Micropatterned model biological membranes composed of polymerized and fluid lipid bilayers

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Micropatterned phospholipid bilayers on solid substrates offer an attractive platform for various applications, such as high-throughput drug screening. The authors have developed a photopolymerization-based methodology for generating micropatterned bilayers composed of polymerized and fluid lipid bilayers. Lithographic photopolymerization of a diacetylene-containing phospholipid (DiynePC) allowed facile fabrication of compartmentalized arrays of fluid lipid membranes. Herein, the authors report on the present state of research and discuss on the prospective application of the model membranes. © *2008 American Vacuum Society.*
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I. INTRODUCTION

Substrate-supported planar lipid bilayers (SPBs) provide unique possibilities for reconstituting cellular membranes on solid surfaces.[1–3] They also provide an attractive platform for various new applications, such as high-throughput drug screening.[4–6] However, one pertinent shortcoming of SPBs is their inherent limited stability. Possible pathways to improve the stability include the use of self-assembled monolayers,[7] tethered lipopolymers to the surface,[8–10] and polymerizable lipids.[11–13]

To realize stable SPBs, we have recently developed a methodology to create micropatterned SPBs composed of polymerized and fluid lipid bilayers, where the polymerized bilayer forms an integrated matrix with embedded fluid bilayer corrals (Fig. 1).[14–17] For the polymerization of bilayers, a commercially available diacetylene phospholipid, 1,2-*bis*(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine (DiynePC), has been used (Fig. 1). After the UV polymerization, monomers were removed by treatment with a detergent solution, and various phospholipid bilayers could be incorporated into the lipid-free regions by a self-assembly process (vesicle fusion method), forming a hybrid membrane composed of polymeric and fluid lipid bilayers. In this configuration, polymeric bilayers act both as a barrier for the lateral diffusion of membrane-associated molecules and as a stabilizing framework for embedded SPBs.

There have been several lines of micropatterning approaches reported to date, including use of mechanical scratching,[18] prepatterned substrates,[19–22] microcontact printing,[23] microfluidics,[24,25] and ink-jet printer.[6] Compared with these approaches, micropatterning of SPBs by polymerized lipid bilayers is unique in that polymeric and fluid bilayers are integrated as a continuous membrane. This architecture gives various unique features to the model membrane. For example, it was observed that the transformation of vesicles into SPBs was accelerated by preformed polymeric bilayers.[26] This observation suggests that preformed polymeric bilayers could also facilitate the incorporation of membranes derived from biological samples. We have also demonstrated that the lateral diffusion of membrane-associated molecules could be controlled by the presence of polymeric bilayers.[17] Such obstructed lateral diffusion is commonly observed in cellular membranes, and, in fact, nonrandom distribution of membrane components and their confinement in microdomains are regarded as a prerequisite for numerous vital functions such as signal transduction and trafficking. Therefore, designed model membranes composed of polymeric and fluid bilayers may provide unique tools for studying the membrane functions. In this short account, we describe these unique features and their potential utility for basic and applied researches by summarizing the previous work from our research group.

II. LITHOGRAPHIC POLYMERIZATION OF LIPID BILAYERS

For the lithographic polymerization of lipid bilayers, monolayers of DiynePC (monomer) were deposited onto solid substrates from the air/water interface by the Langmuir–Blodgett and Langmuir–Schaefer methods, successively. Micropatterning of the bilayers was achieved by placing a contact lithography mask on the bilayer surface during the photopolymerization. After the UV irradiation, nonreacted monomers were selectively removed by immersing the sample in a detergent solution (0.1*M* sodium dodecylsulfate) for 30 min (at 25 °C) and new lipid bilayers [phosphatidylcholine from egg yolk (egg-PC) containing 1 mol % Texas Red 1,2-dihexadecanoyl-*sn*-glycerophosphoethanolamine (TR-PE)] were introduced into the wells surrounded by polymeric bilayers by the vesicle fusion technique.[27–32] Figure 2 shows fluorescence micrographs of a patterned bilayer. In Fig. 2(a), the polymerized bilayer is observed due to the fluorescence from a conjugated polymer backbone. Lipid bilayers of egg-PC containing 1 mol % TR-PE were selectively incorporated into the square-shaped areas (corrals) where monomers had been protected with the

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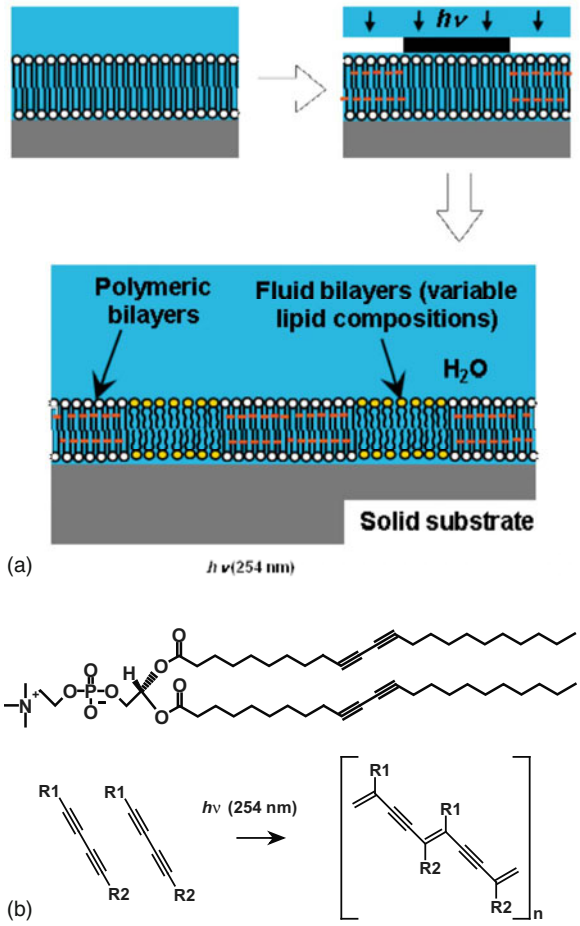

FIG. 1. (a) Schematic of the pattering method. (b) The structure and polymerization scheme of DiynePC.

mask during the lithographic UV exposure and selectively removed [Fig. 2(b)]. The bilayers are continuous and fluid within the areas surrounded by the polymeric bilayers (corrals) as has been clearly demonstrated by the local photobleaching of TR-PE with a focused intense illumination (data not shown).

One of the critical steps for the generation of micropatterned model membranes is the formation of polymeric bilayers in a controlled manner. We have recently reported that the homogeneity of polymerized bilayers from a diacetylene-containing phospholipid (DiynePC) was very sensitively influenced by the film deposition temperature and alternatively

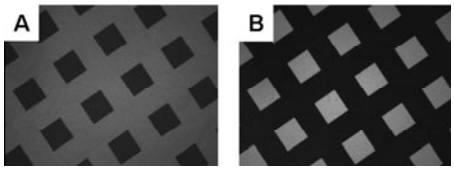

FIG. 2. Fluorescence micrographs of a patterned bilayer on a glass substrate. (a) Fluorescence from polymerized DiynePC bilayers (UV irradiation dose: 4.4 J/cm^2). (b) Fluorescence from TR-PE doped egg-PC bilayers incorporated in the wells between polymerized bilayers (corrals). Two images were obtained at the same position by using different filters.

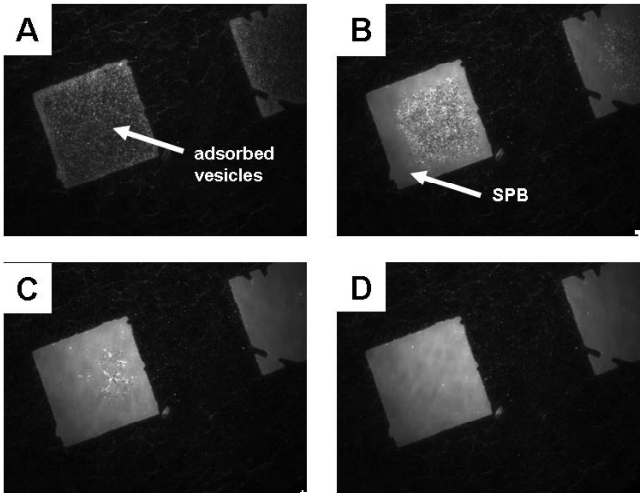

FIG. 3. TIR-FM images of the vesicle fusion process on a glass substrate with patterned DiynePC bilayer. Time lapse images were obtained after the addition of egg-PC vesicles containing 1 mol % TR-PE: (a) adsorbed vesicles started to form homogeneous SPBs at the boundaries of DiynePC bilayer and [(b) and (c)] expanded to the central region, (d) finally covering the glass surface completely. The size of the corral was 50 μm.

by the annealing/quenching protocols.[33] Whereas DiynePC bilayers (monomer) deposited at a temperature below the triple point temperature of DiynePC monolayers (~20 °C) formed homogeneous polymeric bilayers, those deposited at a temperature higher than the triple point temperature showed a markedly increased number of line defects. The differences were attributed to the domain structures in the monolayer on a water surface. Since the progress of polymerization as detected by the polymer backbone conjugation (UV/visible absorption spectra) and the residual film thickness [atomic force microscopy (AFM) and ellipsometry] was unaffected by the film deposition temperatures, the domain size, rather than the molecular packing in each domain, was postulated to play a critical role. Changes in the spontaneous curvature and film area are the plausible source of destabilization and detachment of the films upon polymerization. These results highlight the importance of controlling the domain structures of polymerized bilayers for generating homogeneous polymeric bilayers.

III. ENHANCED INCORPORATION OF FLUID BILAYERS BY PREFORMED POLYMERIC BILAYERS

The fact that the polymeric scaffolds and incorporated fluid membranes have the same bilayer structure gives various unique features to the model membrane. For example, we have observed that the incorporation of SPBs by the vesicle fusion method was significantly accelerated by the presence of preformed polymeric bilayers.[26] Figure 3 shows total internal reflection fluorescence microscopy (TIR-FM) images during the vesicle fusion process on a patterned DiynePC bilayer substrate (50 μm grid). The images were obtained after adding vesicle suspensions of egg-PC containing 1 mol % TR-PE. The formation of planar bilayers near

the boundaries was visible by the appearance of homogeneously fluorescent domains [Fig. 3(a)]. The SPB domains subsequently expanded to the central regions [Figs. 3(b) and 3(c)] and finally covered the whole surface between DiynePC bilayers [Fig. 3(d)]. The kinetics of vesicle fusion in the presence of preformed polymeric DiynePC bilayers was also studied by the quartz crystal microbalance with dissipation (QCM-D). The results from TIR-FM and QCM-D measurements have shown that the formation of SPBs was promoted by the presence of preformed DiynePC bilayers. The TIR-FM observations revealed that SPBs were preferentially formed at the boundary of DiynePC bilayers and subsequently propagated to the central part of the lipid-free regions. QCM-D results showed that patterned bilayer substrates accelerated the formation of SPB, as indicated by the reduced accumulation of transiently adsorbed vesicles. The acceleration was more prominent for patterned substrates with a higher density of boundaries between DiynePC bilayers and lipid-free regions (a smaller stripe width).[26]

Facilitated incorporation of fluid SPBs by preformed DiynePC bilayers is a strong indication that it is energetically favorable to incorporate guest SPBs into the matrix of DiynePC bilayers. In order to obtain an energetic gain, these two types of bilayers should form a continuous hybrid membrane, sealing the edges of DiynePC and fluid lipid bilayers. The formation of such hybrid membranes is the most important feature of the present micropatterning strategy, because the polymeric bilayers can act both as barriers for the lateral diffusion of membrane-associated molecules and as a scaffold to stabilize incorporated fluid bilayer membranes. This unique property should enable various extensions in the architecture of micropatterned model membranes such as separation of the membrane from the substrate with a spacer and incorporation of membrane proteins in a native state. The enhanced vesicle fusion by the presence of a preformed polymeric bilayer scaffold also suggests the possibility that a wider variety of lipid membranes, including native cellular membranes, may be incorporated into micropatterned bilayers.

IV. REGULATION OF LATERAL MOBILITY IN THE COMPOSITE MEMBRANES

One of the unique features of the present micropatterning strategy is the fact that the area fraction of polymeric bilayers can be regulated by changing the UV irradiation dose applied for the photopolymerization. Figures 4(a) and 4(b) compare micropatterned bilayers, where the polymerization of DiynePC was made with different UV irradiation doses (4.4 and 0.9 J/cm^2). Polymeric and fluid bilayers were completely segregated if a large UV irradiation dose was applied upon photopolymerization [Fig. 4(a)]. For the sample with a smaller UV dose, fluorescence of TR-PE was observed not only in the corrals but also within the polymerized DiynePC bilayer domain [Fig. 4(b)], suggesting that fluid lipid bilayers of egg-PC/TR-PE penetrate into the region of polymerized DiynePC. This result suggests that membrane-associated molecules can penetrate into defects of polymeric domains.

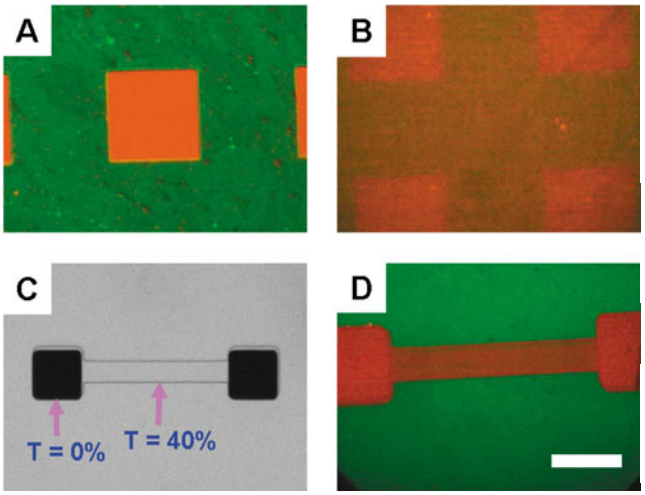

FIG. 4. Fluorescence micrographs of micropatterned lipid bilayer with varied UV irradiation doses. (a) Complete separation of polymeric and fluid bilayer domains, in the case of a large UV irradiation dose (4.4 J/cm^2). Fluorescence image of polymerized DiynePC (green) and egg-PC/ TR-PE (red) were superimposed. (b) A smaller UV dose (0.9 J/cm^2) was applied for the polymerization of DiynePC. Egg-PC/ TR-PE bilayers were observed both in the corrals and within the polymeric bilayer domains. (c) A photomask that has two chromium squares connected by a narrow stripe of thin glass layer that transmits 40% of UV light. (d) A membrane channel of permeable membrane domain connects two fluid lipid bilayer membranes. The scale bar corresponds to 50 μm.

The diffusion coefficient of lipid molecules in the fluid bilayer was found to be 1.54 ± 0.28 $\mu m^2/s$ for samples without polymeric bilayers (homogeneous bilayers of egg-PC). On the other hand, it was progressively suppressed with heightened UV irradiation dose of the photopolymerization and reached zero at the UV dose of about 1.3 J/cm^2.[17] The increased coverage of the substrate surface with the polymeric bilayer domains, as indicated by the domain fraction in the AFM images, has most likely acted as an effective obstacle for the long distance diffusion of lipid molecules.[16] For a sufficient UV irradiation dose, the lateral diffusion of lipid molecules was completely hindered by the polymer domains.

The present results suggest that one could modulate the lateral mobility of membrane-associated molecules by purposefully designing the geometry and degree of polymerization. In this way, one should be able to construct arrays of lipid bilayers that are not completely isolated but partially connected. A conceptual demonstration is given in Figs. 4(c) and 4(d). By using a mask, in which two chromium squares are connected by a narrow channel that transmits 40% of UV light, patches of fluid lipid bilayers could be connected by a membrane channel that has partially polymerized bilayers. The channel connects two fluid membrane domains with a reduced lateral mobility of molecules.

V. INCORPORATION OF BIOLOGICAL MEMBRANES

An important step for the development of micropatterned model membranes is the incorporation of membrane-associated proteins in their functional form. However, native

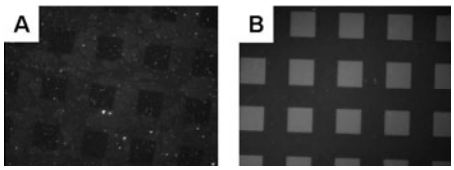

FIG. 5. Incorporation of SR membranes into the corrals surrounded by polymeric bilayers using DHPC mixtures: (a) application of SR membranes; (b) SR membranes incorporated with DHPC [1%(w/v)=22 mM]. The patterned membrane was observed after rinsing the solution (removing DHPC). (In all samples, membranes contained a fluorescence dye, 3,3′-dioctadecyloxacarbocyanine perchlorate.)

biological membranes contain various lipids and proteins, and their incorporation into model membrane systems via vesicle fusion is generally very difficult. As an alternative approach for incorporating membranes derived from biological samples, we studied the incorporation of biological membrane fractions into a micropatterned polymeric bilayer scaffold together with a detergent [1,2-Dihexanoyl-sn-glycero-3-phosphocholine (DHPC)]. We used sarcoplasmic reticulum (SR) membrane vesicles prepared from rabbit skeletal muscle as a model system.[34] Although SR membrane vesicles could not be incorporated into the corrals of a micropatterned bilayer substrate [Fig. 5(a)], they could be homogeneously incorporated into the corrals in the presence of DHPC [Fig. 5(b)]. The concentration of DHPC [1%(w/v) =22 mM] was higher than its critical micelle concentration of 11 mM.[35] SR membranes, which were partially solubilized by DHPC, adsorbed onto the substrate surface. This result suggests that a purposeful use of detergents significantly improves the incorporation of membranes obtained from biological samples. It should also be noted that polymeric bilayer matrices are a useful tool for studying the incorporation of various components derived from cellular membranes by providing a framework with a defined thickness. Since polymeric bilayers of DiynePC have a defined thickness of about 4.4 nm and a relatively low roughness of the surface, this bilayer scaffold can be used as a reference system for analyzing the thickness and structures of incorporated membranes. For example, it should be possible to study the structures of adsorbed membranes in detail by using surface plasmon resonance and surface plasmon fluorescence spectroscopy.[36,37] We are currently investigating the structures of adsorbed membranes from biological samples.

VI. PROSPECTIVE APPLICATIONS

By immobilizing various membrane proteins in an array format, it should be possible to study their functions in a systematic and quantitative manner. For example, we have recently reported on a methodology for immobilizing cytochrome P450 enzymes that play critical roles in the oxidation of xenobiotics, including drugs and environmental pollutants.[38–40] We could detect the enzymatic activity on the substrate. Furthermore, competitive assay experiments between two substrates demonstrated the feasibility of bioassays based on immobilized P450s. The polymeric bilayer played multiple roles in this configuration, i.e., stabilization of incorporated bilayers, suppression of nonspecific binding to the surface, and patterning of P450s. Although the structure of immobilized membranes was not yet well defined as in the case of pure lipid bilayers, this result represents an important step toward the high-throughput screening of P450 activities.

Another important direction of the development is the construction of complex model membranes with a well defined architecture. Compared with other approaches that have been demonstrated to be effective in creating patterned lipid membranes, the present method has a unique feature, in that the pattern is imprinted in the bilayer membrane as polymerized domains. This fact allows the patterned two-dimensional structure to be independent of the substrate, and one could possibly construct a patterned bilayer separated from the substrate by a thin layer of soft polymeric cushion. Such polymer layers have been used as a spacer in order to accommodate membrane proteins in solid-supported bilayers in a functionally active form. It should also be possible to covalently attach the polymerized bilayer to the underlying polymer cushion by using a chemically reactive head group. The polymeric lipid bilayer might contribute to the mechanical stabilization of the membrane system, similar to the conjunctions between cytoskeleton and membrane proteins.

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