

Micropatterning of Lipid Bilayers in a Microfluidic System via Forced Vesicle Fusion

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Abstract

Substrate-supported planar lipid bilayers (SPB) are excellent model for bio-membranes, and have potential scientific and technological applications. Due to these features, SPBs have been studied to determine if their application can be useful for biological analyses, such as the evaluation of cell to cell interactions or as cell based biosensors. In this study, the patterning of 1, 2-bis(10, 12-tricosadiyno-yl)-sn-glycero-3-phosphocholine (diynePC) bilayer that is a polymerizable lipid was evaluated via forced vesicle fusion in a microfluidic system. The formation of diynePC lipid bilayers in the microfluidic channel was sensitive to flow rate, and homogeneous diynePC lipid bilayers were formed when diynePC was applied at a flow rate of 0.1 μ L/min for 10 min. These layers were patterned via selective photopolymerization using a UV dose of 4.1 J/cm². Because lipids in areas blocked from UV can be selectively removed by ethanol, new lipid layers, such as egg yolk phosphatidylcholine (egg PC), that retain some characteristic features of their native cellular membrane, can be incorporated into the areas that were cleared by ethanol. This micropatterning of diynePC lipid bilayers within the microfluidic system can be used to provide a platform for a lipid-based immunoassay chip, and may therefore be a useful tool for cell based high-throughput systems.

Keywords: Forced vesicle fusion, Infusion rate, Photopolymerization, Microfluidic system, Micropatterning

Introduction

Cellular functions such as cell adhesion, migration, and proliferation are controlled by interactions between molecules within the extracellular matrix (ECM)

and receptors on the cell surface¹. Substrate-supported planar lipid bilayers (SPB) that have been absorbed to a solid surface by physical interactions or chemical bonds make an excellent model for bio-membranes, and have potential scientific and technological applications^{2,3}. However, in order to study the actual biological membrane systems, the complexity of the artificial systems must be increased. Patterning of SPBs is one approach that can be used to mimic the actual biological function of a cell membrane. Because the fabrication of lipid-associated biomolecules on controlled surfaces is possible⁴⁻¹¹ via SPB patterning, the use of this process for the development of high-throughput drug screening and establishing chip-based cellular interactions based on lipid bilayers has increased¹²⁻¹⁸. Microfluidic systems also have an advantage because they require only a small reaction volume, it is easy to control their channel design, and they provide the opportunity to integrate on-chip detection schemes into their systems. Therefore, the usefulness of SPB patterning would be further increased if it were combined with a microfluidic system. However, traditional patterning techniques, such as microcontact printing⁶, microfluidics^{9,19} and polymer lift-off²⁰, can not be used to generate pattern in microfluidic systems because there is no accessible space in which to contact the SPB in the microfluidic channel.

Photopolymerizable lipids may be used to overcome the aforementioned fabrication problem because they can be patterned in a microfluidic channel without direct contact through photopolymerization of the lipids via UV irradiation. This remote control system for the patterning of lipid bilayer was first suggested by Ross *et al.*, who used bis-SorbPC (1, 2'-bis(10-(2', 4'-hexadienoyloxy) decanoyl)-sn-glycero-3-phosphocholine) as a UV photopolymerizable lipid in fused silica capillaries²¹. This lipid, however, is hard to synthesize and very difficult to purify. Morigaki *et al.* fabricated an SPB pattern using diacetylene phospholipids (1, 2-bis(10, 12-tricosadiynoyl)-sn-glycero-3-phosphocholine (diynePC)), which are commercially available products that are easy to photopolymerize by UV irradiation^{8,22,23}. The SPB pattern produced by diynePC, however, is still difficult to manage because photopolymerization of this lipid is only possible when these molecules are in a highly ordered state. Although Morigaki produced a highly-ordered SPB comprised of diacetylene by packing the lipid mole-

cles using the Langmuir-Blodgett method (LM method)²⁴, this method can only be used in an open system that allows the consecutive transfer of two lipid monolayers onto a solid surface. This implies that the primary challenge in constructing a micropattern on the lipid bilayer in a microfluidic channel is to construct a photopolymerizable lipid bilayer in the microfluidic channel.

In this study, we have successfully fabricated a polymerizable lipid bilayer in the microfluidic system by a method referred to as, “forced vesicle fusion”, which controls the shear stress created by the diynePC vesicle over the solid surface in the microfluidic devices.

Materials and Methods

Substrate Cleaning

To prepare the SPB substrate, cover glasses (No 1, Marienfeld, Germany) and quartz glasses (Electron Microscopy, USA) were cleaned with piranha solution ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ (7 : 3)) using a sonicleaner for 1 h. After cleaning, the substrates were rinsed with deionized water extensively, and then dried with N_2 gas. This procedure resulted in a hydrophilic surface that was suitable for the adsorption of lipid bilayers. The cleaned substrates were then stored in 25% ethanol at 4°C for up to 2 weeks.

Microfluidic Channel Fabrication

Photoresist, SU-8 2080 (Microchem Co., USA), was spin-coated onto silicon wafers to a thickness of 50 μm . The photoresist-coated wafers were then prebaked at 65°C for 10 min, and at 95°C for 30 min. The wafers were then exposed to UV (365 nm wavelength) for 7 s through a photomask that contained a predesigned micropattern, then baked at 65°C for 10 min, followed by baking at 95°C for 30 min. The micropattern was developed on the wafers by removing the unpolymerized photoresist using an SU-8 developer. After washing the wafers with methanol, they were dried with N_2 gas. Polydimethylsiloxane (PDMS) Sylgard 184 (Dow corning corporation, USA) was then poured on the micropatterned wafers and the bubbles were removed using a vacuum pump. Flat PDMS substrates were then formed by curing Sylgard 184 onto planar silicon wafers for 1 h at 80°C. Next, the PDMS mold was carefully peeled off, washed with methanol, and the PDMS surface was then rendered hydrophilic by oxygen plasma treatment for 1 min with plasma cleaner (Plasma cleaner PDC-32G, Harrick Scientific, USA). The hydrophilic substrates were then placed in contact with the hydrophilic

PDMS surface and cured for more than 3 h at 80°C.

Preparation of Supported Planer Bilayers

The diynePC was dissolved in chloroform, and evaporated with N_2 gas. The plates were dried in desiccators under a vacuum for more than 6 h. To hydrate the lipid film, the desired amount of degassed phosphate buffered saline (PBS) solution was mixed with the lipid film by vortexing for 1 h. Seven freeze/thaw cycles were then conducted to completely dissolve all of the lipids. The lipid solution was then extruded using a mini extruder (Avanti Polar Lipids, USA) equipped with a 50 nm membrane at 60°C (over transition temperature) to make a small, unilamellar vesicle solution (SUV). The transition temperature for egg yolk phosphatidylcholine (egg PC) and diynePC (Avanti Polar Lipids, USA) is -18°C and 40°C , respectively. After extrusion, the SUV solution was stored at 4°C for up to 1 week. Next, the vesicle fusion method was used to adhere the SPB to the substrate, and then 1 mol% Texas Red[®] 1, 2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Texas Red[®] DHPE, Molecular Probes, USA) was used to detect the SPB. The lipid vesicles were then dropped on a crystallizing dish and ruptured into cleaning substrates, followed by removal of the excess vesicles by extensive washing with deionized water.

For the preparation of the SPB in the microfluidic channel, diacetylene lipid vesicles that contained 1 mol% TR-PE were injected into the microfluidic channel at various flow rates, which were controlled using a Harvard syringe pump (Harvard apparatus, USA). Excess lipid vesicles were then removed by rinsing the channel with deionized water for 20 min at a flow rate of 0.5 $\mu\text{L}/\text{min}$. Because flow rate and incubation time are the critical factors for the formation of lipid bilayers in the microfluidic channel, various combinations of reaction conditions were evaluated. The formation of lipid bilayers under each set of experimental conditions was then evaluated by fluorescence recovery after photobleaching (FRAP), which shows the lateral diffusion of lipid bilayer membranes.

Micropatterning of Lipid Bilayers via Photopolymerization in Microfluidic Channel

The following fabrication processes were used to patternize lipid bilayer in the microfluidic channel (Figure 1): (i) formation of an SPB on a solid substrate, (ii) lithographic photopolymerization using UV as a light source, (iii) removal of the unpolymerized bilayers, and (iv) refilling the empty areas with new lipid bilayers. After formation of the SPB, a small low pressure mercury lamp (UVP, Inc., USA) that emitted strong UV light at 254 nm was used as a light

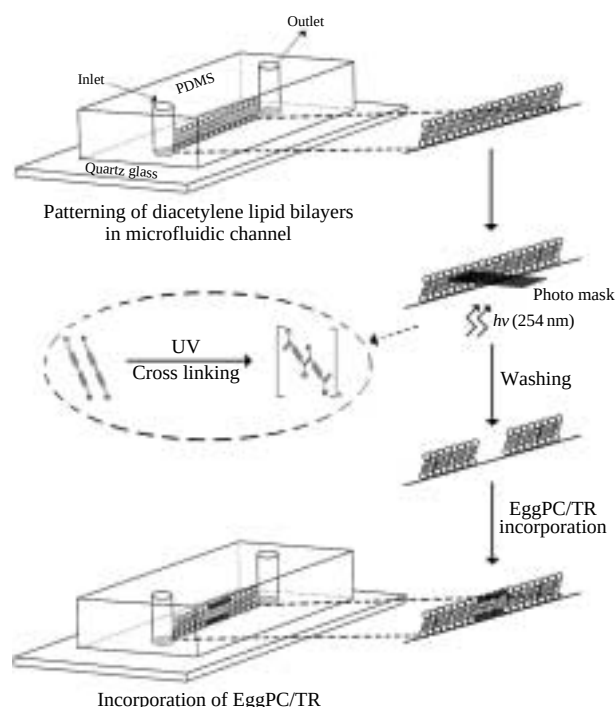


Figure 1. Schematic view of the fabrication of the supported planer bilayer comprised of diynePC in a microfluidic channel via forced vesicle fusion.

source for photopolymerization²⁵. UV photopolymerization was achieved by illuminating the supported planer bilayers through a physical mask (transparency sheet), which was placed directly onto the clean quartz glass on which the SPB was formed. Following UV irradiation, the unpolymerized lipid bilayers were removed with ethanol or 1% SDS followed by extensive rinsing with milliQ water. To incorporate the new lipid bilayers into the lipid-free wells, the vesicle fusion method was applied. Small Unilamellar Vesicle (SUV) that was obtained from eggPC and contained 1 mol% Texas-Red, was extruded through a filter membrane with a 50 nm pore diameter 10 times using a mini-extruder. The eggPC/TR DHPE vesicles were then incorporated into lipid-free wells that were enclosed by the polymeric bilayers (10 min, 5 μ L/min). The channel was then washed with deoxygenated water at room temperature for 30 min to remove excess vesicles (Figure 1).

Results and Discussion

Formation of DiynePC Supported Planer Bilayers in an Open System

Vesicle fusion, which does not require sophisticated

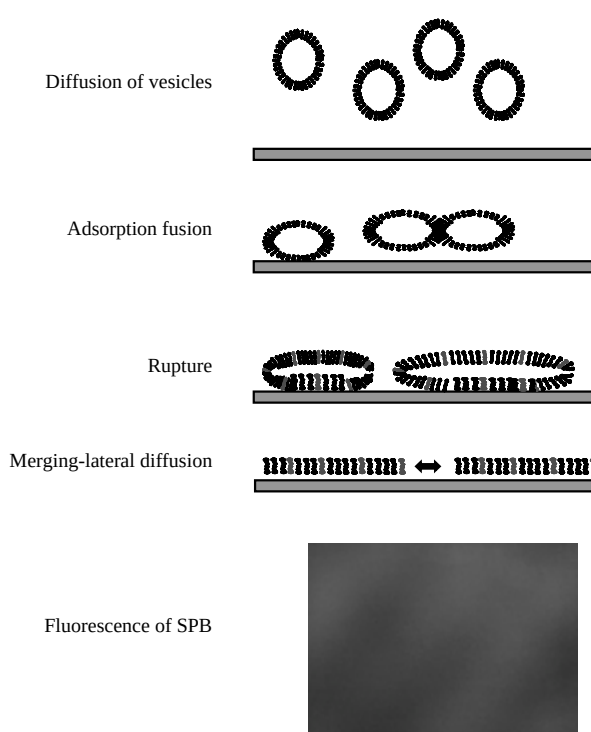


Figure 2. Formation of the supported planer bilayers via vesicle fusion: 1) diffusion of vesicles, 2) adsorption and fusion, 3) rupture, and finally 4) merging of the disks. The SPB fluorescence image on glass as a result of vesicle fusion with eggPC containing 1 mol % TR-DHPE labeled lipid.

equipment, is generally used for the formation of SPB on solid surfaces, such as glass, mica, self-assembled monolayers, polymers, and quartz²⁶. As shown in Figure 2, the first step in the formation of SPB is the diffusion of vesicles from the bulk solution onto the solid surface. The vesicles near the surface then adsorb to the solid surface or fuse to each other to form larger vesicles, which are subsequently ruptured, resulting in bilayer disks. The continuous bilayers developed in this study were formed by merging these disks. The formation of the lipid bilayers, which contained Texas Red labeled lipids, could be proven via the FRAP.

The prerequisite step for construction of the phospholipid bilayer pattern is to form SPB on the solid surface. Because we intended to use diynePC to generate the pattern inside the microfluidic channel, the possibility of the formation of SPB using diynePC via vesicle fusion was first tested in an open system. Various concentrations (0.5 mg/mL, 1 mg/mL, and 2 mg/mL) of 100 μ L of diynePC that contained 1 mol % TR-DHPE vesicles were dropped onto the crystallizing dish at room temperature for 1 min. After re-

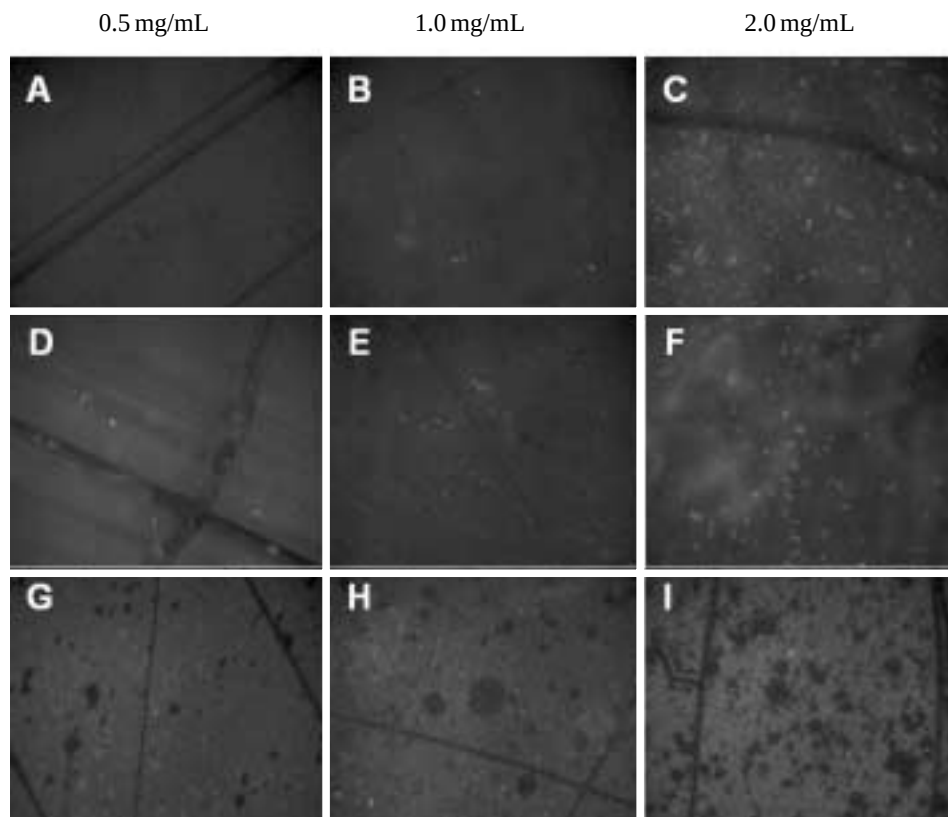


Figure 3. Fluorescence image of the diynePC layer on a glass surface. Various concentrations of diynePC were used for the vesicle fusion, 0.5 mg/mL (A, D, and G), 1 mg/mL (B, E, and H), and 2 mg/mL (C, F, and I). Vesicle fusion was carried out at room temperature for 1 min (A, B, and C), at room temperature for 30 min (D, E, and F), and above the transition temperature for 1 min (G, H, and I).

removal of the excess vesicles by rinsing extensively with deionized water, the lipid layers were observed using an inverted fluorescence microscope (ECLIPSE TE 2000, Nikon, Japan). Fluorescence microscopy images were obtained with a CCD camera, and then quantified using Image-pro plus 5.1 (Olympus) and a DPC controller (Olympus). All images were processed using Adobe Photoshop (Adobe, USA). As shown in Figure 3, no homogeneous SPB formation was observed after 1 min (A, B, and C), or when the reaction time was extended to 30 min (D, E, and F). Phospholipids usually self-assemble spontaneously to form a bilayer structure with hydrophobic tails directed toward the center and hydrophilic head groups exposed to the aqueous phase. Each lipid has its own transition temperature (T_m) that is determined by the aliphatic chain length and the presence and location of any unsaturations in the aliphatic chains. Lipid hydrocarbon tails change from the solid to liquid state at their T_m ²⁷⁻²⁹. Below their T_m , the lipid aliphatic tails are in a well-ordered solid state and the translational mobility of the bilayer constituents is limited, however, above the T_m , the phospholipid aliphatic tails are in a more disordered fluid state, which results in more lateral freedom^{30,31}. DiynePC has a transition temper-

ature of 40°C, however, the experiments were performed at room temperature, and therefore, the limited translational mobility of the bilayer constituents at that temperature may explain the failure of the SPB formation. Additionally, although a more intense fluorescent image was observed above the T_m (G, H, and I), scattered dust particles and discernible defects were also recognized in the layer. Overall, these results imply that the traditional vesicle fusion method is not adequate for the formation of an SPB using diynePC.

Formation of DiynePC Supported Planer Bilayers in the Microfluidic System

As shown in previous section, it was not possible to create an SPB via the traditional vesicle fusion method using diynePC in an open system. Morigaki *et al.* used the Langmuir-Blodgett (LB) method to preorganize diacetylene moieties on a glass surface²² where the first monolayer was deposited by dipping and withdrawing the substrate vertically, and the second leaflet was then deposited onto the hydrophobic surface of the first monolayer by pressing the substrate horizontally through the monolayer at the air/water interface, and then dropping it into the subphase. The

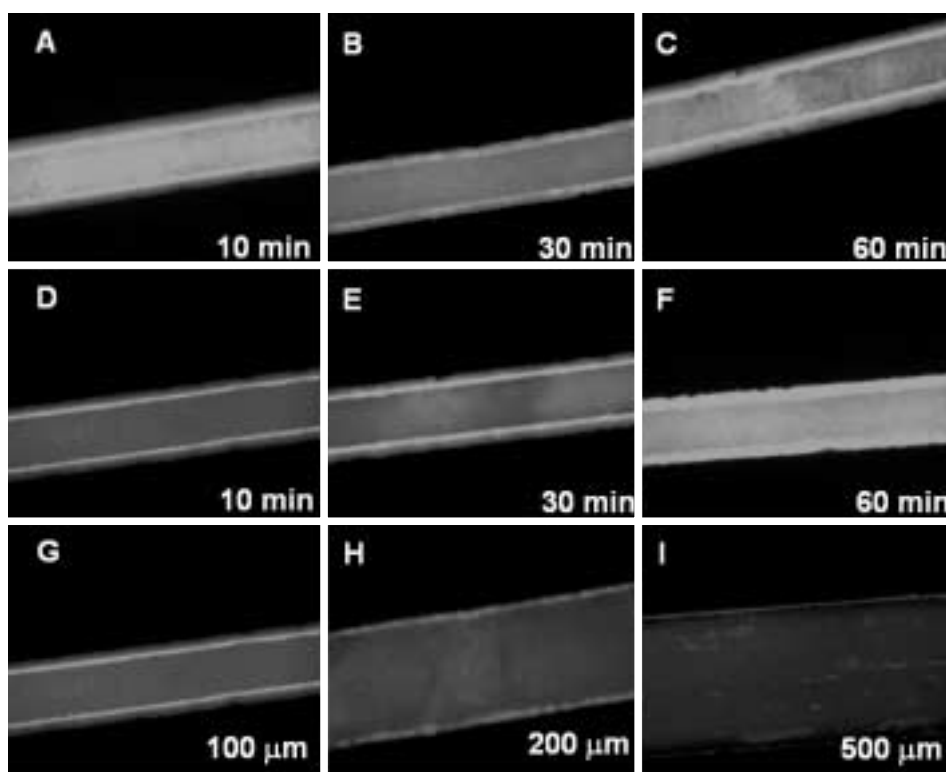


Figure 4. Fluorescence image of the diynePC layer in the microfluidic channel. The diynePC was incubated while in the static state in the channel for the times indicated (A, B, and C). For forced vesicle fusion, the diynePC was continuously infused into the microfluidic channel at 0.1 $\mu\text{L}/\text{min}$ for 10 min, 30 min, and 60 min (D, E, and F). Forced vesicle fusion using channels that were 100 μm , 200 μm , and 500 μm wide (G, H, and I) with a flow rate of 0.1 $\mu\text{L}/\text{min}$ for 10 min.

critical point in the LB method is the packing of the poly (diacetylenes). Because the LB method cannot be used for the formation of an SPB in a microfluidic channel, it was necessary to develop a novel method to overcome the aforementioned problem and pack the diynePC into the microfluidic channel. Compared to an open system, the microfluidic channel has a constrained surface, and it has been reported that self-assembled monolayers in the constrained surface favor direct adsorption of lipids that are standing-up, thus facilitating their packing³². This implies that an SPB could be made by packing diynePC into the microfluidic channel. Initially, diynePC lipid vesicles containing 1 mol% TR-PE were injected into the microfluidic channel, which was 100 μm wide, at a flow rate of 0.1 $\mu\text{L}/\text{min}$. The vesicles were then incubated for between 10 min and 60 min. After incubation, excess vesicles were removed from the channel by deionized water, and the channels were then observed using fluorescence microscopy. The design of this experiment was very similar to the vesicle fusion method used in the open system; however the lipid is injected into the microfluidic channel, which is a constrained area. As shown in Figure 4 (A, B, and C), more fluorescence was observed in the layer formed using this method than the one formed using the open system (Figure 3). Some defects, however, were still

observed, and the layers did not show the fluorescence recovery observed during the FRAP experiment. Although the layers in the microfluidic channel were not SPBs, these results indicated that the constrained surface may be helpful for adsorption of the diynePC vesicle.

Because adsorption and rupture of the vesicle is the prerequisite step for the formation of SPB, we attempted forced vesicle fusion inside the microfluidic channel to produce shear stress on the surface. For this method, instead of incubating the vesicle inside the channel after it was filled, we forced the diynePC over the surface continuously. The flow rate was adjusted to 0.1 $\mu\text{L}/\text{min}$ using a Harvard syringe pump and the diynePC was maintained at room temperature during the fusion process. After 10 min, 30 min, and 60 min (D, E, and F) of continuous infusion into the microfluidic channel, the excess vesicles were removed by water, and the channel was purged with N_2 gas. The fluorescence intensity increased as the forced infusion time increased, and the FRAP data indicated that bilayers were formed inside the microfluidic channel after 10 min of forced vesicle fusion in the 100 μm wide channel. However, when we increased the flow rate to 10 $\mu\text{L}/\text{min}$, SPB formation was not observed (data not shown). The channel size effect on this forced vesicle fusion was also tested at a

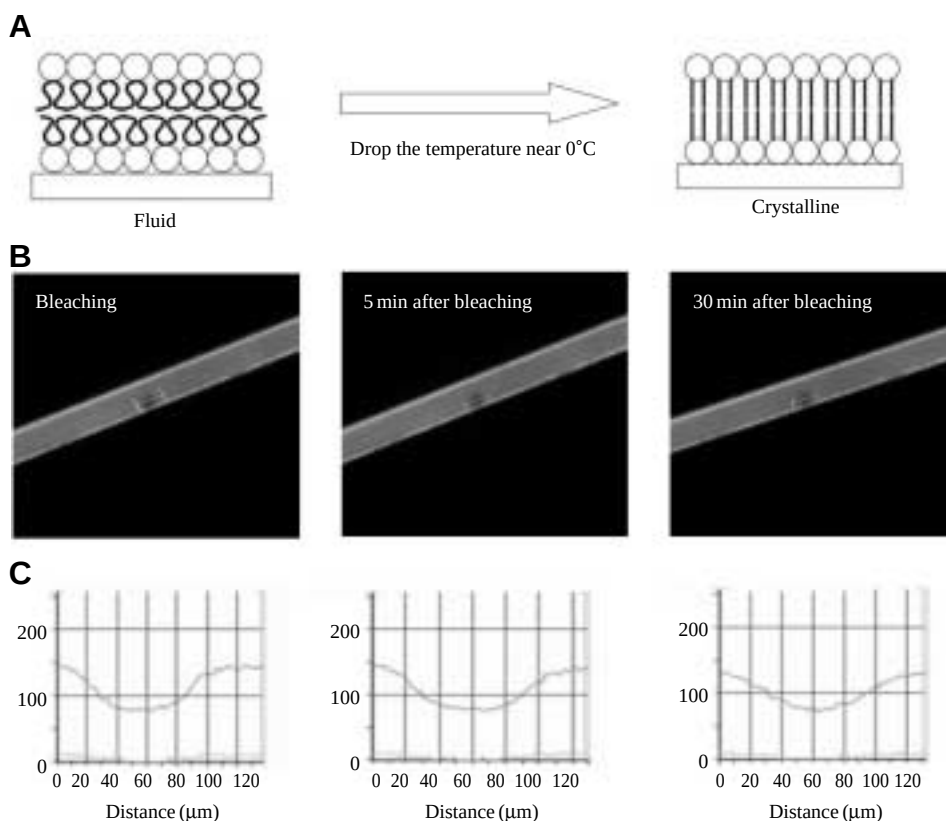


Figure 5. State change of diynePC as the temperature dropped to near 0°C . The diynePC changed from the fluid state to the crystalline state as the temperature dropped (A). In the FRAP experiment, a $40\text{ }\mu\text{m}$ spot was bleached for 6 min, and then the fluorescence recovery was monitored after 5 min and 30 min (B and C).

fixed infusion time of 10 min with a flow rate of $0.1\text{ }\mu\text{L}/\text{min}$ using channels that were 100, 200 and $500\text{ }\mu\text{m}$ wide. As shown in Figure 4 (G, H, and I), as the channel size increased, more defects were observed in the channel, which indicated that formation of the diynePC SPB was possible as a result of forced vesicle fusion.

Micropatterning via Photopolymerization of DiynePC in a Microfluidic System

It was reported that diynePC is only light sensitive when this lipid was prepared below the phase transition temperature, and lose its photosensitivity if the lipid undergo over phase transition temperature. In our study, the SUV for diynePC was prepared above the transition temperature, and ruptured on the solid surface via forced vesicle fusion. This means that the produced diynePC bilayers are in fluid state, which is not photosensitive. The state change of diynePC is possible via temperature drop near 0°C (Figure 5), and only the crystalline state is sensitive to photopolymerization. Because the photosensitivity of diynePC was correlated with the lipid state, which can be controlled via temperature change, diynePC bilayers need cooled down near 0°C to restore the photosensitivity. The FRAP experiment which shows the lateral dif-

fusion of the phospholipids bilayers are good indicator for this state change of diynePC. If the lipid bilayers were changed to the crystalline state, the recovery of fluorescence will be retarded. When the temperature dropped to near 0°C , the bilayer showed an extended recovery time, which indicated that lateral diffusion of the SPB had decreased dramatically (Figure 5B and C).

SPB patternizing was conducted by placing a contact mask directly on the bilayers during UV irradiation. Polymerization of the diynePC bilayers was then conducted via UV irradiation using a small low pressure mercury lamp (UVP, Inc., USA) as the light source. The desired patterns were then transferred to the diynePC bilayer during the polymerization process by illuminating the sample through a mask, which was placed directly on the diynePC bilayer. After the photopolymerization, nonpolymerized bilayers were removed selectively by allowing ethanol to flow through the microfluidic channel for 10 min (at room temperature). The UV dose used is important when conducting photopolymerization of diynePC. Because the lipids in the area blocked from UV were nonpolymerized, they were removed by the ethanol wash, and the eggPC/TR was then selectively incorporated into these regions, as shown in Figure 6.

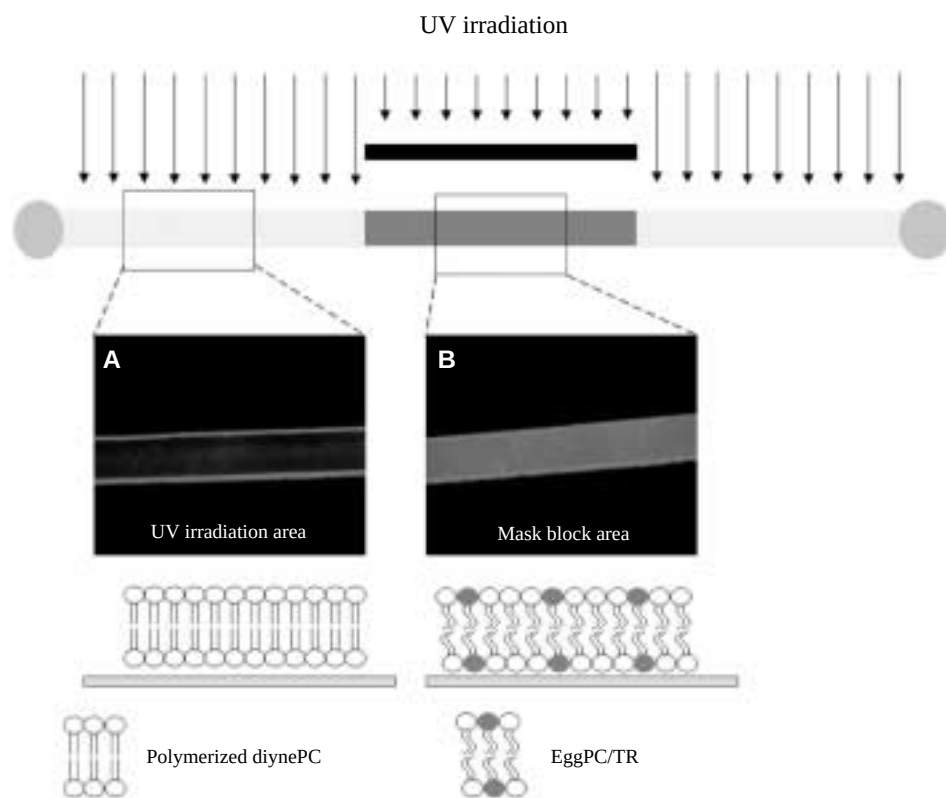


Figure 6. Photopolymerization of diynePC in the microfluidic channel.

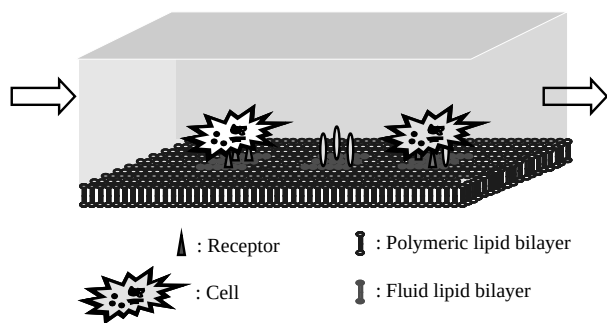


Figure 7. Construction of complex and versatile biomimetic membrane systems based on the bilayer micropattern in the microfluidic channel, and its application for cell-lipid membrane interactions.

Conclusions

SPBs have recently attracted attention as a biomimetic platform with various potential applications, such as cell-cell interaction, lipid-associated biochips and biosensors; therefore, patterning of SPBs has been used to study cellular functions on controlled surfaces. In this study, we patterned diynePC SPBs in a microfluidic system via forced vesicle fusion by

controlling the infusion rate. The micropatterning strategy, which was based on lithographic photopolymerization of lipid bilayers in the microfluidic channel, made it possible to control the lateral diffusion of the membrane associated molecules without the need for microfabricated barriers or lift-off stamps in the microfluidic channel²¹. For this purpose, patterning of the membranes is a feasible approach because various components can be integrated into the membrane system using defined spatial controls. Further, the lipid bilayers retain some of the features characteristic to their native cellular membranes (e.g., lateral fluidity), and can therefore be used for further biological applications. This method also provides an alternative platform for the fabrication of lipid-based immunoassay chips¹⁵ and a useful device for the study of lipid membranes within microfluidic devices used for processes such as high throughput drug screening (Figure 7)^{33,34}.

Acknowledgements

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