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Surface Functionalization of a Polymeric Lipid Bilayer for Coupling a Model Biological Membrane with Molecules, Cells, and Microstructures

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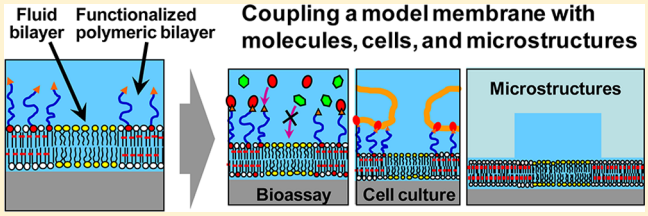
Supporting Information

ABSTRACT: We describe a stable and functional model biological membrane based on a polymerized lipid bilayer with a chemically modified surface. A polymerized lipid bilayer was formed from a mixture of two diacetylene-containing phospholipids, 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine (DiynePC) and 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphoethanolamine (DiynePE). DiynePC formed a stable bilayer structure, whereas the ethanolamine headgroup of DiynePE enabled functional molecules to be grafted onto the membrane surface. Copolymerization of DiynePC and DiynePE resulted in a robust bilayer. Functionalization of the polymeric bilayer provided a route to a robust and biomimetic surface that can be linked with biomolecules, cells, and three-dimensional (3D) microstructures. Biotin and peptides were grafted onto the polymeric bilayer for attaching streptavidin and cultured mammalian cells by molecular recognition, respectively. Nonspecific adsorption of proteins and cells on polymeric bilayers was minimum. DiynePE was also used to attach a microstructure made of an elastomer (polydimethylsiloxan: PDMS) onto the membrane, forming a confined aqueous solution between the two surfaces. The microcompartment enabled us to assay the activity of a membrane-bound enzyme (cyochrome P450). Natural (fluid) lipid bilayers were incorporated together with membrane-bound proteins by lithographically polymerizing DiynePC/DiynePE bilayers. The hybrid membrane of functionalized polymeric bilayers and fluid bilayers offers a novel platform for a wide range of biomedical applications including biosensor, bioassay, cell culture, and cell-based assay.



1. INTRODUCTION

Biological membranes play critical roles in cells owing to their two-dimensional (2D) and three-dimensional (3D) structural organizations.[1,2] They are 2D fluids that allow membrane-bound molecules to laterally diffuse and efficiently encounter each other.[3] At the same time, they are located in a 3D environment, forming boundaries between inner and outer spaces of cells and organelles. The structural order of lipids and proteins in the normal direction of the membrane is essential for establishing functions associated with separation and transport of molecules across the membrane. Specific interactions of the membrane with the 3D structures inside and outside of the cell, including cytoskeleton and extracellular matrices, are realized by molecular recognition on the surface of the biological membrane, which is enhanced by the unique property of a phospholipid bilayer to suppress nonspecific adsorption of proteins.

Various types of model membrane systems have been developed to mimic a part of these structural and functional features. The model systems include lipid vesicles, black lipid membranes (BLMs), and substrate supported planar lipid bilayers (SPBs).[4−6] Among these model systems, SPBs are currently attracting heightened attention due to the fact that they can be analyzed by various sensitive techniques that are specific to the solid−liquid interface (e.g., total internal reflection fluorescence (TIRF), surface plasmon resonance (SPR), and quartz crystal microbalance with dissipation monitoring (QCM-D)).[7−11] Furthermore, micropatterned SPBs can be generated by applying the microfabrication techniques, forming an integrated array of model membranes.[12−15]

One critical drawback of lipid membranes is their inherent instability due to the fact that they are formed by the self-assembly of many lipid molecules. In order to improve the stability of model membranes, attempts have been made to polymerize lipid bilayers, while retaining their structures.[16−20] However, polymerized lipid molecules are immobile and compromise an important feature of the biological membrane (i.e., fluidity). As an alternative approach, we developed a methodology to combine stable polymeric lipid bilayers and

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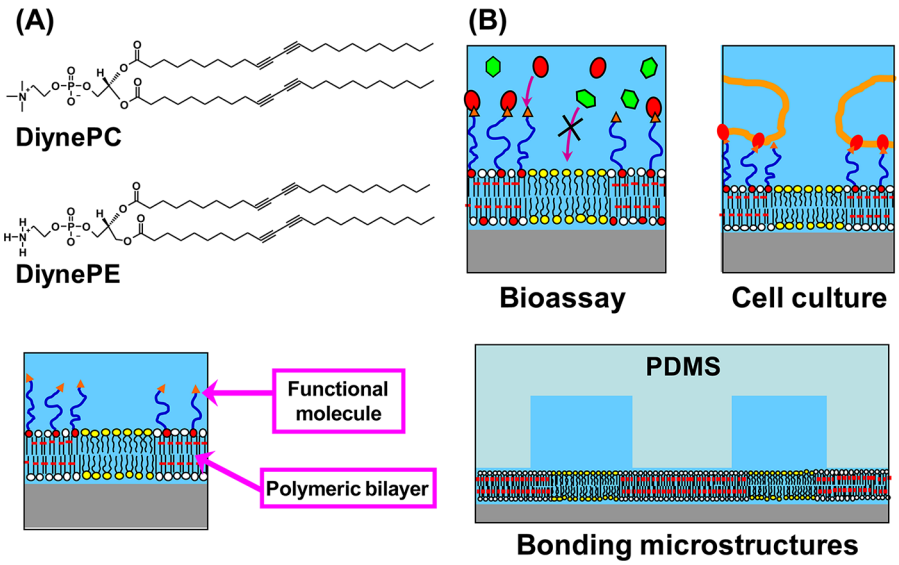

Figure 1. (A) Structures of DiynePC and DiynePE (top) and a schematic drawing of a functionalized polymeric bilayer (bottom). Polymeric and fluid lipid bilayers are lithographically combined, and functional molecules are grafted to the surface of the polymeric bilayer. (B) Potential applications of the functionalized model membrane: bioassay platform with minimal nonspecific adsorption (upper left), cell culture (upper right), and bonding with an elastomer microstructure (bottom).

fluid lipid bilayers by lithgraphic polymerization of a diacetylene phospholipid.[21−25] We generated micropatterned hybrid SPBs composed of polymeric and natural lipid bilayers. The polymeric bilayer acts as a framework that stabilizes the model membrane, whereas embedded lipid membranes are composed of natural phospholipids and retain physicochemical properties of the biological membrane.

In the present work, we developed a methodology to chemically functionalize the surface of a polymeric bilayer and use it as a basis for attaching various objects (molecules, cells, and microfabricated structures; Figure 1). This work was partially motivated by the fact that SPBs are generally used in a 3D environment, facing a solution (with a finite volume) or 3D objects such as cells, although SPBs have a 2D membrane structure. Therefore, it would be advantageous, if we could establish a means to effectively couple SPBs with 3D microstructures (e.g., microchannels and microwells) to confine the solution to a limited volume and control the supply of molecules. Such microstructures are most widely made of polydimethylsiloxane (PDMS). However, bonding PDMS and fluid lipid bilayers in an aqueous solution is difficult due to the poor adhesion between these materials, although bonding PDMS and glass substrate in a dried state is well established. Fluid bilayers are also not suitable for the attachment with other materials due to the lateral mobility of molecules and the instability of the bilayer structures. To avoid these technological limitations, we generated polymeric bilayers that can provide a stable platform for connecting 2D membranes with 3D objects.

To realize a functional surface based on polymeric bilayers, we polymerized phospholipid bilayers having a reactive ethanolamine group at the headgroup (DiynePE; Figure 1). Although DiynePE does not form a stable bilayer structure due to the fact that the occupied area of the headgroup is significantly smaller than the hydrophobic tails,[26] stable bilayers could be formed by mixing DiynePE with a polymerizable phospholipid having a choline headgroup (DiynePC). Mixed DiynePC/DiynePE bilayers could be photopolymerized, resulting in robust polymeric bilayers. Functional molecules were attached onto the surface via the reaction between an amine group and an activated ester. The functionalized surface allowed the model biological membrane to couple with proteins, cells, and microstructures by molecular recognition, extending the model membrane into the vertical direction and enabling its integration with various analytical platforms.

2. MATERIALS AND METHODS

2.1. Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-hexyanoyl-*sn*-glycero-3-phosphocholine (DHPC), 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine (DiynePC), and 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphoethanolamine (DiynePE) were purchased from Avanti Polar Lipids (Alabaster, AL). *N*-(6-Tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TRITC-PE) and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) were purchased from Molecular Probes (Eugene, OR). Albumin from bovine serum (BSA) was purchased from Sigma-Aldrich. Nicotinamide adenine dinucleotide phosphate ($NADP^+$), dithiothreitol (DTT), magnesium chloride, and glucose-6-phosphate dipotassium (G6P) were purchased from Nacalai Tesque (Kyoto, Japan). Glucose-6-phosphate dehydrogenase was purchased from Toyobo (Osaka, Japan). 7-Ethoxyresorufin (7-ER) was purchased from Toronto Research Chemicals (Toronto, Canada). Photoprotected G6P (caged-G6P) was prepared according to the previously reported method.[27] Peptides were synthesized by the Fmoc chemistry using benzotriazolyloxy-tris-pyrrolidino-phosphonium hexafluorophosphate (1 equiv.), hydroxybenzotriazole (1 equiv.), and *N*-methylmorpholin (1.5 equiv.), and fractions purified by semi-preparative RP-HPLC were characterized by the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDITOF MS, Voyager): RGD peptide, YAVTGRGDSPASSC-amide, calcd. 1369.48, obsd. ($[M+H]^+$) 1369.11; DGR peptide, CSSAPSDGRGT-VAY-amide, calcd. 1369.48, obsd. ($[M+H]^+$) 1369.67. Deionized water used in the experiments was ultrapure Milli-Q water (Millipore) with a resistance of 18.2 MΩ cm. It was used for cleaning substrates, preparing buffer solutions (0.01 M sodium phosphate buffer with 0.15 M NaCl, pH 6.6 (PBS) and 0.1 M potassium phosphate buffer, pH 7.4 (KPB)), and all other experiments.

2.2. Substrate Cleaning. Microscopy glass slides and coverslips (Matsunami, Osaka, Japan) were used as substrates for fluorescence microscopy observation. The substrates were cleaned with a commercial detergent solution, 0.5% Hellmanex/water (Hellma,

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Mühlheim, Germany), for 20 min under sonication, rinsed with deionized water, treated in a solution of NH_4OH (28%)/H_2O_2 (30%)/H_2O (0.05:1:5) for 10 min at 65 °C, rinsed extensively with deionized water, and then dried in a vacuum oven for 30 min at 80 °C. Before use, these substrates were further cleaned by the UV/ozone treatment for 20 min (PL16−110, Sen Lights Corporation, Toyonaka, Japan).

2.3. Preparation of Patterned Polymeric Bilayers. A detailed description of the fabrication method of patterned polymeric bilayers is given in previous papers.[23,25,28] Briefly, bilayers composed of monomeric DiynePC and DiynePE were deposited onto substrates from the air/water interface by the Langmuir−Blodgett (LB) and subsequent Langmuir−Schaefer (LS) methods using a Langmuir trough (HBM-AP, Kyowa Interface Science, Asaka, Japan). The temperature of the subphase (deionized water) was controlled at 16 °C by circulating thermostatted water. The surface pressure was controlled at 30 mN/m. Polymerization of DiynePC/DiynePE bilayers was conducted by UV irradiation using a mercury lamp (UVE-502SD, Ushio, Tokyo, Japan) as the light source. The applied UV intensity was typically 10 mW/cm^2 at 254 nm, and the dose was modulated by the illumination time. After UV irradiation, nonpolymerized DiynePC and DiynePE molecules were removed from the substrate surface by immersing in 0.1 M sodium dodecylsulfate (SDS) solution at 30 °C for 30 min and rinsing with deionized water extensively. The polymerized bilayers were stored in deionized water in the dark at 4 °C for the experiments.

2.4. Surface Functionalization of Polymeric Bilayers. For modifying the surface of the DiynePC/DiynePE bilayer with biotin, we applied a conjugate of *N*-hydroxysuccinimide (NHS), polyethylene glycol (PEG) having four monomer units, and biotin (NHS-PEG_4-biotin: Thermo Scientific). NHS-PEG_4-biotin was dissolved in a $NaHCO_3$ buffer (0.1 M, pH 8.4) with the concentration of 1 mg/mL. NHS-PEG_4-biotin solution was applied onto a DiynePC/DiynePE polymeric bilayer sample and incubated for 30 min. After rinsing with deionized water, the sample was immersed in 0.1 M SDS solution at 30 °C for 30 min and extensively rinsed with deionized water. For the attachment of cells, two peptides (the RGD and DGR ptptides) were grafted onto polymeric bilayers. A conjugate of NHS, PEG, and maleiminde (NHS-PEG_4-maleimide: Thermo Scientific) was first applied onto the DiynePC/DiynePE bilayer, and a peptide having a cysteine terminal group was subsequently added to form the thiol-maleimide linkage.

2.5. Preparation of Vesicle Suspensions and Incorporation into Patterned Bilayers. Lipids dissolved in chloroform were mixed in a round-bottom flask, dried under a stream of nitrogen, and subsequently evaporated for at least 4 h in a vacuum desiccator. The dried lipid films were hydrated in PBS (the lipid concentration was 1 mM) overnight. Lipid membranes were dispersed by five freeze/thaw cycles, and the suspension was extruded by using a Liposofast extruder (Avestin, Ottawa, Canada) with 100 nm polycarbonate membrane filter (10 times) and 50 nm polycarbonate filter (15 times). Extruded vesicles were applied onto the surface of the substrate with a patterned polymeric bilayer to form supported planar lipid bilayers (SPBs) in the corrals surrounded by the polymer.[24]

2.6. Fluorescence Microscopy Observation. Fluorescence microscopy observations were performed by using an Olympus BX51WI upright microscope with a 20x water-immersion objective (NA 0.95, Olympus). Three types of filter sets were used: (1) excitation 470−490 nm/emission 510−550 nm (green fluorescence), (2) excitation 540−550 nm/emission 575−625 nm (yellow fluorescence), and (3) excitation 545−580 nm/emission >610 nm (red fluorescence). Fluorescence images were collected with a CCD camera (DP30BW, Olympus) and processed with the MetaMorph program (Molecular Devices, Sunnyvale, CA).

2.7. Cell Adhesion onto the Polymerized Bilayer. Adhesion of cells onto the chemically modified polymeric bilayer surface was studied using mouse fibroblast NIH3T3 cells (RIKEN Cell Bank, Tsukuba, Japan). Cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (ICN Biochemicals, Aurora, OH) in a humidified atmosphere containing 5% CO_2 at 37 °C. Suspended cells were applied onto the surface of the micropatterned bilayer. After sedimentation of the cells, nonadsorbing cells were rinsed by a gentle flushing of the culture medium. Adhesion of the cells onto the surface was monitored by using the Olympus BX51WI upright microscope.

2.8. Activity Assay of Cytochrome P450 in Microcompartments. The activity of human cytochrome P450 (h-CYP1A1) was measured in microcompartments formed between a PDMS (polydimethylsiloxane) chip having regularly positioned microwells (diameter: 100 μm, depth: 50 μm) and a micropatterned bilayer. h-CYP1A1 was expressed in *E. coli* cells together with human P450 reductase (reductase) and purified as membrane fragments. Detailed procedures for the expression and purification are given in the previous literature.[27] The membrane fragments were stained with DiO and applied onto a micropatterned bilayer of polymeric DiynePC/PE with grafted PEG_4-biotin (DHPC (5 mM) was added to enhance the incorporation into the corrals).[29] After rinsing the surface with KPB buffer to remove membrane fragments that were not incorporated into the corrals, a reaction solution having a fluorogenic substrate of h-CYP1A1 (7-ER) was applied onto the surface (dealkylation of 7-ER forms a fluorescent product, resorufin[30]). To photoregulate the initiation of enzymatic reaction, we controlled the supply of NADPH (cofactor of P450) with photoprotected G6P (caged-G6P).[27] The reaction solution contained 0.1 M KPB, 7-ER (1.5 μM), 0.3 mM caged-G6P, 0.4 U/ml G6P dehydrogenase, 3 mM magnesium chloride, 0.1 mM $NADP^+$, and 1 mM DTT. Subsequently, we confined the reaction solution in PDMS microwells by overlaying a PDMS chip onto the patterned bilayer. The surface of PDMS had adsrobed BSA with grafted PEG_4-biotin and streptavidin. PDMS and micropatterned bilayer were attached by the biotin-streptavidin molecular recognition (vide infra). The enzymatic reaction of h-CYP1A1 was triggered by photoactivating caged-G6P with UV illumination (330−385 nm).[27] The conversion of 7-ER into resorufin was observed with the red fluorescence (excitation 545−580 nm, emission >610 nm).

3. RESULTS AND DISCUSSION

3.1. Formation of Polymeric Bilayers from DiynePC and DiynePE. Polymeric bilayers containing DiynePC and DiynePE were generated by depositing mixed monolayers onto a glass substrate by the LB/LS technique and subsequently polymerizing the bilayer.[28] Pure DiynePE did not form a stable polymeric bilayer (we could not observe polymeric bilayers after the UV irradiation). It is presumably due to the imbalance of the hydrophilic headgroup and hydrophobic tails (it is known that phosphatidylethanolamine lipids tend to form inverted structures).[26] Formation of a Langmuir monolayer at the air/water interface was monitored with the surface pressure, and the film was deposited onto a substrate at 30 mN/m. The $\pi-A$ isotherm showed a direct transition from the gas phase into the liquid condensed phase, in agreement with the previous observations of DiynePC monolayers (Supporting Information, Figure S1).[28,31] As the content of DiynePE increased, the isotherm deviated from that of pure DiynePC. For the DiynePE content of 40%, we observed that the monolayer started to collapse above 25 mN/m. Since it is an indication of the film destabilization, we prepared mixed bilayers below this DiynePE content. Micropatterned polymeric bilayers could be obtained by the lithographic UV polymerization (Supporting Information, Figure S2).

3.2. Attachment of Biotin onto the Polymeric Bilayer Surface. We attached biotin with a PEG linker (NHS-PEG_4-biotin) onto the surface of a polymeric bilayer containing DiynePE. Biotin was chosen as a model biological molecule because of its well-known specific and strong binding to streptavidin.[32] The attachment of biotin on the surface was assessed by monitoring the binding of fluorescently labeled

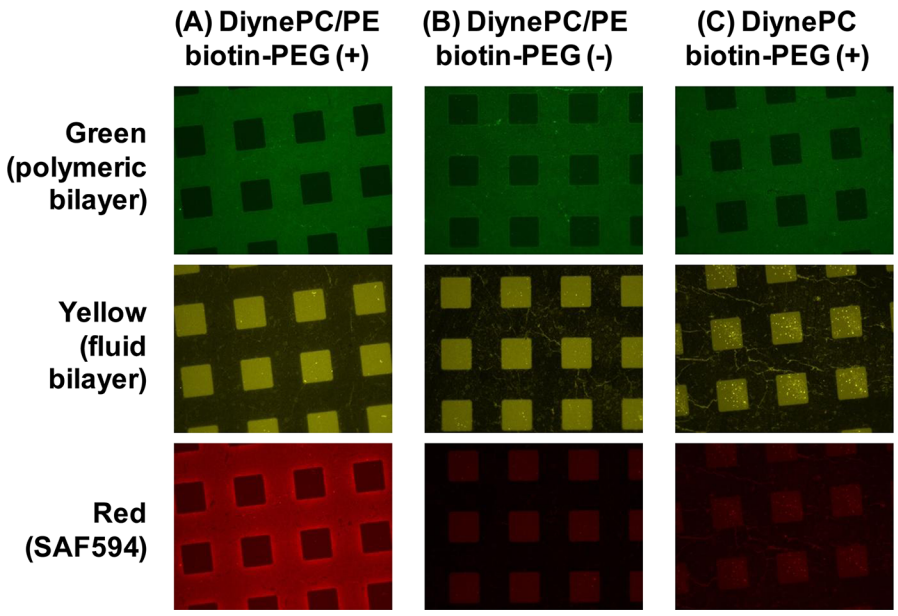

Figure 2. Binding of fluorescently labeled streptavidin (SAF594) onto micropatterned polymeric bilayers with or withiout attached biotin: (A) DiynePC/DiynePE (98.5:1.5) polymeric bilayer functionalized with biotin. (B) DiynePC/DiynePE (98.5:1.5) polymeric bilayer without biotin. (C) DiynePC polymeric bilayer with the functionalization procedure of biotin. Top row: green fluorescence from polymeric bilayer. Middle row: Yellow fluorescence from fluid bilayer (DOPC/TRITC-PE). Bottom row: Red fluorescence from adsorbed SAF594. The size of corrals was 20 μm.

streptavidin (SAF594). Figure 2 summarizes the microscopic observations obtained with polymeric bilayers composed of DiynePC and DiynePE. Fluorescence of SAF594 was observed for a DiynePC/DiynePE bilayer that was functionalized with biotin (left column). SAF594 did not adsorb onto DiynePC/DiynePE bilayer without biotin (central column). It should be noted that the weak red fluorescence observed in the corrals was due to the fluorescence of fluid bilayers (TRITC-PE). If we used a DiynePC bilayer and applied the functionalization procedure, no SAF594 binding was observed, supporting the premise that biotin can be grafted only in the presence of reactive molecules (DiynePE). The negligible attachment of SAF594 on polymeric bilayers without biotin suggests that the surface is resistant toward nonspecific adsorption of proteins.

The density of functional molecules (biotin) on the polymeric bilayer could be modulated by three variables. First, the ratio of DiynePC and DiynePE had a direct influence on the biotin density on the polymeric bilayer. Figure 3A shows the fluorescence intensity of adsorbed SAF594 on polymeric bilayers with varied compositions of DiynePC and DiynePE. The fluorescence intensity increased linearly with the DiynePE content up to 2%. For a higher DiynePE content, the fluorescence intensity was constant, suggesting that the surface of the polymeric bilayer was fully covered with SAF594. This result is reasonable considering the fact that streptavidin occupies a much larger area per molecule compared with phospholipid (streptavidin, 64 nm^2/ molecule; diacetylene phospholipid, 0.66 nm^2/ molecule).[33,34] Since a streptavidin molecule occupies about 100 times larger area compared with a phospholipid molecule and one streptavidin can bind to two biotin molecules on one side, saturation at about 2% of DiynePE is very close to the theoretically predicted value for a streptavidin 2D crystal fully covering the surface.[33] Black squares in Figure 3A are the fluorescence intensities in the corrals (DOPC bilayer without TRITC-PE) which was the same level as the background noise. The fluorescence intensity for pure DiynePC was very close to that of the DOPC bilayer

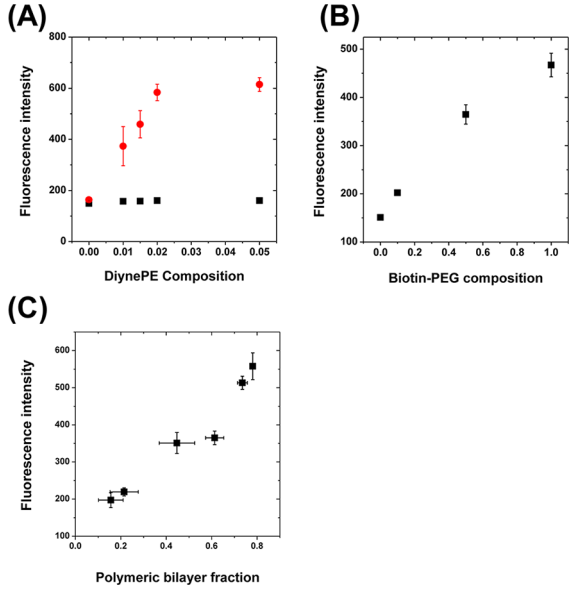

Figure 3. Modulating the surface density of functional moieties: The amount of adsorbed SAF594 on biotin-functionalized polymeric DiynePC/PE bilayers was quantified. The following parameters were varied. (A) Polymeric bilayer compositions (DiynePC/DiynePE): Red circles and black squares are fluorescence intensities of the polymeric region and the corrals (DOPC bilayers), respectively. (B) Composition of grafted molecules (mixtures of NHS-PEG_4-biotin and NHS-PEG_4 was added): The polymeric bilayer had the same composition (DiynePC/DiynePE = 98.5: 1.5). (C) Ratio of polymeric and fluid bilayers: The amount polymeric bilayers (DiynePC/DiynePE = 95: 5) was modulated with the applied UV dose.

(and background), clearly showing that adsorption of SAF594 on polymeric bilayer was negligible. The second variable to modulate the surface density of biotin is the composition of grafted molecules. We mixed NHS-PEG_4-biotin and an amine-reactive molecule having a methyl terminal group (NHS-PEG_4-

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CH_3). Figure 3B shows the effect of compositional variation of grafted molecules. As the composition of NHS-PEG_4-biotin increased, the amount of adsorbed SAF594 increased accordingly. The third variable parameter is the ratio of polymeric and fluid bilayers (Figure 3C). The coverage of the surface with a polymeric bilayer increases by increasing the UV dose applied for the polymerization.[23,25] We generated composite membranes with varied polymer/fluid bilayer ratio. The fraction of fluid bilayer (DOPC bilayer containing 1 mol % of TRITC-PE) was estimated by measuring the fluorescence intensities of TRITC-PE (weak fluorescence arising from diacetylene polymers was subtracted as a background). The polymeric bilayer fraction was determined by subtracting the fluid bilayer fraction from the unity. As shown in Figure 3C, the amount of SAF594 increased linearly with the coverage of polymeric bilayer. It should be noted that we did not observe a saturation of SAF594 coverage like in Figure 3A because each polymeric bilayer domain was saturated with SAF594 (the DiynePE content was 5 mol %) and the number of domains increased with the polymeric bilayer fraction. The results in Figure 3 demonstrate that we can control the surface density of the functional moiety by three methods, resulting in different distribution of the functional moieties on the surface. Changing the compositions of DiynePC/DiynePE or grafted molecules should result in a homogeneous density modulation of functional molecules, whereas changing the polymer/fluid bilayer ratio should modulate the density of domains with clustered functional groups.

3.3. Asymmetric Modification of a Polymeric Bilayer. Biological membranes are generally located in an asymmetric environment, with their two monolayer leaflets facing exterior and interior sides of the membrane and having asymmetric lipid compositions. Asymmetric lipid distribution has been generated in SPBs to mimic the biological membrane.[35,36] For some applications, asymmetric modification of polymeric bilayer should be advantageous. For example, if one applies an SPB to bioassay, one usually assumes that analyte molecules in the solution have access to receptor molecules only in the monolayer facing the aqueous solution (binding to the monolayer facing the substrate should be hindered due to the membrane barrier and the narrow gap between the membrane and the substrate). However, the real situations can be more complex, because molecules may migrate to the opposite leaflet by the diffusion through defects and permeate into the cleft between the substrate and the membrane. Therefore, an asymmetric bilayer having the functional moiety only in the monolayer facing the aqueous phase should be beneficial to avoid the complication caused by the different accessibilities of the two monolayers. To realize a polymeric bilayer with asymmetric compositions, we fabricated hybrid bilayers of pure DiynePC and DiynePC/DiynePE (98.5:1.5) by the successive LB and LS film deposition. By combining two types of monolayers, we could construct four types of polymeric bilayers (Figure 4A). We modified these bilayers with NHS-PEG_4-biotin, and subsequently SAF594 was added. The fluorescence intensities of adsorbed SAF594 are compiled in Figure 4B. Adsorption of SAF594 onto the polymeric bilayer was observed, only if DiynePE was present in the upper monolayer facing the aqueous phase. If DiynePE was present only in the bottom monolayer facing the substrate, no detectable fluorescence of SAF594 was observed (the fluorescence intensity was the same level as the background). Since the distance between the bilayer membrane and the substrate is

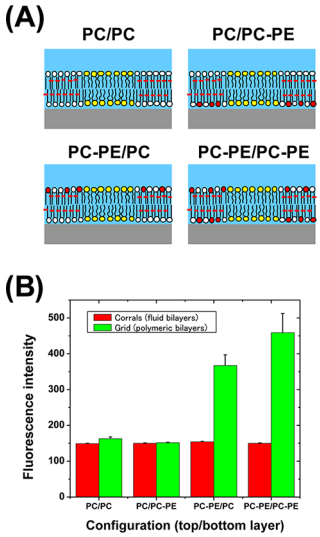

Figure 4. Asymmetric functionalization of polymeric bilayers: Symmetric and asymmetric bilayers were generated by combining DiynePC and DiynePC/DiynePE (98.5: 1.5) monolayers via independent LB and LS deposition. (A) Schematic of four types of bilayers. Red and white circles represent the head groups of DiynePE and DiynePC, respectively. (B) The amount of SAF594 bound onto the surface of four types of polymeric bilayers. The green columns and red columns are fluorescence intensities of SAF594 in the polymeric regions and corrals, respectively. The corrals were filled with DOPC bilayer (without fluorescence marker) to prevent nonspecific binding of SAF594 onto the substrate.

expected to be 1 nm or less,[37,38] it is unlikely that SAF594 molecules could reach biotin moiety in the bottom monolayer. In addition, the corrals were filled with fluid lipid bilayers, blocking the penetration of SAF594 molecules to the opposite side of the membrane and preventing SAF594 from approaching the cleft between bilayer and substrate. The results in Figure 4B also suggest that nonspecific adsorption of SAF594 onto the surface of polymerized DiynePC bilayer was effectively suppressed. The amount of adsorbed SAF594 on a bilayer with DiynePE in both monolayer leaflets was slightly higher compared with that of a bilayer having DiynePE only in the upper monolayer. A possible mechanism of this discrepancy between the homo and hetero bilayers is the difference in the membrane morphologies such as defect densities and surface roughness. However, the drastically different attachment of SAF594 to two types of hetero bilayers (PC/PC-PE and PC-PE/PC in Figure 4) clearly demonstrates that we can control the orientation of functional moieties by selectively incorporating DiynePE in one of the two monolayer leaflets.

3.4. Cell Adhesion onto a Polymeric Bilayer. One important application of the functionalized polymeric bilayer is cell culture. There have been a number of studies that utilized functionalized biomimetic membranes for controlling the adhesion of cells.[39−41] Micropatterned lipid membranes also proved to be a useful tool for studying cellular responses toward external stimuli.[42,43] A micropatterned polymeric bilayer offers the possibility to control the adhesion and growth of cells on the surface by presenting molecules that regulate cell adhesion. As a proof of the concept, we grafted a peptide that contained an arginine-glycine-aspartic acid (RGD) sequence (YAVTGRGDSPASSC), which is a major ligand motif found in extracellular matrices such as fibronectin and laminin.[44] The synthetic peptide had a single cysteine at the N-terminal end,

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which could be covalently coupled to maleimide. We grafted a bifunctional linker molecule (NHS-PEG$_4$-Maleimide) to polymerized DiynePE, and attached the RGD peptide onto the surface. Functionalized polymeric bilayers and fluid bilayers (DOPC/TRITC-PE) were constructed as stripe patterns (width, 100 μm), and mouse NIH3T3 cells were applied onto the membrane (Figure 5). We compared the adsorption

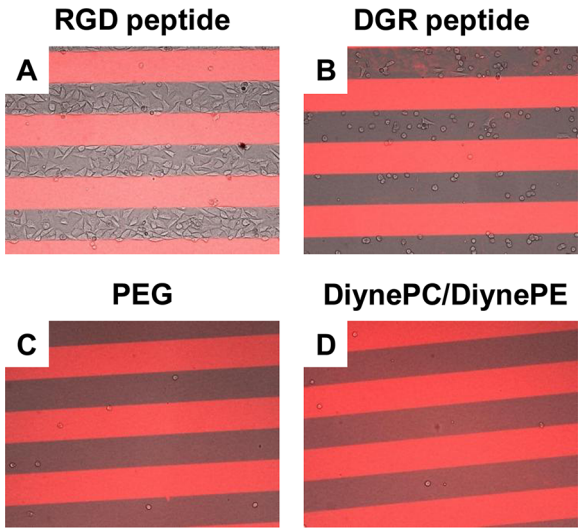

Figure 5. Adhesion of NIH3T3 cells onto polymeric bilayers of DiynePC/DiynePE (98.5:1.5) with different surfaces: (A) RGD-peptide brush; (B) DGR-peptide brush, (C) PEG chains, and (D) no chemical modification (bare surface of a polymeric bilayer). A bright field image was superimposed with the fluorescence image from fluid bilayers of DOPC/TRITC-PE (red). The width of stripes was 100 μm.

of the cells on four different types of polymeric bilayers: (A) modified with the RGD peptide, (B) modified with the DGR peptide (a peptide containing the reverse sequence of RGD (CSSAPSDGRGTVAY)), (C) modified with methyl terminated PEG brushes, and (D) bare DiynePC/DiynePE surface (no modification). NIH3T3 cells were found to adhere only onto the polymeric bilayer having RGD peptide on the surface. Cells did not adhere onto fluid bilayers or polymeric bilayers without a suitable functional moiety on the surface. These results demonstrate the possibility to use a patterned bilayer with chemically functionalized polymeric bilayer as a substrate for the spatially controlled cell culture. Since polymeric bilayers are more robust compared with fluid lipid bilayers, they should be able to support the tensile forces exerted by the cells.[39] On the other hand, fluid bilayers can contain functional molecules that can laterally migrate and give stimuli to the cells. Therefore, the combination of polymeric and fluid lipid bilayer membranes should offer an attractive platform for long-term cell culture of various cell types, including neuron and stem cells.

3.5. Bonding a Three-Dimensional (3D) Microstructure with a Polymeric Bilayer. Another potentially important application of the functionalized polymeric bilayer is to attach model biological membranes to 3D objects such as microfabricated elastomer sheets having microstructures. Complex structures can be easily generated by the soft lithography using erastomeric materials, most notably polydimethylsiloxane (PDMS), and such microstructures have been extensively used for miniaturized analytical devises (lab-on-a-chip).[45,46] By integrating PDMS and model membrane, one should be able to confine membrane-bound and water-soluble molecules in predesigned 2D and 3D compartments. However, combined use of PDMS microstructures and model membranes has been rather limited, except for blotting and stamping of SPBs[47] or using microchannels to generate SPBs.[48−50] It is partially because PDMS and phospholipid membranes cannot be tightly attached. PDMS can be attached onto a glass substrate, if both surfaces are dry, but it is generally difficult to attach PDMS and glass in a wet state. Attaching PDMS and a fluid SPB is even more challenging, because a fluid lipid membrane can be easily disrupted by contact with the hydrophobic surface of PDMS. In the present work, we applied chemically modified polymeric bilayers as a stable and functional molecular platform for bonding a model biological membrane and PDMS.

To demonstrate the membrane-PDMS bonding, we measured the activity of cytochrome P450 monooxygenase (human CYP1A1, h-CYP1A1) in a hybrid compartment comprising a micropatterned model biological membrane and PDMS microwells (Figure 6). The P450 enzyme super family plays a crucial role in the metabolism of drugs and dietary materials in the human body.[51,52] P450 isoforms (57 wild types in human and many single nucleotide polymorphs) are being assayed for identifying the toxicity of drug candidates at an early stage of drug development. Although mammalian P450s are membrane-bound enzymes, some of their substrates and

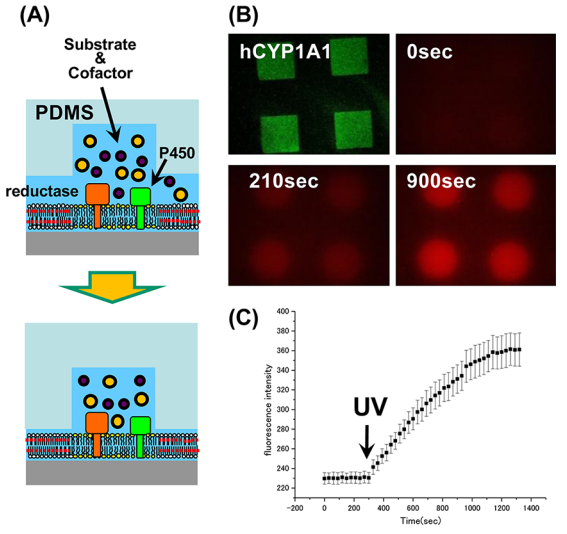

Figure 6. Bonding a PDMS microstructure with a polymeric bilayer: The surfaces of PDMS and the polymeric bilayer were functionalized with biotin and bonded via the biotin- streptavidin binding. (A) Schematic of the P450 assay in microcompartments: P450 (h-CYP1A1) and reductase were incorporated into the membrane and the reaction solution was encapsulated in the microcompartments formed between the membrane and PDMS. (The molecules and microstructures are not drawn in the real scale.) (B) Fluorescence observation of the P450 assay. PDMS microwells (diameter, 100 μm; depth, 50 μm) and a patterned membrane (polymeric bilayer (DiynePC/DiynePE = 95:5) and fluid bilayer corrals (size 100 μm)) were attached. Membrane fragments containing h-CYP1A1 were incorporated into the corrals (observed with DiO) (upper-left). The enzymatic reaction was initiated by UV illumination, and conversion of 7-ER into resorufin was observed (the elapsed time after the UV illumination is given in the images). (C) Time course of the resorufin fluorescence intensity in the microwells. The timing of the UV illumination is indicated with an arrow.

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coenzymes have relatively high water solubilities. Therefore, assaying immobilized P450 on the surface is difficult, if the membrane is in contact with a bulk aqueous solution and the reaction products can diffuse away into the solution. In order to confine the water-soluble molecules to the vicinity of the surface, we bonded a PDMS sheet having microwells (diameter, 100 μm; depth, 50 μm) with a micropatterned membrane that had a functionalized polymeric bilayer and incorporated h-CYP1A1 and reductase (P450 requires electrons provided by reductase for the enzymatic activity), as schematically depicted in Figure 6A. As a simple and biocompatible way to attach PDMS and the membrane, we used the specific binding of biotin and streptavidin. We grafted biotin onto polymeric bilayer and PDMS, and bound them by using streptavidin as a linker. (Streptavidin has four binding sites and can bind with two biotins on polymeric bilayer and two biotins on PDMS, forming a bridge structure.[32]) The surface of the polymeric bilayer was biotinylated by grafting NHS-PEG_4-biotin, whereas the surface of PDMS was functionalized by physically adsorbing BSA functionalized with NHS-PEG_4-biotin. (Adsorption of biotinylated BSA onto PDMS was confirmed by adsorbing fluorescently labeled streptavidin (SAF594) onto the surface (Supporting Information, Figure S3).)

Membrane fragments containing h-CYP1A1 and reductase were stained with DiO and incorporated into the corrals (Figure 6B). (It should be noted that membrane fragments may not be incorporated in the form of a planar membrane, but rather adsorbing onto the surface as vesicles. Since the present study focuses on the confinement of the enzyme in a microspace, this possibility does not change the main result shown in Figure 6.) Nonspecific adsorption of membrane fragments on the functionalized polymeric bilayers was minimum, as determined with the fluorescence intensity of DiO. For measuring the activity of P450, we added a reaction solution containing a fluorogenic substrate, 7-ethoxyresorufin (7-ER), and other necessary ingredients onto the surface of the patterned membrane. The reaction solution also contained a photoprotected derivative of G6P (caged-G6P), which was added to control the initiation of the enzymatic reaction by photoregulating the supply of its cofactor (NADPH) (G6P was needed for the conversion of $NADP^+$ into NADPH).[27] The use of caged-G6P helped to avoid uncontrolled initiation of the enzymatic reaction during the incorporation and confinement of the reaction solutions into microwells, resulting in a significantly heightened accuracy of the assay.[27]

As we overlaid the PDMS sheet onto the micropatterned membrane, the cleft between the two surfaces was kept in an aqueous solution to prevent the dehydration and destruction of lipid bilayer and P450. We attached the polymeric bilayer and PDMS by gently pressing them. (The positions of square-shaped corrals in the membrane and microwells in PDMS were not aligned. We rather chose positions where the corrals and wells were overlapping.) After attaching PDMS and the membrane, the enzymatic reaction was initiated by illuminating the microwells with UV light (Figure 6, panels B and C). Prior to the UV illumination, no fluorescence was observed in the microwells. Upon UV illumination, conversion of 7-ER into resorufin was observed in the microwells as an increase of red-fluorescence. Without surface modification of the polymeric bilayer and PDMS, it was not possible to observe the enzymatic activity in microwells, presumably because the polymeric bilayer and PDMS did not adhere tightly and solutes diffused between compartments (Supporting Information, Figure S4). These results clearly demonstrate that functionalized polymeric bilayers can be applied to attach model membranes and 3D microstructures, enabling to confine both water-soluble and membrane-bound molecules in microcompartments. It should be possible in the future to conduct parallel assays of different types and concentrations of molecules in individual corrals and wells. We can trigger enzymatic reactions in desired positions (even in a single well) by using caged-G6P.[27] On the other hand, repeated assays in a single well with different solutions pose significantly more difficult challenges, because one needs to attach and detach PDMS and the substrate reversibly for solution exchanges.

4. CONCLUSIONS

Mixtures of diacetylene-containing phospholipids having choline and ethanolamine head groups, DiynePC and DiynePE, formed a robust polymeric bilayer, whose surface could be chemically modified with functional molecules. Functionalized polymeric bilayer offers several unique advantages compared with other formats of functionalized surfaces. First, it possesses the same surface structure as the biological membrane and can suppress nonspecific binding of proteins, enhancing the detection of specific binding of molecules to the surface. Second, the polymeric bilayer is robust and can sustain the structure for a much longer period compared with fluid lipid bilayers (we can typically store polymeric bilayers for several months (kept in water at 4 °C) and do not see any significant changes in the structure and surface properties), which is an important feature for biomedical applications, including biosensors, bioassays, and cell culture. By lithographically combining the DiynePC/DiynePE bilayer with a pure DiynePC bilayer, one can generate a micropatterned "all polymeric bilayer" containing both functionalized and nonfunctional regions (Supporting Information: Figure S5). This configuration should maximize the stability of the bilayer membrane and provide a nonfouling platform for long-term bioassays and cell culture. Third, the polymeric bilayer can be combined with natural (and fluid) lipid bilayers in an arbitrary spatial pattern, which enables the membrane bound proteins to be incorporated into the model membrane and their functions to be studied. Finally, as an important extension of the surface functionalization, bonding PDMS and a polymeric bilayer with a molecular glue, allows us to combine two important materials, PDMS microstructures and substrate supported biomimetic membranes. We demonstrated the assay of h-CYP1A1 with its water-soluble substrate in microcompartments between PDMS and micropatterned model membrane. Although we used biotin-streptavidin bonding in the present study, it should be possible to design the structure of molecular glues to realize a molecularly controlled interface between a biomimetic membrane and 3D objects. By combining the unique properties of biomembranes with the control of the liquid flow and confinement, we should be able to create a new generation of biosensing and bioassay platforms.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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