Black lipid membranes stabilized through substrate conjugation to a hydrogel

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Recent research in stabilizing lipid bilayer membranes has been directed toward tethering the membrane to a solid surface or contacting the membrane with a solid support such as a gel. It is also known that the solvent annulus plays an important role in lipid bilayer stability. In this work, the authors set out to stabilize the solvent annulus. Glass substrates with ~ 500 μm apertures were functionalized with 3-methacryloxypropyltrimethoxysilane to allow cross-linking with a surrounding polyethyleneglycol dimethacrylate hydrogel. The hydrogel makes a conformal mold around both the lipid bilayer and the solvent reservoir. Since the hydrogel is covalently conjugated with the glass substrate via vinyl groups, the solvent annulus is prevented from leaving the aperture boundary. Measurements of a membrane created with this approach showed that it remained a stable bilayer with a resistance greater than 1 GΩ for 12 days. Measurements of the ion channel gramicidin A, α-hemolysin, and alamethicin incorporated into these membranes showed the same conductance behavior as conventional membranes. © *2008 American Vacuum Society.*
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I. INTRODUCTION

Artificial planar lipid bilayers are important tools for investigating physical properties of biological membranes[1] and for many applications utilizing channel proteins.[2,3] Freestanding membranes [bilayer lipid membranes or black lipid membranes (BLMs)] are of particular interest due to the accessibility allowed to both sides of the membrane by ions and analytes. Although a freestanding membrane can be created rather simply, the fragility and short lifetime characteristic of these membranes limit their use in technological applications and make long-term channel measurements problematic. To increase membrane stability and longevity, previous workers have introduced preformed hydrogels.[4] We recently demonstrated encapsulation of lipid bilayers *in situ* within a hydrogel,[5] which has been extended by others,[6,7] and shown to extend membrane lifetime to several weeks. Single molecule measurements of protein incorporation into these gel-encapsulated membranes following diffusion through the gel were also shown. Covalent conjugation of the lipid bilayer membrane to the encapsulating hydrogel was also observed to increase preservation of the solvent reservoir and extend membrane lifetime.[8] This work suggested that the resulting enhanced membrane longevity was linked to stabilization of the lipid-containing solvent reservoir[9] surrounding the membrane. Specifically, membranes in which the reservoir depleted more slowly were also coincident with extended lifetimes.

Therefore a strategy for extending membrane lifetime may be to focus on maintenance of the solvent annulus and to prevent it from leaving the aperture boundary at the interface between the bilayer and the plastic partition surface. A typical solvent used is decane, which wets most materials well due to its low surface energy, including the commonly used partition materials Teflon and Delrin. Although hydrogel encapsulation in our recent work stabilized the membrane by conformal molding of the hydrogel to the shape of the solvent annulus, the annulus could still drain through the small space between the hydrogel and Teflon partition. In this work, we investigate a strategy to eliminate this small space entirely by using glass as an alternative partition material and directly bonding the hydrogel to it.

Hydrophilic materials such as glass have been conventionally avoided in freestanding lipid bilayer membrane fabrication. The relatively high surface energy between the hydrophilic glass and nonpolar solvents makes it difficult to spread the solvent/lipid solution, and, once spread, the quality of electrical seal can be poor. In the present work, we have chemically modified glass apertures to lower the sur-

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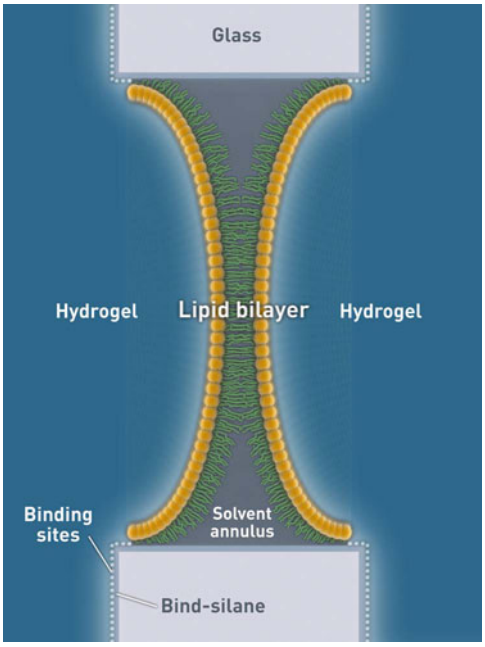

FIG. 1. Schematic illustration of a lipid bilayer membrane formed on a glass substrate. The glass substrate functionalized with bind silane was covalently attached to the hydrogel, trapping the solvent annulus at the orifice boundary.

face energy and create good insulating membrane seals. The modified glass can also participate in the hydrogel cross-linking reaction, enabling its covalent attachment to the encapsulating hydrogel (Fig. 1). This attachment decreases the free space between the gel and minimizes the drainage of solvent from the reservoir surrounding the membrane, prolonging the metastable equilibrium of the lipid bilayer.

II. MATERIALS AND METHODS

A. Materials

All chemicals unless specified were purchased from Sigma-Aldrich. Standard glass cover slides (Fisher Scientific) were machined to create a 500 μm aperture for membrane formation. Bonding wax (McMaster-Carr Inc.) was used to secure the glass cover slide to a large substrate prior to drilling. A 500 μm diamond grinding mandrel (Starlite Industries) was used to create a hole in the glass cover slide. The drilling was done on a MAXNC 15CL2 (Maxnc Inc.) computer numerical control (CNC) drilling device. Chloroform, acetone, methanol, isopropyl alcohol, and a sulfuric acid: hydrogen peroxide mixture were used to clean and activate the glass cover slides for silanization. Ethanol, deionized (DI) water, acetic acid, and methacryloxypropyltrimethoxysilane (Gelest Inc.) were used to create the silanization bath. 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and used to make a lipid solution without further purification. *n*-decane was obtained from MP Biomedicals (Solon, OH). Chambers for membrane formation were purchased from Eastern Scientific (Rockville, MD). PE 90 tubing was used to make agar bridges and obtained from Becton Dickinson (Franklin Lakes, NJ).

To make a hydrogel precursor solution, 7.5% *w/v* poly(ethylene glycol) dimethacrylate (PEG1000-DMA) from Polysciences (Warrington, PA) was included in a buffer solution containing 1*M* KCl buffered with 10 mM tris-HCl at *p*H 8.5. The initiator used to trigger the photopolymerization was 1% Irgacure 2959 photoinitiator obtained from Ciba Specialty Chemicals (Basel, Switzerland). For the photopolymerization reaction, UV Spot Curing System was used (UVP, Upland, CA). Optical observations of the membrane were made with a digital microscope (Prime Entertainment, Marietta, GA). Electrical measurements were made using Axopatch and a Digidata 1322A, with PCLAMP and CLAMPFIT software (Axon Instruments, Foster City, CA) with Ag/AgCl electrodes (World Precision Instruments). Alamethicin (Sigma-Aldrich) was used without further purification and stored in an ethanolic stock solution at 0.1% (*w/v*); for channel measurements, it was added to the measurement chamber at a final concentration of 10 ng/ml. Heptameric α-hemolysin (αHL) in an aqueous solution of 200 mM NaCl and buffered with 100 mM tris-HCl (*p*H 8.2) was added to a final concentration of 17 ng/ml. Gramicidin A (Sigma) was resuspended in ethanol and used for single channel measurements without further purification; it was added to the measurement chamber at a final concentration of 1 ng/ml.

B. Fabrication of aperture on the glass substrate

Although suitable apertures in glass could be made by etching or pipette pulling, the apertures in this work were made by drilling the glass with a diamond mandrel. A glass cover slide was first mounted on a larger glass substrate using bonding wax. This substrate provides a backing during drilling which reduces blow out and cracking of the glass. This is accomplished by placing the large glass substrate on a hot plate at 90 °C. The bonding wax is placed on top of this large substrate and allowed to melt. Next the glass microscope cover slide is placed on the substrate. The substrate, with the glass cover slip, is removed from the hot plate and allowed to cool before drilling. The drilling is done with a 500 μm diamond grinding mandrel. A CNC with a 1/5 horsepower spindle is programed to drill at 2400 rpm with a 0.8 gear ratio. The drilling went to a depth of 0.007 in. with a feed rate of 0.005 in./min. During the drilling the glass was submerged under water for cooling and lubrication. After the drilling was complete, the glass substrate and bonded cover slip were placed on a hot plate at 90 °C. The wax was melted and the cover slide, with hole, was removed. To remove any additional wax from the glass cover slide, it was sonicated in chloroform for 20 min and then rinsed with acetone, methanol, and isopropyl alcohol followed by a DI rinse and drying. Resultant holes were 550–750 μm in diameter.

C. Silanization of the glass substrate

Silanization of the glass slide took place after removing the wax. The glass cover slides were placed in Piranha solution ($3:1(v/v)H_2SO_4:H_2O_2$) at 100 °C for 10 min. This process activates the surface by increasing the hydroxyl concentration allowing for better silanization. The glass slides are then rinsed with DI water and blown dry under nitrogen. A 95:5 (*v*/*v*) ethanol:DI water solution was adjusted to *p*H 5 using acetic acid. 3% (*v*/*v*) of methacryloxypropyltrimethoxysilane was added to the ethanol:water mixture. This mixture was allowed to stir gently for approximately 15 min. The glass slides are then added to the silanization bath and left for 2–3 min. After this, the slides are removed and rinsed briefly with ethanol and placed in an oven at 100 °C for 30 min. The slides are then removed, allowed to cool, and used.

D. Lipid bilayer formation and substrate conjugation

A glass substrate containing a 500 μm diameter aperture was placed between two Teflon chambers. Each chamber was filled with the hydrogel precursor solution described above. A lipid bilayer was formed on the glass aperture using standard techniques.[10] Briefly, a lipid solution consisting of 3% (*w*/*v*) DPhPC in *n*-decane was applied to the orifice and dried in a pretreatment step and then applied again. Upon electrical and optical observation of membrane formation,[11] hydrogel polymerization was triggered by illumination with broad spectrum UV at 400 W for 5–6 min, leading to hydrogel polymerization and substrate conjugation with the hydrogel. Following hydrogel polymerization, channel proteins were added to the top gel surface. Glass lids were then placed on both chambers to prevent evaporation.

E. Electrical and optical measurements

Electrical measurements were made using an Axopatch amplifier with Ag/AgCl electrodes attached. To avoid direct contact of pellet electrodes to the gel, agar bridges were used to connect the electrodes to each chamber. The agar bridges were made using PE90 tubing filled with 2% (*w*/*v*) Agar containing 3*M* KCl. To measure membrane resistance, increasing potentials were applied across the membrane and the resistance was calculated by fitting a line on a current versus voltage plot. Optical observations of the membrane were made using a digital microscope at 200*x* magnification with transmitted light source.

III. RESULTS AND DISCUSSION

In this work, glass was explored as a replacement for a conventional Teflon partition for the ability of its surface to be chemically modified. The glass substrate was covalently functionalized with 3-methacryloxypropyltrimethoxysilane (bind silane) to provide cross-linkable vinyl groups on the glass surface. Bind silane is commonly used with glass slides to attach polyacrylamide gels for gel electrophoresis.[12] Similarly, the PEG-DMA polymers in our hydrogel precursor can covalently bind to the vinyl groups on the silane-coated glass substrates during the process of hydrogel polymerization. Figure 1 depicts glass substrate conjugation to the encapsulating hydrogel, resulting in direct molecular attachment of the hydrogel to the glass substrate. As a result of this attachment, the flow of organic solvent away from the membrane may be greatly reduced, stabilizing the solvent reservoir at the boundary of the orifice.

With the bare, unconjugated glass substrate, it was difficult to conventionally paint a lipid solution across the aperture. In rare cases where a thin solvent plug could be placed to span the aperture, either a lipid bilayer did not result or the membrane failed within several minutes. With no hydrogel present, membranes were able to be formed on glass substrates functionalized with the silane similarly to conventional Teflon films; the resultant bilayers typically had typical resistances in the GΩ range and lifetimes on the order of 1 day. Ion channels were successfully incorporated into these membranes and their measured conductance properties were similar to those in conventionally formed membranes.

For lipid bilayers formed in the presence of the hydrogel precursor solution, following the photoinitiation of polymerization, the GΩ membrane resistance and capacitance (discussed below) were unchanged from their prior values. We were also able to successfully incorporate the previously described ion channels into these membranes (Fig. 2), and the measured channel conductances were similar to those measured prior to polymerization as well as to conventionally formed membranes.[5,13–15]

To probe membrane lifetime, we measured the resistance and capacitance while optically monitoring a membrane formed on a 726 μm diameter hole (Fig. 3). The optical measurements were used to determine the bilayer area (the bilayer boundary is shown by the inner ring) and from this area the bilayer thickness was derived from capacitance measurements.[1] We considered the bilayer to be intact and of high quality if the membrane thickness was determined to be 4–5 nm and the membrane resistance was greater than 1 GΩ. Specific capacitances of the membrane on day 0 and day 11 were 0.386 and 0.389 $\mu F/cm^2$, respectively, which is similar to that reported previously.[16] Figure 4 shows measurements of $>$GΩ resistance for 12 days. From these values we can calculate the specific conductance, 3×10^{-8} to 4.5×10^{-8} mho/cm^2. These values are larger than that previously reported;[17] since the resistance was unchanged following gelation and the resistances measured here are also consistent with previous work with painted bilayers on silanated glass, SiO_2, and Si_3N_4 surfaces,[18–20] it seems that the presence of the gel is not responsible for this. It is possible that the glass surface or its silanation contribute to a leakage current that increases the conductance.

The micrograph in Fig. 3(a) shows the solvent annulus surrounding a membrane on day 0. By day 11 [Fig. 3(b)], the solvent annulus remains almost identically in place and the membrane intact. For lipid membranes spread on conventional Teflon films, the solvent annulus changes relatively quickly and membrane failure occurs within a few hours to 1 day.[8] The stabilization of the solvent annulus by the hydro-

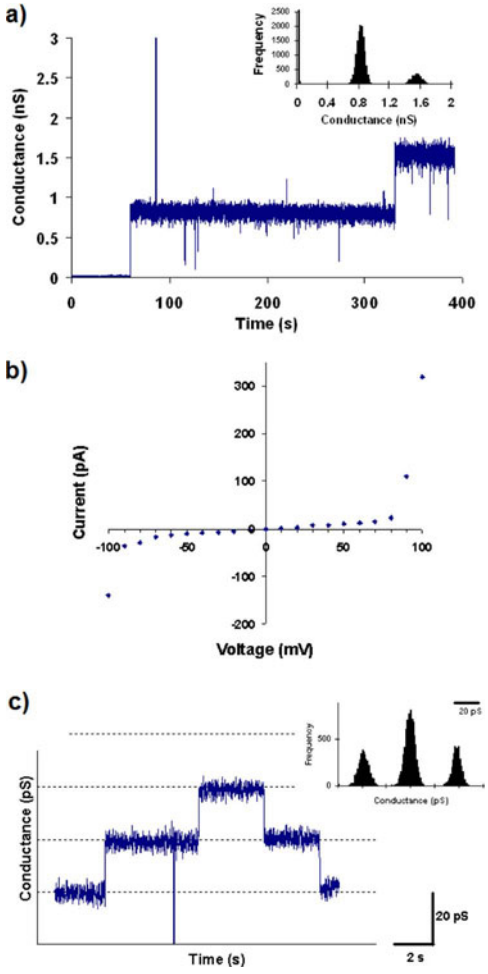

FIG. 2. Ion channels incorporated into membranes formed on the glass substrate functionalized with a cross-linkable silane. Ion channels were recorded in the presence of the encapsulating hydrogel. (a) Conductance trace of αHL channels and the corresponding conductance histogram. The data shown here were acquired with the sampling frequency at 5 kHz and the current trace was filtered digitally at 30 Hz. (b) *I-V* curve of alamethicin pores, showing a typical exponential behavior activated by voltage. Step function was generated from -100 to 100 mV with 10 mV increments. Each data point represents the mean value of currents recorded for 1 s at each holding potential. (c) Measurement of the association/dissociation of gramicidin A dimers. The applied electrical potential was −60 mV. The data were sampled at 10 kHz and further filtered digitally at 30 Hz. Inset: a histogram of these data.

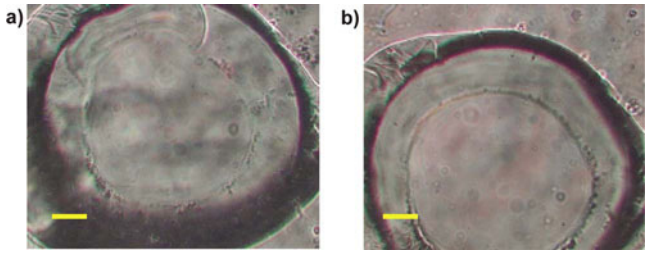

FIG. 3. Transmission microscopy images of a membrane. Each membrane image was taken on (a) day 0 and (b) day 11, respectively. By day 11, the solvent annulus remained unchanged and the membrane intact. Resistances of each membrane were also measured.

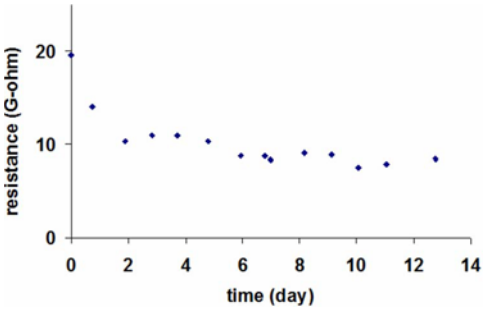

FIG. 4. Resistance of a membrane formed on a gel-conjugated glass substrate as a function of time. This membrane retained resistance in the GΩ range for over 12 days.

gel was so great that the solvent annulus remained unchanged around the aperture boundary even after membrane failure (Fig. 5). When a conventional freestanding membrane surrounded by aqueous solution fails, the solvent annulus immediately returns to the surface, as a result of surface tension. In the current work, we explain the annulus' persistence because, with the presence of the encapsulating hydrogel and the conjugation of the glass to the hydrogel, it has no place to go.

Previously, we suggested that the maintenance of the solvent annulus was the key to extended membrane lifetime.[8] The system described here is physically similar to our previous work in which the membrane was encapsulated by hydrogels[5] (HEM) except for the conjugation of the hydrogel to the substrate. However, the conjugation of the hydrogel to the glass substrate resulted in roughly doubling the lifetime of the HEM.

IV. CONCLUSIONS

In order to achieve increased stability of lipid bilayer membrane, researchers have focused on the stabilization of the bilayer. Previously, we have explored ways to extend membrane lifetime of BLMs by encapsulating them within hydrogels and conjugating them directly to the hydrogels. Observations from this work suggested that the maintenance

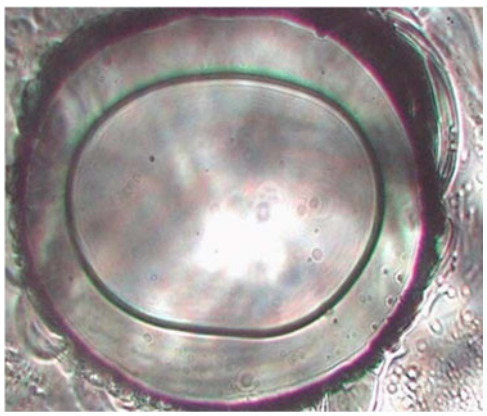

FIG. 5. Solvent annulus was stabilized by hydrogels. The annulus remained in place around the aperture boundary even after the membrane failure due to the attachment of hydrogels to the substrate. There is no membrane present in the inside of the inner ring. The area between the inner ring and aperture boundary contains the solvent annulus.

of the solvent annulus at the membrane/interface boundary was key to increasing membrane stability. This is also in accord with our previous work with automated membrane formation in a microfluidic device through solvent extraction; there, the membrane lifetimes were significantly extended when an unextractable fluorocarbon solvent was included in the lipid mixture, thereby also creating a more stable solvent annulus.[21]

Using this insight we have designed and demonstrated a system which increases membrane lifetime by preserving the solvent annulus. By chemically conjugating a hydrogel to the substrate, two stabilizing conditions are achieved: (1) the mechanical stability of the membrane itself and (2) the trapping of the organic solvent annulus at the orifice boundary. Using this approach, continuous electrical and optical monitoring of membranes was achieved for 12 days.

This system also represents a long-lasting, robust membrane system on glass, a material which has historically been problematic with respect to BLM but is attractive for a number of reasons including robustness, cost, noise performance, functionalization, and compatibility with microfabrication processes. Future work using membranes on glass or other functionalizable substrates such as silicon could extend the annulus stabilization concept by micromachining topographic features or changing the surface chemistry at the aperture boundary to further stabilize the solvent annulus and improve BLM lifetime or ease of formation on these substrates.

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