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Microcontact printing for creation of patterned lipid bilayers on tetraethylene glycol self-assembled monolayers

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Abstract

Supported lipid bilayers (SLBs) formed on many different substrates have been widely used in the study of lipid bilayers. However, most SLBs suffer from inhomogeneities due to interactions between the lipid bilayer and the substrate. In order to avoid this problem, we have used microcontact printing to create patterned SLBs on top of ethylene glycol-terminated self-assembled monolayers (SAMs). Glycol-terminated SAMs have previously been shown to resist adsorption of biomolecules including lipid vesicles. In our system, patterned lipid bilayer regions are separated by lipid monolayers, which form over the patterned hexadecanethiol portions of the surface. Furthermore, we demonstrate that α -hemolysin, a large transmembrane protein, inserts preferentially into the lipid bilayer regions of the substrate.

Introduction

Supported lipid bilayers (SLBs) are widely utilized to gain insight into phospholipid bilayers.¹ SLBs are advantageous for the study of phospholipid bilayers due to their ease of formation and increased stability relative to black lipid membranes. Moreover, the formation of the bilayer within close proximity to a solid substrate allows for easy characterization using a broad array of surface techniques. Consequently, SLBs have been used to investigate lipid rafts in phospholipid bilayers,² activity and structure of transmembrane proteins,^{3, 4} and have even shown promise as biosensor platforms.⁵ However, maintaining the 2-dimensional fluidity of the lipid bilayer has been a significant problem in these systems due to interaction of proteins and lipids with the underlying substrate. This problem is further exacerbated in the presence of large transmembrane proteins, which likely denature on the substrate.¹

In order to circumvent interactions between the substrate and the lipid bilayer, substrate modification has been used to increase the thickness of the water layer between the bilayer and the substrate. Common modifications implement large polymers or lipid tethered polymers as a spacer between the bilayer and the surface.^{1, 6} While these systems have shown promise, a more robust method of preventing biomolecule adhesion to surfaces is through use of ethylene glycol-terminated self-assembled monolayers (SAMs).^{7–10} However, spontaneous vesicle rupture atop glycol terminated surfaces does not occur.^{11, 12} In order to induce bilayer formation, Reich *et al.* developed a method to spin coat phospholipid bilayers on to polyethylene glycol polymers, but this method uses harsh organic conditions which are not compatible with most biomolecules.¹³

Another advantage of SLBs is their ability to be patterned on the microscale by a variety of methods. Patterning of SLBs has been accomplished through physical barriers such as

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scratching the substrate,¹⁴ or using a microfluidic channel,^{11, 15} direct microcontact printing of lipid bilayers on to glass,¹⁶ and microcontact displacement of SLBs in preformed bilayers.¹⁷ While these systems successfully create patterned SLBs, they are not easily compatible with cushioned or tethered systems.

Jenkins *et al.*¹⁸ and Wang *et al.*¹⁹ have applied microcontact printing of mercaptoethanol to fabricate patterned bilayer wells. However, the hydroxyl termination of mercaptoethanol mimics a glass surface and absorbs proteins and lipids. SLBs have also been patterned by changing the hydrophobicity of the surface. Howland *et al.* patterned hydrophobic terminated SAMs to create lipid monolayers and lipid bilayers adjacent to one another on a glass substrate.²⁰ This strategy was also recently used to create lipid bilayers on top of gold, a material that traditionally adsorbs intact vesicles.²¹

In this study, we show that a similar strategy to that of Howland *et al.* can be employed to create patterned fluid lipid bilayers over glycol-terminated SAMs. These compartmentalized SLBs incorporate α -hemolysin, a large self-inserting transmembrane protein, preferentially into the lipid bilayer regions.

Experimental

Materials

Tail-labeled NBD-PC (Molecular Probes, Carlsbad, CA) was dissolved in chloroform to a concentration of 1 mg/ml. Azolectin (Sigma-Aldrich, St. Louis, MO) was dissolved in chloroform to a final concentration of 25 mg/ml. α -hemolysin, from *Staphylococcus aureus*, (Sigma-Aldrich, St. Louis, MO) was diluted with nanopure water to a concentration of 0.5 mg/ml. Hexadecanethiol (Alfa Aesar, Ward Hill, MA) was dissolved in ethanol to a final concentration of 10 mM. (1-mercaptopundec-11-yl)tetra(ethyleneglycol) was synthesized as previously described.²² AZ9245 photoresist and 400K developer were purchased from Mays Chemical Company (Indianapolis, IN). All organic solvents were reagent grade, purchased from commercial sources, and used without further purification.

Fabrication of polydimethylsiloxane (PDMS) stamp

The polydimethylsiloxane stamp was produced as previously described.²³ Briefly, a master was fabricated by coating a silicon wafer with AZ9245 photoresist (nominally 4 μ m) and patterned using direct-write photolithographically on a LaserWriter (Microtech, Palermo, Italy) system equipped with a 325 nm He:Cd laser. The master was developed using 1:2 400K developer diluted in water. In order to form the PDMS stamp, Sylgard 182 was mixed at a ratio of 10:1 resin to catalyst, poured over the master, degassed under vacuum and allowed to cure at 50° C for several hours.

Patterned self-assembled monolayers

Glass coverslips (25 mm or 12 mm, No. 1, VWR, Batavia, IL) were cleaned by oxygen plasma using a Diener Femto plasma oxidizer (Diener Electronic, Nagold, Germany) at 100% power for 20 min. The slides were subsequently rinsed with ethanol, deionized water, and ethanol; drying with nitrogen gas in between each rinse. Titanium (50 Å) and gold (150 Å) were deposited using a PVD-75 (Kurt J Lesker, Pittsburgh, PA) electron beam evaporator at 0.1 Å/s. The PDMS stamp was inked with 10 mM hexadecanethiol in ethanol and left in contact with the gold coverslip for 30 s. The slide was rinsed three times with ethanol; drying with nitrogen gas between rinses. The patterned slide was then immediately immersed in a 1 mM ethanolic solution of (1-mercaptopundec-11-yl)tetra(ethyleneglycol) for 12–14 h in the dark. After SAM formation, the slide was rinsed three times with ethanol; drying with nitrogen gas between each rinse.

Unilamellar vesicle formation

Chloroform was removed from the azolectin solution by drying overnight in a vacuum dessicator. For fluorescence experiments, NBD-PC was added to the azolectin solution prior to drying to give a final concentration of 1% by weight. The resulting lipid film was resuspended in 10 mM HEPES/150 mM NaCl (pH 7.4) to give a final lipid concentration of 1 mg/ml. The solution was subjected to five freeze-thaw cycles in a dry ice/acetone bath and passed 24 times through a lipid extruder (Mini-Extruder, Avanti Polar Lipids, Alabaster, AL) with a 100 nm membrane (Whatman). Vesicles were diluted 1:20 in 10 mM HEPES/150 mM NaCl (pH 7.4) for all experiments.

Supported lipid bilayer formation

The diluted vesicles were added to patterned SAMs on gold and allowed to incubate for 3 h. Afterwards, the slides were rinsed 8 times with 10 mM HEPES/150 mM NaCl (pH 7.4). During rinsing care was taken to prevent the slide from going completely dry. The slide was incubated for up to 12 h at room temperature and then rinsed an additional three times.

Fluorescent labeling and incorporation α -hemolysin

The labeling reaction was initiated by combining 1 M sodium bicarbonate (1 μ l), 10 mg/ml rhodamine succinimide ester (1 μ l), and 0.5 mg/ml α -hemolysin (20 μ l). Reaction was allowed to occur at room temperature for one hour, and then quenched by addition of 1.5 M hydroxyl amine (3 μ l). The fluorescently labeled protein was added to a rinsed patterned lipid bilayer, prepared as previously described. The protein was allowed to incubate with the bilayer at room temperature for 12 hours, and the bilayer was then rinsed 8 times with 10 mM HEPES/150 mM NaCl (pH 7.4) buffer.

Fluorescence microscopy

All images were acquired on a Nikon A1 confocal microscope with a photomultiplier tube detector in confocal mode using a 60X Plan Apo oil objective (1.4 NA). NBD-labeled lipids were imaged using a 488 nm laser and a band pass emission filter (500–550 nm). Rhodamine labeled protein was imaged with a 561.5 nm laser and a band pass emission filter (570–620 nm). All images were captured with the pinhole at its largest setting (255.4 μ m).

Fluorescence recovery after photobleaching (FRAP) was carried out by acquiring 4 images, followed by bleaching with the 488 nm laser at 100% power for 6 s. Recovery was monitored by acquiring images every 2 s after bleaching. The half-life of fluorescence recovery was determined by fitting the recovery of fluorescence intensity to an exponential in Origin (Northampton, MA). The diffusion coefficient was then calculated from the following equation:²⁴

$$D=0.224\omega^2/\tau_{1/2} \quad (1)$$

Where ω is the radius of the bleach spot and $\tau_{1/2}$ is the half-life of the fluorescence recovery.

Quartz crystal microbalance (QCM)

QCM experiments were carried out using a QCM25 5 MHz Crystal Oscillator (Stanford Research Systems, Sunnyvale, CA) in conjunction with a QCM200 digital controller. All QCM runs were performed in 10 mM HEPES/150 mM NaCl (pH 7.4) and data was captured at 10 s intervals. QCM electrodes (5 MHz, AT-cut, Stanford Research Systems, Sunnyvale,

CA) were patterned using the same method described for the gold coverslips. Control experiments were carried out by immersing the electrode in a 1 mM solution of the appropriate molecule for 12–14 h. A closed flow cell was used for addition of buffer and the system was allowed to equilibrate for 2 h before lipids were added. Azolectin vesicles were added via syringe and allowed to incubate for 15 min.

Atomic force microscopy (AFM)

All AFM images were obtained on a Multimode VIII with Peak Force Quantitative Nanomechanical property mapping (Bruker, Santa Barbara, CA) using a silicon tip on a silicon nitride cantilever with a nominal spring constant of 0.7 N/m (Scanasyt-Fluid+, Bruker Probes, Camarillo, CA). The images were captured using fluid quantitative nanomechanical mapping mode with a fluid cell (without the o-ring). Gold coverslips (12 mm) were patterned as described previously. Rubber cement (Weldwood, Baltimore, MD) was used to coat a magnetic puck by spinning at 3000 RPMs for 30 s to ensure uniformity. The dried patterned gold coverslip was placed on the puck and allowed to dry for 30 min. The patterned SLB was produced as described above, and the substrate was transferred to the sample holder taking care to keep the surface hydrated. The images were obtained with 2560 points per line and 2560 lines per viewing area at a frequency of 0.195 Hz.

Results and Discussion

Patterned substrate and SLB formation

The procedure for formation of patterned SLBs on glycol-terminated SAMs is outlined in Figure 1. The hexadecanethiol areas of the pattern act as a seeding point for vesicles to attach to the surface. This attachment is followed by rupture of the vesicles which will form lipid monolayers on the alkane-terminated areas of the surface, but will form lipid bilayers on the glycol-terminated areas. The different lipid structures that are formed from the rupture of the vesicles is dependent on the surfaces ability to retain water. Hexadecanethiol monolayers typically have a water contact angle greater than 100°, while glycol-terminated SAMs have a water contact angle of 30°. ^{25, 26} The difference in contact angle demonstrates the ability of the glycol surface to retain water, which should lead to a lipid bilayer structure.

Figure 1 also shows a typical image of the patterned bilayer by fluorescence microscopy, where the lines are the hexadecanethiol portions and the squares are the glycol-terminated portions. The squares consistently have a greater fluorescence intensity than the lines, with an average ratio of 1.5. While this is not the expected 2:1 ratio for a bilayer versus a monolayer, values obtained by others for patterned lipid bilayers on glass next to lipid monolayers on octadecylsilane were also lower than expected. ²⁰

Another key feature observed in the fluorescent images of the patterned SLB is the slightly more intense ring around the edge of the squares. Howland *et. al.* observed a similar ring on lipid bilayers patterned adjacent to lipid monolayers on glass. ²⁰ They showed that the ring is due to the lipids at the interface adopting a gel phase structure as opposed to the liquid disordered phase. ²⁷ Wang *et. al.* observe a similar ring, but depict the ring as a lipid multilayer associated with the transition from lipid monolayer to lipid bilayer. ¹⁹ A key difference found between these two models, is the presence of a moat region, where no lipids are observed, between the monolayer and bilayer regions in the Howland system. ²⁰ Conversely, there is no moat region in the Wang model, which is consistent with our results. Currently we are not able to assess the structure of the ring, however further investigation into this phenomenon are ongoing.

QCM

QCM has been used extensively for following both vesicle absorption and rupture on substrates to form SLBs.²⁸ An increase in mass at the QCM electrode is observed as a decrease in frequency. When the azolectin vesicles were added to a hexadecanethiol-terminated surface the frequency decreased, which indicates that vesicles attached to the electrode (Figure 2a). However, a decrease in frequency only indicates that vesicles have attached to the QCM electrode and is not necessarily indicative of vesicle rupture and lipid bilayer or monolayer formation.²⁹ Also observed in Figure 2a, is an increase in the resistance of the electrode at the surface. This increase indicates that the vesicles have ruptured to form a lipid bilayer or monolayer. Due to this formation, a greater physical barrier resides on top of the electrode, as compared to unruptured vesicles, which decreases the flow of ions to the electrode and gives rise to the larger impedance. The increase in resistance for hexadecanethiol coated electrodes indicates that the azolectin vesicles rupture on the hexadecanethiol surface to form lipid monolayers.³⁰ The data in Figure 2a agrees with similar measurements of vesicle rupture as a change in energy dissipation, which is inversely proportional to resistance.³¹

Conversely, azolectin vesicles do not attach nor rupture on a glycol-terminated QCM electrode. Azolectin vesicles were added to the glycol-terminated QCM electrode, but no significant change in either the frequency or the resistance was observed (Figure 2b). The lack of a signal change confirms that vesicles do not spontaneously adhere on a glycol-terminated SAM.

Finally, The QCM electrode was patterned by microcontact printing and subjected to azolectin vesicles (Figure 2c). The change in frequency decreased and the change in resistance increased, which indicates that vesicles both attached and ruptured on the patterned QCM electrode. Also, the resistance increase in the patterned QCM electrode is greater than the resistance change for the hexadecanethiol-terminated surface. The larger change in resistance is an indicator that the patterned QCM electrode contains a mixture of lipid bilayer and monolayer, since a lipid bilayer would bring about greater impedance than a lipid monolayer.

FRAP

FRAP measures the two-dimensional lipid diffusion coefficient by monitoring the recovery of fluorescence intensity into a spot on the sample that has been photobleached. SLBs have a characteristic two-dimensional diffusion coefficient of approximately $1 \mu\text{m}^2/\text{s}$ on glass.³² Images of the patterned SLBs were acquired before bleaching and the intensities in the pre-bleached regions were averaged to give F_{max} . A spot in the lipid monolayer (line) and lipid bilayer (square) were bleached and monitored simultaneously for recovery. The recovery of fluorescence intensity (F) into the bleached spots was monitored every 2 s. Figure 3 shows plots of F/F_{max} versus time; which were fit to exponentials to give the half-lives for fluorescence recovery ($\tau_{1/2}$) and converted to diffusion coefficients (D) (Equation 1). The line (lipid monolayer) gave a diffusion coefficient of $0.12 \pm 0.05 \mu\text{m}^2/\text{s}$ and the square (lipid bilayer) showed a diffusion coefficient of $0.27 \pm 0.02 \mu\text{m}^2/\text{s}$. The mobility of the lipid bilayer is approximately two times greater than the lipid monolayer which is in good agreement with previous studies.³³ The difference in mobilities also indicates that the bilayers are isolated from one another by the lipid monolayers.

While the comparison between the mobilities of the two different lipid structures is congruent with previous data, the overall mobilities are slower than what is expected for SLBs. However, the fluorescence intensity data in Figure 3 shows a recovery curve that looks similar to typical recovery curves for SLBs. The slower diffusion coefficients may be

due to the size of the bleach spot. The bleach spots for both the line and the square were 6 μm , which was selected due to the relatively small area of the square. Unfortunately due to the small bleach area, $\tau_{1/2}$ is short and our measurement is limited by our capture speed. Nevertheless, the recovery for the square (lipid bilayer) returns to 100% indicating that the lipids do not interact with the gold substrate.

Also, the overall ability to perform FRAP experiments on this system is hampered due to the gold substrate. The gold substrate is able to quench fluorescence and act as a neutral density filter making FRAP experiments on gold substrates rare.³⁴ These issues give rise to long bleach times, which create uncertainty in the value of the diffusion coefficient as well as limit the ability to rapidly monitor fluorescence recovery.³⁵ A prerequisite for the use of Equation 1 is that half-life of fluorescence recovery must be 10 times greater than the bleach time.²⁴ In our system the half-life of fluorescence recovery for the line is 68.7 s, which is barely greater than 10 times the bleach time. Unfortunately the half-life of fluorescence recovery for the square is 30.4 s which does not fulfill the requirement. Therefore, our numbers represent lower limits of diffusion with the actual diffusion coefficients likely being much larger.

Fluorescent images and incorporation of α -hemolysin

α -hemolysin is a membrane associated toxin that inserts into lipid bilayers.³⁶ The incorporation of rhodamine labeled α -hemolysin into our patterned SLBs was monitored fluorescently. Figure 4b shows that the α -hemolysin fluorescence intensity is greater in the squares than the lines. Also, fluorescence intensity of the protein is greatest in the ring structure that surrounds the square (lipid bilayer). The localization of α -hemolysin in the ring structure is consistent with both the Howland and Wang models described previously. While α -hemolysin does not contain a large intermembranous domain, it does possess a stem portion which spans the lipid bilayer and protrudes into the aqueous reservoir.³⁶ This stem portion can be easily accommodated in the fluid lipid bilayer, but not in the lipid monolayer. This is likely due to the ordered nature of the hexadecanethiol monolayer below the lipid monolayer. The fact that α -hemolysin is found predominantly in the squares, indicates lipid bilayer formation, and shows that our patterned lipid bilayers can support transmembrane proteins.

AFM of patterned SLBs

Nanomechanical mapping of SLBs has recently become an area of interest.^{37, 38} Analysis of the force curves leads to a number of different properties of the interacting material that provide an in depth look at the patterned SLB system. The force of the AFM tip is measured as it contacts the lipid bilayer and gives an accurate depiction of the lipid system and contributions from the underlying substrate. Analysis of height does not show a big difference between the lipid monolayer and bilayer portion (Figure 5), which is most likely due to squishing of the bilayer under the force of the tip. However, the lipid bilayer and lipid monolayer regions have significantly different nanomechanical properties.

The observed dissipation is determined by integrating the area between the trace and the retrace of the force curve. The dissipation of the square (lipid bilayer) is less than the dissipation of the lines (lipid monolayer). The dissipation of the system is dependent on loss of energy of the tip as it probes the material. Since the square contains the lipid bilayer, which is more fluid than the lipid monolayer,³³ the material does not absorb as much energy from the tip. This leads to a lower observed dissipation for this region relative to the lipid monolayer. The dissipation measured by AFM is also consistent with the dissipation measured by QCM. As previously mentioned, dissipation is related inversely to the change in resistance measured by the QCM. The resistance change was greater for the patterned

substrate compared to the lipid monolayer substrate, which means the dissipation is less for the lipid monolayer. As clearly seen in Figure 5, the dissipation of the square is less than that of the lines.

The adhesion of the system is determined by integrating the force below the zero point of the force curve. Qualitatively, adhesion measures the force required to pull the tip away from the substrate. Adhesion is less in the square (lipid bilayer) than the lines (lipid monolayer). The difference in adhesion most likely arises from the lipid monolayer being formed on a hexadecanethiol monolayer which is covalently attached to the substrate. Also, in the bilayer region there is a layer of hydration, similar to SLBs on glass,^{39, 40} between the lipid bilayer and the glycol-terminated SAMs that does not exist for the lipid monolayer. The layer of hydration will reduce the adhesion of the lipid bilayer to the substrate.

The DMT modulus is determined from the retracted force curve and the logDMT modulus is the logarithmic fit of the DMT modulus. Essentially, the logDMT modulus is a measure of the elasticity and related to the Young's modulus of a material. The supported lipid bilayer has a greater DMT modulus than the lipid monolayer. This is a result of the ability of the lipid bilayer to bend and distort when it interacts with the tip. The lipid monolayer is coupled to the substrate which causes it to be stiffer and therefore it cannot distort as easily when it interacts with the tip. Analysis of the force-curve for patterned lipid bilayers allows for simple discrimination of a lipid bilayer versus a lipid monolayer.

Conclusion

Formation of supported lipid bilayers on glycol-terminated SAMs does not happen spontaneously. However, we have shown that microcontact printing areas of alkane-terminated SAMs adjacent to glycol-terminated SAMs facilitate vesicle attachment and rupture. This vesicle rupture forms a lipid monolayer over the alkane-terminated areas and induces lipid bilayer formation over the glycol-terminated areas of the surface. We have characterized lipid monolayer and bilayer formation in this system using fluorescence microscopy, FRAP, QCM, and by measuring the quantitative nanomechanical properties of the system. Furthermore, the lipid bilayer portions of the pattern are able to accommodate the transmembrane protein α -hemolysin. Creation of the lipid bilayer on glycol-terminated SAMs keeps unwanted interactions of lipids and proteins with the substrate to a minimum.

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Figure 1. Method for creating patterned SLBs on glycol-terminated SAMs. The fluorescent image shows the fluorescent intensity differences for the two different lipid structures.

Figure 2. QCM frequency (—) and resistance changes (---) for a) hexadecanethiol SAM b) glycol-terminated surface and c) microcontact printed surface.

Figure 3.
The recovery of fluorescence into the bleached spot for the square (—) and the lines (---) in NBD-labeled patterned SLBs.

Figure 4. Fluorescent images for patterned SLBs containing α -hemolysin; (a) NBD labeled lipids, (b) rhodamine labeled α -hemolysin, and (c) overlay.

Figure 5. SPM analysis of a single square (lipid bilayer) in a patterned SLB; height, dissipation, adhesion, and logDMT modulus are shown for the system.