH 7.2) for one hour followed by at least seven freeze–thaw cycles. The hydrated solution was extruded through an Avanti miniextruder (Avanti, Alabaster, AL). At least ten extrusion cycles were performed through 0.05 μm polycarbonate membrane filters at the proper temperature. The average size of the vesicles from the extruder, determined by dynamic light scattering, was 70 nm. Buffer solution was added to dilute the vesicles to their final concentration (2 mg mL^{-1}). Vesicle solutions were stored at 4 °C and used within three days. Bilayers were formed by vesicle fusion and rupture on clean glass coverslips. Glass coverslips were cleaned in "piranha" solution [3:7 (v/v) mixture of 30% H_2O_2 and H_2SO_4, caution: this mixture reacts violently with organic materials] for 30 minutes followed by extensive rinsing with pure water from a Milli-Q Gradient A10 system (Millipore, Billerica, MA).

Patterned PDMS (Dow Corning Sylgard Silicone Elastomer-182) was used as a post to protect areas from the formation of lipid bilayer

or as a stamp to transfer bilayer to glass coverslips. Patterning on PDMS was obtained by standard photolithographic techniques. A thin photoresist layer (15 μm, Shipley S1813) was spin-coated onto a silicon wafer and patterned photolithographically to make a master. To imprint the patterns, PDMS monomers were poured on the photoresist and carefully peeled off after 4 h of curing at 100 °C. For posting, the PDMS stamp was sonicated in ethanol overnight to remove any residual siloxane monomers which could contaminate the glass. A 50 g weight was loaded onto the PDMS stamp to contact the planar glass coverslip in solution. To prevent formation of air bubbles on the PDMS during posting, and to aid in the stamping of DPPC lipids, the PDMS was subject to a one-minute treatment in an oxygen plasma cleaner (Harrick Scientific, Ossing, NY) to render it hydrophilic.

Images of the supported lipid bilayers and streptavidin were obtained by inverted epifluorescence microscopy (Eclipse TE300, Nikon, equipped with a CCD camera, CoolSNAP-HQ, Roper Scientific, Tucson, AZ) using a ×10 objective. The images were stored using the Metamorph software from Universal Imaging (Downington, PA) and transferred to Adobe Photoshop for processing.

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