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Automated Formation of Lipid-Bilayer Membranes in a Microfluidic Device

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ABSTRACT

Although membrane channel proteins are important to drug discovery and hold great promise as engineered nanopore sensing elements, their widespread application to these areas has been limited by difficulties in fabricating planar lipid-bilayer membranes. We present a method for forming these sub-5-nm-thick free-standing structures based on a self-assembly process driven by solvent extraction in a microfluidic channel. This facile automatable process forms high-quality membranes able to host channel proteins measurable at single-molecule conductance resolution.

Membrane channel proteins are of great interest as subjects of basic biophysical study, targets of high-throughput assays for drug discovery and drug safety screening,[1,2] and atomically precise nanopores for engineering applications such as molecular sensing[3,4] and oligonucleotide analysis.[5,6] Microfluidic tools for manipulating and analyzing channel proteins would greatly benefit all of these applications: the miniaturization and single-device integration of microfluidic systems bring with them a vast array of advantages, including portability, decreased analysis times, minimized use of precious reagents and scarce analytes, and automation.[7,8] Realizing these advantages requires a method for fabricating free-standing (as opposed to tethered or supported) planar lipid-bilayer membranes in a microfluidic format. Unfortunately, established methods for fabricating free-standing lipid membranes in the laboratory are operator-intensive and not amenable to automation. We have devised a method for automatically forming free-standing planar lipid bilayers in microfluidic channels based on solvent extraction into poly(dimethylsiloxane) (PDMS). Using this method, we have fabricated high-quality lipid-bilayer membranes and incorporated membrane channel proteins into them, as observed in single-channel conductance data. This is a novel mechanism of lipid-bilayer fabrication; it occurs spontaneously as a function of material properties. The combination of automated membrane formation with microfluidic delivery of channel proteins and analytes to these membranes may result in highly parallel devices dramatically increasing throughput for ion channel drug discovery, screening, and sensing at high speed and low cost.

Most previous microfluidic approaches to ion channel analysis have adapted patch clamp technologies, which require expensive and time-consuming cell culture conditions.[9,10] Furthermore, cell-based systems cannot isolate and control the environment of an ion channel to the extent possible in a cell-free system and cannot be used in ion channel-based molecular sensor applications. In this context, free-standing lipid bilayers are more appealing. In a technique commonly used to fabricate free-standing lipid bilayers in the laboratory, lipids dissolved in an organic solvent are manually "painted" over an orifice in a hydrophobic sheet submerged in an aqueous solution. This lipid solution spontaneously thins to form a bilayer membrane.[11,12]

Previous approaches to microfluidic lipid membrane formation have adapted this technique to microfabricated orifices in microfluidic channels made from poly(methyl methacrylate)[13,14] but suffer from a number of disadvantages. Attention from an operator is necessary to manipulate the device during membrane formation. These systems rely on a three-phase interface of lipid solution, aqueous buffer, and air; air can be problematic in microfluidic systems. Finally, the solvent in the membrane precursor solution forms an annulus around the membrane that limits the degree to which the membrane can be miniaturized.[12]

With the goals of speed, miniaturization, and automation in mind, we have developed a facile system for planar lipid-bilayer formation in a microfluidic format. This system is based on a novel mechanism of membrane formation driven by solvent extraction from a lipid-containing organic droplet.

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This approach takes advantage of the material properties of poly(dimethylsiloxane) (PDMS), a hydrophobic elastomer commonly employed to fabricate microstructures using soft lithography techniques.[15] Organic solvents partition into PDMS,[16] removing them from the fluidic stream. These solvents are therefore typically avoided in PDMS microfluidic applications, and significant work has gone into modifying PDMS microfluidic devices to eliminate solvent extraction.[17,18] Rather than avoid extraction, the devices described here take advantage of this property to drive lipid-bilayer formation.

Existing approaches to planar lipid-bilayer fabrication utilize the removal of solvent to drive the self-assembly of bilayer structures. In the painting method described above, surface forces drive solvent into an annulus around the membrane.[11] In the Montal−Mueller method, solvent is evaporated from a lipid solution spread at a aqueous−air interface prior to deposition of the lipids over an orifice.[19] In the method described here, solvent is extracted from the organic phase by bringing that phase in contact with a selectively absorbent material. Figure 1 shows the principle of operation of lipid-bilayer formation by solvent extraction. It requires first the establishment of a biphasic flow[20,21] in a PDMS channel. A droplet of nonpolar solvent containing dissolved lipid is formed in an aqueous flow stream. The amphiphilic lipids assemble on the aqueous−organic interface (Figure 1a, inset). Because the organic solvent is in contact with the PDMS from which the channel is fabricated, the solvent will partition into the bulk of the device. Lipids, however, do not partition into PDMS.[22] As solvent is extracted from the lipid solution droplet, the droplet becomes smaller, bringing the two lipid-coated aqueous−organic interfaces closer together (Figure 1b). Finally, the solvent is completely extracted, bringing the lipid layers together, forming a bilayer membrane (Figure 1c).

Applied to a microfluidic system, such a method of lipid-bilayer formation would have several key advantages. The device manufacture is simple, requiring only standard soft lithography techniques and no special fabrication steps; this allows for fast, cheap manufacture and rapid prototyping. It relies on only two (organic and aqueous) phases, without requiring an air phase. Because the extraction process forms membranes without surrounding excess solvent, membranes can be miniaturized arbitrarily simply by using smaller channels, allowing for the construction of highly parallel devices. Finally, because the process is driven by material properties rather than by the intervention of an expert operator, it is amenable to automation: an essential quality in applications calling for autonomous sensor operation or high-throughput capability.

Initial experiments demonstrated the feasibility of lipid-bilayer membrane formation via PDMS-mediated solvent extraction in wire-molded channels. Stand-alone channels were fabricated by molding a liquid 10:1 PDMS base/cross-linker mixture (Dow Corning, Midland, MI) around 200-μm-diameter silver wires (Ted Pella, Inc., Redding, CA) and curing at 60 °C for 2 h. Plastic cylinders (cut from the body of a syringe, approximately 1.5 cm diameter and 3 cm long)

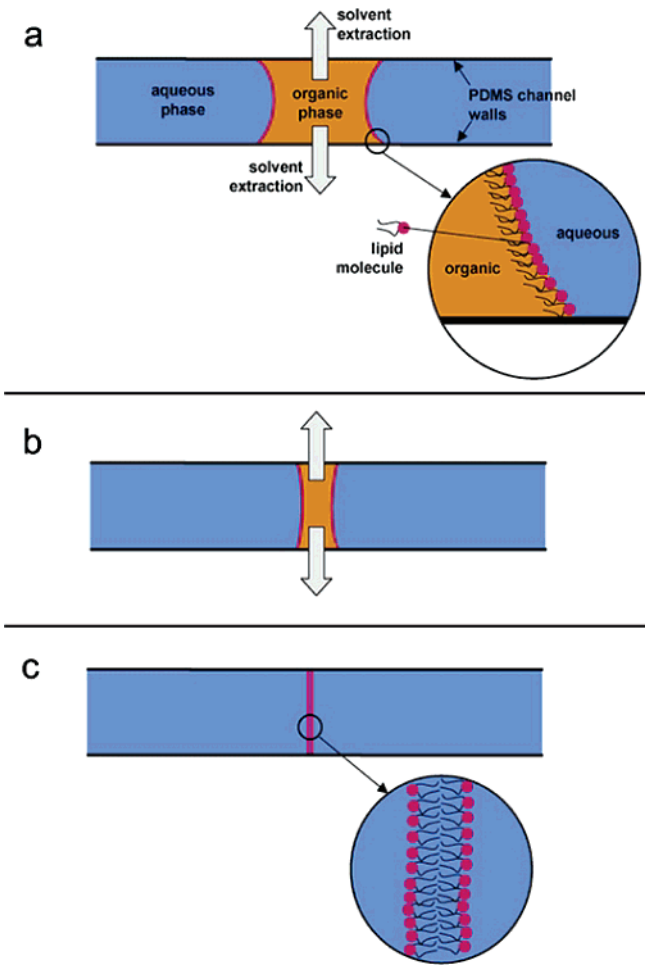

Figure 1. Mechanism for lipid-bilayer formation by microfluidic solvent extraction. (a) A droplet of organic solvent with dissolved lipid is formed in an aqueous stream of fluid. Lipids are organized on the hydrophobic−hydrophilic interface (inset). (b) As solvent enters the PDMS, the two interfaces approach one another. (c) Finally, only the lipid layers are left behind, forming a bilayer membrane.

were incorporated into the PDMS block in a second curing step. Following curing, wires were removed, leaving circular pores 1 cm long and 235 μm in diameter. The plastic cylinders defined a boundary isolating the top and bottom of the channel; when the entire PDMS block was submerged in buffer, the interior of the cylinder was in contact with the top of the channel while the bulk of the fluid was in contact with the bottom of the channel. A cross-sectional diagram of this device is shown in Figure 2a.

The entire PDMS block containing the channel was submerged in aqueous buffer (1 M KCl, 5 mM HEPES, pH 7.0). One Ag/AgCl electrode (World Precision Instrument, Inc., Sarasota, FL) was placed in the reservoir and another was placed within the cylinder, such that there was one electrode on either side of the channel. The electrodes were connected to a home-built transimpedance amplifier,[23] which in turn was connected to a computerized data acquisition system (LabVIEW, National Instruments, Austin, TX). During the experiment, the channel was observed side-on using a stereomicroscope at 10× magnification (Nikon Model SMZ800, Kawasaki, Kanagawa, Japan).

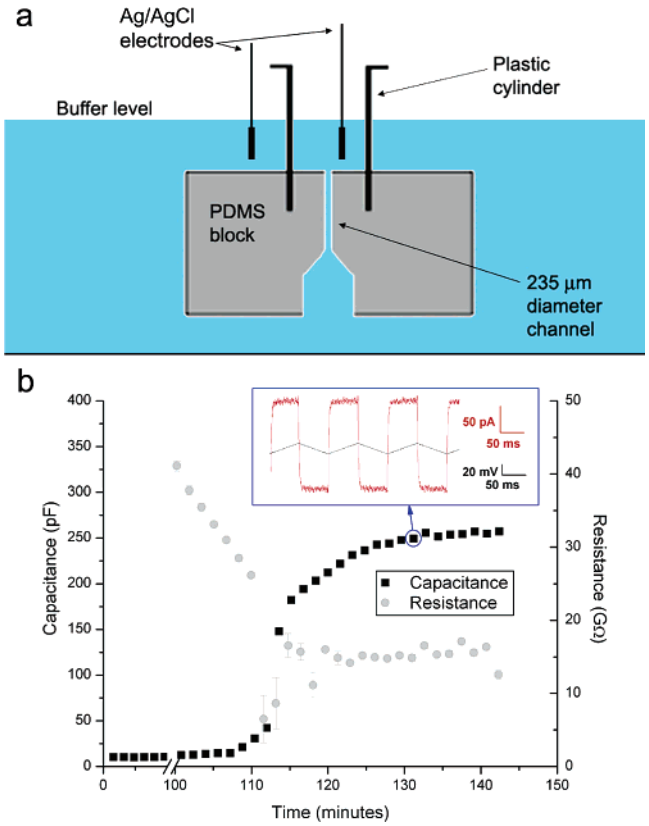

Figure 2. (a) Cross-sectional view of the apparatus for performing PDMS extraction in a wire-molded channel. A plastic cylinder is molded into the PDMS block to provide electrical isolation between the two sides of the channel when the apparatus is submerged in buffer. A droplet of organic lipid solution is injected into the 235-μm-diameter chamber to allow for solvent extraction into the PDMS and membrane formation. (b) Electrical properties of a membrane formed in this 235-μm-diameter channel during solvent extraction. Capacitance (black squares) begins at a constant low background level, with high resistance. Eventually, the capacitance jumps to a level consistent with a lipid bilayer. Resistance (grey circles) stabilizes at ~15 GΩ upon membrane formation: an excellent membrane seal. Inset: The voltage protocol used to measure a single point on the capacitance plot. A triangle-wave voltage signal (black) is applied; the current signal measured across the capacitive membrane is a square wave (red). The data shown are processed with a 1 kHz low-pass filter and correspond to a membrane capacitance of about 250 pF, or a membrane thickness of 4.1 nm. Error bars on the resistance data represent the standard error of the slope of a least-squares straight line fit to a current/voltage curve. Errors on each capacitance point (the standard deviation of the mean for 2000 points from a capacitance trace as shown in the inset) are within the size of the displayed points.

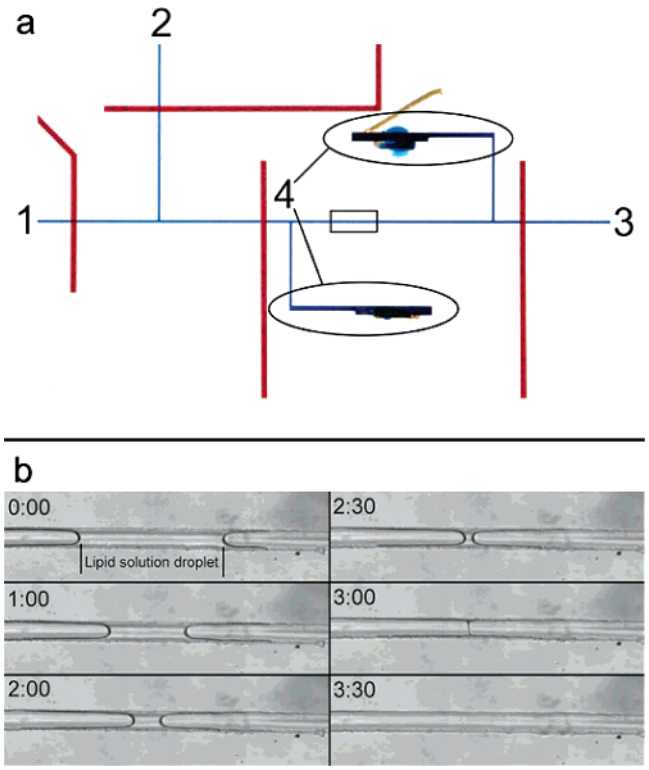

Figure 3. Membrane formation in a microfluidic channel. (a) A photograph of a microfluidic device used for lipid-bilayer membrane formation by solvent extraction. The channels have been filled with food dye for the purposes of illustration. The device consists of two layers: a fluidic layer (blue) and a pneumatically controlled actuation layer forming valves (red). In addition to these valves, this image shows the separate input channels for aqueous (1) and organic (2) phases, the common fluidic outlet (3), and the side channels with integrated Ag/AgCl electrodes (4). Fluidic channels are 100 μm wide; actuation channels are 200 μm wide. The box indicates the region shown in the micrographs in Figure 3b. (b) A sequence of images showing solvent extraction from a lipid solution droplet in a microfluidic channel (channel width of 100 μm). All times are in minutes:seconds. Although no feature within the channel is visible in the last frame, electrical measurements indicated the presence of a bilayer membrane (see the text).

A droplet of organic lipid solution consisting of 0.025% (w/v) diphytanoylphosphatidylcholine (DPhPC, Avanti Polar Lipids, Alabaster, AL) and 50 ppm (v/v) perflurooctane (Sigma) in 1:1 *n*-decane/squalene (both from Sigma) was injected into the top of the microchannel using a syringe attached to a fine (~200 μm diameter) needle. A volume of approximately 20 nL was dispensed. The needle was then withdrawn.

The resistance and capacitance of the channel were monitored continuously during the course of the solvent extraction. Resistance was measured by measuring membrane current at a constant voltage for 10 different voltage points and least-squares fitting a line to this current/voltage curve. Capacitance was calculated from the capacitive current measured resulting from an applied ramp voltage signal, as shown in the inset of Figure 2b. Immediately following deposition of a lipid solution droplet in the channel, the current through the channel dropped to an immeasurable level, indicating that the nonconductive organic solution disrupted electrical connectivity between the two channel halves, completely spanning the channel width. Microscopic observation of the channel showed that the droplet began to shrink almost immediately as the organic phase was absorbed into PDMS (this process is shown for the microfluidic channels in Figure 3b). Figure 2b shows a plot of conductance and capacitance versus time for a typical droplet. As solvent is extracted from the droplet, the resistance remains high and the capacitance remains at a constant background level for about 110 min, at which point the capacitance increases rapidly and resistance drops rapidly. After an

additional 10 min, capacitance and resistance stabilize. This corresponds to the coming together of the two organic/aqueous interfaces and the formation of a lipid bilayer. This interpretation is supported by the fact that capacitance stabilized at about 250 pF. The capacitance of a membrane can be modeled simply as $C = \epsilon_0 \kappa A/d$, where ϵ_0 is the permittivity of free space (8.85×10^{-12} F/m), κ is the dielectric constant of the membrane material, A is the area of the membrane, and d is the thickness of the membrane.[23] On the basis of a lipid dielectric constant of 2.7 [24] and the measured channel diameter of 235 μm, we can conclude that a 250 pF membrane has a thickness of 4.1 nm, consistent with a molecular lipid bilayer. This membrane had a stable resistance of 15 GΩ , indicating an excellent seal.

To adapt this system to a more flexible platform, we designed and fabricated microfluidic devices for lipid-bilayer membrane formation. Devices were fabricated using standard PDMS molding techniques[15] against masters of lithographically patterned photoresist on silicon wafers. The devices consisted of two layers of channels: a fluidic layer and an actuation layer, which served as a valve system on the fluidic layer and was connected to a source of pressurized nitrogen via computer-controlled solenoid valves (Lee Company, Westbrook, CT). Fluidic and actuation layers were separated by a thin membrane of PDMS, as described by Unger et al.[25] To allow for proper valve function, fluidic channels were fabricated with a semi-elliptical cross section.[26] This channel shape was generated on the molds by using an aggressive developer (MF-322, Rohm and Haas), which created photoresist features with a curved rather than rectilinear cross section. On the molds, the fluidic channels had a major axis of 100 μm and a minor axis of $\sim$70 μm, as measured by profilometry (using a Dektak 6 profilometer from Veeco Instruments, Woodbury, NY). Microfluidic devices also incorporated integrated Ag/AgCl electrodes. These electrodes were fabricated by dipping the tips of 200-μm-diameter gold wires into a polymer-based Ag/AgCl ink (Ercon, Inc., Wareham, MA), allowing the ink to dry at 60 °C, and then modifying the remaining bare gold surfaces with 1-mercaptoundecanol (Sigma-Aldrich, St. Louis, MO) by soaking overnight in a 1 mM ethanol solution. Modifying the gold surface to display hydroxyl groups allowed for good bonding to PDMS as it cured. Device layers were molded separately, cured for 2 h each at 60 °C, and bonded following oxygen plasma treatment (150 W, 500 mTorr, 18 s).

Figure 3a is a photograph of a microfluidic membrane formation device. The inlet streams (one each for the aqueous and organic phases) come together at a junction at which lipid solution droplets are formed. Each inlet stream is controlled by a pneumatically actuated valve. Upstream of the inlet junction, there are two additional valves that isolate the region of the device in which membranes are formed. This region is also flanked, interior to the isolating valves, by two blind side channels that lead to silver/silver chloride electrodes. These channels can be filled with buffer prior to experiments by pressurized outgas priming.[27] This allows electrical conductivity to the electrodes while preventing flow into the side channels.

The compositions of organic and aqueous phases were as described for the stand-alone microchannel experiments above. Throughout all of the microfluidic experiments, the device rested on the stage of an inverted microscope (Leica model Leitz DMIRB, Wetzlar, Germany), and it was observed in brightfield mode.

The entire device was filled with aqueous buffer (as above), and organic lipid solution was injected into the device so as to form a droplet of approximately 2 nL in the aqueous flow stream. This was accomplished by driving each stream with a syringe pump (from KD Scientific, Holliston, MA, fitted with 50 μL syringes from Hamilton, Reno, Nevada) at 15−45 μL/h and actuating the input control valves. Pressure was then applied to the aqueous stream in order to push the droplet downstream to the membrane formation region of the device, between the electrodes. The valve immediately upstream of this region was closed, and the solvent extraction process began. Immediately prior to the completion of the extraction process, a valve downstream from the lipid solution droplet was closed, isolating the bilayer formation region from the rest of the device. The Ag/AgCl electrodes were attached to the same amplifier and computer data acquisition system described above.

The process of solvent extraction from a lipid solution droplet is shown in Figure 3b. In this case, complete extraction occurred in about 3.5 min. Typical extraction times in the range of 3−10 min were observed, depending on the size of the lipid solution droplet. A mean membrane capacitance of 17 ± 1.7 pF was measured. Using the method described above, based on a 3500 μm^2 channel cross-sectional area (measured from the mold by profilometry as described above), the calculated thickness of the membranes in the microfluidic channels was 4.8 ± 0.49 nm, consistent with bilayer thickness. Detailed current/voltage curves consistently gave resistance values in excess of 70 GΩ . Channels were reusable immediately: membranes could be formed repeatedly in the same channel with no intervening cleaning step. In a typical experimental session, at least five membranes were formed consecutively in a device. Further, devices could be flushed with buffer, stored overnight at room temperature, and used the next day to form membranes. Devices were reused in this manner for up to 5 days. For both of the PDMS channels constructed, we found that the addition of small amounts of perfluorooctane (50 ppm), which is not expected to partition into PDMS,[16] in the organic phase improved membrane stability but had no deleterious effect on the membrane's thickness or ability to incorporate protein (below).

Although electrical measurements indicate that PDMS solvent extraction forms high-quality lipid-bilayer membranes, the utility of this technique is best demonstrated by the capacity of these membranes to support a channel protein. We therefore inserted α-hemolysin into a microfluidic membrane by including assembled α-hemolysin pores in the aqueous phase at a concentration of 1 ng/mL. Alpha-hemolysin is a water-soluble protein from *Staphylococcus aureus* forming heptameric pores that spontaneously insert into lipid-bilayer membranes with conductance in the range

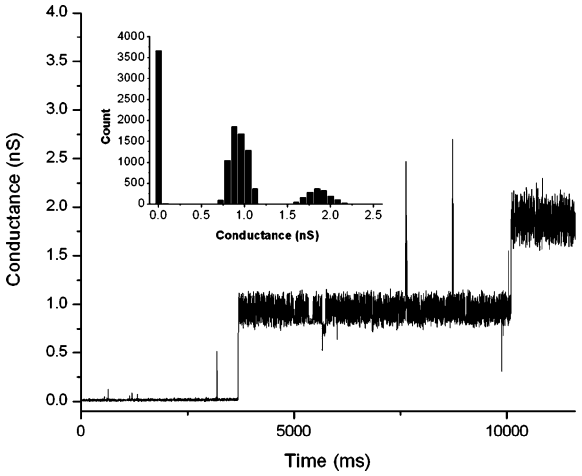

Figure 4. Raw single-channel conductance data for α-hemolysin molecules inserted into a microfluidic lipid-bilayer membrane formed by PDMS solvent extraction. Inset: a histogram of these data, showing the three discrete conductance states corresponding to zero, one, and two protein molecules inserted into the membrane.

of 0.8−1.0 nS.[28] Following membrane formation, the spontaneous insertion of these channels into the membrane was observed by clamping the voltage across the membrane at 80 mV and monitoring the current. Figure 4 shows the resulting trace of membrane conductance with time. The discrete jumps in conductance correspond to the incorporation of single α-hemolysin channels into the membrane; the magnitude of these jumps match the values of the single-channel conductance in the literature.[29]

The work presented here demonstrates a new fundamental mechanism of lipid-bilayer fabrication in which amphiphilic molecules can be guided to form self-assembled structures by aligning them at fluid-phase interfaces and selectively extracting one of the phases. We have demonstrated that this approach is capable of forming true molecular bilayers that sustain high-quality resistive seals with the PDMS structures in which they are formed. We have further demonstrated that these bilayers are capable of supporting membrane channel proteins and revealing single-channel conductance through these proteins. The microfluidic format is especially exciting because it is easily miniaturized and adapted to highly parallel applications. It has direct applications to high-throughput screening for drug discovery and drug safety testing. In its simplest form, however, as presented here, it provides researchers with a facile method for studying lipid membranes and membrane proteins using only the most basic microfabrication techniques.

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