Polymer-Supported Lipid Bilayers on Benzophenone-Modified Substrates

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Solid-supported lipid membranes are important for their roles in fundamental biophysical research as well as in applications such as biosensors. In our study, lipopolymers containing alkyl side chains were synthesized and a mixture of the lipopolymer and free lipids was preorganized at the air−water interface and then transferred to a solid substrate using the Langmuir−Blodgett technique. A photochemical reaction between a substrate-functionalized benzophenone and C−H bonds on the lipopolymer was used to attach the lipopolymers to the substrate. The final assembly of the membrane was completed by vesicle fusion. Langmuir film experiments at the air−water interface indicate tighter molecular packing for the lipopolymers with 28 mol % alkyl side chains than for the ones with 22 mol %. Atomic force microscopy images point to phase separation of lipopolymers on the substrates due to their dewetting from hydrophobic surfaces. However, a mixture of lipopolymers and free lipids formed a smooth film on the same substrate. After the addition of the second lipid layer on the lipopolymer/free lipid layer, the fluorescence images of the polymer-supported bilayer suggested that the distal lipid layer is homogeneous on the micrometer scale. The relaxation of the fluorescent probe lipids was analyzed after application of an electric field to determine their diffusion coefficient; the distal lipid layer was mobile with an average diffusion coefficient of ~0.1 μm^2/s. Moreover, the immobile fraction of the lipids in the distal layer was estimated to be around 15%.

Introduction

The coupling of functional lipid bilayers to solid substrates has been a widely studied topic in recent years.[1−5] The planar configuration of these supported membranes allows easy access to study biological systems. A supported membrane was first used to investigate cellular immune responses;[6] many other studies on fundamental biophysical phenomena have then followed.[7] The possibility of combining artificial membranes and natural membrane proteins on technologically relevant substrates has also been explored to create a novel class of biosensors.[8] Lipid bilayers that are directly supported on glass or quartz are separated from the solid surface by a thin (10−20 Å) lubricating layer of water.[9] This water layer seems to be sufficient to support the lateral mobility of lipids in both layers. However, if one deposits a lipid bilayer containing transmembrane proteins directly onto the solid substrate, the substrate can interact with the proteins due to insufficient separation between the lipids and solids. In turn, this may result in loss of protein lateral mobility.[10] To circumvent this problem and still use phospholipids as the binding matrix for the proteins, a hydrophilic polymer cushion may be inserted between the lipids and the solid substrate. This polymer layer is intended to create enough separation between the membrane and the hard substrate so that large integral membrane proteins may be accommodated in the supported lipid bilayer. Furthermore, the polymer layer can potentially stabilize the whole assembly.

One strategy for creating a polymer-supported lipid bilayer is the spontaneous self-assembly of organic molecules adsorbed from solution onto a variety of solid surfaces. Self-assembly systems based on low-molar-mass organic compounds can be readily extended to assemblies that involve polymers. The two most popular systems are organic disulfides,[11] thiols,[12] and sulfides[13] on gold surfaces and silanes[14,15] on various oxide surfaces.

Polymer systems with different designs have been previously incorporated into the supported bilayers. In one approach, either a telechelic or multifunctional terpolymer system can be used to form tethered lipid monolayers.[16] In another approach, the polymer-supported lipid bilayer is built onto the substrate in a stepwise fashion.[17] This starts with the modification of the solid substrate by attaching an anchoring molecule. The anchor is usually bifunctional— one end to be used during the surface modification and the other one to be available for the chemisorption of the polymers. After the polymer attachment, the lipids will be covalently attached to the polymer layer.

Another viable approach to create polymer-supported lipid bilayers utilizes Langmuir−Blodgett (LB) techniques.[18,19]

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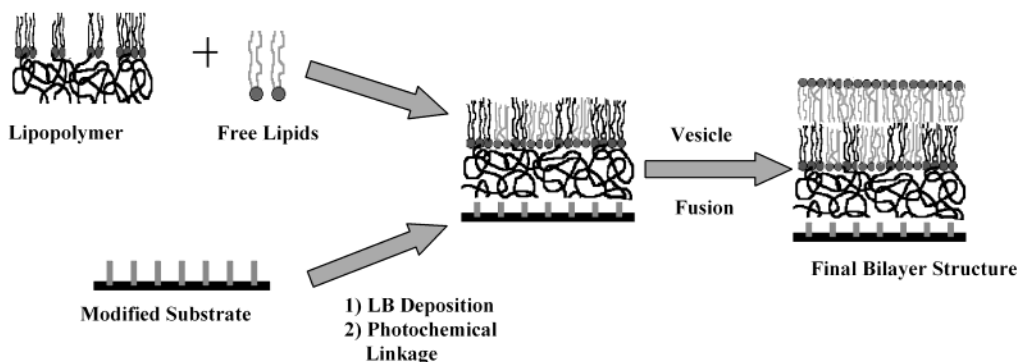
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Figure 1. Overview of the assembly process.

This involves assembling the necessary components at the air−water interface and transferring the preorganized films onto a solid substrate. Compared to self-assembly, LB techniques are relatively time-consuming and require elaborate instrumentation; however, they offer considerable control over the state of organization of the polymers and lipids. The amphiphilic nature of the lipids and properly designed polymers can be exploited to preorganize them at the air−water interface before they are transferred to the substrate. Many factors need to be considered before the incorporation of any transmembrane proteins into the polymer-supported bilayer: polymer thickness, interaction between polymers and lipids, and lateral mobility of the lipid bilayers are of upmost importance to the success of the concept. LB techniques and carefully designed polymer−lipid systems can directly influence the polymer layer thickness and polymer−lipid interaction. These two factors will determine the lateral mobility and the general fluidity of the membrane.

In this study, we have created a tethered polymer-supported lipid monolayer on a solid substrate utilizing LB techniques. Vesicle fusion[20] was used to add the second lipid layer and to complete the assembly (Figure 1). Lipopolymers consisting of a poly(ethyloxazoline-*co*-ethyleneimine) (PEOX-PEI) backbone and alkyl-chain side groups were synthesized. PEOX-PEI was chosen because of its hydrophilicity, nontoxicity, and well-characterized physical properties. The alkyl side chains were covalently attached to the polymer backbone via active ester chemistry, which involves the reaction between *N*-hydroxysuccinimide (NHS) functional groups on the alkyl chain and the secondary amine group provided by the ethyleneimine units on the polymers. The mixture of lipopolymer and free phospholipid was oriented at the air−water interface, with the expectation that the alkyl chains would dispersively interact with the free lipids in the proximal layer to promote the stability of the polymer-supported lipid bilayer. The preorganized lipopolymer/ phospholipid mixture is then transferred onto substrates modified by a molecule containing a benzophenone functional group. The light-induced reaction between the benzophenone and C−H bonds was employed for the covalent attachment of polymers to the substrates. This system was chosen because it is well-known[21] and because benzophenone attaches to C−H bonds in a large number of chemical environments. In addition to the efficiency and the speed of the reaction, the inertness of the photochemical reaction
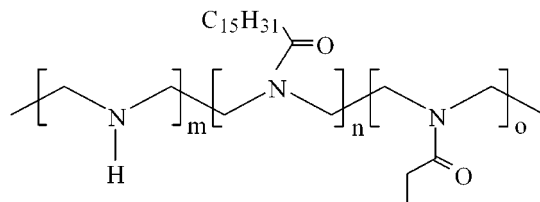

Figure 2. Poly(ethyloxazoline-*co*-ethyleneimine-*co*-pentadecanyl-oxazoline). In lipopolymer #1, $m = 41\%$, $n = 22\%$, $o = 37\%$; in lipopolymer #2, $m = 30\%$, $n = 28\%$, $o = 42\%$.

involving benzophenonone gives us great control over the timing and the extent of the reaction.

This paper describes the assembly of a polymer-supported lipid bilayer architecture on benzophenone-modified substrates. The properties of the polymer cushion and lipid bilayers will be discussed. We will be focusing on polymer thickness, polymer morphology on solid substrates, the swelling behavior during water uptake, the homogeneity of the lipid bilayer, and the diffusion behavior of the lipids in the distal layer.

Experimental Section

Materials. The structure of the lipopolymer, poly(ethyl-oxazoline-*co*-ethyleneimine-*co*-pentadecanyloxazoline), used in this study is shown in Figure 2. The synthesis of the lipopolymer began with commercially available poly(ethyl-oxazoline) (PEOX) with MW of 50 000 (polydispersity 1.9) and MW of 500 000 (polydispersity 3.4); both polymers are from Aldrich Chemical, Milwaukee, WI. The PEOX was first partially hydrolyzed[22] to render a copolymer with both ethyloxazoline and ethyleneimine units. The ethyleneimine "comonomer" units of the polymer were then reacted with pentadecanyl-*N*-hydroxysuccimide, utilizing active ester chemistry,[23] to yield the final lipopolymers. Lipopolymer #1 has side-group alkyl chains on 22% of the polymeric units; lipopolymer #2 has 27.5% alkyl-chain-containing units.

Dimyristoylphosphatidylcholine (DMPC) (Avanti Polar Lipids, Alabaster, AL) was used as the free lipid in the proximal layer. Small unilamellar vesicles (SUVs) composed of L-α-phosphatidylcholine from egg (egg-PC) (Avanti Polar Lipids, Alabaster, AL) with 1 mol % of the fluorescent probe *N*-(Texas Red sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (Texas Red DHPC) (Molecular Probes, Eugene, OR) were made according to the Barenholz procedures.[24] 4-(3′-Chlorodimethylsilyl)-
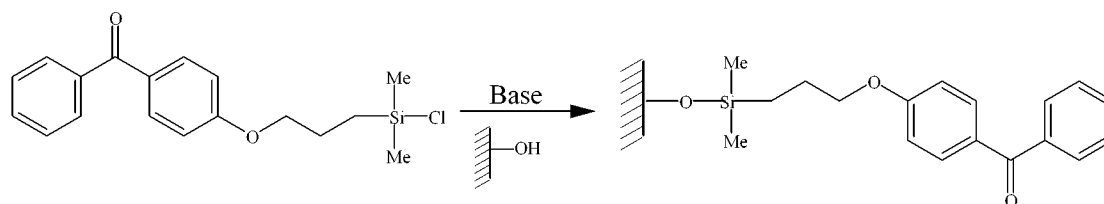
Figure 3. 4-(3′-Chlorodimethylsilyl)propyloxybenzophenone) and its structure after immobilization on the silicon oxide surfaces.

propyloxybenzophenone (see structure in Figure 3) was used to modify the silicon oxide surfaces. This molecule can be prepared by a two-step synthetic route.[25]

Substrate Cleaning. Three types of substrates were used in this study, depending on the experiment. For ellipsometry and atomic force microscopy (AFM), silicon wafers were used. The wafers were treated with Piranha solution (70% sulfuric acid and 30% hydrogen peroxide, v/v) for 3 h. Milli-Q water was used to rinse the substrates extensively before they were dried under nitrogen. BK7 glass slides were used as substrates for surface plasmon spectroscopy. Silver (~50 nm) and silicon dioxide (~30 nm) films were evaporated onto the glass slides before the benzophenone modification. SiO_2 layers were prepared by thermal evaporation of SiO in a partial pressure of O_2 of ca. 10^{-4} Torr. Glass cover slips were used for fluorescence experiments. They were pretreated in 7X cleaning solution (Costa Mesa, CA) with 1:4 dilution in water at 85 °C for 10 min, then extensively rinsed with Milli-Q water, and finally baked at 400 °C for 4 h. At room temperature, there are about eight hydroxyl groups per 100 Å^2 of surface.[26] With the substrates heated to 400 °C, the surface is partially dehydrated; however, it still retains a little more than half of the hydroxyl groups.[26] Furthermore, the dehydration is completely reversible up to 400 °C. The surface will most likely regenerate the −OH groups through exposure to the moisture in the air; hydroxyl groups will thus be available for the substrate modification step.

Substrate Modification. The benzophenone−silane solution with a concentration of 25 g/L was prepared in dry toluene. The immobilization of benzophenone on the SiO_2 surface was carried out in benzophenone−silane solution for ~12 h at room temperature. Freshly distilled triethylamine (Aldrich Chemical, Milwaukee, WI) was added to the solution as a catalyst and acid scavenger (2 mL of triethylamine for every 25 mL of benzophenone−silane solution). The substrates were rinsed extensively with dry toluene after immobilization and dried under vacuum. The immobilization step is shown in Figure 3.

Film Balance Measurements and Langmuir−Blodgett Transfer. A 50 cm × 15 cm symmetric-compression KSV-5000 Langmuir−Blodgett trough (KSV, Helsinki) was used for the isotherm measurements. The subphase was deionized water, purified with the Milli-Q system (Millipore Corp.) and held at a constant temperature of 26 °C to within ±0.2 °C. Solutions of approximately 1 mg/mL of lipopolymer and mixtures of lipopolymers and DMPC in HPLC grade chloroform were prepared for spreading. The molar ratio between the DMPC and the alkyl side chains on the lipopolymer is 9:1; however, since each DMPC molecule has two hydrocarbon tails, the actual molar ratio of the *alkyl*

chains from DMPC and from the lipopolymers is 18:1. The spreading solution was added dropwise onto the water surface using a microsyringe. After a 5−10 min waiting period to allow for solvent evaporation, the barriers were compressed at a constant rate of 10 mm/min. The surface pressure was measured using the Wilhelmy plate method[27] with an experimental error of approximately ±0.1 mN/m. The films of lipopolymer−DMPC mixture were transferred onto benzophenone-modified silicon oxide surfaces during the vertical withdrawal of substrates out of the water subphase. The transfer was carried out at constant pressure of 30 mN/m with transfer rate of 0.5−1 mm/min.

Spin-Casting Polymer Films onto BP-silane Modified Substrates. Spin-casting was used to deposit thick polymer overcoats (>100 nm) of poly(ethyloxazoline) (MW of 50 000 or 500 000) and poly(ethyloxazoline-*co*-ethyleneimine) onto the silicon substrates modified with benzophenone−silane molecules. Typically, a spin speed of 2000 rpm was used for 1 min during the spin-casting (the concentrations of the polymer solutions range from 0.5 to 1 mg/mL). The samples were dried in air and irradiated with UV light (λ > 340 nm) for 5 min. They were then extracted in a Soxhlet apparatus with methanol for at least 10 h to remove polymers not covalently bonded to the substrates.

Photochemical Attachment. After Langmuir−Blodgett transfer, the lipopolymers were covalently linked to the solid substrate under illumination with UV light (λ > 340 nm) for 5 min. Benzophenone triplets are extremely reactive toward CH bonds: however carrying out the reaction in ambient atmosphere should not cause any problems. The light source is a low-pressure mercury UV lamp (200 W, Oriel). The photochemical linkage is based on the reaction between the benzophenone functional group and the C−H bonds in the polymer backbone.[28] Figure 4 depicts the attachment of the benzophenone to a polymer chain leading to the covalent attachment of the chains to the substrate surface.

The thickness of transferred polymer layer after photo-immobilization was measured by ellipsometry and surface plasmon spectroscopy (SPS) with and without the Soxhlet extraction. The numbers obtained from both cases are very similar. The LB deposition only transfers monolayer-thick films onto the substrates, and it is likely that most of the polymers are covalently attached to the surface after immobilization. Soxhlet extraction was not used for the LB films composed of both lipopolymers and lipid, because the organic solvent (methanol) used in Soxhlet extraction may remove lipids that only interact with the lipopolymers through hydrophobic means.

Surface Plasmon Spectroscopy. The glass slide with the silver and silicon dioxide films and lipopolymers on top of
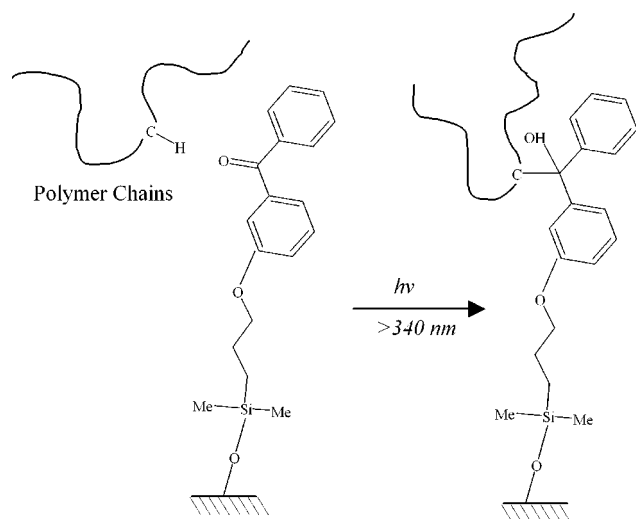
Figure 4. Photochemical reaction involves the benzophenone moiety and a C−H bond.

Figure 5. Water contact angle measurement during the modification process.

it was refractive index matched to a 90° glass prism used as a surface plasmon coupler in the Kretschmann configuration.[29] Resonant excitation of the surface mode, which is very sensitive to the actual interfacial architecture, was then monitored by recording the totally internally reflected light from a He−Ne laser ($\lambda = 633$ nm) as a function of the angle of incidence. An SPS holder with a humidity chamber was used during the swelling experiment. The relative humidity was controlled by placing different concentrated salt solutions in the chamber and allowing the equilibrium water vapor pressure to develop above these solutions. The polymer films were initially dried to 0% relative humidity with potassium hydroxide. Saturated potassium carbonate and ammonium chloride solutions were used to obtain relative humidities of 43% and 79%, respectively. Finally, 100% relative humidity was reached by placing pure deionized water in the humidity chamber. The time-dependent response of the film to various relative humidities was measured by fixing the angle of incidence and recording the change of reflectivity as a function of time. The reflectivity is measured at a fixed angle during the swelling experiment; this angle is usually slightly lower than the resonance angle of the film in ambient humidity. The resonance angle of the film will shift upon changing in the humidity, causing the reflectivity to increase or decrease, depending on the shift direction. The change in reflectivity shows whether the films gained water or lost water. The final thickness is calculated by performing a Fresnel fit on the entire surface plasmon spectrum.

Atomic Force Microscopy. The samples were imaged using a Nanoscope III scanning probe microscope (Digital Instruments, Santa Barbara, CA) in the tapping mode. All roughness data were obtained from sectional analyses.

Bilayer Formation. The second lipid layer was introduced by placing the substrate with lipopolymers and lipids over and in contact with a 50 μL drop of vesicle suspension in a Petri dish for 10 min. The substrate was washed by three injections of 500 μL buffered salt solution (5 mM tris-hydroxymethylaminomethane hydrochloride, pH 8.0, 50 mM NaCl) underneath the substrate. Distilled water was carefully added to the Petri dish, and scratches were made on the supported bilayer with a pair of tweezers. These scratches
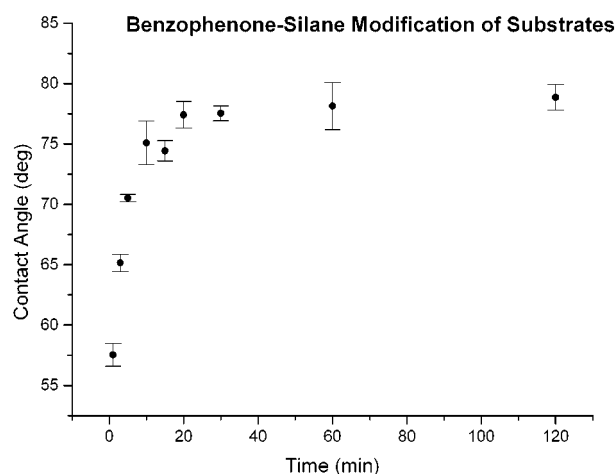
have been shown to function as effective barriers to lipid lateral diffusion.[30]

Fluorescence Experiments. After use of vesicle fusion to add the second lipid layer, which contains the fluorescently labeled lipids, the polymer-supported lipid bilayer was assembled into a sandwich with another cover slip. Membranes were observed in a temperature-controlled room (21 °C) with an epifluorescence microscope (Zeiss, Oberkochen, Germany) using 10× and 40× objectives. For electrophoresis experiments, the cover slip sandwich was mounted in a membrane electrophoresis cell[31] and a field strength of 60 V/cm was applied. Typical currents during the electrophoresis were less than 4 μA, which keeps the resistive heating at a minimum. The electric field was turned off after the formation of an appreciable fluorescence gradient between scratches. Images of the bilayers between the barriers taken during the relaxation process after electrophoresis were analyzed to yield the diffusion coefficient of the lipids in the distal layer of the polymer-supported bilayer.

Results and Discussion

BP−Silane Modification of Substrates. The film thickness of the benzophenone modifier was measured by both ellipsometry and surface plasmon spectroscopy; typical values of 8 ± 2 Å were obtained, assuming a refractive index of $n = 1.5$. The modification process of the substrates was monitored by measuring the advancing water contact angle of the substrate at different times during the process (Figure 5).

Before modification, the substrates were very hydrophilic with negligible advancing contact angles ($\theta \sim 5°$); within the first 5 min in benzophenone−silane solution, however, the contact angle of the substrate reached 70°. The surface modification seems to be completed in the first 1−2 h with the contact angle leveling off around 80°.

Polymer Spin-Coating. To verify the covalent attachment of a polymer layer to the benzophenone-modified surfaces, we spin-coated thick polymer overcoats on the substrates followed by UV irradiation for 10 min. After Soxhlet extraction, we employed ellipsometric measurement to determine the final dry thickness of the polymer layers.
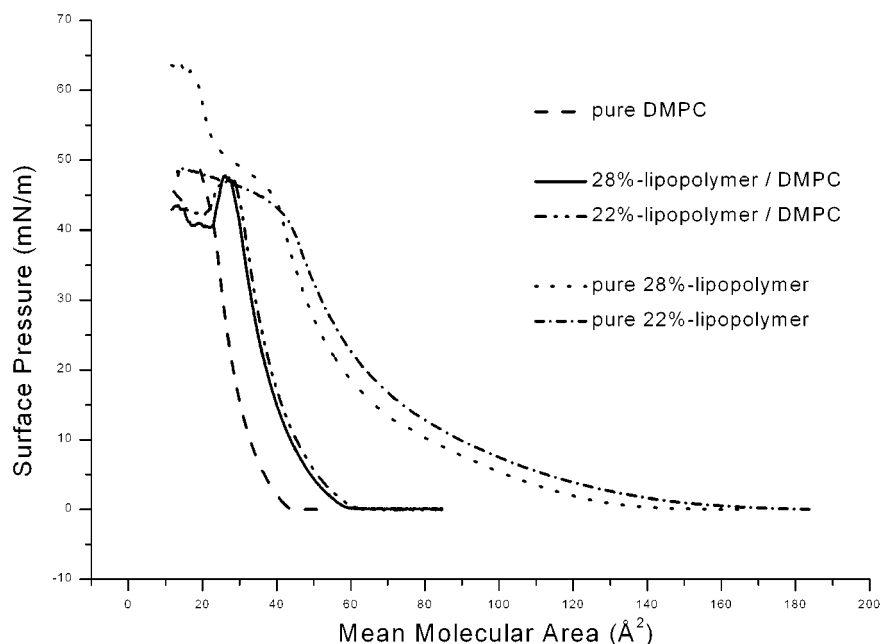
Figure 6. Pressure−area isotherms of the mixtures of lipopolymers and free DMPC lipids, pure lipopolymers, and pure DMPC lipids. The experiments were conducted at 26 °C. The phase transition temperature of DMPC is just below 24 °C.

Several different polymer solutions were prepared for the spin-coating. They are PEOX (MW = 50 000 and MW = 500 000) solutions with concentration of 1 mg/mL in methanol, PEOX (MW = 500 000) with concentration of 0.5 mg/mL, and PEOX-PEI copolymer solution (concentration = 1 mg/mL, MW = 500 000). Previous research has indicated that both the molecular weight of the polymer[32] and the concentration of the polymer solution[22] in spin-coating influence the outcome of the experiment significantly. The ellipsometric results from our study showed that reducing the MW of the polymer and lowering the concentration of the polymer solution both decrease the final dry thickness of the polymer layer. The thickness of the PEOX layer (MW = 500 000, concentration = 1 mg/mL) is 43 Å. The thickness of the film was reduced to 11 Å if the molecular weight of the polymer was changed to 50 000. If the spin-coating solution concentration was decreased from 1 to 0.5 mg/mL, the thickness of the polymer film was 13 Å. Finally, PEOX with MW of 500 000 was partially hydrolyzed (35% hydrolysis) to yield a copolymer of poly-(ethyloxazoline-*co*-ethyleneimine) (PEOX-PEI). PEOX-PEI is more relevant to the lipopolymers used in this study. The thickness of the PEOX−PEI copolymer layer is 61 Å, compared to 43 Å for PEOX homopolymer layer of comparable molecular weight and spin-coating solution concentration. The polymer spin-coating experiment demonstrates that PEOX and PEOX−PEI can both be successfully attached to the silicon oxide surfaces using the photochemical reaction between the polymers and the benzophenone moiety on the substrates.

Langmuir Isotherms. The LB isotherms of the pure lipopolymers #1 and #2 are very similar (Figure 6). The mean molecular area of alkyl side chains on the 28 mol % lipopolymer is slightly smaller than the ones on the 22 mol % lipopolymer. This indicates that lipopolymers with higher lipid content pack tighter at the air−water interface. Lipopolymers were then mixed with pure DMPC lipids; the

isotherms of the mixtures for both lipopolymers as well as the isotherm for pure DMPC are shown in Figure 6. All isotherms in Figure 6 have similar shape and comparable collapse pressure. However, the mean molecular area of the mixtures of lipopolymer/DMPC is about 10 Å² greater than those of pure DMPC molecules; the difference in the area presumably comes from the polymer−polymer interaction in the water subphase.

The mixture preoriented at the air−water interface was transferred to the substrate under constant pressure of 30 mN/m, which is slightly below the collapse pressure. As a result of the film deposition being at relatively high surface pressure, the conformation of the polymers is fairly extended; this may create more separation between lipids and substrate than depositing at low surface pressure where the polymers are not as compressed. The transfer ratio during the Langmuir−Blodgett deposition varied between 0.7 and 0.9.

AFM Images. The polymer film morphology has been shown to be important for the formation of a second lipid bilayer via vesicle fusion. A smooth surface can significantly increase the quality of the fused lipid bilayer. AFM images of the silicon wafer were taken in the height mode beginning prior to treatment and after the photochemical reaction between benzophenone and polymer (see Figure 7). Figure 7a is taken from the clean silicon wafer. The average roughness of the clean silicon wafer is 0.15 nm, which is typical for this type of substrate. Figure 7b indicates that the modification of the solid with benzophenone−silane modifier did not change the surface dramatically; the average roughness increased only slightly to 0.18 nm.

An interesting morphology was observed in Figure 7(c) when pure lipopolymers (28% alkyl-substituted polymer) were grafted to the substrate. A line profile of the image was taken and the results are shown in Figure 8.

It is evident that the features in Figure 7c separate into two distinct heights; the peaks and valleys differ by ap-
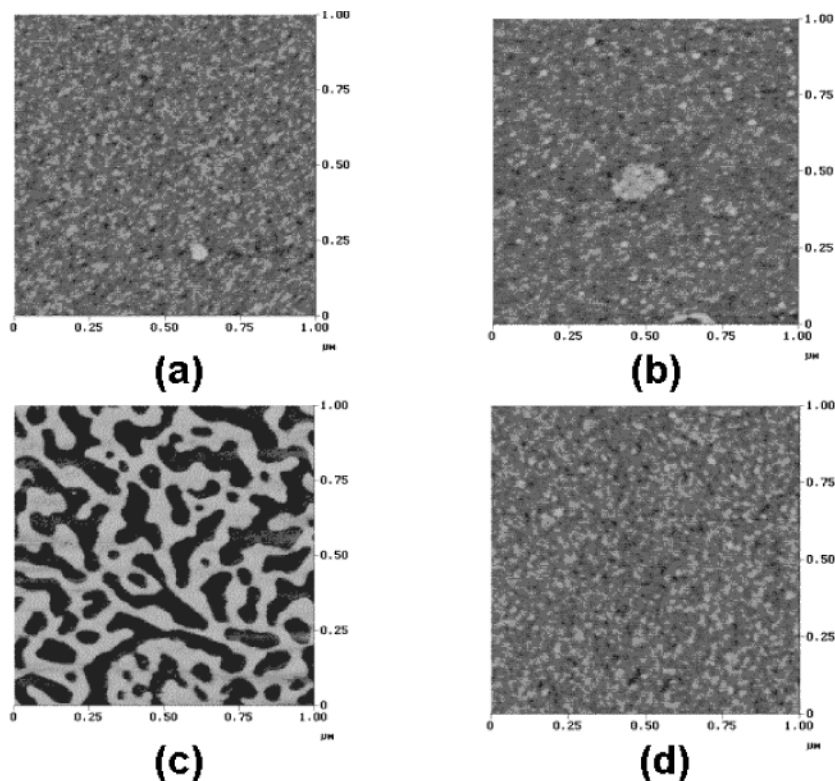
Figure 7. All images were taken in the height mode: (a) clean silicon wafer substrate, $R_a = 0.15$ nm (R_a is the average roughness obtained from the sectional analysis); (b) after benzophenone (BP) modification, $R_a = 0.18$ nm; (c) pure lipopolymer (28%) transferred onto BP-modified silicon wafer by LB deposition and covalently attached to the surface, $R_a = 1.39$ nm; (d) mixture of lipopolymer: free lipids (1:9) on BP-modified silicon wafer, $R_a = 0.15$ nm.

proximately 4 nm. The hydrophilic polymers are very different from the hydrophobic benzophenone-modified substrates in surface energy; this mismatch in surface energy may induce lipopolymers to dewet on the substrate and result in the morphology observed in Figure 7c. When the same lipopolymer was transferred onto the hydrophilic, clean (water contact angle <5°) silicon wafers instead of hydrophobic, benzophenone-modified (water contact angle ∼80°) silicon substrates, a smooth film was obtained when imaged with tapping mode AFM ($R_a = 0.23$ nm, image not shown). The dramatic difference in AFM images for polymers deposited on silicon wafers with different surface energy indicates that the hydrophobicity/hydrophilicity of the substrates is closely related to the film morphology. The film was also examined in the phase mode and a similar morphology was observed (image not shown). The similarity between the images taken in the height and phase modes suggests that the regions of different topography are also very different in their viscoelastic properties. It is plausible that the brighter regions in Figure 7c taken in the height mode are the lipopolymer aggregates on the substrate and the darker regions could simply represent the bare substrate. There exists a certain connectivity in the higher region, which can be important in preventing protein denaturation when transmembrane proteins are eventually incorporated into the polymer-supported lipid bilayer assembly.

When the mixture of lipopolymer (10 mol %) and free lipids (90 mol %) was transferred to the surface by LB deposition, the film observed under the AFM became smooth again in Figure 7d. The average roughness of the mixture on the silicon wafer ($R_a = 0.15$ nm) is identical to that of

the clean substrate. Even though AFM images such as Figure 7c can be used to determine the microphase separation of the film, information on the polymer/lipid arrangement on a molecular level cannot be inferred from AFM images. However, since smooth surfaces (on micrometer scales) have been shown to promote good bilayer formation during vesicle fusion, the mixture of free lipids and lipopolymers was chosen to create the polymer-supported lipid *monolayer* on the solid substrates.

Swelling of Polymer Layers. The swellability of the polymer film is an important property in the assembly; it is directly related to the amount of separation attainable between the lipid bilayer and the solid substrate. The polymer layers were placed under different relative humidities, and surface plasmon spectroscopy was employed to monitor the change in layer thickness as a function of time. The lipopolymers covalently attached to the silicon oxide substrates were first dried above KOH for 1 h. The dry polymers were then placed under relative humidities of 0%, 40%, 80%, and 100%. Figure 9 shows the kinetic measurement of lipopolymer with 28 mol % alkyl side chains covalently linked to the silicon oxide substrate during swelling. Whenever there is an increase in relative humidity, the layer thickness increases sharply within a very short period of time (∼1−2 min). This initial sharp increase is followed by a gradual increase until the layer thickness plateaus. At 0% relative humidity, the lipopolymers have a thickness of 14 Å. The thickness jumps to 21 Å in 40% relative humidity, representing an increase of 50%. In 80% relative humidity, the layer thickness increased to 25 Å. Finally, the polymer layers swell to around 30 Å in 100% relative humidity. This
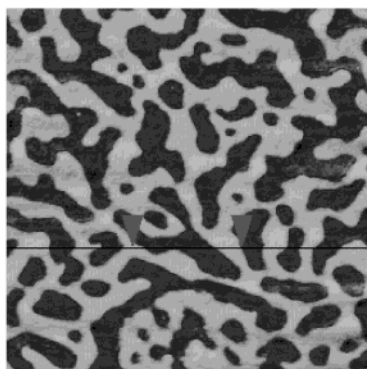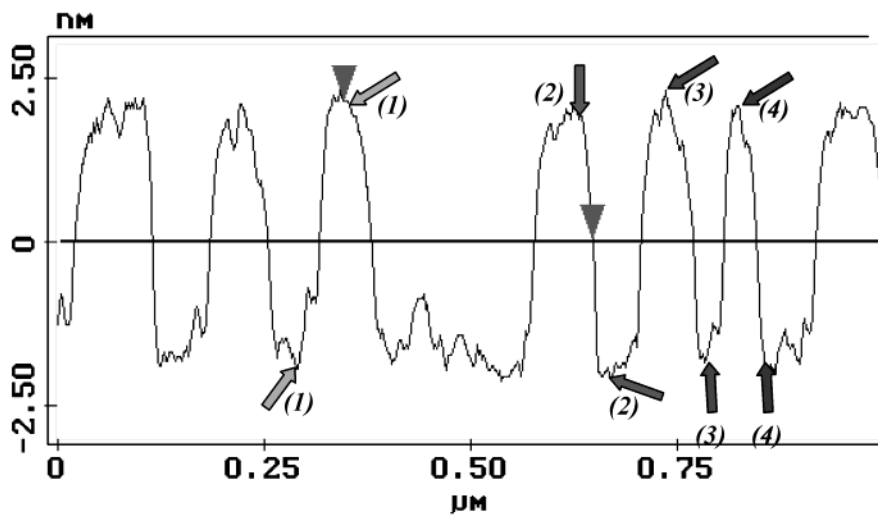
Vertical Dist. (nm)

(1)	4.23
(2)	4.18
(3)	4.11
(4)	4.03

Average = 4.14
Std. Dev.= 0.08

Figure 8. Line profile taken from image c in Figure 7. The vertical distance was measured using the analysis associated with the AFM.
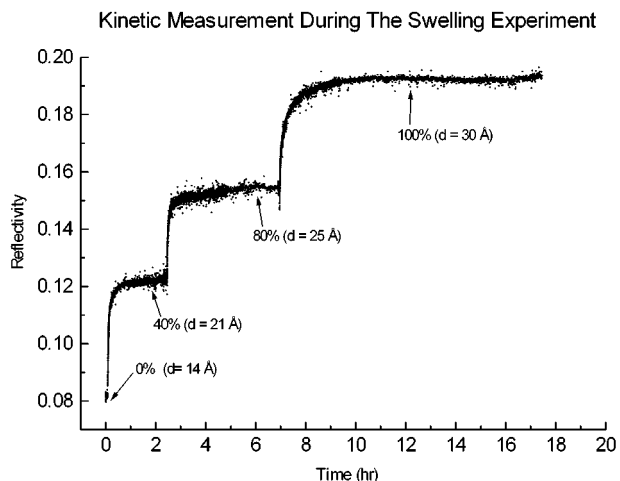

Figure 9. Kinetic measurement during the swelling experiments of the lipopolymer with 28 mol % alkyl side chains covalently linked to the silicon oxide surface.

can be used as a conservative estimate for the polymer layer thickness in water.

Fluorimetric Measurement of the Distal Lipid Layer. A quantitative fluorescence measurement was used to determine the amount of lipids added to the assembly by vesicle fusion. A control experiment was conducted by first performing the vesicle fusion of small unilamellar vesicles (SUVs) made from egg-PC containing 1 mol % Texas Red DHPC on a plain glass cover slip. The membrane on the substrate was dissolved off the surface with a buffer solution (50 mM Tris) containing 0.1% *N,N*-dimethyldodecylamine *N*-oxide (LDAO). The fluorescence intensity of the Texas-Red DHPC was measured at 608 nm with a fluorimeter. The result from this control experiment compares favorably to that obtained from a previous publication.[10]

The same resolubilization/fluorimeter experiments were carried out for the lipid layers assembled on a polymer cushion of the same area. The fluorescence intensity was almost half (~45%) of that obtained from the control system. Since only the distal lipid was made up of egg-PC and the dye molecules in the polymer-supported system, one would expect the fluorescence intensity to be half of that from the bilayers on plain glass, which were deposited entirely by the vesicle fusion. Thus, the experimental result agrees qualitatively with the schematics proposed in Figure 1 of this paper.

Fluorescence Microscopy and Electrophoresis Experiments. Figure 10 shows a number of pictures taken during the electrophoresis experiments as well as from the relaxation of the lipids after the electric field was turned off. The dark
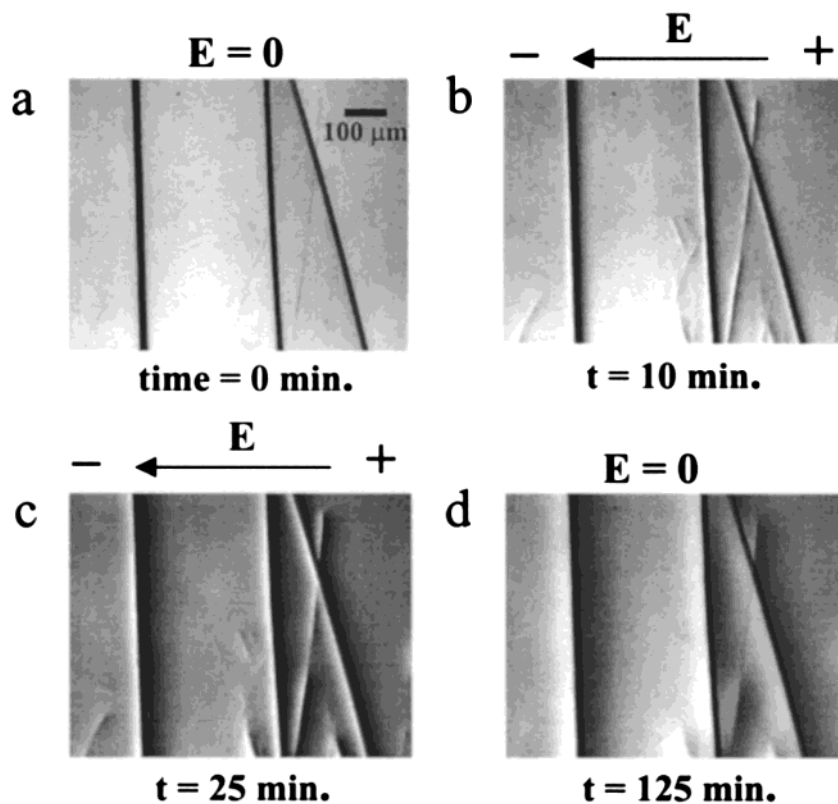
Figure 10. All images were taken for samples on glass cover slips. (a) Epifluorescence image of the lipid in the absence of electric field. The fluorescence is from 1 mol % Texas Red-PE doped in egg-PC. (b, c) Epifluorescence images taken during the electrophoresis experiment (total time of electrophoresis is 25 min) under an applied field of 60 V/cm. The negatively charged component has built-up concentration gradients toward the positive end. (d) Image of the membrane 120 min after the electric field was turned off.

lines are scratches made by a pair of normal tweezers on the bilayer surface. These scratches function as barriers and prevent lipids from moving across. The first image was taken without the application of an electric field; the fluorescence intensity is fairly uniform. This suggests that the second lipid layer (distal layer) was successfully added to the polymer-supported monolayer and that the distal lipid layer appears homogeneous.

The fluorescence image (Figure 11a) of the distal lipid layer before electrophoresis indicates uniform distribution of the lipid molecules. A line profile from the picture was taken (the dashed line), and the fluorescence signal along that line is shown in Figure 11b. Except for the two dips from the scratches, the intensity of the signal stays fairly constant on the micrometer scale.

When an electric field was applied to the lipid bilayer, the negatively charged Texas Red fluorophores should migrate toward the positive end of the field if the lipids are mobile. Several minutes after the beginning of the electrophoresis, depletion of lipids on one side and enrichment of lipids on the other side were evident. After $\sim$30 min, the field was turned off. The image after the electrophoresis shows the relaxation of the lipids and partial recovery of the original state. A diffusion coefficient can be calculated from the images taken during the relaxation by spatial Fourier analysis of the fluorescence intensity distribution. The analysis was loosely based on the method outlined by Stelzle et al.,[33] although the electrophoretic contribution was not included in our fitting. The fit was performed with the Levenberg−Marquardt algorithm. It was determined that the

diffusion coefficient of the distal lipid layer in the polymer-supported bilayer is around 0.1 μm^2/s. Even though this number is 1−1.5 orders of magnitude lower than the diffusion coefficient of lipid bilayers on plain glass (typically ranging from 4 to 8 μm^2/s[34]), this result still shows that some lipid mobility is associated with our system.

In addition to the diffusion coefficient, the lateral mobility of the polymer-supported bilayer can be further characterized by estimating the immobile fraction of the lipids. Under normal conditions, the electric-field-induced molecular reorganization and the thermal energy of the dye molecule result in a smooth distribution of fluorescence intensity between the two barriers after reaching the steady state.[35] The fluorescence signal at the barrier closer to the cathode (negative end) should be negligible, if all lipids in the distal layer are mobile. However, it appears that there is a significant immobile fraction in the polymer-supported lipid layers.

The following formula can be used to approximate the fraction of immobility

immobile fraction =

$$\frac{(\text{intensity after electrophoresis}) - (\text{dark noise})}{(\text{intensity before electrophoresis})}$$

Fluorescence intensity was measured at the scratch closer to the cathode.

The immobile fraction in Figure 10c was calculated using the above equation. The percentage of immobile fraction for lipids in the distal layer for that particular system (the first
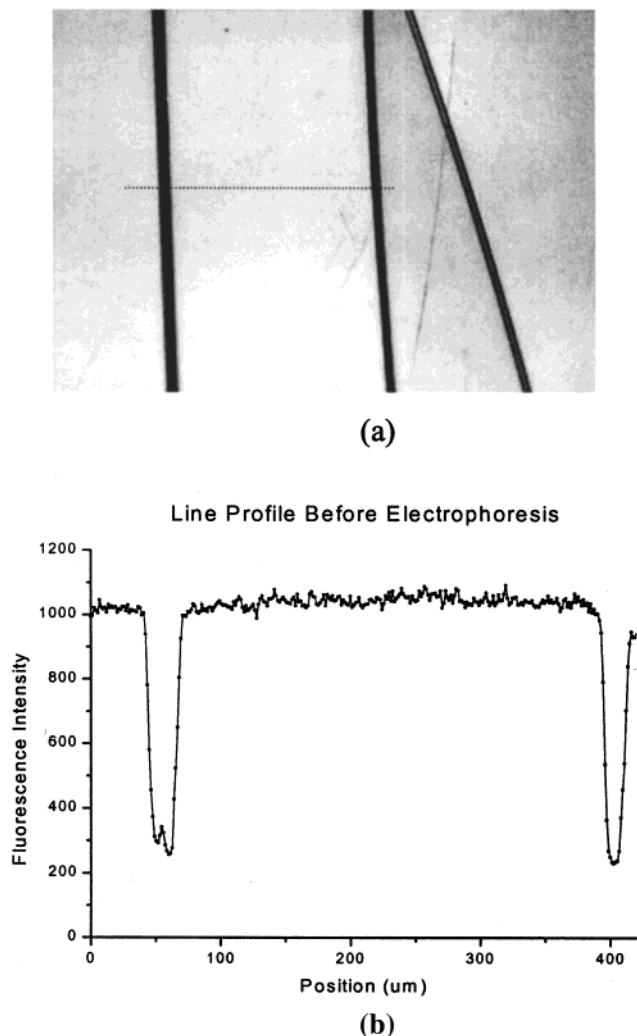
(a)

(b)

Figure 11. (a) Image reproduced from Figure 10a. (b) Line profile taken from the dash line in the image.

lipid layer is composed of 10% lipopolymer and 90% DMPC) was estimated to be ~15%. Naumann et al. reported that the immobile fraction of the lipids in a similar system[36] was 20% from FRAP measurement.[37] It is possible that the alkyl chains on lipopolymer penetrate into the distal layer of the polymer-supported membrane; the interaction between the two lipid layers may introduce obstacles for lipids in the distal layer and result in the significant fraction of immobile lipids.

It should be noted that the fluorescence experiments were conducted on distal lipid layers that are composed of mainly egg-PC, which has a phase transition temperature of −10 °C. However, the asymmetry of the lipid bilayer, with egg-PC in the distal layer and DMPC in the proximal layer, is an important issue to address. The coupling of the two layers may influence the fluidity of the distal layer, potentially due to the rigidity of the underlying DMPC layer at room temperature. This is an area we intend to explore further in future studies.

Summary

We have demonstrated in this paper the successful assembly of a polymer-supported lipid membrane on a solid substrate. The photochemical reaction of benzophenone

proved to be an easy, efficient, and versatile way to covalently link the polymer layer to the solid support. The monolayer of lipopolymer and free lipid mixture oriented at the air−water interface was transferred onto various solid substrates; AFM images showed that the monolayer was quite smooth on the micrometer scale. By introducing a small degree of covalent interaction between lipid layer and the polymer layer, the overall stability of the tethered lipid membrane can be improved. Finally, the distal lipid layer was incorporated successfully via vesicle fusion. To study cellular activities in vitro and to create biosensors, the lipids on solid supports should ideally retain as many of their biological properties as possible. We have attempted to study one of the properties, the fluidity of the membrane. The diffusion coefficient calculated from the relaxation of the probe lipid molecules after electrophoresis can be related to the lipid fluidity. The distal lipid layer of the polymer-supported membrane in our system yielded a diffusion coefficient of 0.1 μm^2/s. The strategy of inserting a hydrophilic polymer cushion between lipid membrane and solid substrates is a feasible approach toward a better understanding of many biophysical phenomena and the creation of a novel class of biosensors.

References and Notes

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